BEHAVIOURAL EFFECTS OF ANCESTRAL STRESS AND POSTNATAL ENVIRONMENT

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Dedicated to my amazing family:

To Nicholas & Kylie,
All this science may not mean much to you, but you were the inspiration for it.
I hope that, in some small way, it will improve the world you will one day inherit.
You have enriched my life in ways I can't begin to express.

To the love of my life, Hilary,
Thank you for all your sacrifice and work to make this thesis possible.
You are the best thing that has ever happened to me.

I love you!

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ABSTRACT

This thesis examines the effects of both ancestral stress and postnatal environment on behaviour in male Long Evans rats. We hypothesized that ancestral stress has a significant impact on the development of the stress response, cognition, and behaviour. In this study, we examined the effects of two types of ancestral stress: transgenerational prenatal stress (TPS), which investigates the consequences of maternal exposure to prenatal stress across generations; and multigenerational prenatal stress (MPS), which investigates the effects of multiple consecutive generations exposed to prenatal stress that may be seen within a hostile natural environment. In addition, we tested the influence of postnatal environment on ancestral stress-induced behavioural changes using both negative (demonstrated here as the consumption of artificial food dye) and positive (demonstrated here as environmental enrichment via complex housing) stimulation. Our results suggest that MPS rats present a more refined phenotype when compared to TPS rats, and that multiple consecutive generations of stress serve as a more consistent environment in which to "calibrate" the developing brain. In addition, our findings suggest that EE is beneficial to all male rats, independent of the experience of ancestral stress. We discuss the implications from an evolutionary perspective, and how they relate to both rats and humans. We feel that our results provide sufficient evidence that working to improve developmental environments is a worthwhile endeavour, and may reduce risk for mental illness in affected populations.

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LIST OF ABBREVIATIONS

ADHD	Attention Deficit Hyperactivity Disorder
ADI	Acceptable Daily Intake
AFD	Artificial Food Dye
ANOVA	Analysis of Variance
EE	Environmental Enrichment
GC	Glucocorticoid
GR	Glucocorticoid Receptor
HPA Axis	Hypothalamic-Pituitary-Adrenal Axis
LG	Licking-and-Grooming
MPS	Multigenerational Prenatal Stress
MR	Mineralocorticoid Receptor
TPS	Transgenerational Prenatal Stress

CHAPTER 1

Introduction

1.1 Introduction

The study of environmental influence on neurodevelopment is critical to our understanding of the brain and behaviour. Both before and after birth, there are a number of factors that contribute to the development of many psychopathologies. It is suggested that the recent rapid change in environmental circumstances for humans may be at least partially contributing to the acceleration of mental illness in highly developed cultures (Crespi and Denver, 2004; Glover, 2011). It has been suggested that the frequent exposure to excess psychosocial stress in Western societies may contribute to the development of many of the different mental illnesses that are on the rise today (Glover, 2011).

While direct exposure to environmental stress is potentially harmful, excess stress during pregnancy may result in life-changing disruptions of behaviour in the developing infant that last into adulthood (Harris and Seckl, 2011), and even subsequent generations (Zucchi et al., 2012). These changes may be mediated by postnatal environmental factors, either by further compromising brain development (Arnold et al., 2012), or by developing a level of resilience to environmental stressors (Simpson and Kelly, 2011). Throughout the course of this thesis, the author will attempt to do the following in a rodent model: (1) study the impact of a family history of prenatal stress on the development of different aspects of behaviour, including locomotor activity, stress response, affective behaviour, and cognition; (2) study the mediating influence of environmental factors, in terms of consumption of artificial food dye and environmental enrichment (EE), on behaviour in

naïve and stressed aging animals; and (3) study the synergistic effects of stress and environment, with a specific interest in how environment may alter the effect of stress on behavioural development.

1.2 Prenatal Stress and Programming of the Stress Response

Stress is defined as a disruption of homeostasis, which triggers a response from the organism's hypothalamic-pituitary-adrenal (HPA) axis (Seckl, 2008). Once activated by higher-level brain structures such as the prefrontal cortex and limbic system, the HPA axis triggers a cascade of neurotransmitter releases that results in the release of stress hormones collectively known as glucocorticoids (GCs). The cascade includes the release of corticotropin-releasing hormone by the hypothalamus, which activates the release of adrenocorticotropic hormone from the anterior pituitary into the blood stream. The adrenal cortex is subsequently activated by the adrenocorticotropic hormone, resulting in the secretion of GCs (cortisol in humans, corticosterone in rats). Additionally, glucocorticoid receptors (GRs) and mineralocorticoid receptors (MRs) are located throughout the brain, which trigger a negative-feedback loop, and deactivate the HPA axis once the desired effect has been reached. As MRs have a higher affinity for GCs, it is believed that MRs serve as the feedback mechanism during baseline secretion, and GRs serve as a trigger for periods of increased stress response activity (Matthews, 2002).

While exposure to GCs *in utero* is beneficial and even necessary, excess GC exposure can be detrimental to brain development. Furthermore, chronic maternal stress can result in a decreased expression of placental enzyme 11β-hydroxysteroid dehydrogenase type 2, which catalyzes the inactivation of GCs into inert forms (cortisone

in humans and 11-dehydrocorticosterone in rats) (Edwards et al., 1993; Holmes et al., 2006). This results in increased GC exposure of the developing fetus, leading to increased risk of long-lasting programming effects on the brain and behaviour (Weinstock, 2011). Prenatal stress-related programming effects include low birth weight, glucose intolerance (Lesage et al., 2004), permanent changes in GR expression in the amygdala and hippocampus (Welberg et al., 2000), and increased HPA axis reactivity (Henry et al., 1994). These effects manifest in a wide variety of behavioural pathologies, including decreased cognitive performance (Igosheva et al., 2007, Vallee et al., 1999), altered social behaviour (O'Connor et al., 2003; Takahashi et al., 1992a; Weinstock, 2008), and increased stress reactivity, anxiety, and depression (Alonso et al., 2000; Estanislau and Morato, 2005; Murmu et al., 2006; Vallee et al., 1997; Van Den Bergh and Marcoen, 2004; Weinstock 2008).

The effects outlined above differ in males and females (Darnaudery and Maccari, 2008; Glover and Hill, 2012). For example, when exposed to prenatal stress, females show more depression-like behaviour in the forced swim task when compared to males (Frye and Wawrzycki, 2003). Additionally, Biala et al. (2011) found that prenatal stress reduced gender differentiation in elevated plus maze and novel object recognition tasks. They also found a reduction of sex differentiation in a number of key genes and proteins that act as sex-dependent molecular sensors, suggesting that prenatal GC overexposure may result in changes to the timing of sex-specific hormones such as testosterone (Biala et al., 2011). Interestingly, Perez-Laso et al. (2013) showed that prenatal stress induced maternal-like behaviour in male rats, further emphasizing the demasculinization of stressed male rats (Perez-Laso et al., 2013).

In addition to physiological effects of early-life stress, postnatal maternal care, which is influenced by gestational stress, can also significantly alter the structure and functioning of the developing brain in the offspring. For example, licking-and-grooming (LG) behaviour is used as an observable measure for maternal care during early postnatal life (Champagne et al., 2003). Both prenatally stressed (Champagne et al., 2006) and low LG rats (Weaver et al., 2004) result in "epigenetic" programming effects to the HPA axis, i.e. heritable changes in gene expression patterns without direct changes to the genomic sequence. Such epigenetic changes may alter hippocampal glucocorticoid receptor (GR) gene methylation, which results in decreased hippocampal GR density and gene expression (Champagne et al., 2006; Weaver et al., 2004; Zucchi et al., 2012). The decrease in hippocampal GR, which is essential for HPA axis regulation, results in higher GC secretion and anxiety-like behaviour in response to a stressor (Weaver et al., 2004, 2006). Interestingly, cross-fostering studies have confirmed that the effects occur in the absence of stress-related changes in postnatal maternal care (Schneider et al., 2002), suggesting that these heritable changes in gene expression are sufficient to alter the stress response.

1.3 Prenatal Stress Effects Beyond the F_1 Generation

Excessive exposure to elevated levels of GCs during pregnancy not only affects stress response and behaviour in the first generation (the filial F_1 generation), but subsequent generations as well (Dunn et al., 2011; Franklin et al., 2010; Skinner, 2008). The study of these effects (referred to collectively in this thesis as *Ancestral Stress*) can be divided into two categories: (1) the effects of a single generation of prenatal stress on the development of multiple subsequent generations (referred to in this thesis as

Transgenerational Prenatal Stress, or TPS); and (2) the study of multiple consecutive generations of prenatal stress, as potentially experienced in a stressful habitat (referred to in this thesis as *Multigenerational Prenatal Stress*, or MPS). These configurations are admittedly an oversimplification of the wide variety of possible situations that may occur in nature, but the distinction between TPS and MPS represents a valuable paradigm in establishing a distinction in ancestral stress effects.

Exposure to excess glucocorticoids may, through mechanisms such as DNA methylation, alter gene expression without directly altering the DNA sequence (Zucchi et al., 2012). The definition of epigenetic factors includes heritability, thus genuinely epigenetic changes are able to be passed on from a parent to several generations of offspring, and can therefore influence the development of future generations (Skinner, 2008). However, in order to study epigenetic effects of TPS in the female lineage independent of the effects of direct exposure to elevated GCs, it is essential to extend study into the third generation of offspring, or F_3 generation (Skinner, 2008; Zucchi et al., 2012). When a pregnant dam is stressed, three generations are directly exposed to the effects of prenatal stress (Zucchi et al., 2012). First, the pregnant dam (F_0) is directly exposed to the stressor, which results in an increase in GC secretion, as well as possible changes in feeding behaviour, weight loss, and increased anxiety related behaviour (Harris and Seckl, 2011). The developing fetus (F_1) is exposed to elevated levels of maternal GCs, as well as secondary effects such as disruptions in maternal feeding behaviour (Harris and Seckl, 2011). If the fetus is a female, reproductive cells (F_2) are already present during development, and they too may be exposed to the direct consequences of prenatal stressor. As a result, the F_3 generation is the first generation in an ancestral line that can confidently

be assumed to have not experienced direct exposure to maternal stress. Therefore, the F_3 generation is the first generation in the ancestral line in which we can confidently assume that phenotypic changes induced by TPS are transferred by genuine epigenetic mechanisms (Skinner, 2008; Zucchi et al., 2012).

In contrast to the epigenetic effects in TPS rats, the study of MPS can provide insight into how stress can change the behaviour of a species over time. A number of researchers have suggested that in multiple consecutive generations of prenatal stress, offspring are exposed to compounding effects of repeated stress occurring across generations (Crespi and Denver, 2004; Harris and Seckl, 2011). Conversely, however, some have suggested that repeated experience of stress may play a role in the adaptation of a species to their environment (Glover, 2011), or that maladaptive behavioural pathologies may be the result of a mismatch between prenatal stress-related programming and postnatal environment (Schmidt, 2011). Additionally, others have suggested that prenatal stress signals the development of a "trendy" phenotype in offspring, in which a rat's metabolism and behaviour are optimized for survival and reproduction (Harris and Seckl, 2011). However, there has been no study exploring the consequences of recurrent multigenerational stress under controlled laboratory conditions yet. Regardless of how the topic of ancestral stress is approached, it is clear that further investigation into the effect of postnatal environment is equally critical to understanding how stress modulates behavioural traits. Furthermore, a study of postnatal environmental factors can contribute to the development of possible interventions and mediating treatments that may alleviate the effects of prenatal stress.

1.4 Postnatal Environmental Factors Contributing to Behavioural Development

Postnatal environment, like the prenatal environment, has a strong impact on behavioural development. In fact, environmental factors play a mediating role in the development of behavioural pathologies with an ancestral component (Holmes et al., 2005). For example, consumption of artificial food dye (AFD) has been associated with modulation of the stress response, inhibition of serotonergic activity, and histamine release (Arnold et al., 2012). Mainly, these effects have been shown to promote hyperactivity in children (Bateman et al., 2005; Kanarek, 2011; Lau et al., 2006; Park et al., 2009). However, it remains unclear if the effects of AFDs are more pronounced in offspring genetically predisposed to ADHD-like behaviour (Arnold et al., 2012). Our study (Chapter 2) indicated that AFD consumption resulted in hyperactivity regardless of a history of ancestral stress (Erickson et al., 2014).

Environmental enrichment (EE) also is a highly influential and clinically relevant determinant of brain and behavioural development and widely investigated in experimental models. EE has been defined as the introduction of additional physical or social stimuli that would not be normally encountered in standard laboratory housing conditions (Rosenweig and Bennett, 1996; Simpson and Kelly, 2011). EE has been well studied as a positive intervention treatment for brain injury (Kolb and Muhammed, 2014; Kolb and Whishaw, 1998), which results in altered epigenetic regulation of gene expression in the brain (Rampon et al, 2000). More specifically, EE promotes neuroplasticity by promoting the expression of genes associated with the synthesis of neurotrophic factors (Johansson, 2000). Furthermore, EE has been shown to promote an increase in brain size, cortical thickness, and synaptic density (Andra et al., 2006; Kolb et al., 2003). In addition, although

EE is understood to reduce GC secretion in response to stress (Pena et al., 2009; Welberg et al., 2006), baseline GC levels have been shown to both increase (Moncek et al., 2004) and decrease (Belz et al., 2003) in EE rats. These effects can also be seen in offspring of EE rats, further suggesting that EE may result in heritable epigenetic changes (Mychasiuk et al., 2012).

The above mentioned effects result in a host of behavioural changes. For example, EE has been shown to decrease anxiety-like behaviour. Using the elevated plus maze (EPM), Galani et al. (2007) demonstrated that EE resulted in higher time spent in the open arms, which is an indicator of decreased anxiety. Moreover, Pena et al. (2009) found that EE rats showed increased exploration in the EPM. In addition to changes in anxiety, Brenes et al. (2009) showed decreased learned helplessness in terms of reduced floating time in the forced swim task (Porsolt et al., 1979), which represents an indicator of depression-like behaviour. Finally, EE has also been shown to improve performance in learning and memory tasks using both the Morris water maze (Leggio et al., 2005; Zhong et al., 2009) and the object recognition task (Bruel-Jungerman et al., 2005; Pamplona et al., 2009).

To our knowledge, no studies have yet been conducted into the investigation of the effects of ancestral stress (both TPS and MPS) and EE, while looking at both adolescence and adulthood. Furthermore, while many studies investigate the effects of baseline GC levels, they do not study GC levels following both acute and chronic stress. Due to the interactive nature of ancestral stress and postnatal environment, an in-depth behavioural study of these effects in a single cohort would be valuable to further understanding of their impact on behaviour.

1.5 Thesis Objectives and Outline

The purpose of the present thesis is as follows: (1) to study the behavioural effects of ancestral prenatal stress, in both its TPS and MPS forms, using a standardized rat model of maternal stress; (2) to study the role of environmental factors (specifically, AFD and EE) in mediating behavioural improvement in naïve and stressed aging rats; and (3) to study the synergistic effects of ancestral prenatal stress and environment, and the possible benefit of EE as an intervention for prenatal stress. This will be accomplished in three chapters, each presenting one set of experiments, which make up the body of the thesis. Chapter 2 contains a manuscript published in PLoS One by Erickson, Falkenburg, and Metz (2014), in which we studied the effects of MPS and AFD consumption on the development of hyperactivity and anxiety in male rats. In this study, we hypothesized that MPS and AFD would both result in hyperactivity, and that MPS would result in increased anxiety in the open field task. In Chapter 3, the effect of MPS and TPS on aging rats' ability to learn an odor discrimination task is investigated. In this study, we hypothesized that ancestral stress would result in a decrease in observable effort to learn the task. In Chapter 4, the effect of MPS, TPS, and EE on exploration, affective behaviour, stress response, learning and memory, and social behaviour is investigated. The experimental design in this chapter provides an excellent opportunity to examine the effects of multiple different factors that contribute to the development of behavioural abnormalities in a single cohort of animals. Furthermore, the study includes examination of the stress response both at baseline and following acute and chronic stress points, providing a comprehensive look at programming effects in the stress response. In this study, we hypothesized that ancestral stress would result in increased locomotor activity, learning and memory deficits, stress responsivity,

and social aggression. We also hypothesized that EE would decrease stress-associated changes in locomotor activity, learning and memory deficits, stress responsivity, and social aggression. Finally, in Chapter 5 contains a general discussion of findings and implications. We discuss the role of both ancestral stress and postnatal environment in the development of adaptive behavioural change across multiple generations.

CHAPTER 2

Study #1: Lifespan Psychomotor Behaviour Profiles of Multigenerational Prenatal Stress and Artificial Food Dye Effects in Rats

Erickson, Z.T., Falkenberg, E.A., Metz, G.A. (2014). Lifespan Psychomotor Behaviour Profiles of Multigenerational Prenatal Stress and Artificial Food Dye Effects in Rats. PLoS One, 9(6), e92132. doi:10.1371/journal.pone.0092132

2.1 Abstract

The consumption of artificial food dye (AFD) during childhood and adolescence has been linked to behavioural changes, such as hyperactivity. It is possible that the vulnerability to AFDs is modified by prenatal stress. Common consequences of prenatal stress include hyperactivity, thus potentially leading to synergistic actions with AFDs. Here, we investigated the compounding effect of multigenerational prenatal stress (MPS) and AFD consumption on the development of hyperactivity and anxiety-related behaviours across the lifespan in male rats. MPS treatment involved a family history of four consecutive generations of prenatal stress (F_4 generation). AFD treatment included a 4%-concentration of FD&C Red 40, FD&C Yellow 5, FD&C Yellow 6, and FD&C Blue 1 in the drinking water from postnatal days 22 to 50 to resemble juvenile and adolescent dietary exposure. Using several exploration tasks, animals were tested in motor activity and anxiety-like behaviours from adolescence to 13 months of age. MPS resulted in hyperactivity both early (50 days) and later in life (13 months), with normalized activity patterns at reproductive age. AFD consumption resulted in hyperactivity during consumption, which subsided following termination of treatment. Notably, both MPS and AFD promoted risk-taking behaviour in young adults (3 months). There were few synergistic effects between MPS and AFD in this study. The findings suggest that AFDs

exert the most noticeable effects at the time of exposure. MPS, however, results in a characteristic lifespan profile of behavioural changes, indicating that development and aging represent particularly vulnerable periods in life during which a family history of prenatal stress may precipitate.

2.2 Introduction

During critical periods of neurodevelopment, both before and after birth, environmental and endocrine factors can have long-lasting effects on brain plasticity and behaviour. One of the most critical influences on neurodevelopment is prenatal stress. Prenatal stress will expose the developing fetal brain to elevated levels of glucocorticoid hormones, which may lead to increased risk of psychological disruptions later in life, including hyperactivity and anxiety-related behaviours (Harris and Seckl, 2011; Holmes et al., 2006; O'Connor et al., 2003; Van Den Bergh and Marcoen, 2004). These stress-induced behavioural changes may be influenced by an organism's family history as well, as many behavioural effects of stress may be transmitted from one generation to the next (Korosi and Baram, 2009; Ward et al., 2013; Zucchi et al., 2012). In a continuously stressful environment, however, prenatally stressed females may themselves be exposed to stress during pregnancy, thus generating several generations of prenatally stressed offspring. While the significant sequelae of prenatal stress in a single generation have been investigated in detail, the influence of multigenerational prenatal stress (MPS) on behaviour and brain development have not been shown yet. In turn, such family history of prenatal stress may also raise the vulnerability to environmental risk factors (Matthews and Phillips, 2010).

An environmental risk factor that was suggested to influence behavioural development is the consumption of artificial food dyes (AFDs). Although the contribution to behavioural disturbances is still controversial (Arnold, Lofthouse, and Hurt, 2012), evidence suggests that AFD consumption increases the risk of behavioural change in children (Bateman et al., 2005; Kanarek, 2011; Lau et al., 2006; Park et al., 2009). Earlier reports indicated that children with ADHD may show above-average sensitivity to AFDs (Stevents et al., 2011), while other findings suggested that AFDs may affect healthy populations as well (Schab and Trinh, 2004). In particular, previous studies showed that AFD consumption results in increased motor activity (Schab and Trinh, 2004; Tanaka et al., 2008). For example, a randomized study showed that AFD exposure in 3-year old and 8/9-year old healthy children results in hyperactivity, with the greatest effect in the 8/9-year age group (McCann et al., 2007). Very few studies, however, used animal models to pursue further mechanistic studies of the behavioural effects of AFD exposure (Reisen and Rothblat, 1986).

The purpose of this study was to examine the effects of MPS and chronic AFD consumption during adolescence on locomotor activity and anxiety-like behaviours in aging rats. We hypothesized that both MPS and AFDs represent experiences that may modulate brain development in adolescence and alter lifespan behavioural profiles in an age-dependent manner. The study used a standard exploratory task (open field) and a newly developed test of affective state and exploration. The purpose of this study was (1) to investigate the behavioural consequences of MPS, (2) to investigate the behavioural effects of chronic consumption of commonly used certified AFDs during neurodevelopment, and

(3) to determine potential synergistic effects of MPS and AFDs from adolescence to late adulthood.

2.3 Methodology

2.3.1 Animals

Thirty-two male Long-Evans hooded rats, weighing an average of 46 g at the beginning of the study, were used. The animals were housed in pairs in standard shoebox polycarbonate cages on corn cob bedding (Bed O Cobs 1/8", Anderson, OH, USA). The housing room was maintained at 20^{o}C and relative humidity at 30% on a 12-hour dark/ light cycle with light starting at 7:30 AM. The experimental procedures were approved by the University of Lethbridge Animal Welfare Committee according to guidelines set forth by the Canadian Council on Animal Care.

2.3.2 Experimental Design

The present study was conducted with a two-by-two factorial design, with multigenerational prenatal stress ("Stress") and adolescent consumption of artificial food dye ("Dye") as the two treatments (see Figure 2.1). The male F_4 generation born to four generations of stressed dams was used in this study. The experimental design resulted in four groups of rats (n=8 per group): (1) non-treated controls; (2) Stress without Dye consumption; (3) Dye consumption without stress; (4) Stress combined with Dye Consumption. Dye solution was provided from postnatal day (P) 22 (infancy) to P 50 (adolescence). At P 50, all animals given the dye treatment were switched to standard tap water. The animals were allowed to drink *ad libitum*, and fluid consumption and body mass

were monitored daily. Animals were tested in locomotor activity and emotional behaviours at P 50, and at the age of 3 months, 7 months, and 13 months. Behavioural analyses were performed by an experimenter blind to the experimental conditions.

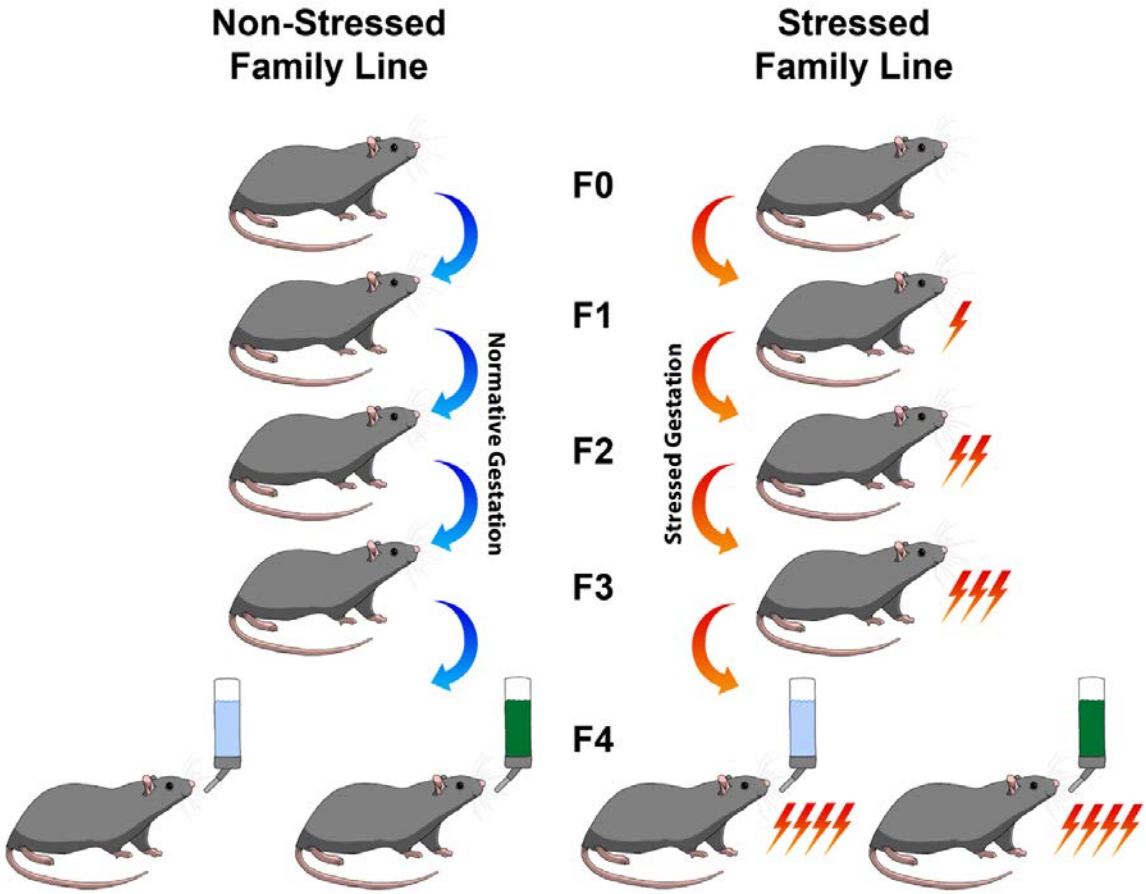

Figure 1. Experimental design. The present study used male rats in a two-by-two factorial design. The independent variables were multigenerational prenatal stress and artificial food dye (AFD) consumption. The multigenerational prenatally stressed rats were the fourth generation (F_4) of an ancestral line in which dams in each generation were given stress during pregnancy. Both the stressed and non-stressed animals were given either normal tap water or a 4%-AFD solution for regular consumption.

2.3.3 Multigenerational Prenatal Stress

Four consecutive generations of rat dams were stressed daily during pregnancy from gestational day 12 to 18 (Ward et al., 2013). The stress treatment involved two

different stressors, which previously have been validated as effective stress procedures (Flores et al., 1990; Metz, Jadavji, and Smith, 2005): (1) restraining the dam in a Plexiglas tube for 20 minutes and (2) placing the dam in a barrel of room temperature water (~25°C) for 5 minutes. The dams received each of the two treatments daily at 08:00 or 16:00, and varied in a semi-random fashion to avoid habituation. As in the F_0 - F_2 generations before, nulliparous females from the F_3 generation were bred with naïve, unstressed Long Evans males (obtained from Charles River Laboratories International Inc., Wilmington, MA, USA) to generate the F_4 generation. Breeding occurred at the age of P 110 and P 180. Dams only delivered a single litter before being euthanized. The pups were weaned at P 21 and housed in pairs with siblings by sex. Maternal corticosterone levels were monitored before pregnancy, gestational day 11 (before stress), gestational day 18 (the final day of stress), and one day following delivery. Maternal blood sampling occurred between 8:00 am and 10:00 am, and no more than 0.6 mL was taken from a rat on each sampling day.

2.3.4 Artificial Food Dye Treatment

Animals received an AFD solution in place of regular drinking water from P 22 to P 50. The AFD solution consisted of 1 gram per litre of four common AFDs: FD&C Red 40, also known as Allura Red AC; FD&C Yellow 5, also known as Tartrazine; FD&C Yellow 6, also known as Sunset Yellow FCF; and FD&C Blue 1, also known as Brilliant Blue FCF (all from Sigma-Aldrich, St. Louis, MO). Fluid consumption and body mass were monitored and recorded. Acceptable Daily Intake (ADI) in mg/day for each dye was calculated using body mass and suggested levels from the Food and Drug Administration

in mg/kg/day (Food and Drug Administration/Center for Food Safety and Applied Nutrition, 2011), and compared to the actual amount of dye consumed daily.

2.3.5 Behavioural Assessments

2.3.5.1 Open Field Task

The animals were tested using an open field task (Jadavji, Supina, and Metz, 2011; Smith et al., 2008) at P 50, 3 months, 7 months, and 13 months of age. The open field task allows the quantification of motor activity, anxiety-like behaviours, and exploration in an open arena. In this study, the open field task was conducted using the VersaMax Legacy Open Field activity box (Omnitech Electronics, Inc., Dartmouth, NS, Canada), which measured an animal's activity for 10 minutes using an array of infrared sensors connected to a computer. This test was conducted at P 50, 3 months, 7 months, and 13 months of age.

Behavioural measures included total distance travelled during the testing period (Distance Travelled), the total time spent moving during the test interval (Movement Time), and the amount of time spent within the margins of the open field (Margin Time).

2.3.5.2 Affective Exploration Task

The animals were tested at 1.5 months, 3 months, and 13 months in a new task developed for this experiment, the affective exploration task. This task combines the features of a light-dark test (Fernandez et al., 2004) with an open field arena. A rat was placed inside a refuge (a 10 cm x 10 cm x 20 cm plastic tube attached to a small platform) on the top of a large table (75 cm x 150 cm) for 5 minutes. The test was video recorded for

later scoring and analysis. After each 5 minute session, the testing environment was cleaned to remove any olfactory cues.

The video footage was scored for three main measures of affective state and exploration: the time before initial emergence from the refuge (Emergence Latency), the total time the rat spent within the refuge (Refuge Time), and number of exits from the refuge (Refuge Exits) were recorded. Finally, each animal was categorized on the basis of whether or not it left the refuge during the testing period (Binary Exploration).

2.3.6 Statistical Analysis

Collected data were analyzed using the SPSS version 20 software package. Primary analysis consisted of analysis of variance (ANOVA) and independent sample t-tests to investigate effects at a post-hoc level. Correlation analyses were also conducted between pairs of variables, controlling for Stress Treatment, Dye Treatment, and Age. In partial correlations, including the affective exploration task, the Binary Exploration Score was also controlled for. A p-value less than 0.05 was considered as significant. All data in figures are shown as mean values and standard error of the mean (SEM).

2.4 Results

2.4.1 General Observations

An independent sample t-test revealed that there was no significant difference ($t(5) = 1.07$) in litter size between stressed ($M = 14.25$, $SD = 1.7$) and unstressed dams ($M = 16.00$, $SD = 2.6$). A one-tailed independent t-test showed that maternal corticosterone levels at gestational day 18 were significantly higher ($t(3.49) = -2.39$, $p < 0.05$) in stressed

dams (M = 1630.58 ng/mL, SD = 579.6) when compared to unstressed control dams (M = 910.03 ng/mL, SD = 146.0). Maternal corticosterone levels did not differ between groups on non-stress days, such as baseline, gestational day 11, or one day following delivery.

The administration of AFDs in the drinking water did not affect daily water consumption or weight gain. Averaged over the last four days of the dye consumption period, rats that consumed dye (M = 48.1 mL, SD = 2.7) did not differ from rats that consumed water (M = 49.7 mL, SD = 5.3; $F(1,28) = 1.044$, $p = 0.32$). Similarly, rats that consumed dye (M = 281.7g, SD = 17.2) did not significantly differ in body mass at P 50 when compared to rats that consumed water (M = 285.5g, SD = 25.9; $F(1,28) = 0.424$, $p = 0.52$). In both cases, stress did not have a significant main effect, and there was no interaction effect with dye. Each dye was ingested on average at a rate of 1.71 mg/day (SD = 1.71). The calculated ADI for each dye were as follows: Allura Red had a mean ADI of 2.0 mg/day (SD = 0.12), Tartrazine had a mean ADI of 1.41 mg/day (SD = 0.08), Sunset Yellow had a mean ADI of 1.06 mg/day (SD = 0.06), and Brilliant Blue had a mean ADI of 3.38 mg/day (SD = 0.21).

2.4.2 Multigenerational Prenatal Stress and Dye Consumption Alter Open Field Activity Profiles

Overall, the ANOVA revealed a significant main effect of Stress ($F(1,125) = 7.11$, $p < 0.01$) and Age ($F(3,125) = 13.61$, $p < 0.001$) for Distance Travelled, as well as a significant main effect for Age in Movement Time ($F(3,125) = 19.17$, $p < 0.001$). There was no significant interaction between Stress, Dye, and Age. At 1.5 months old, a

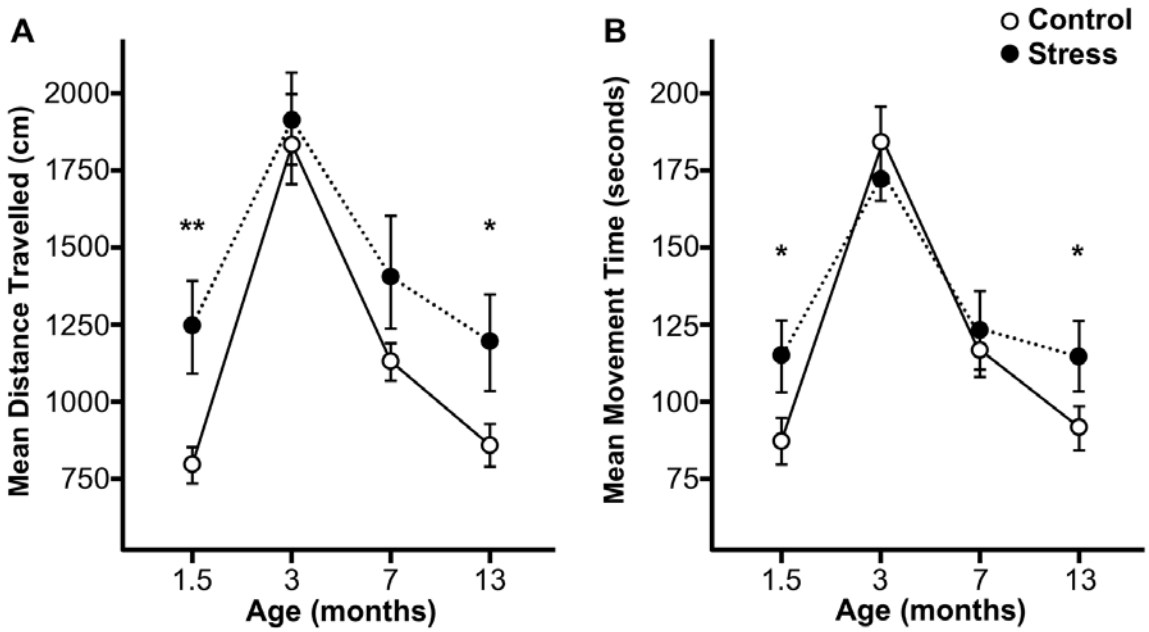

Figure 2.2. A lifespan profile of the effect of multigenerational prenatal stress on motor activity in the open field task. (A) Multigenerational prenatal stress (n = 16) resulted in a significant increase in the mean distance travelled at 1.5 months and 13 months of age, but not at 3 or 7 months of age, when compared to non-stress animals (n = 16). (B) Multigenerational prenatal stress (n = 16) resulted in a significant increase in mean movement time at 1.5 months and 13 months of age, but not at 3 or 7 months of age, when compared to non-stress animals (n = 16). Thus, motor activity of MPS rats resembles that of non-stressed rats during peak sexual reproductive age, but differs early in life and in late adulthood. Asterisks indicate significances: * $p < 0.05$; ** $p < 0.01$, compared to non-stress animals.

significant main effect of Stress was present in Distance Travelled ($F(1,30) = 8.316$, $p < 0.01$) and Movement Time ($F(1,30) = 5.61$, $p < 0.05$), and a significant main effect of Dye was present in Movement Time ($F(1,30) = 4.45$, $p < 0.05$). At both 3 and 7 months old, there were no significant main effects. At 13 months old, trends in the main effect of Stress were present in Distance Travelled ($F(1,29) = 3.65$, $p = 0.067$) and Movement Time ($F(1,29) = 2.83$, $p = 0.10$), but not for the main effect of Dye. Both Distance Travelled and Movement Time showed a characteristic age profile, with Distance Travelled and Movement Time being highest at 3 months postnatal, and lower both before and after that age. It should be noted that no significant effect was found for dye consumption after the

animals were returned to standard tap water. Additionally, there were no significant effects in the analysis of Margin Time in the open field.

When comparing treatment groups, stress rats, independently of dye treatment, showed significantly higher Distance Travelled at 1.5 months old ($t(30) = -2.86$, $p < 0.01$) and 13 months old ($t(29) = -2.00$, $p < 0.05$; one-tailed; see Figure 2A). Stress rats also showed significantly more Movement Time than unstressed controls at 1.5 months old ($t(29) = -2.26$, $p < 0.05$), and 13 months old ($t(29) = -1.75$, $p < 0.05$; one-tailed; see Figure 2B).

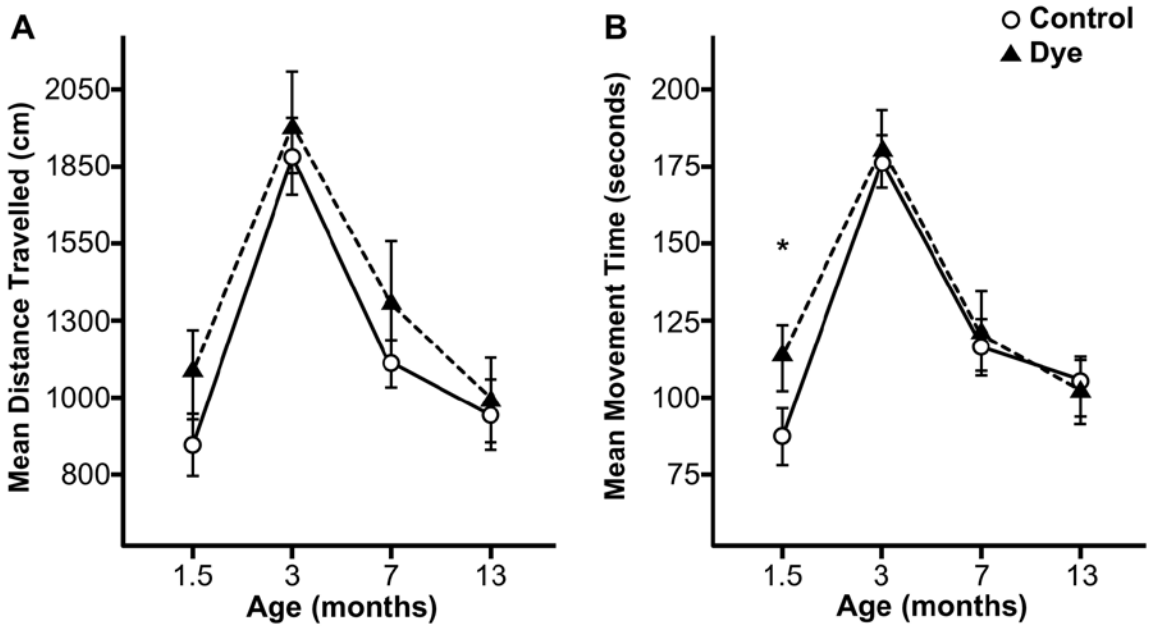

Figure 3. A lifespan profile of the effect of artificial food dye consumption on motor activity in the open field task. (A) AFD consumption (n = 16) from postnatal days 22 through 50 did not increase the mean distance travelled when compared to untreated animals (n = 16). (B) AFD consumption (n = 16) resulted in an increase in the mean movement time while the animals were placed on the AFD-containing diet when compared to untreated animals (n = 16). However, these effects were not found after the AFD was removed from the diet. Asterisks indicate significances: * $p < 0.05$, compared to untreated animals.

There was no difference in dye treatment groups when comparing Distance Travelled (see Figure 3A). However, independently of stress treatment, dye rats showed significantly higher Movement Time at 1.5 months old (following dye consumption) than rats that did not consume dye ($t(30) = -1.98$, $p < 0.05$; one-tailed; see Figure 3B).

2.4.3 Dye Consumption Alters Affective Behaviour and Exploratory Behaviours

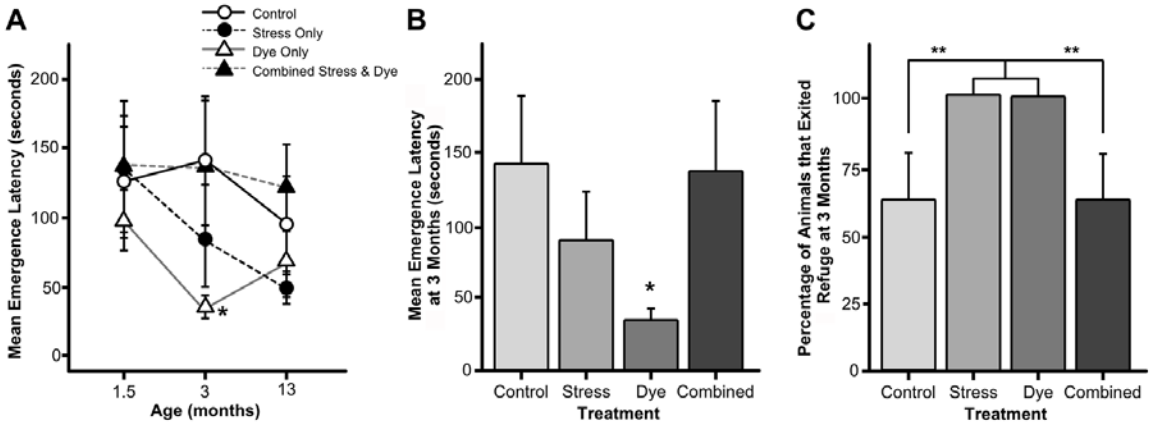

Figure 2.4. Behavioural effects of multigenerational prenatal stress and artificial food dye consumption in the affective exploration task. (A) At both 1.5 and 13 months of age, there were no effects of treatment with regard to the latency to emerge from the refuge. However, at 3 months of age, AFD-only animals (n = 8) were less reluctant to exit the refuge than any other group (n = 8 per group). (B) At 3 months of age, the AFD-only group (n = 8) was the fastest to exit the safe refuge. (C) Percentage of animals in each group that left the safe refuge at the age of 3 months. Both the prenatally stressed and AFD-treated groups left the refuge in 100% (n = 8) of the test sessions, while both control and combined prenatal stress and AFD groups left the safe refuge only in 62% of the time (n = 5 out of n = 8). Asterisks indicate significances: * $p < 0.05$; ** $p < 0.01$, compared to controls.

When considering Emergence Latency, the ANOVA across the three testing periods showed a significant interaction effect for Stress*Dye ($F(1,92) = 5.25$, $p < 0.05$; see Figure 4). However, significant main effects, including a main effect of Age, were not found for Emergence Latency when analyzing all three testing periods cumulatively. When conducting ANOVA at individual time points, only the 3-month period showed a trend (p

= 0.054) for the same Stress*Dye interaction. It is interesting that a significant effect of Dye was found only at the 3-month time point, i.e., 40 days after the cessation of Dye consumption. At 3 months of age, Dye-only animals emerged much faster from the refuge than untreated controls ($t(14) = 2.24$, $p < 0.05$; Figures 4A and 4B). The analysis of both Refuge Time and Number of Refuge Exits showed no significant effects.

An analysis of the Binary Exploration Score showed a significant interaction effect for Stress*Dye across all ages when controlling for age ($F(1,92) = 9.05$, $p < 0.01$). When comparing at 3 months of age, a similar interaction effect was found ($F(1,31) = 8.40$, $p < 0.01$; Figure 4C). More specifically, at 3 months of age, both Stress-only and Dye-only groups left the refuge at a rate of 100%, whereas the control and combined Stress and Dye groups left the safe refuge at a rate of 62.5%.

2.4.4 Correlations

When controlling for Stress, Dye, Age, and Binary Exploration Score, the negative correlation was significant between Centre Time in the open field and Time Spent in the refuge in the affective exploration task ($R^2 = 0.068$, $p < 0.05$). Thus, animals that spent more time in the open field centre also showed the shortest latency to exit the safe refuge in the affective exploration task.

Additionally, the Distance Travelled and Movement Time in the open field were significantly correlated ($R^2 = 0.887$, $p < 0.01$), as well as the Refuge Time and Emergence Latency in the affective exploration task ($R^2 = .449$, $p < 0.01$). Thus, animals that travelled farther in the open field spent more time moving, and animals that spent more time in the

refuge also showed the longest emergence latency. All other pairings were not statistically significant, including the pairing of Total Distance and Refuge Time ($R^2 = 0.007$, $p = 0.51$).

2.5 Discussion

The purpose of this study was to investigate the consequences of multigenerational exposure to prenatal stress and AFD consumption during development on the vulnerability to hyperactivity and anxiety-related and risk-taking behaviours in male rats. We found that MPS promoted motor hyperactivity during particularly vulnerable periods in life, during adolescence (P 50) and aging (13 months). Furthermore, AFD consumption from postnatal day 22 to 50 resulted in hyperactivity and reduced anxiety-like behaviour and greater tendency for risk taking in adolescence. The combination of MPS and AFD, however, did not exaggerate anxiety-like behaviours. Interestingly, the MPS animals showed no significant manifestation of anxiety-like behaviour.

In this experiment, pregnant rat dams were exposed to a semi-random combination of restraint and swim stress, which may represent an ecologically valid rat model of moderate stress (Flores et al., 1990; Ward et al., 2013) with effects on motor behaviour (Jadavji, Supina, and Metz, 2001; Smith et al., 2008). We used the open field task, a standard test to effectively assess motor activity and anxiety-related behaviour (Fernandez et al., 2004; Nosek et al., 2008). Moreover, we developed a new task, the affective exploration task, which is based on a combination of light/dark and open field tasks (Bourin and Hascoët, 2003). Time spent in the refuge in the new affective exploration task was negatively correlated with time spent in the centre of the open field, which serves as a demonstration of its validity to assess anxiety-related and risk-taking behaviours.

The present study explored the effects of multigenerationally recurrent prenatal stress. We hypothesized that the recurrent prenatal stress would enhance the predisposition to hyperactive behaviours and the vulnerability to potential psychomotor effects of environmental compounds, such as AFDs. The exposure of the developing brain to elevated glucocorticoid levels during prenatal stress programs hypothalamic-pituitary-adrenal (HPA) axis activity, which may in turn alter locomotor activity profiles and increase anxiety-related behaviours (Harris and Seckl, 2011; Holmes et al., 2006; Lupien et al., 2009). Prenatal exposure to elevated glucocorticoid levels has been shown to alter mesolimbic dopamine (MesoDA) system activity and increase motor activity and ADHD-like behaviours in animal models (Field et al., 2008; Gatzke-Kopp, 2011; Li et al., 2007; Metz, 2007). In our study, the MPS rats showed an increase in hyperactivity both in early and late adulthood, suggesting a transgenerationally cumulative programming of the MesoDA system by prenatal stress that becomes exposed during the most vulnerable periods in life.

A common consequence of exposure to prenatal stress in a single generation, in the male F_1 generation, is the elevation of anxiety-like behaviours (Hao and Metz, 2013; Laloux et al., 2012; Wilson, Vazdarjanova and Terry, 2013). The compounding influences of multigenerational exposure to prenatal stress across four generations of individuals have not been previously studied. Although many endocrine and behavioural responses may adapt or become resilient to recurrent mild maternal/prenatal stress across generations (Crespi and Denver, 2004), we were able to show changes in psychomotor profiles across the lifespan. Interestingly, MPS did not increase anxiety-related behaviour in the open field or the affective exploration tasks, but rather promoted risk-taking behaviours. It is possible

that the experience of a continuously stressful environment across generations may promote some form of stress resilience or coping. This is suggested by previous findings that indicated stress-mediated adaptation to a stressful environment (Crespi and Denver, 2004; Glover, 2011). Moreover, MPS rats may display enhanced anxiety-like responses in other tasks or environments not tested here. Moreover, the behavioural profiles of MPS rats do not exactly resemble those of non-stressed rats either, as seen in previous findings (Ward et al., 2013). It is possible that fear-related responses interact with risk-taking behaviours thus masking a conclusive profile of anxiety-like behaviours under the present testing conditions. Regardless, additional research is needed to determine how the extent to which MPS and single-generation prenatal stress differ.

Our findings are consistent with previous findings that prenatal stress results in programming effects both early (Harris and Seckl, 2011; Jankford, et al., 2011; Sandman, Glynn, and Davis, 2013) and later in life (Vallee et al., 1999). It has been suggested that the deleterious effects of prenatal stress seen in juvenile male rats may serve to eliminate weaker potential mates, resulting in a net benefit toward the survival of the species in a stressful environment (Sandman, Glynn, and Davis, 2013). By contrast, early behavioural changes may assist survival until reproduction is secured (Harris and Seckl, 2011). Additionally, some of the cognitive effects of prenatal stress may not manifest until later in adulthood (Vallee et al., 1999). Further studies are necessary to reveal the mechanisms that explain why a family history of prenatal stress resembles non-stressed behaviours during peak sexual reproductive age, but differ both early in life and in late adulthood.

The present AFD administration procedure was based on a previous protocol by Tanaka (2006). In our study, we used FD&C Red 40, FD&C Yellow 5, FD&C Yellow 6,

and FD&C Blue 1, which are among the most commonly used AFDs that are approved by the Food and Drug Administration in the United States. Although the AFD-containing drinking water was readily accepted by the animals, the freely available solution in group housing bears limitations by preventing the measurement of individual dose-response relationships. Additionally, it is still unclear if individual AFDs result in interactions when consumed alongside other AFDs (Lau et al., 2006). However, it is common for more than one AFD to be used in food products and at concentrations similar to the present ones. It should also be noted, however, that Tartrazine and Sunset Yellow consumption exceeded the ADI in the present study. Given the suggested exponential increase in the average intake of AFDs over the past decades (Arnold, Lofthouse, and Hurt, 2012), we believe that the present approach may be representative of actual AFD exposure encountered by humans.

Our findings suggest that AFD consumption at least contributes to motor hyperactive behaviours independently of underlying stress-induced programming. These results are similar to other experiments that linked AFD consumption to increased motor activity (Schab and Trinh, 2004). However, an interesting finding of the present study was observed following dye treatment cessation at three months of age in the affective exploration task. While all of the MPS-only and AFD-only rats left the refuge, only two-thirds of the animals treated with combined MPS and AFD left the refuge, consistent with the control group. Based on the parameters collected in the present study an unambiguous explanation of this observation is not possible. One explanation may be that MPS and AFD have synergistic, potentially stimulating effects on cognitive performance, as previously shown for caffeine (Prediger et al., 2005) or L-amphetamine (Sagvolden, 2011) in rat

models of ADHD. Additionally, the binary exploration score and activity in the open field were not significantly associated with each other, suggesting that the binary exploration score may not reflect motor hyperactivity but aspects of cognitive functions or fear-related behaviour not assessed in the present study. The leaving of the refuge, however, may suggest a reduced fear of the open, brightly lit surface area in AFD-treated rats.

Although the present study indicates behavioural changes as a function of cumulative AFD consumption as expected to occur in human populations, the use of a cocktail of compounds does not allow dissociation of the effects of individual dyes and their dose-response relationships. Some dyes may be more potent in their actions on behaviour (Reisen and Rothblat, 1986; Vorhees et al., 1983) and genotoxicity (Lau et al., 2006; Vallee et al., 1999). A well-studied compound is Allura Red, which produces physical and behavioural toxicity in rats (Vorhees et al., 1983). Notably, these effects can be partially passed on to the offspring and increase post-weaning open field vertical activity (Vorhees et al., 1983). The mechanisms of specific AFDs on behaviour may include modulation of stress response, inhibition of serotonergic activity, and histamine release (Arnold, Lofthouse, and Hurt, 2009).

The findings of the present study emphasize the critical role of the early environment on behavioural development, adulthood and aging. An important observation is that motor activity profiles of MPS rats resemble those of non-stressed rats during the peak sexual reproductive age, but differ during the most vulnerable periods in life, during development and aging. Although long-term consequences of prenatal and transgenerational stress and AFDs have not yet been systematically studied, the present data suggest that aging processes may unmask the effects of early exposure to adverse

experiences. It is important to note that even statistically small effects may have clinically important consequences and interactions (Arnold, Lofthouse, and Hurt, 2009). The present evidence suggests that AFD effects are universal, and do not just affect developmentally compromised individuals. Interactions of various adverse experiences may provide new insights into predisposing and precipitating factors of mental illness and new or refined interventions.

CHAPTER 3

Study #2: Ancestral Stress Effects on Adult Male Rats Learning an Odor Discrimination Task

3.1 Abstract

Maternal stress during pregnancy can influence the developing fetus, resulting in delayed cognitive development and associated lifelong consequences in the offspring. The aim of this study was to examine the effects of transgenerational prenatal stress (TPS; n = 7) and multigenerational prenatal stress (MPS; n = 8) on performance in an odor discrimination task in male adult rats as compared to unstressed controls (n = 8). Once the rats had successfully associated a single odor to a sucrose reward solution, a second odor, which was associated with a quinine punishment solution, was introduced. Rats were given six 30-minute sessions to distinguish between the two odors. The number of attempts, as well as success rates in odor discrimination were recorded and analyzed. TPS rats showed significantly more attempts than MPS and controls, as well as a higher quinine success rate, i.e. they gave a “No-Go” response more often when presented with the odor signalling a quinine punishment stimulus. Additionally, MPS rats showed a lower “No-Go” success rate, i.e., they gave inappropriate “No-Go” responses more often. Our results suggest that TPS rats were more successful in learning the odor discrimination task due to increased persistence in returning to the odor well. Our results also suggest that learning and memory in MPS rats is impaired. We conclude that an ancestral history of prenatal stress may contribute to the development of cognitive changes, which may consequently play a role in the development of learning deficits.

3.2 Introduction

Prenatal stress, caused by severe or chronic maternal distress or trauma, during pregnancy can result in cognitive impairments in developing offspring with consequences that last into adulthood. Exposure to increased maternal glucocorticoids while in utero has been shown to result in increased anxiety and depression-like behaviour, as well as disrupted cognitive ability (Bergman et al., 2007; Meaney and Szyf, 2005; Raikkonen et al., 2008; Smith, 1981). Additionally, prenatal stress has been shown to result in adverse changes to factors that may contribute to learning in the presence of aversive stimuli, including motivation (Slattery and Cryan, 2012) and inhibition (Wilson et al., 2012). This seems to be at odds with suggestions that prenatal stress serves as a preparatory warning signal to the developing offspring (Schmidt, 2011), as decreased motivation and inhibition would most likely decrease survivability.

With respect to learning and memory, prenatal stress is characterized by task-specific changes that may facilitate certain cognitive performance in a stressful environment. Research suggests that learning may be either enhanced (Cory-Slechta et al., 2012; Fujioka et al., 2001; Gutteling et al., 2006) or reduced (Bergman et al., 2012; Vallee et al., 1999) by prenatal stress. For example, three days of mild prenatal stress was reported to facilitate active avoidance and spatial navigation (Fujioka et al., 2001). Some of this conflict may be due to the wide range of contributing affective factors, such as motivation and stress-associated anhedonia, involved in learning and cognition. Furthermore, prenatal stress induced impulsivity may hinder the learning process by providing an increase in negative stimuli (Weston et al., 2014). The present study involves the study of motivation and impulsivity in a “Go, No-Go” odor discrimination task. To our knowledge, this task

has not yet been used to investigate the effects of prenatal stress on learning. However, a similar task has been used to investigate the connection between prefrontal cortex disinhibition and cognitive deficits (Gruber et al., 2010). This task, therefore, may provide insight into whether prenatal stress serves as an adaptive preparation for learning in an adverse environment later in life.

The aim of the present study is to determine whether ancestral prenatal stress, either as transgenerational prenatal stress (TPS) or multigenerational prenatal stress (MPS), influence cognition as measured by an odor discrimination task. Furthermore, this study aims to determine whether any differences in performance are the result of motivation, impulsivity, or overall stress-associated higher cognitive functions.

3.3 Methods

3.3.1 Animals, Experimental Design, and Experimental Treatments

Twenty-three Long Evans male rats were used in the current study, and were tested at between 10 and 11 months old. Each rat was taken from one of three experimental treatments: naïve controls (n = 8), transgenerational prenatal stress (TPS; n = 7) and multigenerational prenatal stress (MPS; n = 8). The control and MPS rats were taken from the cohort that was used in the study detailed in Chapter 2, and published in PLoS One (Erickson et al., 2014). Only the rats that did *not* receive food dye during adolescence were used in this study. The TPS rats were an additional cohort added to investigate the effects of transgenerational stress.

TPS rats were born in the same cohort and during the same time period as the control and MPS rats. The family history of rats included gestational stress; the MPS rats

had a SSSS family history of stress (i.e., four consecutive generations of prenatal stress), while TPS rats had a SNNN (S, stress; N, no stress) family history of stress. Additionally, it should be noted that the TPS rats were part of a separate study that included some cognitive training, including the Morris water maze and single pellet reaching. Rats were placed on water restriction for five days a week over the course of the experiment, during which they were given access to water for 30 minutes each day at least one hour after a session.

3.3.2 Behavioural Assessment

The purpose of this study was to determine the effect of TPS and MPS on performance of a modified odor discrimination task. A rat is required to learn which of two odors is associated with a rewarding stimulus (i.e., a small amount of sucrose solution), and which odor is associated with an aversive stimulus (i.e., a small amount of bitter quinine solution; Roman et al., 2004; Schoenbaum et al., 2002). A diagram of the testing environment (30 cm x 30 cm x 30 cm) can be seen in Figure 3.1. The training and testing in this task was divided into two phases, the training phase and the odor discrimination phase. The goal of the training phase was for the rat to associate inserting its nose in an odor port with both the delivery of a 100-ms puff of 4-allylanisole (the “Go Odor”) in the odor port, followed by the delivery of a sucrose solution at the reward well. Each session lasted 30 minutes, with five sessions per week. The training phase was considered a success when the rat completed at least 200 trials within 30 minutes, and consistently obtained the sucrose reward from the reward well.

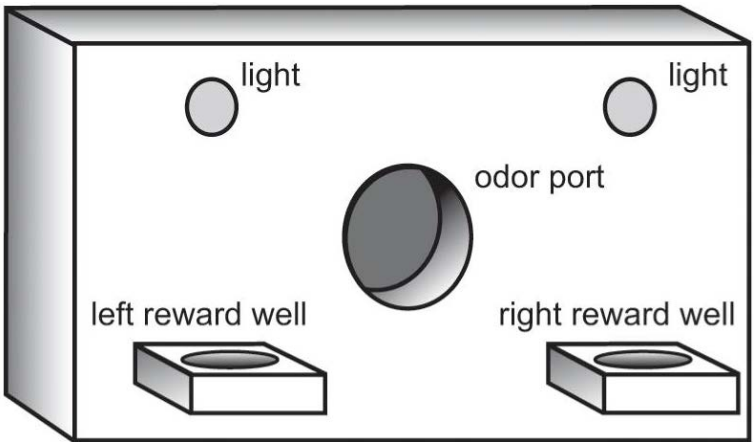

Figure 3.1 The behavioural apparatus used to perform the odor discrimination task. Odor cues were received when the rat inserted its nose into the odor port. Either sucrose or quinine was delivered in the left reward well. The right reward well as not used for this task. (Gruber et al., 2010)

The goal of the odor discrimination phase was for the rat to learn to discriminate between the "Go Odor" and a second odor, 100 ms of geranyl formate (the "No-Go Odor"), which was introduced at a 1:1 randomized frequency. When the "No-Go Odor" was presented to the rat, quinine punishment solution was given in the well, which was aversive to the rat due to its bitter taste. The number of attempts during the 30-minute period was considered a measurement of motivation and persistence in learning the task. Additionally, success rates for appropriate matching of Go Odor to Go Response and No-Go Odor to No-Go Response were also considered. The odor discrimination phase was completed after the sixth session.

3.3.3 Statistical Analysis

Data were analyzed using the SPSS version 20 software package. Primary analysis consisted of analysis of variance (ANOVA) and independent sample t-tests to investigate effects at a post-hoc level. A two-way multivariate analysis of covariance (MANOVA) was

conducted to evaluate the relationship between behavioural measures and the different configurations of prenatal stress. The independent variables were day of trial (from Day 1 to Day 6), and the stress treatment group (control, MPS, or TPS). The dependent variables were Number of Attempts (i.e., number of times the rat inserted its nose into the odor port), as well as Sucrose Success Rate (percent of "Go" responses when the Go Odor was given compared to the total number of Go Odor trials), Quinine Success Rate (percent of "No-Go" responses when the No-Go Odor was given compared to the total number of No-Go Odor trials), Go Success Rate (percent of appropriate "Go" responses compared to total number of "Go" responses), No-Go Success Rate (percent of appropriate "No-Go" responses compared to total number of "No-Go" responses). There were two univariate outliers in the data set for Sucrose Success Rate, two univariate outliers for Go Success Rate, and three in No-Go Success Rate; these outliers were removed from the data pool. There was no skewness in the data set.

3.4. Results

Means and standard deviations are found in Table 3.1, the results of the MANOVA are summarized in Table 3.2, and results are shown in Figure 3.1. It is notable that a significant main effect of stress was found for Number of Attempts, Quinine Success Rate, Go Success Rate, and No-Go Success Rate. Additionally, no significant main effect of trial day or stress*dye interaction effect was found. Follow-up Tukey tests were conducted to evaluate pairwise differences among the treatment groups. For Number of Attempts, TPS rats showed significantly more attempts than control or MPS rats (see Figure 3.2). For Quinine Success Rate, there were no significant differences between stress

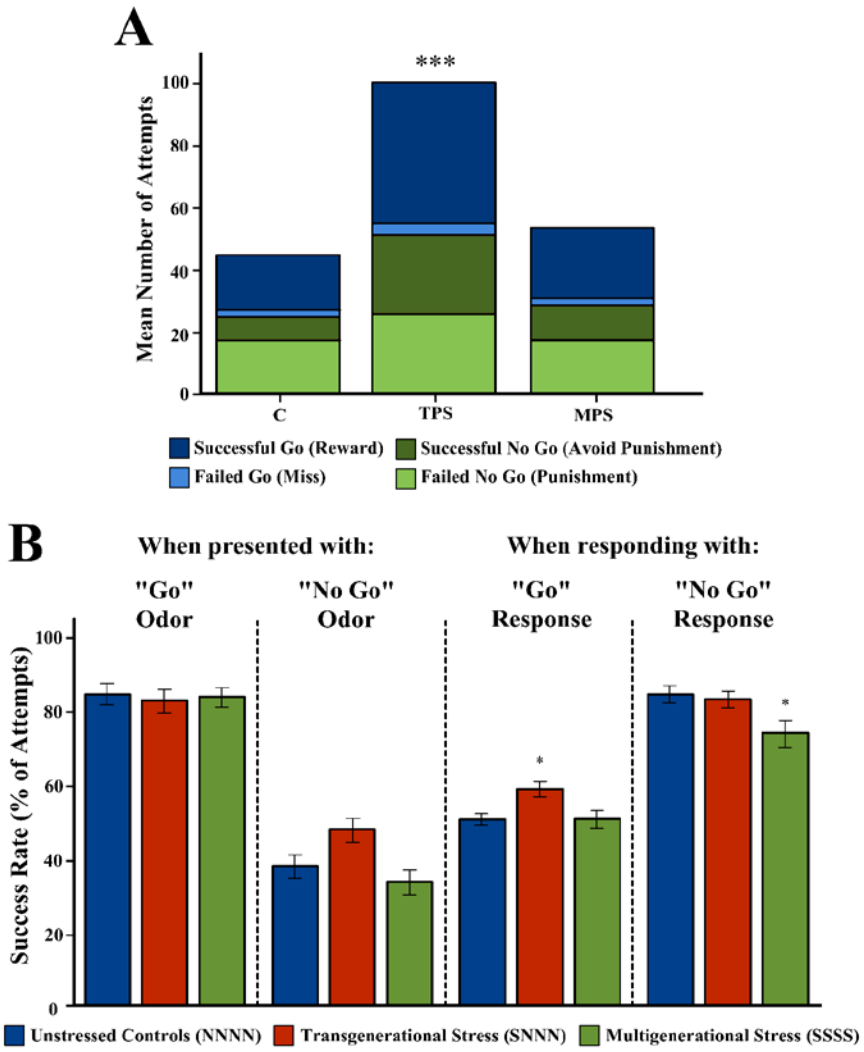

Figure 3.2 Behavioural effects of ancestral stress on learning the odor discrimination task. (A) Transgenerationally stressed rats made significantly more attempts than multigenerationally stressed rats or unstressed controls, resulting in more failed "No-Go" attempts ($p < 0.001$). (B) Transgenerational stress caused higher success rates during trials in which animals gave a "Go" response ($p < 0.05$), and multigenerational stress led to lower success rates in trials during which animals gave a "No-Go" response ($p < 0.05$).

groups, although TPS rats trended to be higher than MPS rats ($p < 0.1$, two-tailed). For Go Success Rate, TPS rats displayed significantly higher success rates than MPS rats or controls. For No-Go Success Rate, MPS rats had significantly lower success rates than controls.

3.5 Discussion

3.5.1 Ancestral Stress Alters Learning and Memory Functions

The purpose of the present study was to determine if transgenerational prenatal stress (TPS) and multigenerational prenatal stress (MPS) results in a disruption in the learning of an odor discrimination task by altering motivation and learning success rate. More specifically, we were interested in how the introduction of quinine punishment solution would affect prenatally stressed rats. Prenatal stress may result in decreased motivation and increased depression-like behaviour (Brenes et al., 2009), which has been associated with decreased performance in learning tasks (Yau et al., 2011). In the present study, TPS rats were more persistent in attempting to learn the Go/No-Go Task when the quinine punishment solution was introduced, and this persistence resulted in improved performance in learning the Go/No-Go Task. The increased Number of Attempts in TPS rats may represent less depression-like behaviour, as well as a higher number of potential learning experiences during each session. Compared to unstressed controls and MPS rats, TPS rats also had a higher Go Success Rate, i.e., when a TPS rat gave a "Go" response, it was more likely to be preceded by a "Go" odor cue. This suggests that TPS rats were more judicious about when they gave a "Go" response, and that minimal associative learning had occurred. These results are consistent with previous research that has demonstrated that increased stress resilience is associated with improved cognitive performance (Richter et al., 2012).

Additionally, MPS rats had a lower "No-Go" success rate, i.e., when MPS rats gave a "No-Go" response, it was less likely to be preceded by a "No-Go" odor cue. These results demonstrate that MPS rats inappropriately produced a "No-Go" response when presented

with an odor associated with a sucrose reward. Possible explanations may include that prenatal stress alters amygdala function (Welberg et al., 2000), which plays a role in associative learning and memory (Gruber and McDonald, 2012). Furthermore, prenatal stress has programming effects on the hippocampus, including decreased glucocorticoid receptor (GR) expression and density, resulting in deficits in learning and memory (Champagne et al., 2006; Igosheva et al., 2007; Weaver et al., 2004; Zhang et al., 2013). These results may provide evidence that epigenetic programming by ancestral stress results in cognitive deficits that effect associative encoding in odor discrimination. Additionally, the over-use of the "No-Go" response in MPS rats seems may contradict previous findings that prenatal stress results in impulsivity. Instead, these results may suggest that MPS rats show a reduction in impulsivity, while a single generation produces an increase.

3.5.2 Limitations and Reservations

In interpreting the results of this study, it is crucial to note that none of the rats were highly successful at the Go, No-Go Task. Even the TPS rats, which had significantly higher success rates, only performed at chance when presented with a No-Go Odor; the control and MPS rats gave a Go response more often that a No-Go in the same situation. Additionally, the Learning Phase normally consists of as many days as needed for the rats to successfully associate the No-Go Odor with a No-Go response and the Number of Trials to return to the frequency demonstrated at the end of the Training Phase. In the present study, however, the Learning Phase was discontinued after six days because one of two behaviours characterized a large majority of the rats: (1) the initial odor port-to-reward well behaviour had been extinguished, or (2) the rat's behaviour did not change over time, and

gave a “Go” response regardless of which odor cue had been given. In short, while there are some differences between stress groups, all groups performed poorly, particularly on the No-Go trials. It is unclear what role ancestral stress may have as a mediating factor on the subjective aversiveness of the quinine solution, and its impact on behaviour in this study.

Additionally, these results need to be replicated without the confounding factors involved in the TPS group. As mentioned, the TPS rats were exposed to additional behavioural tasks that the control and MPS did not have experience with. Exposure to learning opportunities in one domain has been shown to improve performance on subsequent learning tasks (Threlkeld et al., 2012). We cannot, therefore, rule out the possibility that exposure to prior learning experiences did not contribute to the increased Number of Attempts in the TPS group.

3.5.3 Conclusions

The results of the present study provide insight into the effects of ancestral stress on learning in adult F_4 offspring. TPS may result in increased resilience to aversive stimuli that otherwise may prevent a rat from attempting to learn a behavioural task, although it is difficult to conclude this due to the confounding factors in the current TPS group. Additionally, MPS may result in more rapid extinction of previously formed associations, as seen in the increased use of the “No-Go” response in MPS rats, although potential mediating factors such as subjective aversiveness of quinine may also play a factor.

3.6 Tables

Table 3.1 Means and Standard Deviations

			Number of Attempts		Sucrose Success Rate		Quinine Success Rate		Go Success Rate		No Go Success Rate	
Effect	**Treatment**		**M**	**SD**	**M**	**SD**	**M**	**SD**	**M**	**SD**	**M**	**SD**
Main Effect of Stress	**Control**		45.02	9.07	0.86	0.03	0.39	0.03	0.51	0.02	0.83	0.03
	SNNN		101.37	9.99	0.84	0.04	0.49	0.04	0.60	0.02	0.81	0.03
	SSSS		59.63	9.54	0.78	0.03	0.38	0.03	0.50	0.02	0.73	0.03
Main Effect of Day	**Day 1**		72.91	13.50	0.88	0.05	0.38	0.05	0.53	0.03	0.81	0.05
	Day 2		60.71	13.40	0.79	0.05	0.49	0.05	0.56	0.03	0.76	0.05
	Day 3		64.14	12.83	0.80	0.05	0.40	0.05	0.48	0.03	0.78	0.04
	Day 4		62.67	13.86	0.74	0.05	0.36	0.05	0.51	0.03	0.66	0.05
	Day 5		67.42	13.50	0.87	0.05	0.48	0.05	0.59	0.03	0.87	0.05
	Day 6		84.17	13.86	0.89	0.05	0.42	0.05	0.55	0.03	0.85	0.05
Stress*Dye Interaction	**Control**	**Day 1**	52.00	38.87	0.87	0.09	0.45	0.27	0.56	0.11	0.83	0.08
		Day 2	32.57	15.97	0.86	0.15	0.43	0.21	0.51	0.07	0.84	0.18
		Day 3	41.88	42.60	0.90	0.12	0.34	0.24	0.48	0.12	0.90	0.12
		Day 4	48.50	37.35	0.76	0.29	0.40	0.20	0.49	0.15	0.76	0.17
		Day 5	41.38	41.40	0.93	0.14	0.32	0.22	0.53	0.09	0.90	0.15
		Day 6	49.00	41.94	0.83	0.23	0.37	0.22	0.49	0.10	0.76	0.34
	SNNN	**Day 1**	104.86	101.46	0.96	0.06	0.33	0.21	0.54	0.14	0.89	0.11
		Day 2	102.57	70.19	0.72	0.26	0.52	0.18	0.58	0.14	0.73	0.20
		Day 3	102.29	82.60	0.82	0.20	0.52	0.18	0.57	0.13	0.82	0.15
		Day 4	69.67	68.93	0.77	0.28	0.42	0.28	0.59	0.12	0.67	0.36
		Day 5	92.50	82.12	0.90	0.13	0.61	0.24	0.70	0.18	0.90	0.12
		Day 6	121.17	81.14	0.90	0.11	0.47	0.14	0.59	0.13	0.86	0.15
	SSSS	**Day 1**	41.75	25.66	0.85	0.17	0.27	0.19	0.46	0.10	0.71	0.21
		Day 2	42.13	51.89	0.82	0.12	0.45	0.30	0.57	0.19	0.72	0.18
		Day 3	48.25	69.40	0.67	0.33	0.33	0.18	0.39	0.21	0.63	0.28
		Day 4	59.00	75.34	0.76	0.37	0.20	0.22	0.44	0.22	0.56	0.38
		Day 5	63.63	55.88	0.79	0.30	0.47	0.25	0.53	0.16	0.80	0.16
		Day 6	62.38	70.07	0.96	0.12	0.31	0.23	0.53	0.12	0.94	0.13

Table 3.2 Results of Multivariate Analysis of Variance

	Main Effect of Stress	**Main Effect of Day**	**Stress*Day Interaction**
Number of Attempts	***$F(2,108) = 9.09$ $p < 0.001$**	$F(5,108) = 0.39$ $p = 0.86$	$F(10,108) = 0.27$ $p = 0.99$
Sucrose Success Rate	$F(2,108) = 0.362$ $p = 0.70$	$F(5,108) = 1.14$ $p = 0.34$	$F(10,108) = 0.68$ $p = 0.74$
Quinine Success Rate	***$F(2,108) = 3.14$ $p < 0.05$**	$F(5,108) = 0.94$ $p = 0.46$	$F(10,108) = 0.69$ $p = 0.74$
Go Success Rate	***$F(2,108) = 4.10$ $p < 0.05$**	$F(5,108) = 1.20$ $p = 0.31$	$F(10,108) = 0.67$ $p = 0.75$
No Go Success Rate	***$F(2,108) = 3.88$ $p < 0.05$**	$F(5,108) = 2.03$ $p = 0.08$	$F(10,108) = 0.89$ $p = 0.54$

CHAPTER 4

Behavioural Effects of Ancestral Stress and Environmental Enrichment on Adolescent and Adult Male Rats

4.1 Abstract

Early-life experiences have a significant impact on behaviour. In this study, the effects of transgenerational prenatal stress (TPS), multigenerational prenatal stress (MPS), and interactions with the exposure to environmental enrichment (EE) are investigated. TPS and MPS treatment involved a family history of prenatal stress. EE treatment included being housed in a large arena with seven other cage mates, and being provided with frequent novel toys and foods for rich social, sensory and motor stimulation. Using a number of behavioural tasks, we assessed locomotor activity, anxiety and depression-like behaviour, learning and memory, and social aggression and dominance. TPS and MPS resulted in distinct phenotypes, with MPS appearing to be more adaptive to survival in a stressful environment. In animals with ancestral stress, EE decreased locomotor activity, anxiety- and depression-like behaviour, learning and memory deficits, and attenuated social aggression and dominance. These findings suggest that behavioural change is mediated by the interaction of environmental factors both before and after birth.

4.2 Introduction

The development of brain and behaviour are significantly impacted by pre- and post-natal environmental factors. Ancestral prenatal stress exposes the developing fetal brain to detrimental levels of glucocorticoids (GCs) (Harris and Seckl, 2011). Excessive levels of GCs during brain development may result in an increased risk of a host of long-lasting behavioural pathologies, including altered cognition, social behaviour and affective state, and motor hyperactivity later in life (Cory-Slechta et al., 2012; de Souza et al., 2013; Holmes et al., 2006; O'Connor et al., 2003; Van Den Bergh and Marcoen, 2004). While the specific effects of prenatal stress within a single generation are well understood, the effects of prenatal stress across multiple generations are a relatively novel and emerging field of research. Previous studies, including the two preliminary studies outlined in Chapters 2 and 3 of this thesis, have suggested that behavioural effects of prenatal stress may be transmitted beyond a single prenatally stressed generation (Erickson et al., 2014; Korosi and Baram, 2009; Ward et al., 2013; Zucchi, Yao, and Metz, 2012). Additionally, ancestral prenatal stress may exacerbate the vulnerability and response to an adverse environment on behaviour in future generations (Matthews and Phillips, 2010).

In addition to ancestral prenatal stress, the postnatal environment is extremely influential in the development (or alleviation) of behaviour and potential pathological consequences of altered stress response (Simpson and Kelly, 2009). Environmental enrichment (EE), within the context of experimental animal research, is defined as the introduction of additional physical and/or social stimuli beyond normative animal housing (Rosenzweig and Bennett, 1996). The beneficial effects of EE on developmental outcomes

are well established (Comeau et al., 2008; Simpson and Kelly, 2011). For example, EE has been shown to decrease anxiety-like behaviours in an elevated plus maze (Galani et al., 2007; Pena et al., 2006; Pena et al., 2009), decrease depression-like behaviour in a forced swim task (Brenes et al., 2009), and improve learning and memory functions in tasks like the Morris water maze (Leggio et al., 2005; Zhong et al., 2009) and the object recognition task (Bruel-Jungerman et al., 2005; Pamplona et al., 2009). Considering the impact of prenatal stress on these functions, EE may ameliorate the adverse consequences of early adverse experience (Zhang et al., 2012), although cases to the contrary have been published (Emack and Matthews, 2011).

While both transgenerational prenatal stress (TPS; i.e., a single generation of prenatal stress, followed by multiple generations of unstressed gestations) and multigenerational prenatal stress (MPS; i.e., multiple consecutive generations of prenatal stress) have been studied individually in the past (Erickson et al., 2014; Ward et al., 2013; Zucchi, Yao, and Metz, 2012), there have been no studies comparing both ancestral stress configurations within the F_3 generation. In a model of transgenerational inheritance, the female F_3 generation is the first which germline has not been directly exposed to the F_0 maternal stress (Skinner, 2008). Therefore, any behavioural effects in the F_3 generation can be assumed to be of epigenetic origin (Zucchi, Yao, and Metz, 2012). Furthermore, there have been no studies investigating the impact EE on the behavioural development of rats with an ancestral history of prenatal stress.

The purpose of this study was to examine if ancestral prenatal stress exposure in either a single generation or multiple generations will induce behavioural changes in the F_3 generation, and if EE will be able to attenuate the consequences of programming by

ancestral stress in the F_3 generation. We hypothesized (1) that TPS and MPS rats would alter behaviour and the stress response; and (2) that EE would result in an improved, more adaptive behavioural phenotype. The findings will provide insights if EE is particularly important to offset the effects of stress in developmentally compromised offspring.

4.3 Methods

4.3.1 Animals, Treatments, and Timeline

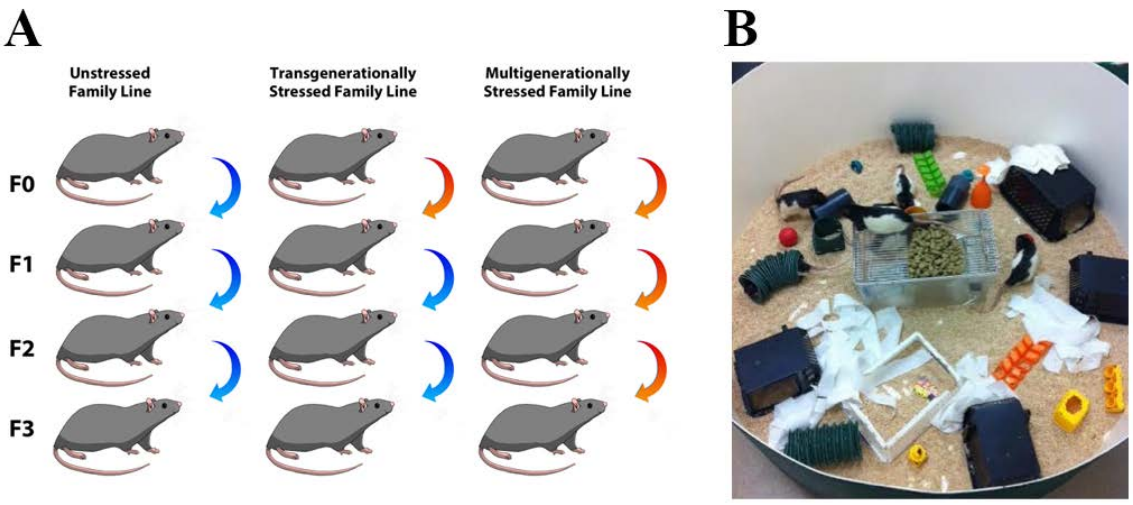

Figure 4.1 Experimental treatments. The present study used male rats in a two-by-three factorial design. The independent variables were stress treatment (unstressed, transgenerational prenatal stress (TPS) and multigenerational prenatal stress (MPS)) and environmental enrichment (EE).***(A)*** The TPS rats were the third generation (F_3) of an ancestral line in which dams in the F_0 were given stress during pregnancy, and MPS rats were the F_3 of an ancestral line in which dams in each generation were given stress during pregnancy. ***(B)*** All rats were housed in either standard shoebox housing (n = 2 per cage), or in large empty Morris water maze pools that were filled with a variety of toys, shelters, and novel foods (n = 8 per cage).

Figure 4.1 outlines the experimental design for the current study. Forty-eight Long Evans male rats were taken from one of three stress treatments: controls (n = 16), transgenerational prenatal stress (TPS; n = 16), and multigenerational prenatal stress (MPS; n = 16). TPS rats were the F_3 generation of a filial line in which only the F_1 generation was

stressed prenatally (i.e., an SNN filial line). MPS rats were the F_3 generation of a filial line in which each consecutive generation was prenatally stressed (i.e., an SSS filial line). Pregnant dams were stressed using a social isolation stressor, which has been shown to result in mild psychosocial stress in rats (Hawkley et al., 2012). Each dam was housed alone and did not experience any direct contact with any other rats from P 90 until her offspring were weaned. Control rats were housed in pairs until gestational day 21.

In addition to prenatal stress, half of each stress group was randomly assigned to one of two environmental conditions: standard housing or enriched environment (EE). In the standard housing condition rats were housed in non-sibling pairs in a standard shoebox housing unit. From post-natal (P) day 21 to P 35, the EE rats were housed in groups of four living in a standard housing unit. At P 35, each group of four EE rats was moved to large circular condominiums (i.e., empty water maze pool, [DIMENSIONS]). In addition to the increased social interactions and living space, the EE was filled with multiple shelters and enrichment toys. In addition to standard rat chow, EE rats were regularly provided with novel types of foods, including raw pasta, non-sweetened breakfast cereal, and seeds. In total, three identically arranged EE were used in the study, each housing all of the rats from a single stress treatment group.

Body weight was regularly recorded as the rats aged. During adolescence (P 21 to P 60), rats were assessed in the open field, elevated plus maze, and object recognition tasks. In adulthood starting from P 100, the rats were tested in the Morris water maze and water competition task in addition to open field, elevated plus maze, and object recognition tasks. The stress response phase, which began approximately 20 weeks post-partum, consisted of a 14-day period in which the rats were exposed daily to 20 minutes of restraint stress.

Corticosterone levels were assessed the day before the stress began (pre-stress time point), day 1 of stress (acute stress time point), and day 14 of stress (chronic stress time point). Additionally, animals were tested in the open field task and elevated plus maze on days 2 and 13 of stress exposure, and the forced swim task on days 3 and 12 of stress exposure. All behavioural tests were initiated within 10 minutes following to stress.

4.3.2 Behavioural Assessments

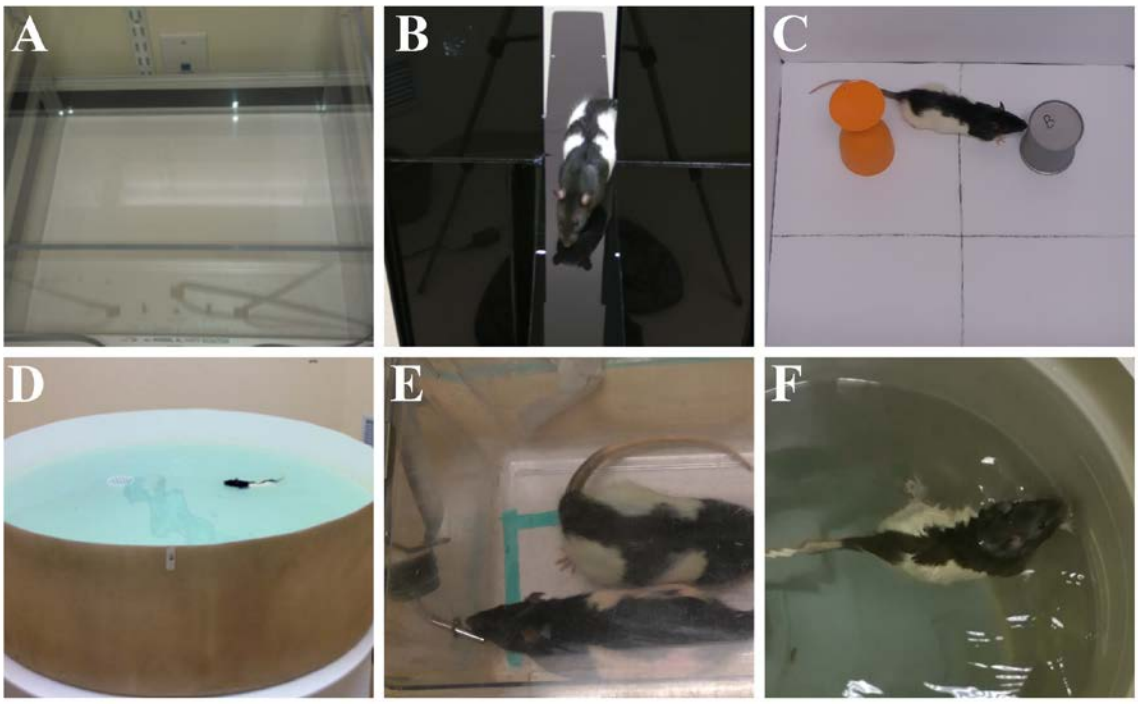

Figure 4.2 Behavioural tasks. (A) The open field task was used to assess locomotor and anxiety-related behaviour; (B) The elevated plus maze was used to assess locomotor and anxiety-related behaviour; (C) the object recognition task was used to assess memory; (D) the Morris water maze was used to assess learning and memory; (E) the water competition task was used to assess social aggression and cooperation; and (F) the forced swim task was used to assess depression-like behaviour.

4.3.2.1 Open Field Task

The open field task, shown in Figure 4.2A, allows the quantification of motor activity, anxiety-like behaviours, and exploration in an open arena (Jadavji et al., 2011; Smith et al., 2008). In this study, the open field task was conducted using the VersaMax

Legacy Open Field activity box (Omnitech Electronics, Inc., Dartmouth, NS, Canada), which measured an animal's activity for a period of 10 minutes using an array of infrared sensors connected to a computer. Behavioural measures included total time spent moving during the test interval (*Movement Time*), the amount of time spent within the margins of the open field (*Margin Time*), and time spent rearing (*Vertical Time*). Unfortunately, due to a malfunction of the vertical sensors, *Vertical Time* data was not collected during the Acute and Chronic Stress Phases.

4.3.2.2. Elevated Plus Maze

The elevated plus maze, shown in Figure 4.2B, allows the quantification of motor activity and anxiety-like behaviours (Lister, 1987). In this study, the elevated plus maze was conducting use an opaque black Plexiglas maze suspended 50 cm from of the ground. Two arms (50 cm x 10 cm) were enclosed by Plexiglas walls 40 cm high ("*closed arms*"), and two identically sized arms without walls ("*open arms*"). Individual rats were placed in the center of the maze and allowed to explore freely for a period of five minutes. Behavioural measures included *total time* spent *i*n the closed arms, open arms and end of open arms; *number of entries* into closed arms, open arms, and end of open arms; and *latency to enter* the closed arms.

4.3.2.3. Object Recognition Task

The object recognition task, shown in Figure 4.2C, allows the quantification of declarative memory (Bruel-Jungerman et al., 2005; Pamplona et al., 2009). In this study, the object recognition task was conducted in three phases. In Phase 1, rats were placed

individually into the testing box (a 60 cm x 60 cm x 60 cm) made of corrugated plastic) for 10 minutes to habituate to the testing environment. After 24 hours, Phase 2 was conducted by placing each rat into the testing environment with two identical objects for a period of four minutes. After 30 minutes, Phase 3 was conducted by placing each rat into the testing environment for a period of four minutes with two objects: one familiar object, which was identical to the objects used in Phase 2, and one novel object, which the rat had not been exposed to. Phase 3 was video recorded and scored at a later time.

Behavioural measures included time investigating the familiar and novel objects, time interacting with (but not investigating) the familiar and novel objects, and time spent in each quadrant. Analysis was conducted for four variables per rat: time spent with the novel object (*Novel Object Time*), the total time spent with both objects (*Total Object Time*), the percentage of time spent investigating the novel object when compared to the total time investigating both objects (*Investigation Novel Percentage*), and the percentage of time that the rat spent interacting (i.e., investigation and interacting combined) with the novel object when compared to the total time interacting with both objects (*Total Novel Percentage*).

4.3.2.4. Morris Water Maze

The Morris water maze, shown in Figure 4.2D, allows the quantification of learning, memory, and motor activity (Leggio et al., 2005; Zhong et al., 2009). In this study, the Morris water maze protocol was conducted over the course of nine days using a pool filled with room temperature water. The water was made opaque by adding non-toxic white tempura paint. Additionally, visual cues were placed on the walls to further assist the rat in

spatial orientation. Rat movement was tracked using a video camera connected to a computer, which recorded performance for later analysis.

Days 1, 3, 5, and 7 were considered *Learning Days*; days 2, 4, 6, and 8 were considered *Memory Days*; and on day 9 a probe session was conducted. On *Learning Days*, a submerged platform was placed into one of four quadrants, and the rat was given eight attempts to find the platform within 60 seconds (two attempts beginning at south, north, east, and west). If the rat did not find the platform within 60 seconds, the experimenter would assist the rat to the platform, where it was given 10 seconds to familiarize itself with the location. On *Memory Days*, the platform was placed in the same location as it was on the previous day, and the rat was given eight attempts to find the platform. On day 9, the platform was not placed in the pool, and the rat swam freely until it was removed from the pool after 30 seconds. Behavioural measures in this task included the *time to reach the platform*, *swim speed*, and *distance travelled* before reaching the platform.

4.3.2.5 Water Competition Task

The water competition task, shown in Figure 4.2E, allows the quantification of social navigation and aggression. The task used in the present study was a modified version of the task outlined by Lucion and Vogel (1994), in which rats competed for access to a single water spout after being given water restriction for 24 hours. Unlike previously published studies using the water competition task, no alterations were made to the water spout to force animals to drink one at a time. The reason for this was to assess the likelihood of a rat to share access to the spout.

Testing was divided into two days to test for the effect of stress and environment independently. To test the effect of environment, rats were paired within stress groups against differing environmental treatments (e.g., standard housing TPS rats were paired with enriched TPS rats). To test the effect of stress, rats were placed in groups of three within enrichment groups (e.g., one enriched control, one enriched TPS, and one enriched MPS were grouped together). Rats were ranked by body weight within-groups, and were paired with other rats of similar ranking. It should be noted that weight between groups was not controlled for in this task. The task was video recorded and scored at a later time.

Video scoring was divided into two separate components: time spent drinking from spout (*Drinking Time*) and competitive interactions with other rats (*Social Interaction*). Drinking Time was further divided into *Alone Time* at the spout vs. *Group Time*. During *Group Time,* the position of the rat was also recorded, whether in the dominant position (in a normal drinking position, with its left side against the side of the cage) or submissive (drinking either from the side or beneath the spout). Social Interaction consisted of three different actions performed by the rats: *Push with Paw*, in which the rat used its forelimbs to push an intruding rat away from the spout; *Push with Head or Body*, in which the rat attempted to displace a rat using its head or shoulders; and *Pouncing*, in which the rat jumped on top of another rat in an attempt to gain access to the spout. Behavioural measurements used in the analysis included *Drinking Time*, *Alone Drinking Time* (expressed as a percentage of total drinking time), *Group Dominance* (expressed as a percentage of time drinking with other rats), and total number of *Social Interactions*.

4.3.2.6 Forced Swim Task

The Forced Swim Task, shown in Figure 4.2F, allows the quantification of anxiety and depression-like behaviour (Brenes et al., 2009). In this task, the rats were forced to swim in a barrel of room-temperature water for four minutes. The task was video recorded and scored at a later time. Behavioural measures included *time spent swimming*, *time spent passively floating* in the water, and *number of dives* underwater.

4.3.3 Statistical Analysis

All data analysis was conducted using SPSS version 20 software for Mac (IBM Corporation, Armonk, New York, USA). All behavioural measures, except for the water competition task, were analyzed on a per-variable basis using a mixed-design analysis of variance (ANOVA). The between-group independent variables included stress treatment (unstressed controls, TPS, and MPS) and environmental enrichment (standard housing and enriched rats). The within-group variable was time. In cases where a behavioural task was conducted across multiple phases of the experiment, a separate analysis was conducted for Maturation and Stress Response Phases. For the sake of analysis, the Adulthood Phase of the experiment was considered to be both the final stage of the Maturation Phase, as well as the pre-stress time point of the Stress Response Phase. In cases where Machley's test for sphericity was significant, the Geisser-Greenhouse correction was used. Post-hoc comparisons were done using Tukey HSD for between-enrichment group differences in stress groups, and independent sample t-tests for between-stress group differences for enrichment. Additionally, non-parametric correlation was conducted using Kendall Tau B test to determine the relationship between the dependent variables; Pearson's r was not

used because several variables in the data set were skewed when not controlling for stress and enrichment conditions.

The water competition task data were analyzed using a multivariate analysis of variance (MANOVA) for each session separately (Two-Rat Session vs. Three-Rat Session), with stress and enrichment being the two independent variables. Also, a Pearson's correlation was conducted to test the hypothesis that total drinking time was negatively correlated with the rate at which the rat shared access to a common water source. It should be noted that the interpretation of the data was different for each session. When interpreting the main effects for the two-rat, within-stress session, the effect of enrichment was considered to be representative of the behaviour of animals on an individual level, whereas the effect of stress was only considered at the group level. Similarly, during the three-rat, within-enrichment session, the effect of stress was considered to be representative of individual behaviour, whereas the effect of enrichment was considered only at the group level.

4.4 Results

4.4.1 Body Mass

A mixed design ANOVA was used to determine whether stress or enrichment influenced body mass over time. The between-group factors were Stress Treatment (control, SNN, and SSS) and Enrichment (Standard, Enriched Housing), and the within-group factor was Age (P 21, P 35, P 60, P 90, P 120, P 140, P 160, and P 180). There was no skewness, kurtosis, or missing values in the data set. The average body weights (in grams) are listed in Table 4.1.1.

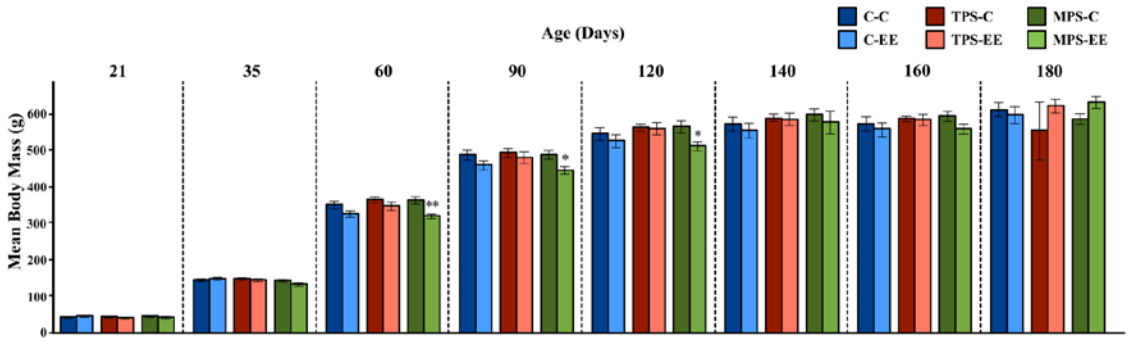

Figure 4.3. A lifespan profile of the effect of ancestral stress and environmental enrichment on body mass. At 60, 90, and 120 days old, enriched MPS rats weighed significantly less than standard housing MPS rats. Significance markers: * $p < 0.05$, ** $p < 0.01$

Table 4.1.2 displays the main effects and interaction effects, and results can be seen in Figure 4.3. As can be seen, the only significant main effect was that of Age, which had a partial η^2 of 0.99. Additionally, there was a significant Age*Enrichment interaction ($F(3.18,130.17) = 3.06$, $p < 0.05$), and further pairwise comparisons revealed that enriched MPS rats weighed significantly less than standard housed MPS rats at P 60, P 90, and P 120 ($p < 0.01$, 0.05, and 0.05, respectively).

4.4.2 Open Field Task

Means and standard deviations for each dependent variable are found in Table 4.2.1, and results are shown in Figure 4.4. During the Maturation Phase (see Table 4.2.2), a statistically significant main effect of Enrichment was found in all variables, indicating that Enriched rats had decreased Movement ($F(1,42) = 19.45$, $p < 0.001$) and Vertical Time ($F(1,42) = 18.45$, $p < 0.001$) and increased Margin Time ($F(1,42) = 15.25$, $p < 0.001$). The statistically significant effect of Age indicates an age-related increase in Movement Time ($F(2,84) = 15.28$, $p < 0.001$) and Vertical Time($F(2,84) = 48.22$, $p < 0.001$), and an age-related decreased Margin Time ($F(2,84) = 48.06$, $p < 0.001$). The statistically significant

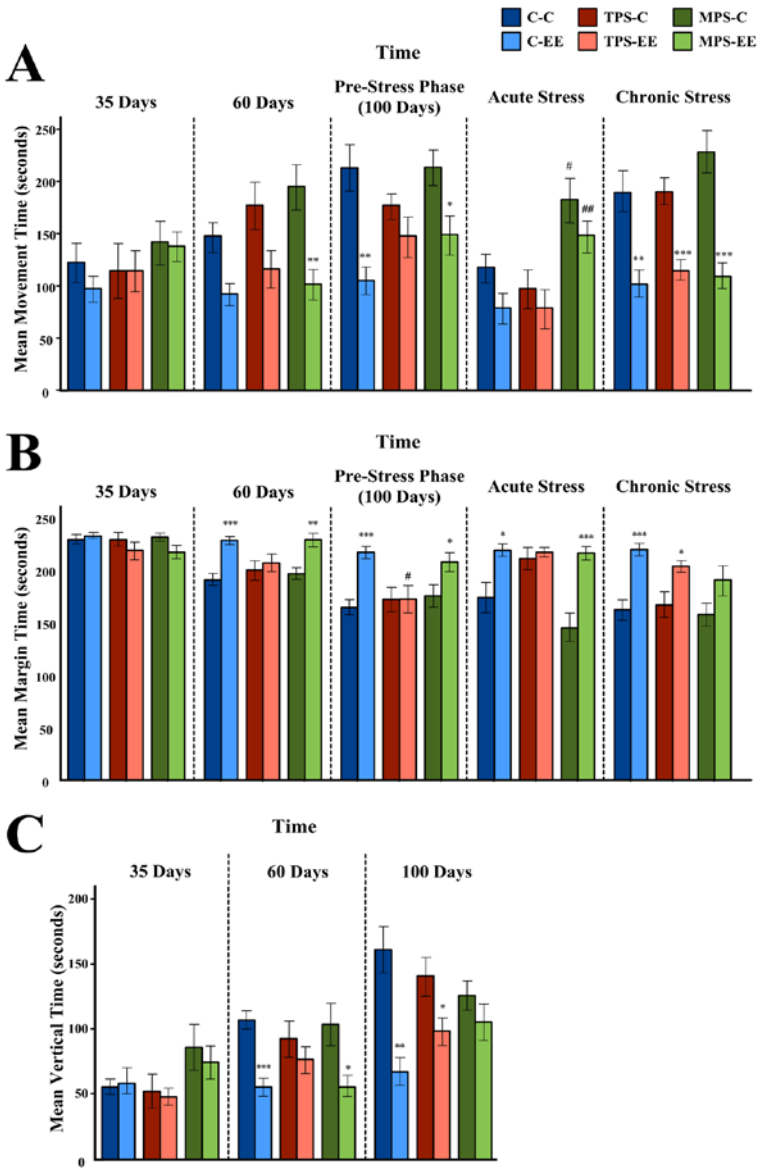

Figure 4.4. A profile of the effect of ancestral stress and environmental enrichment on performance in the open field task. (A) Movement Time, (B) Margin Time, and (C) Vertical Time. Significance markers: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, Enriched rats compared to Standard Housing rats, within-stress treatment; : # $p < 0.05$, ## $p < 0.01$, Stressed rats (either TPS or MPS) compared to unstressed rats, within-enrichment treatment.

interaction between Age*Environment indicates that Enriched rats showed a greater age-related increase in Movement ($F(2,84) = 8.11$, $p < 0.001$) and Vertical Time ($F(2,84) = 9.70$, $p < 0.001$), as well as a greater age-related decrease in Margin Time ($F(2,84) =$

10.68, $p < 0.001$). Additional post-hoc comparisons at showed that enriched unstressed and MPS rats showed decreased activity in all behavioural measures at P 60 and P 100 (see Figure 4.5).

During the Stress Response Test Phase (see Table 4.2.3), the statistically significant main effect of Enrichment indicates that overall Enriched rats had decreased Movement ($F(1,42) = 35.35$, $p < 0.001$) and increased Margin Time ($F(1,42) = 34.68$, $p < 0.001$). Additional pairwise comparisons at the each phase indicated that enrichment resulted in increased Margin Time at all phases for unstressed rats, during pre-stress and acute stress time points for MPS, and at the chronic stress time point for TPS rats (see Figure 4.5). Enriched TPS rats showed significantly lower Margin Time than unstressed enriched rats during the pre-stress phase. Additionally, Enrichment resulted in decreased Movement Time in unstressed and MPS rats during the pre-stress time point, and a decrease in all three stress groups during the chronic stress phase.

4.4.3 Elevated Plus Maze

Means and standard deviations for each dependent variable are summarized in Table 4.3.1, and results are shown in Figure 4.5. During the Maturation Phase (see Table 4.3.2), the Time Spent in the open arms showed a significant main effect of Age, with older rats spending more time in the open arms than younger rats ($F(1.49,62.58) = 4.60$, $p < 0.05$). Additionally, a significant main effect of Enrichment in the number of closed arm entries indicates that enriched rats had less entries into the closed arms than standard housing rats ($F(1,42) = 7.12$, $p < 0.05$). Further pairwise comparisons revealed that, at P 35, Enrichment resulted in an increase in time spent in open arms in MPS rats ($p < 0.05$);

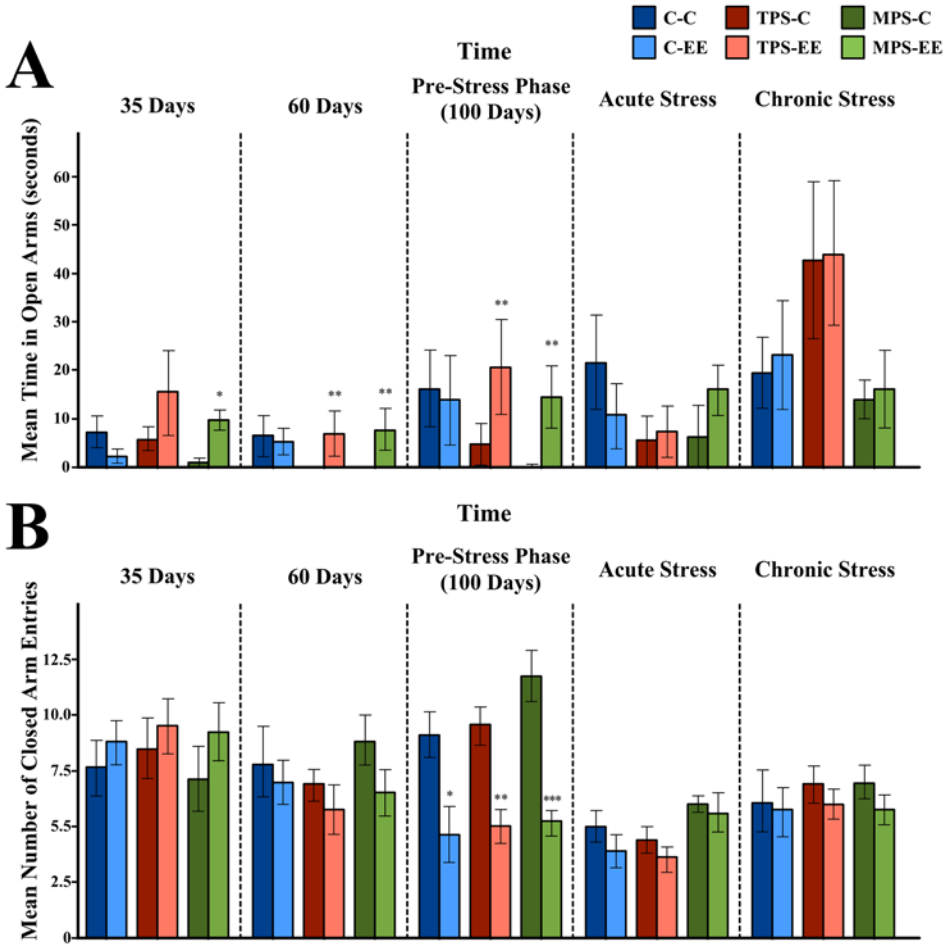

Figure 4.5. A profile of the effect of ancestral stress and environmental enrichment on performance in the elevated plus maze. (A) Time in open arms, (B) Number of Closed Arm Entries. Significance markers: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, Enriched rats compared to Standard Housing rats, within-stress treatment.

at P 60 Enrichment resulted in an increase in time in the open arms in TPS and MPS rats ($p < 0.01$); and, at P 100, Enriched TPS and MPS rats spent more time in the open arms ($p < 0.01$). Analysis for Stress Response Phase showed no significant effects of stress or enrichment (Table 4.3.3). However, there was a significant Age*Stress interaction effect on the time in open arms ($F(3.18,66.72) = 3.91$, $p < 0.05$) and Age*Enrichment interaction effects on the number of closed arm entries ($F(1.64,68.93) = 17.53$, $p < 0.001$). Follow-up

pairwise tests showed that TPS rats had the largest increase in Time Spent in the Open Arms between the Acute and Chronic Stress phases ($p < 0.01$).

4.4.4 Object Recognition Task

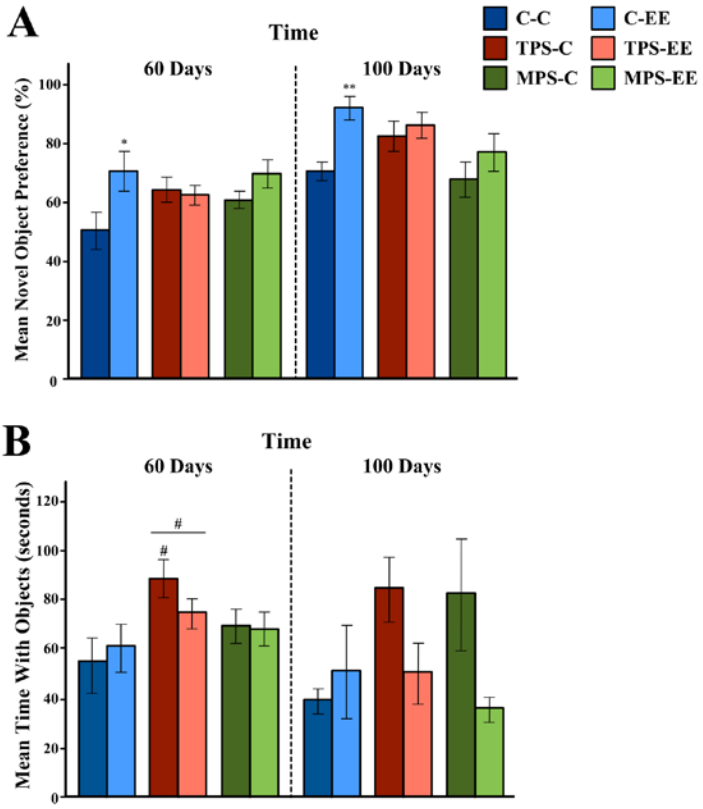

Figure 4.6. A profile of the effect of ancestral stress and environmental enrichment on performance in the object recognition task. (A) Novel Object Preference, (B) Total Object Time. Significance markers: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, Enriched rats compared to Standard Housing rats; : # $p < 0.05$, ## $p < 0.01$, Stressed rats (either TPS or MPS) compared to unstressed rats.

Means and standard deviations for each dependent variable are summarized in Table 4.4.1, main and interaction effects can be found in Table 4.4.2, and results are shown in Figure 4.6. A significant main effect of enrichment on Novel Object Preference ($F(1,84) = 13.16$, $p < 0.001$) indicates that enriched rats spent a higher percentage of time investigating the novel object instead of the familiar object. A significant main effect of stress on Total Object Time was also found ($F(2,84) = 3.96$, $p < 0.05$). Further pairwise

comparisons revealed that TPS rats showed a higher Total Object Time when compared to unstressed controls at P 60. Enrichment increased Novel Object Preference in unstressed rats at both P 60 ($p < 0.05$) and P 100 ($p < 0.01$), but not in TPS or MPS rats.

4.4.5 Morris Water Maze

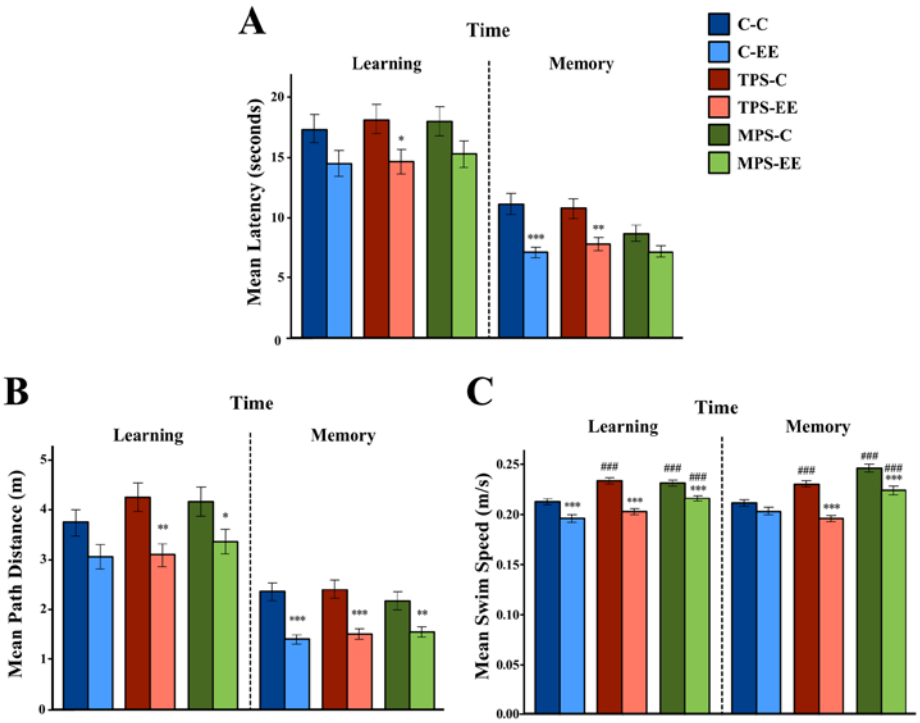

Figure 4.7. A profile of the effect of ancestral stress and environmental enrichment on performance in the Morris water maze. (A) Latency to reach the platform, (B) Path distance, and (C) Swimming speed. Significance markers: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, Enriched rats compared to Standard Housing rats, within-stress treatment; : # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$, Stressed rats (either TPS or MPS) compared to unstressed rats, within-enrichment treatment.

The within-group variable was task type (Memory versus Learning Days) instead of Time. Means and standard deviations for each dependent variable are summarized in Table 4.5.1, main and interaction effects can be found in Table 4.5.2, and results are shown in Figure 4.7. A significant main effect of enrichment on each of the variables indicates that enriched rats showed lower Latency to reach the platform ($F(1,3061) = 34.94$, $p < 0.001$), shorter Path Distance ($F(1,3061) = 60.78$, $p < 0.001$), and slower Swim Speed

($F(1,3050) = 144.90$, $p < 0.001$). There was a significant effect of stress on Swim Speed ($F(2,3050) = 50.78$, $p < 0.001$), and a significant effect of Task Type on Latency ($F(1,3061) = 240.60$, $p < 0.001$) and Path Distance ($F(1,3061) = 221.16$, $p < 0.001$). Additionally, there were significant interaction effects of Task Type*Stress and Stress*Enrichment on Swim Speed ($F(2,3050) = 4.99$, $p < 0.01$; $F(2,3050) = 10.20$, $p < 0.001$, respectively). Further pairwise comparisons revealed that Enrichment reduced latency in TPS rats on both learning ($p < 0.05$) and memory days ($p < 0.01$) and in unstressed rats on memory days ($p < 0.001$). Enrichment reduced Path Distance in all stress groups during memory days, and in TPS ($p < 0.01$) and MPS ($p < 0.05$) rats during learning days. Enrichment reduced Swim Speed in all stress groups during learning days ($p < 0.001$), and in TPS and MPS during memory days ($p < 0.001$). There was also a significant difference between unenriched unstressed controls and unenriched TPS ($p < 0.001$) and MPS rats ($p < 0.001$) during both learning and memory days.

4.4.6 Water Competition Task

Means and standard deviations for each dependent variable are found in Table 4.6.1, main and interaction effects can be found in Table 4.6.2, and results are shown in Figure 4.8. During the two-rat, within-stress session, there was a significant effect of Enrichment on Group Dominance ($F(1,42) = 30.04$; $p < 0.001$), which demonstrated that enriched rats spent less time in the dominant position while drinking with other rats. Additionally, there was a main effect of stress on Spout Time ($F(2,42) = 6.22$; $p < 0.01$) and Alone Spout Time Percentage ($F(2,42) = 3.67$; $p < 0.05$), and further Tukey comparisons revealed that TPS ($p < 0.05$) and MPS ($p < 0.01$) sessions had higher Spout

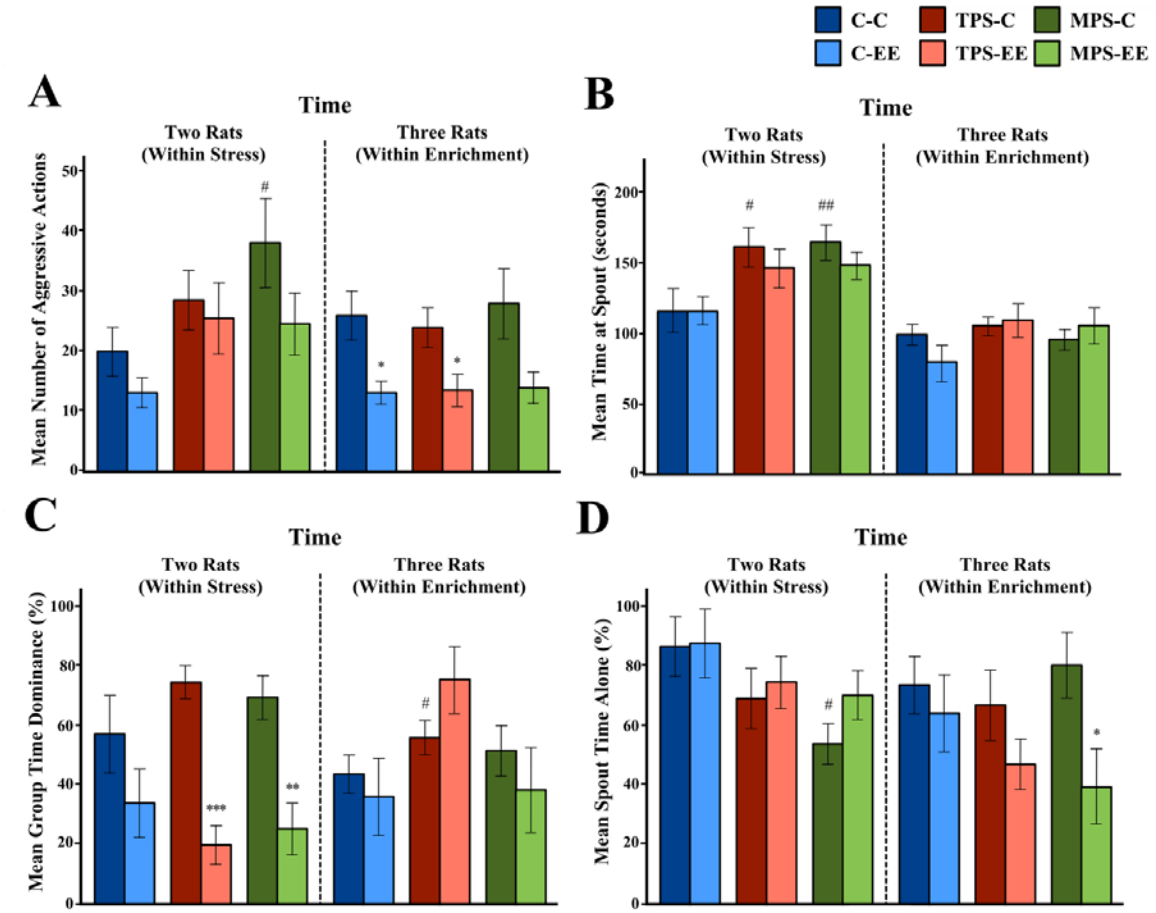

Figure 4.8. A profile of the effect of ancestral stress and environmental enrichment on performance in the water competition task. (A) Number of social interactions, (B) Time at spout, (C), Portion of time holding the dominant position while drinking from spout with two or more rats (percent), and (D) Portion of time that the rat was drinking from the spout alone (percent), (E) Alone Time at Spout vs Spout Time Scatter Plot. Significance markers: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, Enriched rats compared to Standard Housing rats, within-stress treatment; : # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$, Stressed rats (either TPS or MPS) compared to unstressed rats, within-enrichment treatment. (E) Scatterplot/correlation of Spout Time x Along Spout Time Percentage.

Time than unstressed sessions, and MPS rat sessions had lower Alone Spout Time Percentage than unstressed sessions ($p < 0.05$). During the three-rat, within-enrichment sessions, there was a significant main effect of stress on Group Dominance ($F(2,42) = 3.59 = p < 0.05$), and follow-up Tukey comparisons found that unenriched TPS rats had a higher Group Dominance than unenriched unstressed rats ($p < 0.05$). There was also a significant

main effect of Enrichment on Social Interactions ($F(1,42) = 15.90$; $p < 0.001$) and Alone Spout Time Percentage ($F(1,42) = 8.98$; $p < 0.01$), demonstrating that enriched sessions contained less Social Interactions and a lower percentage of alone time during drinking when compared to unenriched rats.

Additionally, the Pearson's r correlation between Drinking Time and Alone Spout Time Percentage showed a significant negative correlation ($r = -0.256$, $p < 0.05$, $n = 96$). The relationship was even stronger when conducting the analysis separately on the two-rat ($r = -0.46$, $p < 0.01$, $n = 48$) and three-rat ($r = -0.43$, $p < 0.01$, $n = 48$) sessions. These results indicate that as rats spent more of their time sharing access to the spout, their total time drinking from the spout also increased. There was no correlation between Drinking Time and Group Dominance ($r = -0.12$, $n = 0.41$, $n = 96$).

4.4.7 Forced Swim Task

Means and standard deviations for each dependent variable are shown in Table 4.7.1, main and interaction effects can be found in Table 4.7.2, and results are shown in Figure 4.9. A significant main effect of Enrichment on Floating Time ($F(1,84) = 15.03$, $p < 0.001$) and Swimming Time ($F(1,84)=11.28$, $p < 0.01$) indicated that enriched rats had a lower Floating Time and a higher Swimming Time. There was a significant main effect of stress on Number of Dives ($F(2,84) = 3.76$, $p < 0.05$), and a significant main effect of Time on Floating Time ($F(1,84) = 31.17$, $p < 0.001$), Number of Dives ($F(1,84) = 4.79$, $p < 0.05$), and Swimming Time ($F(1,84) = 24.69$, $p < 0.001$). There was also a significant Enrichment*Time interaction effect on Floating Time ($F(1,84) = 7.85$, $p < 0.01$) and Swimming Time ($F(1,84) = 4.47$, $p < 0.05$). Additional pairwise comparisons revealed

that Enrichment reduced Floating Time in TPS rats at the chronic stress time point ($p < 0.01$).

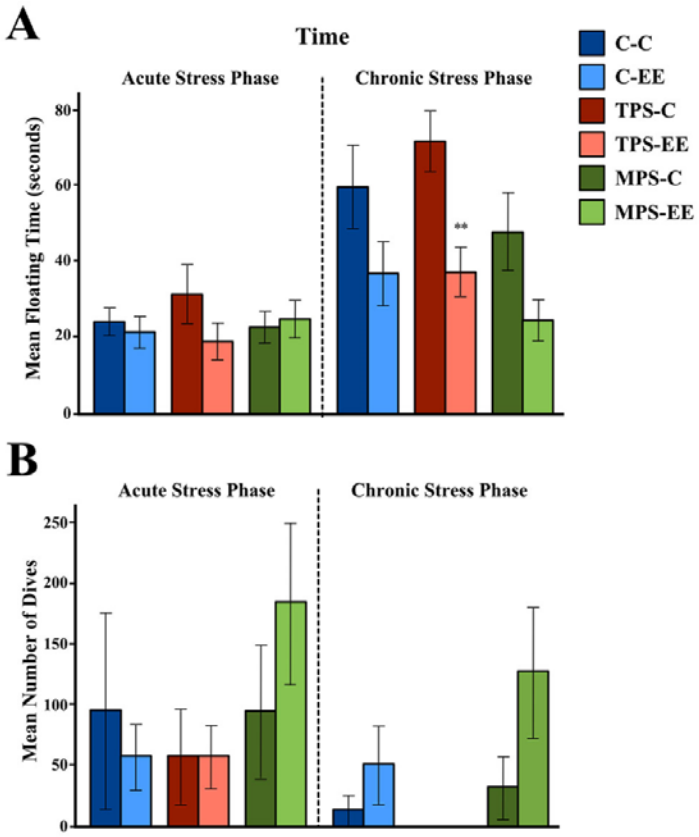

Figure 4.9. A profile of the effect of ancestral stress and environmental enrichment on performance in the forced swim task. (A) Floating Time, (B) Number of Dives, Significance markers: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, Enriched rats compared to Standard Housing rats, within-stress treatment; : # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$, Stressed rats (either TPS or MPS) compared to unstressed rats, within-enrichment treatment.

4.4.8 Circulating Corticosterone Levels

Means and standard deviations for circulating corticosterone can be found in Table 4.8.1, main and interaction effects can be found in Table 4.8.2, and results are shown in Figure 4.9. A significant main effect of Enrichment on CORT ($F(1,42) = 16.16$, $p < 0.001$) indicates that enriched rats had lower levels of circulating CORT than unenriched rats. A

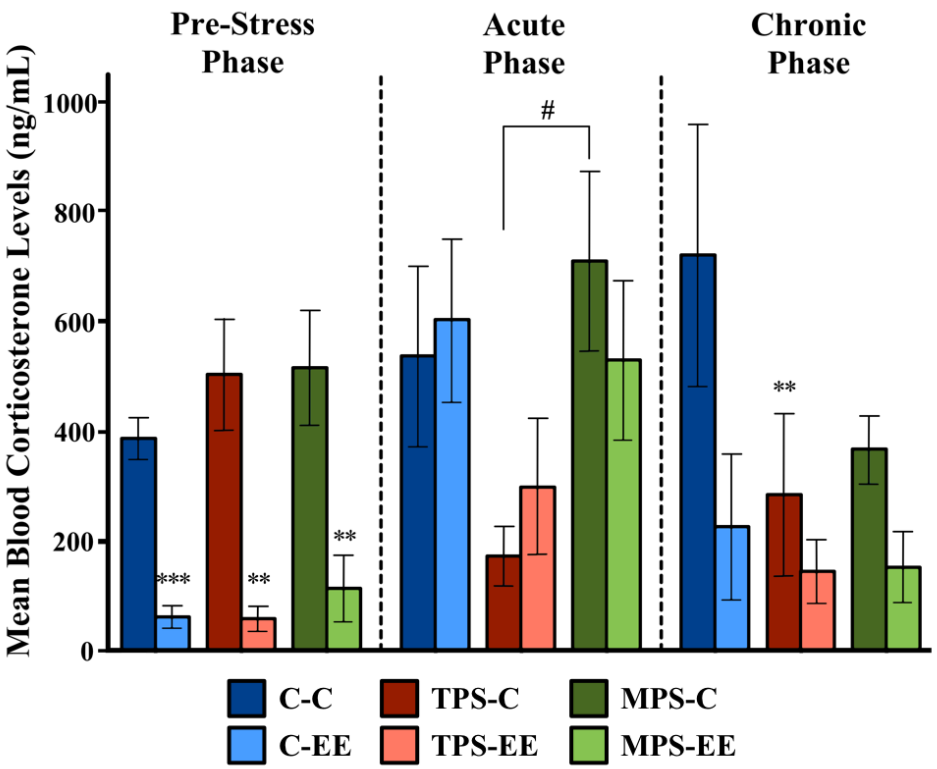

Figure 4.10. A profile of the effect of ancestral stress and environmental enrichment on circulating corticosterone. Significance markers: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, Enriched rats compared to Standard Housing rats, within-stress treatment; : # $p < 0.05$, Stressed rats (either TPS or MPS) compared to unstressed rats, within-enrichment treatment.

significant main effect of stress on CORT levels was also present ($F(2,42)=4.09$, $p < 0.05$), and further Tukey comparisons indicated that unenriched MPS rats had significantly higher CORT levels than unenriched TPS rats during the acute stress time point ($p < 0.05$), and lower CORT levels than unenriched unstressed rats during the chronic stress time point ($p < 0.01$). A significant main effect of Time was also found ($F(1.76, 73.70)$ 4.89, $p < 0.05$, Greenhouse Geisser correction). Additional pairwise comparisons indicated that enriched rats had significantly lower CORT levels at the pre-stress time point, but not at the acute or chronic stress time points.

4.4.9 Correlations

A full list of cross-task correlations is found in Table 4.9. Many variables within each behavioural task were significantly correlated, although they are not listed. Notably, time investigating the objects in the object recognition task was significantly correlated with the number of closed arm entries in the elevated plus maze ($r = 0.34$, $p < 0.01$). Additionally, swim speeds in the Morris water maze was significantly correlated to number of closed arm entries in the elevated plus maze ($r = 0.273$, $p < 0.01$).

4.5 Discussion

The purpose of this study was to examine the effects of an ancestral history of prenatal stress on the development of locomotor activity, anxiety and depression-like behaviours, and learning and memory. Furthermore, this study investigated if postnatal EE is able to attenuate the effects of prenatal stress transmitted through the maternal ancestral lineage. The findings revealed that ancestral prenatal stress, either occurring once in the F_0 parental generation (TPS) or occurring repeatedly in each generation (MPS) results in drastic behavioural changes as compared to unstressed controls. Notably, qualitative and quantitative impairments caused by TPS and MPS did not differ from each other. By contrast, EE reduced the adverse consequences of ancestral prenatal stress in locomotor behaviour, anxiety and depression-related behaviour, improved learning and memory, and decreased social aggression and dominance. Interestingly, TPS rats did not appear to "benefit" from EE as much as MPS rats and unstressed controls did.

In the present study, pregnant rat dams were socially isolated for the duration of their pregnancy, which may be a reliable model of moderate stress (Hawkley et al., 2012).

The severity of the stress was comparable to an established semi-random stress procedure published earlier (Zucchi et al.,2014; Erickson et al.,2014). We used three standard behavioural tasks to measure motor activity, anxiety- and depression-like behaviours in the rats using the open field task (Smith et al., 2008; Jadavji et al., 2011), the elevated plus maze (Lister, 1987), and the forced swim task (Brenes et al., 2009). We used two standard behavioural tasks to measure learning and memory, including the novel object recognition task (Bruel-Jungerman et al., 2005; Pamplona et al., 2009), and the Morris water maze (Leggio et al., 2005; Morris 1984; Sutherland and Rodriguez, 1989). Finally, we used a modified water competition task (Lucion and Vogel, 1994) to assess social aggression and cooperation. In addition, to address the complex nature of interpreting results from multiple behavioural tasks that are affected by multiple internal factors (Zimmerman et al., 2001), a correlation analysis was conducted to elucidate the relationship between behavioural variables and tasks. The results revealed that ancestral stress induces a suite of characteristic modifications in the behavioural responses, as discussed in the following.

4.5.1 Effects of Ancestral Stress

4.5.1.1 Motor Activity, Affective Behaviour, and the Stress Response

The present study investigated the effects of a family history of prenatal stress on exploratory and affective behaviours. Based upon our previous findings (Erickson et al., 2014), we hypothesized that the stress exposure would increase locomotor activity, but not anxiety-related behaviour, in the TPS and MPS rats. The prenatal exposure to excessive glucocorticoid (GC) levels is known to program activity of the developing brain and hypothalamic-pituitary-adrenal (HPA) axis with consequences that extend into adulthood,

which may result in changes in motor activity and affective behaviour (Harris and Seckl, 2011; Holmes et al., 2006; Lupien et al., 2009). Prenatal stress has also been shown to alter mesolimbic dopamine (MesoDA) system activity, resulting in increased motor activity and ADHD-like behaviours in animal models (Field et al., 2008; Gatze-Kopp, 2011; Li et al., 2007; Metz, 2007). In the present study, both TPS and MPS generated elevated motor activity as reflected in several of the tests. For example, TPS rats spent more time investigating the objects in the object recognition task, which was significantly correlated with the number of closed arm entries in the elevated plus maze. Additionally, MPS rats showed a higher tendency to diving in the forced swim task, demonstrating increased motor activity. Both TPS and MPS rats had faster swim speeds in the Morris water maze, which was also significantly correlated to the number of closed arm entries in the elevated plus maze. Taken together, these findings support the notion that TPS- and MPS-related exposure to excessive GCs *in utero* may increase exploratory behaviour, which may be the result of an alteration of MesoDA system functioning (Baier et al., 2012).

While there was no significant effect of stress on baseline CORT secretion, TPS rats showed a significant reduction in CORT secretion at the acute stress time point. This is consistent with existing research suggesting a down-regulation of GC secretion in response to acute stress following prenatal stress (Lui et al., 1997). Furthermore, there were no significant main effects of stress in the open field task or elevated plus maze before the two-week stress phase of the experiment. As rats were exposed to both tasks multiple times, habituation may have resulted in the behaviour being less representative of anxiety. However, MPS rats did not show the same decrease in movement time in the open field as

TPS and unstressed rats during acute stress, suggesting that MPS rats may demonstrate increased stress resilience.

4.5.1.2 Learning and Memory

This study also investigated the effects of ancestral prenatal stress on learning and memory functions in male rats. We hypothesized that increased exposure to prenatal GCs would result in deficits in learning and memory. Prenatal stress has been shown to alter neurological architecture in the hippocampus (Lucassen et al., 2013), including reduced GR receptor density (Welberg et al., 2000) and diminished neurogenesis (Odagiri et al., 2008). These effects may result in deficits in learning and memory (Modir et al., 2014). Interestingly, there were no significant effects of TPS or MPS in the object recognition task or Morris water maze, aside from the variables associated with motor activity and exploration as mentioned previously.

The lack of between-group stress effects may be a reflection of the altered locomotor behaviour in the TPS and MPS. For example, increased swimming speed in the Morris water maze may compensate for decreased cognitive ability, resulting in similar alterations in performance times and thus prevent a conclusive interpretation of a specific deficit in an animal's ability. Similarly, spending more time investigating objects in the object recognition task due to a greater exploratory drive may compensate for potentially impaired cognitive ability, thus masking the effects of prenatal stress on cognitive functions. This hypothesis is supported by the fact that both water maze escape latency and novel object preference were significantly correlated with rearing and movement time, respectively, in the open field. Therefore, we suggest that stress-related hyperactivity may

demonstrative of a compensatory change in behaviour. Although not investigated in the present study, increased disinhibition/impulsivity, which has been associated with prenatal stress (Van Den Bergh and Marcoen, 2004), may also contribute to the more "frantic" approach to cognition in rats affected by ancestral stress.

4.5.1.3 Social Behaviour

The present study also investigated the effect of ancestral stress on social behaviour. We hypothesized that stressed rats would display increased aggression and drinking time in the water competition task. According to previous notions, early-life stress may increase social dominance and aggression, which may result in greater access to resources and subsequent survivability of the rat to reproductive success (Parent et al., 2013). Accordingly, in the present study, TPS rats showed a higher percentage of time drinking in the dominant position while in a group, suggesting increased dominance during the course of the task.

Additionally, when comparing group behaviour, both TPS and MPS groups drank more than unstressed rats. Notably, in contrast to unstressed or TPS rats, the MPS animals displayed a higher percentage of time sharing access to the spout with other rats during the task. However, there was no significant effect of stress on the drinking time, indicating that altered dominance or sharing behaviours in stressed rats did not increase their drinking time. These findings of a remote ancestral history of stress are partially in line with previous research in which stressful early-life events resulted in increased dominance and drinking behaviour (Parent et al., 2013). Increased exposure to GCs *in utero* has been associated

with elevated testosterone levels in male pups, which is associated with enhanced aggression that may promote survivability (Quinn et al., 2014).

4.5.2 Environmental Enrichment as a Treatment for Ancestral Prenatal Stress

4.5.2.1 Motor Activity, Affective Behaviour, and the Stress Response

A major focus of the present study was to determine if EE would offset some of the effects of programming by ancestral stress. We hypothesized that EE would result in decreased anxiety- and depression-like behaviours, and also reduce locomotor activity. Exposure to a more stimulating physical and social environment may decrease GC production, which may directly relate to a reduction in anxiety-like behaviour (Belz et al., 2003). Previously, EE has also been shown to enhance the expression of serotonin (5HT), which may play a role in the reduction of depression-like behaviour (Brenes et al., 2009). In the present study, EE rats spent more time in the open arms of the elevated plus maze, suggesting decreased anxiety-related behaviour in EE-treated groups. EE rats also spent less time moving and rearing in the open field task, and performed fewer closed-arm entries, demonstrating a decrease in locomotor activity. These results are similar to results using the elevated plus maze, further supporting the claim that EE reduces anxiety-related behaviour (Galani et al., 2007). Additionally, EE-treated rats spent more time swimming, and less time passively floating in the forced swim task, indicating reduced learned helplessness and depression-like behaviour compared to non-EE rats. These results also are comparable to previous findings that supported the claim that EE reduces depression-like behaviour (Brenes et al., 2009). These findings were associated with drastically reduced CORT levels in EE-treated animals before the beginning of the two-week stress

phase of the experiment, demonstrating that EE may have a significant impact on basal GC secretion. A confounding factor, however, may be that EE rats were more familiar with the experimenters, resulting in a more significant decrease in corticosterone in EE rats.

Interestingly, at P 100, TPS rats did not display an increased margin time in the open field task, as was seen in both MPS and unstressed rats, suggesting TPS rats benefited less from EE. One possible explanation may be found in the *Mismatch Hypothesis* (Schmidt, 2011), which states that if programming by prenatal stress is not followed by stressful environmental stimuli later in life, the result may lead to a maladaptive phenotype. Expanding this concept further, it is reasonable to assume that multiple generations of stress (as modelled in the MPS rats) may be required to maintain a fully adaptive phenotype. Within the context of the present study, while additional epigenetic changes likely occurred in the ancestral line between the F_1 and F_3 offspring, it is reasonable to predict that some of the changes propagated to the F_3 generation. However, the parental F_0 generation of prenatal stress in the TPS lineage was not followed by any additional stress in subsequent generations and may have been insufficient to produce an adaptive phenotype in the F_3 generation. Conversely, the ancestral line of MPS rats has experienced a consistent message preparing the offspring for a stressful environment, resulting in a more "refined" and adaptive phenotype. Interestingly, Vojta and Zoldos (2013) suggested that multigenerational stress changes in behaviour may be the result of increased risk of genetic mutation of 5-methylcytosine, the methylated variant of cytosine. The longer gene expression is suppressed via methylation, the more likely that 5-methylcytosine mutates into thymine, possibly resulting in loss of the gene sequence. This mechanism may serve to explain, at least in part, the cumulative nature of MPS.

Importantly, the adaptation to a stressful environment does not come without its drawbacks. Potentially adaptive changes in early adulthood in these stressed animals may come at the expense of healthy aging. Persistently physiological expenditure induced by recurrent prenatal stress may challenge cellular homeodynamic processes and potentially accelerate aging (Ambeskovic et al., 2013). Consequently, prenatal stress is associated with the increased risk for several complex diseases later in life, including hypertension, type 2 diabetes, and cardiovascular disease (Harris and Seckl, 2011). Furthermore, while stress results in earlier onset of puberty and reproductive viability (Parent et al., 2013), this also results in increased risk-taking behaviour and cognitive deficits, which may increase risk to predation later in life (Adriani and Laviola, 2004)). It could be argued that stress-related programming effects may be adaptive because they prioritize reproductive success over long-term survivability of the individual. Experimental causal studies to provide clear conclusive associations, however, still remain to be pursued.

4.5.2.2 Learning and Memory

The present study also investigated the effect of EE on learning and memory. We hypothesized that EE treatment would favor improved learning and memory. Indeed, in the present study, EE rats showed decreased escape latency and path distance on both learning and memory days in the Morris water maze. These results support previous findings that EE enhances cognitive functioning as measured by this task (Leggio et al., 2005; Zhong et al., 2009). Additionally, EE rats showed an increased novel object preference in the object recognition task, supporting previous findings that EE improved performance in the novel object recognition task (Bruel-Jungerman et al., 2005; Pamplona et al., 2009). Our results

lend even further evidence that EE helps to improve the cognitive ability of developing rats.

4.5.2.3 Social Behaviour

The present study also investigated the effect of EE on social behaviour. We hypothesized that EE rats would show decreased social aggression and a greater tendency to cooperate with other rats when competing for access to water. Increased social enrichment reduces circulating basal GC levels (Pena et al., 2009; Welberg et al., 2006), which may favor submissive or cooperative behaviour when competing for a limited water source (Quinn et al., 2014). As predicted, the EE rats in our study were less aggressive, less dominant, and spent a higher percentage of spout time sharing with other rats. These findings are consistent with previous research in which EE promoted social behaviour in rats (Morley-Fletcher et al., 2003). Interestingly, it appears that cooperation with other rats may be an adaptive behaviour, as rats that shared access to the spout also had a higher total time at the spout.

4.5.3 Conclusions

In conducting this study, we aimed to more fully understand the role of ancestral prenatal stress and EE in the development of a number of psychopathological disruptions. We conclude that an ancestral history of stress results in increased exploratory behaviour and some changes in social behaviour, but it does not exacerbate anxiety-like behaviours or cause measurable changes in learning and memory. Furthermore, we conclude that EE resulted in increased decreased exploratory behaviour, decreased anxiety- and depression-

like behaviours, improved learning and memory, and decreased social aggression. We conclude that EE is a valuable treatment for both stressed rats, as well as unstressed controls, as both benefited from the experimental manipulation. It is reasonable to assume that an increased endeavour to enrich the developmental environment in humans would have similar effects, which is especially important for families and homes with an increased risk for psychopathological disorders such as ADHD, learning disabilities, anxiety, and depression. Finally, we feel that further investigation is needed to understand the role of the Mismatch Hypothesis in understanding how prenatal stress and EE truly interact, and if there are any special considerations needed when providing early-life interventions with children predisposed to HPA-axis programming effects.

4.6 Tables

Table 4.1.1 Means and Standard Deviations of Body Mass

Stress Treatment	Enrichment Treatment	p21		p35		p60		p90		p120		p140		p160		p180	
		M	SD	M	SD	M	SD	M	SD	M	SD	M	SD	M	SD	M	SD
Control	Unenriched	42.1	3.5	145.3	8.2	352.3	31.0	487.1	40.1	546.4	50.1	574.8	56.1	576.1	51.0	613.9	56.1
	Enriched	43.9	6.4	147.9	13.9	326.0	24.9	461.6	34.5	528.6	51.1	556.9	59.8	558.9	54.7	598.6	63.8
SNN	Unenriched	41.9	4.2	148.2	12.5	365.8	24.7	494.9	33.8	564.9	27.6	591.1	27.3	589.5	21.5	635.3	19.2
	Enriched	41.2	3.8	145.7	12.4	348.9	34.8	481.8	42.5	561.6	47.4	586.8	50.9	585.0	45.6	623.5	53.9
SSS	Unenriched	44.0	3.9	143.4	7.4	363.4	27.4	490.4	35.9	566.9	45.3	598.8	47.9	596.0	44.2	588.1	41.0
	Enriched	42.3	5.9	132.5	12.5	320.6	17.6	446.8	29.9	512.5	34.4	579.3	90.4	559.5	40.2	632.8	48.2

Table 4.1.2 Mixed Design ANOVA Results of Body Mass

Effect	ANOVA Results	Partial Eta Squared
Stress	$F(2,41) = 1.46$, $p = 0.24$	partial $\eta^2 = 0.07$
Enrichment	$F(1,41) = 3.18$, $p = 0.08$	partial $\eta^2 = 0.07$
Age***	$F(3.18,130.17) = 3017.26$, $p < 0.001$	partial $\eta^2 = 0.99$
Stress * Enrichment	$F(2,41) = 0.12$, $p = 0.89$	partial $\eta^2 = 0.01$
Age x Stress	$F(6.35,130.17) = 1.08$, $p = 0.38$	partial $\eta^2 = 0.05$
Age x Enrichment*	$F(3.18,130.17) = 3.06$, $p < 0.05$	partial $\eta^2 = 0.07$
Age x Stress x Enrichment	$F(6.35,130.17) = 1.60$, $p = 0.15$	partial $\eta^2 = 0.07$

Table 4.2.1 Means and Standard Deviations of the Open Field Task

Stress Treatment	Enrichment Treatment	Variable	p35		p60		Adult		Acute Stress		Chronic Stress	
			M	SD	M	SD	M	SD	M	SD	M	SD
Control	Unenriched	Movement Time	121.9	53.2	146.9	40.4	213.4	63.4	117.1	38.3	189.7	55.7
		Vertical Time	55.2	17.1	106.3	19.8	160.1	50.8	70.2	35.4	116.6	58.4
		Margin Time	552.3	29.3	459.2	40.5	395.9	51.6	418.6	96.8	390.5	63.6
	Enriched	Movement Time	97.0	34.9	91.6	30.6	105.2	37.4	78.6	41.7	101.1	36.6
		Vertical Time	57.7	22.4	54.6	19.7	66.9	30.7	49.4	34.5	58.9	31.0
		Margin Time	560.5	15.0	548.6	27.7	521.1	39.3	526.2	40.4	528.3	41.0
SNN	Unenriched	Movement Time	114.4	74.0	177.1	65.0	175.7	34.6	96.9	53.4	189.9	36.7
		Vertical Time	51.8	35.9	91.5	38.7	139.2	41.8	53.9	32.9	119.1	19.2
		Margin Time	551.3	45.8	481.2	62.6	413.8	81.3	508.3	71.8	402.1	82.3
	Enriched	Movement Time	114.0	55.4	115.6	50.5	146.4	55.3	77.3	52.1	114.2	27.7
		Vertical Time	47.5	18.0	75.4	28.8	97.2	29.5	37.0	22.0	64.3	18.9
		Margin Time	525.7	55.0	498.6	57.4	415.1	91.3	522.7	26.7	489.8	38.2
SSS	Unenriched	Movement Time	141.5	58.7	194.4	61.1	213.4	48.0	182.4	60.3	228.0	57.2
		Margin Time	557.0	27.0	473.2	41.6	422.3	71.9	349.3	93.3	380.1	72.5
	Enriched	Movement Time	137.2	41.3	100.9	41.6	148.6	53.1	147.5	42.8	108.6	35.0
		Margin Time	523.2	42.4	550.6	42.9	500.2	62.3	520.1	43.3	457.1	97.3

Table 4.2.2 Mixed Design ANOVA Results of Open Field Task (Maturation Phase)

Effect	Dependent Variable	ANOVA Results	Partial Eta Squared
Stress	Movement Time	$F(2,42) = 1.93$, $p = 0.158$	partial $\eta 2 = 0.08$
	Margin Time	$F(2,42) = 2.88$, $p = 0.067$	partial $\eta 2 = 0.12$
	Vertical Time	$F(2,42) = 0.46$, $p = 0.632$	partial $\eta 2 = 0.02$
Enrichment	Movement Time***	$F(1,42) = 19.45$, $p < 0.001$	partial $\eta 2 = 0.32$
	Margin Time***	$F(1,42) = 15.25$, $p < 0.001$	partial $\eta 2 = 0.27$
	Vertical Time***	$F(1,42) = 18.45$, $p < 0.001$	partial $\eta 2 = 0.31$
Age	Movement Time***	$F(2,84) = 15.28$, $p < 0.001$	partial $\eta 2 = 0.27$
	Margin Time***	$F(2,84) = 48.22$, $p < 0.001$	partial $\eta 2 = 0.53$
	Vertical Time***	$F(2,84) = 48.06$, $p < 0.001$	partial $\eta 2 = 0.53$
Stress * Enrichment	Movement Time	$F(2,42) = 0.76$, $p = 0.475$	partial $\eta 2 = 0.04$
	Margin Time**	$F(2,42) = 5.33$, $p < 0.01$	partial $\eta 2 = 0.20$
	Vertical Time	$F(2,42) = 1.22$, $p = 0.306$	partial $\eta 2 = 0.06$
Age x Stress	Movement Time	$F(4,84) = 0.57$, $p = 0.684$	partial $\eta 2 = 0.03$
	Margin Time	$F(4,84) = 1.00$, $p = 0.413$	partial $\eta 2 = 0.05$
	Vertical Time	$F(4,84) = 2.12$, $p = 0.086$	partial $\eta 2 = 0.09$
Age x Enrichment	Movement Time**	$F(2,84) = 8.11$, $p < 0.01$	partial $\eta 2 = 0.16$
	Margin Time***	$F(2,84) = 10.68$, $p < 0.001$	partial $\eta 2 = 0.20$
	Vertical Time***	$F(2,84) = 9.70$, $p < 0.001$	partial $\eta 2 = 0.19$
Age x Stress x Enrichment	Movement Time	$F(4,84) = 1.53$, $p = 0.201$	partial $\eta 2 = 0.07$
	Margin Time	$F(4,84) = 1.17$, $p = 0.328$	partial $\eta 2 = 0.05$
	Vertical Time*	$F(4,84) = 3.32$, $p < 0.05$	partial $\eta 2 = 0.14$

Table 4.2.3 Mixed Design ANOVA Results of Open Field Task (Stress Response Phase)

Effect	Dependent Variable	ANOVA Results	Partial Eta Squared
Stress	**Movement Time****	$F(2,42) = 5.38, p < 0.01$	partial $\eta 2 = 0.20$
	Margin Time	$F(2,42) = 1.05, p = 0.36$	partial $\eta 2 = 0.05$
Enrichment	**Movement Time*****	$F(1,42) = 35.35, p < 0.001$	partial $\eta 2 = 0.46$
	Margin Time***	$F(1,42) = 34.68, p < 0.001$	partial $\eta 2 = 0.45$
Age	**Movement Time*****	$F(2,84) = 27.14, p < 0.001$	partial $\eta 2 = 0.39$
	Margin Time**	$F(2,84) = 5.35, p < 0.01$	partial $\eta 2 = 0.11$
Stress * Enrichment	**Movement Time**	$F(2,42) = 1.13, p = 0.33$	partial $\eta 2 = 0.05$
	Margin Time*	$F(2,42) = 3.33, p < 0.05$	partial $\eta 2 = 0.14$
Age x Stress	**Movement Time****	$F(4,84) = 4.20, p < 0.01$	partial $\eta 2 = 0.17$
	Margin Time***	$F(4,84) = 6.05, p < 0.001$	partial $\eta 2 = 0.22$
Age x Enrichment	**Movement Time*****	$F(2,84) = 9.89, p < 0.001$	partial $\eta 2 = 0.19$
	Margin Time	$F(2,84) = 1.33, p = 0.27$	partial $\eta 2 = 0.03$
Age x Stress x Enrichment	**Movement Time**	$F(4,84) = 1.58, p = 0.19$	partial $\eta 2 = 0.07$
	Margin Time*	$F(4,84) = 2.95, p < 0.05$	partial $\eta 2 = 0.12$

Table 4.3.1 Means and Standard Deviations of Elevated Plus Maze

Stress Treatment	Enrichment Treatment	Variable	p35		p60		Adult		Acute Stress		Chronic Stress	
			M	SD	M	SD	M	SD	M	SD	M	SD
Unstressed	Unenriched	Closed Arm Entries	7.6	3.5	7.9	4.5	9.1	2.9	5.0	2.0	6.1	4.0
		Time in the Open Arms	7.4	9.5	6.5	12.1	16.3	22.2	21.8	27.4	19.6	20.4
		Time in End of the Open Arms	1.9	2.8	2.4	4.6	5.1	8.0	3.8	5.2	6.1	12.4
		End of Open Arm Entries	0.5	0.8	0.6	1.2	1.1	2.1	0.9	1.1	0.5	0.9
		Latency to Ented Closed Arm	23.4	32.1	5.5	3.6	7.9	7.0	9.9	6.8	6.3	9.0
	Enriched	Closed Arm Entries	8.8	2.8	7.0	2.8	4.6	3.6	3.9	2.1	5.6	3.2
		Time in the Open Arms	2.1	4.4	5.5	7.7	14.0	26.4	10.6	19.0	23.3	31.8
		Time in End of the Open Arms	0.0	0.0	2.6	4.1	6.1	12.4	3.1	6.7	9.1	15.5
		End of Open Arm Entries	0.0	0.0	0.4	0.5	1.0	2.1	0.4	0.7	1.3	2.1
		Latency to Ented Closed Arm	13.9	16.2	11.1	8.2	6.8	5.7	16.0	17.5	14.3	20.2
SNN	Unenriched	Closed Arm Entries	8.5	3.8	6.9	2.0	9.5	2.4	4.4	1.7	6.9	2.4
		Time in the Open Arms	6.0	7.0	0.0	0.0	4.8	12.3	5.5	14.8	42.8	45.4
		Time in End of the Open Arms	1.5	3.2	0.0	0.0	1.8	5.0	2.0	5.7	18.5	24.7
		End of Open Arm Entries	0.4	0.7	0.0	0.0	0.3	0.7	0.4	1.1	2.0	2.7
		Latency to Ented Closed Arm	9.8	11.2	11.0	12.9	9.0	3.0	5.5	5.6	13.3	11.6
	Enriched	Closed Arm Entries	9.5	3.5	5.8	3.2	5.0	2.1	3.5	1.6	6.0	1.9
		Time in the Open Arms	15.4	24.6	7.0	13.2	20.8	27.4	7.5	15.1	44.1	42.1
		Time in End of the Open Arms	7.8	12.7	3.4	7.2	6.4	14.3	0.0	0.0	11.6	11.4
		End of Open Arm Entries	1.1	1.6	0.5	0.9	1.1	2.4	0.0	0.0	1.8	1.8
		Latency to Ented Closed Arm	43.6	86.5	14.0	13.4	16.4	22.9	7.8	6.3	14.5	25.7
SSS	Unenriched	Closed Arm Entries	7.1	4.2	8.9	3.2	11.8	3.3	6.0	1.1	7.0	2.2
		Time in the Open Arms	1.0	2.8	0.0	0.0	0.3	0.7	6.5	18.4	14.0	11.3
		Time in End of the Open Arms	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.5	3.5
		End of Open Arm Entries	0.1	0.4	0.0	0.0	0.0	0.0	0.0	0.0	0.3	0.5
		Latency to Ented Closed Arm	16.8	11.3	3.5	2.1	10.8	8.2	16.0	17.0	8.3	8.6
	Enriched	Closed Arm Entries	9.3	3.7	6.5	2.9	5.1	1.6	5.6	2.6	5.8	1.9
		Time in the Open Arms	9.9	5.9	8.0	12.1	14.5	18.1	16.0	14.6	16.1	22.3
		Time in End of the Open Arms	1.9	2.7	4.3	8.7	5.1	8.6	2.9	3.6	4.4	8.1
		End of Open Arm Entries	0.5	0.8	0.8	1.5	0.8	1.2	0.5	0.5	0.6	1.2
		Latency to Ented Closed Arm	22.8	22.4	6.1	5.6	10.0	11.5	9.8	12.2	9.4	10.1

Table 4.3.2 Mixed Design ANOVA Results for Elevated Plus Maze (Maturation Phase)

Effect	Dependent Variable	ANOVA Results	Partial Eta Squared
Stress	Closed Arm Entries	$F(2,42) = 0.37$, $p = 0.697$	partial $\eta 2 = 0.02$
	Time in Open Arms	$F(2,42) = 0.48$, $p = 0.621$	partial $\eta 2 = 0.02$
	Time in End of Open Arms	$F(2,42) = 0.42$, $p = 0.658$	partial $\eta 2 = 0.02$
	End of Open Arms Entries	$F(2,42) = 0.35$, $p = 0.705$	partial $\eta 2 = 0.02$
	Latency to Closed Arms	$F(2,42) = 0.90$, $p = 0.415$	partial $\eta 2 = 0.04$
Enrichment	Closed Arm Entries*	$F(1,42) = 7.12$, $p < 0.05$	partial $\eta 2 = 0.15$
	Time in Open Arms	$F(1,42) = 3.93$, $p = 0.054$	partial $\eta 2 = 0.09$
	Time in End of Open Arms	$F(1,42) = 3.63$, $p = 0.064$	partial $\eta 2 = 0.08$
	End of Open Arms Entries	$F(1,42) = 1.78$, $p = 0.190$	partial $\eta 2 = 0.04$
	Latency to Closed Arms	$F(1,42) = 1.67$, $p = 0.204$	partial $\eta 2 = 0.04$
Age	Closed Arm Entries	$F(2,84) = 2.93$, $p = 0.059$	partial $\eta 2 = 0.07$
	Time in Open Arms*	$F(1.49,62.58) = 4.60$, $p < 0.05$	partial $\eta 2 = 0.10$
	Time in End of Open Arms	$F(1.52,63.76) = 1.75$, $p = 0.188$	partial $\eta 2 = 0.04$
	End of Open Arms Entries	$F(1.59,66.69) = 1.60$, $p = 0.212$	partial $\eta 2 = 0.04$
	Latency to Closed Arms*	$F(1.17,49.22) = 4.09$, $p < 0.05$	partial $\eta 2 = 0.09$
Stress * Enrichment	Closed Arm Entries	$F(2,42) = 0.17$, $p = 0.841$	partial $\eta 2 = 0.01$
	Time in Open Arms	$F(2,42) = 2.10$, $p = 0.135$	partial $\eta 2 = 0.09$
	Time in End of Open Arms	$F(2,42) = 1.09$, $p = 0.346$	partial $\eta 2 = 0.05$
	End of Open Arms Entries	$F(2,42) = 1.51$, $p = 0.232$	partial $\eta 2 = 0.07$
	Latency to Closed Arms	$F(2,42) = 1.47$, $p = 0.242$	partial $\eta 2 = 0.07$
Age x Stress	Closed Arm Entries	$F(4,84) = 1.14$, $p = 0.342$	partial $\eta 2 = 0.05$
	Time in Open Arms	$F(2.98,62.58) = 1.00$, $p = 0.398$	partial $\eta 2 = 0.05$
	Time in End of Open Arms	$F(3.04,63.76) = 1.16$, $p = 0.331$	partial $\eta 2 = 0.05$
	End of Open Arms Entries	$F(3.18,66.69) = 1.31$, $p = 0.277$	partial $\eta 2 = 0.06$
	Latency to Closed Arms	$F(2.34,49.22) = 0.11$, $p = 0.926$	partial $\eta 2 = 0.01$
Age x Enrichment	Closed Arm Entries***	$F(2,84) = 17.69$, $p < 0.001$	partial $\eta 2 = 0.30$
	Time in Open Arms	$F(1.49,62.58) = 0.66$, $p = 0.477$	partial $\eta 2 = 0.02$
	Time in End of Open Arms	$F(1.52,63.76) = 0.20$, $p = 0.758$	partial $\eta 2 = 0.01$
	End of Open Arms Entries	$F(1.59,66.69) = 0.27$, $p = 0.711$	partial $\eta 2 = 0.01$
	Latency to Closed Arms	$F(1.17,49.22) = 0.37$, $p = 0.577$	partial $\eta 2 = 0.01$
Age x Stress x Enrichment	Closed Arm Entries	$F(4,84) = 0.50$, $p = 0.738$	partial $\eta 2 = 0.02$
	Time in Open Arms	$F(2.98,62.58) = 0.21$, $p = 0.891$	partial $\eta 2 = 0.01$
	Time in End of Open Arms	$F(3.03,63.76) = 0.31$, $p = 0.819$	partial $\eta 2 = 0.02$
	End of Open Arms Entries	$F(3.18,66.69) = 0.12$, $p = 0.956$	partial $\eta 2 = 0.01$
	Latency to Closed Arms	$F(2.34,49.22) = 0.96$, $p = 0.400$	partial $\eta 2 = 0.04$

Table 4.3.3 Mixed Design ANOVA Results for Elevated Plus Maze (Stress Response Phase)

Effect	Dependent Variable	ANOVA Results	Partial Eta Squared
Stress	Closed Arm Entries	$F(2,42) = 1.93$, $p = 0.157$	partial $\eta 2 = 0.08$
	Time in Open Arms	$F(2,42) = 1.20$, $p = 0.311$	partial $\eta 2 = 0.05$
	Time in End of Open Arms	$F(2,42) = 1.71$, $p = 0.193$	partial $\eta 2 = 0.08$
	End of Open Arms Entries	$F(2,42) = 1.54$, $p = 0.226$	partial $\eta 2 = 0.07$
	Latency to Enter Closed Arms	$F(2,42) = 0.04$, $p = 0.957$	partial $\eta 2 = 0.00$
Enrichment	Closed Arm Entries***	$F(1,42) = 19.57$, $p < 0.001$	partial $\eta 2 = 0.32$
	Time in Open Arms	$F(1,42) = 0.58$, $p = 0.450$	partial $\eta 2 = 0.01$
	Time in End of Open Arms	$F(1,42) = 0.30$, $p = 0.584$	partial $\eta 2 = 0.01$
	End of Open Arms Entries	$F(1,42) = 0.60$, $p = 0.443$	partial $\eta 2 = 0.01$
	Latency to Enter Closed Arms	$F(1,42) = 0.65$, $p = 0.425$	partial $\eta 2 = 0.02$
Age	Closed Arm Entries***	$F(1.64,68.93) = 21.44$, $p < 0.001$	partial $\eta 2 = 0.34$
	Time in Open Arms**	$F(1.59,66.72) = 9.49$, $p < 0.01$	partial $\eta 2 = 0.18$
	Time in End of Open Arms**	$F(1.68,70.40) = 6.67$, $p < 0.01$	partial $\eta 2 = 0.14$
	End of Open Arms Entries*	$F(2,84) = 3.79$, $p < 0.05$	partial $\eta 2 = 0.08$
	Latency to Enter Closed Arms	$F(2,84) = 0.07$, $p = 0.937$	partial $\eta 2 = 0.00$
Stress * Enrichment	Closed Arm Entries	$F(2,42) = 0.20$, $p = 0.823$	partial $\eta 2 = 0.01$
	Time in Open Arms	$F(2,42) = 0.50$, $p = 0.612$	partial $\eta 2 = 0.02$
	Time in End of Open Arms	$F(2,42) = 0.52$, $p = 0.597$	partial $\eta 2 = 0.02$
	End of Open Arms Entries	$F(2,42) = 0.31$, $p = 0.734$	partial $\eta 2 = 0.02$
	Latency to Enter Closed Arms	$F(2,42) = 0.64$, $p = 0.531$	partial $\eta 2 = 0.03$
Age x Stress	Closed Arm Entries	$F(3.28,68.93) = 0.98$, $p = 0.412$	partial $\eta 2 = 0.05$
	Time in Open Arms*	$F(3.18,66.72) = 3.91$, $p < 0.05$	partial $\eta 2 = 0.16$
	Time in End of Open Arms	$F(3.35,70.40) = 2.54$, $p = 0.057$	partial $\eta 2 = 0.11$
	End of Open Arms Entries	$F(4,84) = 2.18$, $p = 0.078$	partial $\eta 2 = 0.09$
	Latency to Enter Closed Arms	$F(4,84) = 1.42$, $p = 0.236$	partial $\eta 2 = 0.06$
Age x Enrichment	Closed Arm Entries***	$F(1.64,68.93) = 17.53$, $p < 0.001$	partial $\eta 2 = 0.29$
	Time in Open Arms	$F(1.59,66.72) = 0.72$, $p = 0.461$	partial $\eta 2 = 0.02$
	Time in End of Open Arms	$F(1.68,70.40) = 0.68$, $p = 0.483$	partial $\eta 2 = 0.02$
	End of Open Arms Entries	$F(2,84) = 0.77$, $p = 0.468$	partial $\eta 2 = 0.02$
	Latency to Enter Closed Arms	$F(2,84) = 0.15$, $p = 0.859$	partial $\eta 2 = 0.00$
Age x Stress x Enrichment	Closed Arm Entries	$F(3.28,68.93) = 0.60$, $p = 0.630$	partial $\eta 2 = 0.03$
	Time in Open Arms	$F(3.18,66.72) = 0.48$, $p = 0.711$	partial $\eta 2 = 0.02$
	Time in End of Open Arms	$F(3.35,70.40) = 0.60$, $p = 0.634$	partial $\eta 2 = 0.03$
	End of Open Arms Entries	$F(4,84) = 0.78$, $p = 0.542$	partial $\eta 2 = 0.04$
	Latency to Enter Closed Arms	$F(4,84) = 0.57$, $p = 0.684$	partial $\eta 2 = 0.03$

Table 4.4.1 Means and Standard Deviations of Object Recognition Task

Stress Treatment	Enrichment Treatment	Variable	p60		p100	
			M	SD	M	SD
Control	Standard Housing	Total Object Time	53.8	31.9	39.3	14.4
		Investigation Novel Percentage	0.5	0.2	0.7	0.1
	Enriched Environment	Total Object Time	60.8	27.8	51.1	53.7
		Investigation Novel Percentage	0.7	0.2	0.9	0.1
SNN	Standard Housing	Total Object Time	88.6	21.8	84.4	37.1
		Investigation Novel Percentage	0.6	0.1	0.8	0.1
	Enriched Environment	Total Object Time	74.8	17.0	50.6	34.7
		Investigation Novel Percentage	0.6	0.1	0.9	0.1
SSS	Standard Housing	Total Object Time	69.6	19.6	82.4	64.3
		Investigation Novel Percentage	0.6	0.1	0.7	0.2
	Enriched Environment	Total Object Time	68.5	19.1	35.9	13.2
		Investigation Novel Percentage	0.7	0.1	0.8	0.2

Table 4.4.2 Mixed Design ANOVA Results for Object Recognition Task

Effect	Dependent Variable	ANOVA Results	Partial Eta Squared
Stress	Total Object Time*	$F(2,84) = 3.96, p < 0.05$	partial $\eta 2 = 0.09$
	Investigation Novel Percentage	$F(2,84) = 1.03, p = 0.360$	partial $\eta 2 = 0.02$
Enrichment	Total Time Spent with Objects	$F(1,84) = 3.52, p = 0.064$	partial $\eta 2 = 0.04$
	Investigation Novel Percentage***	$F(1,84) = 13.16, p < 0.001$	partial $\eta 2 = 0.14$
Age	Total Time Spent with Objects	$F(1,84) = 3.16, p = 0.079$	partial $\eta 2 = 0.04$
	Investigation Novel Percentage***	$F(1,84) = 33.03, p < 0.001$	partial $\eta 2 = 0.28$
Stress * Enrichment	Total Time Spent with Objects	$F(2,84) = 2.67, p = 0.076$	partial $\eta 2 = 0.06$
	Investigation Novel Percentage*	$F(2,84) = 4.19, p < 0.05$	partial $\eta 2 = 0.09$
Stress * Age	Total Time Spent with Objects	$F(2,84) = 0.03, p = 0.968$	partial $\eta 2 = 0.00$
	Investigation Novel Percentage	$F(2,84) = 2.66, p = 0.076$	partial $\eta 2 = 0.06$
Enrichment * Age	Total Time Spent with Objects	$F(1,84) = 2.20, p = 0.142$	partial $\eta 2 = 0.03$
	Investigation Novel Percentage	$F(1,84) = 0.19, p = 0.667$	partial $\eta 2 = 0.00$
Stress * Enrichment * Age	Total Time Spent with Objects	$F(2,84) = 1.14, p = 0.324$	partial $\eta 2 = 0.03$
	Investigation Novel Percentage	$F(2,84) = 0.07, p = 0.930$	partial $\eta 2 = 0.00$

Table 4.5.1 Means and Standard Deviations for Morris Water Maze

Stress Treatment	Enrichment Treatment	Variable	Learning		Memory	
			M	SD	M	SD
Control	Standard Housing	Latency	17.4	18.3	11.1	13.0
		Path Distance	3.7	4.1	2.3	2.8
		Speed	0.212	0.048	0.212	0.046
	Enriched Environment	Latency	14.5	16.7	7.1	7.2
		Path Distance	3.1	3.9	1.4	1.5
		Speed	0.196	0.053	0.204	0.057
SNN	Standard Housing	Latency	18.2	19.2	10.7	12.7
		Path Distance	4.2	4.7	2.4	2.9
		Speed	0.234	0.051	0.231	0.051
	Enriched Environment	Latency	14.7	15.8	7.8	8.6
		Path Distance	3.1	3.6	1.5	1.8
		Speed	0.203	0.044	0.196	0.046
SSS	Standard Housing	Latency	18.0	19.6	8.7	10.4
		Path Distance	4.2	4.7	2.2	2.9
		Speed	0.231	0.046	0.246	0.061
	Enriched Environment	Latency	15.3	17.5	7.2	7.6
		Path Distance	3.4	4.1	1.6	1.7
		Speed	0.216	0.044	0.224	0.069

Table 4.5.2 Mixed Design ANOVA Results for Morris Water Maze

Effect	Dependent Variable	ANOVA Results	Partial Eta Squared
TaskType	Latency***	$F(1,3061) = 240.60$, $p < 0.001$	partial $\eta 2 = 0.07$
	Path Distance***	$F(1,3061) = 221.16$, $p < 0.001$	partial $\eta 2 = 0.07$
	Speed	$F(1,3050) = 0.88$, $p = 0.348$	partial $\eta 2 = 0.00$
Stress	Latency	$F(2,3061) = 1.45$, $p = 0.236$	partial $\eta 2 = 0.00$
	Path Distance	$F(2,3061) = 1.14$, $p = 0.321$	partial $\eta 2 = 0.00$
	Speed***	$F(2,3050) = 50.78$, $p < 0.001$	partial $\eta 2 = 0.03$
Enrichment	Latency***	$F(1,3061) = 34.94$, $p < 0.001$	partial $\eta 2 = 0.01$
	Path Distance***	$F(1,3061) = 60.78$, $p < 0.001$	partial $\eta 2 = 0.02$
	Speed***	$F(1,3050) = 144.90$, $p < 0.001$	partial $\eta 2 = 0.05$
TaskType * Stress	Latency	$F(2,3061) = 1.37$, $p = 0.254$	partial $\eta 2 = 0.00$
	Path Distance	$F(2,3061) = 0.72$, $p = 0.487$	partial $\eta 2 = 0.00$
	Speed**	$F(2,3050) = 4.99$, $p < 0.01$	partial $\eta 2 = 0.00$
TaskType * Enrichment	Latency	$F(1,3061) = 0.30$, $p = 0.584$	partial $\eta 2 = 0.00$
	Path Distance	$F(1,3061) = 0.06$, $p = 0.807$	partial $\eta 2 = 0.00$
	Speed	$F(1,3050) = 1.12$, $p = 0.290$	partial $\eta 2 = 0.00$
Stress * Enrichment	Latency	$F(2,3061) = 0.94$, $p = 0.390$	partial $\eta 2 = 0.00$
	Path Distance	$F(2,3061) = 0.75$, $p = 0.475$	partial $\eta 2 = 0.00$
	Speed***	$F(2,3050) = 10.20$, $p < 0.001$	partial $\eta 2 = 0.01$
TaskType * Stress * Enrichment	Latency	$F(2,3061) = 0.43$, $p = 0.652$	partial $\eta 2 = 0.00$
	Path Distance	$F(2,3061) = 0.31$, $p = 0.735$	partial $\eta 2 = 0.00$
	Speed	$F(2,3050) = 1.21$, $p = 0.299$	partial $\eta 2 = 0.00$

Table 4.6.1 Means and Standard Deviations of Water Competition Task

Stress Treatment	Enrichment Treatment	Variable	Two Rats (EE Comparison)		Three Rats (Stress Comparison)	
			M	SD	M	SD
Control	Standard Housing	Social Interactions	19.50	25.63	11.55	11.35
		Time at Spout	116.13	99.13	44.11	21.11
		Paired Time Dominance (%)	0.57	0.44	0.37	0.18
		Spout Time Alone (%)	0.61	0.52	0.20	0.19
	Enriched Environment	Social Interactions	12.88	12.75	6.98	5.39
		Time at Spout	115.88	78.63	28.31	36.35
		Paired Time Dominance (%)	0.34	0.36	0.32	0.34
		Spout Time Alone (%)	0.62	0.45	0.23	0.26
SNN	Standard Housing	Social Interactions	28.13	23.63	14.05	9.40
		Time at Spout	161.00	105.13	39.70	18.74
		Paired Time Dominance (%)	0.75	0.56	0.16	0.16
		Spout Time Alone (%)	0.48	0.47	0.20	0.23
	Enriched Environment	Social Interactions	25.25	13.13	16.78	7.49
		Time at Spout	146.25	109.13	38.64	33.96
		Paired Time Dominance (%)	0.20	0.75	0.19	0.32
		Spout Time Alone (%)	0.52	0.33	0.17	0.17
SSS	Standard Housing	Social Interactions	37.50	27.50	21.06	16.62
		Time at Spout	164.38	95.13	35.60	20.93
		Paired Time Dominance (%)	0.70	0.51	0.20	0.24
		Spout Time Alone (%)	0.38	0.56	0.14	0.22
	Enriched Environment	Social Interactions	24.13	13.75	14.67	7.32
		Time at Spout	148.00	104.88	26.98	36.01
		Paired Time Dominance (%)	0.25	0.38	0.24	0.40
		Spout Time Alone (%)	0.49	0.28	0.16	0.25

Table 4.6.2 ANOVA Results for Water Competition Task

Session	Source	Dependent Variable	ANOVA Results	Partial Eta Squared
Two Rats (EE Effect)	Stress	Social Interactions	$F(2,42) = 4.14$, $p = 0.02$	partial $\eta 2 = 0.17$
		Drinking Time**	$F(2,42) = 6.22$, $p < 0.01$	partial $\eta 2 = 0.23$
		Group Dominance	$F(2,42) = 0.03$, $p = 0.98$	partial $\eta 2 = 0.00$
		AloneTimePercent*	$F(2,42) = 3.67$, $p < 0.05$	partial $\eta 2 = 0.15$
	Enrichment	Social Interactions	$F(1,42) = 3.17$, $p = 0.08$	partial $\eta 2 = 0.07$
		Drinking Time**	$F(1,42) = 1.01$, $p = 0.32$	partial $\eta 2 = 0.02$
		Group Dominance***	$F(1,42) = 30.04$, $p < 0.001$	partial $\eta 2 = 0.42$
		AloneTimePercent	$F(1,42) = 1.02$, $p = 0.32$	partial $\eta 2 = 0.02$
	Stress * Enrichment	Social Interactions	$F(2,42) = 0.52$, $p = 0.60$	partial $\eta 2 = 0.02$
		SpoutTime	$F(2,42) = 0.24$, $p = 0.79$	partial $\eta 2 = 0.01$
		Group Dominance	$F(2,42) = 1.58$, $p = 0.22$	partial $\eta 2 = 0.07$
		AloneTimePercent	$F(2,42) = 0.35$, $p = 0.71$	partial $\eta 2 = 0.02$
Three Rats (Stress Effect)	Stress	Social Interactions	$F(2,41) = 0.19$, $p = 0.83$	partial $\eta 2 = 0.01$
		SpoutTime	$F(2,41) = 0.95$, $p = 0.40$	partial $\eta 2 = 0.04$
		Group Dominance*	$F(2,41) = 3.59$, $p < 0.05$	partial $\eta 2 = 0.15$
		AloneTimePercent	$F(2,41) = 0.23$, $p = 0.79$	partial $\eta 2 = 0.01$
	Enrichment	Social Interactions***	$F(1,41) = 15.90$, $p < 0.001$	partial $\eta 2 = 0.28$
		SpoutTime	$F(1,41) = 0.02$, $p = 0.89$	partial $\eta 2 = 0.00$
		Group Dominance	$F(1,41) = 0.01$, $p = 0.95$	partial $\eta 2 = 0.00$
		AloneTimePercent**	$F(1,41) = 8.98$, $p < 0.01$	partial $\eta 2 = 0.18$
	Stress * Enrichment	Social Interactions	$F(2,41) = 0.10$, $p = 0.90$	partial $\eta 2 = 0.01$
		SpoutTime	$F(2,41) = 0.58$, $p = 0.57$	partial $\eta 2 = 0.03$
		Group Dominance	$F(2,41) = 1.43$, $p = 0.25$	partial $\eta 2 = 0.07$
		AloneTimePercent	$F(2,41) = 0.68$, $p = 0.51$	partial $\eta 2 = 0.03$

Table 4.7.1 Means and Standard Deviations of Forced Swim Task

Stress Treatment	Enrichment Treatment	Variable	Acute Stress Phase		Chronic Stress Phase	
			M	SD	M	SD
Control	Unenriched	Floating Time	23.88	9.83	59.50	31.03
		Number of Dives	1.88	4.52	0.25	0.71
		Swimming Time	211.75	13.08	178.88	30.86
	Enriched	Floating Time	21.00	11.95	36.38	24.14
		Number of Dives	1.13	1.55	1.00	1.77
		Swimming Time	216.88	13.53	194.50	21.17
SNN	Unenriched	Floating Time	30.88	22.14	71.38	22.47
		Number of Dives	1.13	2.23	0.00	0.00
		Swimming Time	206.13	27.48	168.63	22.47
	Enriched	Floating Time	18.75	13.50	37.00	18.50
		Number of Dives	1.13	1.46	0.00	0.00
		Swimming Time	219.38	14.35	203.00	18.50
SSS	Unenriched	Floating Time	22.25	11.83	47.50	28.29
		Number of Dives	1.88	3.09	0.63	1.41
		Swimming Time	213.25	18.03	191.88	29.17
	Enriched	Floating Time	24.25	13.91	24.25	15.47
		Number of Dives	3.63	3.70	2.50	3.02
		Swimming Time	211.00	17.04	212.75	18.48

Table 4.7.2 Mixed Design ANOVA Results for Forced Swim Task

Source	Dependent Variable	ANOVA Results	Partial Eta Squared
Stress	Floating Time	$F(2,84) = 2.04$, $p = 0.14$	partial $\eta 2 = 0.05$
	Number of Dives*	$F(2,84) = 3.76$, $p < 0.05$	partial $\eta 2 = 0.08$
	Swimming Time	$F(2,84) = 1.31$, $p = 0.28$	partial $\eta 2 = 0.03$
Enrichment	Floating Time***	$F(1,84) = 15.03$, $p < 0.001$	partial $\eta 2 = 0.15$
	Number of Dives	$F(1,84) = 1.55$, $p = 0.22$	partial $\eta 2 = 0.02$
	Swimming Time**	$F(1,84) = 11.28$, $p < 0.01$	partial $\eta 2 = 0.12$
TimePeriod	Floating Time***	$F(1,84) = 31.17$, $p < 0.001$	partial $\eta 2 = 0.27$
	Number of Dives*	$F(1,84) = 4.79$, $p < 0.05$	partial $\eta 2 = 0.05$
	Swimming Time***	$F(1,84) = 24.69$, $p < 0.001$	partial $\eta 2 = 0.23$
Stress * Enrichment	Floating Time	$F(2,84) = 0.92$, $p = 0.40$	partial $\eta 2 = 0.02$
	Number of Dives	$F(2,84) = 1.55$, $p = 0.22$	partial $\eta 2 = 0.04$
	Swimming Time	$F(2,84) = 1.17$, $p = 0.32$	partial $\eta 2 = 0.03$
Stress * TimePeriod	Floating Time	$F(2,84) = 1.58$, $p = 0.21$	partial $\eta 2 = 0.04$
	Number of Dives	$F(2,84) = 0.04$, $p = 0.96$	partial $\eta 2 = 0.00$
	Swimming Time	$F(2,84) = 1.82$, $p = 0.17$	partial $\eta 2 = 0.04$
Enrichment * TimePeriod	Floating Time**	$F(1,84) = 7.85$, $p < 0.01$	partial $\eta 2 = 0.09$
	Number of Dives	$F(1,84) = 0.31$, $p = 0.58$	partial $\eta 2 = 0.00$
	Swimming Time*	$F(1,84) = 4.47$, $p < 0.05$	partial $\eta 2 = 0.05$
Stress * Enrichment * TimePeriod	Floating Time	$F(2,84) = 0.03$, $p = 0.97$	partial $\eta 2 = 0.00$
	Number of Dives	$F(2,84) = 0.25$, $p = 0.78$	partial $\eta 2 = 0.01$
	Swimming Time	$F(2,84) = 0.21$, $p = 0.81$	partial $\eta 2 = 0.01$

Table 4.8.1 Means and Standard Deviations for Circulating Corticosterone (ng/mL)

Stress Treatment	Enrichment Treatment	Pre-Stress Phase		Acute Stress Phase		Chronic Stress Phase	
		M	SD	M	SD	M	SD
Control	Unenriched	387.69	107.00	537.15	461.07	719.11	674.40
	Enriched	61.43	56.96	601.17	416.02	224.60	371.94
SNN	Unenriched	501.05	287.48	170.43	146.87	281.86	418.26
	Enriched	58.69	68.16	297.77	348.42	142.86	160.35
SSS	Unenriched	514.23	295.84	707.48	462.30	364.50	178.36
	Enriched	111.38	174.65	526.01	409.33	151.79	182.02

Table 4.8.2 ANOVA Results for Circulating Corticosterone (ng/mL)

Source	ANOVA Results	Partial Eta Squared
Stress*	$F(2,42) = 4.09$, $p < 0.05$	partial $\eta 2 = 0.16$
Enrichment***	$F(1,42) = 16.16$, $p < 0.001$	partial $\eta 2 = 0.28$
Testing Period*	$F(1.76,73.70) = 4.89$, $p < 0.05$	partial $\eta 2 = 0.10$
Stress * Enrichment	$F(2,42) = 0.42$, $p = 0.66$	partial $\eta 2 = 0.02$
Testing Period x Stress*	$F(3.51,73.70) = 2.64$, $p < 0.05$	partial $\eta 2 = 0.11$
Testing Period x Enrichment*	$F(1.76,73.70) = 4.50$, $p < 0.05$	partial $\eta 2 = 0.10$
Testing Period x Stress x Enrichment	$F(3.51,73.70) = 0.97$, $p = 0.42$	partial $\eta 2 = 0.04$

Table 4.9 Kendall Tau Correlations

Behavioural Task	Reference Variable	Correlated Variable	Correlation Results
-	Corticosterone Levels	Movement Time (Open Field Task)	r = 0.296, p < 0.01 , n = 48
		Margin Time (Open Field Task)	r = -0.255, p < 0.05 , n = 48
		Number of Closed Arm Entries (Elevated Plus Maze)	r = 0.293, p < 0.01 , n = 48
		Time in Open Arms (Elevated Plus Maze)	r = -0.312, p < 0.01 , n = 48
		Novel Object Preference (Object Recognition)	r = -0.305, p < 0.01 , n = 48
		Latency (Morris Water Maze)	r = 0.246, p < 0.05 , n = 48
		Path Distance (Morris Water Maze)	r = 0.262, p < 0.05 , n = 48
		Swimming Speed (Morris Water Maze)	r = 0.252, p < 0.05 , n = 48
		Percent of Group Time in Dominant Position (Water Competition - 2 Rats)	r = 0.267, p < 0.01 , n = 48
		Social Interactions (Water Competition - 3 Rats)	r = 0.326, p < 0.01 , n = 48
Open Field Task	Movement Time	Corticosterone	r = 0.296, p < 0.01 , n = 48
		Margin Time (Open Field Task)	r = -0.254, p < 0.05 , n = 48
		Vertical Time (Open Field Task)	r = 0.560, p < 0.01 , n = 48
		Number of Closed Arm Entries (Elevated Plus Maze)	r = 0.255, p < 0.05 , n = 48
		Number of Dives (Forced Swim)	r = -0.263, p < 0.05 , n = 48
		Total Time with Objects (Object Recognition)	r = 0.242, p < 0.05 , n = 48
		Novel Object Preference (Object Recognition)	r = -0.226, p < 0.05 , n = 48
		Social Interactions (Water Competition - 3 Rats)	r = 0.301, p < 0.01 , n = 48
		Percent of Spout Time Alone (Water Competition - 2 Rats)	r = 0.246, p < 0.05 , n = 48
	Margin Time	Corticosterone	r = -0.255, p < 0.05 , n = 48
		Movement Time (Open Field Task)	r = -0.254, p < 0.05 , n = 48
		Vertical Time (Open Field Task)	r = -0.237, p < 0.05 , n = 48
Open Field Task	Margin Time	Number of Dives (Forced Swim)	r = 0.271, p < 0.05 , n = 48
		Percent of Spout Time Alone (Water Competition - 2 Rats)	r = -0.251, p < 0.05 , n = 48
	Vertical Time	Movement Time (Open Field Task)	r = 0.560, p < 0.01 , n = 48
		Margin Time (Open Field Task)	r = -0.237, p < 0.05 , n = 48
		Number of Closed Arm Entries (Elevated Plus Maze)	r = 0.317, p < 0.01 , n = 48
		Latency (Morris Water Maze)	r = 0.218, p < 0.05 , n = 48

		Path Distance (Morris Water Maze)	r = 0.299, p < 0.01 , n = 48
Elevated Plus Maze	**Number of Closed Arm Entries**	**Corticosterone**	r = 0.293, p < 0.01 , n = 48
		Movement Time (Open Field Task)	r = 0.255, p < 0.05 , n = 48
		Vertical Time (Open Field Task)	r = 0.317, p < 0.01 , n = 48
		Total Time with Objects (Object Recognition)	r = 0.343, p < 0.01 , n = 48
		Novel Object Preference (Object Recognition)	r = -0.235, p < 0.05 , n = 48
		Latency (Morris Water Maze)	r = 0.348, p < 0.01 , n = 48
		Path Distance (Morris Water Maze)	r = 0.405, p < 0.01 , n = 48
		Swimming Speed (Morris Water Maze)	r = 0.273, p < 0.01 , n = 48
		Percent of Group Time in Dominant Position (Water Competition - 2 Rats)	r = 0.415, p < 0.01 , n = 48
		Social Interactions (Water Competition - 3 Rats)	r = 0.349, p < 0.01 , n = 48
	Time in Open Arms	**Corticosterone**	r = -0.312, p < 0.01 , n = 48
		Floating Time (Forced Swim)	r = -0.251, p < 0.05 , n = 48
		Social Interactions (Water Competition - 2 Rats)	r = -0.321, p < 0.01 , n = 48
Forced Swim Task	**Floating Time**	**Time in Open Arms (Elevated Plus Maze)**	r = -0.251, p < 0.05 , n = 48
		Swimming Time (Forced Swim)	r = -0.889, p < 0.01 , n = 48
		Percent of Group Time in Dominant Position (Water Competition - 2 Rats)	r = 0.285, p < 0.01 , n = 48
	Number of Dives	**Movement Time (Open Field Task)**	r = -0.263, p < 0.05 , n = 48
Forced Swim Task	**Number of Dives**	**Margin Time (Open Field Task)**	r = 0.271, p < 0.05 , n = 48
	Swimming Time	**Floating Time (Forced Swim)**	r = -0.889, p < 0.01 , n = 48
		Percent of Group Time in Dominant Position (Water Competition - 2 Rats)	r = -0.229, p < 0.05 , n = 48
Novel Object Recognition Task	**Total Time with Objects**	**Movement Time (Open Field Task)**	r = 0.242, p < 0.05 , n = 48
		Number of Closed Arm Entries (Elevated Plus Maze)	r = 0.343, p < 0.01 , n = 48
		Time Drinking from Spout (Water Competition - 2 Rats)	r = 0.248, p < 0.05 , n = 48
		Percent of Group Time in Dominant Position (Water Competition - 2 Rats)	r = 0.200, p < 0.05 , n = 48
		Percent of Spout Time Alone (Water Competition - 2 Rats)	r = -0.222, p < 0.05 , n = 48
		Social Interactions (Water Competition - 3 Rats)	r = 0.278, p < 0.01 , n = 48
Novel Object Recognition Task	**Novel Object Preference**	**Corticosterone**	r = -0.305, p < 0.01 , n = 48
		Movement Time (Open Field Task)	r = -0.226, p < 0.05 , n = 48
		Number of Closed Arm Entries (Elevated Plus Maze)	r = -0.235, p < 0.05 , n = 48

		Social Interactions (Water Competition - 3 Rats)	r = -0.204, p < 0.05 , n = 48
Morris Water Maze	**Latency**	**Corticosterone**	r = 0.246, p < 0.05 , n = 48
		Vertical Time (Open Field Task)	r = 0.218, p < 0.05 , n = 48
		Number of Closed Arm Entries (Elevated Plus Maze)	r = 0.348, p < 0.01 , n = 48
		Path Distance (Morris Water Maze)	r = 0.777, p < 0.01 , n = 48
		Percent of Group Time in Dominant Position (Water Competition - 2 Rats)	r = 0.291, p < 0.01 , n = 48
	Path Distance	**Corticosterone**	r = 0.262, p < 0.05 , n = 48
		Vertical Time (Open Field Task)	r = 0.299, p < 0.01 , n = 48
		Number of Closed Arm Entries (Elevated Plus Maze)	r = 0.405, p < 0.01 , n = 48
		Latency (Morris Water Maze)	r = 0.777, p < 0.01 , n = 48
		Swimming Speed (Morris Water Maze)	r = 0.291, p < 0.01 , n = 48
		Social Interactions (Water Competition - 2 Rats)	r = 0.216, p < 0.05 , n = 48
Morris Water Maze	**Path Distance**	**Time Drinking from Spout (Water Competition - 2 Rats)**	r = 0.215, p < 0.05 , n = 48
		Percent of Group Time in Dominant Position (Water Competition - 2 Rats)	r = 0.325, p < 0.01 , n = 48
		Social Interactions (Water Competition - 3 Rats)	r = 0.204, p < 0.05 , n = 48
	Swimming Speed	**Corticosterone**	r = 0.252, p < 0.05 , n = 48
		Number of Closed Arm Entries (Elevated Plus Maze)	r = 0.273, p < 0.01 , n = 48
		Path Distance (Morris Water Maze)	r = 0.291, p < 0.01 , n = 48
		Time Drinking from Spout (Water Competition - 2 Rats)	r = 0.286, p < 0.01 , n = 48
		Percent of Group Time in Dominant Position (Water Competition - 2 Rats)	r = 0.269, p < 0.01 , n = 48
		Percent of Spout Time Alone (Water Competition - 2 Rats)	r = -0.225, p < 0.05 , n = 48
		Percent of Spout Time Alone (Water Competition - 2 Rats)	r = 0.225, p < 0.05 , n = 48
Water Competition Task (2 Rats)	**Social Interactions**	**Time in Open Arms (Elevated Plus Maze)**	r = -0.321, p < 0.01 , n = 48
		Path Distance (Morris Water Maze)	r = 0.216, p < 0.05 , n = 48
	Time Drinking From Spout	**Total Time with Objects (Object Recognition)**	r = 0.248, p < 0.05 , n = 48
		Path Distance (Morris Water Maze)	r = 0.215, p < 0.05 , n = 48
		Swimming Speed (Morris Water Maze)	r = 0.286, p < 0.01 , n = 48
		Percent of Spout Time Alone (Water Competition - 2 Rats)	r = -0.325, p < 0.01 , n = 48
		Corticosterone	r = 0.267, p < 0.01 , n = 48

	Percent of Group Time in Dominant Position	**Number of Closed Arm Entries (Elevated Plus Maze)**	r = 0.415, p < 0.01 , n = 48
		Floating Time (Forced Swim)	r = 0.285, p < 0.01 , n = 48
		Swimming Time (Forced Swim)	r = -0.229, p < 0.05 , n = 48
		Total Time with Objects (Object Recognition)	r = 0.200, p < 0.05 , n = 48
		Latency (Morris Water Maze)	r = 0.291, p < 0.01 , n = 48
		Path Distance (Morris Water Maze)	r = 0.325, p < 0.01 , n = 48
		Swimming Speed (Morris Water Maze)	r = 0.269, p < 0.01 , n = 48
Water Competition Task (2 Rats)	**Percent of Group Time in Dominant Position**	**Social Interactions (Water Competition - 3 Rats)**	r = 0.326, p < 0.01 , n = 48
	Percent of Spout Time Alone	**Total Time with Objects (Object Recognition)**	r = -0.222, p < 0.05 , n = 48
		Swimming Speed (Morris Water Maze)	r = -0.225, p < 0.05 , n = 48
		Time Drinking from Spout (Water Competition - 2 Rats)	r = -0.325, p < 0.01 , n = 48
Water Competition Task (3 Rats)	**Social Interactions**	**Corticosterone**	r = 0.326, p < 0.01 , n = 48
		Movement Time (Open Field Task)	r = 0.301, p < 0.01 , n = 48
		Number of Closed Arm Entries (Elevated Plus Maze)	r = 0.349, p < 0.01 , n = 48
		Total Time with Objects (Object Recognition)	r = 0.278, p < 0.01 , n = 48
		Novel Object Preference (Object Recognition)	r = -0.204, p < 0.05 , n = 48
		Path Distance (Morris Water Maze)	r = 0.204, p < 0.05 , n = 48
		Percent of Group Time in Dominant Position (Water Competition - 2 Rats)	r = 0.326, p < 0.01 , n = 48
	Time Drinking From Spout	**Percent of Spout Time Alone (Water Competition - 2 Rats)**	r = -0.242, p < 0.05 , n = 48
	Percent of Spout Time Alone	**Movement Time (Open Field Task)**	r = 0.246, p < 0.05 , n = 48
		Margin Time (Open Field Task)	r = -0.251, p < 0.05 , n = 48
		Swimming Speed (Morris Water Maze)	r = 0.225, p < 0.05 , n = 48
		Time Drinking from Spout (Water Competition - 2 Rats)	r = -0.242, p < 0.05 , n = 48

CHAPTER 5

General Discussion & Conclusions

5.1 Summary

The main objective of this thesis was to investigate the behavioural effects of ancestral prenatal stress, in the form of transgenerational prenatal stress (TPS) or multigenerational prenatal stress (MPS), and postnatal environmental factors, in the form of artificial food dye (AFD) consumption or environmental enrichment (EE). The present body of work was the first to investigate the behavioural consequences of TPS and MPS in a rat cohort, and to consider how adverse (AFD) and beneficial (EE) postnatal experiences may interfere with programming by ancestral stress. In *Chapter 1*, the existing literature and knowledge surrounding ancestral stress and postnatal environment were discussed. In *Chapter 2*, the effects of fourth-generation MPS and AFD consumption on lifespan locomotor and anxiety-related behavioural profiles were investigated using the open field task, as well as a novel affective exploration task (Erickson et al., 2014). In *Chapter 3*, the effect of TPS and MPS on performance in a cognitive odor discrimination task was investigated. In *Chapter 4*, the effect of TPS and MPS, as well as EE, on locomotor, cognitive, affective, and social behaviour were conducted using a multitude of established and novel paradigms. Together, these studies present a novel platform to understand the programming of behaviour and lifespan health by ancestral experiences.

5.1.1 Ancestral Stress as a Signalling Mechanism for Adaptive Change

Our results suggest that early-life experiences play a critical role in behavioural

development. As described in previous chapters, a family history of prenatal stress resulted in changes in locomotor and exploratory behaviour. MPS, but not TPS, rats showed hyperactive behaviour in the open field task, as well as increased diving behaviour in the forced swim task. Furthermore, stressed rats showed increased swim speed in the Morris water maze and increased time investigating objects within a novel object recognition task. Interestingly, TPS, rats did not show an increase in anxiety- or depression-like behaviour, while MPS rats showed a down-regulation of anxiety-related behaviour in the open field when exposed to acute stress. Finally, ancestral stress did not result in any deficits in learning and memory.

Prenatal exposure to elevated glucocorticoids (GCs) has been suggested as an early-life environmental signal to the developing offspring that the environment it is about to encounter will be stressful and dangerous (Glover, 2011). We suggest that this developmental signal is not binary in nature, but rather that the signalling is requisitely cumulative across multiple generations in order to produce a truly adaptive phenotype. This is supported by the results found in our study that MPS rats showed a phenotype more adaptive for a hostile environment. For example, MPS rats showed a more consistent increase in locomotor/exploratory behaviour across behavioural tasks when compared to TPS rats, as well as increased locomotor behaviour during acute stress. However, TPS rats showed a decrease in circulating corticosterone during the acute stress period, while MPS rats were more similar to controls. These results suggest that GCs are not the sole mechanism of stress response programming.

Our results suggest that behaviour is heavily affected by ancestral stress. Even a single generation of prenatal stress in the F_1 generation, as demonstrated in the TPS rats,

resulted in significant behavioural effects in F_3 generation. Observed TPS effects included an increase in locomotor activity, changes to stress response, and increased aggression. As these results are seen in the F_3 generation of the TPS rats, we can be relatively confident that the behavioural effects of TPS are the result of epigenetic changes in gene expression (Skinner, 2008). However, it should be noted that maternal care may also have influenced the development of the intervening generations, as stress-related changes in maternal care have been shown to be affected by transgenerationally stress (Ward et al., 2013).

In contrast to the single generation of stress induced in rats of the TPS lineage, MPS is more representative of the culmination of many different environmental factors all influencing the gradual adaptation of a family line across generations. Admittedly, a careful investigation of each generation would be required to confidently state that MPS represents the cumulative change of multiple generations of gene-environment interactions. However, the only difference between TPS and MPS experimental conditions was the absence of prenatal stress in the F_2 and F_3 generations. We can, therefore, reasonably postulate that the increased frequency of prenatal stress exposure resulted in the more adaptive phenotype seen in MPS rats. We may expect hippocampal GR density to be lower in MPS rats than in TPS rats, as prenatal stress results in down-regulation of hippocampal GR gene expression (Champagne et al., 2006; Weaver et al., 2004; Welberg et al., 2000).

5.1.2 Postnatal Environment as a Mediating Factor in Behavioural Development

In addition to ancestral stress, our results suggest that postnatal environment has a significant impact on behaviour. As described in Chapter 2, consumption of artificial food dyes (AFDs) results in an increase in locomotor behaviour. This effect is independent of

ancestral stress effects, suggesting that AFD effects are universal, and do not only alter behaviour in developmentally compromised offspring in cases such as ADHD. Furthermore, consumption of AFDs did not produce hyperactivity beyond the period of consumption during adolescence, suggesting that AFD consumption during critical neurodevelopmental periods does not increase the risk for behavioural symptoms later in life. In fact, AFD consumption in prenatally stressed rats may have reduced hyperactive behaviour in adult rats, potentially due to the usefulness of stimulants in treating dopamine-related hyperactive symptoms (Prediger et al., 2005; Sagvolden et al., 2011).

Furthermore, our results suggest that environmental enrichment (EE) results in a number of beneficial effects. In fact, EE resulted in beneficial changes in behaviour in every behavioural test used in Chapter 4, including decreased exploratory behaviour, decreased anxiety-related behaviour, decreased depression-like behaviour, improved learning and memory, and decreased social aggression. It is reasonable to conclude that EE was beneficial regardless of ancestry, as even unstressed rats showed beneficial changes in many behavioural measures. In addition, circulating corticosterone levels were also reduced in EE rats, suggesting that the HPA axis is sensitive to postnatal environmental effects.

The present results relate well to human application, as early-life experience in humans has been shown to play a large role in the development of a host of psychopathological conditions (Glover, 2011). For example, the risk for anxiety and depression are significantly higher in neighbourhoods with higher poverty rates (Santiago et al., 2011). It is reasonable to assume that an increased endeavour to enrich the developmental environment in humans would have similar effects, which is especially

important for families and homes with an increased risk for psychopathological disorders such as ADHD, learning disabilities, anxiety, and depression. Our study further supports efforts that facilitate the development of programs within communities to empower parents and educators to enrich the lives of the children under their stewardship. Furthermore, our study suggests that such endeavours may reduce the risk of mental illness in the children affected by such programs, allowing them to live happier and more productive lives.

5.2 Adaptive Change in Response to Environmental Factors

The present body of work represents a novel platform to further understand the interaction of ancestral stress and postnatal factors. Given the results mentioned previously, we conclude that ancestral stress plays a role in behavioural change across generations. Furthermore, we conclude that MPS results in more adaptive changes than TPS, and that this may be due to a more consistent exposure to prenatal stress in the ancestral line. We suggest that the development of an adaptive behavioural phenotype in a stressful environment can be explained well by first considering the Three-Hit Concept of vulnerability and resilience (Daskalakis et al., 2013) and the Mismatch Hypothesis (Schmidt, 2011). Within the Three-Hit Concept, environmental factors are most influential during three critical periods of life: (1) pre-conception via ancestral predisposition (i.e. genetics or epigenetics), (2) early-life environment, and (3) later-life environment (Daskalakis et al., 2013). According to the Mismatch Hypothesis, if environmental conditions during one of these critically susceptible periods result in a developmental signal that does not coincide with a later realm, the mismatch results in a phenotype that is ill-prepared for the environment in which they must survive (Schmidt, 2011). While this

paradigm appears to be beneficial for the long-term survival of a species in a natural habitat, it is important to consider the implications in modern-day *Homo sapiens.* The mismatch of environmental conditions during critical periods has been suggested to be at the heart of many of the psychopathologies in humans in the 21st century, including attention deficit hyperactivity disorder (ADHD), depression, and anxiety disorders (Glover, 2011). The apparent mismatch between genetic predisposition of early-life environment with later-life environment may be an adequate explanation for maladaptive symptoms.

In the early *Homo sapiens* hunter/gatherer, early-life environmental stress, such as famine or increased competition for resources, over time may have resulted in epigenetic and neuroendocrine events that resulted in a hyperactive phenotype. A single generation of prenatal stress during famine most likely would not have been sufficient to produce phenotypic optimization, but would instead result in many of the symptoms seen in the study of prenatal stress to date, i.e. increased anxiety-related behaviour, decreased cognitive flexibility, etc. (Weinstock, 2008). After multiple generations of "recalibration" via constant gene-environment interactions, the result would be a phenotype better adapted for survival in a hostile environment.

This same mechanism of transgenerational epigenetic adaptation has been preserved in modern humans. The rapid sociocultural evolution of *Homo sapiens* has resulted in a mismatch between the behavioural outcomes of ancestral stress and the behavioural requirements of the typical environment of an adult in a modern Western society. As a result, early-life stress produces a phenotype that is maladapted to the later-life environment, which favours individuals with a lower level of impulsivity, anxiety, and cognitive impairment. Unfortunately, this mismatch only serves to increase the level of

stress in the individual, which increases the risk of epigenetic predisposition in future generations. This results in a multigenerational "snowballing" of psychopathic symptoms. Fortunately, our results provide evidence that these effects can be mediated through optimizing early-life environment via enrichment.

While it has been confirmed by rodent research that a single generation of prenatal stress produces a distinctive phenotype (Weinstock, 2008), to our knowledge, the present case is the first demonstration of multiple generations of stress producing a less anxious phenotype in a rodent model. However, our results may relate to results in both plants (Boyko and Kovalchuk, 2011; Molinier et al., 2006) and bacteria (Rosenberg and Hastings, 2003), in which multiple generations of stress increased the likelihood of adaptive change in later generations (Lopez-Maury et al., 2008). While still controversial, some researchers have suggested that stress may actually accelerate evolution via a combination of epigenetics and mutation (Rosenberg and Hastings, 2003; Vojta and Zoldos, 2013). In short, the longer a gene sequence is methylated, the greater the risk for mutation at the genetic level. Therefore, if gene-environment interactions across multiple generations result in consistently methylated areas of the genome, those sequences would be at a higher risk for mutation and potential change. The implications of such a mechanism are tremendous, as it would drastically reduce the role of *random* mutation in evolution, and instead favour *environment-directed* mutation. Our results support the notion of environment-directed mutation, as MPS rats were better suited for a stressful environment than TPS rats. However, change within a few generations of ancestral stress is more likely to be the result of more proximal causes, such as elevated GC exposure *in utero*, changes in maternal care, and epigenetic regulation of gene expression.

5.3 Limitations & Considerations for Future Research

In interpreting the results of this thesis, there are a number of important factors to consider. First, many of the effects of both ancestral stress and postnatal environment may differ in female offspring. Secondly, it is important to consider the complex nature of both ancestral stress and postnatal environment when applying to other species, including humans. Human-specific factors (e.g. socioeconomic status, access to education, cultural difference) may influence environmental stress or enrichment in ways that cannot be explored in a rodent model.

We feel that further investigation is needed to understand the causal role of epigenetics in behavioural changes. A complete analysis of each generation of both TPS and MPS rats may further demonstrate the role of both heritable and environmental factors in the development of mental illness. Furthermore, a similar study investigating transgenerational enrichment and multigenerational enrichment may also provide valuable insight into the nature of environment-driven changes in behaviour. We feel that further investigation is needed to understand the role of the Mismatch Hypothesis in understanding how prenatal stress and EE truly interact, and if there are any special considerations needed when providing early-life interventions with children predisposed to HPA-axis programming effects.

5.4 Conclusions

The results of this body of work suggest that adaptive behavioural change is the result of multiple interacting factors. Both *ancestral stress* and *postnatal environment* have significant effects on behaviour. The interaction of these factors may serve to optimize the

organism for survival in a wide range of environments. This constant calibration process may operate both within individuals and across generations via transgenerational epigenetic mechanisms. Our results suggest that endeavours to optimize and enrich the lives of children, adolescents, and even adults are beneficial and may significantly reduce the cost of mental illness.

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