ANCESTRAL PRENATAL STRESS IN F3 AND F4 RATS: THE MATCH/MISMATCH HYPOTHESIS

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ABSTRACT

Cumulative lifetime stress can have detrimental impacts on developmental trajectories and life outcomes. The impacts of stress over multiple generations become a more complex issue. This thesis aimed to understand the impacts of prenatal stress exposure over four generations of rats and worked to develop a maternal stress index (MSI) that adequately quantifies the physiological dysregulation induced by stress. This study assessed the impacts of two different protocols of ancestral prenatal stress in rats. The models included transgenerational prenatal stress (TPS) where the filial 0 generation (F0) dams experienced mild gestational stress, and each subsequent generation afterwards experienced unstressed pregnancies, and multigenerational prenatal stress (MPS), in which each generation experienced stress during gestation. The first study investigated the impacts on F2 dams and F3 young offspring and revealed that the novel MSI was sensitive to maternal stress and predicted the behavioural phenotype in the offspring. The TPS lineage was linked to more adverse pregnancy and developmental outcomes than the MPS lineage. The second study measured the impacts of TPS and MPS on the F4 generation. It was determined that some of the negative impacts found in the F3 generation had disappeared, but reduced exploration in MPS animals was observed. The third and final study focused on the aging F4 generation of the MPS lineage and found that anxiety behaviour at postnatal day 110 was able to predict anxiety at 12 months of age. It was also determined that the lifespan of MPS animals lengthened compared to controls. These studies reveal patterns of generational adaptation to stress and postulate models that predict lifetime health trajectories.

PREFACE

Contribution Of Authors

Literature Review

Teddi Reynolds and Gerlinde Metz wrote the manuscript.

Study 1, 2 and 3

Teddi Reynolds, Mirela Ambeskovic and Gerlinde Metz designed the experiment; Teddi Reynolds, Mirela Ambeskovic and Erin Falkenberg performed the data collection; Teddi Reynolds, Mirela Ambeskovic, Erin Falkenberg, and Oluwagbohunmi Awosoga participated in data analysis; Teddi Reynolds, Mirela Ambeskovic and Gerlinde Metz wrote the manuscript; all authors provided edits and approved the final version of the manuscript.

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LIST OF ABBREVIATIONS

11β-HSD2 – 11 beta-hydroxysteroid dehydrogenase type 2
ACTH – Adrenocorticotropin hormone
AL – Allostatic load
CORT - Corticosterone
CRH – Corticotropin releasing hormone.
CVD – cardiovascular disease
ELISA - enzyme-linked immunosorbent assay
F – Filial Generation
G – Gestational day
HPA – Hypothalamic-Pituitary Adrenocortical
ICPMS – Inductively coupled plasma mass spectrometry.
LBW- Low birth weight
MPS – Multigenerational Prenatal Stress
MSI – Maternal Stress Index
NA/K – Sodium potassium ratio
PTB – Preterm Birth
P – Postnatal day
rCALM – Rat Cumulative Allostatic Load Measure
TSST – Trier social stress test
TPS – Transgenerational Prenatal stress

Models of Prenatal Stress: A Literature Review

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Introduction

All factors that disrupt the body's physiological balance, known as homeostasis, are considered stressors [1]. Stress is not a single process within the body, but a wide range of reactive factors that influence health. The physiological stress response is elicited by external stimulation that is perceived as a threat. This response mainly involves stimulation of the sympathetic nervous system and hypothalamic-pituitary adrenocortical (HPA) axis while suppressing the parasympathetic nervous system.

The sympathetic nervous system and the HPA axis together comprise the sympathoadrenal system. The activation of the sympathoadrenal system causes the release of catecholamines, such as adrenalin and noradrenalin, from the sympathetic nerve terminals and the adrenal medulla [2-4]. This in turn stimulates the secretion of corticotrophin from the anterior pituitary. Corticotropin mediates the release of glucocorticoids from adrenal cortex. The major glucocorticoid in humans is cortisol, and the rodent equivalent is corticosterone. Glucocorticoids are part of an essential negative feedback mechanism that prevents their over-production, and subsequent damage to the nervous system [5]. After the sympathoadrenal system is repeatedly activated, adrenal steroid receptors in the hippocampus are often damaged, which reduces sensitivity of the

glucocorticoids' negative feedback system, resulting in the over production of glucocorticoids [6, 7].

Chronically elevated glucocorticoid levels can facilitate the pathogenesis of disorders and malfunctioning of the sympathetic nervous system [8, 9]. For example, chronic stress can re-program the blood glucose regulatory system. Elevated glucocorticoids increase the rate of gluconeogenesis [10], which results in higher levels of circulating glucose [3-5]. This can lead to metabolic disorders such as type 2 diabetes [8]. Also, an excess of glucocorticoids has an impact on neuronal cytoarchitecture, including dendritic pruning in the hippocampus and prefrontal cortex, and dendritic expansion and synaptic formation in the amygdala [11]. This dendritic expansion results in superfluous emotional memory formation and reduced episodic and semantic memory formation [12-16]. There is also evidence of suppressed immune function due to long periods of elevated circulating glucocorticoid levels [3, 17, 18]. It has been shown that elevated levels of glucocorticoids can also interfere with the development and further functioning of the circadian rhythm [6, 12]. Since rodents serve as the prime models in exploratory studies of prenatal stress and health outcomes, this review will summarize some of the most common parameters and their clinical equivalents. In addition, this review aims to identify some of the gaps in basic and clinical approaches in prenatal stress research.

Prenatal Stress as a Risk Factor in Lifetime Health Trajectories

Due to the widespread effects of stress on the body, it is not surprising that stress during gestation can negatively impact the development of the fetus. Prenatal stress is defined as stress before birth, during gestation. Even a normal pregnancy by itself induces a slight stress response in the mother, as detected in markers such as elevated corticotropin releasing hormone (CRH), and adrenocorticotrophic hormone and cortisol [3, 19, 20]. Stress reportedly further elevates these markers, entering the fetal circulatory system through the placenta and potentially programming the fetal stress response with lasting consequences. It is thought that maternal stress can lead to higher risk of gestational diabetes [21] and preterm birth (PTB) [22-24], low birth weight (LBW) [25, 26] and other adverse pregnancy outcomes. In the newborn, prenatal stress can lead to developmental delays [27, 28], and long-term disabilities [29-33]. In fact, it has been postulated that PTB may be an adaptive response to maternal stress, to minimize the effects of the pregnancy on the mother and in order to remove the fetus from the stressful environment before its health is further affected [3]. It is not yet fully understood how the stress experienced by the mother and her subsequent elevated cortisol levels affect the HPA axis of the fetus, though it is proposed that the stress affects the placenta in a way that alters the flow of cortisol to the fetus [34]. The amount of maternal cortisol that the fetus is exposed to is at least partially regulated by 11beta-hydroxysteroid dehydrogenase type 2 (11β-HSD2), which is an enzyme within the human placenta and catalyzes the conversion of glucocorticoids to an inactive form, cortisol [35, 36], and prenatal stress has been shown to down-regulate 11β-HSD2 through methylation [37]. While much is

known in regard to prenatal stress in humans, studying rodents enables researchers to study the effects of various stressors more rigorously.

Rodent Models of Gestational Stress

There are multiple methods by which to induce stress in pregnant rats, such as swimming, environmental heat, social isolation or exposure to unpredictable light or noise [4, 38]. A large variety of studies regarding prenatal stress in rats have used the paradigm of restraint stress. The timing and duration of the restraint stress in most protocols varies from 20 minutes a day up to 9 hours a day, for 3 days up to 10 days, at different times during gestation [28, 39-42]. While there are many congruencies, there is also a wide range of variability in the results, suggesting the ways in which the studies differ has an impact on various health outcomes, as outlined in the following.

Maternal Weight

Many studies that tracked weight loss in mothers undergoing restraint stress during gestation demonstrated a reduction in weight gain compared to the naïve controls [28, 39]. The immobilisation and subsequent elevation in body temperature of stressed rats consistently produced weight loss, or in pregnant mothers a reduction in weight gain. This reduction in maternal weight gain during pregnancy is related to a reduction in birth weight, Baker et al. [39] found that the gestationally stressed mothers had female offspring that were significantly smaller than controls, whereas Barlow et al. [28] found that stressed mothers had smaller male and female pups. It is also important to note that

this reduction in weight gain is likely not common to all types of gestational stress [43-45].

Corticosterone

In Mothers. Smith [18], using rats, showed an elevation of corticosterone in the pregnant mothers due to restraint stress for 1 hour a day, from gestation days 10-20. An earlier study by Barlow et al. [28], however, found no change in maternal corticosterone during restraint stress for 9 hours a day over 3 consecutive days of gestation. These two studies are the only viewed in this review that measured maternal corticosterone. This is a major failing of many studies thus far, in that few examined the changes in the mothers beyond maternal behaviour. If changes in the offspring due to prenatal stress are to be fully understood, it is crucial to better understand the metabolic, immune, neuroendocrine, and affective state of mothers that are being stressed during gestation. Important pieces of information can be determined through stress studies in non-pregnant rats, such as a study by Zardooz et al. [5], which showed an elevation of corticosterone due to restraint stress. However, as this is not studied in pregnant rats, inferences cannot easily be made about the effect of restraint stress during gestation.

In Offspring. Corticosterone is a common assessment of stress especially in prenatally stressed rodents. Maccari et al. [6] suggested that the elevation of corticosterone in prenatally stressed offspring is regardless of sex. This is also seen in a study by Smith [18], but in a different pattern, where the levels initially started off lower than controls, but rose faster and became elevated after a swim stress task [18]. Other

studies performed after prenatal restraint stress have shown sexual dimorphism in terms of corticosterone levels, with males demonstrating similar levels of corticosterone compared to controls, or even a reduction in corticosterone [40, 41]. McCormick et al. [42] also demonstrated sexually dimorphic results in that the female prenatally stressed rats showed elevated corticosterone levels 20-70 minutes after an additional stress treatment. By contrast, male corticosterone levels were not significantly altered by the prenatal stress. A study by Weinstock et al. [46], which used noise and light stress throughout gestation showed similar dimorphic effects with females having significantly elevated levels of corticosterone and the males having a small, non-significant increase. Studies on the effect of maternal separation and handling reveal an elevation of corticosterone after maternal separation of over 3 hours, and a mediating effect of handling [47, 48]. This suggests that the understanding that males are more sensitive to prenatal stress [49, 50] may not be relevant when measuring corticosterone [51-53]. While corticosterone is frequently measured in the offspring, it is not a biomarker that is commonly measured in pregnant females.

Glucose

Glucose levels are a potentially revealing marker rarely measured in prenatal stress studies in rats. Only one study by Maccari et al. [6] revealed the effect on glucose levels after prenatal stress and found higher rates of hyperglycemia. Many studies viewing the direct effects of restraint and swim stress on non-pregnant rats examined glucose levels, and every one found that glucose levels were increased in the adult rats [5,

10, 54]. There was also an upregulating effect on glucose while undergoing foot-shock stress, and while witnessing foot-shock stress [4].

The effect of stress on glucose levels has been proposed to be due to two potential mechanisms; 1) the hyperglycemic effect of catecholamines and glucocorticoids released by the sympathetic adrenal medullary system and pituitary adrenocortical systems, respectively; and 2) the alpha-adrenergic inhibition of insulin [4, 5]. These physiological markers serve as important indicators of stress vulnerability, resilience, and wellbeing, in addition to several behavioural markers that can indicate an animal's stress and anxiety levels, including maternal behaviour and maternal affective state.

Maternal Behaviour

Maternal behaviour is widely acknowledged as an important marker predicting offspring health and development [55, 56], although it still is not widely measured when studying gestational stress. When maternal behaviour is recorded, relevant behaviours include licking and grooming, nursing, retrieving behaviour, and tail chasing [55, 56]. These will be discussed in the following.

Licking. In rodents, evidence suggests that the duration of maternal contact with an offspring has significant influence on long-term offspring behaviour [57, 58]. Time spent licking, as well as nursing are the most commonly measured indicators of maternal time spent with offspring, with significant positive influences on offspring development [59]. Prenatal stress studies of licking behaviour yielded mixed results, however. Several

studies suggested no change in licking behaviour in stressed mothers [18, 60]. On the other hand, several studies saw a reduction in licking behaviour due to restraint and maternal separation stress [39, 48].

Nursing. Several studies found a reduction in arched back nursing due to maternal stress, which reflects the active form of nursing, during both restraint stress as well as maternal separation [18, 48]. However, a study by [45], measuring the effects of chronic social stress by an unfamiliar male intruder, found that social stress diminishes nursing efficiency, which then resulted in increased total nursing time. The discrepancy between these results is likely due to the type of stressor applied and because Murgatroyd and Nephew did not differentiate among the type of nursing, only their duration. Moreover, this observation emphasizes the need to assess an array of different behaviours from the mothers instead of focusing on only one type.

Offspring Retrieval. Offspring gathering is generally assessed by scattering the offspring throughout the cage and timing the latency of the mother to retrieve all the offspring and place them back into the nest [61, 62]. A study by Smith [18] suggested that dams undergoing restraint stress gathered their offspring much more quickly than controls. A cross-fostering experiment by Meek et al. [60] suggested that stressed moms retrieved non-stressed offspring very fast, and non-stressed moms retrieved stressed offspring very slowly. In Meek's study, it was discovered that non-stressed mothers raising non-stressed fostered offspring retrieved their offspring faster than stressed mothers retrieving stressed fostered offspring [60], contrary to the previously stated

literature in this field. Murgatroyd and Nephew [45] performed a study using chronic social stress in rat dams and found that the socially stressed moms also retrieved their offspring very quickly.

Tail Chasing. Tail chasing in pregnant dams represents a behaviour that is thought to prepare mothers for post-partum pub retrieval. It is characterized by a chasing of the tail, picking up the tail with the mouth, and carrying the tail to the nest [63]. Tail chasing was first characterized by Ward et al. [56] in pregnant rat dams as a unique indicator of post-delivery behaviours. Tail chasing was found to increase in frequency around 12 hours before parturition. Ward et al. [63] observed that the tail chasing duration and frequency decreased in rats that were stressed, as well as in rats which had great grandmothers that were stressed during their gestation suggesting this behavioural marker serves as a biomarker of transgenerational programming by ancestral adverse experience.

Affective State

It has been well documented that stress negatively programs the affective state of pregnant mothers [18, 39, 64]. A common and consistent marker of affective state in rodents is immobility in the forced swim task [65, 66]. Several investigations have found that mothers stressed during pregnancy via gestational restraint stress, show increased immobility during the forced swim task [18, 39]. Immobility serves as an indicator of depression-like behavior and learned helplessness, and this effect can be moderated via anti-depressant drug treatments [66].

Anxiety-like behaviours are often measured with the elevated plus maze, which consists of two closed arms and two open arms of a Plexiglas structure raised above the ground [67]. The rats' natural desire to explore competes with its fear of heights and novel environments. If an animal responds anxiously to the novel environment of the EPM it will tend to spend most of the time in the closed arms. With lower levels of anxiety-like behaviour, it will tend to spend more time in the open arms [67]. A study by Baker et al. [39] revealed increased anxiety-like behaviours in young rodents, but later showed that as the offspring age, they started to exhibit less anxiety-like behaviours than age-matched controls [68]. The literature on anxiety due to stress is relatively divided. One study demonstrated that rats that were prenatally stressed through injections and then stressed directly through restraint stress, were more anxious at postnatal day 45 (P45), but not at P25 [69]. With various stressors, some of the same fearful and anxious behaviours in the EPM have been demonstrated [70, 71], but there are studies that claim the opposite effect, such as the rats becoming more active after prenatal stress induced by social conflict [40]. The variations of anxiety measured are likely determined by the type of stressor used, such as injection, restraint, or social conflict prenatal stress.

Offspring Developmental Health

Offspring and litter characteristics are routinely measured to determine the developmental health trajectories. The classic measurements include offspring weight and litter size, plus other measures generally focused on developmental stages, including eye opening, teething time, and vestibular and reflex response. Offspring's weight at birth commonly is not found to be significantly altered by prenatal restraint stress [39], though

found a significant reduction in weight in older males and a non-significant trend of a weight reduction in females as they age [39, 68]. This observation contrasts with Barlow et al. [28] and Patin et al. [72], who found low birth weight in the prenatally stressed offspring regardless of sex, likely due to elevated glucocorticoids, which has been shown to reduce birth weight [7]. Moreover, Brunton et al. [43] found that prenatal stress was associated with reduced birth weight in females but not males. Amugongo and Hlusko [73] found prenatal stress caused faster physical development in offspring in terms of bone formation. Patin et al. [72] also reported a drop in litter survival in the repeated prenatal stress group. Barlow et al. [28] found an effect of prenatal restraint stress on offspring development in terms of slower ear opening, delayed teething, a retarded startle response, and a longer latency to jump into the home cage when the animals' eyes were still closed. Though the animals were developmentally delayed, they were still born after regular gestation length. In terms of offspring numbers and mortality, Baker et al. [39] reported a reduction in the number of offspring born as well as an increase in offspring mortality, while Smith [18] reported no change in litter size.

Previous reports have also pointed out learning deficits in prenatally stressed offspring when performing the Morris water maze [6, 41]. The effect of prenatal stress on learning is further supported through a study by Yang et al. [74] that found an impairment in the development of long-term potentiation and long-term depression in hippocampal neurons in offspring that were 5 weeks old. This developmental change in the hippocampus supports the deficit in the Morris water maze task. Furthermore, in the lateral and basolateral nucleus of the amygdala researchers have found

neurodevelopmental changes in prenatally stressed rats as early as 7 days after birth. It is possible that this developmental trajectory induces the alterations in anxiety related behaviour mentioned previously in this review [75].

Sodium/Potassium Ratio

A novel marker of health that has not been very rigorously studied in animal models is the sodium potassium ratio (Na/K). This can be determined by analyzing the individuals' urine, hair, or various other tissue samples [76]. This marker has not been used in prenatal stress studies, but there are studies using chronic acceleration stress that can aid in understanding this phenomenon, including findings if stress-induced hypertension [77]. After chronic immobilisation stress, researchers have seen a decrease in sodium consumption and a reduction in sodium (Na^+) and potassium (K^+) urinary excretion, possibly facilitated through an alteration in kidney activity. It was concluded that stress could cause hypertension due to impaired sodium excretion [77], although the internal Na^+ levels were not measured. It is possible that internal Na^+ levels could be elevated due to the reduction in eliminated Na^+. It is also notable that other researchers have also found stress to be an antidiuretic, particularly in dogs, supporting the theory that stress may affect kidney function [78].

Gell et al. [79] compared acceleration stress to tied-down stress and controls. They found that the acceleration stress increased K^+ levels in the brain tissue, in contrast to its expected reduction due to a general shift of intracellular K^+ into extracellular fluid. This may have been due to the fact that physical activity and trauma has been found to

leach K^+ out of muscles, but during the acceleration stress paradigm the rats were unable to move, therefore resulting in more K^+ in the tissues than when they were mobile (control) or were struggling against the ties (tied) [79].

These findings suggest that Na^+ and K^+ levels are relevant to stress studies, and suggest an interesting biomarker for future prenatal stress studies, particularly with the relevance of the Na/K ratio for cardiovascular disease (CVD) [80], and the relevance of CVD as a consequence of prenatal stress [81]

Common Challenges Presented by the Use of Rodent Models

While rodent studies of prenatal stress are often complementary in nature, challenges in the use of variables and their limited clinical validity are evident. Below we will discuss some of the most critical variables that determine the results of prenatal stress studies: the duration of stress, the timing and nature of the stressor, and age.

Duration of Stress

Each study varies drastically with how long the stressor is applied, and whether it is applied in a single session or divided between multiple sessions throughout the day. Some of the differences may seem minor, but they still may significantly affect the results and the subsequent conclusions drawn. For example, Baker et al. [39] performed an investigation on the effects of maternal restraint stress on the health of the offspring. The stress consisted of one 75-min session a day from gestational day (G) 10-19. The results showed a reduction in maternal care behaviours of licking, hovering, carrying, and

sniffing, as well as a smaller litter size born to the gestationally stressed rats. A study by Smith [18] used an almost identical restraint stress protocol, from G 10-20 for 1 hour a day, but found no differences in grooming and licking behaviour, and no change in the litter size. A small change of a reduction of 15 minutes of stress a day may have been enough to change the maternal behaviour in the mothers, but also other factors may be responsible for this discrepancy, such as rat strain, Baker et al. [39] used Long-Evans from Charles River, Quebec, and Smith [18] used Hooded Listers, bred in house.

Stressors differ in terms of the physiological response, depending on whether it is interpreted as chronic or acute [82, 83]. Thus, the main discussion about the duration of stress has centred on the comparison of acute vs. chronic stress [2, 83]. These studies support the notion that acute and chronic stress affect the body in different ways.

Timing of Stressor

There is no consensus of which timing of the onset of stress during gestation is the ideal model to reproduce the consequences of human gestational stress. The timing of stress ranges from application throughout the entirety of gestation to stressing dams only right before parturition. There are various sensitive periods in fetal neural and physiological development that occur throughout pregnancy and during early childhood [15], and disruption by stress during different stages may affect the development of various different systems and organs. Stress during different periods of fetal development and the potential associated health effects have been demonstrated in a study by Barlow et al. [28]. Here, pregnant dams were stressed by restraint at four different times during

gestation: G9-11, G12-14, G15-17, and G18-20. It was found that there was a greater effect on the weight of the dams and gestational length when they were stressed late during pregnancy, in addition to greater effects on all offspring markers including low birth weight, and delayed development of the startle response. For the rodents this suggests that the later the stress is applied during gestation, the more drastic the effects on offspring. An alternative theory is that stress later in pregnancy does not allow much recovery time for the mother, or the fetus, before birth [28].

Nature of the Stressor

Different stressors vary according to their nature and intensity. Thus, it is challenging to compare their salience, and neither should they be expected to affect the body in identical ways. Different types of stress allow for different types of confounds. For example, as previously mentioned, restraint stress has been criticized for consistently producing weight loss in the dams, which might induce some of the changes attributed to the stress. Other stressors also have confounds inherent in the procedure. For instance, swim stress involves strenuous exercise which induces another range of effects on the body through mechanical, thermal, and chemical stimuli, leading to extensive changes in body temperature and metabolic responses in various organs. Other stressors such as light or injection stress do not require any movement or reaction besides that of the stress response. The consequences of these stressors reach far beyond the physiological responses associated with purely psychological stressors. Thus, different stressors and their corresponding confounds may produce inconsistencies in the results, and confusion about the actual effects of stress. These features represent a challenge in the comparison

of the physiological and health consequences of different stressors, their intensities, and their effectiveness.

The Role of Age

As prenatal stress affects the health trajectory across a lifetime, its impact also depends on the age of the offspring when observations are being recorded. Baker et al. [39], [68] provide critical insight on this subject with two of their studies examining the effects of prenatal stress induced by maternal restraint on offspring health and development. The first study by Baker et al. [39] investigated the effects of G10-19 restraint stress for 75 minutes a day on the behavioural phenotype of P30 rat offspring. There was a significant increase in activity in stressed females as measured through total arm entries in the elevated plus maze. An emergence test was also performed, and it was discovered that the females born to stressed mothers took slightly longer to emerge from an opaque box that served as a refuge, suggesting a non-significant trend for more anxious behavior [39].

In their follow-up study with the same rats, Baker et al. [68] observed that at the later age of P85, prenatally restrained offspring showed shorter emergence latencies and more unprotected head dips, which is indicative of reduced anxiety-like behaviour. The authors discussed that anxiety-like behaviour likely does not appear until adolescence at about P60, after maturation of underlying neural circuits [39]. The emergence and progression of age-related disorders may be equally programmed by prenatal stress, and symptoms would become overt only after a critical age has passed [8, 84, 85]. This is

particularly the case in diseases more prevalent in older age such as CVD, diabetes mellitus, and cancer [86] or in mental health disorders [49].

Since rodents are altricial species, their newborns are relatively immobile, and their eyes are still closed. This poses the challenge of certain behaviours and responses not being apparent at early age and unable to be recorded in experimental studies [87, 88]. It can be expected that prenatal stress may delay certain developmental milestones while accelerating the maturation of sensory and organ systems that are critical for survival in a stressful environment. To avoid misinterpretation of experimental findings critical periods of development and maturation need to be considered, especially in pre-clinical studies. Although humans are also an altricial species, translation of experimental rodent data to human studies comes with certain limitations. Ideally, the impact of prenatal stress needs to be studied in humans directly in order to make an assumption on the underlying physiological and molecular pathways.

Prenatal Stress in Humans

The main body of human studies of prenatal stress is divided into retrospective and prospective studies. Retrospective studies are performed after individuals have been exposed to natural disasters or traumatic events, and through questionnaires or other assessment tools the aftermath of these events is being evaluated. The consequences are monitored for several years or even decades after the event occurred [89, 90]. In studies involving prenatal stress, the health of the mother and her child are evaluated and correlated to the objective or subjective impact of the reported stress [91]. Prospective

studies usually focus on more naturally occurring stress due to everyday stress, or the use of a mild to moderate experimental stressors, and consequences are recorded through self-questionnaires or biofluid sampling before, during, and after the stressor [90]. In the case of prenatal stress, often the mother would be evaluated before pregnancy, during pregnancy, and after parturition, and children are evaluated for developmental milestones throughout their childhood. In prospective studies the long-term investigation of consequences of stress become challenging due to financial and time constraints. The vast majority of prenatal stress studies focus largely on the health and development of the child, rather than on the preceding behavioural, physiological or molecular alterations in the mother or even previous generations. The following will provide a few key gestational stress studies that exemplify the main alterations.

Preterm Birth Risk and Low Birth Weight

Some studies have considered the effects of gestational stress on gestational length. One such study performed by Lederman et al. [92] discovered that women pregnant during the 2001 World Trade Center attacks tended to have shortened gestation lengths as well as children with smaller head circumferences. Another study performed by Nkansah-Amankra et al. [25] revealed the effects of low socioeconomic status and poverty during pregnancy on preterm birth risk and low birth weight, showing that the latter correlated strongly with low socioeconomic status and being of African American decent. There was a relatively minimal effect on gestation length. In line with the first study, Wainstock et al. [26] found a reduction in birth weight where the mothers were subjected to frequent air raids during pregnancy. These studies suggest that certain forms

of prenatal stress can influence the length of the gestation period as well as the size of the infant, resulting in a health compromised infant.

Cortisol

Cortisol is the most commonly adopted marker in studies of gestational and prenatal stress in humans. In studying the salience of adverse maternal experiences on offspring cortisol levels, self-rated general mental and physical health in the mothers are often used as a complementary outcome to identify the nature of the stressor. Occasionally, these subjective measurements of stress are also combined with measurements of objective hardship [91]. Retrospective assessments can also be implemented, such as identifying the impact of the 1985 Chernobyl nuclear accident in Ukraine [53]. This study investigated the effects of the Chernobyl accident on mothers who were pregnant during the event, and discovered elevated cortisol levels in infants that were prenatally exposed in the second trimester or later [53].

A common finding is that children born to mothers who self-evaluated as stressed during their pregnancy display elevated cortisol levels, though the diurnal patterns of elevation may vary. Research from O'Connor et al. [93] revealed that basal waking and afternoon cortisol was higher in 10-year-old prenatally stressed children, while a study by Van den Bergh et al. [94] discovered a lower cortisol rating in the morning for 14- to 15-year-old female prenatally stressed children, but a higher cortisol reading in the evening [94]. This discrepancy may stem from different gestational ages of exposure; while O'Connor et al. [93] included mothers who were in the top 15% of self-rated anxiety in

the 32nd week of pregnancy, the study by Van den Bergh et al. [94] included mothers who were most stressed during the 12-22nd week of pregnancy. Moreover, another factor that explains the discrepancies may be the ages of the children at the time of sample collection. Accordingly, a study performed by Grant et al. [95] measured the offspring at the much younger age of 7 months and found that while baseline levels of cortisol did not differ, the prenatally stressed offspring had a higher cortisol response 40 minutes after interacting with a non-responsive and expressionless mother. Gutteling et al. [96] measured the stress of the mother directly. The level of stress experienced was based on the levels of morning cortisol in the mother during pregnancy, as well as on the mother's self-rated fear of bearing a handicapped child. They found that children at 5-years of age had higher cortisol levels on their first day of school, if, during pregnancy, their mother had higher levels of morning cortisol. Entringer et al. [97] researched the effect of severe life events such as the death of a loved one and found that the offspring of stressed mothers were not significantly altered in basal cortisol levels but had lower cortisol after a Trier social stress test (TSST). Entringer et al. [97] also revealed that the reduction in cortisol levels was paired with higher levels of adrenocorticotropic hormone (ACTH).

A selection of studies measured the effects of gestational depression in mothers on cortisol levels in offspring. Brennan et al. [98] examined whether depression before, during, or after pregnancy influences infant cortisol and concluded that depression during pregnancy has the most impact on raising infant cortisol reactivity, and mothers with a history of depression were associated with increased baseline cortisol levels. Furthermore, Oberlander et al. [99] were able to confirm these results by demonstrating

that mothers who experienced depression during their pregnancy gave birth to children with higher stress-induced cortisol levels. Oberlander et al. [99] associated this increase in cortisol reactivity with an increased methylation of the promoter region of glucocorticoid receptor NR3C1.

Glucose and Insulin

The greater stress sensitivity in prenatally stressed children may also be associated with greater risks of metabolic disease. Entringer et al. [85] performed a study viewing the effects of high stress family events during pregnancy on the offspring's glucose and insulin levels. The findings revealed that while there was no change in glucose, there were elevated levels of insulin, which reflects relative insulin resistance [85]. This suggests that prenatal stress can lead to an increase in susceptibility to metabolic disorders, likely due to an alteration to the regulation of various metabolic indicators [21, 43].

Sodium/Potassium Ratio

The Na/K ratio in humans is thought to play a major role in the onset of hypertension, which is a common symptom of stress and a risk factor in cardiovascular, cerebrovascular, and renal diseases. Diets that are high in sodium and low in potassium cause smooth muscle contractions and increased blood pressure, leading to hypertension. Accordingly, a low Na/K ratio is linked to reduced risk of stroke [100], and supplementation with potassium can reduce blood pressure [101-105]. Moreover, the

Na/K ratio has also recently been implicated in metabolic disease risk, including diabetes [106].

Common Challenges Presented by Human Studies

Essentially, human studies are faced with issues similar to the ones faced in animal studies, in addition to dealing with complex lifestyle and environmental factors that differ between populations. Common variables include the type of stressor, the nature of the stressor, the timing of the stress during the pregnancy, and the age of the offspring when tested. Some challenges which are unique to human studies are outlined below.

Retrospective Studies

When dealing with naturally occurring stressors such as the aftermaths of a hurricane or flood, or when investigating individuals exposed to war and violence, it is difficult to measure stress on the mother objectively at the time of her pregnancy. There is an inherent limitation in human stress studies in that the effects of stress on the mother are usually assessed retrospectively, and often through subjective means such as questionnaires. After a natural disaster, researchers may retrospectively evaluate the mother's stress indirectly through objective measures in her child, such as cortisol reactivity. They may also consider the delay of developmental milestones in children and attempt to correlate the results to the stress the mothers claimed to have experienced. It is imperative to realize the limitations of these measures, as they are correlations, not giving insights about causation. Thus, studies of prenatal stress in humans require careful

consideration of the use of controls, and it is challenging to make an assumption on the absolute impact of isolated prenatal stress on behaviour and health outcomes.

Any number of variables may potentially influence the effect of stress on mothers and their children. Dancause et al. [23] performed a study to determine the effects of timing and severity of a natural disaster, an ice storm that hit Quebec in 1998, and the consequences in gestation length. The authors discovered that when retrospectively a mother is much more likely to identify the pregnancy as stressful if the resulting health of her baby was sub-optimal, or if the environment was abnormal [23]. This demonstrates a flaw in subjective measures, such as stress questionnaires, in that they may not be fully accurate and provide limited understanding of the mothers' observable experience and physiological sequelae. Thus, the same team adopted assessments of the mother's objective hardship in subsequent studies, such as the numbers of hours or days without electricity [91].

Another disadvantage of the retrospective evaluation of maternal stress is that it can be challenging to accurately pinpoint the timing of the stressor with the timing of the pregnancy. The mother may not report a specific event that caused stress, but instead a general state of anxiety or stress over a long period of time. Similar to rodent experimental studies, the timing of the stressor during pregnancy represents an important variable in determining the impact on the health of the offspring [107, 108].

Nature of Stressor

While the adversity of events can be confirmed as being stressful, through physiological changes or subjective questionnaires, the impact on the psychological and physiological state of the mother shows significant individual differences. Depending on the mother's personal stress vulnerability, her coping styles and intrinsic resilience, stressful experiences are also going to produce symptoms of variable severity in her offspring. For example, a woman's emotional reactivity will influence her response to stressors such as the loss of a job, low socio-economic status, or divorce [109]. As previously mentioned, the multitude of life stressors and their common and unique risk factors are problematic to control when studying humans.

Different environmental, lifestyle and even ancestral factors affect the intensity of the stressor of interest. An example for the heterogeneity of stress outcomes is provided by studies examining the aftermath of the 9/11 World Trade Center attacks in 2001. This event affected women pregnant at the time and their distress being passed on to their children [110]. In particular, changes in maternal diurnal cortisol fluctuations were predictive of infant distress and response to novelty [110]. Moreover, trajectories of disaster-related post-traumatic stress disorder also revealed significant heterogeneity in their symptoms among first responders [111]. Notably, pregnant mothers responded even when they lived in remote areas at the time, such as the Netherlands, and were at greater risk of giving birth to lower birth weight babies [112]. In addition to the traumatic situation, an added barrage of pollutants and toxic residue from the collapsing structures has been identified to cause long-term adverse health outcomes [113]. This complexity illustrates how a single event can affect pregnancy outcomes at various levels.

External Factors

Numerous environmental factors can modify the results, and it is impossible to keep these consistent between studies or identify all of them explicitly. It is widely acknowledged that factors such as the extent and subjective helpfulness of the mothers' social support system has mediating effects on gestational stress [114, 115]. In support of this finding, a community-based prenatal care strategy, CenteringPregnancy®, has been shown to promote maternal mental health, prenatal education, and behaviours to ultimately reduce adverse birth outcomes [116]. Other factors, such as the mothers' own stress-relieving practices, such as mindfulness and meditation, are also known to mediate of the effect of stress [117, 118]. In line with these concepts, the increasing awareness of the effectiveness of alternative prenatal care strategies has provided more opportunities for pregnant mothers to rely on support systems that may promote healthy pregnancy outcomes.

Maternal Care

The quality of maternal care has a large impact on the health and development of the offspring [55]; [56]. Maternal care can be robustly measured and controlled for in an animal study, but less so in a human cohort. While the vulnerability of a women to lifetime or gestational stress may be apparent, we cannot easily measure day-to-day quality of maternal care in each mother-child dyad, and therefore, cannot be certain whether the health outcomes are due to postnatal or prenatal factors. The question of prenatal stress being altered by postnatal nurturing is explored in several studies [119,

120] which demonstrate that adoption and cross-fostering mediates the effects of prenatal stress, emphasizing the importance of knowing the quality of the maternal care for any prenatal stress study. For example, Brennan et al. [98] studied the effect of prenatal and postnatal depression and found that postnatal depression plays an important role in cortisol reactivity in children. Postpartum depression is known to alter maternal care, thus the attachment formed between parent and child [121].

Ancestral Stress

In order to understand the concept of transgenerational stress programming, we have to consider the concept of epigenetic regulation. Epigenetics is the interface between the environment and the genome and is the mechanism by which stress influences development. Stressors in the environment induce changes in epigenetic markers such as methyl or acetyl groups, or changes to histone structure. These epigenetic markers encourage or discourage transcription, effectively turning on and off genes [122, 123]. Stress during pregnancy affects the developing fetus representing the first filial generation (F1). If the child is female, the same prenatal stress will also directly affect the second filial generation (F2), because the gametes that will generate the grandchildren, the F2 generation, are already present in the womb. This intergenerational impact of stress may create a germ-line dependent epigenetic change [123, 124]. In the maternal lineage, the female great-grandchildren i.e., the third filial generation (F3), therefore represents the first generation that is not directly affected by the prenatal stress. Therefore, if the F3 offspring turns out to be developmentally delayed or behaviorally altered as a consequence of that previous stress, it is not any more a direct effect of stress,

but instead is due to heritable epigenetic changes that have been passed on from previous generations [31, 125]. Transgenerational programming by ancestral stress may provide a mechanism for how mothers could acquire adaptive traits and resilience to stress, but also greater vulnerability. Many experimental studies to date have provided evidence for vulnerability to anxiety and depressive-like behaviours and greater risk of complex diseases in individuals exposed to transgenerational stress [49, 86, 126].

Allostatic Load

A coordinated operation of physiological processes is at work to help maintain the complex balance of intricate physiological functions in the body, also coined homeostasis [3]. The concept of allostasis has been developed to describe the optimal responsiveness to physiological challenges such as stress, or the mechanism by which homeostatic systems are maintained and adjust to ongoing environmental challenges [3, 127]. Allostasis is achieved through various mediators of the stress response such as adrenal hormones, inflammatory cytokines, and neurotransmitters [128, 129]. In the case of repeated or long-term activation of the stress response, the physiological systems that deal with the increased demand in energy and other resources across the body can experience “wear and tear”. The accumulation of this wear and tear can disturb the orchestrated function of physiological and immune systems and result in chronically elevated or suppressed levels of markers such as: blood pressure, glucose, corticosterone, inflammatory cytokines, catecholamines, and others [128]. This dysregulation is called allostatic load (AL) and will eventually lead to disease if left untreated.

The AL concept was initially studied in healthy individuals undergoing various types of stress and was used to understand how complex non-communicable diseases such as cardiovascular disease, type 2 diabetes mellitus, and immune disorders can present in seemingly healthy people [3]. In addition, AL has recently been introduced as a conceptual construct to explain and evaluate the effects of gestational stress in pregnant woman, and to explain and predict offspring health [3, 21, 130, 131]. AL is still considered as an explorative measure and thus as a potential preventative tool is not being utilized to its full potential yet. To assist with translational success from preclinical models, very recently AL assessments have been introduced to rodent models [132, 133]. These tools will be essential in evaluating the complex effects of prenatal stress in rodent models, for testing the validity of AL concepts in experimental contexts, and for lending credence to a translation in humans, leading to predicting and preventing adverse birth outcomes.

Conclusion

Prenatal stress can have serious detrimental effects on offspring development. Prenatal stress studies have been done extensively with humans as well as on rodent models. When studying the effects of preterm stress on newborns the most frequently measured parameters, in rodents, include glucocorticoid levels, glucose, maternal behaviour affective state, and physical development. It was determined that the effect on each parameter is varied alterations in the methodology, including the timing and duration of the stress, the form the stressor takes, and the age of the animals at the time of the study. In humans, prenatal stress studies measure similar parameters, including

glucocorticoids, body weight, and glucose, but can include other measures such as the sodium potassium ratio. In human studies, a key challenge are the inherent restraints of human research, in which one cannot induce stress on pregnant mothers to objectively measure the outcomes of prenatal stress on infants, and therefore cannot indisputably determine a causal relationship between prenatal stress and any health outcome. Instead, human studies are often retrospective, observational studies, in which scientists observe mothers who have already been through a stressful event such as a natural disaster and determine the health outcomes in their children. It is also almost impossible to determine the exact severity of the stress due to other external factors such as socioeconomic status and social support, especially considering the research indicating a mediating effect of enriched environment in rats, and social support in humans [134-136]. When comparing animal and human prenatal stress studies, it is also necessary to bring up the question of translation. It is difficult to produce stress in a model organism that is equivalent to stress experienced in humans and conclusions about stress models need to be made with caution.

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Study 1

Ancestral Trauma and Maternal Health as Predictors of Adverse Pregnancy Outcomes and Offspring Development in the F3 Generation

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Declarations

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Introduction

Gestation is a period of particular vulnerability to stress and other adverse experiences for both the mother and her unborn child [1-4]. Prolonged gestational stress has been recognized as a prime risk factor for adverse pregnancy outcomes, such as preterm birth [5]. Moreover, maternal stress can also affect hypothalamic-pituitary-adrenal (HPA) axis function of the fetus, thus raising the risk of metabolic disorder, such as diabetes, and cardiovascular disease in the child's later life [6-8]. Maternal stress during pregnancy alters programming of the fetal stress response leading to altered brain development and risk of bipolar disorder, impairments in intellect and language development, autism, schizophrenia, anxiety disorders, and depression [9-12].

The consequences of prenatal stress also propagate to subsequent generations via heritable epigenetic mechanisms [13, 14] and may contribute to adverse reproductive and birth outcomes, as well as mental and metabolic disorders in the unexposed offspring [5, 15-19]. Moreover, prenatal stress occurring repeatedly across several subsequent generations may promote new behavioural traits [20] and sex-specific stress resilience [21] in spite of a vulnerability to anxiety-like behaviours [22, 23]. These findings support the match/mismatch hypothesis stating that individuals fare better when adaptation to prenatal or early postnatal experiences, such as stress, is met with similar lifetime experiences [24-27].

The theoretical construct of allostatic load (AL) has been developed to estimate the impact of cumulative lifetime stresses on health in mothers and their children [28].

AL refers to prolonged or repetitive stress that challenges allostasis and the adaptive capacity of the autonomic nervous system and HPA axis, thus inducing cumulative damage to the body [28]. AL indices provide a theoretical basis for understanding the complex and multi-layered biological mechanisms of the impact of chronic and toxic stress on health outcomes [29]. The combination of assessments of multiple biological systems and endocrine variables provides a particularly robust predictor of adverse health outcomes as opposed to any single marker. High AL has been recognized as a primary cause of adverse pregnancy outcomes, inflammation, and other adverse health conditions [30]. While AL has become the most popular clinical framework for risk prediction, the use of animal models is vital to understand the mechanistic aspects of stress programming. The recent development of the rat cumulative AL measure (rCALM) became the first AL-related assessment tool for rodent models [31]. The rCALM provides an effective translational assessment of cumulative stress burden and the therapeutic effectiveness of interventions [31].

Both clinical and experimental research indicates that maternal distress during pregnancy is causally related to offspring health [32, 33]. To extend previous research, we propose a new maternal stress index (MSI) in rats as a translational AL measure based on markers that are assessed in human pregnancy. In this proof-of-concept study, we validate individual maternal biomarkers and the MSI using two rat models of chronic generational stress, to demonstrate their predictive power of adverse pregnancy and offspring development outcomes. The two models of stress included transgenerational prenatal stress (TPS) and multigenerational prenatal stress (MPS), which both produce

characteristic changes in maternal and offspring behaviour and physiology in the F2 and F3 generations [16, 18-20, 22, 23, 31]. The TPS lineage experiences gestational stress within the first generation, followed by non-stressed pregnancies afterwards. The MPS lineage experiences gestational stress in each generation. Moreover, the present study investigated age-dependent sex differences in response to maternal TPS and MPS in the young and adult F3 generation.

Methods

Animals and Experimental Design

This research involved 15 pregnant female Long-Evans hooded rats of the filial 2 (F2) generation and their filial 3 (F3) male and female offspring (n=188; CCC=60, TPS=54, MPS=74) born to three lineages raised under closely controlled housing and testing conditions. A local breeding colony was used to generate (1) a transgenerational prenatal stress (TPS) lineage, in which only the F0 parental generation was stressed during pregnancy; (2) a multigenerational prenatal stress (MPS) lineage in which each parental generation (F0, F1, F2) was stressed during pregnancy; and (3) a non-stressed Control lineage consisting of naïve, handled animals that were not experimentally stressed (Figure 1A; [19]). In each generation nine naïve males were bred with 23-37 stressed or naïve females to generate the F1, F2 and F3 generations, respectively (Figure 1A). The JAX Colony Management System (JCMS; Jackson Laboratory, Bar Harbour, ME, USA) was used for pedigree tracking [34]. Males were paired with females for 1 hr/day to allow for timed pregnancy. The exact timing of gestational length, prepartum

and postpartum maternal behaviour were determined using an infrared security system (CCTV Cameras, Panasonic, Newark, NJ, USA) [18, 19].

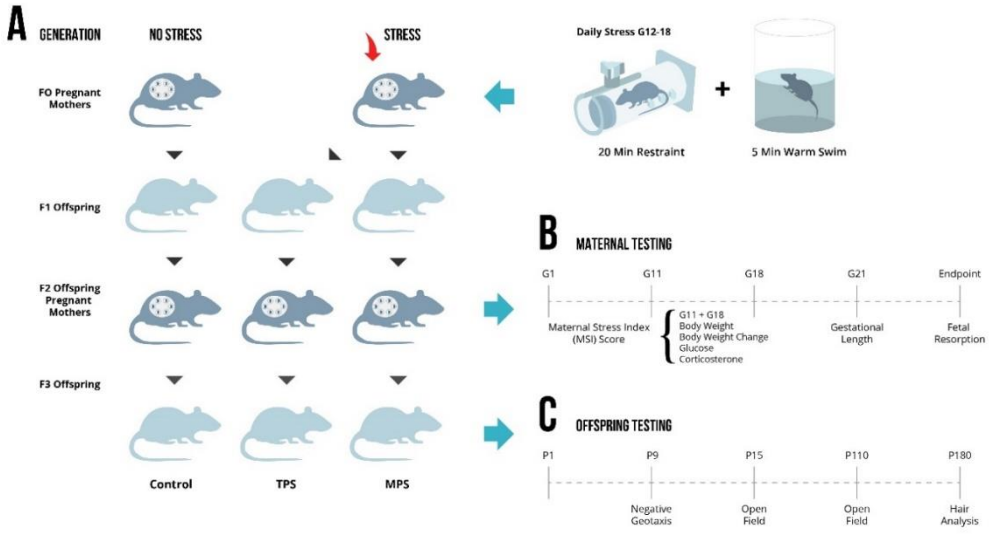

Figure 1. Ancestral stress paradigm and maternal and offspring testing timelines. A) Pregnant dams were stressed G12 to G18 with daily 5 min swim and 20 min restraint to generate ancestral stress lineage or left undisturbed to generate non-stressed lineage. Ancestral stress lineage was generated by stressing (red arrows) pregnant dams over three consecutive generations (F0, F1, F2) to produce F3 multigenerational stress (MPS) or only in first generation (F0) to produce transgenerational stress (TPS) offspring. This experiment used F2 generation pregnant mothers and their F3 generation Control, MPS and TPS offspring. (B) F2 mothers were assessed across pregnancy G11 and G18 for body weight, glucose, and corticosterone markers. Gestational length and fetal reabsorptions were also documented. (C) F3 male and female offspring were assessed for developmental trajectories longitudinally at P9, P15, P110, and P180 in negative geotaxis, open field, and hair mineral analysis, accordingly.

Gestational stress was induced daily during gestational days (G) 12-18 using a semi-random sequence of morning or afternoon 20-min restraint and 5-min swim in room-temperature water (Figure 1A; [19, 22]). Bystander effects of stress were avoided by using designated testing and housing spaces. A maximum of three offspring per litter of each sex were randomly selected for behavioural phenotyping in the F3 generation.

Offspring were tested from postnatal day 9 (P9) into adulthood as outlined below (Figure 1C). All housing, handling, testing and tissue sampling conditions were kept consistent across generations. All rats and their ancestry were raised at the University of Lethbridge local vivarium and housed in standard polycarbonate shoebox cages (45.5 x 25.5 x 20 cm) on corn cob bedding. The light cycle was 12:12 h with lights on at 7:30 am. All procedures in this study were approved by the University of Lethbridge Animal Care Committee in compliance with the standards set out by the Canadian Council for Animal Care.

Antepartum Analysis of Maternal Parameters

Maternal weight was recorded after mating activity was observed. Pregnancy was confirmed by a weight increase of approximately 30g by G11 and it was determined if the pregnant dam requires assignment to a stress regimen. The baseline pregnancy weight was recorded on G1, and weight gain was continuously monitored throughout gestational days 11 (G11), 18 (G18) and 21 (G21; Figure 2A). Blood samples (0.6 ml) were also collected prior to pregnancy, on G11, and G18 (Figure 1B) from the tail vein while under isoflurane anaesthesia. First, the blood samples were used to measure glucose using an Ascensia Breeze Blood Glucose Meter (Bayer, Toronto, ON, Canada) with test strips. The remaining blood was used to determine corticosterone (CORT) levels using an enzyme-linked immunosorbent assay (ELISA) commercial kits (Cayman Chemical, Ann Arbor, MI, USA). The time of delivery was recorded and used to calculate the gestation length in hours [19].

Postpartum Analysis of Pregnancy Outcomes

Resorption and uterine tissues. Litter sizes were recorded at parturition, and each pups' sex was determined measuring anogenital distance. Following pup weaning approximately at postnatal day 21-22 the dams were euthanized, and their uterine horn was collected in order to determine the number of implantation sites. The difference between the initial implantation sites and the resulting litter size was calculated to determine the number of pups that we resorbed during gestation. These results are presented as resorption rate.

*Maternal Stress Index (MSI) Calculatio*n. A MSI was calculated throughout gestation by comparing the individual data points to the control group distribution at each timepoint [G1 (baseline), G11, G18, and G21] using calculated z scores, which were then converted into a percentile. Markers included maternal total weight and weight change, where a marker in the 25^{th} percentile was considered dysregulated and given a score of 1, as well as glucose and corticosterone, where the 75^{th} percentile was considered dysregulated. A hard cut-off was used here as the distributions for these metrics (measurements) were normal. If a marker was within a healthy range, the animal was given a score of 0 for that marker. The individual scores were added up to get a number between 0 and 4 for each animal at each time period, with a score of 0 denoting the lowest amount of dysregulation due to stress and a score of 4 showing the highest amount of dysregulation.

Offspring Health and Behaviour Analyses

Immediately after birth, the number of pups and their sex were recorded. Special note was taken of any stillbirths. The health of the litters was checked daily and counted to determine any missing pups.

Negative Geotaxis. The negative geotaxis task (Figure 3A) was used to monitor early sensorimotor development of the offspring [19, 35]. On P9 pups were placed face down on a plastic mesh sheet maintained at a 40° angle. The time it took for the pup to turn around 180° from a downward facing angle to an upward facing angle was recorded. In addition, the total time facing up and the number of falls off the apparatus were recorded [36].

Exploratory Motor Activity in an Open Field. On P15 offspring was placed individually in the centre of an open field apparatus (Figure 2C) black Plexiglas, 42cm x 42cm, with 9 fields to measure horizontal exploratory activity and anxiety-like behaviour. The behaviour was video recorded for an interval of 5 min. The video recordings were analyzed for the number of centre vs. wall fields entered.

In adult offspring on P110, exploratory open field activity was recorded using an automated open field apparatus (Figure 3A; AccuScan Instruments Inc., OH, USA). Rats were placed individually into transparent Plexiglas boxes for a period of 10 minutes. Infrared sensors connected to a computer recorded the animal's horizontal and vertical

exploratory movements in the open field. The time in centre square, total distance travelled, and total number of vertical movements were recorded.

Hair Trace Element Analysis

Hair was collected upon animal's perfusion (at P180) with stainless steel scissors and stored in Ziploc bag. Hair mineral analysis was performed by CanAlt Health Laboratories (Concord, ON, Canada), to determine the levels of K and Na. Here we calculated and reported Na/K ratio, which has been shown to predict adrenal activity and related health outcomes, in particular function of stress response system and the risk for hypertension [37, 38]. For the hair analysis, 300 mg of finely cut hair was transferred into centrifuge tubes. To each sample 3.0 ml of nitric acid (HNO_3) was added. After an incubation period of 25 minutes, samples were subjected to acid microwave digest to stabilize the elements. The solution was analyzed by inductively coupled plasma mass spectrometry (ICPMS). Sample results were quantified by comparison with calibration solutions of known concentrations [37].

Statistical Analysis

Statistical analysis was performed using SPSS 20 for Windows 11.5.0 (IBM Corporation, Armonk, NY, USA). After confirming that the data was normally distributed by a Shapiro-Wilk test, ANOVA was used. A mixed measures ANOVA was performed for the maternal MSI, and a two-way ANOVA was performed for the offspring health with Treatment and Sex as the independent variables and the behavioural health outcome as the dependent variable. In case outliers were revealed, the data were winsorized, and in the

case of a breach in the assumption of normality necessary for an analysis of variance, the appropriate transformation was used. In every case where significance was found a Tukey post-hoc analysis was performed to specify the change. The data are available upon request to the authors. All results are shown as the means ± standard error of the mean (±SEM).

Results

Ancestral Stress Alters Maternal Weight Gain and Uterine Receptivity

Pregnancy Weight Gain. A mixed measures ANOVA was performed to determine the effect of stress on maternal weight during pregnancy on F2 dams (n=5 control, n=4 TPS, n=6 MPS). There was no significant effect of Time or Treatment, but there was a significant interaction of Time x Treatment ($F(4,66)=7.83$, $p<0.001$, $\eta^2=0.32$; Figure 2A). It is important to highlight the extremely high effect size of this finding. A pairwise comparison revealed slower weight gain in MPS dams starting at G18; the total weight gain during pregnancy in control dams was 98.9g, 99.9g in TPS dams and only 81g in MPS dams.

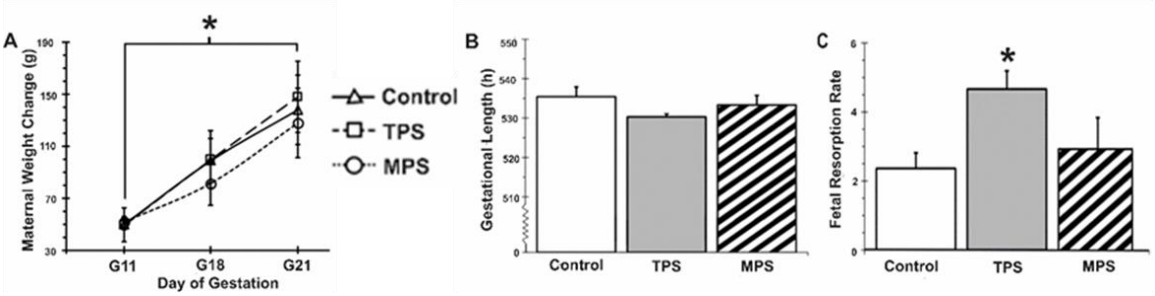

Figure 2. Antepartum F2 generation maternal health outcomes. A) Maternal weight change during gestation altered by TPS and MPS treatment. B) Gestation length in Control, TPS and MPS F2 dams. C) Resorption rates in Control, TPS, and MPS F2 dams. Error bars show SEM. Asterisk * denotes a significance difference at $p<0.05$.

Gestational Length. A one-way ANOVA showed no effects of prenatal stress and ancestral stress on the gestation length in F2 dams ($F(2,32)=1.30$, $p=0.29$, $\eta^2=0.08$; Figure 2B).

Uterine Receptivity. A one-way ANOVA was performed in order to evaluate the effect of stress on offspring birth outcomes (n=5 control, n=4 TPS, n=6 MPS). Inspection of uterine tissues revealed resorption of fetuses during pregnancy across all groups (Figure 2C). TPS mothers resorbed more of their offspring than control and MPS animals ($F(2,30)=3.35$, $p<0.05$, $\eta^2=0.18$; Figure 2C). It is worthy to note that the effect size here is considered large despite a small sample size. Nevertheless, the resultant litter size was not significantly changed by stress treatment, with control animals having an average of 11.8 pups, TPS dams having an average of 12.2 pups and MPS dams having an average of 12.8.

Ancestral Stress Alters Offspring Developmental Trajectories

P9 Negative Geotaxis. Early development of motor and vestibular functions in negative geotaxis (Figure 3A) were assessed in F3 offspring on postnatal (P) 9, while the eyes were still closed, using a two-way ANOVA for Treatment and Sex. There was an overall significant effect of Treatment ($F(2,116)=5.24$, $p<0.05$, $\eta^2=0.08$; Figure 3B). Pairwise comparisons demonstrated that there was an effect of ancestral stress in females ($F(2,57)=4.43$, $p<0.05$, $\eta^2=0.14$) with a large effect size, but not in males ($F(2,54)=2.71$, $p=0.08$, $\eta^2=0.09$; Figure 3B) in the number of falls off of the tilted platform. Follow-up

tests showed that the number of falls for TPS females, 6.76, was significantly higher compared to controls, 4.67, but not MPS females, 6.63 (Figure 3B).

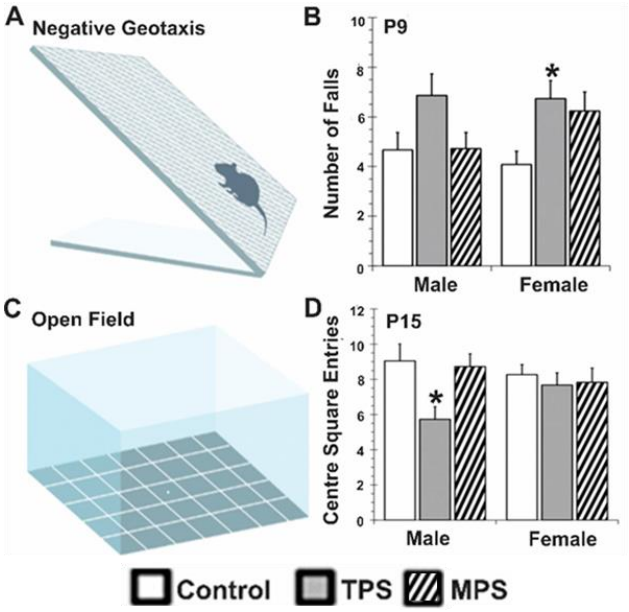

Figure 3. Early offspring development. A) Illustration of negative geotaxis task. B) Number of falls of the inclined platform in F3 male and female offspring at P9. C) Illustration of the open field task. D) Number of entries into the centre square of the open arena field in F3 male and female offspring at P15. Error bars show SEM. Asterisk * denotes a significance of $p<0.05$.

P15 Exploratory Motor Activity. An open field (Figure 3C) was used to assess exploratory activity and response to novelty at the age of P15, just after eye opening was completed. A two-way ANOVA for the number of centre square entries revealed a significant overall effect of Treatment ($F(2,105)=3.46$, $p<0.05$, $\eta^2=0.06$; Figure 3D). A pairwise comparison showed a significant effect, with a large effect size, of ancestral stress in males ($F(2,50)=4.39$, $p<0.05$; $\eta^2=0.15$; Figure 3D), but not in females ($F(2,55)=0.23$, $p=0.79$, $\eta^2=0.01$). TPS males entered the centre square significantly less frequently (6 entries) than the control (9 entries) and MPS (8.5 entries; Figure 2D). There was no group difference for the number of wall squares entered.

P110 Automated Exploratory Open Field. An automated version of the open field task (Figure 4A) was used to assess exploratory and anxiety-like behaviours in the adult F3 offspring at P110. Centre time and margin time in an open field provide critical measures of anxiety-like behaviours. TPS females spent 22.06 seconds in the centre of the field, while MPS females spent 24.04 seconds, and controls spent 27 seconds. While there was no overall effect of Treatment in males and females, a pairwise comparison showed a significant increase in time spent in the centre square in TPS females ($F(2,60)=3.68$, $p=0.03$, $\eta^2=0.11$; Figure 4B). The males showed no significant group difference ($F(2,53)=0.02$, $p=0.98$, $\eta^2=0.001$; Figure 4B), each group spending approximately 32 seconds in the centre square. There was a significant effect of Sex ($F(1,119)=22.67$, $p<0.001$, $\eta^2=0.17$), which reflects that male (32.0 seconds) remained significantly longer in the centre of the open field, compared to (24.9 seconds) females.

Vertical activity describes the number of rearing movements during open field exploration. While control females reared 6.11 times on average, TPS females reared 5.62 times, and MPS females reared 6.57 times. These data represent a significant increase in rearing movements among MPS females compared to TPS females ($F(2,60)=3.97$, $p<0.05$, $\eta^2=0.12$; Figure 4C). There was no significant difference in males ($F(2,56)=0.40$, $p=0.67$, $\eta^2=0.02$; Figure 4C). There was also a significant effect of Sex because females on average reared 6.1 times and males reared only 5.3 times ($F(1,119)=17.36$, $p<0.001$, $\eta^2=0.13$; Figure 4C).

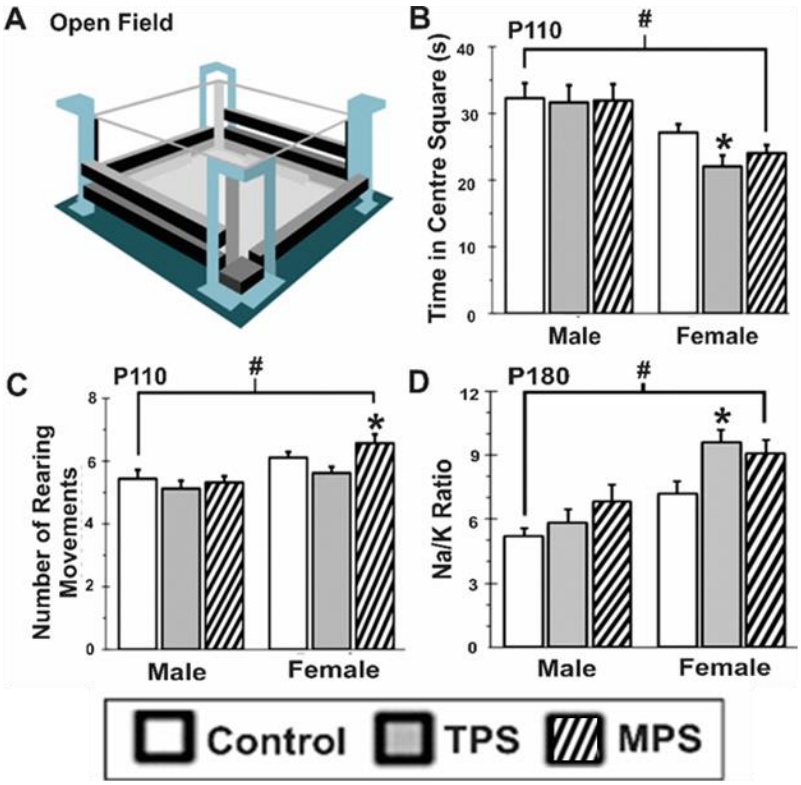

Figure 4. Adulthood offspring behavioural and physiological stress response**.** A) Illustration of an automated open field task. B) Time spent in centre square in the open field in F3 male and female offspring at P110. C) Number of rearing movements in automated exploratory field in F3 male and female offspring at P110. D) Hair Na/K ratio as a function of stress response in F3 male and female offspring at P180. Error bars show SEM. Asterisk * denotes a significance of $p<0.05$ between groups, # denotes a significance of a significance of $p<0.05$ between males and female.

The Na/K Ratio Reveals Altered Stress Responses in Female F3 Offspring

Indicators of HPA axis function in hair were based on the relative sodium (Na) and potassium (K) concentration in F3 male and female offspring (Table 2). Female TPS offspring revealed lower Na and elevated K levels, indicating a low Na/K ratio ($F(2,29)=4.63$, $p<0.05$, $\eta^2=0.24$; Figure 4D). This effect is emphasized through a very large effect size. Sex differences were found due to an overall male Na/K ratio of 5.96, and an overall female ratio of 8.61 ($F(1,65)=29.04$, $p<0.001$, $\eta^2=0.33$; Figure 4D). This effect of sex was large, as demonstrated by the extremely large effect size of 0.33.

Markers and their comparable contribution to MSI

Pearson's R correlation was used to examine the relationship between the gestational day (G) 11 and G18 MSI and each individual biomarker in order to determine the extent to which each individual marker was contributing to the MSI score (Figure 5A-H). The MSI at G11 was positively correlated to blood glucose ($r = 0.60$, $p<0.001$; Figure 5A) and corticosterone levels ($r=0.44$, $p<0.05$), and negatively correlated to weight change ($r =-0.48$, $p<0.01$; Figure 5D). At G18 the MSI was still positively correlated to glucose ($r=0.49$, $p<0.01$; Figure 5E) and corticosterone ($r=0.48$, $p<0.01$; Figure 5F), and negatively correlated to both absolute body weight ($r=-0.87$, $p<0.001$; Figure 5G & H) and gestational weight change ($r=-0.62$, $p<0.001$).

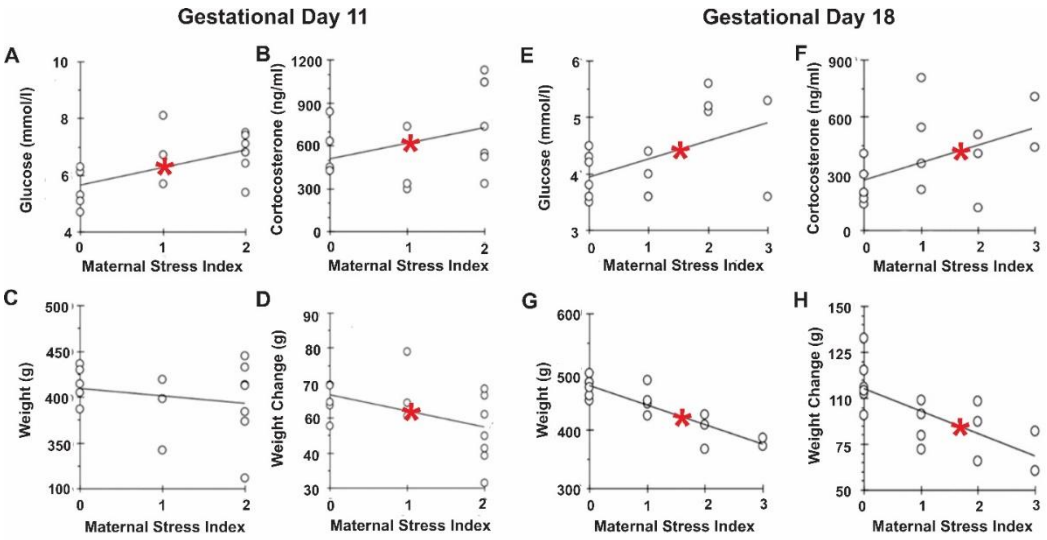

Figure 5. Individual maternal marker contribution to MSI at G11 and G18. A & E) The relationship between MSI and maternal blood glucose level. B & F) The relationship between MSI and maternal blood corticosterone level. C & G) The relationship between MSI and the maternal body weight. D & H) The relationship between MSI and the maternal weight change. Asterisk * denotes a significance at $p<0.05$.

Maternal Biomarkers as Predictors of Offspring Health

The relationship between the individual maternal biomarkers at G11 and G18 and each of the behavioural outcomes in offspring revealed significant changes as a result of ancestral stress (Table 1). Maternal blood glucose levels at G11 were negatively correlated to the vertical exploration in the open field in P110 females ($r=-0.52$, $p<0.05$) but not in males (Table 1). Moreover, maternal circulating corticosterone levels at G11 were negatively correlated to the open field centre square entries in P15 males ($r=-0.56$, $p<0.05$; Table 1), but not in females. Maternal body weight at G11 was negatively correlated to margin time and positively correlated to centre time in P110 females (margin: $r=-0.45$, $p<0.05$; centre: $r=0.45$, $p<0.05$; Table 1). Maternal body weight at G18 was negatively correlated to centre square entries in the open field in P15 males ($r=-0.56$, $p<0.05$; Table 1), but not in females. Maternal weight change was not correlated to any behaviour change at G11 or G18. In summary, these findings reveal that maternal physiological biomarkers may serve as predictors of offspring behavioural outcomes.

Table 1. Correlations of the individual biomarkers that composed the MSI at G11 and G18

	Maternal Marker	**Offspring Behaviour**	**Age**	**Correlation**	**Sig**
MALE					
Gestational Day 11	Glucose	Number of Rearing Behaviours	P110	Neg	ns
	Glucose	Number of Vertical Movements	P110	Neg	ns
	Body Weight	Centre Time	P110	Neg	ns
	Body Weight	Margin Time	P110	Neg	ns
Gestational Day 18	Glucose	Number of Rearing Behaviours	P110	Neg	ns
	Glucose	Number of Vertical Movements	P110	Neg	ns
	Body Weight	Centre Square Entries	P15	Neg	p<0.05 *

	Corticosterone	Centre Square Entries	P15	Pos	p<0.05 *
FEMALE					
Gestational Day 11	Glucose	Number of Rearing Behaviours	P110	Neg	p<0.05 *
	Glucose	Number of Vertical Movements	P110	Neg	p<0.05 *
	Body Weight	Centre Time	P110	Pos	p<0.05 *
	Body Weight	Margin Time	P110	Neg	p<0.05 *
Gestational Day 18	Glucose	Number of Rearing Behaviours	P110	Neg	p<0.05 *
	Glucose	Number of Vertical Movements	P110	Neg	p<0.05 *
	Body Weight	Centre Square Entries	P15	Neg	ns
	Corticosterone	Centre Square Entries	P15	Pos	ns

Furthermore, Pearson's correlations were used to determine the relationship between G11 and G18 MSI values and offspring behavior to identify predictors of offspring health trajectories at various ages (Figure 6). The results indicated that the MSI at G11 was negatively correlated to vertical movements in the open field exploration task for P110 males ($r=-0.514$ $p<0.05$; Figure 6A & C), but not in females. The MSI at G18 was positively correlated to centre square entries in open field exploration in P9 males ($r=0.75$, $p<0.01$), but not in females (Figure 6B & D).

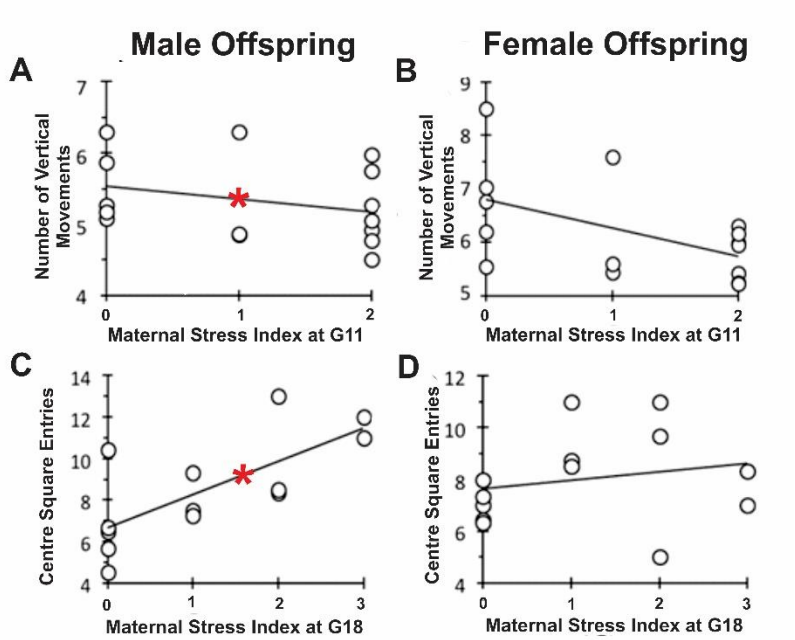

Figure 6. Correlations demonstrating the relationship between MSI at G11 & G18 and behavioural outcomes in F3 males and females. A & C) Correlation between MSI at G11 and the number of the number of vertical movements in the automated exploratory open field at P110 in male and females, respectively. B & D) Correlation between MSI at G18 and the centre square entries in the open field at P15 in males and female, respectively. Asterix * denotes a significance of $p<0.05$.

Discussion

Maternal distress during pregnancy is recognized as one of the main determinants of offspring health. Here we show in a rat model that ancestral maternal prenatal distress reaching back three generations may affect pregnancy outcomes and sex-dependent offspring developmental trajectories. The comparison between a single generational stress exposure (TPS) versus repeated generational stress exposure (MPS) provides a unique framework upon which the cumulative burden of stress can be assessed. Both TPS and MPS induced adverse pregnancy outcomes and delayed offspring development. Despite the limited group size in this proof-of-concept study, the data indicate that

maternal physiological biomarkers serve as valuable predictors of pregnancy outcomes and postnatal offspring development. In addition, the present findings demonstrate sex differences in response to TPS and MPS as a function of age in the F3 generation.

The finding that TPS mothers resorbed their offspring at a higher rate than the control mothers may be an indication that the mothers were at higher risk of adverse birth effects due to their ancestral stress. Resorption of fetuses during pregnancy particularly in the TPS dams may indicate that the environment initially was assessed as plentiful in resources, but later shifted during gestation, leading to loss of offspring. This problem has been addressed, specifically regarding insects and their clutch size, and is referred to as clutch-size optimization [55]. The surrounding assumption is that there is a negative correlation between the number of offspring, and their corresponding survival, primarily due to resource accessibility [55, 56]. Therefore, a large litter is based on sufficiently abundant environmental resources, so as to not limit the growth and development of the offspring.

The present data support previous reports showing that ancestral stress [15, 16, 19] and prenatal stress [39-42] program HPA axis sensitivity, thus generating a lasting impact on physiology and metabolism [43]. Even though the present data are based on a limited sample size, the markers indicate changes in glucose, corticosterone, and weight dysregulation due to ancestral stress in both groups, with largest significant effect observed in the TPS group. Accordingly, other types of gestational stressors have been shown to limit weight gain during pregnancy [44, 45].

The correlational analysis of the longitudinal assessments suggest that maternal metabolic and hormonal status may predict offspring development. For example, maternal gestational glucose levels measured at G11 and G18 predicted adult female offspring exploratory activity levels. Mothers with elevated glucose levels at G11 had female offspring that were less likely to engage in physical activity themselves. These data agree with previous descriptions, showing mothers with gestational diabetes are more likely to have children that are obese or have high blood pressure, or have neurobehavioural changes indicative of anxiety or depression [46-49]. Moreover, higher gestational corticosterone levels on G11 were correlated with higher anxiety-like behaviours, as indicated by fewer open field centre square entries in 15-day old male offspring. This finding agrees with previous data and suggests that higher levels of maternal corticosterone are associated with higher levels of anxiety in young male offspring [50, 51], which may disappear with advanced age, however [52].

Gestational maternal body weight gain also revealed to be a robust predictor of risk taking and affective behaviours in offspring. For example, higher maternal weight at G11 was positively associated with greater risk-taking behaviour in terms of more time spent in the open field centre among adult female offspring. At G18, this association shifted towards higher maternal weight gain predicting lower exploratory activity and lower anxiety-like behaviours in young and adult male offspring. Because higher maternal weight gain may be regarded as an indicator of low maternal stress, this result is opposed to some reports that linked prenatal stress to increased anxiety [22, 53, 54].

The MSI, calculated as a composite score based on these values, may represent a valid index for assessing the impact of chronic HPA axis dysregulation in these models. The correlational analysis suggests that at G11, glucose, corticosterone, and weight change were all strong contributing factors to the MSI, whereas at G18, maternal weight became a robust predictor of the MSI. It is relevant to note that because G11 occurred before the stressor period, these correlations suggest that ancestral stress is potentially measurable using glucose and corticosterone, as they were both strongly contributing to the MSI at this period. This correlation, however, was not significantly altered at G18, when the stressor was applied in MPS animals, but weight gain became the most significantly correlated measure. This suggests that body weight contributed significantly to the MSI during ongoing stress treatment, and glucose and corticosterone were contributors to ancestral stress. Thus, reduced body weight gain during pregnancy may be a marker that significantly predicts offspring resilience, as mentioned earlier, and high glucose and corticosterone levels may be the best predictors of high offspring vulnerability.

The G11 MSI also served as a predictor of exploratory activity in adult females, with a high MSI linked to low rearing activity, whereas the post-stress MSI on G18 was positively related to lower anxiety-like behaviours in young males. The correlational analysis reflects its predictive value for adverse developmental trajectories in the offspring. Including a larger number of variables of maternal health may further grow its predictive power [31].

Ancestral maternal stress in the present study was also accompanied by metabolic changes that reflect altered adrenal activity. Metabolic health through hair samples has been pursued previously revealing that advanced age reduces levels of potassium among other elements in rat hair, and that myocardial infarction and renal failure were associated with elevated levels of sodium and potassium [37]. The finding of higher Na/K ratios in female TPS rats is supported by previous observations that stress caused a reduction in Na consumption, and a corresponding reduction in Na excretion [57]. Moreover, it was found that behavioural stress in dogs resulted in antinatriuresis, or a reduction in urine output, and a corresponding decrease in Na excretion [58]. This reduction in mineral excretion through the urine would likely result in accumulation of Na in organs and hair and leading to altered stress response via hyperactive adrenal glands [21].

Ancestral stress bears the risk of dysregulating the HPA axis in the offspring and alters brain morphology in the prefrontal cortex and amygdala, resulting in long-term alternations in stress reactivity [22]. Nevertheless, developmental programming in response to ancestral stress may expose different mechanisms of shaping stress vulnerability and resilience [21]. Erickson et al. [59] showed that MPS alters exploratory movement patterns particularly in early and late life but appear normal at reproductive age [59]. These findings indicated that early life and older age are particularly vulnerable to the impact of ancestral stress [59]. Overall, the present data also indicate a higher capacity for stress resilience in the MPS lineage as compared to TPS animals. These findings are in line with previous showing the recurrent stress exposure in the MPS

lineage may promote partial tolerance of lifetime stress [21] and health trajectories [43]. Specifically, Ambeskovic et al., [22] showed that TPS young adult females had increased levels of anxiety-like behaviours but not MPS females, nor males at P180 as measured in EPM [22]. The present longitudinal behavioural profiles in the F3 offspring, however, indicate that both, TPS and MPS lineages, were both vulnerable to anxiety-like changes and altered affective states with larger significant effects in TPS offspring.

For better interpretation of the present findings, it is important to note the ways in which the nature of ancestral stress in the TPS and MPS lineages differed. The F2 TPS mothers were directly exposed to F0 grandmaternal stress in *utero* through the gametes [60, 61]. Their F3 offspring, on the other hand, were not exposed directly but influenced by inherited stress-associated epigenetic programming in their F0 great-grandmother. The MPS paradigm, by contrast, exposed each pregnant mother to gestational stress in the F0, F1 and F2 generations. Thus, each MPS generation represents the cumulative prenatal stress burden of their ancestors in combination with their own prenatal stress [62]. It is possible that this repeated presentation of stress is producing partial resilience [21, 63], which has been demonstrated previously [21], where prolonged stress can encourage resilience through epigenetic preparedness [27, 64].

In this study, MPS did not result in any significant changes in behavioural outcomes compared to control, indicating potential resilience due to cumulative effects of prenatal stress. These observations are supported by our previous studies showing resilience in F4 female offspring for multiple behavioural outcomes including fine motor

function [63] and emotional wellbeing [21] in adulthood. Similarly, resilience in the MPS cohorts has also been shown as important moderator of parental trauma effects on the second generations in clinical studies [65].

The present study provides novel insights into sex differences in maturation from early life to young adulthood in response to ancestral stress. Males generally seemed to be most vulnerable to TPS [19, 22, 66] and MPS [19, 67] at infantile ages while females responded more strongly during young adulthood [20, 22, 68]. These findings are supported by previous reports that in later life, females rather than males show a higher risk of anxiety-related behaviours in the elevated plus maze [69-71], and that prenatal stress in females leads to greater risk to anxiety-like and depression-like behaviours [72]. This result is also supported by human studies, which demonstrated that females are twice as likely to develop depression and anxiety disorders, as compared to males [73-75]. Alternatively, in Brunton and Russell [53] anxious behaviour was seen solely in 3-month-old males after exposure to a stressor, while females either showed no increased anxiety [53] or showed attenuated anxiety behaviour [76]. The variability of these findings illustrates that the timing, nature, duration, and intensity of the prenatal stressor, as well as (epi)genetic predisposition determine the outcome and sexual dimorphism of stress-induced outcomes. These findings, however, highlight the value of the use of cumulative stress indices, such as the MSI, which allow to generate an estimate of total stress load at a certain time in life [31] in order to determine the momentary physiological impact of the past lifetime and generational experiences. It needs to be noted that, even when effects are only significant in one sex, the trend frequently seems to be present in

the other sex as well. For example, geotaxis behaviour was significantly altered in TPS females at P9 while TPS males displayed a non-significant trend in the same direction. The non-significant trends may arguably derive from limited sample size and small effect size. Thus, the present sex differences need to be interpreted with caution to prevent misleading conclusions about sex-specific effects.

Ultimately, the present findings support the match/mismatch hypothesis of prenatal stress [24-26], in terms of match/mismatch across generations. Instead of original match/mismatch hypothesis where stress vulnerability and resilience is defined by the match between developmental programming during intrauterine development and the actual conditions of the postnatal environment [27], we observed that MPS offspring who experienced cumulative intrauterine stress across each generation adopted better than TPS offspring that were subject to stress in one generation only. The mismatch in prenatal stress exposure seems to lead to negative health outcomes in adolescents and young adult female offspring. The present proof-of-principle study suggests that MPS animals, though experiencing more cumulative stress across several generations, adopt mechanisms mediating at least partial stress resilience, which amounts to more positive pregnancy outcomes. Through epigenetic mechanisms, MPS may support the formation of new behavioral traits [20] and coherence between brain areas [77] thus supporting behavioural complexity in a dynamically changing environment, but also inducing health risks [43].

The present study used a model of mild maternal stress to better account for ecological validity and translational value to a clinical context. The consequences of mild stress may produce a covert phenotype in single behavioural and physiological measures, while the cumulative effects of such subclinical changes may be advantageous early in life [16, 20, 22, 68] it can produce a cumulative wear and tear with adverse health consequences in later life [43]. Thus, a composite index such as the MSI may provide a more reliable assessment of homeostatic dysregulation than any single parameter. The use of composite indices therefore provides a conceptual framework through which the cumulative physiological burden of past and present adverse experiences can be approximated. This knowledge will facilitate the development of predictive models of lifetime health trajectories within a precision medicine framework.

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Study 2

Ancestral Prenatal Stress on F3 and F4 Young Offspring: The Match-Mismatch Hypothesis

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Consent to Participate. N/A

Consent for Publication. N/A

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Code Availability. N/A

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Introduction

Maternal stress during pregnancy is regarded as one of the most critical predictors of adverse health outcomes in the offspring, including low birth weight, and increased vulnerability to disease in later life [1-3]. It is thought that a primary factor mediating the effects of prenatal stress is the over-activation of the maternal hypothalamic-pituitary-adrenal (HPA) axis, generating excess glucocorticoid levels [4-8] and modulating immune function [9, 10].

The adverse impact of prenatal stress not only reaches the next generation but also propagates further. In the F3 generation, experimental trans- and multigenerational stress puts mothers at risk for preterm birth and other adverse pregnancy outcomes [11], affective and motor impairments [12-14]. In fourth generation offspring, multigenerational stress promoted partial adaptation and resilience of the behavioural phenotype as opposed to transgenerational stress [2, 15-17]. In part these effects can be reproduced by maternal synthetic glucocorticoid exposure [18]. Mechanisms for transgenerational programming by stress include transgenerational epigenetic inheritance of altered miRNA expression, and DNA methylation marks [16, 19, 20], which in the maternal lineage become evident in the third generation removed from the stressor [20, 21].

The complex health risks associated with prenatal and ancestral stress have created the need to develop unifying concepts for risk assessment. Allostatic load (AL) has become a successful theoretical construct for the quantification of stress on the body [22,

23]. AL is generated by disruption of physiological homeostasis caused by the cumulative consequences of chronic stress [23]. AL has been primarily applied to clinical populations, where large differences in study design and health outcomes generate non-standard sets of biomarkers [24, 25]. AL indices enable the inclusion of a large variety of markers to be taken into consideration in a unifying approach [1, 22, 26]. So far only one AL index, the rat Cumulative Allostatic Load Measure (rCALM) has been developed for use in experimental models [27]. Here, we developed a maternal stress index (MSI) as a measure of AL in pregnant rats, to compare the stress burden of transgenerational versus multigenerational maternal stress in a rat model. Using the maternal lineage, it was hypothesized that AL burden is elevated by direct exposure to stress (F2 generation) and epigenetically inherited stress response (F3 generation) that carry on into the F4 generation. Furthermore, we proposed that AL will serve as a sensitive indicator of stress vulnerability and resilience particularly in the multigenerational lineage.

Methods

Animals and Experimental Design

This study used the F2 and F3 generations derived from a lineage of timed-pregnant dams bred under thoroughly controlled conditions. The F2 and F3 generations included three lineages: (1) transgenerational prenatal stress (TPS), where only the F0 parental generation underwent gestational stress (F2-TPS, F3-TPS); (2) multigenerational prenatal stress (MPS) in which dams from each generation underwent gestational stress (F2-MPS, F3-MPS); and (3) non-stressed controls (F2-CONTROL, F3-CONTROL) [11].

Forty-four F3 females were bred with 9 F3 males with the family tree in mind to avoid incest, the JAX Colony Management System (JCMS; Jackson Laboratory, Bar Harbour, ME, USA) was used for pedigree tracking [28]. Males were paired with females for 1 hour/day for timed pregnancy. Gestational length was determined using infrared security system (CCTV Cameras, Panasonic, Newark, NJ, USA). A total of 280 and 260 female and male F4 offspring were birthed respectively.

Gestational stress was induced in pregnant dams over gestational days 12-18 through a semi-random sequence of daily restraint stress for 20 minutes, and a 5-minute swim in room temperature water [11]. Bystander effects were mediated with specifically designated testing and housing spaces. All housing, handling, testing and tissue sampling conditions were harmonized across generations. All rats were bred and housed in the local vivarium at the University of Lethbridge in standard polycarbonate shoebox cages (45.5 x 25.5 x 20) using corncob bedding. The light cycle was set at 12:12 with lights on at 7:30 a.m. All procedures in this study were approved by the University of Lethbridge Animal Care Committee in compliance with the standards set out by the Canadian Council for Animal Care. All animals were tested on postnatal day 9 (P9) into adulthood as described in further detail below.

Antepartum Maternal Physiology Analysis

Maternal weight was measured after mating activity and weight gain over seven consecutive days was taken as confirmation of pregnancy. Weight gain was continuously monitored throughout pregnancy and on gestational days 11 (G11), 18 (G18), and 21

(G21). The pregnant rats were monitored by video surveillance cameras (CCTV Cameras, Panasonic, Newark, NJ, USA), to determine maternal behaviour, the duration of labour and time of parturition for calculation of gestational length.

Between 10-12 hrs. in the morning, blood samples (0.6ml) were taken at baseline, G11, G18, and G21, from the lateral tail vein while under isoflurane anaesthesia. Blood glucose levels were measured using test strips with a blood glucose meter (Ascensia Breeze, Bayer, Toronto, On, Canada). The remaining blood sample was used to assess corticosterone (CORT) levels using an enzyme-linked immunosorbent assay (ELISA) commercial kit (Cayman Chemical, Ann Arbor, MI, USA) according to manufacturer's directions.

Postpartum Analysis of Pregnancy Outcomes

Adverse birth outcomes were recorded in terms of gestational length, the number of uterine implantation sites, reabsorption, still births, litter size for newborns, and offspring deaths within the first two weeks of life.

Maternal Stress Index (MSI) Calculation

Physiological markers were used to calculate the maternal stress index (MSI) as an indicator of allostatic load. The MSI involved weight, weight change, glucose, and corticosterone at baseline (G1), G11, G18, and G21. For glucose and corticosterone, scores above the 70^{th} percentile were given a score of 1, otherwise it was given a score of 0. For body weight and weight change, values below the 30^{th} percentile were given a score of 1. Scores were then summed together for a score between 0 and 4 for each

animal at each time period, with 4 representing the highest dysregulation possible, and 0 representing the lowest.

Offspring Behavioural Development and Maturation

Immediately at the time of birth, the number and sex of pups was recorded, and note was taken of any still births.

Negative Geotaxis. Negative geotaxis was measured on postnatal day (P)9 to monitor physical development. Pups were placed face down on a 40-degree angled mesh ramp [11]. Animals were video recorded and experimenters blind to the experimental conditions scored the data for (1) latency to turn around 180° to the face up position, and (2) the number of times animals fell off the ramp [29].

Open Field Exploratory Activity. Exploratory activity in an open field was recorded on P15. The animals were placed in a black Plexiglas box (42cm x 42cm) in which the floor was subdivided into 9 equal squares and recorded from a vertical perspective for 5 minutes. The number of novel squares, the total number of squares entered, the number of outside squares, the number of inside squares were recorded based on the video recordings.

On P110 an automated open field task (VersaMax 4.0 software, AccuScan Instruments Inc., OH, USA) was used to monitor exploration for 10 mins. While animals explored the Plexiglass open field (42cm x 42 cm) framed by 4 infrared sensors a

computer interface recorded overall activity and resting time, the time spent in each centre and wall areas, stereotypic activity, and rearing activity in 1-minute time bins.

Affective Exploration Task. The affective exploration task measured anxiety and motor activity on P110. Animals were placed in a black Plexiglass tube (10 x 10 x 20cm) placed on a dark open table surface (75 x 150cm) [15]. Top-down vertical perspective video recordings recorded the latency to leave the tube, and the time spent exploring the open area of the table.

Statistical Analysis

Statistical analysis was performed using SPSS 20 for Windows 11.5.0 (IBM Corporation, Armonk, NY, USA). For the maternal and offspring behaviour an ANOVA was performed with the treatment as the independent variable and the behavioural health outcome as the dependent variable. In case outliers were revealed, the data were winsorized, and in the case of a breach in the assumption of normality necessary for an analysis of variance, the appropriate transformation was used. For the generational offspring behaviour an ANCOVA was performed with generation as the covariate, in order to determine the extent that generation was altering the affecting the results. In every case where significance was found a post-hoc analysis was performed to specify the change.

Results

Antepartum Maternal Physiology and Maternal Stress Index

A mixed design measure ANOVA of the MSI for F3 dams determined the stress impact of transgenerational and multigenerational stress on maternal health (F3-CONTROL n=8, F3-TPS n=5, F3-MPS n=7). There were no missing values and no evidence of skewness, kurtosis, or outliers. As summarized in Table 2 in Appendix B, the MSI in controls increased from baseline to G11 and showed the largest effect on G21, indicating an overall physiological burden of pregnancy. The TPS and MPS animals showed higher baseline values than controls, reflecting the burden of chronic stress, followed by a steeper incline of MSI scores during pregnancy. Scores in the MPS group exceeded those among TPS animals indicating a larger impact of cumulative stress. The MPS MSI scores on G21 were over 100% higher (3.0±0.82) than those of non-stress controls (1.38±0.32). Table 2 in Appendix B summarizes the averages and standard deviations of the MSI at each time point. Table 4 shows a significant effect of Time, but not of treatment for the MSI, $F(3,51) = 2.965$, $p = 0.041$, $\eta^2 = 0.149$. Note this large effect size for the effect of time. A follow-up pairwise comparison determined that the MSI at G21 was significantly higher than the baseline MSI.

As for the F2 MSI, it is summarized on table 3 in Appendix A, within the MPS group, MSI increased over the G11 to G18 period, but comparatively, was a much smaller increase in MSI compared to the F3 group. The MPS MSI scores at G18 were also over 100% higher (1.67±1.21) than non-stress controls (0.60±0.89). The MSI at G21 was still elevated, but less so, showing a 75% increase (1.33±1.37) compared to non-

stressed controls (0.80±0.84). Table 5 shows a non-significant effect of time, nor treatment.

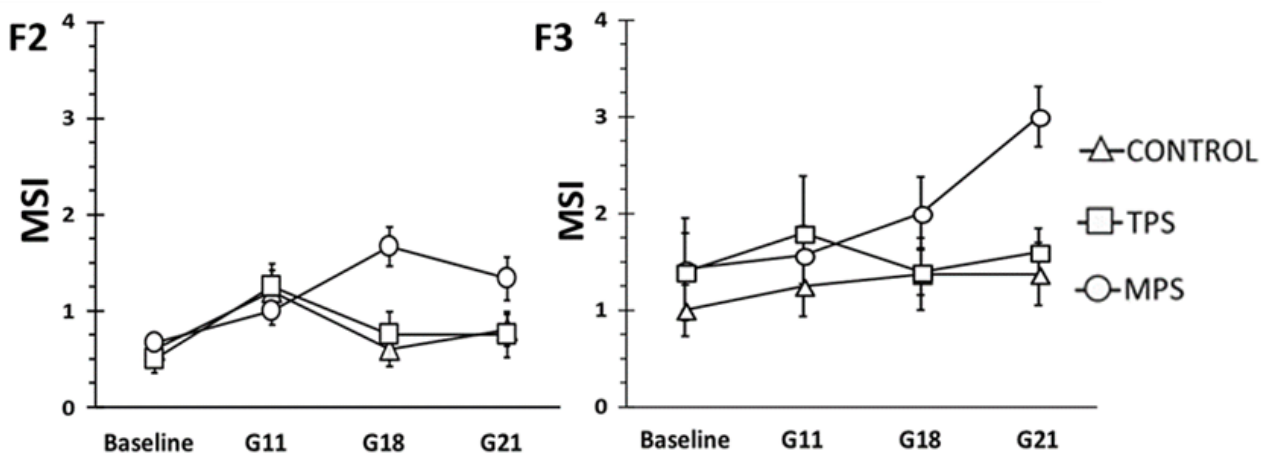

Figure 7. F2 and F3 cumulative MSI over the duration of gestation. Error bars show SEM.

An ANCOVA for the MSI among the dams of the F2 (F2-CONTROL n=5, F2-TPS n=4, MPS n=6; total=15) and F3 (F3-CONTROL n=8, F3-TPS n=5, MPS n=7; total=20) generations revealed a generational effect. There were no missing values and no evidence of skewness, kurtosis, or outliers. With treatment as the independent variable and total MSI as the dependent variable, Table 3 shows a significant incline in MSI index scores for the F3-MPS group. Table 7 in Appendix B displays the main and interaction effects for the F2 vs F3 comparison. There was no significant difference in treatment, however, there was a significant effect of generation indicating an increased MSI in each of the groups, including controls, $F(1,31) = 5.319$, $p = 0.028$, $\eta^2 = 0.146$. This effect size is also considered quite large.

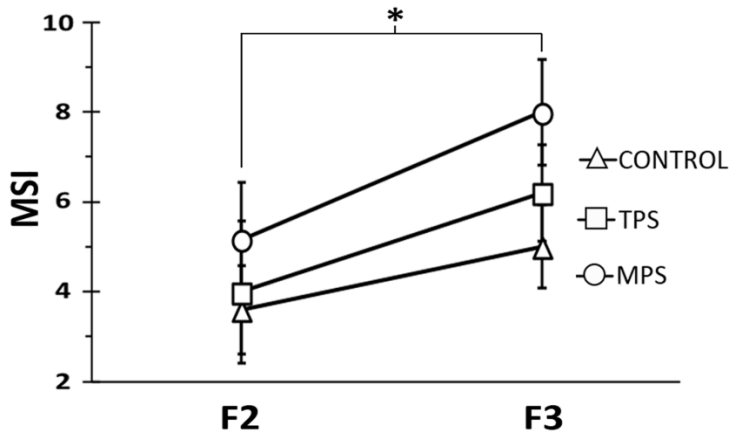

Figure 8**.** Generational cumulative MSI for F2 and F3 generations. Note that in each group the overall allostatic load increased, with MPS conferring the highest risk for allostatic burden. Error bars show SEM. Asterisk marks significance of generation: * $p<0.05$, compared to baseline.

Postpartum Analysis of Pregnancy Outcomes

ANOVAs were performed for gestational length, the number of uterine implantation sites, reabsorption, and litter size for newborns. Kruskal-Wallis tests were performed for still births, and offspring deaths within the first two weeks of life, as they violated the rule of normality (F3-TPS n=11, F3-MPS n=12, F3-CONTROL n=11 litters). Table 9 in Appendix B summarizes the main effects. There was a trend for stress to alter gestational lengths, ($F(2,31) = 3.082$, $p=0.06$, $\eta^2 = 0.166$). This is a non-significant result but does have a large effect size. The control animals had an average of 531.0 hours of gestation, while MPS animals had a slightly reduced gestation length at 528.5 hours, and the TPS had a slightly increased gestation length of 534.4 hours. There was also a significant decrease in uterine implantation sites within the TPS dams, decreasing from an average of 18.3 implantation sites in the control dams to 14.9 implantation sites in the TPS dams. This was a significant change ($F(2,31) = 3.671$, $p < 0.05$, $\eta^2 = 0.191$) with a very large effect size. The overall litter size also decreased in TPS dams, from a control

average of 15.5 to 11.4 pups, ($F(2,31) = 62.094$, $p = 0.001$, $\eta^2 = 0.359$), a high effect size, as illustrated in Figure 9.

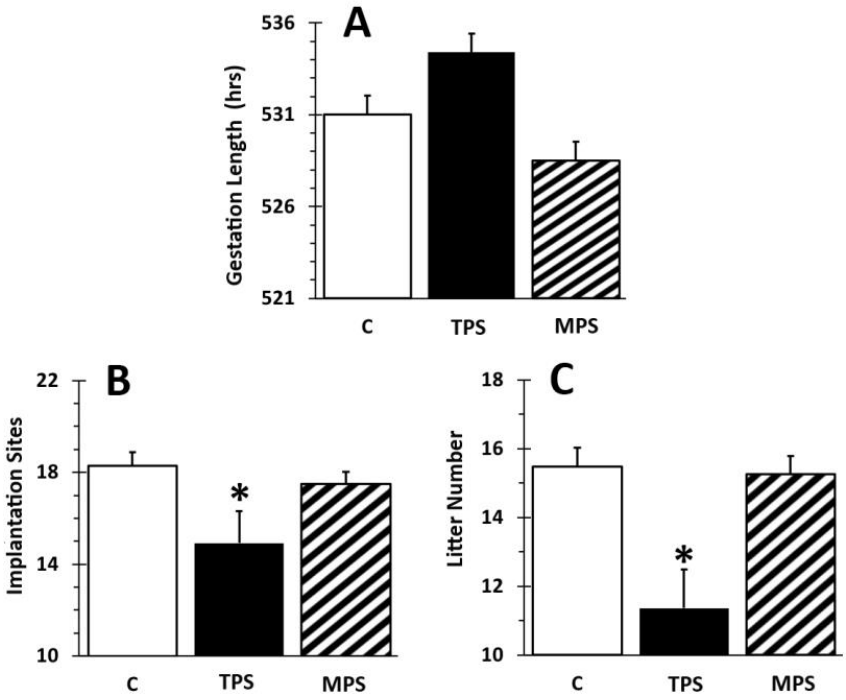

Figure 9. F3 litter health including A) gestational length, B) implantation sites, and C) Litter size. Note that TPS had the most adverse influence and reduced the number of uterine implantation sites and number of viable pups per litter. Error bars show SEM. Asterisk marks significance: * $p<0.05$, compared to CONTROL.

Generational Litter Health

ANCOVAs were performed on each of the significant litter health markers using treatment as the independent variable, and generation as a covariate. The generation did not significantly alter gestational length and the number of implantation sites. The only marker of litter health that was significantly altered by generation was litter number $F(1,69) = 5.049$, $p = 0.028$, $\eta^2 = 0.068$. As can be seen in Figure 10, each treatment within the F2 generation had litters that were roughly 12 pups each. An additional bred

generation increased the size of the litters by 3 pups in the control (15.5±2.06) and in the MPS dams (15.25±1.79). TPS had the opposite effect, of slightly reducing the litter size to roughly 11 pups per litter (±3.55).

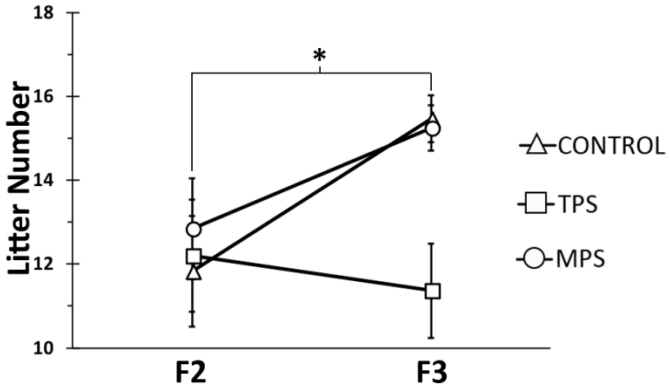

Figure 10. Litter size for F2, control, TPS, and MPS dams, and F3 control, TPS, and MPS dams. Error bars show SEM. Asterisk denotes a significance of generation: * $P<0.05$.

Offspring Behavioural Development and Maturation

Negative Geotaxis. ANOVAs for F4 offspring at P7 sensorimotor response to head-down inclined position revealed sex-dependent changes in the time to turn, time facing up, and number of falls. Table 11 shows the effect of stress treatment on the sensorimotor response among males (CONTROL n=56, TPS n=35, MPS n=55) and females (CONTROL n=63, TPS n=44, MPS n=61). Time to turn for males and females was negatively skewed and transformed using the inverse transformation. The results revealed that there were no significant effects of treatment within negative geotaxis in the F4 generation.

To test the effect of an added generation on sensorimotor development, an ANCOVA was performed with generation as the covariate. In males, there was skewness in time facing up as well as falls, and both were transformed using a log transformation. The number of falls was skewed for females and was corrected using a log transformation as well.

A pairwise comparison in males for the number of falls revealed a significant effect of Treatment with the TPS group making significantly more falls in the F3 generation than controls. This difference disappeared in the F4 generation (Figure 11C).

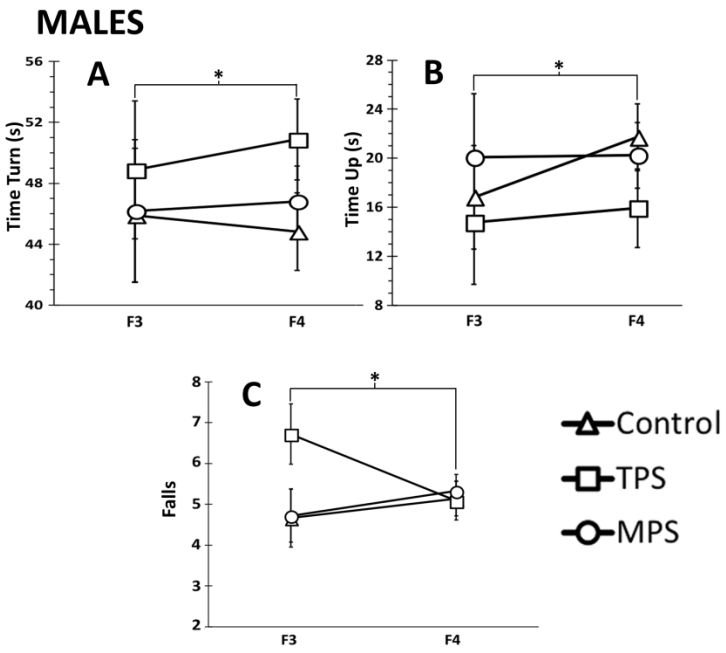

Figure 11. F3 and F4 females at P9 in the negative geotaxis task including A) Latency to face up, B) total time facing up, and C) number of falls. Error bars show SEM. Asterisk denotes a significance of generation: * $p<0.05$.

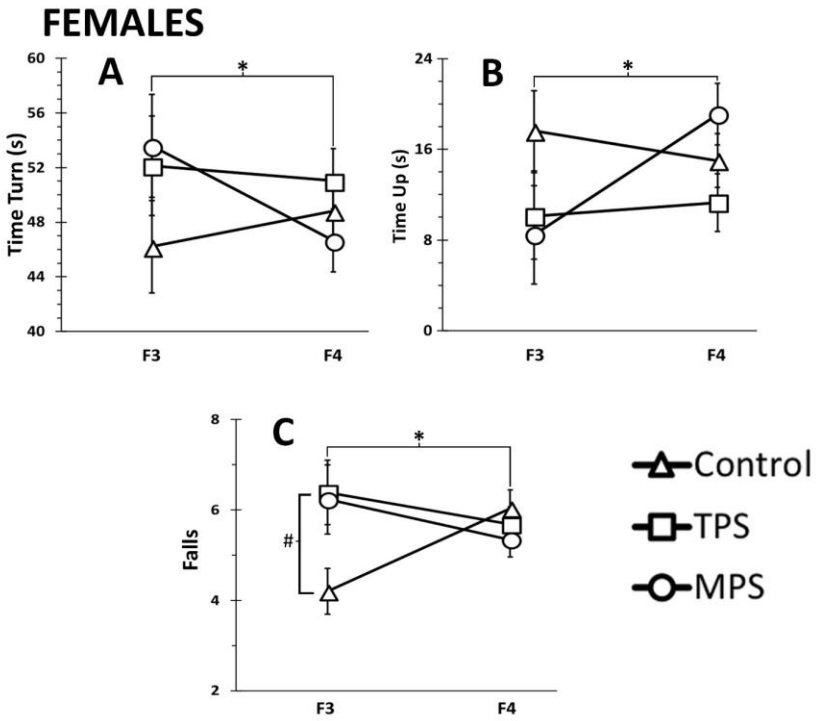

Figure 12. F3 and F4 female negative geotaxis at P9 including A) latency to face upwards, B) total time facing up, and C) number of falls off of the inclined platform. Error bars show SEM. Asterisk denotes a significance of generation: * $p<0.05$. Octothorp denotes a significance of treatment: # $p<0.05$

Exploratory Open Field Activity. ANOVAs for the number of novel squares entered, number of outside squares, the number of inside squares, and the total number of squares, the was performed for F4 males (CONTROL n=56, TPS n=32, MPS n=55) and females (CONTROL n=62, TPS n=44, MPS n=61). Males total and outside squares each had one outlier and was therefore winsorized on both ends. For females, total squares also had one outlier and was winsorized on both ends. While there was no significant effect of treatment in males, in females, significant differences were seen in the number of novel squares ($F(2,164) = 6.692$, $p = 0.002$, , $\eta^2 = 0.075$), outside squares explored ($F(2,164) = 5.883$, $p = 0.003$, $\eta^2 = 0.067$), and total number of squares ($F(2,164) = 3.409$, $p = 0.035$,

$\eta^2 = 0.040$) with MPS animals exploring significantly fewer novel squares, and outside squares, and fewer squares total than both control and TPS females (Figure 13). There was no significance of sex in any of the measures.

ANCOVAs for F3 and F4 animals revealed a main effect of Generation (Table 13). In males, outside squares and inside squares had outliers, therefore were winsorized, and total number of squares was positively skewed, but corrected with a square root transformation. In females, novel and total squares had outliers and were winsorized. Total squares was skewed and corrected with a square root transformation in order to maintain normality. Results shown in Figures 14 and 15 illustrate the main effect of Generation in males and females, respectively. The male F3 TPS animals explored fewer inside squares ($F(1,192) = 6.117$, $p = 0.014$, $\eta^2 = 0.031$) (Fig. 14A), F3 TPS and MPS groups explored significantly fewer total squares ($F(1,192) = 4.407$, $p = 0.037$, $\eta^2 = 0.022$) (Fig. 14B) compared to controls. This difference disappeared in the F4 generation. The number of total squares showed a significant reduction in the MPS group compared to controls. Across generations, all of the treatments increased the number of inside squares explored.

In the ANCOVA for females, treatment altered the number of novel squares ($F(2,221) = 6.809$, $p = 0.001$, $\eta^2 = 0.058$), outside squares ($F(2,221) = 6.410$, $p = 0.002$, $\eta^2 = 0.055$), and total squares ($F(2,221) = 4.084$, $p = 0.018$, $\eta^2 = 0.036$) for the F3 and F4 animals (see table 13 in Appendix B). A pairwise comparison in females revealed that the exploration of novel and outside squares was significantly reduced in MPS animals when

compared to controls or TPS females. There was also a significant difference in the number of inside squares explored across all groups over F3 to F4 generation, with more squares explored in the F4 generation than in the F3 generation ($F(1,221) = 4.075$, $p = 0.045$, $\eta^2 = 0.018$) (fig. 15).

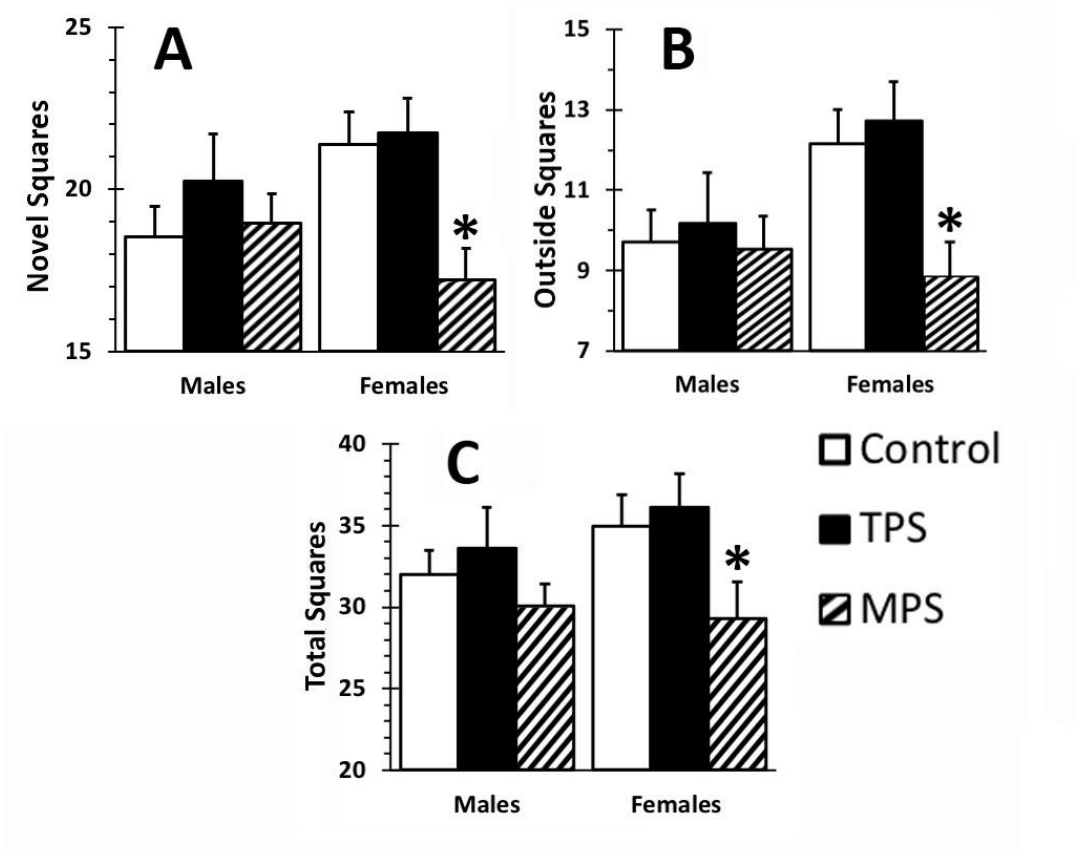

Figure 13. Exploratory open field activity including in P15 F4 animals, showing the effect of stress treatment on exploratory behaviour including A) number of novel squares explored, B) number of outside squares explored, and C) number of total squares explored. Error bars show SEM. Asterisk denotes significance of treatment: * $p<0.05$.

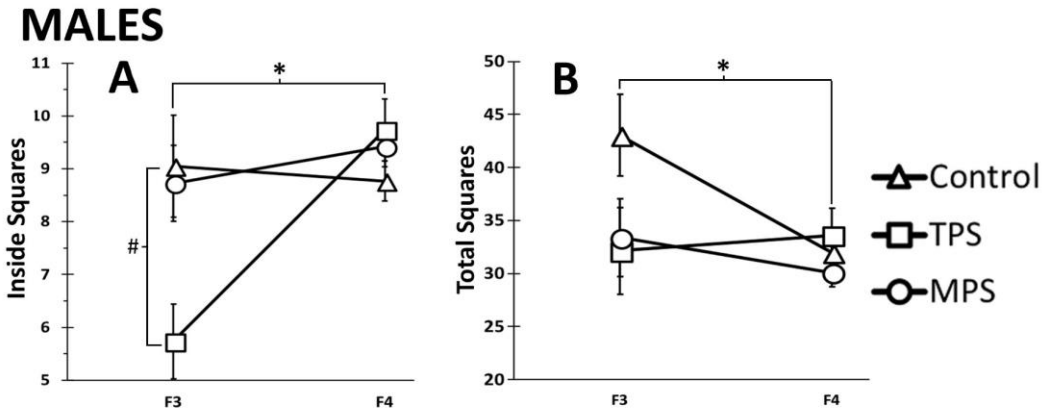

Figure 14. F3 and F4 exploratory open field activity at P15 including A) number of inside squares explored and B) totfal number of squares explored. Error bars show SEM. Asterisk denotes significance of generation: * $p<0.05$, octothorp denotes a significance of treatment: # $p < 0.05$.

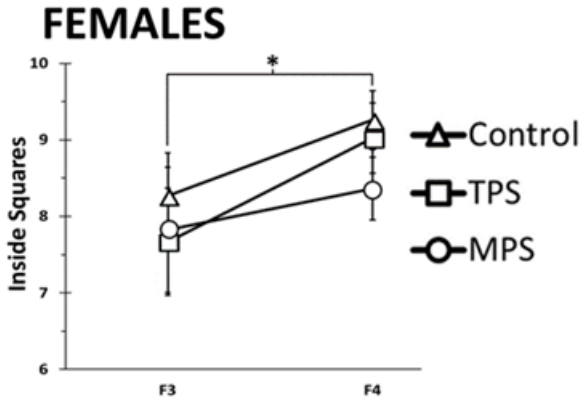

Figure 15. F3 and F4 female exploratory open field activity at P15 including number of inside squares explored. Asterisk denotes SEM. Asterisk denotes a significant effect of generation: * $p<0.05$.

Offspring Adult Behaviour

Automated Exploratory Open Field Activity. ANOVAs were performed for horizontal and vertical locomotor activity and stereotypy in the automated open field, including time spent in each of the areas of the field for male and female F4 offspring. In F4 males (CONTROL n=23, TPS n=12, MPS n 24) there were various significant

changes, all are listed in table 14 in Appendix B. A few of them will be discussed here. There were several significant changes in overall exploration in males, first being movement time ($F(2,56) = 6.060$, $p = 0.004$, $\eta^2 = 0.178$). A Pairwise comparison determined that both TPS and MPS spent less time moving horizontally around the arena compared to control animals. Another measure of exploration and activity included stereotypy, reflected by animals performing repetitive movements where animals spin around in circles. There were several measures of stereotypy that were significant, including stereotypy number ($F(2,56) = 4.801$, $p = 0.012$, $\eta^2 = 0.146$), and stereotypy time ($F(2,56) = 5.446$, $p = 0.007$, $\eta^2 = 0.163$). Both stereotypy number and overall time spent in stereotypy demonstrated similar results, where male MPS rats spend less time compared to controls. Note the high effect sizes in both of these groups. Among F4 females (CONTROL n=24, TPS n=18, MPS n=24) there were several differences across treatment groups, all of which are also listed in table 14 in Appendix B. Stress treatment altered the exploration in the females, as measured several ways including the total movement time ($F(2,63) = 10.176$, $p<0.001$, $\eta^2 = 0.244$), and the number of horizontal movements made in the field ($F(2,63) = 3.156$, $p = 0.049$, $\eta^2 = 0.091$; Fig.16 A and B). For the movement time, we found a reduction in TPS females as compared to controls and MPS females. This effect was large as demonstrated through the effect size. In overall number of horizontal movements there was a reduction of movement in MPS females compared to controls alone. Another measure of exploration is stereotypy, and two measures of stereotypy were significantly altered by stress treatment, including stereotypy number ($F(2,63) = 13.113$, $p<0.001$, $\eta^2 = 0.294$), and time ($F(2,63) = 4.415$, $p = 0.003$, $\eta^2 = 0.169$). Both showed the same trend of TPS females spinning less than

controls. The final measure to be discussed here includes a measure of anxiety-like behaviours, which is indicated by the amount of time the animal spent in the centre of the open field ($F(2,63) = 4.108$, $p = 0.021$, , $\eta^2 = 0.115$; Figure 16, E). This result demonstrated TPS females spending less time in the centre compared to controls and MPS females but was only significantly altered compared to MPS females.

A significant effect of sex was observed in movement time, number of movements, stereotypy number, and centre time. Females displayed longer bouts of continuous horizontal motor activity than males, with a lower number of overall movements, demonstrating the horizontal movements were more continuous, resulting in fewer stops and starts. In terms of stereotypy, females spun fewer times, but spent the same amount of time spinning compared to males. Finally, females spent less time in the centre of the open field compared to males.

ANCOVAs comparing the F3 and F4 generations revealed main effects of generation in males and females. For males, generation affected the stereotypy time ($F(1,111) = 27.61$, $p < 0.001$, $\eta^2 = 0.199$) and number ($F(1,111) = 28.77$, $p < 0.001$, $\eta^2 = 0.201$), as well as overall movement time ($F(1,111) = 17.30$, $p < 0.001$, $\eta^2 = 0.135$). The additional generation of stress treatment increased stereotypy in each of the treatment groups, but the control group increased the most. Furthermore, movement time also increased over the generations within each treatment group. In females, generation affected many variables, see the full complement of significant changes see table 15 in Appendix B. A few of the more relevant changes included movement time ($F(1,125) =$

14.52, $p < 0.001$, $\eta^2 = 0.104$), stereotypy number ($F(1,125) = 14.18$, $p < 0.001$, $\eta^2 = 0.102$) and time ($F(1,125) = 15.13$, $p < 0.001$, $\eta^2 = 0.108$), centre time ($F(1,125) = 10.61$, $p = 0.001$, $\eta^2 = 0.078$), and the number of rearing movements ($F(1,125) = 10.61$, $p = 0.001$, $\eta^2 = 0.113$; Fig. 17 and 18).

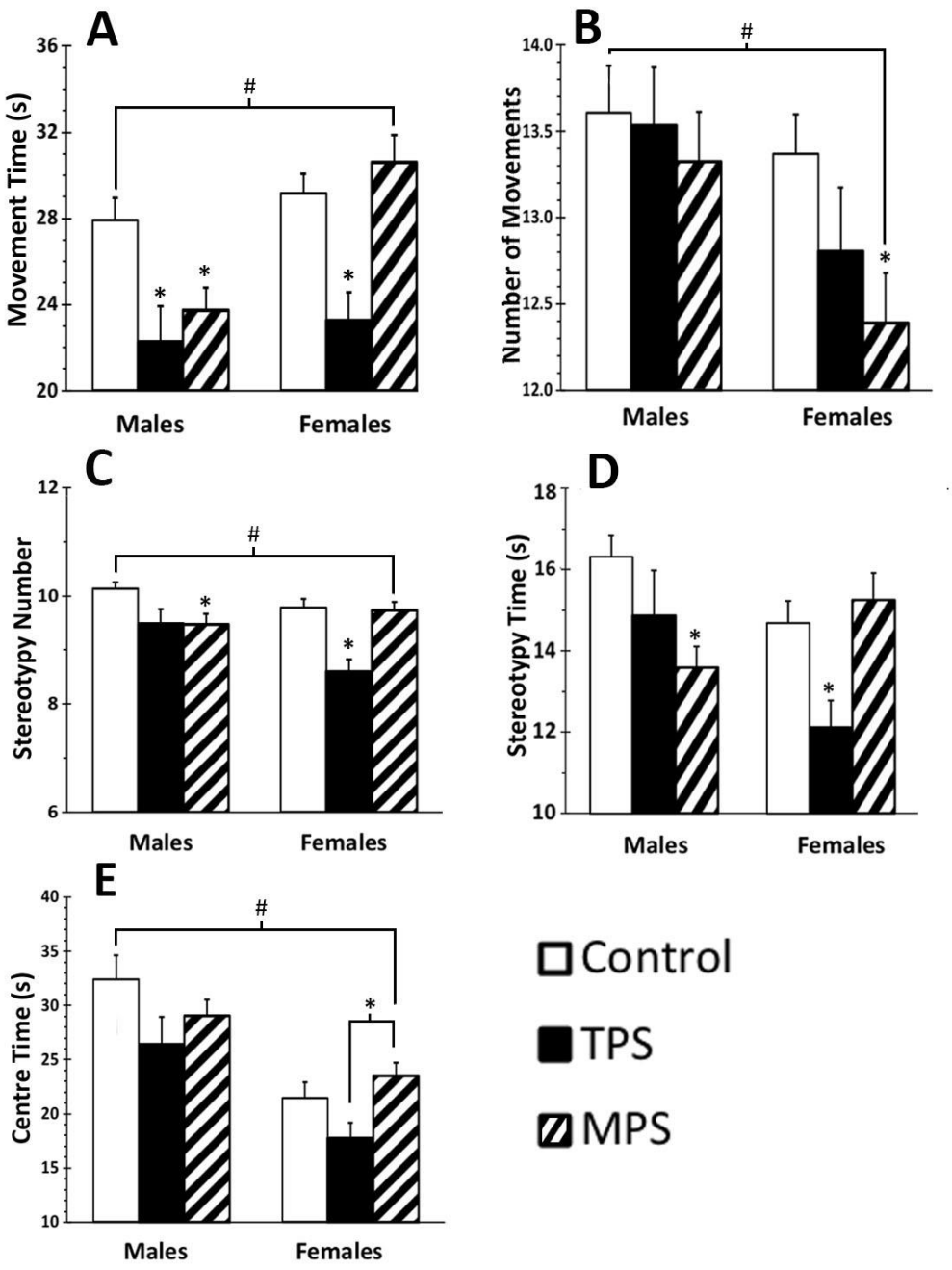

Figure 16. F4 male and female automated exploratory open field at P110 results including A) duration of movements, B) number of movements made, C) Number of bouts of stereotypy, D) total time in stereotypy, E) time spent in the centre of the field. Error bars show SEM. Asterisk denotes a significance of treatment: * $p<0.05$, and octothorp denotes a significance of sex: # $p<0.05$.

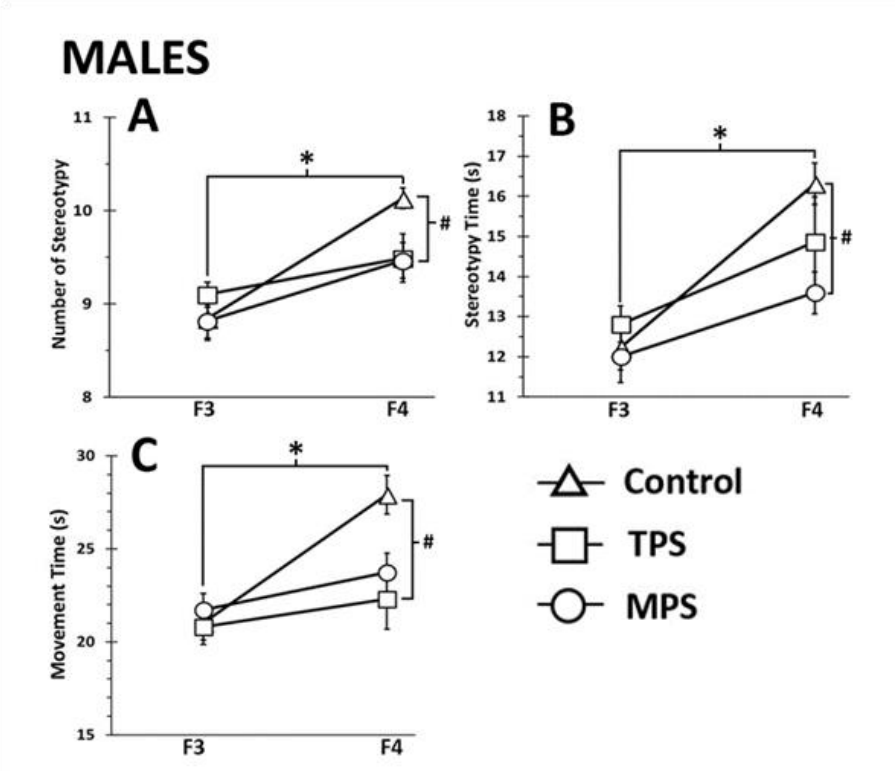

Figure 17. F3 and F4 males at P110 in the automated exploratory open field including A) number of stereotypic bouts B) total duration of stereotypy C) duration of movement. Error bars show SEM. Asterisk denotes significance of generation: * $p<0.05$. Octothorp denotes a significance of treatment: # $p<0.05$.

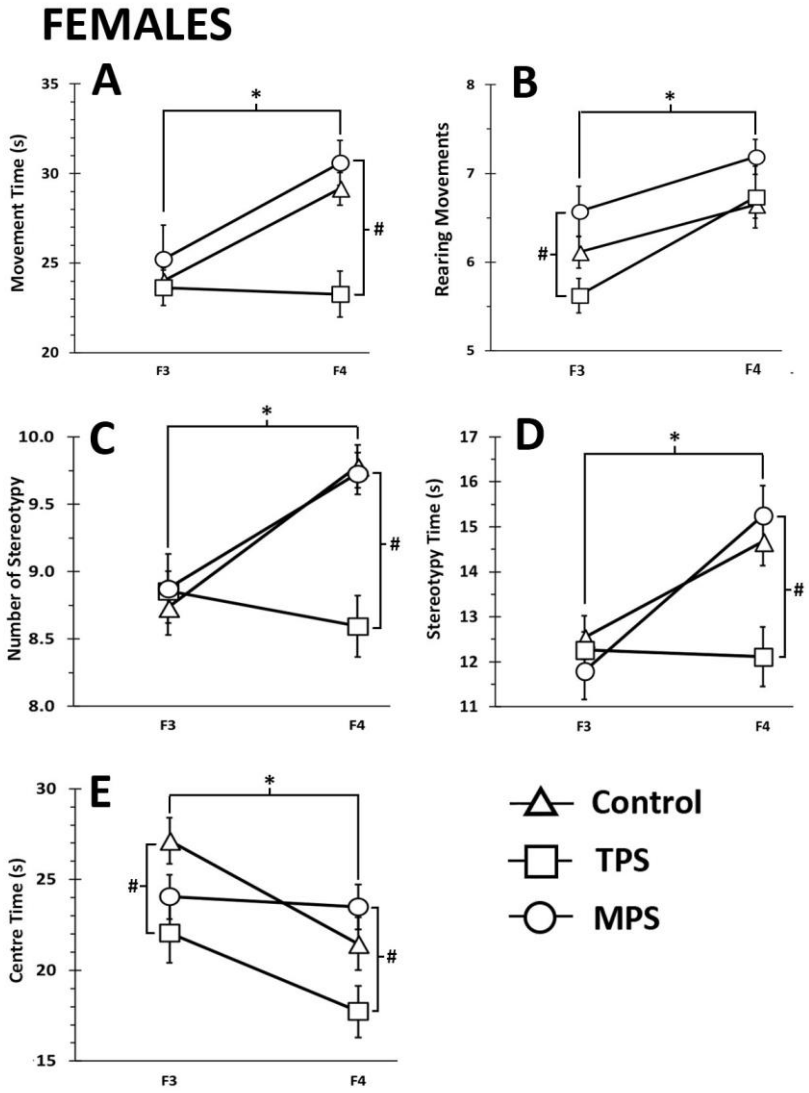

Figure 18. F3 and F4 females at P110 in the activity box including A) duration of movement, B) number of rearing movements, C) number of stereotypic bouts, D) total duration of stereotypy, E) time spent in the centre of the field. Error bars show SEM. Asterisk denotes a significance of generation: * $p<0.05$, octothorp denotes a significance of treatment: # $p<0.05$.

Affective Exploration. ANOVAs in males (CONTROL n=24, MPS n=31) and females (CONTROL n=24, MPS n=24) showed that there was no significant effect of treatment in any of the measures of affective exploration (see Appendix B, table 16.)

ANCOVAs for both the F3 and F4 generations indicated that every single measurement taken in the novel emergence task showed a significant effect of generation. In males, an additional generation from F3 animals to F4 animals increased latency to emerge ($F(1,83) = 6.073$, $p = 0.016$, $\eta^2 = 0.066$), increased the time inside of the tube ($F(1,83) = 15.36$, $p < 0.001$, $\eta^2 = 0.156$), and subsequently percent time in the tube ($F(1,83) = 15.36$, $p < 0.001$, $\eta^2 = 0.156$). Inversely the added generation decreased the amount of time outside of the tube ($F(1,83) = 16.31$, $p < 0.001$, $\eta^2 = 0.164$). In females, the same trend is evident, to increase time inside the tube ($F(1,192) = 11.36$, $p = 0.001$, $\eta^2 = 0.056$), decrease time outside of it ($F(1,192) = 11.97$, $p = 0.001$, $\eta^2 = 0.059$), and increase the latency to exit the tube ($F(1,192) = 2.031$, $p = 0.002$, $\eta^2 = 0.049$).

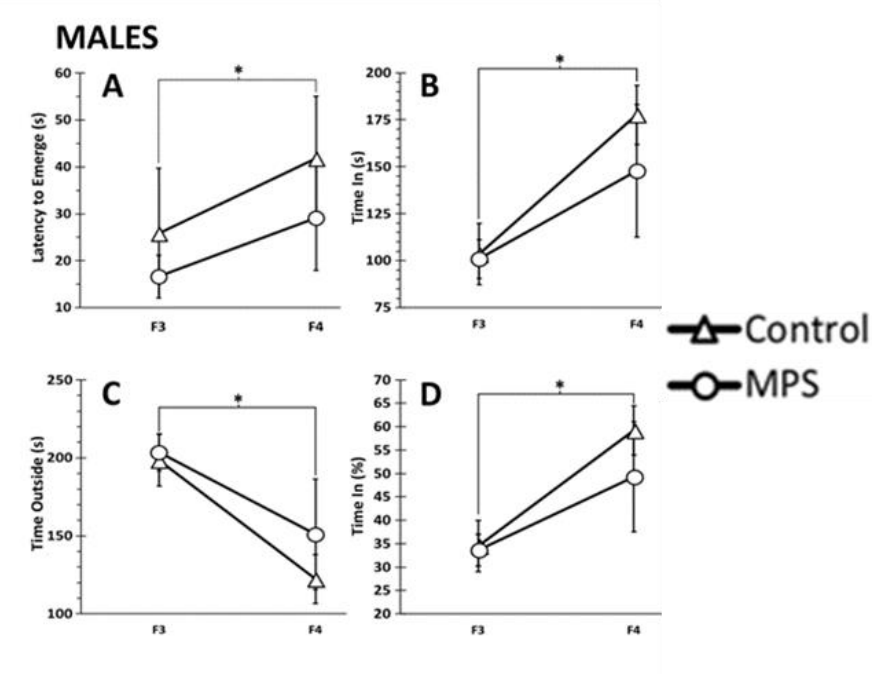

Figure 19. F3 and F4 males at P110 in the affective exploration task including A) latency to emerge from the tube, B) time spent inside the tube, C) time spent outside the tube, D) percent of total time spent inside tube. Error bars show SEM. Asterisk denotes significance of generation: * $p<0.05$.

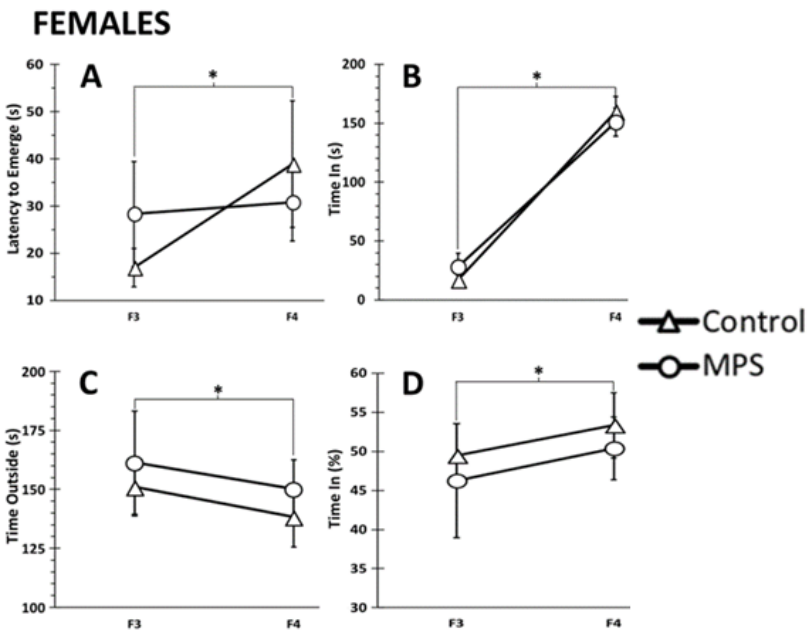

Figure 20. F3 to F4 females in the affective exploration task at P110 including A) latency to emerge from the tube, B) time spend inside the tube, C) time spent outside the tube, D) percent of total time spent inside tube. Error bars show SEM. Asterisk denotes significance of generation: *p<0.05.

Discussion

Here, we demonstrated that ongoing and ancestral distress is causally connected to pregnancy health, pregnancy outcomes, and offspring health. Despite the low power of the study, the findings provide evidence that generational stress caused physiological dysregulation. As generational stress programs HPA axis activity, its effects may accumulate during pregnancy. the resulting stress response and potential HPA axis dysregulation led to persistent increases in corticosterone and glucose values, and reduced weight gain during pregnancy [6, 30, 31]. This preliminary study supports the potential of further research with larger sample sizes. Future studies on this topic should consider including additional markers affected by transgenerational stress, as glucose and body weight were marginally affected by ongoing stress during pregnancy.

The effect of generation when comparing the F2 and F3 mothers within each treatment group revealed an elevated MSI, including among controls. This increase therefore is not reflective of the stress treatment itself, but instead may demonstrate the impact of environmental factors or bystander stress. Alternatively, it is possible that the observations are affected by handling and testing procedures, as each mother was weighed, and their blood drawn four times during pregnancy, while also their offspring received behavioural tests. It is possible that the stress of testing is accumulating in the mothers and can be measured through the MSI.

The litter health measures, on the other hand, demonstrated a significant effect of dysregulation due to ancestral stress. The TPS animals experienced slightly longer gestation times, which may suggest that the mothers have enough resources to extend their pregnancy [32-34]. Resource abundance is not evident though in litter sizes, where TPS litter sizes were negatively impacted by the ancestral stress as a result of a reduction in implantation, but not increased resorption. This is not an unfamiliar phenomenon, as studies of clutch size, particularly in fish and insects, have revealed similar observations [35, 36]. It is possible litter size is a resultant of the perceived resource availability, as each environment may be linked to a specific optimal clutch size, with too few offspring being a waste of resources, but too many resulting in higher offspring mortality [35, 36].

There are two possible explanations for this phenomenon. The first potential explanation for the reduction in the number of offspring in TPS animals assumes that

dangerous or resource-limited environments tend to induce smaller litter sizes, as each additional offspring takes energy and resources to keep alive. The optimal number of pups would be reduced in order to conserve the resources and energy needed for the survival of the whole. This phenomenon was not followed by an increase in pup mortality or infanticide during the first 20 days of life, which may occur when dams experience dangerous environments after parturition [37, 38]. Indeed, research has demonstrated that elevated corticosterone levels and psychosocial stress can lead to early or aborted births, likely as an adaptive response to the perceived danger of the environment [39, 40].

The reciprocal explanation is that that the gestating rat was biologically prepared for a very safe environment, and therefore determined it was likely their offspring would survive until sexual maturity. If offspring have a high chance of survival, less offspring would be required to guarantee the passing of their genetic material. This theory is less likely, as insect and bird studies suggest habitats with bountiful resources, and few stressors, encourage larger litter sizes [57, 58].

The effect of generational stress on litter number can be discussed further when viewing the data comparing the F2 to the F3 mothers. Litter numbers showed a significant effect of generation, which suggests that TPS did not reduce the number of offspring it bore as compared to the previous generation, but control and MPS animals increased the number of offspring they bore. Based on the more likely of the two theories discussed above, this observation would favor the second notion of a stressful environmental favoring larger litters. If the first notion would apply, the findings would

suggest that control and MPS animals were deeming their environment to be safer and more resource abundant than that of TPS animals. Then the TPS animals were not benefiting from this perceived resource boon. These circumstances of litter size therefore need to be investigated further.

In terms of behavioural outcomes, within a single generation there was no indication of motor development delay in the negative geotaxis task. Males in the F3 generation, however, experienced a minor motor development delay in the TPS lineage. This deficit was ameliorated by the F4 generation. For the time spent facing up and time to turn revealed a slight trend in controls to turn over faster and remain facing up a little longer in the F4 as compared to F3 animals. Among females, the MPS animals showed a large reduction in the time it takes to turn and an increase in the time facing up, suggesting a minor motor deficit shown in the F3 generation that is no longer evident in the F4 generation. Also, in the F3 generation there was a slight trend for TPS and MPS females falling off the apparatus more frequently than the controls, yet again this effect disappeared in the F4 generation.

A general feature of increased stress in the literature is the observation of reduced exploratory activity likely due to an increase in fear [41-45]. In the present exploratory open field data, males revealed no effect of stress, but females showed lower levels of exploration as indicated by a reduction in novel squares, and total squares explored in the MPS animals. Nevertheless, the MPS females explored inside and outside squares equally, whereas the controls and TPS animals explored the inside squares less. Therefore, one could argue that MPS females were showing fewer anxiety-like

behaviours, as they did not discern whether they were safe in the outside squares or exposed in the inside squares. These animals may have been either grooming, resting, or rearing which was not measured in the manually scored open field exploratory paradigm. This finding is supported by the generational data, in that the females explored more of the inside squares than the previous generation, demonstrating a reduction in anxiety-like behaviours, but one that is displayed in every treatment group. In the males, the control and MPS groups did not show significant changes between generations. The TPS animals, however, which were significantly different than the controls and MPS males in the previous generation, starkly increased in number of inside squares explored, showing a reduction in anxiety now matching controls and MPS males. The total number of squares males explored was also abnormal in that the F3 controls explored a large number of squares, but in F4 the controls dropped down to explore the same number as the TPS and MPS animals, possibly showing a similar pattern as seen in the MSI and the litter numbers previously discussed.

When considering the automated open field exploratory behaviour, the results demonstrate that across variables such as horizontal activity, and overall movements made and rest time that the MPS and TPS males were exploring less and resting more than the control group. In the literature, reduced exploration serves as an indicator of anxiety behaviour [13, 46-48]. Alternatively, this change could also represent depressive-like behaviour, similar to what was demonstrated in the forced swim task where animals will stop swimming and float, which can be resolved with depression medication [49, 50]. In MPS males there was also a decrease in number of stereotypic bouts, and a

reduction in overall stereotypic time, demonstrating less stereotypy overall. Previous sources have shown that stereotypy can be an indicator of anxiety and can result from different stresses [51, 52], suggesting the MPS males were showing reduced anxiety-like behaviours. For the females, there were two main findings. The only significant change among MPS females was a reduction in the number of movements made, while the movement time remained high. This suggests that when the MPS females got going, they rarely stopped moving, leaving little time for rest. They did not travel significantly longer distances than the controls, so they were moving consistently, but slowly. This corroborates the results of the exploratory open field, where the females did not discern the difference between the inside and outside squares, therefore showing very little fear of the inner squares. In the older animals analyzed with the automated exploratory open field, this lack of fear manifested itself to increased movement and longer distance travelled [41]. Their slowness of movement could be a manifestation of depressive-like behaviour, or lack of motivation [53]. Among TPS females, a pattern of reduced exploration and increased anxiety was seen. Reduced exploration was evident in the horizontal activity, reduced total distance, and reduced movement time. The increased anxiety-like behaviour can be discerned by margin time, centre time, and centre distance, where it was evident that TPS females were spending less time and exploring less in the centre of the apparatus and spending more time in the margin. The TPS females were also displaying fewer, but longer bouts of stereotypic behaviour, in the same pattern as the males.

Reviewing the male generational data demonstrates that while there was a lack of movement and suspected depression-like behaviours in male TPS and MPS animals, all treatment groups were moving more and showing less depressive-like behaviours than the previous generation. The TPS and MPS animals changed less than the controls, therefore resulting in lower levels of activity. TPS females were the only rats that did not increase in activity and exploration from one generation to the next, again demonstrating that the MPS and control animals seems to react similarly, and the TPS animals changing very little from one generation to the next (as seen in litter sizes). As for anxiety-like behaviours, there was an increase among TPS females, and a smaller increase among MPS females.

Given the effects of recurrent stress across generations observed here, the experiment further considered aging among the MPS lineage Both MPS and control rats showed an increase in anxiety from F3 to F4 generations. This result lines up with the litter health and MSI data documenting an increase in general stress in the last generation, in controls and MPS animals alike. This demonstrates that the common condition experienced by all the animals is still influencing the animals' behaviour into adulthood.

These results of the present study support the match-mismatch hypothesis, which states that development is at least partially determined by the match between the conditions experienced in the uterine environment and the conditions experienced during early postnatal development [54, 55], The match-mismatch hypothesis offers a possible explanation for the abnormal behaviour found in TPS F3 animals. According to the

match-mismatch hypothesis, where the F3 TPS rats demonstrated extensive behavioural deficits due to a mismatch in the maternal environment, it is hypothesized that in the F4 generation one should see a slight amelioration of these deficits, as these offspring should be experiencing similar maternal environments as their mothers. The maladaptation of the F3 TPS animals may have been linked to epigenetic changes caused by the previous generations altering the offspring's expected environment. The continuation of this trend depends on the strength and longevity of those biological changes. If prenatal epigenetic regulation is preparing the animal for an adverse postnatal environment, one would expect a mismatch in the TPS animals. As the TPS animals are displaying behaviour that is consistently differing from control and MPS animals the epigenetic regulation of the previous stress may be activated, leading maladaptive phenotype changes. In the TPS lineage, the additional generation of a stress-free pregnancy did not "reset" the epigenetic profile to expect a safe environment. The MPS animals, experiencing another stressful pregnancy, may have allowed for some physiological and behavioural reserve to continue to prepare the animal for an unpredictable life. Instead of displaying increasingly impoverished behaviour from one generation to the next, the MPS lineage showed improved adaptation and phenotypic fitness in some respects.

In conclusion, the similarity between the MPS and control animals, and their subsequent dissimilarity to TPS animals suggests that the match-mismatch theory is a suitable hypothetical framework to explain the present findings. The negative behavioural impacts seen in the F3 TPS animals became partially ameliorated. But while some TPS behaviours were now more similar to controls, many of the behaviours measured were

distinctly different in the TPS animals compared to the control and MPS animals, including litter number, stereotypy, and exploratory activity. By contrast, the MPS animals and controls were both prenatally programmed to adapt to their postnatal environment, either a stressful environment or a non-stressful environment, respectively. Thus, the present findings offer an experimental model which may offer new insights into developmental and generational programming of lifetime health trajectories.

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Study 3

Ancestral Prenatal stress on F4: Aging Outcomes

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Declarations

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Consent for Publication. N/A

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Code Availability. N/A

Authors' Contributions. TR, MA, and GM designed the experiment; TR, MA and EF performed the data collection; TR, MA, EF, and OA participated in data analysis; TR, MA and GM wrote the manuscript; all authors provided edits and approved the final version of the manuscript.

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Introduction

The prenatal period may predict future health and behaviour of the offspring, including stress vulnerability later in life [1, 2], and the health of future generations [3, 4]. One proposed mechanism of gestational transmission of stress involves physiological dysregulation and homeostatic disturbance linked to maternal hypothalamic-pituitary-adrenal (HPA) axis functioning [4-7].

The effects of prenatal stress may not be limited to a single generation, but future generations [3, 4]. Indeed, ancestral stress has been shown to propagate through several generations through epigenetic mechanisms [8-13]. Here, it was hypothesized that the fourth filial generation (F4) of multigenerational prenatally stressed (MPS) animals will show behavioural abnormalities including anxiety and alterations in exploration, and that early behaviour outcomes can be used to predict adverse outcomes in old age .

Methods

Animals and Experimental Design

This study used the filial 4 (F4) generation, bred from a lineage of dams bred in a controlled environment. The F4 generation included 2 lineages, (1) multigenerational prenatal stress (MPS) in which dams from each consecutive generation underwent gestational stress, and (2) non-stressed controls.

Forty-four F3 dams were bred with 9 F3 males to generate the F4 generation. The JAX Colony Management System (JCMS; Jackson Laboratory, Bar Harbour, ME, USA) was used for pedigree tracking [14]. Males and females were paired for 1 hour/day. A

total of 280 females and 280 F4 males were birthed and assigned to either the MPS lineage (MALE = 91, FEMALE = 92, generating F4 SSSSS), or control (n= MALE = 111, FEMALE = 121, generating F4 CONTROL).

The F0, F1, F2, and the 13 F3 dams in the MPS treatment group underwent a semi-random sequence of gestational stress including 20 minutes of daily restraint stress and a 5-minute swim in room temperature water on gestational days (G) 12 to 18 [8, 15]. Animals were bred and housed at the University of Lethbridge local vivarium in standard shoebox cages (45.5 x 25.5 x 20), with corn cob bedding with a partner of the same gender and a piece of PVC tubing as a refuge. At P110 animals were tested on the automated exploratory open field. At the age of 6-, 12-, and 18-months animals were tested in an array of behavioural tests, including a follow-up automated exploratory open field, elevated plus maze, a skilled walking task, and a forced swim task.

Behavioural Analysis

Automated Exploratory Open Field. Exploratory activity was measured using an automated activity monitoring system with VersaMax 4.0 software (AccuScan Instruments Inc., OH, USA). The animals were placed in the left corner of a Plexiglass box (42cm x 42cm). There were 4 bars of infrared sensors around the edges of the box that monitor the location and activity of the animal for 10 sessions of 1 minute. The data were automatically transferred to a connecting computer and analyzed. The distance travelled, time each of the individual areas of the box, and the number of rears and stereotypy were recorded.

Elevated Plus Maze. The elevated plus maze [16] apparatus was made of a black opaque Plexiglass that was in the shape of a plus, with each arm 85 X 17cm. The apparatus was raised 93cm above the ground. Two of the opposite arms were enclosed with side and end walls and two of the arms were open. Animals were placed in the centre of the maze and filmed for 5 minutes. Videos were analyzed and scored by trained individuals. The time spent in the closed versus the open arms of the maze, as well as the number of respective entries were scored.

Ladder Rung Walking Task. The ladder rung walking task was used to assess skilled walking [17, 18]. The task involved a 1m long horizontal ladder and made out of Plexiglass with 20 cm high walls with metal bars each with 3 mm diameter that were placed at random intervals ranging from 1 to 5 cm apart. The ends of the ladder were placed on the edges of Plexiglass boxes that were 30 cm high and parallel to the ground. Rats were placed at one end of the ladder and were left to walk across the ladder. This was done in 2 stages over 2 days. The first day was a training day where the rats were given 5-6 chances to walk across the ladder successfully. On the second day, the animals were filmed from underneath the ladder in order to accurately film the position of fore- and hindlimbs as animals crossed the ladder. Animals were filmed 5 times per session. Videotapes were scored for the number of errors or missteps taken by each of their paws, as well the time taken to cross the device [17].

Forced Swim Test. During the forced swim test [19], animals were placed in a 121-litre were filmed for 5 minutes, which was scored by a trained individual on how long the animals were swimming versus floating and the total number of dives taken.

Statistical Analysis

Statistical analysis was performed using SPSS 20 for Windows 11.5.0 (IBM Corporation, Armonk, NY, USA). For aged offspring behaviour, an ANOVA was performed for each of the behavioural tests in order to determine the effects of mutigenerational prenatal stress on exploratory and anxiety behaviour. In any case where outliers were revealed, the data were winsorized. In the case of a breach in the assumption of normality necessary to perform an ANOVA, the appropriate transformation was performed, and the equivalent non-normal test was performed if appropriate. In order to determine if young exploratory behaviour predicts old age exploratory behaviour, a linear, or where appropriate, non-linear regression was performed.

Results

Animal Behaviour at 6, 12, and 18 Months

Elevated Plus Maze. An ANOVA was performed on each of the variables in the elevated plus maze at each of the time periods. The sample size, the standard error of the mean and the standard deviations can be found in table 1of Appendix C. The data was checked for normality, and in the males, time spent in the open arm was transformed with the log transformation in the first 2 time periods. Time spent in the end of open arms was

winsorized for 1 outlier in the first two time periods. In the 6-month rats, number of open arm entries was winsorized for 1 outlier. For the 12-month rats, time in centre was skewed, and corrected with a square root transformation. Table 2 in Appendix C displays all the main effects of treatment for the elevated plus maze ANOVAS.

The results showed no significant changes in the females. The males at 6 months revealed a significant increase in time spent in the centre of the elevated plus maze in MPS animals ($F(1,18) = 4.577$, $p = 0.046$, $\eta^2 = 0.203$). At 12 months there was a significant increase in the number of closed arm ($F(1,20) = 4.678$, $p = 0.043$, $\eta^2 = 0.190$) and total arm entries ($F(1,20) = 7.665$, $p = 0.012$, $\eta^2 = 0.277$) in the MPS animals. At 18 months the MPS animals showed a decrease in the time spent in the closed arm ($F(1,10) = 7.455$, $p = 0.021$, $\eta^2 = 0.427$). It is worth noting the high effect sizes on all of these findings, even at 18 months despite the small sample sizes.

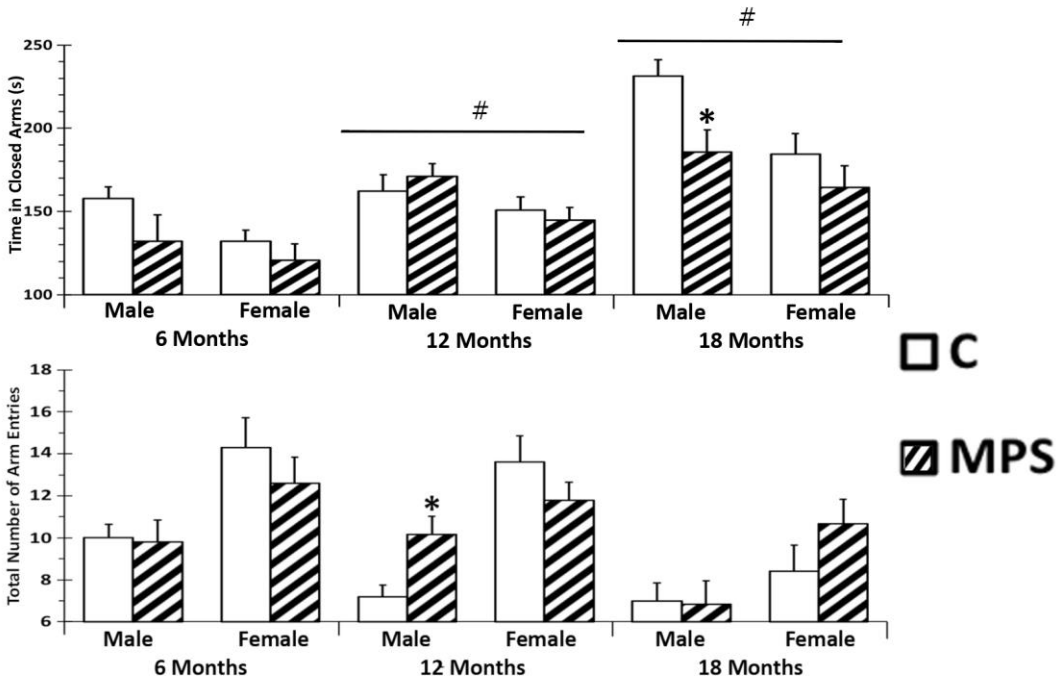

Figure 21. Results of the elevated plus maze in males and females at 6-, 12-, and 18-months including A) time spent in the centre of the plus, B) time spent in the closed arms, C) number of closed arm entries, and D) the total number of arm entries. Error bars show SEM. Asterisk denotes a significance of treatment: * $p<0.05$ compared to CONTROL. Octothorp denotes a significance of sex: # $p<0.05$.

Forced Swim Task. ANOVAs for 6,12, and 18 months revealed sexually dimorphic changes in latency to immobility and the number of dives during the forced swim task. Table 3 in Appendix C shows the main effects among males (CONTROL 6M n = 11, 12M n = 10, 18M n = 5; MPS 6M n = 10, 12M n = 12, 18M n = 7) and females (CONTROL 6M, n = 10, 12M n = 13, 18M = 9; MPS 6M n = 10, 12M n = 15, 18M n = 12). In the 6-month males the number of dives was winsorized to remove the impact of one outlier. In females, number of dives was found to be very skewed, and while the log transformation helped, it was not able to achieve normality, so a Mann-Whitney test was performed instead of the ANOVA.

Three significant results were found. At 6 months the MPS males took longer to become immobile than the CONTROLs ($F(1,19) = 4.695$, $p = 0.043$, $\eta^2 = 0.198$) and at 12 months the MPS males performed more dives ($F(1,20) = 7.435$, $p = 0.013$, $\eta^2 = 0.271$), though it should be noted that the Levene's statistic was significant at $p = 0.033$, so these results may not be totally reliable. In females, the 12- month MPS animals had a longer latency to immobility ($F(1,26) = 5.824$, $p = 0.023$, $\eta^2 = 0.183$).

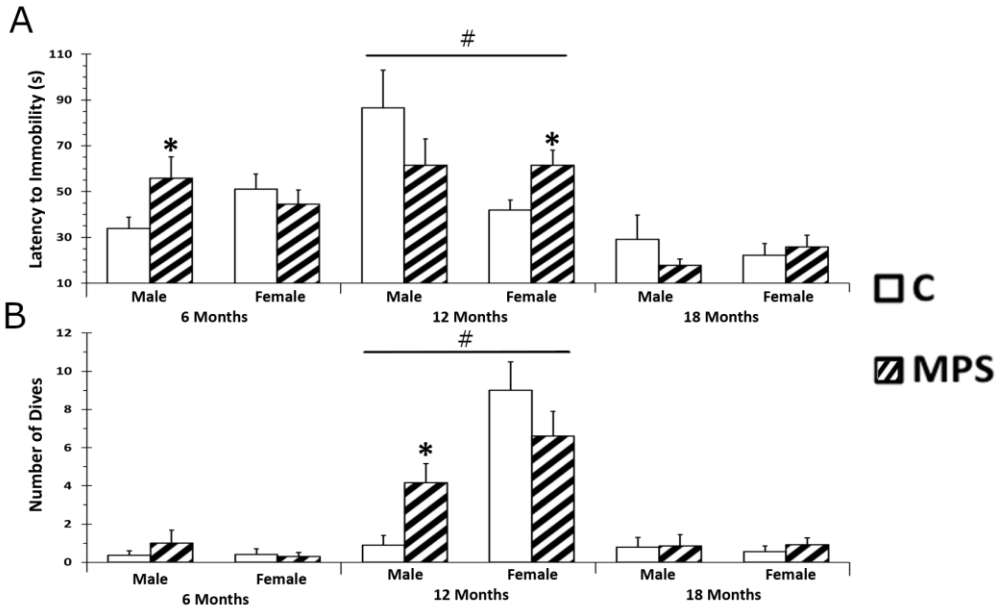

Figure 22**.** Results of the forced swim results in males and females at each 6, 12, and 18 months of age including A) latency to immobility, and B) total number of dives. Error bars show SEM. Asterisk denotes a significance of treatment: * $p<0.05$ compared to CONTROLS. Octothorp denotes a significance of sex: # $p<0.05$.

Automated Exploratory Open Field. ANOVAs for each age group revealed sex-dependent alterations on exploratory and anxiety-related behaviours. Table 5 in Appendix C shows the main effects among males (CONTROL 6M n = 10, 12M n = 9, 18M n = 5; MPS 6M n = 9, 12M = 9, 18M n = 6) and females (CONTROL 6M n = 10, 12M n = 13, 18M n = 10; MPS 6M n = 9, 12M = 14, 18M n = 12). No evidence of skewness or kurtosis was found.

Several significant results were found. Firstly, the 6-month MPS males spent more time, and walked farther, in the margin (Margin Time $F(1,17) = 4.642$, $p = 0.046$, $\eta^2 = 0.214$; Margin distance $F(1,17) = 6.019$, $p = 0.025$, $\eta^2 = 0.261$), with a corresponding significant reduction in the time spent in the centre ($F(1,17) = 4.642$, $p = 0.046$, $\eta^2 = 0.214$). 12-

month MPS males walked a farther total distance ($F(1,16) = 8.095$, $p = 0.012$, $\eta^2 = 0.336$) and reared for a longer period of time ($F(1,16) = 4.879$, $p = 0.042$, $\eta^2 = 0.234$). Among the 18-month MPS males and females there were no significant changes. For 6-month MPS females there was a significant increase in horizontal activity ($F(1,18) = 4.501$, $p = 0.048$, $\eta^2 = 0.200$), movement time ($F(1,18) = 5.743$, $p = 0.028$, $\eta^2 = 0.242$), and a corresponding reduction in resting time ($F(1,18) = 5.772$, $p = 0.027$, $\eta^2 = 0.243$), as well as an increase in total distance travelled ($F(1,18) = 4.976$, $p = 0.039$, $\eta^2 = 0.217$). For 12-month females, MPS reduced the number of movements ($F(1,25) = 7.431$, $p = 0.012$, $\eta^2 = 0.229$) as well as stereotypy ($F(1,25) = 6.085$, $p = 0.021$, $\eta^2 = 0.196$). It is worth mentioning that each of these findings had very high effect sizes, all above 0.190.

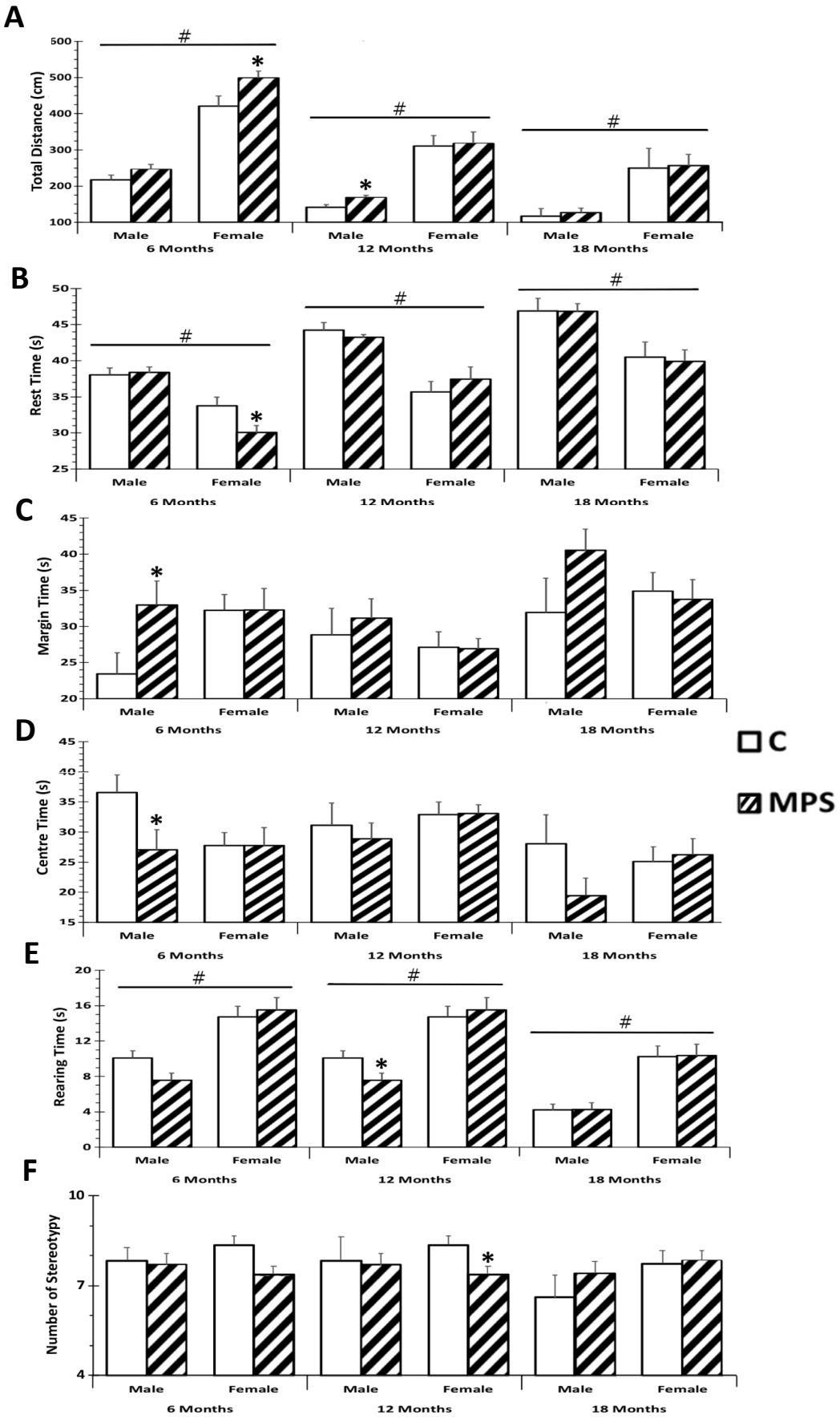
A
Total Distance (cm)
B
Rest Time (s)
C
Margin Time (s)
D
Centre Time (s)
E
Rearing Time (s)
F
Number of Stereotypy
Male
Female
6 Months
12 Months
18 Months
C
MPS

Figure 23. Automated exploratory open field results for males and females at 6, 12, and 18 months of age, including A) total distance travelled, B) amount of time spent resting, C) amount of time spent in the margin, D) amount of time spent in the centre of the field, E) the total time spent rearing, and F) total number of stereotypy. Error bars show SEM. Asterisk denotes a significance of treatment: * $p<0.05$ compared to CONTROLS. Octothorp denotes a significance of sex: # $p<0.05$.

Lifespan

The ANOVA for the offspring lifespan found sex-specific effects. Table 6 in Appendix C lists the descriptive statistics for each treatment and sex. The female lifespan was negatively skewed and transformed using an inverse transformation. MPS treatment in males showed no significant change in lifespan. In females there was a significant increase in lifespan due to MPS ($F(1,26) = 4.791$, $p = 0.038$, $\eta^2 = 0.156$). It should be noted that Levene's statistic was highly significant $p<0.001$.

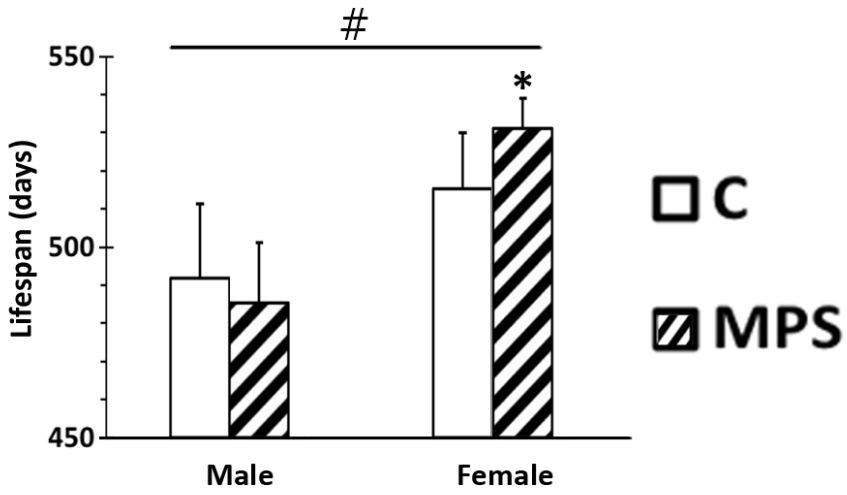

Figure 24. Lifespan of CONTROL and MPS males and females in days. Error bars show SEM. Asterisk denotes a significance of treatment: * $p<0.05$ compared to CONTROLS. Octothorp denotes a significance of sex: # $p<0.05$

Young Offspring Behaviour Predicting Old Age Abnormalities

Regressions were performed for each of the variables in the automated exploratory open field. All of the main results for males (CONTROL P110 by 6 Months n = 10, P110 by 12 Months n = 9, P110 by 18 Months n = 5 ; MPS P110 by 6 Months n = 9, P110 b7 12 Months n = 9, P110 by 18 Months n = 6), and females (CONTROL P110 by 6 Months n = 10, P110 by 12 Months n = 13, P110 by 18 Months n = 10; MPS P110 by 6 Months n = 10, P110 by 12 Months n = 14, P110 by 18 Months n = 8), are summarized in Table 7 in Appendix C, but for the remainder of this chapter we will only be discussing the time points P110 by 12 months.

Males. For 12-month MPS males, the time spent in the centre of the automated exploratory open field demonstrated a significant positive linear correlation ($R = 0.68$, $p = 0.04$) when comparing to P110. Inversely, the amount of time spent in the margin of the automated exploratory open field at P110 also predicted the amount of time spent in the margin at 12 months in a negative linear relationship ($R = 0.68$, $p = 0.04$). Correlations were also calculated for overall lateral activity, including distance travelled, rest time, and movement time, but no significant correlations were discovered. Finally, repetitive behaviour, termed stereotypy, was determined to have a significant negative linear correlation in the 12M to P110 males, but only in the CONTROL group ($R = -0.82$, $p = 0.04$). The significant correlation was lost in the MPS males.

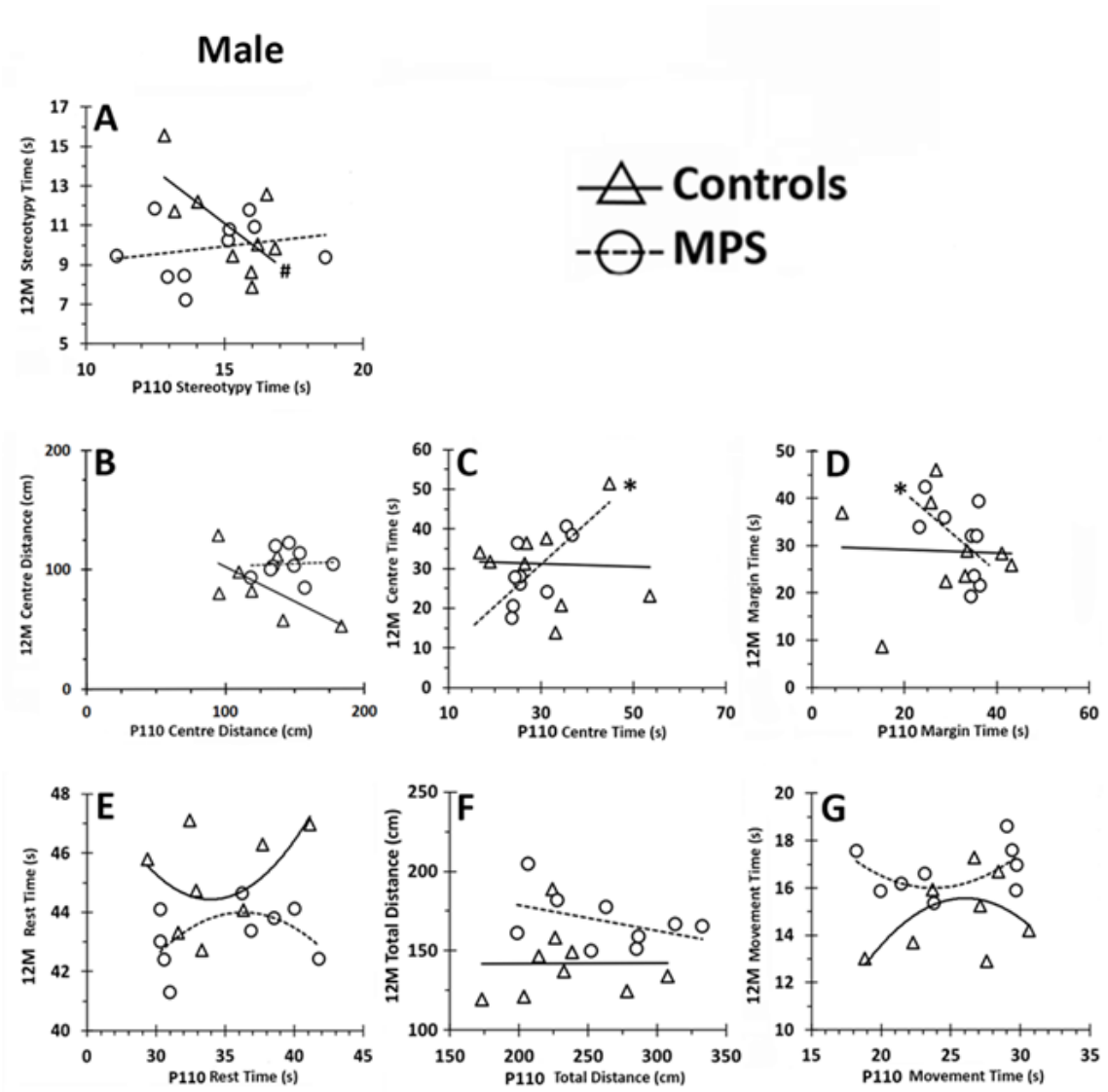

Figure 25. Correlations between P110 and 12-month automated exploratory open field measurements in males including A) stereotypy time, B) distance walked in the centre of the field, C) time spent in the centre of the field, D) time spent in the margin of the field, E) time spent resting, F) total distance travelled, G) and total movement time. Asterisk denotes a significant correlation in the MPS group: * $p<0.05$, and octothorp denotes a significant correlation in the CONTROL: # $p<0.05$.

Females. The amount of time spent resting during the automated exploratory open field at 12 months can be predicted by the amount of time rest at P110 in a positively linear relationship ($R = 0.55$, $p = 0.04$) for the MPS females only. Most of the significances seen in the males were not found in the female MPS animals, but

CONTROL females did show some significance, including a positive linear relationship for centre distance (R = 0.56, p = 0.05), a positive linear relationship for centre time (R = 0.68, p = 0.01), and a positive linear relationship for time spent in the margin (R = 0.68, p = 0.01). Activity levels also showed significance in CONTROL females, but not in MPS females for total distance (R = 0.63, p = 0.02), and movement time (R = 0.57, p = 0.04). Rest time was the only variable that showed a significant relationship in both the MPS females (R = 0.55, p = 0.04) and the CONTROL females (R = 0.57, p = 0.04).

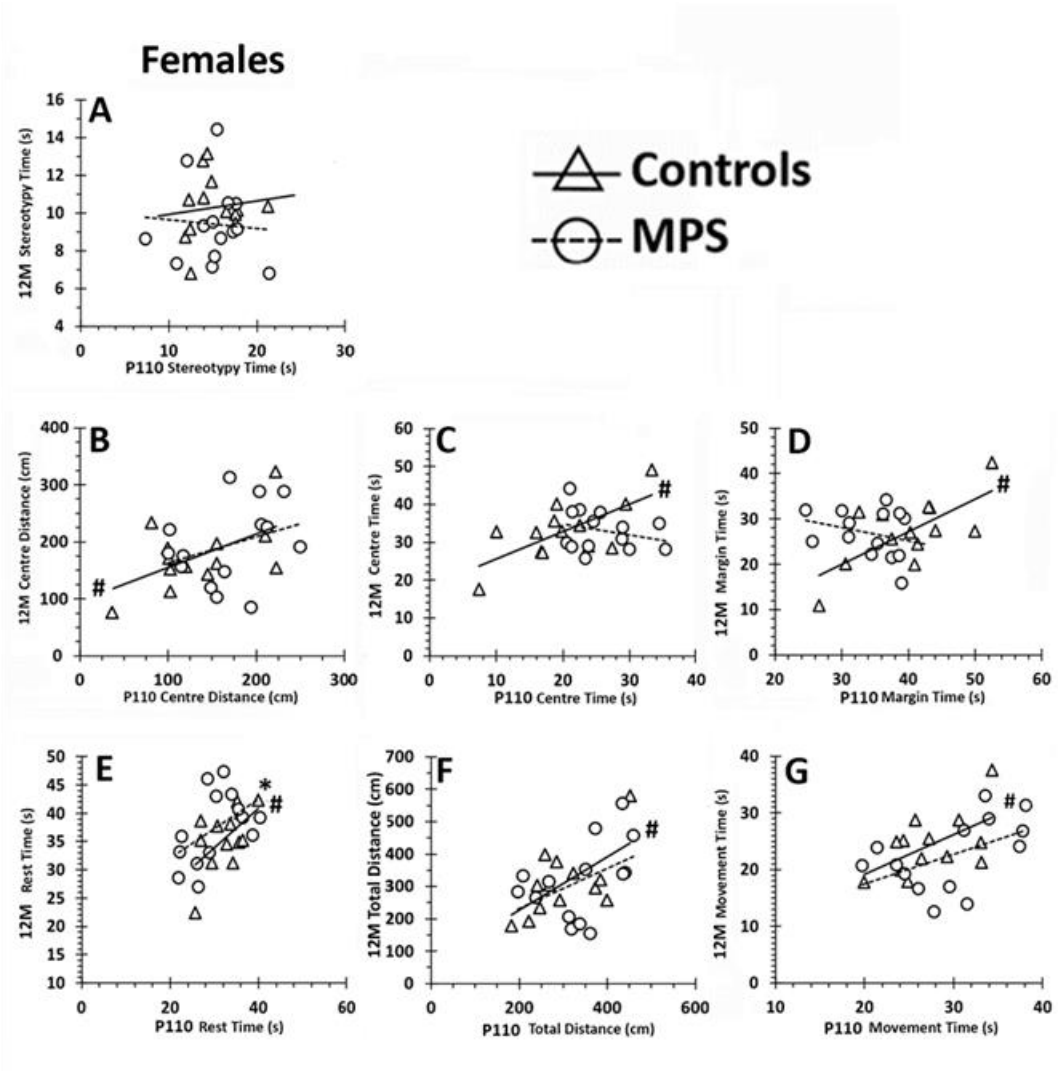

Figure 26. Correlation between P110 and 12 month automated exploratory open field measurements in females including A) stereotypy time, B) distance walked in the centre of the field, C) time spent in the centre of the field, D) time spent in the margin

of the field, E) time spent resting, F) total distance travelled, G) and total movement time. Asterisk denotes a significant correlation in the MPS group: * $p<0.05$, and octothorp denotes a significant correlation in CONTROL: # $p<0.05$.

Discussion

This chapter investigated the aging trajectory of animals exposed to MPS versus non-stressed controls. The results of the behavioural tests suggest sex- and age-related changes as a result of MPS as demonstrated through evidence of anxiety-like behavious, primarily in young males, lower risk for depression-like behaviours in middle aged females and young males, and an increase in overall motor activity in males and females. The expectation that behavioural functions among MPS animals may deteriorate with age due to generations of prenatal stress was not substantiated.

Anxiety-like behaviours and motor activity can be measured through the elevated plus maze by assessing the rodents' contradictory desire to explore a new space, but stay in safe, closed, and protected areas [20, 21]. The elevated plus maze demonstrated in males, a consistent message of low levels of anxiety-like behaviours. At 6 months, the male MPS animals spent significantly more time in the centre of the elevated plus maze, suggesting increased exploration of an open, dangerous, environment. At 12 months the male MPS animals showed a significant increase in the number of closed arm entries. This measure alone could demonstrate an increase in anxiety, but when taken with total arm entries suggests an increase in exploration and a possible subsequent decrease in fear of the unknown areas, as suggested by social isolation literature [22-24]. At 18 months there was a significant reduction in time spent in the closed arms, again, suggesting a

lower level of anxiety-like behaviour. Females showed no significant change, suggesting the females were less affected by the MPS than the males.

The forced swim task is a verified test for depressive-like behaviour in rodents [25, 26]. Experimenters measure how long a rodent remains swimming to escape the water. Rodents will also frequently float, and the latency to immobility is regarded an indicator of depressive-like activity [25, 26]. Anti-depressive medications predictably increase the latency to immobility [27]. When evaluating the forced swim task, the MPS males exhibited a significant increase in the time it takes for the animals to become immobile, suggesting a reduction in depressive-like symptoms in 6-month MPS males. Among the 12-month rats, the immobility latencies were not significantly altered compared to controls, but there was an increase in number of dives, suggestive of an increase in exploratory behaviour, and a possible reduction of fear or anxiety-like behaviours. In MPS females, there was a similar pattern, but with an increase in latency to become immobile occurring at 12 months instead of 6 months, so while there was a reduction in depressive-like symptoms it occured later in life.

The automated exploratory open field is a computer monitored field that allows objective measurement of various indicators of anxiety-like behaviour and exploration [10]. In the F4 MPS males the open field measure presented some contradictory results. 6-month males were spending more time in the margin, and less in the centre of the open field which would indicate anxiety, or at least timid behaviour. This effect disappeared by 12 months, where the males no longer exhibited any significant change in time spent in

the margin or centre of the field, but instead an increase in walking distance, and a decrease in rearing activity. The walking distance is a measure of exploration, typically seen as an adaptive trait for a rat, and the rearing activity, while adaptive, may be seen as a measure of anxiety-like behaviour if significantly elevated as the rat is spending a disproportionate amount of time searching for danger [10]. This suggests any anxiety-like behaviour seen at 6 months had disappeared by 12 months. The 6-months old females displayed increased exploration as well, as demonstrated through in increase in horizontal activity, a spike in time spent moving over resting, and a corresponding increase in distance travelled, which suggests low levels of anxiety-like behaviour. 12-months old females showed a reduction in stereotypy, which previous studies have shown, elevated levels of stereotypy may serve as an indicator of stress induced dysregulation [28, 29], suggesting the present females were experiencing lower levels of stress.

The final measure of health calculated was lifespan. The results demonstrated that female MPS rats had the longest lifespan, indicating an adaptive advantage to MPS. The males, while they did not show an advantage, they were not showing a significant disadvantage due to stress either, as would likely be expected assuming that cumulative stress may impact life- and health span.

In the literature there is no consensus on which sex is more vulnerable to the effects of stress. The results arguably depend on the type of measure use to determine vulnerability or resilience. Several studies have determined females to be more vulnerable [30, 31], as measured with the open field and the elevated plus maze.

Numerous studies have noted males to be more vulnerable, and females more resilient to prenatal stress [32-36] as determined through the skilled reaching task and the elevated plus maze. In this study males demonstrated some resilience, with reduced anxiety-like behaviours in the elevated plus maze, with contradictory results in the activity box, revealing an increase in anxiety-like behaviours. The lifespan data on the other hand suggests that females may have higher resilience.

To determine if anxiety-like behaviour in a rat can predict affective state in an older age, a correlation between measures for anxiety-like behaviours and exploration in the activity box at P110 was performed at 12 months. For males, young age anxiety-related behaviour was able to predict old age anxiety-related behaviour in a linear relationship. This can be determined through the three significant correlations that were related to anxiety-like behaviour, centre time, margin time, and stereotypic behaviour. The male MPS animals that were spending very little time in the centre of the field at P110, were likely to be the animals that were spending very little time in the centre at 12 months. The margin time data is interesting in that it shows the inverse relationship. The animals that spent much time in the margin at 12 months, spent very little time in the margin at 12 months. This contradictory result suggests that one of the measures, either margin or centre time, was not predictive of anxiety-like behaviour. Arguably, spending time in the margin of the field does not necessarily mean the animal is showing low levels of anxiety-like behaviours. It is also worth mentioning that this relationship was not observed in the control animals. Stereotypic behaviour did show a significant

correlation, but only in the controls, suggesting that the MPS reduced the predictability of that behaviour.

The correlation between the ages of P110 and 12 months among females revealed very little significance for the MPS group, except for a single measure, resting time. This measure may not have been connected to MPS as this correlation was also significant for the control group. A notable difference between controls and MPS females can be seen in anxiety-like behaviour and exploratory behaviour in a loss of predictability. Centre distance, centre time, margin time, total distance, and movement time all showed significant correlations in the control animals but non-significant correlations in MPS animals. This demonstrates that the MPS is impacting these rats between the ages of P110 and 12 months, but not necessarily in a predictable or consistent way. Some rats experienced an increase in exploration and anxiety-like behaviours, some experienced a decrease of the same. Therefore, an unknown factor may influence the predictability of this result.

In conclusion, the results of this chapter suggest that the match-mismatch model of ancestral stress is an appropriate model to follow when determining the possible effects of MPS on the fourth generation. The MPS animals have a history of receiving mild stress during pregnancy. The more common cumulative stress model would suggest that this should result in animals that have a large range of behavioural vulnerabilities including anxiety, deficits in development, and learning and cognition [37-39]. The animals in this study showed some signs of vulnerability, primarily in the automated

exploratory open field, with some indication of anxiety in 6-month males, but other observations suggest the males and females adapted to from the multigenerational prenatal stress, with decreased anxiety in elevated plus maze 6, 12 and 18 month males, reduced depressive symptoms in the forced swim in males and females, and females increased exploration in the automated exploratory open field. This is likely due to the accumulation of adaptation that occurred throughout the 4 generations. This isn't a novel finding, there has been consistent research showing that repeated ancestral stress can result in a greater resilience [13, 15, 21, 32, 40-42]. After each subsequent generation of mild prenatal stress, the inherited epigenetic mechanisms allowed the animals to better prepare themselves for the next generations of stress. Further research should examine the mechanisms leading to better mental health, and an extended lifespan. The present findings suggest that the MPS model provides an exciting new venue for the understanding of the biological origins of stress vulnerability versus resilience.

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Final Conclusions

It is generally acknowledged that experiencing stress at any point in life can lead to detrimental health and wellness outcomes, but stress-inducing events occurring during the prenatal period are typically seen as particularly impactful. The fetal period is known to be a "sensitive period" for developmental trajectories, making the embryo extremely vulnerable to disruption in brain development [1, 2]. A certain amount of stress may be beneficial towards building an accurate expectancy and resilience towards the environment, particularly when viewing multiple generations of stress. This theory has been termed the match/mismatch hypothesis [3], which states that mild adversity and stress early in life epigenetically prepares an individual for adversity and stress later in life. In other words, those that experience a prenatal stress event could become more resilient because they are prepared for the environment to which they are exposed. The three studies that comprise my thesis set out to comprehend the sort of effects that exposure to a single hit of prenatal stress has on a four-generation lineage, known as transgenerational prenatal stress (TPS), compared to repeated hits of prenatal stress which formed the multigenerational prenatal stress (MPS) lineage. We also aimed to understand the extent of the cumulative dysregulation that occurs in the maternal line due to stress, and to work towards a quantitative score that could eventually be used to predict the extent of the physiological and behavioural dysregulation expected in the future offspring. This score has been introduced as the maternal stress index (MSI) and the findings suggest it may provide a useful tool to approximate individual stress vulnerability versus resilience.

The first study aimed to develop the Maternal Stress Index (MSI) that would standardize and conceptualize the extent of stress the mothers experienced, and the effects that stress is having on the behaviour of the fourth-generation rats. The study concluded that the MSI measured ongoing stress moderately well, and that it was able to predict reduced anxiety-like behaviour in the young filial generation 3 (F3) male offspring but could not accurately quantify the impact of ancestral stress. The TPS dams resorbed more of the implanted embryos, and the remaining offspring showed delays in sensorimotor development and increases in anxiety-like behaviour. In contrast, the MPS dams had healthy births and produced offspring which exhibited behaviour similar to controls with some evidence of resilience and increases in exploratory behaviour.

The second study aimed to increase the sensitivity of the MSI by comparing the MSI obtained for the F2 and F3 generations to determine the cumulative effects of further generations of stress up to the F4 generation. Maternal physiology, birth outcomes and behaviour in the F3 to F4 generations revealed the developmental delays and resorption evident in F3 were no longer evident in F4, therefore an additional generation without stress seems to have reduced the effects of TPS, making TPS animals developmentally similar to controls. This is not surprising as these F4 TPS animals have not experienced prenatal stress directly. However, the control and MPS animals showed increased litter number and behavioural stereotypy time over the generations, but TPS animals remained with smaller numbers and minimal stereotypy. The only deviation from an adaptive response in the MPS animals was observed in the amount of exploration, where they explored fewer squares, inside compartments, outside compartments, and novel squares

in the exploratory open field at P15. This reduction of exploratory behaviour has been linked to both prenatal stress as well as ongoing stress [4-6], but because there is an equal reduction in exploration across centre and margin squares, this may not be signaling anxiety.

The third and final study focused on the F4 generation to determine if the healthy young MPS animals developed adverse behaviour as they aged, and if the behaviour at a young age provides a suitable predictor of behaviour in older ages. The primary findings were that the MPS animals had generally positive results, showing an indication of increased exploration and reduced depressive-like behaviour in both the males and females as demonstrated in the elevated plus maze and the forced swim test, respectively. In the exploratory open field, there were observable increases in exploration in MPS males and females and increases in anxiety-like behaviours in males. The observations indicate that behaviour at P110 was able to linearly predict anxiety-like behaviour in a positive correlation. Finally, the assessment of lifespan found that female MPS animals lived longer than their control counterparts. This suggests that the repeated prenatal stress in the MPS lineage leads to potentially protective effects on health span and lifespan.

Each of the studies was able to support the match/mismatch theory regarding ancestral prenatal stress, but the study is not definitive. In each group, the animals that were directly exposed to stress in the womb performed better on tests, and the animals that were ancestrally exposed to stress, but were not exposed in their own generation showed a rather higher vulnerability to adverse pregnancy outcomes and impaired

behavioural outcomes. This suggests that the current understanding of the detrimental impacts of mild stress during the prenatal period may need further research and thought. The observations would likely be different if dams in the present study were exposed to more intense stressors during pregnancy [7-9] or exposed to stressors within their adulthood [10], but the use of mild stressors makes this work novel, with the stress experienced being more ecologically valid. Mild stress being harder to measure using subclinical physiological changes further supports the use of a cumulative allostatic like score, like the MSI. This research also showed the sexually dimorphic impact of stress, and the novelty is that we demonstrated impacts in both males and females, and generally found more severe impacts in females which is more unique in the literature [11, 12].

Future steps within this field would be beneficial. Firstly, assessing other measures of stress dysregulation to determine if they would be appropriate to add to the MSI, and strengthening the index to predict offspring development and behaviour more reliably. It would also be beneficial to translate the present findings to human populations at risk by developing a novel stress assessment device that would be practical in a clinical setting, for predicting health risks, intervening in a timely manner, and protecting the health and wellness of future generations.

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APPENDIX A – Study 1

Table 1. Descriptive Statistics for F2 mothers MSI

	Baseline		G11		G18		G21	
	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD
Control	0.6	0.55	1.2	1.10	0.6	0.89	0.8	0.84
TPS	0.5	0.58	1.25	0.96	0.75	0.96	0.75	0.96
MPS	0.67	0.52	1.0	0.89	1.667	1.21	1.33	1.37

Table 2. Main effects and Interaction Effects for the cumulative MSI in the Mixed Design ANOVA

Time	F (3,36)=1.363, p=0.270	η^2=0.102	Power=0.331
Treatment	F (2,12)= 0.531, p=0.601	η^2 = 0.081	Power= 0.118
Time x Treatment	F (6,36) = 0.855, p=0.537	η^2 = 0.125	Power = 0.292

Table 3**.** Descriptive Statistics for falls during the Negative Geotaxis

	n		**$\bar{x}$**		**SD**	
	Male	**Female**	**Male**	**Female**	**Male**	**Female**
Control	21	27	4.67	4.67	3.26	2.51
TPS	18	17	6.72	6.76	3.12	2.66
MPS	18	16	4.72	6.63	6.72	2.80

Table 4. Descriptive Stats for Centre square entries in the Exploratory Open Field

	n		**$\bar{x}$**		**SD**	
	Male	**Female**	**Male**	**Female**	**Male**	**Female**
Control	20	26	9.05	8.27	4.32	2.86
TPS	15	15	5.73	7.67	3.04	2.72
MPS	18	17	8.72	7.82	2.74	3.38

Table 5. Descriptive statistics for Margin Time in the Automated exploratory open field

	n		**$\bar{x}$**		**SD**	
	Male	**Female**	**Male**	**Female**	**Male**	**Female**
Control	21	29	27.71	32.87	10.31	6.76
TPS	18	18	28.35	37.94	10.91	6.96
MPS	17	16	28.06	35.96	10.12	4.87

Table 6. Descriptive statistics for centre time in the automated exploratory open field

	n		$\bar{x}$		SD	
	Male	**Female**	**Male**	**Female**	**Male**	**Female**
Control	21	29	32.29	27.13	10.31	6.76
TPS	18	18	31.65	22.06	10.91	6.96
MPS	17	16	31.94	24.04	10.12	4.87

Table 7. Descriptive statistics for rearing movements in the automated exploratory open field

	n		$\bar{x}$		SD	
	Male	**Female**	**Male**	**Female**	**Male**	**Female**
Control	21	29	5.44	6.11	1.29	0.97
TPS	18	18	5.12	5.62	1.07	0.82
MPS	17	16	5.32	6.57	0.83	1.14

Table 8. Descriptive statistics for vertical movements in the automated exploratory open field

	n		$\bar{x}$		SD	
	Male	**Female**	**Male**	**Female**	**Male**	**Female**
Control	21	29	5.43	6.09	1.29	1.00
TPS	18	18	5.12	5.58	1.08	0.83
MPS	17	16	5.31	6.50	0.85	1.10

Table 9. Descriptive statistics for K/Na Ratio in F3 offspring

	n		$\bar{x}$		SD	
	Male	**Female**	**Male**	**Female**	**Male**	**Female**
Control	11	11	5.19	7.19	1.30	1.98
TPS	10	10	5.86	9.62	1.96	1.71
MPS	11	11	6.82	9.10	2.62	2.10

Table 10. Correlations of MSI at G11 and G18 and the individual biomarkers used

	Biomarker	P Value	PearsonR
MSI G11	***Glucose***	**0.001**	**0.596**
	Corticosterone	**0.015**	**0.439**
	Weight	0.132	-0.281
	Weight Change	**0.007**	**-0.482**
MSI G18	***Glucose***	**0.006**	**0.494**
	Corticosterone	**0.007**	**0.479**
	Weight	**<0.001**	**-0.873**
	Weight Change	**<0.001**	**-0.623**

Table 11. Individual biomarkers correlated to offspring behaviour

				Male		**Female**	
				p Value	*Pearson R*	*p Value*	*Pearson R*
Glucose	*G11*	*Open Field Exploration*	*Centre square entries*	0.288	-0.171	0.219	-0.236
		Negative Geotaxis	*Falls*	0.398	0.073	0.088	0.369
		Automated Open Field Exploration	*Margin Time*	0.398	0.073	0.442	0.041
			Centre Time	0.398	-0.073	0.442	-0.041
			Rearing Movements	**0.061**	**-.417**	**0.023**	**-0.522**
			Vertical Movements	**0.056**	**-0.429**	**0.023**	**-0.523**
		Hair Analysis	*K Na Ratio*	0.484	0.013	0.322	0.167
	G18	*Open Field Exploration*	*Centre square entries*	0.218	0.237	0.400	0.078
		Negative Geotaxis	*Falls*	0.319	-0.122	0.174	0.260
		Automated Open Field Exploration	*Margin Time*	0.236	0.201	0.075	0.391
			Centre Time	0.236	-0.201	0.075	-0.391
			Rearing Movements	**0.25**	**-0.189**	**0.013**	**-0.574**
			Vertical Movements	**0.246**	**-0.192**	**0.015**	**-0.561**
		Hair Analysis	*K Na Ratio*	0.181	-0.276	0.105	0.435
CORT	*G11*	*Open Field Exploration*	*Centre square entries*	0.084	0.407	0.054	0.466
		Negative Geotaxis	*Falls*	0.161	-0.275	0.205	0.230
		Automated Open Field Exploration	*Margin Time*	0.261	-0.180	0.283	-0.161
			Centre Time	0.261	0.180	0.283	0.161
			Rearing Movements	0.196	-0.239	0.092	-0.362
			Vertical Movements	0.197	-0.238	0.086	-0.372

		Hair Analysis	*K Na Ratio*	0.296	0.164	0.493	0.006
	G18	*Open Field Exploration*	***Centre square entries***	**0.013**	**0.610**	**0.140**	**0.325**
		Negative Geotaxis	*Falls*	0.404	0.069	0.076	-0.389
		Automated Open Field Exploration	*Margin Time*	0.430	-0.05	0.220	-0.216
			Centre Time	0.430	0.05	0.220	0.216
			Rearing Movements	0.237	0.20	0.138	0.301
			Vertical Movements	0.238	0.20	0.173	0.262
		Hair Analysis	*K Na Ratio*	0.059	0.455	0.470	-0.028
Weight	*G11*	*Open Field Exploration*	*Centre square entries*	0.053	-0.47	0.147	-0.316
		Negative Geotaxis	*Falls*	0.133	-0.307	0.147	0.290
		Automated Open Field Exploration	***Margin Time***	**0.238**	**-0.199**	**0.045**	**-0.453**
			Centre Time	**0.238**	**0.199**	**0.045**	**0.453**
			Rearing Movements	0.403	-0.069	0.351	0.108
			Vertical Movements	0.402	-0.07	0.328	0.126
		Hair Analysis	*K Na Ratio*	0.193	-0.263	0.266	-0.225
	G18	*Open Field Exploration*	***Centre square entries***	**0.025**	**-0.555**	**0.273**	**-0.184**
		Negative Geotaxis	*Falls*	0.329	-0.125	0.279	0.165
		Automated Open Field Exploration	*Margin Time*	0.129	-0.311	0.072	-0.396
			Centre Time	0.129	0.311	0.072	0.396
			Rearing Movements	0.359	-0.102	0.439	0.043
			Vertical Movements	0.364	-0.098	0.411	0.064
		Hair Analysis	*K Na Ratio*	0.269	-0.189	0.260	-0.231
Weight Change	*G11*	*Open Field*	*Centre square entries*	0.313	-0.150	0.116	-0.356

		Exploration					
		Negative Geotaxis	*Falls*	0.373	-0.092	0.455	0.032
		Automated Open Field Exploration	*Margin Time*	0.409	0.065	0.388	-0.08
			Centre Time	0.409	-0.065	0.388	0.08
			Rearing Movements	0.209	0.226	0.129	0.312
			Vertical Movements	0.204	0.231	0.142	0.296
		Hair Analysis	*K Na Ratio*	0.386	-0.089	0.481	0.018
	G18	*Open Field Exploration*	*Centre square entries*	0.093	-0.392	0.242	0.214
		Negative Geotaxis	*Falls*	0.117	0.327	0.225	-0.211
		Automated Open Field Exploration	*Margin Time*	0.107	-0.341	0.491	-0.007
			Centre Time	0.107	0.341	0.491	0.007
			Rearing Movements	0.356	-0.104	0.332	-0.122
			Vertical Movements	0.372	-0.092	0.348	-0.110
		Hair Analysis	*K Na Ratio*	0.451	-0.038	0.357	-0.133

Table 12. Results for Pearson's R Correlations between MSI and Offspring Behaviour

			Male		**Female**	
			p Value	*PearsonR*	*p Value*	*PearsonR*
MSI G11	*Open Field Exploration*	Centre square entries	0.218	0.366	0.178	0.398
	Negative Geotaxis	Falls	0.626	0.137	0.804	-0.070
	Automated Open Field Exploration	Margin Time	0.412	-0.229	0.459	-0.207
		Centre Time	0.412	0.229	0.459	0.207
		Rearing Movements	0.086	-0.458	0.052	-0.510
		Vertical Movements	**0.084**	**-0.461**	**0.050**	**-0.514**
	Hair Analysis	K Na Ratio	0.406	0.252	0.099	-0.550
MSI G18	*Open Field Exploration*	**Centre square entries**	**0.003**	**0.754**	**0.558**	**0.179**
	Negative Geotaxis	Falls	0.155	0.581	0.567	-0.161
	Automated Open Field Exploration	Margin Time	0.589	0.152	0.790	0.075
		Centre Time	0.589	-0.152	0.790	-0.075
		Rearing Movements	0.888	0.040	0.820	-0.064
		Vertical Movements	0.900	0.036	0.755	-0.088
	Hair Analysis	K Na Ratio	0.656	0.137	0.814	0.086

APPENDIX B – Study 2

Table 1. Means and standard deviations for the F3 dams, measuring various physiological indicators, including glucose, corticosterone, weight, and weight change

	G18							
	Glucose		Corticosterone		Weight		ΔWeight	
	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD
Control	5.20	0.50	997.6	361.4	451.9	29.46	109.99	16.03
TPS	4.68	0.67	1278.8	109.2	436.2	33.64	99.55	26.49
MPS	5.11	0.51	1571.7	626.4	430.6	32.30	91.57	13.71
	G21							
	Glucose		Corticosterone		Weight		ΔWeight	
	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD
	5.95	1.12	1276.4	476.5	505.5	33.92	163.6	21.97
	6.04	0.73	1843.7	588.9	476.4	35.67	139.7	31.96
	6.11	0.72	2080.9	550.0	476.8	34.06	137.7	15.11

Table 2. Means and standard deviations of the MSI for F3 dams in the control, TPS, and MPS groups from baseline until parturition.

	Baseline		G11		G18		G21	
	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD
Control	1.00	0.76	1.24	0.89	1.38	1.06	1.38	0.32
TPS	1.40	0.89	1.80	1.30	1.40	0.55	1.60	0.55
MPS	1.43	1.40	1.57	0.79	2.00	1.00	3.00	0.82

Table 3. Means and standard deviations of the MSI for F2 dams in the control, TPS, and MPS groups from baseline until parturition.

	Baseline		G11		G18		G21	
	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD
Control	0.60	.55	1.20	1.10	0.60	0.89	0.80	0.84
TPS	0.50	.58	1.25	0.96	0.75	0.96	0.75	0.96
MPS	0.67	.52	1.00	0.89	1.67	1.21	1.33	1.37

Table 4. Main and interaction effects for mixed measure ANOVA MSI in F3 dams.

Time	F (3,51) = 2.965, p = 0.041	N^2 = 0.149	Power = 0.669
*Time * Treatment*	F (6,51) = 1.776, p = 0.123	N^2 = 0.173	Power = 0.614
Treatment	F (2,17) = 2.227, p = 0.138	N^2 = 0.208	Power = 0.391

Table 5. Main and interaction effects for mixed measure ANOVA MSI in F2 dams.

Time	F (3,36) = 1.363, p = 0.270	N^2 = 0.102	Power = 0.331
*Time * Treatment*	F (6,36) = 0.855, p = 0.537	N^2 = 0.125	Power = 0.292
Treatment	F (2,12) = 0.531, p = 0.601	N^2 = 0.081	Power = 0.118

Table 6. Means and standard deviations of the total summed MSI in the F2 and F3 generation.

	F2		F3	
	$\bar{x}$	SD	$\bar{x}$	SD
Control	3.60	2.19	5.00	2.62
TPS	4.00	3.16	6.20	2.39
MPS	5.17	3.13	8.00	3.11

Table 7. Main effects and interactions of ANCOVA for the MSI between F2 and F3 generations

Time	F (3,93) = 0.699, p = 0.699	N^2 = 0.022	Power = 0.193
*Time*Generation*	F (3,93) = 0.554, p = 0.646	N^2 = 0.018	Power = 0.160
*Time*Treatment*	F (6,93) = 1.623, p = 0.150	N^2 = 0.095	Power = 0.596
Generation	F (1,31) = 5.319, p = 0.028*	N^2 = 0.146	Power = 0.608
Treatment	F (2,31) = 2.547, p = 0.095	N^2 = 0.141	Power = 0.471

Table 8. Means and standard deviations of birthing outcomes for F3 dams in the control, TPS, and MPS groups.

	Gestation length		Implantation Sites		Litter Number		Reabsorption	
	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD
Control	531.0	5.29	18.27	1.65	15.47	2.06	2.64	1.23
TPS	534.4	7.43	14.91	4.44	11.36	3.55	3.55	2.10
MPS	528.5	2.64	17.50	1.76	15.25	1.79	2.25	1.69

Table 9. Main effects in birth outcomes and litter health (ANOVA, Kruskal Wallis)

Gestation Length (ANOVA)			
Treatment	F (2,31) = 3.082, p=0.06	N^2 = 0.166	Power = 0.552
Implantation Sites (ANOVA)			
Treatment	F (2,31) = 3.671, p = 0.037 *	N^2 = 0.191	Power = 0.632
Litter Number (ANOVA)			
Treatment	F (2,31) = 62.094, p = 0.001 *	N^2 = 0.359	Power = 0.954
Reabsorption (ANVOA)			
Treatment	F (2,31) = 1.563, p = 0.226	N^2 = 0.092	Power = 0.306
Dead at Birth (K-W)			
Treatment	X^2(2, N = 38) = 1.814, p = 0.404		
Missing Pups (K-W)			
Treatment	X^2(2, N = 38) = 2.369, p = 0.306		

Pairwise Comparisons: TPS to Control (*) MPS to Control (#) MPS to TPS (^)

Table 10. Main effects of the ANOVAs for P7 negative geotaxis

Males	*Time to Turn*	F(2,143) = 1.118, p = 0.330	N^2 = 0.015	Power = 0.244
	Time Up	F(2,143) = 0.947, p = 0.390	N^2 = 0.013	Power = 0.212
	Number of Falls	F(2,143) = 0.084, p = 0.920	N^2 = 0.001	Power = 0.063
Females	*Time to Turn*	F(2,165) = 0.588, p = 0.557	N^2 = 0.007	Power = 0.147
	Time Up	F(2,165) = 2.130, p = 0.122	N^2 = 0.025	Power = 0.432
	Number of Falls	F(2,165) = 0.826, p = 0.440	N^2 = 0.010	Power = 0.190

Table 11. Main effects of negative geotaxis ANCOVA

Males	*Time to Turn*		
Treatment	F(2,199) = 2.300, p = 0.103	N^2 = 0.023	Power = 0.463
Generation	F(1,199) = 53.01, p < 0.001	N^2 = 0.210	Power = 1.000
	Time Up		
Treatment	F(2,199) = 1.266, p = 0.284	N^2 = 0.013	Power = 0.273
Generation	F(1,199) = 56.73, p < 0.001	N^2 = 0.222	Power = 1.000
	Number of Falls		
Treatment	F(2,199) = 3.813, p = 0.024*	N^2 = 0.037	Power = 0.688
Generation	F(1,199) = 78.35, p < 0.001	N^2 = 0.282	Power = 1.000
Females	*Time to Turn*		
Treatment	F(2,229) = 0.383, p = 0.682	N^2 = 0.003	Power = 0.111
Generation	F(1,229) = 59.12, p < 0.001	N^2 = 0.205	Power = 1.000
	Time Up		
Treatment	F(2,229) = 0.567, p = 0.568	N^2 = 0.005	Power = 0.143
Generation	F(1,229) = 116.5, p < 0.001	N^2 = 0.337	Power = 1.000
	Number of Falls		
Treatment	F(2,229) = 1.255, p = 0.287	N^2 = 0.011	Power = 0.271
Generation	F(1,229) = 58.90, p < 0.001	N^2 = 0.205	Power = 1.000

Table 12. Main effects of exploratory open field ANOVAs

Male	*Novel Squares*	F(2,140) = 0.352, p = 0.704	N^2 = 0.005	Power = 0.106
	Total Squares	F(2,140) = 0.556, p = 0.575	N^2 = 0.008	Power = 0.111
	Outside Squares	F(2,140) = 0.107, p = 0.899	N^2 = 0.002	Power = 0.066
	Inside Squares	F(2,140) = 1.378, p = 0.255	N^2 = 0.019	Power = 0.293
Females	*Novel Squares*	F(2,164) = 6.692, p = 0.002#^	N^2 = 0.075	Power = 0.911
	Total Squares	F(2,164) = 3.409, p = 0.035#^	N^2 = 0.040	Power = 0.635
	Outside Squares	F(2,164) = 5.883, p = 0.003#^	N^2 = 0.067	Power = 0.870
	Inside Squares	F(2,164) = 1.370, p = 0.275	N^2 = 0.016	Power = 0.292

Pairwise Comparisons : TPS from Control (*) MPS from Control (#) MPS from TPS (^)

Table 13. Main effects of open field ANCOVAs

MALE	*Novel Squares*		
Treatment	F(2,192) = 0.151, p = 0.860	N^2 = 0.002	Power = 0.073
Generation	F(1,192) = 0.054, p = 0.817	N^2 = 0.000	Power = 0.056
	Total Squares		
Treatment	F(2,192) = 1.765, p = 0.174	N^2 = 0.018	Power = 0.367
Generation	F(1,192) = 4.407, p = 0.037*	N^2 = 0.022	Power = 0.551
	Outside Squares		
Treatment	F(2,192) = 0.275, p = 0.760	N^2 = 0.003	Power = 0.093
Generation	F(1,192) = 0.797, p = 0.373	N^2 = 0.004	Power = 0.144
	Inside Squares		
Treatment	F(2,192) = 0.758, p = 0.470	N^2 = 0.008	Power = 0.178
Generation	F(1,192) = 6.117, p = 0.014*	N^2 = 0.031	Power = 0.692
FEMALE	*Novel Squares*		
Treatment	F(2,221) = 6.809, p = 0.001#^	N^2 = 0.058	Power = 0.917
Generation	F(1,221) = 1.318, p = 0.252	N^2 = 0.006	Power = 0.208
	Total Squares		
Treatment	F(2,221) = 4.084, p = 0.018#	N^2 = 0.036	Power = 0.721
Generation	F(1,221) = 0.223, p = 0.637	N^2 = 0.001	Power = 0.076
	Outside Squares		
Treatment	F(2,221) = 6.410, p = 0.002#^	N^2 = 0.055	Power = 0.900
Generation	F(1,221) = 0.346, p = 0.557	N^2 = 0.002	Power = 0.090
	Inside Squares		
Treatment	F(2,221) = 1.407, p = 0.247	N^2 = 0.013	Power = 0.300
Generation	F(1,221) = 4.075, p = 0.045*	N^2 = 0.018	Power = 0.520

Pairwise Comparisons : TPS from Control (*) MPS from Control (#) MPS from TPS (^)

Table 14. Main effects for automated exploratory open field in male and female ANOVAs.

Males	*Horizontal Activity*	F(2,56) = 4.710, p = 0.013*#	N^2 = 0.144	Power = 0.767
	Total Distance	F(2,56) = 2.296, p = 0.110	N^2 = 0.076	Power = 0.447
	Number of Movements	F(2,56) = 0.284, p = 0.747	N^2 = 0.010	Power = 0.093
	Movement Time	F(2,56) = 6.060, p = 0.004*#	N^2 = 0.178	Power = 0.868
	Rest Time	F(2,56) = 6.056, p = 0.004*#	N^2 = 0.178	Power = 0.868
	Stereotypy Count	F(2,56) = 5.579, p = 0.006#	N^2 = 0.166	Power = 0.838
	Stereotypy Number	F(2,56) = 4.801, p = 0.012#	N^2 = 0.146	Power = 0.775
	Stereotypy Time	F(2,56) = 5.446, p = 0.007#	N^2 = 0.163	Power = 0.828
	Clockwise Revolutions	F(2,56) = 3.828, p = 0.028*	N^2 = 0.120	Power = 0.672
	Margin Time	F(2,56) = 1.895, p = 0.160	N^2 = 0.063	Power = 0.378
	Centre Distance	F(2,56) = 0.629, p = 0.537	N^2 = 0.022	Power = 0.150
	Centre Time	F(2,56) = 1.895, p = 0.160	N^2 = 0.063	Power = 0.378
Females	*Horizontal Activity*	F(2,63) = 9.524, p < 0.001*^	N^2 = 0.232	Power = 0.975
	Total Distance	F(2,63) = 5.746, p = 0.005^	N^2 = 0.154	Power = 0.851
	Number of Movements	F(2,63) = 3.156, p = 0.049#	N^2 = 0.091	Power = 0.585
	Movement Time	F(2,63) = 10.176, p<0.001*^	N^2 = 0.244	Power = 0.983
	Rest Time	F(2,63) = 10.144, p<0.001*^	N^2 = 0.244	Power = 0.982
	Stereotypy Count	F(2,63) = 10.803, p<0.001*^	N^2 = 0.255	Power = 0.987
	Stereotypy Number	F(2,63) = 13.113, p<0.001*^	N^2 = 0.294	Power = 0.996
	Stereotypy Time	F(2,63) = 4.415, p = 0.003*^	N^2 = 0.169	Power = 0.889
	Clockwise Revolutions	F(2,63) = 2.895, p = 0.063	N^2 = 0.084	Power = 0.547
	Margin Time	F(2,63) = 4.108, p = 0.021^	N^2 = 0.115	Power = 0.708
	Centre Distance	F(2,63) = 8.466, p = 0.001*^	N^2 = 0.212	Power = 0.958
	Centre Time	F(2,63) = 4.108, p = 0.021^	N^2 = 0.115	Power = 0.708

Pairwise Comparisons: TPS from Control (*) MPS from Control (#) MPS from TPS (^).

Table 15. Main effects of generational automated exploratory open field ANCOVA

MALE	*Horizontal Activity*		
Treatment	F(2,111) = 4.338, p = 0.015*	N^2 = 8.677	Power = 0.742
Generation	F(1,111) = 26.90, p < 0.001*	N^2 = 0.195	Power = 0.999
	Movement Time		
Treatment	F(2,111) = 2.989, p = 0.054	N^2 = 0.051	Power = 0.570
Generation	F(1,111) = 17.30, p < 0.001*	N^2 = 0.135	Power = 0.985
	Resting Time		
Treatment	F(2,111) = 2.998, p = 0.054	N^2 = 0.051	Power = 0.571
Generation	F(1,111) = 17.32, p < 0.001*	N^2 = 0.135	Power = 0.985
	Stereotypy Count		
Treatment	F(2,111) = 4.569, p = 0.012*	N^2 = 0.076	Power = 0.765
Generation	F(1,111) = 28.77, p < 0.001*	N^2 = 0.206	Power = 1.000
	Number of Stereotypy		
Treatment	F(2,111) = 2.176, p = 0.118	N^2 = 0.038	Power = 0.437
Generation	F(1,111) = 28.00, p < 0.001*	N^2 = 0.201	Power = 0.999
	Stereotypy Time		
Treatment	F(2,111) = 3.553, p = 0.032#	N^2 = 0.060	Power = 0.650
Generation	F(1,111) = 27.61, p < 0.001*	N^2 = 0.199	Power = 0.999
	Clockwise Revolutions		
Treatment	F(2,111) = 3.544, p = 0.032*	N^2 = 0.060	Power = 0.649
Generation	F(1,111) = 7.359, p = 0.008*	N^2 = 0.062	Power = 0.767
FEMALE	*Horizontal Activity*		
Treatment	F(2,125) = 8.133, p < 0.001*^	N^2 = 0.115	Power = 0.955
Generation	F(1,125) = 11.55, p = 0.001*	N^2 = 0.085	Power = 0.921
	Total Distance		
Treatment	F(2,125) = 6.363, p = 0.002 ^	N^2 = 0.092	Power = 0.894
Generation	F(1,125) = 4.882, p = 0.029*	N^2 = 0.038	Power = 0.592
	Number of Movements		
Treatment	F(2,125) = 2.677, p = 0.073	N^2 = 0.041	Power = 0.523
Generation	F(1,125) = 0.687, p = 0.409	N^2 = 0.005	Power = 0.130
	Movement Time		
Treatment	F(2,125) = 7.104, p = 0.001*^	N^2 = 0.102	Power = 0.926
Generation	F(1,125) = 14.52, p < 0.001*	N^2 = 0.104	Power = 0.966
	Resting Time		
Treatment	F(2,125) = 7.066, p = 0.001*^	N^2 = 0.102	Power = 0.925
Generation	F(1,125) = 14.61, p < 0.001*	N^2 = 0.105	Power = 0.967
	Number of Vertical Movements		
Treatment	F(2,125) = 4.667, p = 0.011^	N^2 = 0.069	Power = 0.776
Generation	F(1,125) = 16.20, p < 0.001*	N^2 = 0.115	Power = 0.979
	Stereotypy Count		
Treatment	F(2,125) = 8.928, p < 0.001*^	N^2 = 0.125	Power = 0.970

Generation	F(1,125) = 13.07, p < 0.001*	N^2 = 0.095	Power = 0.948
	Number Stereotypy		
Treatment	F(2,125) = 5.663, p = 0.011*^	N^2 = 0.069	Power = 0.776
Generation	F(1,125) = 14.18, p < 0.001*	N^2 = 0.102	Power = 0.962
	Stereotypy Time		
Treatment	F(2,125) = 3.640, p = 0.029*	N^2 = 0.055	Power = 0.663
Generation	F(1,125) = 15.13, p < 0.001*	N^2 = 0.108	Power = 0.971
	Margin Time		
Treatment	F(2,125) = 5.912, p = 0.004*^	N^2 = 0.086	Power = 0.870
Generation	F(1,125) = 10.61, p = 0.001*	N^2 = 0.078	Power = 0.898
	Centre Distance		
Treatment	F(2,125) = 8.852, p < 0.001*^	N^2 = 0.124	Power = 0.969
Generation	F(1,125) = 0.013, p = 0.908	N^2 < 0.001	Power = 0.052
	Centre Time		
Treatment	F(2,125) = 5.912, p = 0.004*^	N^2 = 0.086	Power = 0.870
Generation	F(1,125) = 10.61, p = 0.001*	N^2 = 0.078	Power = 0.898
	Rearing Movements		
Treatment	F(2,125) = 4.628, p = 0.012*	N^2 = 0.069	Power = 0.772
Generation	F(1,125) = 15.94, p < 0.001*	N^2 = 0.113	Power = 0.977

Pairwise Comparisons: TPS from Control (*) MPS from Control (#) MPS from TPS (^)

Table 16. Main effects of ANOVAs in affective exploration in males and females.

Male			
Movement Rating	F(1,29) = 0.281, p = 0.600	N^2 = 0.01	Power = 0.081
Total Areas	F(1,29) = 0.727, p = 0.401	N^2 = 0.024	Power = 0.131
Initial Emergence	F(1,29) = 0.006, p = 0.937	N^2 < 0.001	Power = 0.051
Time in Tube	F(1,29) = 0.051, p = 0.823	N^2 = 0.002	Power = 0.056
Time in Novel Area	F(1,29) = 0.015, p = 0.904	N^2 = 0.015	Power = 0.052
Time Outside Tube	F(1,29) = 0.037, p = 0.85	N^2 = 0.001	Power = 0.054
Female			
Movement Rating	F(1,46) = 0.478, p = 0.493	N^2 = 0.01	Power = 0.104
Total Areas	F(1,46) = 0.512, p = 0.478	N^2 = 0.011	Power = 0.108
Initial Emergence	F(1,46) = 2.078, p = 0.156	N^2 = 0.043	Power = 0.292
Time in Tube	F(1,46) = 0.244, p = 0.624	N^2 = 0.005	Power = 0.77
Time in Novel Area	F(1,46) = 0.702, p = 0.406	N^2 = 0.015	Power = 0.130
Time Outside Tube	F(1,46) = 0.424, p = 0.518	N^2 = 0.009	Power = 0.098

Table 17. Main effects of male and female generational affective exploration. ANCOVAs

MALE	*Initial Emergence*		
Treatment	F(2,83) = 2.020, p = 0.139	N^2 = 0.046	Power = 0.406
Generation	F(1,83) = 6.073, p = 0.016*	N^2 = 0.066	Power = 0.683
	Time In		
Treatment	F(2,83) = 0.815, p = 0.446	N^2 = 0.019	Power = 0.185
Generation	F(1,83) = 15.36, p < 0.001*	N^2 = 0.156	Power = 0.972
	Time Out		
Treatment	F(2,83) = 1.023, p = 0.364	N^2 = 0.024	Power = 0.223
Generation	F(1,83) = 16.31, p < 0.001*	N^2 = 0.164	Power = 0.979
	Percent Time In		
Treatment	F(2,83) = 0.815, p = 0.446	N^2 = 0.019	Power = 0.185
Generation	F(1,83) = 15.36, p < 0.001*	N^2 = 0.156	Power = 0.972
FEMALE	*Initial Emergence*		
Treatment	F(2,192) = 2.031, p = 0.134	N^2 = 0.021	Power = 0.415
Generation	F(1,192) = 2.031, p = 0.002*	N^2 = 0.049	Power = 0.880
	Time In		
Treatment	F(2,192) = 0.551, p = 0.577	N^2 = 0.006	Power = 0.140
Generation	F(1,192) = 11.36, p = 0.001*	N^2 = 0.056	Power = 0.918
	Time Out		
Treatment	F(2,192) = 0.727, p = 0.485	N^2 = 0.008	Power = 0.172
Generation	F(1,192) = 11.97, p = 0.001*	N^2 = 0.059	Power = 0.931
	Percent Time In		
Treatment	F(2,192) = 0.551, p = 0.557	N^2 = 0.006	Power = 0.140
Generation	F(1,192) = 11.36, p = 0.001*	N^2 = 0.056	Power = 0.918

APPENDIX C – Study 3

Table 1. Sample size, means, and standard deviations for Elevated Plus Maze Males

	6 Months			***12 Months***			***18 Months***		
	N	$\bar{x}$	***SEM***	***N***	$\bar{x}$	***SEM***	***N***	$\bar{x}$	***SEM***
Time in Centre									
Control	10	15.40	2.91	10	10.10	5.68	6	1.00	0.82
MPS	10	35.90	9.13	12	15.25	4.33	6	9.98	6.33
Number of Closed Arms Entries									
Control	10	9.90	0.64	10	7.10	0.60	6	7.00	0.86
MPS	10	8.70	0.84	12	9.58	0.92	6	6.50	1.09
Number of Total Arm Entries									
Control	10	10.00	0.63	10	7.20	0.55	6	7.00	0.86
MPS	10	9.80	1.05	12	10.17	0.86	6	6.83	1.11
Time in Closed Arms									
Control	10	157.9	6.80	10	162.20	9.89	6	231.33	10.00
MPS	10	132.2	15.63	12	171.25	7.44	6	185.67	13.41

Table 2. Main effects for EPM ANOVAS effect of treatment

Males	*Time in Centre*	*6M*	F(1,18) = 4.577, p = 0.046*	N = 0.203	Power = 0.526
		12M	F(1,20) = 1.031, p = 0.322	N = 0.049	Power = 0.162
		18M	F(1,10) = 1.917, p = 0.196	N = 0.161	Power = 0.241
	Time in Closed Arms	*6M*	F(1,18) = 2.273, p = 0.149	N = 0.122	Power = 0.298
		12M	F(1,20) = 0.555, p = 0.465	N = 0.027	Power = 0.109
		18M	F(1,10) = 7.455, p = 0.021*	N = 0.427	Power = 0.692
	Number of Closed Arm	*6M*	F(1,18) = 1.283, p = 0.272	N = 0.067	Power = 0.189
		12M	F(1,20) = 4.678, p = 0.043*	N = 0.190	Power = 0.539
		18M	F(1,10) =0.130 , p = 0.525	N = 0.013	Power = 0.062
	Total Arm Entries	*6M*	F(1,18) = 0.027, p = 0.872	N = 0.001	Power = 0.053
		12M	F(1,20) = 7.665, p = 0.012*	N = 0.277	Power = 0.750
		18M	F(1,10) = 0.014, p = 0.908	N = 0.001	Power = 0.051
Females	*Time in Centre*	*6M*	F(1,18) = 0.032, p = 0.860	N = 0.002	Power = 0.053
		12M	F(1,25) = 0.048, p = 0.828	N = 0.002	Power = 0.055
		18M	F(1,20) = 0.002, p = 0.968	N < 0.001	Power = 0.050
	Time in Closed Arms	*6M*	F(1,18) = 0.887, p = 0.359	N = 0.047	Power = 0.145
		12M	F(1,25) = 0.299, p = 0.590	N = 0.012	Power = 0.082
		18M	F(1,20) = 1.167, p = 0.293	N = 0.055	Power = 0.177
	Number of Closed Arm	*6M*	F(1,18) = 0.050, p = 0.826	N = 0.003	Power = 0.055
		12M	F(1,25) = 0.617, p = 0.440	N = 0.024	Power = 0.118
		18M	F(1,20) = 4.265, p = 0.052	N = 0.176	Power = 0.502
	Total Arm Entries	*6M*	F(1,18) = 0.812, p = 0.380	N = 0.043	Power = 0.137
		12M	F(1,25) = 1.891, p = 0.181	N = 0.070	Power = 0.262
		18M	F(1,20) = 1.746, p = 0.201	N = 0.080	Power = 0.242

(*) Signifies a significance at p<0.05

Table 3. Sample size, means, and standard deviations for forced swim

		6 Months			***12 Months***			***18 Months***		
		N	$\bar{x}$	***SD***	***N***	$\bar{x}$	***SD***	***N***	$\bar{x}$	***SD***
	Latency to Immobility									
Males	*Control*	11	33.91	16.28	10	86.60	51.75	5	29.20	23.38
	MPS	10	55.90	29.06	12	61.58	39.70	7	17.71	7.432
	Number of Dives									
	Control	11	0.364	0.809	10	0.900	0.160	5	0.800	1.095
	MPS	10	1.000	2.160	12	4.167	3.486	7	0.857	1.574
Females	***Latency to Immobility***									
	Control	10	51.00	20.97	13	42.00	15.69	9	22.11	15.53
	MPS	10	44.50	19.52	15	61.53	25.22	12	25.92	17.52
	Number of Dives									
	Control	10	0.400	0.966	13	9.000	5.370	9	0.556	0.882
	MPS	10	0.300	0.675	15	6.600	5.040	12	0.917	1.240

Table 4. Main effects for the forced swim task for males and females at 6,12, and 18 months

Males	*Immobility*	*6M*	F(1,19) = 4.695, p = 0.043*	N = 0.198	Power = 0.539
		12M	F(1,20) = 1.648, p = 0.214	N = 0.076	Power = 0.231
		18M	F(1,10) = 1.528, p = 0.245	N = 0.133	Power = 0.201
	Dives	*6M*	F(1,19) = 0.242, p = 0.628	N = 0.013	Power = 0.075
		12M	F(1,20) = 7.435, p = 0.013*	N = 0.271	Power = 0.737
		18M	F(1,10) = 0.005, p = 0.946	N < 0.001	Power = 0.050
Females	*Immobility*	*6M*	F(1,18) = 0.515, p = 0.482	N = 0.028	Power = 0.104
		12M	F(1,26) = 5.824, p = 0.023*	N = 0.183	Power = 0.642
		18M	F(1,19) = 0.267, p = 0.612	N = 0.014	Power = 0.078
	Dives	*6M*	Mann-Whittney, p = 0.971		
		12M	F(1,26) = 1.487, p = 0.234	N = 0.054	Power = 0.217
		18M	F(1,19) = 0.551, p = 0.467	N = 0.028	Power = 0.109

(*) Signifies a significance of $p<0.05$

Table 5. Main effects for Automated exploratory open field ANOVA

Males	*Horizontal Activity*	*6M*	F(1,17) = 0.574, p = 0.459	N = 0.033	Power = 0.110
		12M	F(1,16) = 1.831, p = 0.195	N = 0.103	Power = 0.247
		18M	F(1,9) = 0.529, p = 0.486	N = 0.055	Power = 0.100
	Number of Movements	*6M*	F(1,17) = 1.537, p = 0.232	N = 0.083	Power = 0.216
		12M	F(1,16) = 1.274, p = 0.760	N = 0.074	Power = 0.186
		18M	F(1,9) = 0.111, p= 0.747	N = 0.012	Power = 0.060
	Margin Distance	*6M*	F(1,17) = 6.019, p = 0.025*	N = 0.261	Power = 0.638
		12M	F(1,16) = 2.472, p = 0.135	N = 0.134	Power = 0.315
		18M	F(1,9) = 0.693 , p = 0.427	N = 0.072	Power = 0.116
	Total Distance	*6M*	F(1,17) = 2.115, p = 0.164	N = 0.111	Power = 0.279
		12M	F(1,16) = 8.095, p = 0.012*	N = 0.336	Power = 0.762
		18M	F(1,9) = 0.167, p = 0.693	N = 0.018	Power = 0.066
	Margin Time	*6M*	F(1,17) = 4.642, p = 0.046*	N = 0.214	Power = 0.529
		12M	F(1,16) = 0.254, p = 0.621	N = 0.016	Power = 0.076
		18M	F(1,9) = 2.557, p = 0.144	N = .221	Power = 0.299
	Resting Time	*6M*	F(1,17) = 0.067, p = 0.799	N = 0.004	Power = 0.057
		12M	F(1,16) = 0.774, p = 0.392	N = 0.046	Power = 0.132
		18M	F(1,9) = 0.001, p = 0.971	N < 0.001	Power = 0.050
	Centre Time	*6M*	F(1,17) = 4.642, p = 0.046*	N = 0.214	Power = 0.529
		12M	F(1,16) = 0.254, p = 0.621	N = 0.016	Power = 0.076
		18M	F(1,9) = 2.557, p = 0.144	N = 0.221	Power = 0.299
	Vertical Time	*6M*	F(1,17) = 2.373, p = 0.142	N = 0.122	Power = 0.307
		12M	F(1,16) = 4.879, p = 0.042*	N = 0.234	Power = 0.546
		18M	F(1,9) = 0.003, p = 0.961	N < 0.001	Power = 0.050
	Movement Time	*6M*	F(1,17) = 0.064, p = 0.803	N =0.004	Power = 0.057
		12M	F(1,16) = 0.771, p = 0.393	N = 0.046	Power = 0.131
		18M	F(1,9) = 0.001, p = 0.971	N < 0.001	Power = 0.050
	Rearing Time	*6M*	F(1,17) = 2.392, p = 0.140	N = 0.123	Power = 0.309
		12M	F(1,16) = 4.879, p = 0.042*	N = 0.234	Power = 0.546
		18M	F(1,9) = 0.003, p = 0.961	N < 0.001	Power = 0.050
	Number of Stereotypy Movements	*6M*	F(1,17) = 2.042, p = 0.171	N = 0.107	Power = 0.271
		12M	F(1,16) = 0.075, p = 0.788	N = 0.005	Power = 0.058
		18M	F(1,9) = 1.023, p = 0.338	N = 0.102	Power = 0.148
Females	*Horizontal Activity*	*6M*	F(1,18) = 4.501, p = 0.048*	N = 0.200	Power = 0.519
		12M	F(1,25) = 0.198, p = 0.660	N = 0.008	Power = 0.071
		18M	F(1,20) = 0.010, p = 0.923	N < 0.001	Power = 0.051
	Number of Movements	*6M*	F(1,18) = 0.360, p = 0.556	N = 0.020	Power = 0.088
		12M	F(1,25) = 7.431, p = 0.012*	N = 0.229	Power = 0.745
		18M	F(1,20) = 0.013, p = 0.911	N = 0.001	Power = 0.051
	Margin Distance	*6M*	F(1,18) = 3.361, p = 0.083	N = 0.157	Power = 0.411
		12M	F(1,25) = 0.395, p = 0.535	N = 0.016	Power = 0.093
		18M	F(1,20) = 0.052, p = 0.821	N = 0.003	Power = 0.055
	Total Distance	*6M*	F(1,18) = 4.976, p = 0.039*	N = 0.217	Power = 0.560
		12M	F(1,25) = 0.023, p = 0.881	N = 0.001	Power = 0.052

		18M	F(1,20) = 0.012, p = 0.915	N = 0.001	Power = 0.051
	Margin Time	*6M*	F(1,18) < 0.001, p = 0.998	N < 0.001	Power = 0.050
		12M	F(1,25) = 0.007, p = 0.932	N < 0.001	Power = 0.051
		18M	F(1,20) = 0.093, p = 0.764	N = 0.005	Power = 0.060
	Resting Time	*6M*	F(1,18) = 5.772, p = 0.027*	N = 0.243	Power = 0.623
		12M	F(1,25) = 0.606, p = 0.444	N = 0.024	Power = 0.116
		18M	F(1,20) = 0.051, p = 0.824	N = 0.003	Power = 0.055
	Centre Time	*6M*	F(1,18) < 0.001, p = 0.999	N < 0.001	Power = 0.050
		12M	F(1,25) = 0.007, p = 0.932	N < 0.001	Power = 0.051
		18M	F(1,20) = 0.093, p = 0.764	N = 0.005	Power = 0.060
	Vertical Time	*6M*	F(1,18) = 0.105, p = 0.750	N = 0.006	Power = 0.061
		12M	F(1,25) = 0.148, p = 0.704	N = 0.006	Power = 0.066
		18M	F(1,20) = 0.004, p = 0.948	N < 0.001	Power = 0.050
	Movement Time	*6M*	F(1,18) = 5.743, p = 0.028*	N = 0.242	Power = 0.621
		12M	F(1,25) = 0.613, p = 0.441	N = 0.024	Power = 0.117
		18M	F(1,20) = 0.051, p = 0.823	N = 0.003	Power = 0.055
	Rearing Time	*6M*	F(1,18) = 0.156, p = 0.698	N = 0.009	Power = 0.066
		12M	F(1,25) = 0.179, p = 0.676	N = 0.007	Power = 0.069
		18M	F(1,20) = 0.004, p = 0.952	N < 0.001	Power = 0.050
	Number of Stereotypy Movements	*6M*	F(1,18) = 3.518, p = 0.077	N = 0.163	Power = 0.427
		12M	F(1,25) = 6.085, p = 0.021*	N = 0.196	Power = 0.660
		18M	F(1,20) = 0.030, p = 0.864	N = 0.002	Power = 0.053

(*) Signifies a significant result of $p < 0.05$

Table 6. Sample size, means, and standard error of the mean for lifespan

	N	$\bar{x}$	***SEM***
MALE			
Control	10	491.8	19.46
MPS	12	35.90	15.80
FEMALE			
Control	14	515.3	14.55
MPS	14	531.1	7.96

Table 7. Main effects for P110 by 6-, 12-, and 18-month automated exploratory open field correlations

			P110 – 6Months	***P110 – 12Months***	***P110 - 18Months***
Male	*Control*	*Margin Distance*	R = 0.39, p = 0.26	R =-0.03, p = 0.95	R = 0.85, p = 0.002
		Margin Time	R = 0.48, p = 0.17	R =-0.04, p = 0.92	R =-0.75, p = 0.15
		Centre Time	R = 0.48, p = 0.17	R =-0.04, p = 0.92	R = 0.97, p = 0.07
		Total Distance	R = 0.08, p = 0.82	R = 0.004, p = 0.99	R = 0.80, p = 0.37
		Vertical Time	R = 0.48, p = 0.16	R = 0.06, p = 0.87	R = 0.87, p =0.24
		Rearing Time	R = 0.47, p = 0.17	R = 0.06, p = 0.88	R = 0.88, p = 0.23
		Horizontal Activity	R = 0.14, p = 0.94	R = 0.08, p = 0.84	R = 0.66, p = 0.23
		Movement Time	R = 0.19, p = 0.87	R = 0.56, p = 0.39	R = 0.65, p = 0.24
		Rest Time	R = 0.19, p = 0.87	R = 0.56, p = 0.39	R = 0.65, p = 0.24
		Stereotypy Count	R = 0.25, p = 0.49	R = 0.03, p = 0.94	R = 0.58, p = 0.31
		Number of Stereotypy	R = 0.04, p = 0.90	R =-0.58, p = 0.10	R =-0.44, p = 0.46
		Stereotypy Time	R = 0.41, p = 0.24	R = -0.82, p = 0.04	R =-0.76, p = 0.14
		Clockwise Revolutions	R = 0.81, p = 0.02	R = 0.33, p = 0.70	R = 0.57, p = 0.31
	MPS	*Margin Distance*	R =-0.05, p = 0.91	R =-0.31, p = 0.41	R = 0.22, p = 0.87
		Margin Time	R =-0.06, p = 0.88	R = 0.68, p = 0.04	R = 0.02, p = 0.97
		Centre Time	R =-0.06, p = 0.88	R = 0.68, p = 0.04	R = 0.95, p = 0.04
		Total Distance	R = 0.28, p = 0.47	R =-0.43, p = 0.25	R = 0.96, p = 0.02
		Vertical Time	R = 0.27, p = 0.48	R = 0.21, p = 0.59	R = 0.93, p = 0.045
		Rearing Time	R = 0.27, p = 0.49	R = 0.21, p = 0.58	R = 0.93, p = 0.047
		Horizontal Activity	R = 0.83, p = 0.03	R =-0.21, p = 0.59	R = 0.08, p = 0.88
		Movement Time	R = 0.92, p = 0.004	R = 0.54, p = 0.36	R = 0.39, p = 0.45

		Rest Time	R = 0.92, p = 0.004	R = 0.53, p = 0.37	R = 0.38, p = 0.46
		Stereotypy Count	R = 0.56, p = 0.11	R = 0.13, p = 0.75	R = 0.43, p = 0.40
		Number of Stereotypy	R = 0.13, p = 0.74	R = 0.27, p = 0.49	R = 0.29, p = 0.58
		Stereotypy Time	R = 0.75, p = 0.02	R = 0.25, p = 0.80	R = 0.74, p = 0.15
		Clockwise Revolutions	R = 0.56, p = 0.32	R = 0.33, p = 0.70	R = 0.28, p = 0.81
Female	*Control*	*Number of Movements*	R = 0.31, p = 0.38	R =-0.16, p = 0.60	R =-0.61, p = 0.06
		Margin Time	R =-0.12, p = 0.74	R = 0.68, p = 0.01	R = 0.62, p = 0.06
		Centre Distance	R = 0.44, p = 0.21	R = 0.56, p = 0.05	R = 0.77, p = 0.009
		Centre Time	R =-0.12, p = 0.74	R = 0.68, p = 0.01	R = 0.71, p = 0.02
		Total Distance	R = 0.49, p = 0.15	R = 0.63, p = 0.02	R = 0.74, p = 0.014
		Number of Stereotypy	R = 0.07, p = 0.86	R = 0.04, p = 0.89	R = 0.43, p = 0.21
		Stereotypy Count	R =-0.29, p = 0.41	R = 0.31, p = 0.31	R = 0.55, p = 0.10
		Stereotypy Time	R = 0.84, p = 0.01	R = 0.12, p = 0.69	R = 0.20, p = 0.58
		Horizontal Activity	R = 0.10, p = 0.78	R = 0.50, p = 0.08	R = 0.71, p = 0.02
		Movement Time	R = 0.24, p = 0.50	R = 0.57, p = 0.04	R = 0.78, p = 0.01
		Rest Time	R = 0.24, p = 0.50	R = 0.57, p = 0.04	R = 0.78, p = 0.008
	MPS	*Number of Movements*	R =-0.59, p = 0.07	R = 0.07, p = 0.82	R =-0.46, p = 0.13
		Margin Time	R = 0.08, p = 0.83	R =-0.27, p = 0.35	R =-0.32, p = 0.32
		Centre Distance	R = 0.42, p = 0.23	R = 0.31, p = 0.27	R = 0.43, p = 0.16
		Centre Time	R = 0.08, p = 0.83	R =-0.27, p = 0.35	R = 0.34, p = 0.28
		Total Distance	R = 0.20, p = 0.58	R = 0.45, p = 0.11	R = 0.41, p = 0.19
		Number of Stereotypy	R = 0.31, p = 0.39	R = 0.19, p = 0.51	R = 0.03, p = 0.94
		Stereotypy Count	R = 0.34, p = 0.34	R = 0.31, p = 0.28	R = 0.25, p = 0.43

		Stereotypy Time	R = 0.17, p = 0.90	R =-0.07, p = 0.80	R =-0.19, p = 0.56
		Horizontal Activity	R = 0.13, p = 0.72	R = 0.34, p = 0.24	R = 0.31, p = 0.33
		Movement Time	R = 0.12, p = 0.74	R = 0.51, p = 0.07	R = 0.59, p = 0.04
		Rest Time	R = 0.12, p = 0.73	R = 0.55, p = 0.04	R = 0.56, p = 0.06