**UNIVERSITY OF KWAZULU-NATAL**

# THE INFLUENCES OF PATERNAL PRENATAL CHRONIC STRESS ON OFFSPRING SELECTED METABOLIC, BEHAVIOURAL AND NEURO-CHEMISTRY CHANGES

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**TITLE PAGE**

# THE INFLUENCES OF PATERNAL PRENATAL CHRONIC STRESS ON OFFSPRING SELECTED METABOLIC, BEHAVIOURAL AND NEURO-CHEMISTRY CHANGES:

By

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Date of submission:

## DECLARATION

I, Malishca Perumal, declare as follows:

i. That the work described in this thesis has not been submitted to UKZN or other tertiary institution for purposes of obtaining an academic qualification, whether by myself or any other party.

ii. That my contribution to the project was as follows:

Development and design of research topic and protocol, conduction of research methodology, collection and analysis of data, interpretation of data obtained, formulation of manuscript, and write-up of the final thesis.

iii. That the contributions of others to the project were as follows:

Prof M.V. Mabandla (Supervisor), Dr M. Luvuno (Co-Supervisor):

Development and refining of the research design and plan, assistance with analysis of data and statistical analysis, a review of manuscript and thesis before submission.

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## DEDICATION

*This thesis is dedicated, in memoriam, to my supervisor Ms. Cleopatra Kopaopa.*
*(1989 - 2020)*

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## GLOSSARY OF ABBREVIATIONS

| | |
|---|---|
| 11β-HSD1 | 11β-Hydroxysteroid Dehydrogenase |
| ACTH | Adrenocorticotropic Hormone |
| ANOVA | Analysis of Variance |
| AREC | Animal Research Ethics Committee |
| C | Control |
| EPM | Elevated Plus Maze |
| ELISA | Enzyme-Linked Immunosorbent Assay |
| GND | Gestational Day |
| GAPDH | Glyceraldehyde 3-Phosphate Dehydrogenase |
| IL | Interleukin |
| NS-M | Non-Stressed Mother |
| NS-F | Non-Stressed Father |
| NSM-NSF | Non-Stressed Mother and Non-Stressed Father |
| NSM-SF | Non-Stressed Mother and Stressed Father |
| PND | Postnatal Day |
| Cq | Quantification Cycle |
| qPCR | Real-Time Polymerase Chain Reaction |
| SD | Sprague Dawley |
| SPI | Sociability Preference Index |
| SEM | Standard Error of the Mean |
| S-M | Stressed Mother |
| S-F | Stressed Father |
| SM-NSF | Stressed Mother and Non-Stressed Father |
| SM-SF | Stressed Mother and Stressed Father |
| UKZN | University of Kwazulu-Natal |

## STUDY OUTLINE

The current dissertation is the scientific work authored by M Perumal under the supervision of Prof MV Mabandla and co-supervision of Dr M Luvuno. This is presented in the manuscript format and consists of the sections listed below:

**Chapter 1** consists of the introduction/literature review, aims, objectives and general methodology of the study. This chapter comprises a brief background and relevant literature review about the current knowledge of the study area and shows knowledge gaps. The aims of this study are intended to fill the gaps in the literature and the objectives express how the aims of the study were fulfilled. The general methodology of the study communicates how the aims and objectives of the study were effected.

**Chapter 2** contains the first research study in the manuscript format that investigated the effects of parental prenatal stress on offspring anxiety, depression and sociability. This manuscript has been presented according to the journal's guidelines to authors.

**Chapter 3** contains the first research study in the manuscript format that investigated the effects of parental prenatal stress on offspring body weight, feeding behaviour and stress response. This manuscript has been presented according to the journal's guidelines to authors.

**Chapter 4** is the last section of the study that discusses links between the study in accordance to the aims and objectives of the study. Appendices contain supplementary information that might be useful to the reviewers.

**ABSTRACT**

Exposure to past stress and trauma during early developmental stages can permanently affect the performance and advancements of core systems in humans. Whilst many studies are investigating the lasting effects of maternal prenatal stress, there is a paucity of information on the long-term effects of paternal prenatal stress. Therefore, the present study sought to investigate the effects of parental prenatal stress on the offspring's psychiatric behaviour, particularly the fathers, whether these can be transferred to offspring and a number of parameters commonly associated with prenatal stress. Furthermore, we evaluated the effects of parental prenatal stress on body weight, feeding behaviour and stress response. Animals had access to food and fluids ad libitum during experimentation and were randomly assigned to different groups (n=8 per group). We found that the behavioural and neurochemical manifestations in the offspring of prenatally stressed fathers suggest that stressed fathers can transfer feelings of anhedonia and social anxiety to their offspring mediated, in part, by offspring behavioural changes of depression and social anxiety as well as, a blunted serotonin response. Furthermore, when both parents were prenatally stressed their stress effect to their offspring's behavioural and neurochemistry is augmented. This was confirmed by the behavioural manifestations of extreme anxiety, depression and social anxiety as well as, the subdued serotonin concentration. Additionally, we found that prenatally stressed fathers can impact on offspring feeding behaviour and body weight changes mediated, in part, by the offspring's reduced food intake and body weight as well as, a dysregulated corticosterone response. Moreover, when both parents were prenatally stressed their stress effect to their offspring's development is intermediary. This was confirmed by metabolic manifestations of increased food intake and body weight which may have primarily been accomplished by modifying the glucocorticoid system. Therefore, the prenatally stressed fathers decreased the offspring's sociability and increased anhedonia however, they did not transfer their anxiogenic behaviour. The prenatally stressed fathers also decreased the offspring's appetite and as a consequence their however, they did not affect the stress response.

# CHAPTER 1: LITERATURE REVIEW

Stress is a primary factor in the onset of depression and anxiety [1]. Although several studies have shown and discussed the effects that chronic stress can induce neurochemically and behaviourally during adulthood, the impact of chronic stress conferred from parent to offspring has not been explicitly discussed. In addition, it has not been methodically studied as to which parent confers the stress to its offspring.

Therefore, this study used a standard chronic stress animal model to induce symptoms in the parental generation and study these effects in the first filial generation. From this, we specifically observe if parental stress causes a dysfunction in the offspring stress response and metabolism with corresponding neurochemical fluctuations. Furthermore, we investigate if parental exposure, specifically paternal exposure, to chronic stress triggers neurochemical manifestations and exacerbates the onset of social anxiety, anhedonia and anxiety in the offspring.

## EPIDEMIOLOGY OF STRESS IN SOUTH AFRICA

Stress can be described as a common experience that causes feelings of anxiety and frustration [2]. In a stressful situation, the body reacts by activating the hypothalamic-pituitary-adrenal axis, which stimulates the body's "fight or flight" reaction [3, 4]. An estimated 50% of adults have experienced a traumatic, stressful event in their lifetime, which places a significant burden of disease on society [5]. South Africa's rates of psychiatric morbidity are at a record high due in part to the country's history of violence during the apartheid era [6].

In addition to the remnant physical effects of the apartheid era, such as structural violence, inequality, socioeconomic challenges and poverty, citizens continue to be impacted and affected by declining mental health and behavioural challenges [6]. The older generations of South Africa have further reported memory impairments during their life course, which has been linked to chronic psychosocial stress [7]. Epidemiological studies have also shown that women are more susceptible to depression [8], stress and post-traumatic stress disorder [9, 10].

South African data has suggested that one in six citizens experience anxiety, depression, or substance abuse, 40% of human immunodeficiency virus patients have a comorbid

psychological disorder, 41% of pregnant mothers suffer from depression, approximately 60% may be recovering from post-traumatic stress disorder [11]. In light of this, only 27% of South Africans with severe psychological conditions acquire therapy [11]. The fact that only 27% of South Africans with extreme psychological conditions receive treatment is a sign of the dire health system within South Africa. Furthermore, the Life Esidimeni incident reminds us how brutal and inhumane the South African mental health systems are [12-15]. A global correction is needed, as the lack of mental health institutions and programmes is not unique to South Africa but is a universal trend [15].

A study conducted during the first lockdown period (March 2020) of SARS-CoV-2 showed that 33% of South Africans were depressed, 45% were anxious, and 29% were desolate [16]. This correlated with at least 3 million citizens being retrenched within the first four months of lockdown [17]. A month after the study was conducted, there were approximately 87,000 cases of gender-based violence reported nationally in the first week of lockdown [18].

Evidence has shown that exposure to past stress and trauma during early developmental stages can permanently affect the performance and advancements of core systems in humans, including the neuroendocrine pathways, neurobiological function, immune system and cardiovascular system [6]. Upon encountering a stressor, the body reacts by employing several modifications of the normal homeostatic reaction, including regulating the hypothalamic-pituitary-adrenal axis [19]. Therefore, the following section discusses in detail the pathophysiology of stress.

## PATHOPHYSIOLOGY OF STRESS

Under normal conditions, organisms indulge in the pleasures that drive their species survival, growth and development, such as eating and sex. However, in unfavourable conditions, activation of the stress response can be linked to melancholy and emotional or physical disorders [19]. During a stressed state, the brain redirects its attention to the threat by increasing cardiac output, stimulating the respiratory system and catabolism thereby, redirecting blood flow to mobilise energy in the direction of the active brain, heart and muscles [19]. The two systems involved in reacting to a stressor are the sympathetic adreno-medullary and the hypothalamic-pituitary-adrenal axis [20].

During sympathetic adreno-medullary activity, the cerebral cortex categorizes an unknown threat as a stimulus, information that is sent to the hypothalamus to activate the body's "fight or flight" response. This response stimulates the adrenal medulla to secrete catecholamine, norepinephrine, and epinephrine which produces physiological effects such as increased blood pressure, perspiring, tremors, vasoconstriction etc. [20]. The secretion of norepinephrine stimulates the hypothalamic-pituitary-adrenal axis [21]. This produces a cascade of hormonal activity, commencing with the secretion of corticotropin-releasing hormone from the hypothalamus, which stimulates the pituitary gland to release adrenocorticotropic hormone (ACTH) [21]. This culminates in releasing glucocorticoids (cortisol in humans and corticosterone in rodents) into the bloodstream by adrenal glands. The glucocorticoids stimulate mineralocorticoid receptors and glucocorticoid receptors in the hypothalamus and the pituitary, providing a feedback signal to regulate hypothalamic-pituitary-adrenal axis activity [21-23].

The hypothalamic-pituitary-adrenal axis has been designed to help an organism survive adverse conditions and is regulated by three main structures: the hippocampus, amygdala and medial prefrontal cortex [23, 24]. The amygdala, known for its role in fear detection, activates the hypothalamic-pituitary-adrenal axis compared to the prefrontal cortex and hippocampus, which has an inhibitory role [24]. The hippocampus is the most distinct regulator of the hypothalamic-pituitary-adrenal axis from the three structures attributable to its role in prominent mental health disorders such as depression, post-traumatic stress disorder, and Alzheimer's Disease [24]. The hippocampus is involved in terminating pre-emptive hypothalamic-pituitary-adrenal axis responses, concurrent with its role in memory and emotional processing [22]. Activation of the hypothalamic-pituitary-adrenal axis induced by stress concludes with the adrenal glands releasing glucocorticoids [19]. These processes are keenly regulated by the neuronal activity of the hippocampus and prefrontal cortex, which elicit negative feedback on the hypothalamic-pituitary-adrenal axis activation [25].

An important consideration is that corticosterone and cortisol secretion alone cannot be used to assume the reactivity of the hypothalamic-pituitary-adrenal axis [23]. For example, desensitised adrenal glands can impede the activation of the hypothalamus and pituitary components, thereby miscalculating the stress response [23].

Exposure to stressors in daily life is necessary for the development of the brain as it allows the body to adapt to challenges faced and prepare for bodily harm and injury in the future [26]. However, in cases where an individual cannot react to a stressful encounter or avoid injury, this may have damaging effects and impair cognition later in life [26]. It has been documented that a dysregulated hypothalamic-pituitary-adrenal axis and glucocorticoids secretion can exacerbate the likelihood of developing depression/anxiety disorders [27, 28] and susceptibility to drug abuse; however, further studies are needed to test this statement [28-30]. Therefore, the following section discusses in detail the complex nature of stress hormones.

**STRESS HORMONES**

Glucocorticoids are the final products when activating the hypothalamic-pituitary-adrenal axis, released upon encountering a stressor [24]. Glucocorticoids are lipophilic, meaning they can cross the blood-brain barrier and bind to glucocorticoid receptors in various brain regions [24]. The release of glucocorticoids allows the hypothalamic-pituitary-adrenal axis to mobilise energy and prepare for a real ("reactive" response) or predicted insult ("anticipatory" reaction) [23]. Glucocorticoids signal through their receptor types, namely, mineralocorticoid receptors and glucocorticoid receptors [24].

Mineralocorticoid receptors guide the hypothalamic-pituitary-adrenal axis responsivity in relation to the day and have a superior binding affinity to glucocorticoids than glucocorticoid receptors [23]. Mineralocorticoid receptors sense aldosterone in the kidneys and other tissues via the inactivation of corticosterone (or cortisol) by elevated 11β-hydroxysteroid dehydrogenase (11β-HSD1) enzyme [31]. Contrastingly, in other tissues such as the brain, 11β-HSD1 acts as a reductase and can, in certain conditions, increase glucocorticoid concentrations [31].

Elevated glucocorticoid concentrations activate the lower-affinity glucocorticoid receptors, which mediate the outcome of glucocorticoid secretion by mobilising energy stocks, inflammation and neural action [23]. Glucocorticoid receptors also encourage the forming of memory to prepare for future scenarios [1]. Glucocorticoid receptors decipher and interpret stress levels and, as such, are presumed to be responsible for regulating the feedback loop [23]. During gestation, maternal and foetal glucocorticoid levels increase, as a prenatal developmental mechanism [32]. Foetal exposure to glucocorticoids during the third trimester

is necessary for development of the lungs, brain and to prepare for birth and delivery [32]. Increased levels of glucocorticoids have been linked to diminished cognition and poor sociability, as well as anxiety-like behaviours [32, 33]. Likewise, an upregulation of glucocorticoid receptor influences anxiety and depression-like behaviours in the elevated plus maze [32].

Glucocorticoids have been shown to decrease pro-inflammatory cytokines whilst increasing the expression of anti-inflammatory cytokines [34]. On the other hand, recent studies have shown that glucocorticoids also have a pro-inflammatory influence on the immune system [35]. This was seen in rats, following an acute stress regimen, they presented with an increased basal plasma corticosterone and prostaglandin E2 with less anti-inflammatory factor [36]. Therefore, the following section discusses the role of cytokines in the stress response.

**CYTOKINES**

Cytokines are chemical messengers of immune cells which facilitate inflammatory reactions and immune responses. They are also involved in mediating the signalling in non-immune tissues such as the nervous system and are fundamental players in the natural and pathological systems [37-39]. Cytokines can either be pro-inflammatory or anti-inflammatory, which is dependent on their biological reactions [39]. They each exert different responses on synapsis, neurological development and neurogenesis [37, 39]. An abundance of cytokines can influence the neurodevelopment of the foetus by directly interacting with the foetus glial cells, and is also associated with the transmission of stress between the mother and foetus via cortisol and reactive oxygen species [40].

Elevated serum pro-inflammatory cytokines (including interleukin (IL)-1β) have been noted in patients with major depressive disorder to anti-inflammatory cytokines (such as IL-10, IL-4). Cytokines have complex interactions where they can act in either a synergistic manner or antagonistically. The impact of cytokines within a network is associated with a particular immunological process. Pro-inflammatory cytokines can trigger the HPA axis whilst, cortisol attenuates production of cytokines and other inflammatory indicators [41]. Circulating pro-inflammatory factors directly triggers the HPA axis, producing serum adrenocorticotropic hormone and glucocorticoids, which subsequently prevents the production of these pro-inflammatory factors [42].

Maternal stress has been shown to increase expression of pro-inflammatory placental genes namely, IL-6 [40]. In rat animal studies, the use of Il-6 was shown to produce structural modifications in offspring hippocampus and learning deficiencies as well as increased body weight [40]. Further studies have provided the link between a prolonged increase in IL-6 concentration and acute mental stress in expecting mothers [43]. The exact method of cytokine maternal-foetal transference of cytokines is still being established however; some animal models have documented that the cytokines are able to travel through the placenta towards foetal circulation [44]. It has been suggested that in addition to maternal-foetal transference, the placenta itself may potentially be a source of cytokines and actively secrete cytokines into foetal circulation in response to maternal stress [40].

Chronic stress has been shown to attenuate the production of pro-inflammatory cytokines, which regulate the cellular immune response, whilst it stimulates the secretion of anti-inflammatory cytokines, which mediate the humoral immune response [45, 46] From this, it was hypothesised that chronic stress could lead to disease onset by suppressing the immune system [45-47]. Glucocorticoid's and catecholamines affect glucocorticoid receptors and adrenergic receptors on immune cells extracellularly or intracellularly which subsequently impede the secretion of pro-inflammatory cytokines and stimulate anti-inflammatory cytokine secretion [45, 48]. Contrary to this, some researchers have found that chronic stress stimulates the secretion of pro-inflammatory cytokines [49-51]. This conflict between the role of chronic stress on inflammation is said to be linked to the period and force of the stressor, exposure and phenotypic variances [45, 50]. Therefore, the following section discusses the different types of stressors with specific interest to chronic stress.

## CHRONIC STRESS

Stress can be acute or chronic depending on the duration of and exposure to a stressor [52]. An inability to terminate an acute stress response can lead to persistent changes indicative of a chronically stressed state [52]. Exposure to chronic stress is reported to be highly toxic as it is likely to result in long-term changes in emotional, physiological and behavioural responses, which influence exposure to and the course of disease [53]. Chronic stress is a cumulative process in which the hypothalamic-pituitary-adrenal axis responds to each individual "threat"

through recurrent and hyperbolic exposure and consequently increases the glucocorticoid discharge [23].

Chronic stress has been linked to conformational and physical adaptations of neural networks in the brain [54]. This was reported through imaging studies which showed structural impairments in the brain of patients suffering from stress-related disorders [54]. Consequently, these impairments in one particular brain region can extend to other functionally interconnected areas and result in cognitive, emotional and behavioural dysfunctions associated with chronic stress, which may exacerbate the likelihood of developing psychiatric disorders [54]. A study by Monti Voss, further attested to this by demonstrating that mild head trauma during the early stages of development resulted in impaired memory and diminished hippocampal volumes during adulthood [55].

Chronic stress is a renowned risk factor for numerous psychiatric disorders, such as anxiety and depression [1]. In addition, exposure to uncontrolled chronic stress weakens the reward system through stimulation of the mesocortical dopaminergic system by impeding dopamine release in several terminals, including the hypothalamus [1]. Since the perinatal life is especially sensitive to stressors, as these periods have increased plasticity for the stress system, hostile activity during this time can produce changes to behaviour and physiological mechanisms such as inflammatory response, growth, metabolism and reproduction [28, 56]. Therefore, the following section discusses the physiological responses to stress.

## PHYSIOLOGICAL RESPONSES TO STRESS

### Metabolism

Chronic stress or repeated activation of the stress response is associated with excessive visceral adiposity, reduced body muscle and bone mass and a defeated osteoblastic system [19]. These symptoms are shared by patients who present with a dual-diagnosis of Cushing's syndrome (or hypercortisolism) and depression or chronic-anxiety disorder and metabolic disorders such as diabetes, hypertension, cholesterol [19, 57]. Elevated hepatic gluconeogenesis is attributable to the stress response [19]. Since glucocorticoids increase insulin resistance, it can be assumed that diabetic patients in stressful circumstances may find it harder to regulate their hypothalamic-pituitary-adrenal axis [19].

Obese patients exposed to chronic stress and present with additional signs of depression or anxiety are generally diagnosed with Cushing's syndrome, whilst patients who present with obesity and no indicators of depression or anxiety are eucortisolemic [19]. Recording body weight after birth is important for predicting neuropsychiatric ad metabolic complications later in life [58]. Patients who encounter stressful situations have been shown to adopt unhealthy food patterns with most eventually presenting with obesity, metabolic syndrome and type 2 diabetes [59, 60].

Prolonged stimulation of the hypothalamic-pituitary-adrenal axis has been linked to other conditions, namely, anorexia nervosa, panic attacks, alcoholism, an overactive thyroid, obsessive physical activity, juvenile sexual assault, obsessive-compulsive behaviours and poorly managed diabetes [19]. Therefore, the following section discusses the social problems a dysregulated hypothalamic-pituitary-adrenal axis can produce.

**Sociability**

Chronic stress conducted on rodents using an animal stress model has been reported to lessen social interactions and motivation, which correlates with symptoms expressed by depressed patients [61]. A previous study showed that after exposing their rodents to the chronic restraint stress paradigm, the stressed animals preferred fewer social engagements; however, their social perception, learning, and memory were not significantly affected [61].

In humans, fostering attachment is critical during early development as losing these attachments through neglect, the death of a parent or abuse exacerbates the onset of emotional disorders as the child grows [62].

A previous study reported that social deprivation in male mice could adversely affect childhood, adolescence and adulthood [25]. For example, paternal separation blights sociability and social recognition, reduces parental behaviours, and hinders one resolve to develop social bonds [62]. In humans, separation manipulates mental and psychological development and aggravates the inception of substance abuse and mental disorders [62]. Therefore, the following section discusses the psychiatric problems associated with a dysregulated hypothalamic-pituitary-adrenal axis.

**Memory, Mental Illnesses and Early Life Adversity**

Sequelae develop as the body's exposure to a chronic stressed state increases, such as anxiety, depression and infertility [52]. A previous study showed that unfavourable prenatal environments could affect the development of neuroendocrine systems, thus exacerbating stress-related and physical complications later on in life [63].

Chronic stress has been found to induce cognitive dysfunction in psychiatric patients, subsequently leading to the loss of synaptic connectivity and possibly neuronal networks in brain structures, including the hippocampus and cortex [52]. This further leads to loss of cholinergic neurons and results in a state of dementia [52]. A previous study using a sample of South African older adults, noted that psychosocial stress was more prominent in patients who presented with Alzheimer's Disease than healthy individuals [7]. This study further corroborated that these heightened stress levels were associated with poorer memory performance and a predicted state of Alzheimer's Disease [7]. Building social connections can expedite procreation, improve and modify survival, offer a feeling of security, and lower stress and anxiety in many species [62].

It has been documented that early life adversities are linked to prolonged and detrimental changes to the individuals hypothalamic-pituitary-adrenal axis, producing elevated glucocorticoid responses to a stressor with a diminished glucocorticoid expression in brain regions, incited by maternal neglect [24, 64] early life adversity , through human studies, has also been linked to memory, learning and recall deficits including emotional abuse and neglect [24, 65]. Furthermore, it was reported by Grassi-Oliveira and colleagues (2008) that patients with depressive disorders who were exposed to neglect as children had poor memory recall, thereby validating theories that early life adversity is a critical risk factor for major depressive disorder. The link between early life adversity and depression has been broadly studied, and it was shown that the early life adversity phenotype is remarkably similar to depression. It has also been linked to impaired hippocampus, prefrontal cortex and amygdala functionality which are target brain regions for depression studies and models [24, 66]. Hence, the following section discusses the influences of the perinatal life on anxiety and depression.

**Depression and Anxiety**

The World Health Organisation published a report stating that major depressive disorder will be a primary factor in disability by 2030 globally [67]. The fundamental elements in major depressive disorder are lack of motivation, inability to experience pleasure, mood swings, sleep apnea, loss of appetite and cognition [68]. In animal models, prenatal stress during late gestation has been linked to anxiety- and depression-like behaviours in offspring [69].

Brain imaging studies conducted on humans have reported changes in the blood flow of vessels and other parameters in brain regions of hippocampus, prefrontal cortex, amygdala and more which have further been corroborated by autopsies performed on depressed patients [67, 70]. Relatedly, decreased neuronal activity has been reported in defeat-induced depression mice models [71], structural and neurochemical alterations of the hippocampus have been reported in autopsies conducted on depressed patients [72] and, serotonin innervation in brain regions reportedly related to depression such as the prefrontal cortex and amygdala, to which increased levels have been linked to the antidepressant effect [73]. During the gestational period, the placenta acts as an exogenous source of serotonin to enable the development of the foetal forebrain [40]. Serotonin, which is detectable from seven weeks in human foetuses is vital for the development of the placenta and embryo, as well as foetal and postnatal brain and cardiovascular maturation in humans and animals [74]. Hence, it is conceivable that alterations in the foetal serotonergic homeostasis due to prenatal stress can have sustained physiological ramifications in adulthood [40]. Serotonin is synthesised from tryptophan and is implicated in the pathophysiology of several neuropsychiatric diseases including depression and anxiety [40].

Depressed patients have presented with increased concentrations of cortisol in their urine, an inflated response to psychosocial stress, reduced hippocampal volume and impaired memory [21, 75]. Furthermore, links between inflammatory markers and depressive symptoms such as fatigue, cognitive impairments and abnormal sleep patterns linked to the expression of IL-6 have been described [76]. Pro-inflammatory factors are known to cross the blood-brain-barrier, and either to direct or indirect intervention of the HPA axis, are involved in the pathophysiology of depression [77].

The pro-inflammatory cytokines located in the central nervous system attenuate the secretion of neurotransmitters, serotonin and dopamine [45]. This blockage is significant in the pathogenesis of depression and is used when deciding on suitable therapies [78]. The pro-inflammatory cytokines then activate the corticotrophin-releasing hormone from the paraventricular nucleus, which exacerbates the secretion of ACTH and cortisol [45]. The connection between increased expression of corticotrophin-releasing hormone and chronic stress is being exploited to relate chronic stress and depression [45].

A previous study reported that low birth weight in older men was linked to depression and suggested that permanently amplified cortisol concentrations in depressive illnesses could link the programming of low birth weight and depression in parenthood [79]. Depression is a common comorbidity of anxiety [80]. Hence, the next section looks at the relationship between depression and anxiety.

The diagnostic and statistical manual of mental disorders has linked anxiety to panic attacks, extended anxiety, phobias, post-traumatic stress disorder and obsessive behaviours [81]. Experiments have shown how early-life exposure to stress can be linked and exacerbate the person's likelihood of developing the condition described above [20, 82, 83]. Activation of the hypothalamic-pituitary-adrenal axis produces cortisol which has been shown to affect mood and behaviour [20, 84, 85]. Traumatic childhood events exacerbate the likeliness of developing anxiety disorders by 1.9-3.6 fold [86, 87].

The coexistence of depression-anxiety has been connected to poor health, dysfunctional cognitive and emotional capabilities, and post-traumatic stress disorder [87-89]. A hyperactive amygdala has been shown to exacerbate depressive and anxiety-like indicators [87, 90].

A study examined the link between anxiety proneness after exposure to early life traumas and the consequences in a South African population [91]. This study has shown a noticeable increase in glucocorticoid receptor and corticotrophin-releasing hormone expression. These physiological features correspond with both post-traumatic stress disorder and depression, disorders to which anxiety is a known comorbidity [91, 92]. Therefore, the following section discusses the prenatal maternal stress connexion.

## PRENATAL MATERNAL STRESS

Prenatal stress refers to all types of stress experienced by the parent, e.g., physical or emotional, during the gestational period [2, 93, 94]. When the mother is prenatally exposed to stress, it causes a twofold reaction, that is, an increase in maternal cortisol and a decrease in the expression of 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2), the foetus' natural barrier enzyme to maternal cortisol [95, 96]. 11β-HSD2 converts excess cortisol (corticosterone in rodents) to its inactive form cortisone (dehydrocorticosterone in rodents) [56] however, it does not completely block-off maternal-foetal transmission [32]. 11β-HSD2 decreases naturally during the third trimester to allow for foetal lung maturation and parturition, thereby increasing the risk of exposure of the foetus and placenta to glucocorticoids [95, 96]. Glucocorticoids are lipophilic and can easily cross the placenta, which is further weakened due to decreased 11β-HSD2 hormone [2, 93], where prenatal stress has been shown to increase glucocorticoid receptor in the placenta [32].

Activity of 11β-HSD2 naturally decreases in uncomplicated pregnancies during late gestation, exposing the foetus to programming influences of cortisol during this time [40]. Due to the increased cortisol expression in the placenta, the growing foetus can respond with a dysregulated hypothalamic-pituitary-adrenal axis [28, 96]. This dysregulation leaves the neonate prone to compromised stress response and increased susceptibility to disease [28, 96].

The long-term effects of prenatal stress are unspecified however, literature suggests that prenatal exposure to stress can affect the progression, performance and sensitivity of the stress system [6, 21]. Importantly, it has been noted that the impact of maternal stress on the intrauterine environment is not the only factor in poor child development but rather, maternal involvement during upbringing plays a core role in the child's neurodevelopment [97]. It is therefore conceivable that maternal stress experienced during pregnancy and early parenting can programme the physiological and lifelong course of the infant which ultimately determines their health [97].

Prenatal maternal stress increases the risk of cortisol exposure to the foetus which subsequently produces adverse birth consequences such as, low birth weight, reduced gestational period, and smaller head circumference[6, 21]. Increased neonatal cortisol exposure can permanently alter the growth and physiological sensitivity of the foetal systems such as, the hypothalamic-

pituitary-adrenal axis, immune response, and cognitive functioning development [6]. Prenatal maternal stress has also been shown to exacerbate the onset of febrile seizures [93, 98, 99], and the risk for developing psychiatric disorders such as, autism spectrum disorders, schizophrenia and attention deficit hyperactivity disorder [100], which can durably affect the child's physiological stress response across their life course [6]. These effects depend on the type of stressor, the time during the gestational period, the sex of the foetus and, the stage of foetal brain development [101]. Foetal brain development occurs through three phases namely, embryonic period, early foetal period, and late foetal period [102]. Therefore, the foetal brain is continuously maturing throughout the gestational period and its development can be affected by any biological signal it receives [101, 103].

Whilst many studies are investigating these lasting effects of prenatal maternal stress on infant, child, adolescence and adulthood, there is a paucity of information on the long-term effects of prenatal paternal stress overall. The most recent documented studies on germ cell epigenetic transmission have mainly been studied in paternal models [100]. Therefore, the following section discusses the role of prenatal paternal stress.

**PRENATAL PATERNAL STRESS**

Most documented paternal models have focused on germ cell epigenetic transmission and the phenotypic effects that stress programming on sperm produces in offspring [100]. During spermatogenesis, sperm histones are replaced by protamine's, which are highly charged proteins that reduce sperm chromatin to one-tenth that of somatic cells. Consequently, mature sperm become transcriptionally inactive and are believed to resist external forces [100]. Nonetheless, recent studies have demonstrated that sperm are responsive to homeostatic changes such as dietary changes, stress, trauma, and exposure to drugs [100]. A study showed that male mice exposed to prenatal stress present with an amplified hypothalamic-pituitary-adrenal stress response and altered stress coping behaviours, which were reportedly transferred phenotypically to their male but not female offspring [100].

A significant sex difference has been documented in both the magnitude and duration of the stress response influenced by male (testosterone) and female (oestradiol, progesterone) sex hormones [23]. In particular, testosterone inhibits stress reactivity whilst oestradiol was reported to augment hypothalamic-pituitary-adrenal axis responses [23]. In rat studies, females

in dioestrus (low oestradiol secretion) display inadequate resting glucocorticoid secretion and interchangeable reactions to stressors, similar to those observed in males [23]. Animals in oestrus (high oestradiol ) and proestrus (high oestradiol, high progesterone) have elevated basal corticosterone and exacerbated corticosterone release [23].

The transfer of epigenetic information through multiple generators has been referred to as intergenerational epigenetic inheritance, many of which have been documented in humans [104]. This was seen in the offspring of Holocaust survivors who were deemed more likely to suffer from anxiety and depression, and in the offspring of Australian veterans who fought in the Vietnam war were diagnosed as suicidal [105, 106]. Furthermore, smoking in young males were reportedly linked to the onset of asthma in their offspring, even if the fathers who started smoking before the age of fifteen had quit for five years prior to fertilisation [107]. These studies show the susceptibility of sperm cells to environmental stressors which encourages the onset of disease in the offspring [104].

Prenatal stress models vary in their types and duration of exposures, such as restraint stress, foot shock therapy, social isolation or repetitive social stressors [1]. Restraint stress is the most adaptive and reproducible form of a stressor and directly affects the foetus by limiting all forms of movement [108]. Exposing animals to a prenatal state of chronic stress has increased offspring susceptibility to disease and behavioural dysfunctions; hence we included a stress factor in this study [100]. Therefore, the following section discusses the different types of chronic stress animal models and their significance to clinical studies.

## ANIMAL MODELS AND THEIR SIGNIFICANCE

Developing animal models is critical to the development of drugs, treatments and disease progression. However, because many disorders focus on human pathophysiological responses, it is not always easy to mimic these exact conditions in an animal. To control this, animal models are designed to highlight specific symptoms related to that disease which allows progression in pharmacological treatments [28]. Rodents, rats (Rattus Norvegicus) and mice (Mus Musculus) are the preferred animals used in modelling as they have similar genetic markers to humans, are easier to handle and accommodate, reproduce quickly and have shorter lifespans [109].

**Animal Models of Chronic Stress**

A previous study proposed three principles to be followed when designing animal models; they should have face, construct and predictive validity [110]. Chronic stress models have commonly been designed to mimic depression but are now also being used to reflect and understand the physiological expressions of post-traumatic stress disorder [109].

In the late 1960s, a study led to the "learned helplessness" paradigm, a concept centred on classical conditioning first demonstrated on dogs [111]. Van der Kolk adapted this model, known as the "Inescapable Shock-Learned Helplessness", by exposing the animals to drug-related and non-drug-related stressors [112]. Antelman, in 1988, depicted the time-dependent sensitization protocol, which follows a quick, powerful stimulus followed by a prolonged response to stressors [113]. Rats were subjected to different stressors ranging from restriction to injections of drugs [109, 113].

The foot shock paradigm is commonly depicted in animal models of anxiety and depression [114]. This paradigm exposes the animal to sequential foot shocks of wavering strength and intervals, the effects of which have been shown to last up to 3 weeks [109, 114]. The single prolonged stress model exposes the animal to the forced swim test, then 2 hours of restraint stress, a rest period, and exposure to fumes until the animal loses consciousness [114]. The social defeat model is used to examine behavioural and physiological responses to stress, which has been reported to produce social anxiety and passive behaviours [109, 115].

The model used in this study was the chronic prenatal restraint stress paradigm used by Qulu et al. (2012). This stress model exposes pregnant female rats to restraint stress which has been shown to affect the developing foetus [93]. This paradigm was adapted to fit within the aims and objectives of our study. Our animals were exposed to 1 hour of restraint stress, using a cylindrical plexiglass restraint, for seven days to induce chronic stress symptomology in the offspring. Previous work conducted in our laboratory successfully employed this animal model to induce signs of prenatal stress in rodents. Hence, we replicated the same conditions to ensure comparable indicators of prenatal stress in our animals.

**Justification of the study**

In this study, we investigate physiological repercussions in the first filial generation after exposing the parental generation to a chronic stress regime.

This study will be a first step in elucidating how stressors such as oppression, race and inequality experienced by older generations can be transferred to offspring and how it affects their well-being. These are individuals who have never primarily been exposed to such stressors. It will provide insight into the physiological connection linking lineages exposed to trauma and the effects in the subsequent generations. In addition, it may provide a basis for post-traumatic stress disorder being expressed in respective generations of the same lineage.

It is evident that maternal influences have been extensively documented; however, much less is known regarding the specific role paternal factors play in the programming of offspring. Hence, our study aims to bridge this gap by using a standard chronic stress animal model to induce symptoms in the parental generation and study these effects in the first filial generation. This chronic stress study will enable us to document maternal and paternal influences on the offspring, with specific emphasis on neurochemical and behavioural adaptations. Our findings can be translated to a human cohort to understand the pathophysiological implications surrounding the transference of traumatic experiences in older generations to offspring. This can then be used to improve the diagnosis, prevention and treatment of disease and control the physiological outcomes of posttraumatic generational traumas.

**Aims**

This study uses a standard chronic stress animal model to induce symptoms in the parental generation and study these effects in the first filial generation. Therefore, we will specifically observe if parental stress causes a dysfunction in the offspring stress response and neurochemical changes in the brain. Furthermore, we will investigate if this exacerbates the onset of depression, anxiety, metabolism and anhedonia in the offspring.

**Objectives**

1. To verify if chronic parental stress causes a dysfunction in the offspring stress response, we measured the corticosterone concentration present in the adrenal glands, the expression of IL-6 and glucocorticoid receptor in the prefrontal cortex and ACTH in the hippocampus.

2. To assess offspring metabolism, we compared body weight and food intake across the groups.
3. To determine if chronic parental stress influences depression, we measured dopamine expression in the hippocampus after exposing the offspring to the sucrose preference test.
4. To determine if chronic parental stress influences anxiety in offspring, we measured serotonin expression in the amygdala after exposing the offspring to the elevated plus-maze.
5. To establish if chronic parental stress influences social interaction in offspring, we assessed the expression of serotonin in the amygdala after exposing the offspring to the social novelty test.

**Methodology Overview**

Following approval from the Animal Research Ethics Committee (AREC) of the University of KwaZulu-Natal (UKZN), sixteen male and eight female Sprague Dawley rats weighing approximately 250-300 g were obtained. These rats were housed under standard laboratory conditions of ± 22°C room temperature, 70% humidity and a 12-hour light/dark cycle (lights on at 06h00, off at 18h00). Food and water were available on an *ad libitum* basis for the rats. The rats were first separated into stressed groups, i.e., non-stressed mother (NS-M) and stressed mother (S-M) and non-stressed father (NS-F) and stressed father (S-F). After that, they were assigned to one of four following groups, each group containing four males and two females as follows: (1) control (C) group: non-stressed mother and stressed father (NS-M + NS-F) (n=6); (2) non-stressed mother and stressed father (NS-M+S-F) (n=6); (3) stressed mother and non-stressed female (S-M+NS-F) (n=6); and (4) stressed mother and stressed father (S-M+S-F) (n=6). Once the rats had been assigned their groups, they were left to acclimatise for seven days. Following the acclimatisation period, the male chronic stress protocol was initiated. The NS-F were left in their cages whilst the S-F was exposed to the chronic restraint stress protocol for 1 hour over seven days. Succeeding the stress period, we began mating the animals according to their groups; a female rat was introduced into a male cage to encourage mating. The male rat was removed after successful mating. On gestational day (GND) 14, the S-M was subjected to the chronic restraint stress paradigm for 1 hour over seven days. The NS-F, S-F and NS-M remained in their home cages during the female prenatal restraint protocol. Concurrently, the S-F groups were again exposed to the chronic stress protocol to ensure that the fathers expressed stressed symptoms before exposure to the elevated plus-maze. Both NS-

F and S-F were exposed to the behavioural test. The males were sacrificed 24 hours after the behavioural test to collect the amygdala to measure serotonin concentration and the adrenal glands for ACTH corticosterone concentration. Succeeding birth, the pups were weaned off their mothers for 22 days, and on postnatal day (PND) 23 they were exposed to behavioural tests which were compared across their parental groups (1) C group, (2) NS-M+S-F; (3) S-M+NS-F; and (4) S-M+S-F. The pups were scored on the following behavioural tests: (i) the sucrose preference test; (ii) the elevated plus maze, and (iii) the social novelty test. The pups were sacrificed using a sharpened guillotine 24 hours after the behavioural tests. The adrenal glands were collected to measure the concentration of corticosterone, trunk blood to measure plasma ACTH concentration the hippocampus for dopamine, the amygdala for serotonin concentration and the prefrontal cortex for IL-6 and glucocorticoid receptor expression. All samples were immediately snap-frozen after extraction using liquid nitrogen and then stored in a bio freezer at -80°C until neurochemical and biochemical analysis was performed. Statistical analysis using GraphPad prism was performed to analyse data and confirm statistical significance.

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# CHAPTER 2

## Prologue

## Manuscript 1

Chapter 1 provided an overview into the influences of parental stress on offspring neurochemistry and behaviour, highlighted the gaps in literature regarding the effects on offspring anxiety, depression and sociability as well as defined the clinical relevance of this study.

**"The influences of paternal stress on offspring neurochemistry and behaviour: Effects on offspring anxiety, depression and sociability"**

## Contributions of this chapter

This chapter is comprised of a scientific manuscript that investigated the influences of parental stress on offspring neurochemistry and behaviour, and documented the effects on offspring anxiety, depression and sociability as well as described the paternal contributions to these manifestations. The results of this study were compared across parental groups to determine statistically significant relationships.

Note: This chapter has been prepared according to the guidelines outlined by PONTE Journal (Appendix D).

**The influences of paternal stress on offspring neurochemistry and behaviour: Effects on offspring anxiety, depression and sociability**

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**ABSTRACT**

Stress during gestation has been associated with the exhibition of depression and anxiety-like behaviour in the offspring. However, the effect of a stress father on the offspring is not well understood. The present study sought to investigate the effects of parental stress on the offspring. To achieve this, male and female Sprague Dawley rats, weighing 250 – 300 g, were assigned to one of four groups (n=6 per group) viz: (1) control, (2) non-stressed mother and stressed father, (3) stressed mother and non-stressed father, and (4) stressed mother and stressed father. The fathers were subjected to a chronic restraint protocol after which they were allowed to mate. To observe the influence of chronic parental stress on anxiety, sociability and depression-like behaviour in the offspring, we subjected all offspring to behavioural tests and measured serotonin and dopamine concentration in the hippocampus and amygdala. A stress effect on sociability and anhedonic behaviour in offspring from the NSM-SF group, SM-NSF group and SM-SF group was present. This was accompanied by decreased serotonin concentration in the amygdala. Altogether, our findings show that the prenatally stressed fathers did not transfer their anxiogenic behaviour, however, they affected the offspring's sociability and anhedonia. This was mediated in part by decreased serotonin concentration in amygdala. Furthermore, when both parents are prenatally stressed their stress effect to their offspring's behavioural and neurochemistry is augmented. This was confirmed by the behavioural manifestations of extreme anxiety, depression and social anxiety as well as, the subdued 5-HT concentration.

**Keywords:** prenatal stress, chronic stress, depression, anxiety, sociability, serotonin, dopamine

## 1. INTRODUCTION

In South Africa, psychiatric morbidity is alarmingly high due in part to the country's history of violence during the apartheid era (1). Exposure to stressors such as oppression, racial discrimination, and social inequality has resulted in psychological distress and other mental health issues (1, 2). Despite these challenges, only 27% of patients experiencing severe mental illnesses seek treatment (3). Consequently, the magnitude of the past traumatic experiences on the parents and their progeny is less understood. Furthermore, it is not clear whether the effects of the posttraumatic experiences can be transferred to the offspring.

Chronic stress has been associated with numerous psychiatric disorders, including anxiety and depression (4). The serotonergic system is a key player in modulating social behaviour and depression (5). It has also been suggested that augmented serotonin concentration exacerbates anxiety and amygdala sensitivity in social anxiety (5-7). Serotonin, which is detectable from seven weeks in human foetuses is vital for the development of the placenta and embryo, as well as foetal and postnatal brain and cardiovascular maturation in humans and animals (8). Hence, it is conceivable that alterations in the foetal serotonergic homeostasis due to prenatal stress can have sustained physiological ramifications in adulthood (9). An increased synthesis rate and reuptake may be responsible for the heightened serotonergic function in sociability and anxiety (5). Furthermore, increased cortisol levels decrease the functioning of serotonin in the brain which can lead to a depressive state (10). Permanently increased cortisol concentrations had been linked to low birth weight and depression during adulthood (11). Depression is a common comorbidity of anxiety, seizures, cancer, dementia, Parkinson's disease and pain (12, 13). Exposure to chronic stressors induces dysfunctions in the endocrine and immune system resulting in a permanent low-grade inflammation which has been associated with depression-like behaviour (14, 15). The perinatal life is especially sensitive to stressors; therefore, aggressive activity can elicit changes in behavioural and physiological mechanisms (16, 17). In major depressive disorder, there is an augmented dopamine secretion which activates homeostatic mechanisms (18). These include the overexpression of postsynaptic dopamine receptors and reducing dopamine transporter intensity, which together raise dopamine signal transduction ensuing from amphetamine-induced dopamine secretion into the synapse (18). Animal studies have shown that exposure to acute stressors stimulates the entire dopaminergic system targeting the striatum, particularly the dorsal striatum where object prominence is vital.

On the other hand, exposure to chronic stressors blunts the neurons in the ventromedial striatum, where reward processing is important (19).

The long-term effects of prenatal stress are unspecified however, literature suggests that prenatal exposure to stress can affect the progression, performance and sensitivity of the stress system (1, 20). Elevated cortisol during pregnancy, enters maternal circulation, cross the placental barrier, and influence the gestational environment and the physiological development of the offspring's stress response system (1, 20). Increased neonatal cortisol exposure can permanently alter the growth and physiological sensitivity of the foetal systems such as, the hypothalamic–pituitary–adrenal axis, immune response, and cognitive functioning development (1). These effects depend on the type of stressor, the time during the gestational period, the sex of the foetus and, the stage of foetal brain development (21). Foetal brain development occurs through three phases namely, embryonic period, early foetal period, and late foetal period (22). Therefore, the foetal brain is continuously maturing throughout the gestational period and its development can be affected by any biological signal it receives (21, 23). Whilst many studies are investigating these lasting effects of maternal prenatal stress on infant, child, adolescence and adulthood, there is a paucity of information on the long-term effects of paternal stress on the offspring.

The present study sought to investigate the effects of parental stress on the offspring's psychiatric behaviour, particularly the fathers, and whether these can be transferred to offspring or if they can affect the offspring psychiatric/psychological behaviour. This was executed by evaluating the onset of anxiety, depression, sociability in the offspring through behavioural observations and the assessment of neurochemical markers.

## 2. METHODOLOGY

### 2.1 Materials

All chemicals and reagents used were of analytical grade and were purchased from standard commercial suppliers.

### 2.2 Animals

Sixteen male and eight female Sprague Dawley rats, each weighing between 250 - 300 g, were obtained from the Biomedical Resource Centre of the University of KwaZulu-Natal. The rats

were moved to a new room and housed under standard laboratory conditions of ± 22°C room temperature, 70% humidity and a 12-hr light/dark cycle (lights on at 06h00, off at 18h00), food and water were available ad libitum. The rats were housed in standard conventional polycarbonate 1291H tecniplast (type III) cages (425 x 266 x 185 mm, floor space: 80 cm2). The rats were separated into four groups and allowed to acclimatise for one week. The experimental protocols and procedures performed in this study were approved by the Animal Research Ethics Committee (AREC) of the University of KwaZulu-Natal (AREC/024/020M)

**2.3 Experimental Design**

Chronic stress was induced in parents using the chronic restraint stress protocol (24-26), detailed below. The rats were first separated into non-stressed and stressed groups, i.e., non-stressed mother (NS-M) and stressed mother (S-M) and non-stressed father (NS-F) and stressed father (S-F). After that, they were assigned to the following groups, each group containing four males and two females as follows: (1) control (C) group: non-stressed mother and father (NS-M + NS-F) (n =6); (2) non-stressed mother and stressed father (NS-M+S-F) (n =6); (3) stressed mother and non-stressed father (S-M+NS-F) (n =6); and (4) stressed mother and stressed father (S-M+S-F) (n =6). Following acclimation, the S-F were exposed to chronic restraint stress protocol for 1 hr over seven days. Succeeding the stress period, the animals were mated according to their groups. On gestational day (GND) 14, the S-M group was subjected to the chronic restraint stress protocol for 1 hr over seven days.

On GND 14, the S-F groups were again exposed to the chronic stress protocol, and then the NS-F and S-F were exposed to the elevated plus maze (EPM). The fathers were sacrificed 24 hrs after the behavioural test to collect the amygdala to measure serotonin concentration. Following birth, the pups were weaned off their mothers on postnatal day (PND) 22, and on PND 23, they were exposed to behavioural tests. The pups were scored on the sucrose preference test, EPM and the social novelty test. The pups were sacrificed using a sharpened guillotine 24 hrs after the last behavioural test. Hippocampal and amygdala tissue were collected to measure dopamine and serotonin concentration. All samples were immediately snap-frozen after extraction using liquid nitrogen and then stored in a bio freezer at -80°C until neurochemical analysis was performed.

## 2.4 Paternal chronic stress protocol

The fathers were equally divided into stressed fathers (S-F) (n=8) and non-stressed fathers (NS-F) (n=8). The S-F were exposed to daily chronic restraint stress before mating. The S-F was taken to a separate room and placed in rodent restrainers for one hr between 11 am and 12 pm, for a total of 7 days (24, 27). After the stress protocol for each day had concluded, the S-F were returned to their home cages, and the restrainers were washed and sanitised with 70% ethanol to prevent odour signal interference in the behaviour of the animals. The NS-F remained undisturbed in their home cages.

## 2.5 Mating

The mothers were equally divided into stressed mothers (S-M) (n=4) and non-stressed mothers (NS-M) (n=4). The mothers were paired and allowed to acclimatise for one week to minimise stress and synchronise their oestrous cycles. Following this, vaginal smears were performed daily to assess the females' oestrous cycle, and once in pro-oestrous, a male rat was introduced until mating was successful (28). Vaginal smears were performed to determine if mating was successful by identifying the presence of sperm. The male was moved to its home cage upon positive confirmation, and this marked gestational day (GND) 0.

## 2.6 Maternal Chronic prenatal stress protocol

On GND 14, the pregnant S-M rats were exposed to chronic restraint stress daily. The S-M were exposed to the same chronic restraint stress protocol as the fathers. The stress paradigm occurred on GND 14 as this is the age when neural structure development begins in a foetal brain (29).

## 2.7 Paternal testing and tissue collection

Following successful mating, the S-F groups were again exposed to the same chronic stress protocol to confirm that the fathers were indeed stressed. The NS-F groups remained undisturbed in their home cages throughout this period. After that, the NS-F and S-F were exposed to the EPM to monitor their anxiety (protocol detailed below).

### *2.7.1 Paternal tissue collection*

The fathers were sacrificed 24 hrs after the behavioural test and were taken to the autopsy room 1 hr before decapitation. The fathers were decapitated using a sharpened guillotine, and the

amygdala was collected into 2 ml Eppendorf tubes. All tissue samples were weighed before snap freezing in liquid nitrogen and stored in a bio freezer at -80°C for biochemical analysis.

## 2.8 Postnatal Behavioural Tests

### *2.8.1 Elevated Plus Maze (EPM)*

The apparatus comprised of a maze with two open (50×10 cm) and two enclosed (50x10 cm) arms. The arms radiated from a 10 cm central square. The entire apparatus was elevated by 50 cm off the floor. The fathers were exposed to the EPM 24 hrs after the first chronic restraint stress protocol, and offspring on PND 59. Briefly, a single animal was placed at the junction of the open and closed arms and left to explore the maze for 5 mins. Anti-anxiety behaviour was indicated by more time being spent in the open arms than the closed arms, whilst anxiety-like behaviour was indicated by more time spent in the closed arms of the maze than the open arms (30). An entry was defined only when all four paws of the animal were in the arm. After each session, the maze was cleaned with 70% ethanol in distilled water to prevent odour signal interference in the behaviour of the animals. The time spent in the open and closed arms were recorded and the footage uploaded to the BORIS software (version 7. 10. 7) (31). This behaviour was scored by third-party observants blinded to the study protocol.

### *2.8.2 Sucrose Preference Test (SPT)*

To investigate anhedonia and depression-like behaviour, the pups were exposed to the sucrose preference test on PND 60, using an established protocol with minor modifications (32, 33). Briefly, the pups were habituated over four days, with two bottles of pure water available on days 1 and 2, two bottles of 1% sucrose on day 3, and one bottle of pure water and one bottle of 1% sucrose on day 4. The positions of the two bottles were switched daily to reduce any bias elicited by familiarity. After 12 hrs of food and water deprivation, the pups were separated (one per cage) and presented with 200 mL of pure water and 200 mL of 1% sucrose solution. The quantities of pure water and sucrose consumed were recorded after 12 hrs. According to the equation below, the sucrose preference (SP) was calculated as a percentage of the volume of sucrose intake over the total volume of fluid intake (32).

$$SP = \frac{sucrose\ solution\ (g)}{Sucrose\ solution\ (g) + water\ (g)} \times 100$$

### *2.8.3 Social Novelty Test*

The social novelty test was used to assess the animals' preference for sociability on PND 61 (34, 35). The pups were transferred to a behavioural room 1 hr before the testing for the habituation period. The apparatus used in this test was divided into three chambers (100 × 30 × 30 $cm^3$), one central and two lateral, with wire cups placed on the lateral chambers. The subject rat was placed in the centre compartment to habituate for 5 min and roam the three chambers freely. After that, the subject was removed, and the novel rat was placed inside a wire cup in one of the side chambers, and a familiar rat was placed inside a wire cup in the corresponding chamber. The subject rat was allowed free access to explore all three chambers. After each trial, the chambers were cleaned with 70% ethanol in distilled water to prevent odour signal interference in the behaviour of the animals. The placement of the "stranger" and "familiar" rats was alternated between the left and right lateral sides of each run to eliminate the element of bias by the subject rat. The time spent in each compartment with the novel rat, familiar rat or alone was recorded for 5 min, and the footage was uploaded to BORIS (version 7. 10. 7) (31). To represent inquisitorial behaviours, we noted the total time spent in each chamber, the number of physical interactions with the wire cups and the total time spent with each cup by the subject (36). The behaviour was scored by third-party observants blinded to the study protocol. To evaluate the subject's preference for sociability, we calculated the social preference index (SPI) in the following manner (36):

$$SPI = \frac{time\ spent\ exploring\ novel\ rat - time\ spent\ exploring\ familiar\ rat}{time\ spent\ exploring\ novel\ rat + time\ spent\ exploring\ familiar\ rat}$$

## 2.9 Offspring tissue collection

On PND 30, 24 hrs after the behavioural tests, the pups were taken to the autopsy room and left to acclimatise for 1 hr before decapitation. The pups were decapitated using a sharpened guillotine. The hippocampal and amygdala tissue were collected into 2 ml Eppendorf tubes. All tissue samples were weighed before snap freezing in liquid nitrogen and stored in a bio freezer at -80°C for biochemical analysis.

### *2.9.1 Biochemical Analysis*

Amygdala serotonin concentration from both the fathers and pups was measured using the enzyme-linked immunosorbent assay (ELISA) kit (DEE8900) (Kiel, Germany) according to

the manufacturer's protocol. Hippocampal dopamine concentration from the pups was measured using the ELISA kit (E-EL-0046) (Elabscience, Wuhan, China) according to the manufacturer's procedure.

### 2.10 Analysis of data

All data analyses were performed with GraphPad Prism version 7 (GraphPad Software Inc., California, USA) using the two-way analysis of variance (ANOVA) test followed by the Tukey-Kramer *post hoc* comparison test. An n=8 for offspring analysis and n=4 for paternal analysis were assessed per group. $p < 0.05$ was considered statistically significant. All Data are expressed as the mean ± standard error of the mean (SEM).

## 3. RESULTS

The EPM was employed to evaluate the degree of anxiety in the fathers and offspring, and the time spent in the open arms and closed arms were used to assess their anxiogenic behaviour. The sucrose preference test was used to investigate depression-like behaviour in offspring, by monitoring the quantity of pure water and sucrose consumed. The social novelty test was used to assess the offspring preference for sociability by recording the time spent with a novel rat, familiar rat or alone.

### 3.1 EPM – fathers

There was a difference between the time spent in the open and closed arms of the EPM, which was measured to assess paternal anxiety ($F_{(1, 20)} = 542.8$, $p<0.0001$, Figure 1a). There was a paternal stress effect on time spent in the closed arms in the S-F group compared to the NS-F group $^{\beta}$(S-F closed arms vs NS-F closed arms, $p<0.0001$, Figure 1).

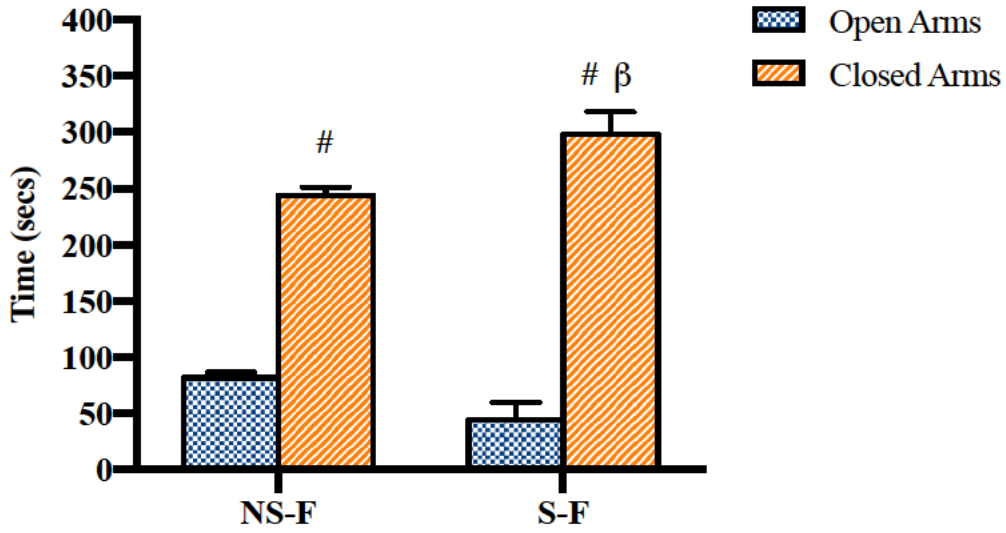


Figure 1: The anxiety levels of non-stressed and stressed from the Control, NSM-SF, SM-NS-F and SM-SF groups. A comparison of time spent in the open and closed arms of fathers and offspring as per the elevated plus-maze. $^{\#}p<0.05$ when compared to open arms; $^{\beta}P<0.05$ when compared to NS-F closed arms.

### 3.2 EPM – offspring

There was a significance between the time spent in the open and closed arms of the EPM, which was measured to assess offspring anxiety ($F_{(1, 34)} = 1543, p < 0.0001$). The offspring had a maternal stress effect on time spent in the open arms in the SM-NSF group compared to the control group $^{*}$(SM-NSF open arms vs Control open arms, $p<0.05$, Figure 2). In addition, there was an offspring stress effect on the time spent in the closed arms in the SM-NSF and SM-SF groups in comparison to the control group $^{\alpha}$(SM-NSF closed arms vs Control closed arms, $p<0.05$, Figure 2), $^{\alpha}$(SM-SF closed arms vs Control closed arms, $p<0.05$, Figure 2).

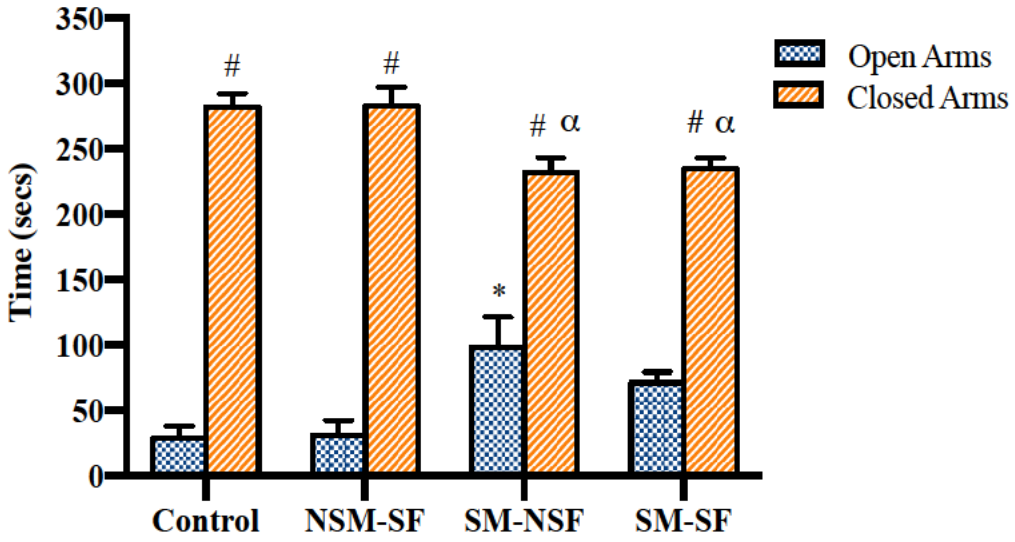


Figure 2: The anxiety levels of offspring from the Control, NSM-SF, SM-NS-F and SM-SF groups. A comparison of time spent in the open and closed arms of offspring as per the elevated plus-maze. *P<0.05 when compared to Control-open arms; $^{\alpha}$ p<0.05 when compared to Control-closed arms.

### 3.3 Social novelty test- offspring

#### *3.3.1 Sociability*

There was a difference in the time spent with the novel rat, familiar rat or alone during the social novelty test, which was measured to assess offspring sociability ($F_{(3,36)} = 76.09$, p<0.0001). There was an offspring sociability effect for the novel rat in the SM-NSF and SM-SF groups when compared to the control group $^{\#}$(SM-NSF novel rat vs Control novel rat, p<0.0001, Figure 3), $^{\#}$(SM-SF novel rat vs Control novel rat, p<0.0001, Figure 3). There was a maternal stress effect on the sociability in the offspring on time spent with the familiar rat in the SM-NSF and SM-SF groups when compared to the NSM-SF group $^{\alpha}$(SM-NSF familiar rat vs NSM-SF familiar rat, p<0.05, Figure 3), $^{\alpha}$(SM-SF familiar rat vs NSM-SF familiar rat, p<0.0001, Figure 3). Additionally, there was a maternal stress effect on the sociability in the offspring on time spent with the novel rat in the SM-NSF and SM-SF groups when compared to the NSM-SF group $^{\beta}$(SM-NSF novel rat vs NSM-SF novel rat, p<0.0001, Figure 3), $^{\beta}$(SM-SF novel rat vs NSM-SF novel rat, p<0.0001, Figure 3).

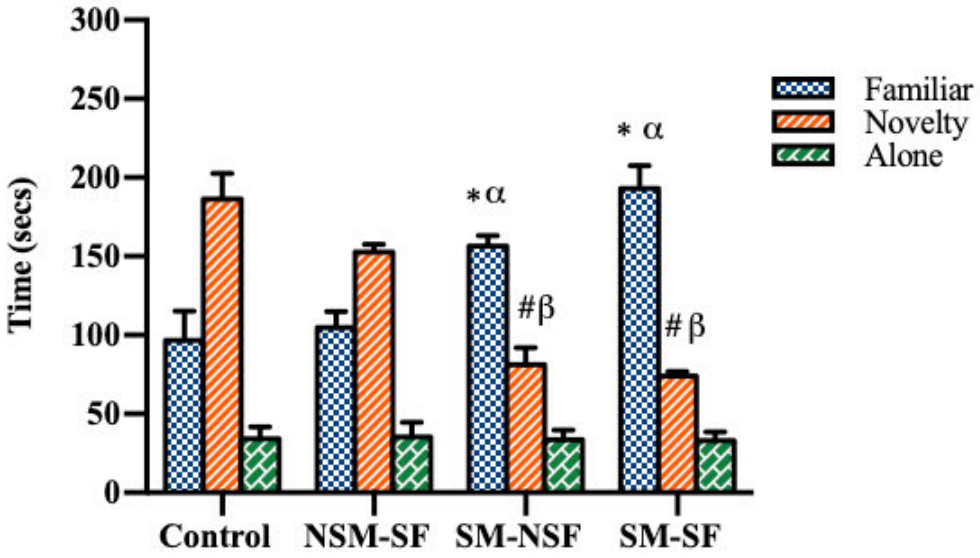


Figure 3: The sociability preference of offspring from the Control, NSM-SF, SM-NS-F and SM-SF groups. A comparison of the time spent by offspring with a familiar rat, novel rat or alone as per the social novelty test. *p<0.05 when compared to Control familiar rat; $^{\#}$p<0.05 when compared to Control novel rat; $^{\alpha}$p<0.05 when compared to NSM-SF familiar rat; $^{\beta}$p<0.05 when compared to NSM-SF novel rat.

*3.1.2 Social novelty preference*

There was a difference in the time spent with the novel rat or familiar rat within each group (Table 1) and the SPI (Figure 3) during the social novelty test, which were measured to assess offspring sociability ($F_{(3,36)} = 76.09$, p<0.0001). There was an offspring sociability effect between the novel rat and the familiar rat within the groups $^{\#}$(Control-novel rat vs Control-familiar rat, P<0.0001, Table 1), $^{\#}$(NSM-SF novel rat vs NSM-SF familiar rat, p<0.05, Table 1), $^{\#}$(SM-NSF novel rat vs SM-NSF familiar rat, p<0.0001, Table 1) and $^{\#}$(SM-SF novel rat vs SM-SF familiar rat, p<0.0001, Table 1). Indeed, the preference index shows that there was an offspring effect on the preference for novelty in other groups when compared to the control group *(NSM-SF vs Control, p<0.0001, Figure 4), *(SM-NSF vs Control, p<0.0001, Figure 4), *(SM-SF vs Control, p<0.0001, Figure 4). There was also an offspring effect on the preference for novelty in the SM-NSF and SM-SF groups when compared to the NSM-SF group $^{\alpha}$(SM-NSF vs NSM-SF p<0,05, Figure 4), $^{\alpha}$(SM-SF vs NSM-SF p<0.0001, Figure 4). There was a parental stress effect on the sociability in the offspring on the preference for novelty in the SM-SF group compared to the SM-NSF group $^{\beta}$(SM-SF vs SM-NSF p<0,05, Figure 4).

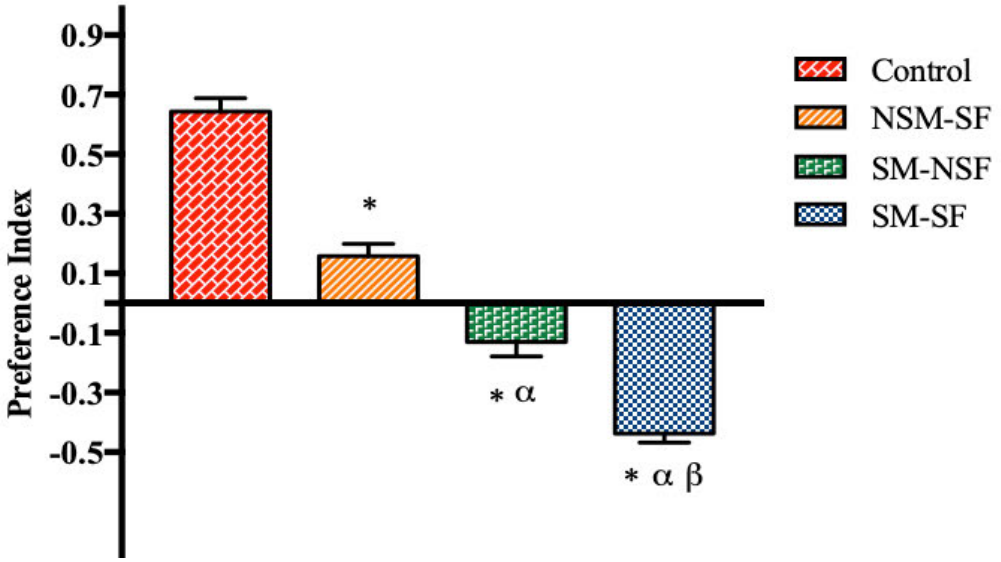


Figure 4: The SPI of offspring from the Control, NSM-SF, SM-NS-F and SM-SF groups. A comparison of the time spent by offspring with a familiar rat, novel rat or alone as per the social novelty test. *p<0.05 when compared to Control; $^{\alpha}$p<0.05 when compared to NSM-SF; $^{\beta}$p<0.05 when compared to SM-NSF.

**Table 1:** Time spent by offspring with a familiar rat or novel rat within each group (n=8, per group).

| **Group (n=8)** | **Interaction (secs)** | |
|---|---|---|
| | **Familiar Rat** | **Novel Rat** |
| **Control** | 101 ± 25,3 | 168 ± 28,5$^{\#}$ |
| **NSM-SF** | 92,3 ± 11 | 133 ± 13,4$^{\#}$ |
| **SM-NSF** | 96,6 ± 12,8 | 163 ± 28,4$^{\#}$ |
| **SM-SF** | 99,4 ± 10,8 | 177 ± 15,2$^{\#}$ |

Values are presented as means ± SEM. $^{\#}$p<0.05 when compared to familiar rat.

### 3.4 Sucrose preference test – offspring

There was a difference in water and sucrose intake between the groups (Figure 4) ($F_{(3, 46)}$ = 13.39, p<0.0001) and the sucrose preference (Table 2) within the groups, which was measured to assess offspring anhedonia ($F_{(3, 18)}$ = 15,11, p<0.0001). There was a control group and SM-NSF group effect on fluid consumption *(Control-SI vs Control-WI, p<0.0001, Table 2), *(SM-NSF SI vs SM-NSF WI, p<0.0001, Table 2). There was a prenatal stress effect on offspring's water intake in the SM-NSF WI and SM-SF WI when compared to the control WI

[#](SM-NSF WI vs Control WI, p<0.05, Table 2), [#](SM-SF WI vs Control WI, p<0.05, Table 2). Similarly, we noted a maternal effect in the offspring preference for sucrose in the NSM-SF SI and SM-SF SI groups when compared to the SM-NSF SI groups [α](NSM-SF SI vs SM-NSF SI, p<0.0001, Table 2), [α](SM-SF SI vs SM-NSF SI, p<0.0001, Table 2). Indeed, the sucrose preference test shows that there was an offspring effect on the preference for sucrose in the NSM-SF AND SM-SF groups when compared to the control group [β](NSM-SF vs Control, p<0.05, Figure 5), [β](SM-SF vs Control, p<0.05, Figure 5). There was also a maternal stress effect in the offspring on the preference for sucrose in the SM-NSF group compared to the NSM-SF [∞](SM-NSF vs NSM-SF, p<0.05, Figure 5). There was a parental stress effect in the offspring on the preference for sucrose in the SM-SF group compared to the SM-NSF group [▽](SM-SF vs SM-NSF, p<0 05  Figure 5).

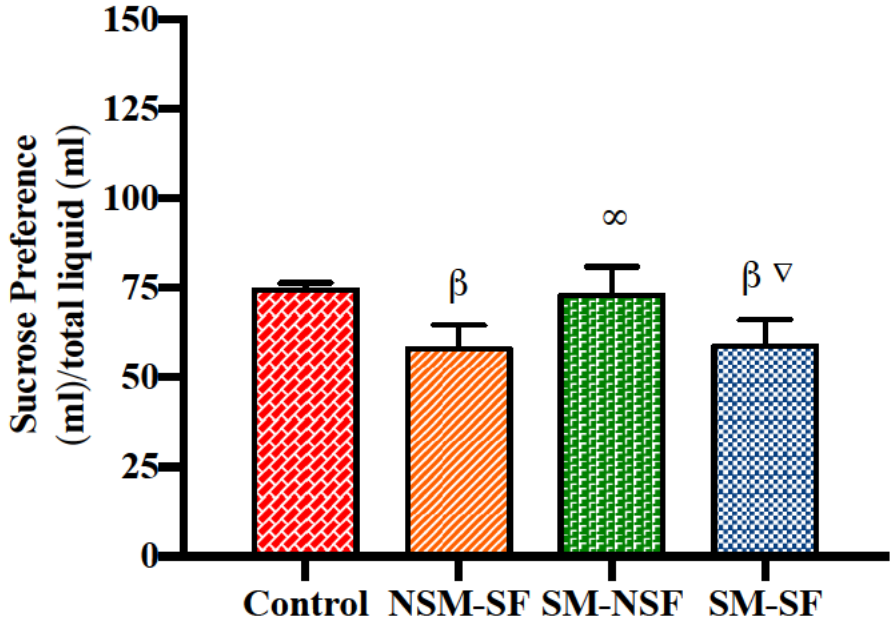


Figure 5:  The sucrose preference of offspring from the Control, NSM-SF, SM-NS-F and SM-SF groups. A comparison of the offspring's consumption of water, sucrose and sucrose preference as per the sucrose preference test. [β]p<0.05 when compared to Control; [∞]p<0.05 when compared to NSM-SF; [▽]p<0.05 when compared to SM-NSF.

**Table 2:** Offspring consumption of sucrose or water within each group (n=8, per group).

| Group (n=8) | Consumption (ml) | |
|---|---|---|
| | **Water Intake (WI)** | **Sucrose Intake (SI)** |
| **C** | 1,75 ± 0,16 | 5,13 ± 0,35* |
| **NSM-SF** | 7,63 ± 1,00 | 4,38 ± 0,71$^{\alpha}$ |
| **SM-NSF** | 2,75 ± 0,70$^{\#}$ | 8 ± 1,05* |
| **SM-SF** | 4,5 ± 1,77$^{\#}$ | 4,38 ± 0,53$^{\alpha}$ |

Values are presented as means ± SEM. *p<0.05 when compared to WI; $^{\#}$p<0.05 when compared to Control-WI; $^{\alpha}$p<0.05 when compared to SM-NSF.

### 3.5 Serotonin concentration in fathers

The amygdala serotonin concentration as above, was measured to assess paternal anxiety ($F_{(5, 6)}$=6,129, p<0.05). There was a stress effect on amygdala serotonin concentration in the fathers *(S-F vs NS-F, p<0.0001, Figure 6).

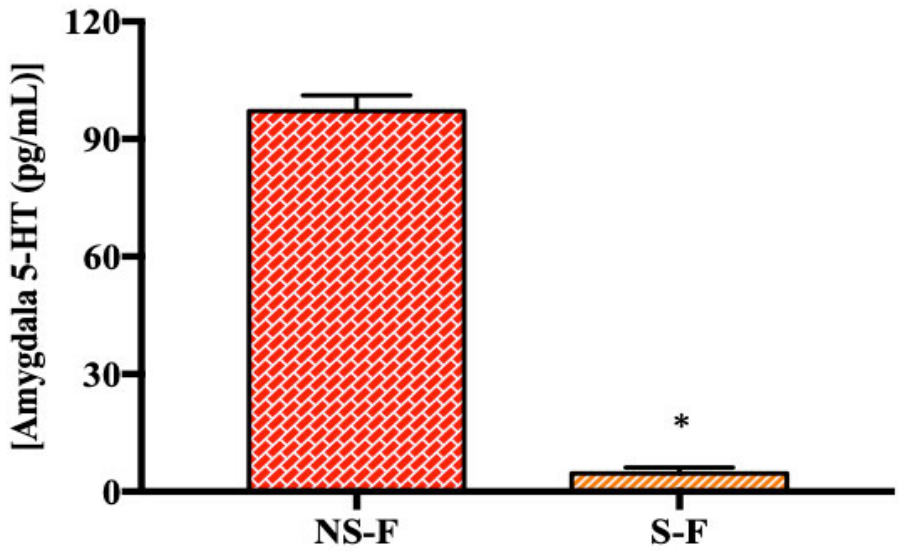


Figure 6: Amygdala serotonin concentration in NS-F and S-F 24 hours following EPM activity. *p<0.05 when compared to NS-F.

### 3.6 Serotonin concentration in offspring

The amygdala serotonin concentration, as above was measured to assess offspring anxiety ($F_{(3, 19)} = 23.67, p<0.0001$). There was a SM-SF group effect on serotonin concentration compared to the control group *(SM-SF vs Control, $p<0.0001$, Figure 7). There was a prenatal stress effect in the offspring on the amygdala serotonin concentration when compared to NSM-SF group $^{\#}$(SM-NSF vs NSM-SF, $p<0.05$, Figure 7), $^{\#}$(SM-SF vs NSM-SF, $p<0.0001$, Figure 7). There was also a SM-SF stress effect in the offspring amygdala serotonin concentration when compared to SM-NSF group $^{\alpha}$(SM-SF vs SM-NSF, $p<0.05$, Figure 7).

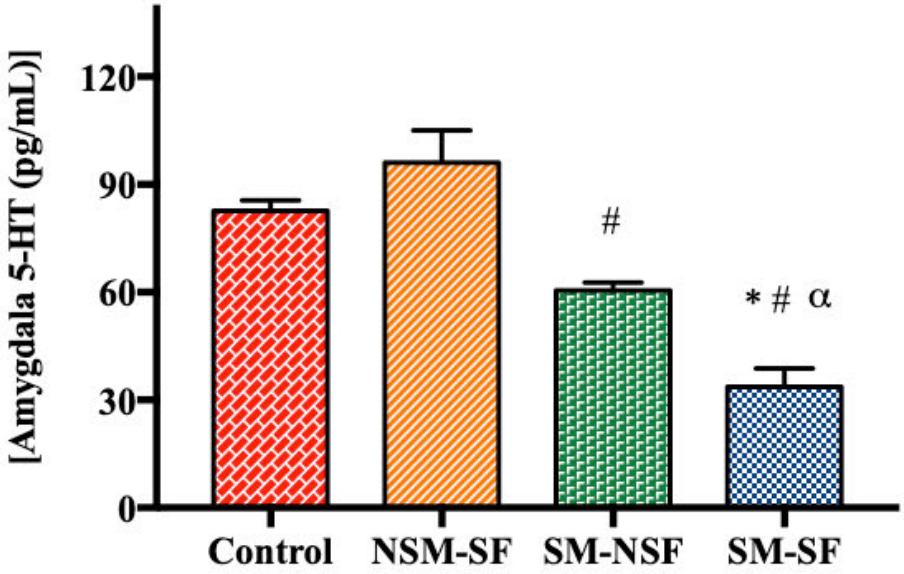


Figure 7: Amygdala serotonin concentration in offspring from the Control, NSM-SF, SM-NS-F and SM-SF groups.*$p<0.05$ when compared to Control; $^{\#}p<0.05$ when compared to NSM-SF; $^{\alpha}p<0.05$ when compared to SM-NSF.

### 3.7 Dopamine concentration in offspring

The hippocampal dopamine concentration was measured to assess offspring depression ($F_{(3, 20)} = 0.1721, p<0.05$). No stress effect on dopamine concentration in the Control, NSM-SF, SM-NSF and SM-SF groups was found (Figure 8).

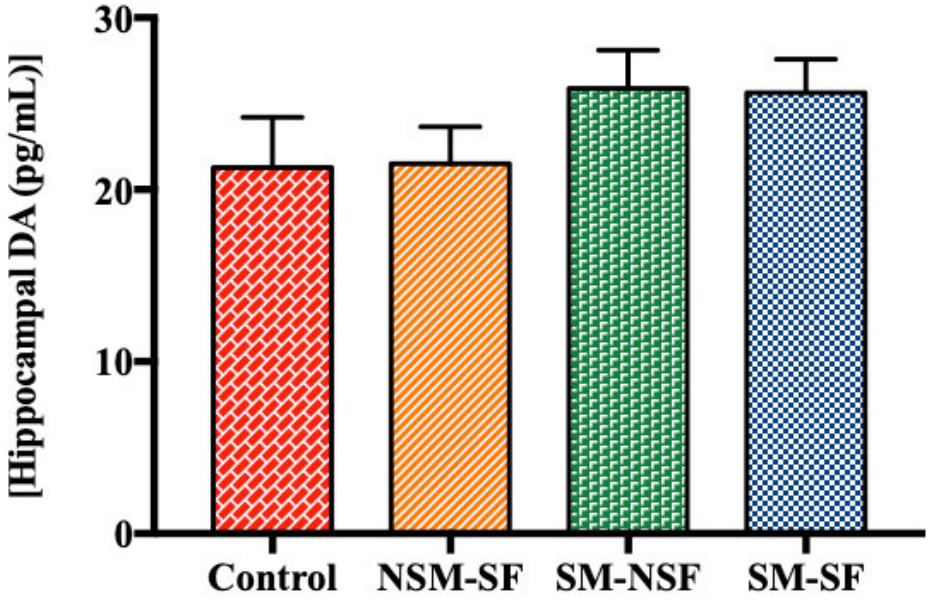


Figure 8: Hippocampal dopamine concentration in offspring from the C, NSM-SF, SM-NS-F and SM-SF groups.

## 4. DISCUSSION

There is enough literature linking maternal prenatal stress to alterations in offspring behavioural, psychiatric and neurological modifications (1, 37). These are associated with the onset of anxiety, depression, impaired memory, sociability, elevated serotonin and cortisol and dysfunctional physiological systems like the HPA axis (1, 37, 38). In addition, to these effects, studies on South Africans and other population groups have shown that stressors and past traumas, especially during early development, can durably impact psychological health and behaviour (1). However, they have not examined the contribution of paternal traumas to adaptations in offspring behavioural, psychiatric and neurological modifications. These findings were matched to changes in brain neurochemistry. Altogether, our findings show that the stressed fathers did not transfer their anxiogenic behaviour however, they did affect sociability and anhedonia. This was mediated in part by decreased serotonin concentration in the offspring. These findings were more prominent when S-F were mated with S-M.

The behaviour observed in the fathers during the elevated plus maze test following exposure to chronic restraint stress was customary of anxiogenic demeanour. This was confirmed by the time spent in the closed arms of the EPM by the S-F when compared to the NS-F. This behaviour signifies the S-F preference for exploring protected areas rather than novel

environments thereby confirming that the fathers were anxious (30). The lack of anxiogenic behaviour observed in the S-F offspring was noted and could in part be due to the intensity of the stress paradigm employed on the fathers which didn't reflect in their offspring. Anxiety is portrayed by increased fear and apprehension; however, this can also be accompanied by a withdrawal from common behaviours and other physiological effects (39). Stressing the mothers during pregnancy resulted in an increase in anxiogenic effects in the offspring, evident by a tendency of the offspring to spend more time in the closed arms when compared to the open arms of the elevated plus maze. A rat animal model has shown that prenatal stress induced signs of anxiety-like behaviour (40). Likewise, an upregulation of glucocorticoid receptor influences anxiety and depression-like behaviours in the elevated plus maze (41). Our EPM results further support previous results which show that prenatal and postnatal stress models have shown a tendency for rats to spend more time in the closed arms, indicating anxious behaviour (38, 42). Furthermore, it has been documented that early life adversities are linked to prolonged and detrimental changes to the individual's hypothalamic–pituitary–adrenal axis (43, 44), which can impact the ability to explore unfamiliar environments (26, 45). Therefore, when mothers are stressed during pregnancy the resultant anxiogenic effects are transferred to the offspring.

Social anxiety was observed in offspring from the SM-NSF and SM-SF groups after exposure to the social novelty test. This was confirmed by SM-NSF and SM-SF pups' preference for engaging with a familiar rat rather than the novel rat when compared to the NSM-SF group. This agrees with studies that have reported behavioural adaptability in chronically stressed rodents (46-48). Furthermore, our findings agree with the results of a different study which reported a reduction in social interaction of prenatally stress rats (49). Exposure to chronic stress has been linked to a reduced motivation for social engagements, which is customary in depressed patients (50, 51). Our results suggest that the offspring of stressed parents attenuated social novelty behaviour, which requires recognition memory. The animals' ability to distinguish familiar from unfamiliar denotes social recognition (52), which is fundamental in novelty-seeking behaviour (46). The preference index value suggests, that on their own prenatally stressed parents are able to affect their offspring's sociability, but this effect is exacerbated when the S-F are mated with S-M. These findings infer that as the number of stressed parents increase, the desire for sociability decreases. This was confirmed by the lower preference for a novel rat when compared to the offspring from the NSM-SF group and SM-

NSF group. Our results concur with previous studies which show that increasing the duration or intensity of chronic restraint stress impairs the animals' sociability (51, 52). This validates our results because the offspring that experienced the lowest sucrose preference index were the progeny of equally stressed parents and therefore, had a higher stress factor. Therefore, we speculate that offspring from prenatally stressed fathers have an apparent aversion to sociability.

The anxiogenic behaviour exhibited by S-F were confirmed by lower serotonin concentration in the amygdala found in the S-F when compared to NS-F. The anxiogenic behaviour exhibited by pups was also confirmed by a decreased serotonin concentration in the amygdala of pups from the SM-NSF and SM-SF. Our findings agree with other studies which reported that prenatal stress selectively decreased serotonin levels (53). Systemic depletion of serotonin, reported in patients with emotional disorders, has been shown to attenuate glutamate activity in the amygdala and exacerbate fear behaviours (54, 55). Furthermore, following a decrease in serotonin, animal models have reported anxiety-like behaviours (56). Therefore, we speculate that offspring from prenatally stressed parents were more susceptible to feelings of fear and anxiety than the NSM-SF offspring. The social anxiety exhibited by the pups were confirmed by lower serotonin concentration in the amygdala of pups from the SM-NSF and SM-SF when compared to NSM-SF. This suggests that offspring from the SM-NSF and SM-SF were predisposed to anxious behaviour when compared to the NSM-SF offspring. Our results agree with a study conducted by Frick et al. which reported an increased serotonin synthesis in patients with social anxiety using neuroimaging of the amygdala when compared to controls (5). Therefore, we speculate that offspring exposed to early life stress present with dysfunctional neural activity in the amygdala, which plays a key role in modulating anxiety (5). For that reason, prenatally stressed parents could have, in part, attenuated serotonin concentration in offspring which exacerbated the onset of social anxiety.

Our sucrose preference test results showed that S-F increased the occurrence of offspring anhedonic behaviour, which is a primary symptom of depression (57). These offspring preferred water over sucrose, which indicates their neutrality to pleasurable activities, in contradiction to offspring from S-M who preferred sucrose. These findings agree with literature which showed that stressed rodents have a lower preference for sucrose than unstressed (53, 58). From these results, we speculate that prenatally stressed fathers exacerbated feelings of

anhedonia in offspring. Paradoxically, higher sugar consumption has also been linked to an increased prevalence of depression in many cross-sectional studies (59-62). Since literature has already reported an association between maternal prenatal stress and risk for depression in offspring (63-66), we do not discredit this result. Our findings instead relate to another cross-sectional study that investigated if there is a positive association between prevalent mood disorders and sugar intake. This study concluded that sugar intake has unfavourable effects on psychological stability, and a lower sugar intake may lead to better psychological well-being (59). Early life adversities are toxic chronic stressors, that result in sustained psychological and biological changes to the body which increase the likelihood of developing depression (15, 67). Therefore, we further speculate that prenatally stressed parents could have, in part, exacerbated the anhedonic behaviour in their offspring.

The anhedonia exhibited by the pups were confirmed by measuring the concentration of dopamine in the hippocampus. Anhedonia has been associated with an altered dopamine reward system which is accompanied by a decrease in dopamine release and lower dopamine binding in depressed patients (62). Our discrepant findings show that there were no significant differences in the hippocampal dopamine concentrations between offspring. Our findings are likely to reflect fluctuations in the stress paradigm used, as exposure to mild stressors intensifies dopaminergic action whilst chronic stressors attenuates dopaminergic action (19). Therefore, we speculate that these discrepant findings are likely a result of the stress paradigm employed and as such, there was minimal effect found in the neural circuitry. We recommend that a follow-up study be conducted to investigate the role of dopamine sensitivity in prenatally stressed parents and in offspring exposed to early life adversities.

Altogether, the behavioural and neurochemical manifestations in the offspring of prenatally stressed fathers suggest that S-F can transfer feelings of anhedonia and social anxiety to their offspring. This was mediated, in part, by offspring behavioural changes of depression and social anxiety as well as, a blunted serotonin response. Furthermore, when both parents are prenatally stressed their stress effect to their offspring's behavioural and neurochemistry is augmented. This was confirmed by the behavioural manifestations of extreme anxiety, depression and social anxiety as well as, the subdued serotonin concentration. Our study adds to the literature on the effects that S-F infer to offspring, which has not been comprehensively explored. It is recommended, that psychological succour be given to fathers and expectant

parents be screened for markers of psychological behaviours to protect their foetus. These findings warrant further investigation into causative mechanisms. Additionally, it is recommended that this study be modelled to investigate the long-term effects of traumatic experiences across multiple generations to, using a larger subset.

## 5. CONCLUSION

Altogether, our findings show that the prenatally stressed fathers did not transfer their anxiogenic behaviour however, they decreased the offspring's sociability and increased anhedonia. This was mediated in part by decreased amygdala serotonin concentration in the offspring. These findings were more prominent when prenatally stressed fathers were mated with prenatally stressed mothers, which yielded an additive effect. Our findings, are beneficial when understanding the relationship between historic human experiences and current societal problems, specifically how this contributes to the pressures and consequences on the current generation. We therefore, suggest that this study be used to further investigate the evolution of historical traumas through multiple generations to understand the mechanisms behind behavioural and neurological behaviours, which will successively advance therapeutic strategies, and minimise the burden of mental illness on society.

## 6. DECLARATION

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### Author Contributions

Miss Malishca Perumal: Project development, Animal work, Data collection, Data analysis, Manuscript writing and editing.

Dr Mluleki Luvuno: Project development, Data analysis, Manuscript editing.

Prof Musa Mabandla: Project development, Data analysis, Manuscript editing.

**Conflict Of Interest**

The authors declare no conflicting interests.

**Ethical Approval**

Ethical approval was obtained from the Animal Research Ethics Committee (AREC/024/020M) of the University of KwaZulu-Natal.

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# CHAPTER 3

## Prologue

## Manuscript 2

Chapter 2 provided a scientific manuscript that investigated the influences of parental stress on offspring neurochemistry and behaviour, by documenting the effects on offspring anxiety, depression and sociability as well as defined the clinical relevance of this study.

**"Paternal prenatal stress alters offspring metabolism and stress regulation"**

## Contributions of this chapter

This chapter is comprised of a scientific manuscript that investigated the influences of parental stress on offspring neurochemistry and behaviour, by documenting the effects on offspring body weight, feeding behaviour and stress response as well as described the paternal contributions to these manifestations. The results of this study were compared across parental groups to determine statistically significant relationships.

Note: This chapter has been prepared according to the guidelines outlined by PONTE Journal (Appendix D).

**Paternal prenatal stress alters offspring metabolism and stress regulation**

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**ABSTRACT**

Studies have shown that the perinatal environment plays a critical role in the development and growth of offspring. However, there remains a paucity in literature on the effects a stressed father confers to the offspring's metabolic, behavioural and neurochemical systems. The present study sought to investigate the influence of chronic parental stress on offspring metabolism and stress regulation. To achieve this, male and female Sprague Dawley rats, weighing 250 – 300 g, were assigned to one of the following four groups (n=6 per group) viz: 1) control, (2) non-stressed mother and stressed father, (3) stressed mother and non-stressed father, and (4) stressed mother and stressed father. The fathers were subjected to a 7 day chronic restraint protocol and then mated. Following pregnancy confirmation, the stressed-mothers were subjected to a chronic restraint stress protocol on gestation day fourteen. Altogether, the metabolic, behavioural and neurochemical manifestations in the offspring of prenatally stressed fathers suggest that stressed fathers can impinge on offspring feeding behaviour, body weight changes as well as disruption of the hypothalamic-pituitary-adrenal axis. This was mediated, in part, by the offspring's reduced food intake and body weight as well as, a dysregulated corticosterone response and upregulated glucocorticoid receptor expression. Moreover, when both parents are prenatally stressed, their stress effect increased offspring food intake but decreased body weight which was mediated, in part, by upregulating corticosterone secretion. In summation, stressed fathers did not explicitly affect the offspring stress response however, they did illicit changes to their appetite and growth.

**Keywords:** perinatal, prenatal stress, metabolism, chronic stress, glucocorticoid receptor, corticosterone

## 1. INTRODUCTION

Exposure to past stress and trauma during early developmental stages can permanently affect the performance and advancements of core systems in humans, including the neuroendocrine pathways, neurobiological and metabolic functioning, and immune system (1). Upon encountering a stressor, the body reacts by deploying several modifications of the normal homeostatic reaction, including regulating the sympathetic adreno-medullary and hypothalamic pituitary adrenal (HPA) axis (2).

Chronic stress is a cumulative process in which the HPA axis responds to each individual "threat" through persistent exposure and consequently results in the release of catecholamines and glucocorticoids (3-5). Glucocorticoids are lipophilic, meaning they can cross the blood-brain barrier and bind to glucocorticoid receptors in various brain regions (6). The glucocorticoids stimulate mineralocorticoid receptors and glucocorticoid receptors in the hypothalamus and the pituitary gland, providing a feedback signal to regulate HPA axis activity (3-5). The feedback process terminates prolonged secretion of glucocorticoids thereby limiting exposure to glucocorticoids, which promote pathological effects in the body (4). Glucocorticoids can influence the brain genomically and non-genomically using various sites and pathways (7).

During gestation, maternal and foetal glucocorticoid levels increase, as a prenatal developmental mechanism (8). Foetal exposure to glucocorticoids during the third trimester is necessary for development of the lungs, brain and to prepare for birth and delivery (8). Increased levels of glucocorticoids have been linked to diminished cognition and poor sociability, as well as anxiety-like behaviours (8, 9). Glucocorticoids and catecholamines affect glucocorticoid receptors and adrenergic receptors on immune cells extracellularly and intracellularly which subsequently impede the secretion of pro-inflammatory cytokines and stimulate anti-inflammatory cytokine secretion (10, 11). An abundance of cytokines can influence the neurodevelopment of the foetus by directly interacting with the foetus glial cells, and is also associated with the transmission of stress between the mother and foetus via cortisol and reactive oxygen species (12). Chronic stress has been shown to decrease the production of proinflammatory cytokines, whilst it stimulates the secretion of anti-inflammatory cytokines (10). From this, it was hypothesised that chronic stress could lead to disease onset by suppressing the immune system (10, 13).

Following a chronic stress period, the elevated glucocorticoids in the bloodstream activate lipoprotein lipase in adipose tissue thereby, increasing fatty deposits (14). Through this mechanism, chronically elevated cortisol concentration leads to an accumulation of fat (14). Glucocorticoids can influence food intake by increasing or decreasing appetite-regulating hormones such as, insulin, ghrelin and leptin and can stimulate food intake by encouraging the desire for "comfort foods (14)." Insulin has an oppressive effect on the reward pathways, this means that food has to be more rewarding for it to have the same effect, therefore, in a stressed state, rats are more inclined to choose foods high in fat and sucrose (14).

Prenatal stress refers to all types of stress experienced by the parent (15-17). The long-term effects of prenatal stress are unspecified however, literature suggests that prenatal exposure to stress can affect the progression, performance and sensitivity of the stress system (1, 5). Maternal prenatal stress increases the risk of cortisol exposure to the foetus which subsequently produces adverse birth consequences (1, 5). Whilst many studies are investigating these lasting effects of maternal prenatal stress, there is a paucity of information on the long-term effects of paternal prenatal stress overall.

Hence, the present study sought to investigate the effects of prenatal stress on metabolism and stress regulation, particularly the fathers, and whether these can be transferred to offspring or if they can affect the offspring development. This was executed by evaluating the stress response, appetite and growth in the offspring through behavioural observations and the assessment of neurochemical markers.

## 2. METHODOLOGY

### 2.1 Materials

All chemicals and reagents used were of analytical grade and were purchased from standard commercial suppliers.

### 2.2 Animals

Sixteen male and eight female Sprague Dawley (SD) rats, each weighing between 250 - 300 g, were obtained from the Biomedical Resource Centre of the University of KwaZulu-Natal. The rats were moved to a new room and housed under standard laboratory conditions of ± 22°C room temperature, 70% humidity and a 12-hour light/dark cycle (lights on at 06h00, off at

18h00), food and water were available *ad libitum*. The rats were housed in standard conventional polycarbonate 1291H tecniplast (type III) cages (425 x 266 x 185 mm, floor space: 80 cm2). The rats were separated into four groups: (1) control (C) group: non-stressed mother and father (NS-M + NS-F) (n =6); (2) non-stressed mother and stressed father (NS-M+S-F) (n =6); (3) stressed mother and non-stressed father (S-M+NS-F) (n =6); and (4) stressed mother and stressed father (S-M+S-F) (n =6), and allowed to acclimatise for one week. The experimental protocols and procedures performed in this study were approved by the Animal Research Ethics Committee (AREC) of the University of KwaZulu-Natal (AREC/024/020M).

**2.3 Experimental Design**

Chronic stress was induced in parents using the chronic restraint stress protocol (15, 18, 19), detailed in the next section. The rats were first separated into non-stressed and stressed groups, i.e., non-stressed mother (NS-M) and stressed mother (S-M) and non-stressed father (NS-F) and stressed father (S-F). After that, they were assigned to the following groups, each group containing four males and two females as follows: (1) control (C) group: non-stressed mother and father (NS-M + NS-F); (2) non-stressed mother and stressed father (NS-M+S-F); (3) stressed mother and non-stressed father (S-M+NS-F); and (4) stressed mother and stressed father (S-M+S-F). Following acclimation, the S-F were exposed to chronic restraint stress protocol for 1 hour daily over seven days. Succeeding the stress period, the animals were mated according to their groups. On gestational day (GND) 14, the S-M group was subjected to the chronic restraint stress protocol for 1 hour daily over seven days.

On GND 14, the S-F groups were again exposed to the chronic stress protocol. Following birth, the pups were weaned off their mothers on postnatal day (PND) 22.

Body weight was measured in offspring from PND 24 until the end of the experimental period and food intake was measured in offspring on PND 24. The prefrontal cortex, adrenal glands and plasma were collected to measure interleukin (IL)-6, corticosterone and adrenocorticotropic hormone (ACTH) concentration respectively. All samples were immediately snap-frozen after extraction using liquid nitrogen and then stored in a bio freezer at -80°C until neurochemical analysis was performed. An n=8 for offspring analysis were assessed per group.

### 2.4 Paternal chronic stress protocol

The fathers were equally divided into stressed fathers (S-F) (n=8) and non-stressed fathers (NS-F) (n=8). The S fathers were exposed to daily chronic restraint stress before mating. The S-F was taken to a separate room and placed in rodent restrainers for one hour between 11 am and 12 pm, for a total of 7 days (15, 16). After the stress paradigm for each day had concluded, the S-F were returned to their home cages, and the restrainers were washed and sanitised with 70% ethanol to remove odour cues. The NS-F remained undisturbed in their home cages.

### 2.5 Mating

The mothers were equally divided into stressed mothers (S-M) (n=4) and non-stressed mothers (NS-M) (n=4). The mothers were paired and allowed to acclimatise for one week to minimise stress and synchronise their oestrous cycles. Following this, vaginal smears were performed daily to assess the females' oestrous cycle, and once in pro-oestrous, a male rat was introduced until mating was successful (20). The non-mated males remained in their home cages until successful mating. Vaginal smears were performed to determine if mating was successful by identifying the presence of sperm. The male was moved to its home cage upon positive confirmation, and this marked GND 0. The offspring in this study were obtained from the successful mating of these animals.

### 2.6 Maternal Chronic prenatal stress protocol

On GND 14, the pregnant S-M rats were exposed to the same chronic restraint stress protocol as the fathers. The stress paradigm occurred on GND 14 as this is the age when neural structure development begins in a foetal brain (21).

### 2.7 Offspring tissue collection

On PND 40, the pups were taken to the autopsy room and left to acclimatise for 1 hour before decapitation. The pups were decapitated using a guillotine. The prefrontal cortex, adrenal glands and plasma were collected into 2 ml Eppendorf tubes. All tissue samples were weighed before snap freezing in liquid nitrogen and stored in a bio freezer at -80°C until biochemical analysis.

*2.7.1 Biochemical Analysis*

*2.7.1.1* Corticosterone concentration from both the fathers and pups was measured using the enzyme-linked immunosorbent assay (ELISA) kit (E-EL-0160) (Elabscience, Wuhan, China)

according to the manufacturer's guidelines. ACTH concentration from the pups was measured using the ELISA kit (E-EL-R0048-96T) (Elabscience, Wuhan, China), according to the manufacturer's guidelines.

*2.7.1.2* IL-6 and glucocorticoid receptor expression from the pups was quantified in prefrontal cortex tissue using the Real-Time Polymerase chain reaction (qPCR). Primers for the target genes and house-keeping gene (HKG) glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were designed on Primer-BLAST (sequences shown in Table 1), and were obtained from Inqaba Biotechnical Industries (Pty) Ltd. Primers were reconstituted to a 100mM stock solution by adding nuclease-free water as per manufacturer instructions. Primers base pairs were designed according to the MIQE guidelines (22).

Total RNA was isolated using the Zymo Research *Quick*-RNA™ MiniPrep Kit (ZR1054). Eluted RNA concentrations were measured for purity using a Nanodrop 2000 (Thermo Scientific, Roche, South Africa). purity ratio ($A_{260}/A2_{80}$) of 1.7 – 2.1 was considered satisfactory for conversion to cDNA. cDNA was synthesised from 1 µg RNA using the BioRad iScript™ cDNA Synthesis Kit (1708891), in 20 µl of reaction volume according the manufacturer's guidelines using a thermocycler 2.0 (Roche, Switzerland). Real-time PCR was performed with the BioRad iTaq™ Universal SYBR® Green Supermix (172-5120. Amplification was run in duplicate in a Roche LightCycler96 (Roche, Switzerland). Expression of IL-6 & GR relative to GAPDH was calculated using the $_2{-}(\Delta\Delta Cq)$ method (49).

Table 1. PCR Target and Reference primers.

| Primer | Forward | Reverse | NCBI Sequence |
|---|---|---|---|
| GAPDH | AGTGCCAGCCTCGTCTCATA | GATGGTGATGGGTTTCCCGT | NM_017008.4 |
| IL-6 | GGGTAGAAGGCAAGGAGTCG | GGACGCACTCACCTCTTGTT | NC_051339.1 |
| GR | TGGGTACTCAAGCCCTGGAA | ACATGTCAGCACCCCGTAAT | NM_012576.2 |

### 2.8 Analysis of data

All data was analysed with GraphPad Prism version 7 (GraphPad Software Inc., CA, USA). The data was subjected to column statistics to determine distribution, and thereafter further analysed using the two-way analysis of variance (ANOVA) test followed by the Tukey-Kramer *post hoc* comparison test. The gene expression was analysed using the one-way ANOVA test

followed by the Tukey-Kramer *post hoc*. An n=8 for offspring analysis were assessed per group. $p < 0.05$ was considered statistically significant. All data are expressed as the mean ± standard error of the mean (SEM).

## 3. RESULTS

### 3.1 Food Intake

There was a difference in food intake between the groups (Table 2) which was measured on PND 39, to assess offspring appetite ($F_{(3, 23)} = 9.596$, $p<0.05$). The prenatal stress effect on offspring food intake was present in the NSM-SF and SM-NSF when compared to the Control *(NSM-SF vs. Control, $p<0.05$, Table 2), *(SM-NSF vs. Control, $p<0.05$, Table 2). There was also a parental stress effect on offspring food intake in the SM-SF when compared to the NSM-SF $^{\#}$(SM-SF vs. NSM-SF, $p<0.05$, Table 2).

**Table 2:** Food intake in the final week by offspring within each group (n=8, per group).

| **Group (n=8)** | **Control** | **NSM-SF** | **SM-NSF** | **SM-SF** |
|---|---|---|---|---|
| **Food Intake (g)** | 32.14 ± 1.44 | 21.4 ± 1.60* | 25.25 ± 1.45* | 29.71 ± 1.32$^{\#}$ |

Values are presented as mean ± SEM. $^{*}p<0.05$ when compared to Control, $^{\#}p<0.05$ when compared to NSM-SF.

### 3.2 Body Weight

There was a difference in body weight between the groups (Table 3) which was measured on PND 39, to assess offspring growth ($F_{(3, 112)} = 79.15$, $p<0.0001$). There was a paternal stress effect on offspring body weight in the NSM-SF and SM-SF when compared to the Control *(NSM-SF vs. Control, $p<0.05$, Table 3), *(SM-SF vs. Control, $p<0.0001$, Table 3). There was a maternal stress effect on offspring body weight in the SM-NSF and SM-SF when compared to the NSM-SF $^{\#}$(SM-NSF vs. NSM-SF, $p<0.05$, Table 3), $^{\#}$(SM-SF vs. NSM-SF, $p<0.0001$, Table 3). There was a parental stress effect on offspring body weight in the SM-SF when compared to the SM-NSF $^{\alpha}$(SM-SF vs. SM-NSF, $p<0.0001$, Table 3).

**Table 3:** Body weights in the final week by offspring within each group (n=8, per group).

| Group (n=8) | Control | NSM-SF | SM-NSF | SM-SF |
|---|---|---|---|---|
| **Body Weight (g) on PND 39** | 163.50 ± 6.19 | 147.13 ± 8.28* | 166.13 ± 4.77# | 112.88 ± 3.32*#α |

Values are presented as mean ± SEM. *p<0.05 when compared to Control, #p<0.05 when compared to NSM-SF, αp<0.05 when compared to SM-NSF.

### 3.3 Corticosterone concentration

The adrenal corticosterone concentration was measured to assess the offspring stress response ($F_{(3, 17)} = 26.38$, p<0.0001). There was a prenatal stress effect on the offspring in the NSM-SF group and SM-NSF when compared to the control group *(NSM-SF vs Control, p<0.05, Figure 1), *(SM-NSF vs. Control, p<0.05, Figure 1). There was a maternal stress effect on the offspring in the SM-NSF group and SM-SF group when compared to the NSM-SF group #(SM-NSF vs NSM-SF, p<0.0001, Figure 1), #(SM-SF vs NSM-SF, p<0.05, Figure 1). There was a parental stress effect on the offspring in the SM-SF group when compared to the SM-NSF group α(SM-SF vs SM-NSF, p<0.05, Figure 1).

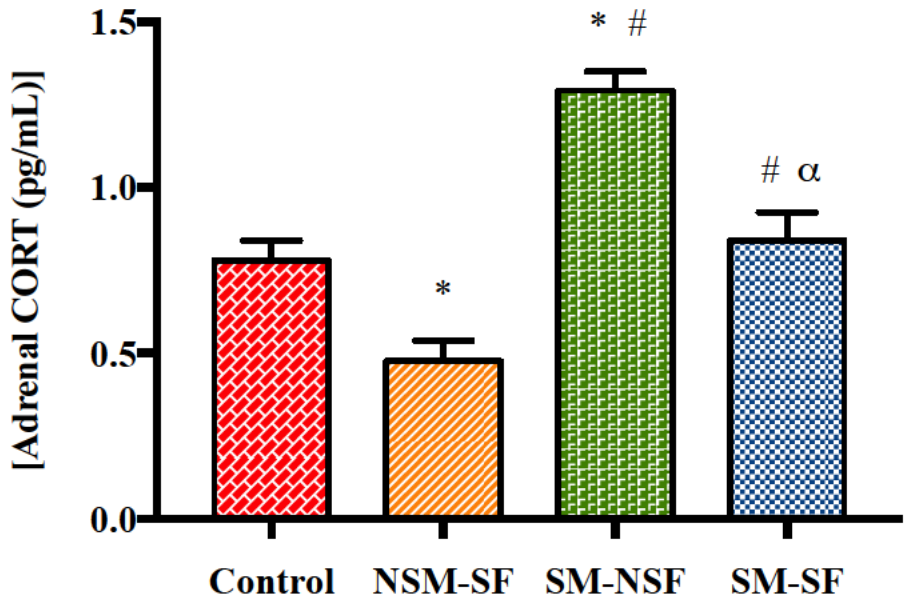


Figure 1: Adrenal gland corticosterone concentration in offspring from the Control, NSM-SF, SM-NS-F and SM-SF groups. *p<0.05 when compared to Control, #p<0.05 when compared to NSM-SF, αp<0 05 when compared to SM-NSF.

### 3.4 Adrenocorticotropic hormone concentration

The plasma ACTH concentration was measured to assess offspring stress response ($F_{(3, 20)}$ = 2.296, $p<0.05$). There was a SM-NSF effect on ACTH concentration compared to the control group *(SM-NSF vs Control, $p<0.05$, Figure 2).

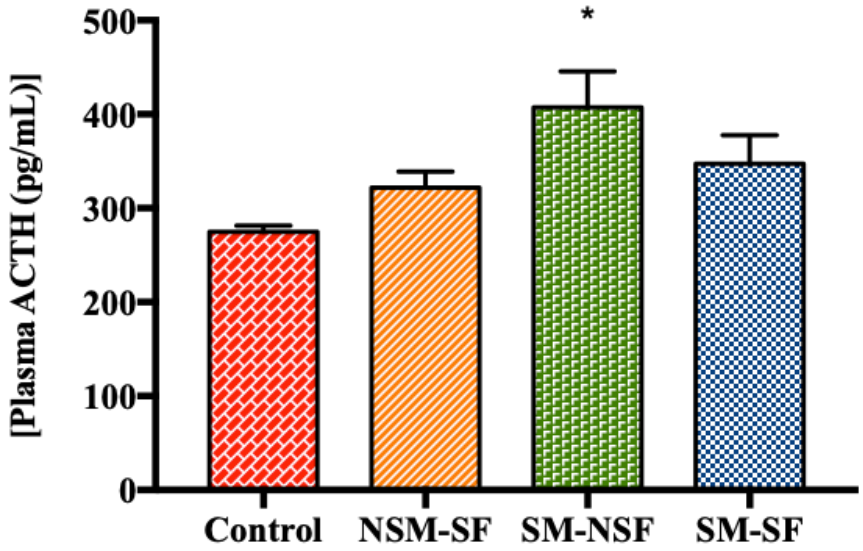


Figure 2: Plasma ACTH concentration in offspring from the Control, NSM-SF, SM-NS-F and SM-SF groups.*$p<0.05$ when compared to Control.

### 3.5 Glucocorticoid receptor expression

The prefrontal cortex glucocorticoid receptor expression was measured to assess offspring stress response ($F_{(3,11)} = 21.27$, $p<0.0001$). There was an increase in the glucocorticoid receptor expression in SM-NSF offspring compared to the control group *(NSM-SF vs Control, $p<0.05$, Figure 3). There was a decrease in the glucocorticoid receptor expression in the SM-NSF group and SM-SF group compared to the NSM-SF group $^{\#}$(SM-NSF vs NSM-SF, $p<0.0001$, Figure 3), $^{\#}$(SM-SF vs NSM-SF, $p<0.05$, Figure 3).

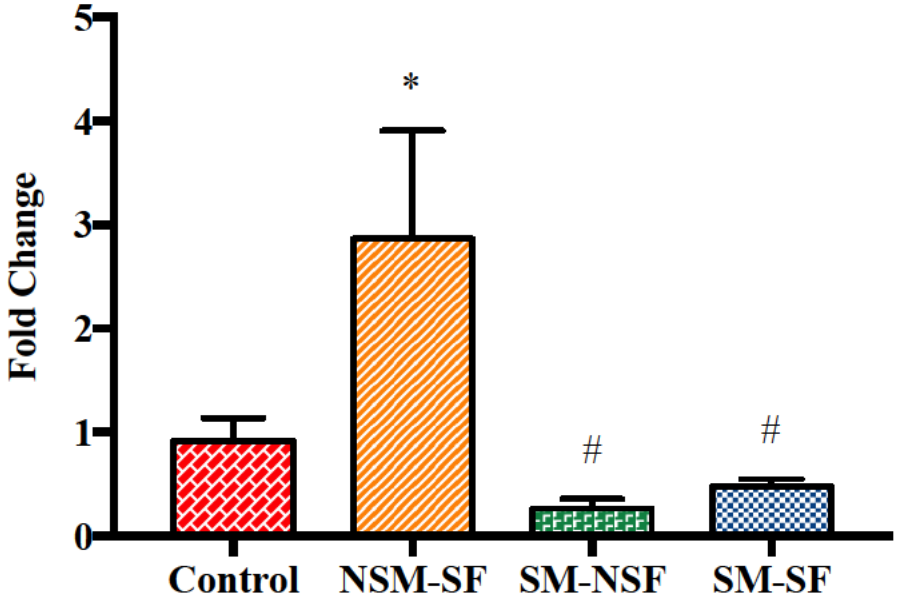


Figure 3: Prefrontal Cortex glucocorticoid receptor expression in offspring from the Control, NSM-SF, SM-NSF and SM-SF groups. *p<0.05 when compared to Control, #p<0.05 when compared to NSM-SF.

### 3.6 Interleukin-6 expression

The prefrontal cortex IL-6 expression was measured to assess offspring inflammatory response ($F_{(3, 16)} = 0.4369$). There was no stress effect on IL-6 expression in the C, NSM-SF, SM-NSF and SM-SF groups (figure 4).

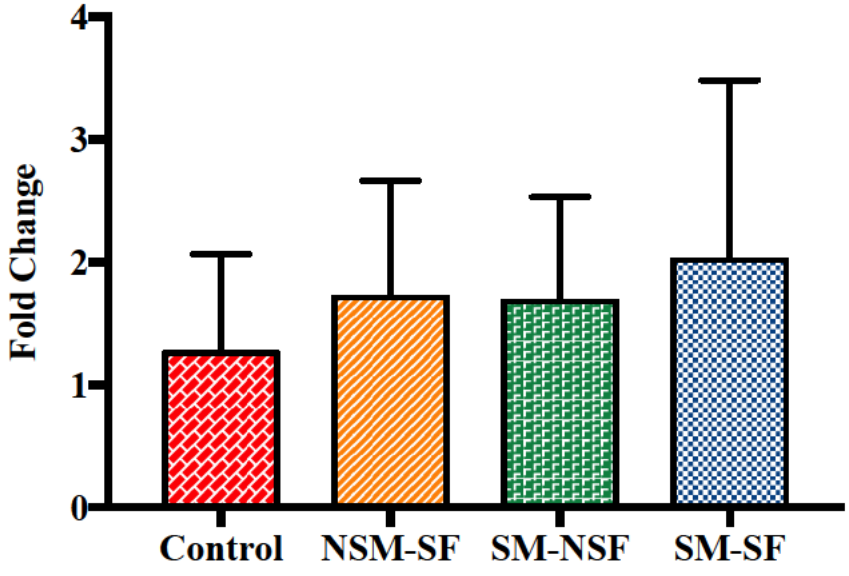


Figure 4: Prefrontal Cortex IL-6 expression in offspring from the Control, NSM-SF, SM-NSF and SM-SF groups.

## 4. DISCUSSION

Prenatal exposure to chronic stress can affect the progression, performance and sensitivity of foetal metabolism and its stress response (1, 5). Maternal prenatal stress increases the risk of cortisol exposure to the foetus which subsequently produces adverse birth effects such as low birth weight, and reduced gestational period (1, 5). However, the contribution of paternal stress to adaptations in offspring birth effects and stress response has not been examined. The present study investigated the stress response, food intake and body weight in the offspring through behavioural observations and the assessment of neurochemical markers.

A lower food intake was observed in offspring of the NSM-SF and SM-NSF groups after, suggesting that stressed fathers influenced food intake behaviour in the offspring. Elevated cortisol concentration is associated with reduced appetite and has further been linked to a depressive state (14). Increased glucocorticoid production after a chronically stressed state is linked to an accumulation of energy and increased appetite (14, 23). Findings in the present study show that offspring from the SM-SF group presented with an increased food intake. Patients who encounter stressful situations have been shown to adopt unhealthy food patterns with most eventually presenting with obesity, metabolic syndrome and type 2 diabetes (24, 25). Changes to the offspring HPA axis during early development can further influence the feeding patterns of offspring by lowering or elevating voluntary food intake and weight (26). Our findings agree with other studies which showed that stressors of specific severity have been shown to affect the appetite of rats (27). Therefore, we speculate that prenatally stressed fathers are capable of modifying their offspring food intake.

The reduced appetite in offspring from the NSM-SF group and SM-SF group was confirmed by the lower body weight when compared to offspring in the control group. Our findings are in agreement with other studies which showed that prenatal exposure to chronic stress is related to anorexia or low body weight (28, 29). Furthermore, it has been suggested that the increased serum cortisol in patients with anorexia is a biological acclimatisation to starvation caused in part by, restricted food intake (28, 30, 31). Hence, we speculate that prenatally stressed fathers are capable of restricting their offspring body weight and feeding behaviour. Noticeably, we observed a higher body weight in offspring from the SM-NSF group when compared to offspring in the NSM-SF group. These findings relate to studies which showed that prenatally stressed pups had a discernibly higher body weight (32, 33). Increased glucocorticoid

production after a chronically stressed state is linked to increased appetite, as consuming high caloric foods leads to weight gain as seen in offspring from the SM-NSF group (14, 23). In humans it has been reported that exposure to stressors during late gestation, the period in which foetus growth is accelerated, produces low birthweight but can also contribute to the development of obesity later on in life (34). Therefore, we speculate that prenatally stressed fathers can predispose their offspring to the risk of developing abnormal feeding behaviours.

The low body weight observed in offspring from the NSM-SF group was confirmed by decreased adrenal corticosterone concentration however, no obvious changes to plasma ACTH were noted. It has been reported that patients with exposure to childhood trauma and stress, exhibited an attenuated cortisol response (35). Children exposed to early life adversities have presented with a smaller hippocampal volume and lower cortisol concentration which have been associated with mental disorders later on in life (36). It was postulated that over-stimulation of glucocorticoids with increased cortisol concentration during extreme trauma contributed to the hypocortisolism during adulthood which may lead to hippocampal atrophy and neuronal damage (36). We also observed elevated adrenal corticosterone concentration in offspring from the SM-NSF group, who presented with increased body weight. Our findings agree with another study which reported increased body weight in offspring exposed to higher than normal corticosterone during pregnancy (37). Both abnormally increased and decreased glucocorticoids lead to behavioural changes similar to depressive behaviour. This can be confirmed by the anhedonia behaviour exhibited by the offspring from the NSM-SF group, SM-NSF group and SM-SF group which was shown in another study, in this project. Therefore, we speculate that offspring from prenatally stressed parents present with a dysfunctional glucocorticoid response which is related to body weight, appetite and depression like behaviours (35). For that reason, prenatally stressed parents could have, in part, influenced plasma corticosterone concentration in offspring which exacerbated the onset of low birth weight, appetite and depression-like behaviour.

The increased adrenal corticosterone concentration observed in offspring from the SM-NSF group when compared to the Control was confirmed by elevated plasma ACTH concentrations. It has been reported that prenatal maternal stress activates the HPA axis which increases cortisol and ACTH secretion (38). Our findings agree with this, as the stressed mothers triggered an increased secretion of both ACTH and corticosterone in the offspring. Our findings

agree with another study which showed that chronic stress stimulates a maximum adrenal secretion as a consequence of ACTH stimulation, in response to encouraging stimuli (39). On the other hand, our results did not confirm that stressed fathers manipulated the offspring ACTH secretion. This could have occurred due to the time elapsed since the trauma exposure; the severity of the stress paradigm and physiological differences in the offspring.

The dysregulated HPA axis stress response was confirmed by measuring the expression of glucocorticoid receptor in the prefrontal cortex in offspring from the C, NSM-SF, SM-NSF and SM-SF. Lesions in the ventromedial prefrontal cortex exacerbate reactions to psychogenic stressors, and stimulation of the prefrontal cortex reduces the scale of the psychogenic glucocorticoid response thereby, implicating the prefrontal cortex in stress inhibition (4, 40). Since the prefrontal cortex is able to manipulate the magnitude and duration of a stress response, prefrontal glucocorticoid receptors may be intricately involved in feedback responses. These interactions imply that the prefrontal cortex when mediated by glucocorticoid feedback can inhibit HPA axis responses (4). The prefrontal cortex expresses glucocorticoid receptors and mineralocorticoid receptors in high abundance (4), implicating it in HPA axis regulation (4). Implants of corticosterone into the prefrontal cortex dampen stress responses (4). Our findings agree with this, as offspring from the stressed fathers exhibited a 3 fold increase in glucocorticoid receptor expression. It has been documented that glucocorticoid receptors in the prefrontal cortex are highly sensitive to disruptions in endogenous glucocorticoid secretion implying that prefrontal cortex dysfunction is present in chronically stressed rats (41). This data is in agreement with this study, as offspring from stressed mothers expressed a fold decrease in glucocorticoid receptor expression. Therefore, we speculate that stressed fathers are able to dysregulate the offspring's glucocorticoid receptor expression. The attenuation of glucocorticoids in response to an upregulation of glucocorticoid receptor indicated that offspring of stressed fathers exhibit a disrupted negative control feedback.

The inflammatory response was confirmed by measuring the expression of IL-6 in the prefrontal cortex in offspring from the C, NSM-SF, SM-NSF and SM-SF. Pro-inflammatory cytokines can trigger the HPA axis whilst, cortisol attenuates production of cytokines and other inflammatory indicators (42). Our findings show that there were no significant differences in the prefrontal cortex IL-6 expression between offspring. Circulating pro-inflammatory factors directly triggers the HPA axis, producing serum adrenocorticotropic hormone and

glucocorticoids, which subsequently prevents the production of these pro-inflammatory factors (43). In rat animal studies, the use of Il-6 was shown to produce structural modifications in offspring hippocampus and learning deficiencies as well as increased body weight (12). Stress-induced cytokine secretion can lead to glucocorticoid resistance (44). Therefore, we speculate that our conflicting findings could be due to a variety of factors such as the stress paradigm employed, gender, age, and emotional well-being which are recognised for affecting cytokine concentration.

Overall, the metabolic, behavioural and neurochemical manifestations in the offspring of prenatally stressed fathers suggest that stressed fathers can impinge on offspring feeding behaviour, body weight changes as well as disruption of the HPA axis. This was mediated, in part, by the offspring's reduced food intake and body weight as well as, a dysregulated corticosterone response and upregulated glucocorticoid receptor expression. Moreover, when both parents are prenatally stressed their stress effect to their offspring's development is exacerbated. This was confirmed by metabolic manifestations of increased food intake and body weight which may have primarily been attained by modifying the glucocorticoid system. Our findings add to the literature on the effects that stressed fathers infer to their offspring, which has not been comprehensively explored. These findings warrant further investigation into causative mechanisms. It is recommended, that this study be modelled to investigate the possible ways stressed fathers affect their offspring, particularly the different life stages in which behavioural, metabolic and neurochemical deviations first appear in offspring and the long lasting physiological adaptions of these stimuli.

## 5. CONCLUSION

Given the anecdotal evidence, the prenatally stressed fathers did not affect the stress response in its entirety however, they affected the offspring's appetite and growth. This was mediated in part by decreased adrenal corticosterone concentration and upregulated glucocorticoid receptor expression in the offspring. On the other hand, when prenatally stressed fathers were mated with prenatally stressed mothers it produced an intermediate effect in the offspring with the behavioural, metabolic and neurochemical outcomes averaging the other two groups. Our findings encapsulates the effects that familial trauma imposes on the growth and development in offspring, specifically how these dysfunctions can manipulate the way in which youth react to challenges and hostilities faced in everyday life. This will aid in advancing therapeutic

strategies for treatment and management of mental illness, and minimise the negative societal impact this might have on society and community.

## 6. DECLARATION


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### Author Contributions

Miss Malishca Perumal: Project development, Animal work, Data collection, Data analysis, Manuscript writing and editing.

Dr Mluleki Luvuno: Project development, Data analysis, Manuscript editing.

Prof Musa Mabandla: Project development, Data analysis, Manuscript editing.

### Conflict Of Interest

The authors declare no conflicting interests.

### Ethical Approval

Ethical approval was obtained from the Animal Research Ethics Committee (AREC/024/020M) of the University of KwaZulu-Natal.

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## CHAPTER 4: SYNTHESIS

Exposure to past stress and trauma during early developmental stages can permanently affect the performance and advancements of core systems in humans. In South Africa, exposure to stressors such as oppression, and racial discrimination, during the apartheid era has resulted in mental anguish and other psychological issues. Furthermore, South Africa's older generation have recounted memory impairments, and one in six citizens experience anxiety, depression, or substance abuse. Stress can be acute or chronic depending on the duration and exposure to a stressor. A chronic stressed state occurs due to prolonged exposure to an acute stressor without cessation. This exposure can be lethal because it results in permanent changes to emotional, physiological and behavioural aspects, which influence the onset and progression of disease. Since the perinatal life is exceptionally sensitive to stressors, adverse conditions can produce abnormal changes to behaviour and physiological mechanisms. These include permanent alterations to the growth and cognitive functioning, the onset of febrile seizures, and the risk for developing psychiatric disorders such as, autism spectrum disorders, schizophrenia and attention deficit hyperactivity disorder.

Prenatal stress refers to any type of stressor experienced by a parent during gestation. The lasting effects of prenatal stress are uncertain however, literature has shown that exposure to stress prenatally can impact the development, functioning and vulnerability of the stress system. Maternal prenatal stress continues to be extensively studied and has been shown to affect the growth and sensitivity of core systems such as the hypothalamic-pituitary-adrenal axis, immune response, and cognitive development as well as, exacerbate the onset of psychiatric conditions such as anxiety and depression. Whilst many are investigating the ramifications of maternal prenatal stress on the infant, child, adolescence and adult, there is a scarcity of information on the long-term effects of paternal stress on the offspring.

The extent of past traumatic experiences on the parents and their progeny is poorly understood. Consequently, it is not certain whether the effects of the experiences can be transferred to the offspring. Hence, this study investigated the physiological repercussions in the first filial generation after exposing the parental generation to a chronic stress regimen. Therefore, we investigated the repercussions of prenatal stress in the offspring by considering the onset of anxiety, depression, and sociability. Furthermore, we looked at the influence of prenatal stress on the offspring stress response and metabolism. This study will be a first step in elucidating

how stressors such as oppression, race and inequality experienced by older generations can be transferred to offspring and how it affects their well-being. It will provide insight into the physiological connection linking lineages exposed to trauma and the effects in the subsequent generations. In addition, it may provide a basis for post-traumatic stress disorder being expressed in respective generations of the same lineage.

The findings of this study, which investigated the effects of paternal prenatal stress on offspring behaviour, were matched to changes in brain neurochemistry. We found that the fathers displayed anxiogenic behaviour which was confirmed by the lower amygdala serotonin concentration. We found that exposing the fathers to chronic restraint stress for one hour over seven days produced significant behavioural and neurochemical changes in the offspring. We noted anhedonia and social anxiety indicators, reduced food intake, and body weight. These instabilities were mediated, in part, by blunted serotonin, a sporadic corticosterone response and upregulated glucocorticoid receptor expression in the offspring. These findings suggest that offspring from prenatally stressed fathers have an apparent aversion to sociability, increased likelihood of developing depression and can also pave the way for eating disorders. We also found that prenatally stressing both parents enhanced the stress effect seen in their offspring's behaviour and neurochemistry. This was evident by the metabolic, neurochemical and behavioural manifestations in the offspring of prenatally stressed parents.

When prenatally stressed fathers were mated with prenatally stressed mothers it produced an intermediate effect in the offspring. We found that when both parents were prenatally stressed, it produced extreme bouts of anxiety, depression, social anxiety, increased food intake and body weight. A suppressed serotonin discharge accompanied these disturbances. This suggests that when both parents are prenatally stressed, they can significantly influence their offspring's onset of psychiatric and physiological changes. The beginning of these physiological disorders can be averted by monitoring any neurochemical fluctuations to baseline serotonin and glucocorticoids in the offspring. This can pre-emptively prepare the parents for any conditions that may arise, allowing for adequate therapeutic interventions.

## CONCLUSION

Given the anecdotal evidence, paternal prenatal stress led to the onset of depression-like behaviour, sociability, food intake and body weight. In addition, prenatal stress preceded the

onset of anxiety, depression, sociability, food intake and body weight in all offspring. Prenatally stressed fathers manipulated offspring's physiological development by way of reduced food intake, and body weight, and behavioural indicators of anhedonia and social anxiety, which was evident in adolescence. Furthermore, when both parents are prenatally stressed, they significantly influence the development of anxiety, depression and sociability in their offspring, which from a very young age was evident through discernible changes in the growth and development of offspring.

**BENEFITS OF THE STUDY**

Our findings can be translated to a human cohort to understand the pathophysiological implications surrounding the transference of traumatic experiences in older generations to offspring. This can then be used to improve the diagnosis, prevention and treatment of disease and control the physiological outcomes of posttraumatic generational traumas.

**RECOMMENDATIONS**

Given the findings of our studies, it would be interesting to further investigate the long-term effects of traumatic experiences across multiple generations, particularly the different life stages in which behavioural, metabolic and neurochemical deviations first appear in offspring and the long lasting physiological adaptations of these stimuli.

We recommend that a follow-up study be conducted to investigate the role of dopamine and glucocorticoid receptor sensitivity in prenatally stressed parents and in offspring exposed to early life adversities.

## APPENDIX A: ETHICAL CLEARANCE

**Department of Agriculture, Land Reform and Rural Development (DALRRD) ethical approval**

agriculture, land reform & rural development

Department:
Agriculture, Land Reform and Rural Development
REPUBLIC OF SOUTH AFRICA

Directorate Animal Health, Department of Agriculture, Land Reform and Rural Development
Private Bag X138, Pretoria 0001

**Enquiries:** Mr Henry Gololo • Tel: +27 12 319 7532 • Fax: +27 12 319 7470 • E-mail: HenryG@dalrrd.gov.za
**Reference:** 12/11/1/5/2 (1685JD)

**Responsible person(s):** Malishca Devani Perumal
**Institution:** Medical Sciences, University of KwaZulu-Natal
**Email:** malishcap@gmail.com

Dear Malishca Devani Perumal

**CONDITIONS FOR RESEARCH IN TERMS OF SECTION 20 OF THE ANIMAL DISEASES ACT, 1984 (ACT NO 35 OF 1984)**

**Title of research project / study: "Influences of maternal and paternal chronic stress on offspring behavior and neurochemistry in a rat model"**

Your application, requesting permission under Section 20 of the Animal Diseases Act, 1984 (Act no 35 of 1984) to perform the research project or study stipulated above, refers.

1. Based on the information provided in your application, the Director of Animal Health has no objection to this study. The study may continue if statement 1.1 to 1.6 hereunder are, and remain, accurate. **Should the scope of your research project change in any way you are required to inform the Section 20 Secretariat and may not proceed with any activities until written permission to do so have been granted by the National Director: Animal Health.**

    1.1. No work will be done with controlled and notifiable animal diseases (list can be obtained / requested from this office), which includes any animal diseases which do not occur in South Africa;
    1.2. No imported material of animal origin or imported animal pathogens will be utilized in the study;

1.3. No samples that originate from a biobank will be used in the study;
1.4. No clinical studies will be performed in the target species, either in a laboratory or in the field;
1.5. The areas where the samples are to be collected are not under restriction for controlled or notifiable diseases to which the species of animal, from which the samples are obtained, is susceptible;
1.6. No samples or products that have not been passed as fit for human consumption will be obtained from an abattoir.

2. In addition to the conditions mentioned in point 1, you are responsible for ensuring that your research project or study complies with all or part of the following, as applicable:

2.1. Permission to perform research under Section 20 of the Animal Diseases Act 1984 (Act no 35 of 84) does not relieve the researcher of any responsibility which may be placed on him/her by any other Act of the Republic of South Africa, including the Veterinary and Para-Veterinary Professions Act 1982 (Act No. 19 of 82), the Fertilizers, Farm Feeds, Agricultural Remedies and Stock Remedies Act 1947 (Act no 36 of 47), the Medicines and Related Substances Control Act 1965 (Act 101 of 65) and the Genetically Modified Organisms Act, 1997 (Act No 15 of 1997);
2.2. No part of the study may begin until valid ethical approval has been obtained in writing from the relevant South African authority;
2.3. All biological or potentially infectious material must be packaged and transported in accordance with International Air Transport Association (IATA) requirements and the National Road Traffic Act, 1996 (Act No. 93 of 1996);
2.4. Any incidence or suspected incidence of a controlled or notifiable disease in terms of the Animal Diseases Act 1984 (Act no 35 of 84), must be reported immediately to the responsible state veterinarian;
2.5. Only Sprague Dawley rats, obtained from the Biomedical Research Centre at UKZN, may be used in this project ;
2.6. An inventory of the stored material must be kept for five years for auditing purposes and the Director of Animal Health reserves the right to conduct an audit on the facility;
2.7. Samples or material may not be outsourced or used for further/other research without prior written approval from the Director of Animal Health;

2.8. All potentially infectious material, utilised or generated during or by the study, is to be destroyed at completion of the study and only a registered waste disposal company may be used for the removal of waste generated during or by the study.

Written permission from the Director of Animal Health must be obtained prior to any deviation from the conditions. Application must be sent in writing to HenryG@dalrrd.gov.za.

Failure to obtain written permission as above may be considered a contravention of the Animal Diseases Act, 1984 (Act no 35 of 1984).

**Expiry date of this permit: 30 November 2023**

Kind regards,

[redacted]

**Dr Mpho Maja**
**DIRECTOR: ANIMAL HEALTH**
**Date:** 2020 -10- 13
**Cc**

## Animal Research Ethics Committee (AREC) ethical approval

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

19 November 2020

**Ms Malishca Devani Perumal (215018723)**
**School of Laboratory Medicine and Medical Sciences**
**Westville**

**Dear Ms Perumal,**

**Protocol reference number: AREC/024/020M**
**Project title:** Influences of maternal and paternal chronic stress on offspring anxiety and depressive-like behaviour in a rat model.

**Full Approval – Research Application**

With regard to your revised application received on 21 October 2020, the Animal Research Ethics Committee has accepted the documents submitted and **FULL APPROVAL** for the protocol has been granted.

**Please note: There must be adherence to national and institutional COVID-19 regulations and guidelines at all times.** Researchers will be personally responsible and liable for non-adherence to national regulations. If in doubt, please contact the Research Ethics Chair and/or the University Dean of Research for advice.

**Please note: Any Veterinary and Para-Veterinary procedures must be conducted by a SAVC registered VET or SAVC authorized person.**

**Any alteration/s to the approved research protocol, i.e Title of Project, Location of the Study, Research Approach and Methods must be reviewed and approved through the amendment/modification prior to its implementation. In case you have further queries, please quote the above reference number.**

Please note: Research data should be securely stored in the discipline/department for a period of 5 years.

**The ethical clearance certificate is only valid for a period of one year from the date of issue. Renewal for the study must be applied for before 18 November 2021.**

**Attached to the Approval letter is a template of the Progress Report that is required at the end of the study, or when applying for Renewal (whichever comes first). An Adverse Event Reporting form has also been attached in the event of any unanticipated event involving the animals' health / wellbeing.**

I take this opportunity of wishing you everything of the best with your study.

[redacted]

**Dr Sanil D Singh, PhD**
**Chair: Animal Research Ethics Committee**

**/kr**
**cc Supervisor:** Ms Cleopatra Kopaopa
**cc BRU Manager:** Dr Jaca

**Animal Research Ethics Committee (AREC)**
**Ms Karen Reinertsen (Administrator)**
**Westville Campus, Govan Mbeki Building**
**Postal Address:** Private Bag X54001, Durban 4000
**Telephone:** +27 (0) 31 260 8850 **Facsimile:** +27 (0) 31 260 4609 **Email:** animalethics@ukzn.ac.za
**Website:** http://research.ukzn.ac.za/Research-Ethics/Animal-Ethics.aspx

## APPENDIX B: COMPETENCE TRAINING LETTER

UNIVERSITY OF KWAZULU-NATAL

*Biomedical Resource Unit*

October 14, 2020

Dear Dr Singh
Chair: Animal Research Ethics Committee
c/o School of Life Sciences

**RE: COMPETENCE TRAINING ON RATS ONLY FOR NON-INVASIVE PROCEDURES**

This letter confirms that Malishca Perumal:215018723 has undergone evaluation for invasive procedures on the 13th October 2020 and shows competence regarding the following:

a. Animal handling and physical restraint.

BIOMEDICAL RESOURCE CENTRE
PRIVATE BAG X54001
2020 -10- 13
DURBAN, 4000

Kind regards

[redacted]

Dr N Jaca
Manager/veterinarian

*To Reduce Replace and Refine Animal Research*

## APPENDIX C: LABORATORY ANIMAL COURSE LETTER

UNIVERSITY OF KWAZULU-NATAL

School of Laboratory Medicine and Medical Sciences
Westville Campus
Private Bag X54001
Durban
4000
Tel; 031 260 7671
Fax: 031 260 7730

*Biomedical Resource Unit*

September 22, 2020

Dear Dr SD Singh
Chair: Animal Research Ethics Committee
c/o School of LMMS

**RE: ATTENDANCE OF LAS COURSE**

This letter certifies that Malishca Perumal: 215018723 has attended the Laboratory Animal Course that was hosted by the Biomedical Resource Unit.
The course was held on the 24-25 February 2020 and entailed the following:

- Introduction to laboratory animal species
- Bioethics and animal experimentation
- Animal research methodology
- Experimental design
- Environmental enrichment
- Occupational safety.

The course was completed satisfactorily, and the student may be allowed to initiate research if the relevant practical procedures were done to a level of competency that was signed off by the veterinarian in charge.

Kind Regards

[redacted]

Dr L A Bester
Course Coordinator

22/9/20
Date

Mrs Ritta Govender, Room 201, 2nd Floor U- Block, E-mail radeber@ukzn.ac.za

*To Reduce Replace and Refine Animal Research*

## APPENDIX D: SUMMARY OF GUIDELINES TO AUTHORS

**PONTE Journal**

**ARTICLE STRUCTURE**

Subdivision - numbered sections

Divide your article into clearly defined and numbered sections. Subsections should be numbered 1.1 (then 1.1.1, 1.1.2, ...), 1.2, etc. (the abstract is not included in section numbering). Use this numbering also for internal cross-referencing: do not just refer to 'the text'. Any subsection may be given a brief heading. Each heading should appear on its own separate line.

**Abstract**

A concise and factual abstract is required. The abstract should state briefly the purpose of the research, the principal results and major conclusions. An abstract is often presented separately from the article, so it must be able to stand alone. For this reason, References should be avoided, but if essential, then cite the author(s) and year(s). Also, non-standard or uncommon abbreviations should be avoided, but if essential they must be defined at their first mention in the abstract itself.

**Keywords**

Immediately after the abstract, provide a maximum of 6 keywords. Be sparing with abbreviations: only abbreviations firmly established in the field may be eligible. These keywords will be used for indexing purposes.

**Introduction**

State the objectives of the work and provide an adequate background, avoiding a detailed literature survey or a summary of the results.

**Material and methods**

Provide sufficient detail to allow the work to be reproduced. Methods already published should be indicated by a reference: only relevant modifications should be described.

**Theory/calculation**

A Theory section should extend, not repeat, the background to the article already dealt with in the Introduction and lay the foundation for further work. In contrast, a Calculation section represents a practical development from a theoretical basis.

**Results**

Results should be clear and concise.

**Discussion**

This should explore the significance of the results of the work, not repeat them. A combined Results and Discussion section is often appropriate. Avoid extensive citations and discussion of published literature.

**Conclusions**

The main conclusions of the study may be presented in a short Conclusions section, which may stand alone or form a subsection of a Discussion or Results and Discussion section.

**Appendices**

If there is more than one appendix, they should be identified as A, B, etc. Formulae and equations in appendices should be given separate numbering: Eq. (A.1), Eq. (A.2), etc.; in a subsequent appendix, Eq. (B.1) and so on. Similarly for tables and figures: Table A.1; Fig. A.1, etc.

**Acknowledgements**

Collate acknowledgements in a separate section at the end of the article before the references and do not, therefore, include them on the title page, as a footnote to the title or otherwise.

**Tables**

- Be sure you have cited each table within the text.
- Enter a short descriptive caption at the top of each table, preceded by an identifying Arabic numeral.

- Columns and their headings are normally used to display the dependent variable(s) being presented in the table.
- Footnotes should be identified by lowercase letters or numbers (e.g., a, b, c; 1, 2, 3) appearing as superscripts in the body of the table and preceding the footnote below the table. The same data should not appear in both tables and figures.

**Figures**

- Each figure should have a caption. The caption should be concise and typed separately, not on the figure area; If figures have parts (for example, A and B), make sure all parts are explained in the caption.
- All figures are to be sequentially numbered with Arabic numerals. Figures should always be cited in text in consecutive numerical order.

**Equation Format**

- Please use earlier versions of Microsoft Word or the legacy equation editor in Word to create equations.
- Long equations should be set apart from the text and numbered sequentially. After an equation is introduced, refer to it by number (e.g., "Eq. 1," "Eqs. 3 and 4").
- If some or all of your equations are simple (on a single baseline), use normal text and fonts.
- Complex equations should be embedded using standard plug-ins like Math type or the Word Equation Editor contained in versions of Microsoft Word up to 2003 or the legacy equation editor in Word 2007, 2008 for Mac, or 2010.
- If the paper includes many equations or schemes, these can be collected in a table of equations.

**References**

References can be listed in any standard referencing style as long as it is consistent with references within a given article. However, key points include:

- Only articles, datasets and abstracts that have been published or are in press, or are available through public e-print/preprint servers/data repositories, may be cited. Unpublished abstracts, papers that have been submitted but not yet accepted, and personal communications should instead be included in the text, and should be referred to as ‘personal communications’ or ‘unpublished reports’ and the researchers involved should be named. It is the responsibility of the authors to ensure they obtain permission to quote any personal communications from the cited individuals.
- The list of references should be arranged alphabetically by authors' names and chronologically per author. If the author's name is also mentioned with co-authors the following order should be used: publications of the single author, arranged chronologically - publications of the same author with one co-author, arranged chronologically - publications of the author with more than one co-author, arranged chronologically. Publications by the same author(s) in the same year should be listed as 2004a, 2004b, etc. Reference lists not conforming to this format will be returned for revision.
- Web links, URLs, and links to the authors’ own websites should be included as hyperlinks within the authors' manuscript, and not as references.