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THE EFFECTS OF EARLY LIFE STRESS ON FACTORS ASSOCIATED WITH FOOD INTAKE AND BODY WEIGHT CHANGES IN SPRAGUE DAWLEY RATS

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A dissertation submitted to the Faculty of Health Sciences, University of Witwatersrand, School of Physiology in fulfilment of the requirements for the degree of Master of Science in Medicine

Johannesburg, 2015

DECLARATION

I, **Maphoko Adelade Masemola**, declare that this dissertation is my own work. It is being submitted for the degree of Master of Science in Medicine in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other University.

……

Maphoko Adelade Masemola

Signed on the……..day of…….2015

DEDICATION

To

GOD

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ABSTRACT

The early years of life are critical for neuroendocrine development. Early life adverse events during this developmental period can alter the Hypothalamic-Pituitary-Adrenal (HPA) axis with consequences on physical and mental health. These consequences may persist into adulthood and result in the dysregulation of the HPA axis and immune system, increased susceptibility to developing obesity, metabolic syndrome and cardiovascular complications. The present study determined the long term effects of early life stress (maternal separation), adolescent social isolation and foot-shock stress on food intake, body mass gain, visceral fat mass, glucose concentrations, insulin concentrations and HOMA-IR (Homeostatic model of insulin resistance), the innate immune response and corticosterone concentrations in male and female Sprague Dawley rats. Our findings show that whilst maternal separation in male rats resulted in significantly ($p<0.05$) increased visceral adiposity, hyperinsulinemia and increased corticosterone; foot-shock stress resulted in decreased body mass gain, visceral fat mass and plasma glucose concentrations and activation of the innate immune response in male rats. Social isolation in female rats resulted in significantly ($p<0.05$) increased feed conversion ratio, body mass gain, visceral adiposity and hyperinsulinaemia. The findings of the current study indicate that social isolation increases the parameters that contribute to the development of metabolic syndrome in female rats. However, in male rats maternal separation increases markers (visceral obesity and hyperinsulinaemia) of metabolic syndrome. This draws attention to the fact that male and female rats respond differently to specific stressors. The mechanisms underlying these variations in gender related stress responses remain unclear.

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LIST OF ABBREVIATIONS

ACE: Adverse Childhood Experience

ACTH: Adrenocorticotropic hormone

CDC: Centres for Disease Control and Prevention

CRH: Corticotrophin releasing hormone

FS: Foot-shock

GC: glucocorticoids

GH: Grouped housing

HOMA-IR: Homeostatic model of insulin resistance

HPA: Hypothalamic-Pituitary-Adrenal

MS: Maternal separation

NFS: Non foot-shock

NMS: Non-maternal separation

PND: Post-natal day

ROS: Reactive oxygen species

SAM: Sympathetic-Adrenal-Medullary

SI: Social isolation

WBC: White blood cell

CHAPTER 1: INTRODUCTION

1.1 Introduction

Early life stressors, such as poverty, abuse and neglect can have long term consequences on both physical and mental health (Springer *et al.,* 2003; Shonkoff *et al.,* 2012). During the early years of life, the brain undergoes rapid development and is most sensitive to environmental influences (Thompson, 2001; Shonkoff, 2009). Vulnerability to early life stress may result in dysfunction of the child's stress responses and development (Knudsen, 2004; Flaherty *et al.,* 2006). Problems in adjusting to stress later in development may also occur as a result of abnormalities in the systems that govern the response to stress (Zannas and West, 2014).

Stress is an important part of human development and is a challenge to the natural homeostasis of an organism (Bose *et al.,* 2009), resulting in behavioural and physiological responses to regain the homeostasis (Wheatley *et al.,* 1997; McEwan 1998; Muller and Holsboer, 2006; Bose *et al.,* 2009; Strekalova and Steinusch, 2010). Exposure to psychological stress may occur at any stage throughout the lifespan and at different intensities (Maniam and Morris, 2012). The body's response to stressful stimulation is mediated by complex systems including the Sympathetic-Adrenal-Medullary (SAM), Hypothalamic-Pituitary-Adrenal (HPA) axes and the immune system.

Toxic stress, which is described as frequent or prolonged adversity often leading to prolonged activation of the stress response, may result in psychopathologies and physical health disorders (National Scientific Council on the Developing Child 2005; Gunner *et al.,* 2009). Childhood trauma, maltreatment and neglect are examples of such early life stressors. The consequences of these toxic early life stressors include impaired memory and learning, anxiety disorders, depression, susceptibility to disease and infection, obesity,

cardiovascular disease, metabolic syndrome and dysregulation of the HPA axis (Heim *et al.,* 2000; VanItallie, 2002; Bentley and Widom 2009; Lupien *et al.,* 2009). Dysregulation of the HPA axis has been implicated in a number of chronic illnesses, including mental health issues (Springer *et al.,* 2003; Hulme, 2010; Shonkoff *et al.,* 2012,) and the metabolic syndrome (VanItallie, 2002; Bose *et al.,* 2009).

Metabolic syndrome is a cluster of symptoms that include abdominal obesity, atherogenic dyslipidaemia, hypoalbuminaemia, increased blood pressure, insulin resistance, glucose intolerance and hypercholesterolaemia (Alberti *et al.,* 2006; Despres and Lemiex 2006). It is well documented that obesity is the main contributor and risk factor in the development of metabolic syndrome (Eckel *et al,* 2005; Wahba and Mak, 2007; Jung and Choi, 2014). Furthermore, stress related eating has been linked to an increased risk of developing obesity (Dallman, 2010). Stress-related feeding behaviours are learned responses to stressful events and are thought to be a mechanism of coping with stress and emotions (Dallman, 2010; Pretlow, 2011) and are associated with the consumption of comfort foods which are high in calories (Pecoraro *et al.,* 2004; Dallman *et al.,* 2005). The consumption of these high caloric foods in response to stressor is associated with the development of obesity (Dallman *et al.,* 2005). Adult women with a history of child abuse had increased likelihood of developing obesity (Rohde *et al.,* 2008). A thirty year follow-up study found that adults who were abused as children had higher BMI scores (Bentley and Widom 2009).

In addition, chronic inflammation (as indicated by the presence of pro-inflammatory cytokines in adipose tissue; Karalis *et al.,* 2009) is a characteristic of obesity and the metabolic syndrome. Since stress has effects on both the HPA axis and the immune

response, dysregulation of these systems may be associated with the development of obesity (Raison *et al.,* 2006). Thus, it can be proposed that there is a three-way relationship between the HPA axis, the immune system and the development of obesity. Furthermore, activation of the stress response has been shown to have inhibitory effects on the inflammatory/immune system.

The effects of stress on the consumption of palatable food (Adams and Epel, 2007; Begg and Woods 2013; Aschbacher *et al.*, 2014), and the immune system (Dunn, 2007; Blume *et al.,* 2011), have been well documented, however the effects of early life stress on feeding behaviour, the development of obesity and on the immune system in later life is not well known. Understanding these complex associations may have important implications for improved identification of who is at risk of obesity as well as potential targets for intervention

1.2 Justification of study

The impacts of stress in later life have been well documented over the past several decades and this is due in part to the growing interest in the pathophysiology of stress and its psychopathological effects (McEwen, 2007). There is a dearth of information regarding the impacts of stress in early life on psychopathology. Early life stress is known to have detrimental effects on adult mental health (Springer *et al.*, 2003; Shonkoff *et al.*, 2012), it is therefore important to evaluate the effects of early life stress on physical health (Reppeti *et al.*, 2002).

Early life stress in the form of maternal separation in rodents is well documented in literature (Ryu *et al.,* 2008; Neigh *et al.,* 2005); however the period of childhood extends

beyond the weaning period and well into adolescence. Adolescence is an important part of childhood development and thus stress during adolescence is considered an early life stress. Thus, we hypothesize that the combined stressors of neonatal maternal separation, adolescent social isolation and further acute stress in adolescence may lead to development of feeding behaviours with life-long pathophysiological consequences in the offspring. Since maternal separation stress also programmes the response to stress in adolescence (Reynolds, 2010), it is important to understand what effects stressors at this critical stage of neurodevelopment might have on feeding behaviours. The findings will augment existing knowledge on the role of early-life stress on the response to stress incurred at later stages of life.

A large volume of literature has focused on the combined effects of chronic stress and a high caloric diet on the development of obesity and the metabolic syndrome (Dullman *et al.,* 2003; Pecoraro *et al.,* 2004; Dullman *et al.,* 2005; Adam and Epel, 2007; Torres and Nowson 2007). In chronically stressed females, the consumption of highly palatable food was associated with greater abdominal adiposity, oxidative stress and insulin resistance (Aschbacher *et al.,* 2014). We believe that such studies do not accurately evaluate the effects of the stressor on the development of obesity, because it is well documented that the frequent consumption of highly palatable food is the main driver of the development of obesity (Vickers *et al.,* 2011). In addition, chronic stress in adulthood is commonly used to evaluate the effects of stress, while the effects of early life stress on the development of obesity is a fairly new subject. It is therefore important to evaluate the effect that early life stress has on the development of obesity.

There is limited information regarding the mechanism of feeding responses to glucocorticoids, with specific focus on glucocorticoid concentrations and the subsequent changes in feeding neuropeptides. Since food intake and body mass changes were recorded weekly with everyday examination of glucocorticoids, the current study serves to provide information about the effects that glucocorticoids have on food intake and body mass.

The interaction between cortisol and adiposity is influenced by gender (Larsson *et al.*, 2009). It was shown that stress related eating behaviours were three times more prevalent amongst 16 year old girls than amongst boys aged 16 years old (Jääskeläinen *et al.*, 2014). With evidence that women can be more susceptible to stress induced eating, it is important to describe the gender differences in food intake and body mass changes in response to early life stress.

It is believed that glucocorticoids play a role in the regulation of the immune system (Chrousos, 1995; Dunn, 2007). Activation of the HPA axis can activate and/or suppress the immune system (Dunn, 2007; Blume *et al.*, 2011). The innate immune response is the first line of defence against pathogens. Thus, it is important to determine the innate immune response in order to understand the effect of the early life stress, adolescent social isolation and foot-shock stress on the immune system.

The endocrine, neurobiological and immune system responses to maternal separation are highly conserved in humans and rats (Gray *et al.*, 2004). Hence we used rats to investigate the changes that take place in children, and further develop this system as a model for investigating early life stressors. Furthermore rodent models can mimic more closely the type, intensity and duration of stress experienced by individuals in everyday life.

1.3 Aims of study

The broad objective of the current study was to determine the effect of early life stress on components that may contribute to the development of metabolic syndrome and the innate immune response in male and female Sprague Dawley rats.

The specific objectives were to determine the effects of:

1. early life stress (maternal separation) on food intake, body mass gain, visceral fat mass, glucose concentrations, insulin concentrations and HOMA-IR, the innate immune response and corticosterone concentrations in male and female rats.
2. adolescent social isolation on food intake, body mass gain, visceral fat mass, glucose concentrations, insulin concentrations and HOMA-IR, the innate immune response and corticosterone concentrations in male and female rats.
3. foot-shock stress on food intake, body mass gain, visceral fat mass, glucose concentrations, insulin concentrations and HOMA-IR, the innate immune response and corticosterone concentrations in male and female rats.
4. early life stress (maternal separation), adolescent social isolation and foot-shock stress on food intake, body mass gain, visceral fat mass, glucose concentrations, insulin concentrations and HOMA-IR (Homeostatic model of insulin resistance), the innate immune response and corticosterone concentrations in male and female rats

1.4 Hypothesis

The null and alternate hypotheses of the study were:

i. H_0: Neonatal maternal separation, adolescent social isolation and foot-shock stress contribute to the development of metabolic syndrome and the dysregulation of the innate immune response in male and female Sprague Dawley rats.

ii. H_1: Neonatal maternal separation, adolescent social isolation and foot-shock stress contribute to the development of metabolic syndrome and the dysregulation of the innate immune response in male and female Sprague Dawley rats.

CHAPTER 2: LITERATURE REVIEW

2.1 Early life stress

The concept of stress dates as far back as 1936, when Selye defined stress as the "non-specific response of the body to any demand for change", a definition that continues to be used. Selye (1936) noted that animals subjected to acute physical stimuli exhibited pathologic changes which later resulted in various diseases (Selye, 1936). The impacts of stress in later life have been well documented over the past several decades (McEwen, 2007) and this is due in part to the growing interest in the pathophysiology of stress and its psychopathological effects. Currently there is great deal of interest in the impact of stress in early life (McEwen, 2007).

Early life stress refers to the negative emotional experiences and or adverse life events that an individual may become exposed to in early childhood (Baum, 1990; Petchel and Pizzagalli, 2011). Be it acute or chronic, early life stress can result in physiological, psychological and cognitive changes. Early life stresses are experienced during the developmental stages between gestation and adolescence (La Prairie *et al.*, 2010). It is during this period that children are most vulnerable to adverse life events that may affect their development (Knudsen, 2004; Flaherty *et. al*, 2006).

There is increasing evidence indicating that conditions experienced during early life can influence an individual long after the resolution of the situation (Maniam and Morris, 2012). Adverse early life events can have long lasting impacts on physical and mental health that may persist well into adulthood (Torres *et al.*, 2007). A study by Edwards *et al* (2003) reported a dose-dependent relationship between the number of forms of maltreatment reported and the mental health scores of adults that experienced adversity in

childhood. This suggests that the co-occurrence of different types of childhood maltreatments has a serious effect on the long term mental health of an individual.

Early life experiences occur during a time of brain plasticity, often resulting in early life programming (Knudsen, 2004). It is believed that there is a 'window period' during which physiological changes occur and affect the subsequent development (Reynolds 2010). These physiological changes are often associated with psychological effects. In humans, a stressful event such as loss of a parent or sexual abuse during childhood increases the risk of developing anxiety disorders and depression, accompanied by abnormal HPA axis responses (Heim *et al.,* 2000). It is also important to note that not all children who experienced childhood adversity develop health and or psychological problems (National Scientific Council on the Developing Child 2005). The developing brain is influenced by factors that may contribute to resilience and vulnerability to stress related diseases (Franklin et al., 2012; Zannas and West, 2014).

Individual susceptibility to stress has been implicated in the development of disease, and often genetic factors play a role (Franklin *et al.,* 2012). Susceptible individuals respond poorly to stressors and may exhibit signs of stress vulnerability, while those with resilient traits may develop appropriate adaptive mechanisms (Franklin *et al.,* 2012; Zannas and West, 2014). Resilience is a positive adaptation despite adverse life events. How individuals cope with stress has implications for their future wellbeing and adaptation (McEwen, 1998). Coping mechanisms play a role in whether or not stress related disease may develop or not, however the basis of coping mechanism is not clearly defined.

There is also an indication that responses to later life stress may be programmed by early life stress (Reynolds 2010). Heim and co-workers (2000) reported that adults who

experienced abuse in early life have increased endocrine and autonomic responses to stress. In adulthood, juvenile stress exposure resulted in anxiety-like behaviours and depressive-like behaviours (Tsoory *et al.,* 2006) as well as reduced exploratory behaviour (elevated plus maze) of rats in the elevated plus maze (Jacobson-Pick and Richter-Levin, 2010). This suggests that early life stress may predispose an individual to become vulnerable to stressors. However, the vulnerability of individuals to stressors is dependent on the type of stressor (Franklin *et al.,* 2012).

2.1.1 Types of early life stress

Stresses vary in intensity, duration and the effect they have on the individual. Three types of stress responses have been determined, namely positive, tolerable and toxic stress (National Scientific Council on the Developing Child 2005). Positive stress responses refers to short duration responses that result in minor physiological changes that return to baseline with the help of responsive adult (National Scientific Council on the Developing Child 2005). Physiological changes to positive stress are brief; they include an elevation in heart rate, blood pressure and stress hormone levels (McEwen, 2007). Positive stressors form part of the normal development of children, for example the anxiety associated with a visit to the doctor/dentist and the first day of school.

Tolerable stress results from a temporary change, such as the loss of a family member or natural phenomenon (Skonhoff *et al.,* 2012). Although tolerable stress elicits a greater physiological response, with a good emotional support system the physiological response to tolerable stress may be reduced and will return to baseline (National Scientific Council on the Developing Child, 2005). For example, human handling of rat pups results in an increased nurturing behaviour (licking and grooming) by the mother which in turn

enhances the HPA axis functioning of the pup (Liu *et al.,* 1997). As much as tolerable stress can progress to positive stress; it can also become toxic stress. Without a proper support structure, tolerable stress can become toxic stress.

Toxic stress results from strong, frequent or prolonged adversity often leading to prolonged activation of the stress response. Prolonged activation of the stress response affects different body systems, eventually leading to psychological and health impairments (Shonkoff *et al.,* 2012). Toxic stress response is often cause by child abuse and neglect, loss of a parent, maternal depression and household dysfunction (Springer *et al.,* 2003; Shonkoff *et al.*, 2012).

2.1.2 Environmental factors of early life stress

Genetic variability plays an important role in the susceptibility and resilience to early life stress. Environmental influences can have an impact on stress reactivity. The environment in which a child is brought up in is important; it influences the child's development (Walker *et al.,* 2007). The relationship between parent and child is considered one of the most important with regards to childhood development. Bowlby (1982) showed that the relationship between the parent and child was especially important for the development of a psychologically healthy adult.

Abuse, neglect, parental loss and household dysfunction are among the most common factors of early life stress (Anda *et al.*, 2006; Bruwer *et al.*, 2014; Suzuki and Tomoda, 2015). A Adverse Childhood Experience (ACE) study done by the Centre for Disease Control and Prevention (CDC) reported that adverse childhood experiences are related to risky health behaviours in childhood and adolescence. The CDC found that children exposed to adverse childhood experiences had increased incidences of pregnancies, suicide

attempts, early initiation of smoking, sexual activity and illicit drug use during adolescence (Middlebrooks and Audage, 2008). It was also found that the number of adverse childhood experiences were proportional to the number of co-occurring health conditions (Middlebrooks and Audage, 2008). Furthermore, an emotionally abusive family environment was found to attenuate the decline in mental health scores of adults who experienced various form of childhood adversity (Edwards *et al.,* 2003).

2.1.3 The stress response (HPA axis)

The hormone mediators of the stress response are essential for adaptation, survival and the maintenance of homeostasis (McEwen 1998). Upon activation by stress the hypothalamus is activated and a stress response is initiated. Rapid responses are produced by the 'fight or flight' response as mediated by the Sympathetic-Adrenal-Medullary (SAM) axis while slow but long acting responses are regulated by the Hypothalamic-Pituitary-Adrenal (HPA) axis (Compass, 2006).

The HPA axis is the neuroendocrine system that regulates the production of glucocorticoids from the adrenal cortex that is cortisol in human beings and corticosterone in rodents. Activation of the HPA axis is the main homeostatic response in mammals. The stress response is initiated by the release of corticotrophin releasing hormone (CRH), from the paraventricular nucleus. CRH in turn results in secretion of adrenocorticotropic hormone (ACTH) from the anterior pituitary, which stimulates the release of glucocorticoids from the adrenal cortex (figure 2.1). Unlike the acute stress response (fight or flight), which results in the release of catecholamines (adrenalin and noradrenalin), glucocorticoid production may take several minutes and is estimated to peak at 25 minutes post stressor with impacts lasting for a longer duration. Glucocorticoids such as cortisol are

thus a direct measure of the level of stress in an individual (Marti and Armario, 1998; Bhatia *et al.*, 2011).

Glucocorticoids interact with receptors of several systems in the body in order to achieve homeostasis. They (glucocorticoids) mobilize stored energy, increase cardiovascular tone, suppress anabolism, and many other protective mechanisms. However, prolonged glucocorticoid secretion has been implicated in a number of clinical disorders (Chrousos, 1995; Torres *et al.*, 2007; Maniam and Morris 2012) (Figure 2.1).

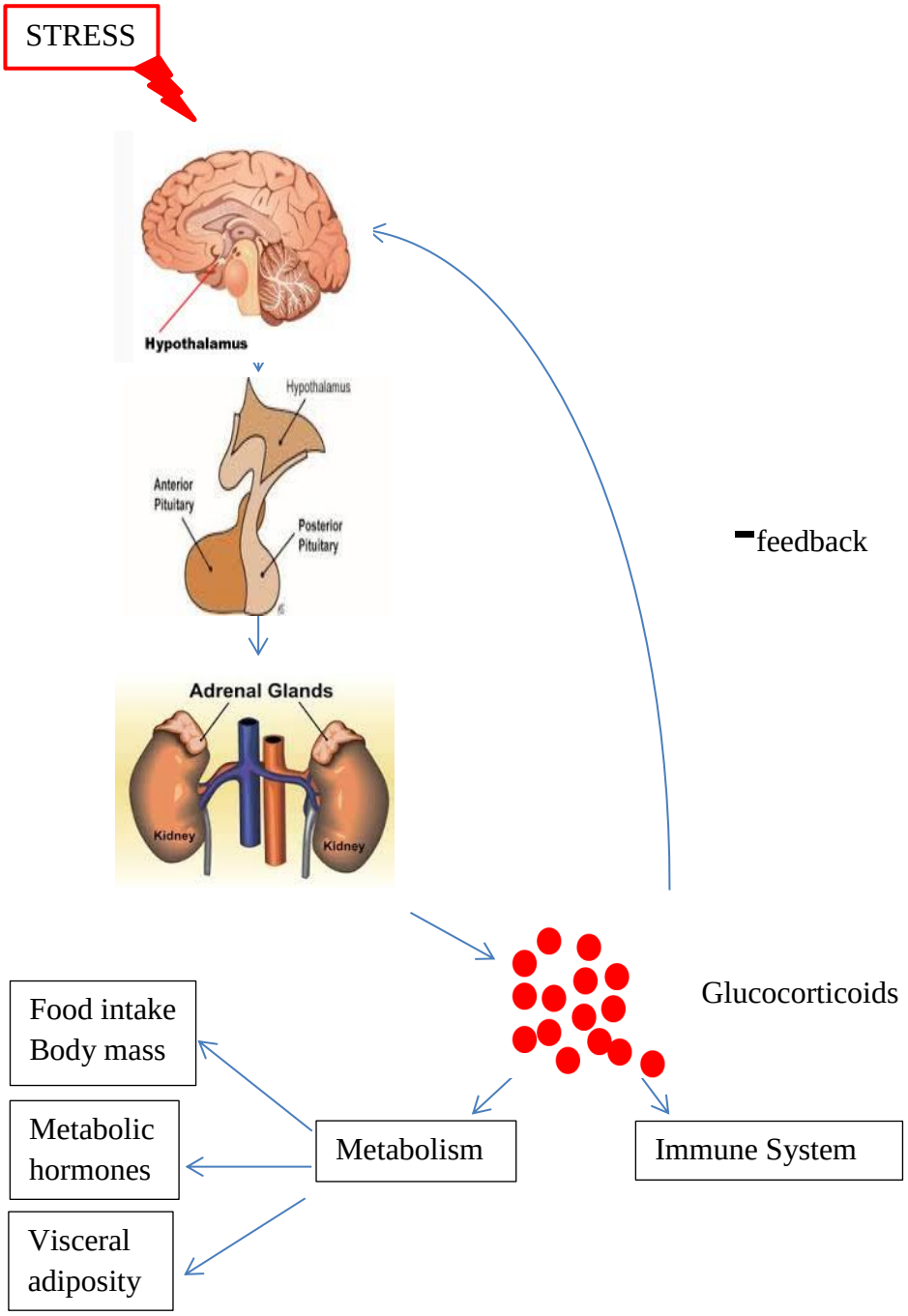


Figure 2.1 A schematic representation of HPA axis activation

Stress activates the hypothalamus resulting in the release of CRH from the paraventricular nucleus, which then stimulates the release of ACTH in the anterior pituitary gland. ACTH stimulates the adrenal cortex to release glucocorticoids which modulate the HPA axis activity via the negative feedback. Under chronic stress, GC can modulate the immune system and metabolism. Copyright: Maphoko Masemola (2015) School of Physiology, Health Sciences, University of the Witwatersrand.

2.1.4 Stress and gender

Historically gender differences in medical and psychiatry disorders were largely ignoredMales and females differ in their response to psychological and physiological stressors. For example men are more likely to suffer substance related disorders such asalcohol and drug abuse (Degenhardt *et al.,* 2008). Gender studies documenting differences in stress response have demonstrated that sex hormones play a role in the regulation of the HPA axis (McCormick *et al.,* 2002). Thus it is of great importance to determine the stress responses of both male and female rats.

2.2 Stress and feeding behaviour

The altered HPA axis activity and the symptoms of anxiety and depression are implicated in the pathophysiology of eating disorders (Ryu *et al.,* 2009; Jahng 2011, Goodman and Whitaker, 2002). Stress has been shown to induce either increased or decreased food intake (Torres *et al.,* 2007). The effects of stress on feeding behaviour are dependent on the severity of the stressor (Maniam and Morris 2012; Torres *et al.,* 2007).

The paraventricular nucleus provides the overlap between stress and the feeding system. The paraventricular nucleus not only contains the cell bodies of CRH containing neurones but is also the centre of convergence of the neural circuits that regulate energy intake, thus regulating the HPA axis activity and also responds to insulin and glucocorticoids (Maniam and Morris 2012; Nieuwenhuizen and Rutters 2008).

The mechanisms involved in the development of obesity via stress-induced hyperphagia are complex and involve multiple pathways. These pathways include the cross communication between glucocorticoids and the neuropeptides and hormones associated

with feeding (Adams and Epel, 2007). It is widely accepted that glucocorticoids have an orexigenic effect through the effect of Neuropeptide Y (Nieuwenhuizen and Rutters 2008; Pernavidou and Chrousos, 2012). Glucocorticoids stimulate feeding responses by increasing the release of Neuropeptide Y, inhibiting CRH, inducing leptin resistance and hyperinsulinemia (Maniam and Morris, 2012; Foss and Dyrstad, 2011; Adams and Epel, 2007). The interactions between these hormones may facilitate the storage, distribution and release of energy (Adams and Epel 2007; Sinha and Jastreboff, 2013).

In addition to altered HPA axis activity being implicated in the regulation of feeding behaviour, disturbed eating behaviours and lifestyle parameters play a role in the pathogenesis of obesity. Stress related feeding behaviours, including emotional eating and the consumption of "comfort foods", are implicated in the development of obesity. Emotional eating is the 'tendency to overeat in response to negative emotions such as anxiety or irritability and is thought to be a learned response in coping with stress and emotions' (Bruch, 1973). A study by the American Psychological Association reported that 43% of people use food to cope with stress (American Psychological Association Press Release, 2007). Furthermore, it was shown that anxiety and depression were associated with emotional eating in overweight children and adolescents (Goossens *et al.,* 2009). Emotional eating is often associated with the consumption of comfort foods, in a process termed the reward processing.

The reward system plays an essential role in the feeding behaviour. It is believed that the consumption of highly palatable foods may activate the reward pathway and result in "neurobiological adaptations" leading to compulsive overeating (Adams and Epel, 2007). Increased consumption of palatable foods may reflect a pleasurable activity that reduces or

counteracts the discomfort of the stress, presumably via the same mechanism as alcohol and drugs of abuse (Dallman, 2009; Mutschler and Kiefer 2011; Adams andEpel, 2007). Maniam and Morris (2012) suggested that these are regulated by the reward pathway involving dopamine and opioids and are not sensitive to satiety, leading to overconsumption and the development of obesity.

2.3 Stress and obesity

The earliest indication of HPA axis involvement in body weight gain and metabolic disorders was obtained from the clinical observations of Cushing's syndrome patients (Bose *et al.*, 2009; Reynolds 2010; Foss and Dyrstad 2011). Cushing's syndrome is characterised by increased cortisol concentrations, increased body weight as well as metabolic disease. Hence, Cushing's syndrome demonstrates that increased HPA axis activity/cortisol concentrations are involved in body weight regulation. Vicennati *et al.,* (2009) found that free urinary cortisol concentrations were greater in women who had gained weight after a stressful event, when compared to women whose obesity was not stress related and to women of normal weight. The study also found that women who gained weight after a stressful event gained a greater weight in a shorter time period, providing further evidence that cortisol concentrations are involved in the development of obesity (Vicennati *et al.,* 2009). Infusion and administration of glucocorticoids have also been shown to increase caloric intake in humans and animals (Dallman *et al.,* 2007).

Stress-induced eating often leads to increased body fat mass and eventually obesity. Obesity results from an energy imbalance, with energy intake exceeding energy expenditure leading to the storage of energy as fat. Co-morbidities such as coronary heart disease, type two diabetes mellitus, hypertension and dyslipidemia cluster together to

develop the metabolic syndrome. Abdominal obesity and insulin resistance are the major underlying factors that contribute to the development of metabolic syndrome with detrimental consequences (Despres and Lemieux, 2006; Standl, 2005). Obesity results in increased fasting plasma total cholesterol, triglycerides but reduces high density lipoprotein cholesterol (HDL-C) resulting in increased risk of atherosclerosis and cardiovascular disease (Standl, 2005). Also implicated with the increase in triglyceride, cholesterol and non-high density lipoproteins is the increase in proinflammatory cytokines and C-reactive protein (Wajchenberg *et al.,* 2009).

2.4 Immune response

The immune response in rodents is affected by stress (Sacerdote *et al.,* 1994) and it is believed that glucocorticoids play a role in the regulation of the immune system (Chrousos, 1995). Reactive oxygen species (ROS) are produced by activated neutrophils and macrophages in response to pathogens (Forman and Torres, 2002; Apel and Hurt, 2004; Fialkow *et al.,* 2007). Adams (1994) argued that the production of ROS may be influenced by psychological stress. There is wide variation in the literature with regards to ROS production in response to stressful stimuli. Repeated exposure to foot-shock stress has previously resulted in the suppression of superoxide production in alveolar macrophages of male Fisher rats (Oishi *et al.,* 2003). In another study, foot-shocked mice were found to have decreased natural killer, lymphokine activated killer and cytotoxic T lymphocyte activities compared to non foot-shocked mice (Li *et al.,* 2005). There have been reports of increased ROS production by blood monocytes in response to foot-shock stress (Coe *et al.,* 1988).

The over production of ROS can lead to oxidative stress resulting in tissue damage (Coe *et al.,* 1988; Halliwell *et al.,* 1992). Such effects on the immune response may result in unfavourable consequences such as delayed healing, the reduced response to vaccines, the shortening of telomere lengths as well as the development and progression of cancer (Glaser *et al.,* 2000; Cole-King and Harding, 2001; Cohen *et al.,* 2001; Epel *et al.,* 2004;Reiche *et al.,* 2004; Gouin and Kiecolt-Glaser, 2011). Thus, it is important to monitor the innate immune response in order to understand the effect of the HPA axis response on the immune system.

2.5 Experimental animal models

Conducting studies to assess the effects of childhood adversity have limitations because of the ethical implications that come with human experimentations. It is problematic to determine early programming of the stress response in humans, because of the invasive processes that may have detrimental consequences. Furthermore, human stress studies have relied on retrospective self-reported incidences of childhood adversity. These retrospective self-reports often lack accuracy since they are influenced by factors such as forgetting, repression and reporting biases (Heim *et al.,* 2008). Hardt and Rutter (2004) found that retrospective self-reports are more likely to yield underestimates of abuse and neglect. It was found that a third of adults that were asked about well-documented serious cases of abuse and or neglect that they experienced in childhood, did not report its occurrence (Hardt and Rutter, 2004).

Animal models of human processes offer the ability of simulating a human condition under controlled conditions, are less complex and can include a large sample size. Animal models of early stress have been widely used to study the effects of early life stress on the

development of stress related disorders. Thus, these models can provide insight into the long term effects of early life stress in animals and, by extrapolation, in humans as well.

Animal models of early life stress have been established. Early life stress has been studied in non-human primates, rabbits, pigs, guinea pig, birds, and most extensively in rats and mice. The latter, rats and mice are consistently used due to their availability, cost-effectiveness and common use in physiological and molecular studies.

2.5.1 Maternal care

In rodents, the early relationship between mother and offspring is essential in the development of the offspring, given that rodents are completely dependent on their mothers for the first three weeks of life (Jahng, 2011). Maternal care in rodents occurs in the form of nursing, licking and gathering the pups (Stern and Johnson, 1990).

Strong maternal behaviour, such as licking and grooming of the infant produces animals that are less emotionally reactive, whilst poor maternal care results in increased emotional reactivity in the animals (Liu *et al.*, 1997). Thus the interaction between mother and pups has major effects on the developmental process and maternal absence can be considered a harmful agent (Oliveira *et al.,* 2011). Attachment is important for the normal development of offspring.

2.5.2 Mother-infant separation

As mentioned earlier, rat pups rely on their mothers for their nutrition, warmth and grooming, and thus separation from their mothers may be considered stressful. There are two forms of separation, neonatal maternal separation and neonatal maternal isolation. Neonatal maternal isolation is a process whereby the rat pups are not only removed from

their mother but are also kept separate from one another. This form of separation evokes very high levels of stress in the rat pups. For the purpose of this dissertation the focus was more on neonatal maternal separation.

Neonatal maternal separation is a process whereby neonates are repeatedly separated from their mother for a certain period of time, resulting in the disruption of normal attachment. This disruption of normal attachment causes intense stress to the neonates which may affect the response to later life stress and may cause health problems (Oliveira *et al.*, 2011). Neonatal maternal separation is a well-established animal model of early life stress, causing permanent changes in HPA axis (Ryu *et al.,* 2008) which may contribute to growth, behavioural and cognitive disturbances (Lupien *et al.,* 2009). The age at which separation occurs and the number of days during which separation occurs varies between laboratories.

2.5.3 Adolescent social isolation

Adolescence is an important period of human development and involves the maturation of several systems including the physiological, cognitive, emotional and social development (Traslaviña *et al.,* 2014). In humans, the interactions with peers during adolescence are thought to be important for social development, because individuals spend more time interacting with peers during adolescence than any other developmental period (Jahng *et al.,* 2012). Early (adolescent) disruption of social interaction between the same species may also affect the brain development leading to prolonged aberrant behaviour (Jahng *et al.,* 2012). Social interaction in the form of play fighting and social grooming is important for normal social, emotional and cognitive development in rats (Jahng *et al.,* 2012).

In rats, the period from post-natal day (PND) 21 is comparable to early adolescence in humans (Whitaker *et al.,* 2012). In a typical social isolation rearing procedure, the rat pups are weaned from the dam between PND21-PND 28 and are housed individually. From this point, the socially isolated rats are not to be handled more than once a week (Fone and Porkess, 2008). Socially isolated rats do not have any further social interaction with other rats. However, they are reared in a room with other socially isolated and group-housed animals and thus have visual, auditory and olfactory contact with other rats (Fone and Porkess, 2008).

In social species, such as the rat, social isolation is aversive and stressful and increases the HPA activity (Jahng *et al.,* 2012). Furthermore Weiss and colleagues (2003) concluded that social isolation increased emotional reactivity to stress and produced a hyper-function of the HPA axis in adult rats (Weiss *et al.,* 2003).

2.5.4 Electric foot-shock stress

Rodents are very susceptible to stress and elicit a rapid response to stressful stimuli. There are several standard animal models of stress including immobilization, forced swim test, novelty, water immersion and foot-shock. Researchers have used electric foot shock of varying degrees (mild, acute, chronic) to evoke stressful conditions in rats (Bhatia *et al.,* 2011). Psychological stressors were shown to have lower influences than foot-shock stress as measured by increase in body temperature and corticosterone concentrations during stress exposure (Endo *et al.*, 2001). The major advantage of using foot-shock stress is that it causes high degrees of stress in animals (Bhatia *et al.,* 2011).

CHAPTER 3: MATERIALS AND METHODS

3.1 Ethical approval

Ethical approval for the study was obtained from the Animal Ethics Screening Committee (AESC) of the University of the Witwatersrand, (Ethics clearance number: 2013/04/05). The experiments were thus conducted in accordance with the laws and regulations controlling the use of animals in experiments.

3.2 Study setting

3.2.1. Housing and feeding of animals

All the rats used in the current study were obtained from the University of the Witwatersrand Central Animal Services unit. The rats were housed in Plexiglass rat cages containing wood shavings and shredded paper for bedding. They were allowed *ad libitum* access to standard rat feed (Epol®, Mice cubes, South Africa) and water. The room ambient temperature was kept at 21±2°C. Lights were kept on a 12hr light-dark cycle with the lights on from7:00hrs to19:00hrs.

3.3 Animals

A total of 96 Sprague Dawley rat pups, with equal numbers of males and females (males= 48, females =48), obtained from 11 Dams, were used in the study. The pregnant dams were reared in a standardized controlled environment to minimize unwanted external stimuli (such as noise). Twelve hours after confirming delivery, (PND 1), the rat pups were left undisturbed with their dams until PND7.

3.4 Experimental Design

To achieve the desired goal of assessing the effects of continuous stressors during development this study was conducted in three experimental phases. The first phase

consisted of daily periods of separation of the neonates from their dams. The second phase was the differential housing phase following weaning (that is, socially isolated or group housed) and then the third phase was the electric foot-shock in the juvenile phase.

The maternal separation phase was implemented to investigate the effect that early life stress initially has on food intake and body mass, followed by investigating the subsequent immune system and HPA-axis response to a secondary stressor. The social isolation phase was implemented to investigate whether, following maternal separation, social isolation has any effect on the food intake, body mass, immune response and the HPA axis response. Electric foot-shock phase was applied to investigate whether social isolation followed by a secondary stressor (foot-shock) differentially affects food intake, body mass, immune response and the HPA axis response.

Experimental Phase 1: Maternal separation

From PND 7 to PND 21 the rat mothers were equally and randomly allocated to a group that was subjected to maternal separation (MS; n=48) or to a group that was not subjected to maternal separation (NMS; n = 48), with equal numbers of male and each female pups allocated to each group. Maternal separation (MS) consisted of removing rat pups from their home cages and placing them in a new cage with clean bedding for 180 min daily (between 09:00hrs – 12:00hrs) from PND 7 to PND 21. No additional treatment to keep the pups warm during the separation period was offered, so limited pup cooling during neonatal maternal separation was expected. The non-maternally separated group (NMS) remained undisturbed with their dams until weaning, except for the routine changing of the cages.

Experimental Phase 2: Weaning and social isolation

On PND 21, the rat pups were weaned and the male and female pups were separated and housed in separate rooms. Following weaning the rats from the NMS and MS groups were equally and randomly allocated to the grouped housing (GH) or socially isolated groups (SI). The GH rats were housed in pairs while the SI rats were housed individually. Thus four groups (n=12) for both sexes were established:

1. Maternally separated and socially isolated (MS + SI)
2. Maternally separated and group housed (MS + GH)
3. Non maternally separated and socially isolated (NMS + SI)
4. Non maternally separated and group housed (NMS + GH)

Experimental Phase 3: Electric foot-shock

The third phase took place between PND 42 and PND 69. On PND 42, the rats from each group i.e. MS + SI, MS + GH, NMS + SI and NMS + GH were further, equally and randomly, allocated to the foot-shock group (FS) or Non foot-shock group (NFS). Between PND 42 and PND 52, rats were either foot-shocked or non foot-shocked.

Eight groups (n=6) for both sexes were established

i) Maternal separation+ individual housing + foot-shock = MS + SI+ FS

ii) Maternal separation+ individual housing + non foot-shock = MS + GH + FS

iii) Maternal separation+ group housing + foot-shock = MS + SI + NFS

iv) Maternal separation+ group housing + non foot-shock = MS + GH + NFS

v) Non-Maternal Separation + individual housing + non foot-shock = NMS + SI + FS

vi) Non-Maternal Separation+ individual housing + foot-shock = NMS + GH+FS

vii) Non-Maternal Separation+ group housing + foot-shock = NMS + SI + NFS

viii) Non-Maternal Separation+ group housing + non foot-shock = NMS + GH + NFS

Stress by electric foot shock was applied in a Plexiglas foot-shock box (34x32x31cm). The floor of the foot-shock box consisted of 18 stainless steel rods (Modular foot-shock floor, Habitest Operant Cage, Coulbourn Instruments, Pennsylvania, USA) connected to a shock generator (Precise Animal Shocker, Coulbourn Instruments, Pennsylvania, USA) set to deliver 2mA at 1.6Hz for one second. The FS rats were placed in the foot-shock box individually and allowed to explore for 2 minutes, thereafter foot-shock was applied in three successions each being 20 seconds apart. All foot-shock sessions took place during the light phase. The animals in the non foot-shock groups were placed in the foot-shock box for 3 minutes but did not receive the foot-shock stress.

From PND53 to PND 69, all rats were left in their respective cages for 17 days to allow sufficient time for the body mass fat mass and hormonal changes to take place (Figure 3.1).

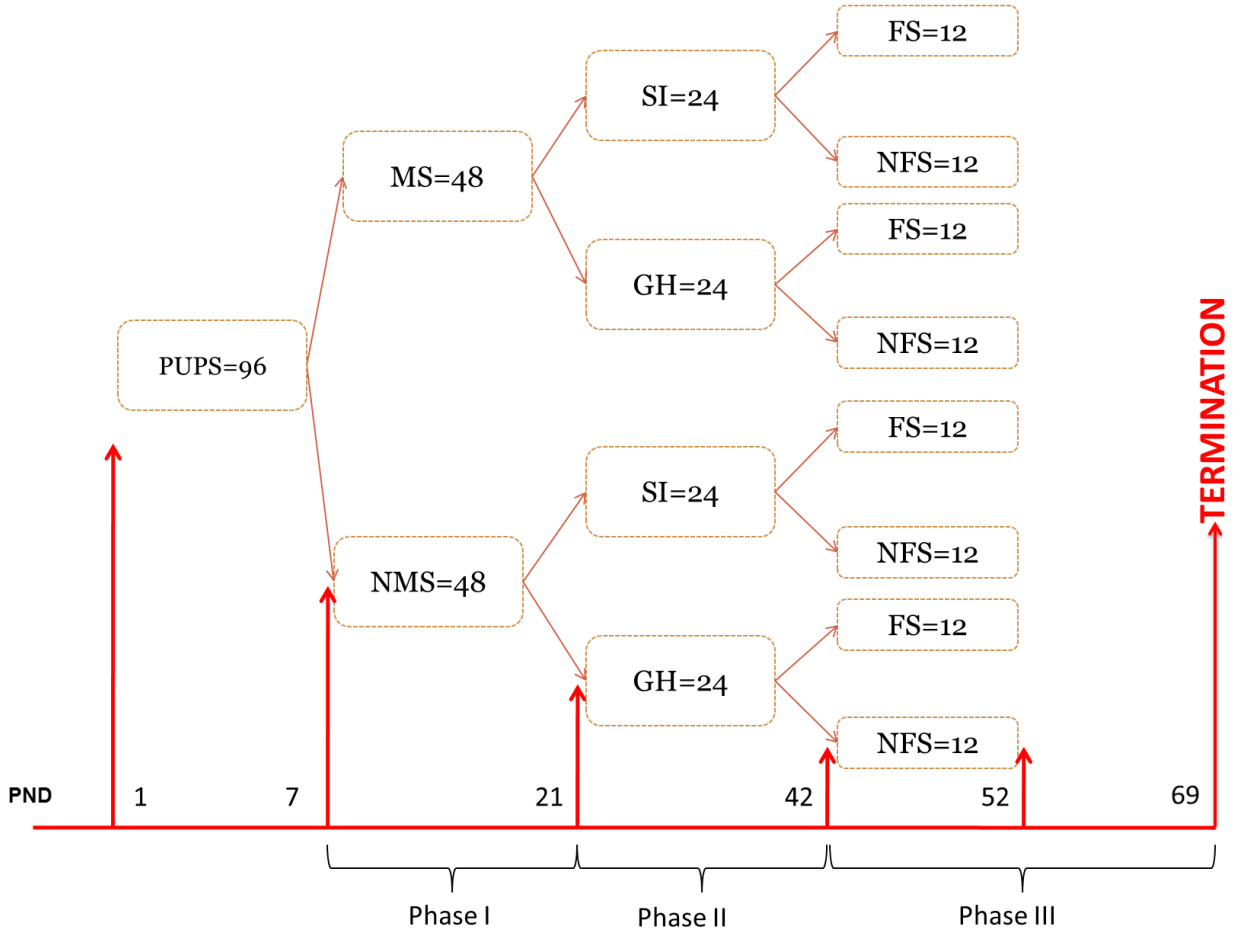


Figure 3.1: A schematic representation of the order of events of the study design.

MS = maternal separation; NMS = non-maternal separation; SI = social isolation; GH= group housing; FS = foot-shock; NFS = non foot-shock; PND = post-natal day

3.5. Body mass and food intake measurements

The rats were weighed (Snowrex Electronic Scale, Clover Scales, Johannesburg) weekly to monitor growth. To evaluate feeding behaviour of rats during the duration of the study, feed was weighed using a balance scale (Snowrex Electronic Scale, Clover Scales, Johannesburg) at the beginning and at the end of each week and then weekly food intake calculated. Feed conversion ratio was calculated to determine food intake relative to body mass gain from the formula;

Feed conversion ratio= feed intake (g)/body mass gain (g)

3.6 Termination procedures

At the end of phase 3 (PND 69), the rats were fasted overnight and then terminated by decapitation using a guillotine. Each decapitation was performed by a trained and authorized Principal Animal Technologist of the Central Animal Service in an isolated room away from all the other animals. The guillotine was placed upon a sterile and stable bench top and sterilized before introducing any rats. Euthanasia by decapitation minimizes the stress inflicted on the animal (IACUC Guidelines, Boston University Medical School, 2007).

3.6.1 Blood collection, processing and plasma storage

Trunk blood was collected from the site where the animal was decapitated. A funnel was used to direct the blood into 6ml Lithium Heparin blood tube (Greiner bio-one,) and 6ml Z Serum Sep Clot Activator blood tubes (Greiner bio-one,Kremsmunster, Austria). One millilitres of whole blood was aspirated from the Lithium Heparin tube and was used for reactive oxygen species determination and white blood cell counts. The remainder of the blood samples were spun for 15 min at 5000g at 20 ^{0}C in a Sorvall RT® 6000B centrifuge (Pegasus Scientific Inc., Rockville USA). The plasma and serum were collected and then stored at -70 ^{0}C until the analysis of insulin, glucose and corticosterone was performed. The analysis of these biochemical assays were done in duplicate.

3.6.2 Determination of reactive oxygen species

To quantify the production of reactive oxygen species (ROS), 100 µl of whole blood was added to 500 µl of phosphate buffered saline (PBS). The blood and PBS were mixed in a plastic test tube and immediately placed in a water bath (37° C). 100 µl of luminol was

then added and a blank or zero reading was taken prior to the addition of the phorbylmiristate acetate (PMA) (PMA, Sigma P8139). To stimulate ROS release 100 ul of PMA was added to the samples. The amount of ROS in the sample was then determined using a Clean-Trace Luminometer (UNI-LITE NG, Biotrace, South Africa) at 5 minutes intervals for 60 minutes. Each data point is a measurement of relative light units (RLUs) generated by the interaction of the endogenous peroxide and the luminol.

3.6.3 White blood cell determination

To determine white blood cell (WBC) counts, 40 µl of whole blood was diluted with 360 µl Turks solution to burst red cells and thereby increasing visualization of leukocytes. The diluted blood solution was then added to a haemocytometer chamber (Neubauer Chamber Cell Counting,) and the white blood cells were counted according to manufacturer instructions (Neubauer Chamber Cell Counting, Celeromics,Valencia, Spain). A detailed description of the protocol is attached in the appendix. The white blood cell count was calculated from the following formula;

WBC count (cells/ml) = (average cell count x dilution factor) / (volume of the counting chamber)

3.6.4 Determination of visceral fat mass

Following blood collection, the thorax and abdomen was cut open by midline incision. The viscera fat (abdominal fat pad, peri-renal and epididymal fat) were the dissected out and their combined mass determined using a balance (Mettler Toledo International Inc., Greifensee, Switzerland).

3.7 Determination of plasma glucose and insulin profiles

3.7.1 Determination of plasma glucose concentration

Plasma glucose concentrations were determined using a glucose assay kit (GLUC-PAP, Randox Laboratories Limited, County Antrim, United Kingdom) according to the manufacturer's instructions. The assay employed an enzymatic oxidation technique in which glucose is oxidised by glucose oxidase to gluconic acid and hydrogen peroxide. In the presence of peroxidase, the hydrogen peroxide reacts with phenol and 4-aminophenazone to form a quinoneimine dye indicator. Absorbances of the samples were read at 500 nm using a spectrophotometer (DU 720, General Purpose UV/Vis Spectrophotometer, Beckman Coulter Inc, California, USA).

A standard calibration serum was used as reference and the concentrations were calculated according to the following formula;

Glucose concentration (mmol/l) = (Sample absorbance/ Standard absorbance) ×Standard concentration

3.7.2 Determination of plasma insulin concentration

Plasma insulin was determined by sensitive rat insulin radioimmunoassay (RIA) kit (SRI-13K Sensitive Rat Insulin RIA, Millipore Corporation, Missouri, USA). The principles of procedure for this assay included the use of a radioactively labelled antigen incubated with an antiserum such that the antigen binding sites on the antiserum are limited. The unlabelled plasma antigen was then introduced to the system and thus the labelled and unlabelled antigens compete for binding sites on the antibody, the bound antigen was then quantified in a scintillation counter. A standard curve was constructed using standard calibrators.

3.7.2.1 Computation of the HOMA IR index

To assess whole body insulin resistance, HOMA-IR index was calculated as per the equation shown below;

HOMA-IR = [26 × fasting insulin (ng.ml-1) × fasting glucose (mg.dl-1)/22.5] (Murakami *et al.*, 2014)

3.9 Determination of corticosterone

3.9.1 Plasma corticosterone concentration determination

Plasma concentrations of corticosterone were determined using a commercially available double-antibody ^{125}I-corticosterone radioimmunoassay kit (ICN Pharmaceuticals; California, USA) as per manufacturer's instructions on all plasma samples. The assay employed the use of radioactively labelled corticosterone to quantify the amount of endogenous corticosterone. Radioactively labelled corticosterone and the plasma (which contains the endogenous corticosterone) were added to a test tube; thereafter a serum specific antibody was added. The radioactively labelled corticosterone and the endogenous then compete for binding sites on the antibody; the unbound (supernatant) corticosterone was then discarded, while the bound (in the form of a pellet) was evaluated in a scintillation counter. A standard curve was constructed using the provided calibrators and the plasma corticosterone concentrations in the samples were determined.

3.10 Statistical analysis

All data are expressed as mean ± SD. Data analysis was done using STATISTICA version 12.5. Statistical significance was considered when $P \leq 0.05$. The computerised multifactorial analyses starts with the assessment of three-way interactions, followed by

two-way effects and finally one-way analyses of variance to analyse the change in body mass, fat mass, feed intake, white blood cell counts and plasma corticosterone. Data for ROS was analysed by calculating the area under curve (AUC) for each sample, followed by a one-way analysis of variance (ANOVA) for the grouped data.

CHAPTER 4: RESULTS

4.1: Results for experiments on female rats

Section 4.1 deals with the results for female rats.

4.1.1 Effect of housing on body mass gain

Figure 4.1.1 shows the effect of housing on the terminal body mass gain as a percentage increase from the initial body mass. A three-way ANOVA showed no significant interactions between early life stress, housing and foot-shock stress on body mass gain ($p=0.554$). However, a one-way ANOVA showed that rats that were socially isolated had a significantly greater increase in body mass compared to rats that were housed in pairs (GH) ($p=0.039$).

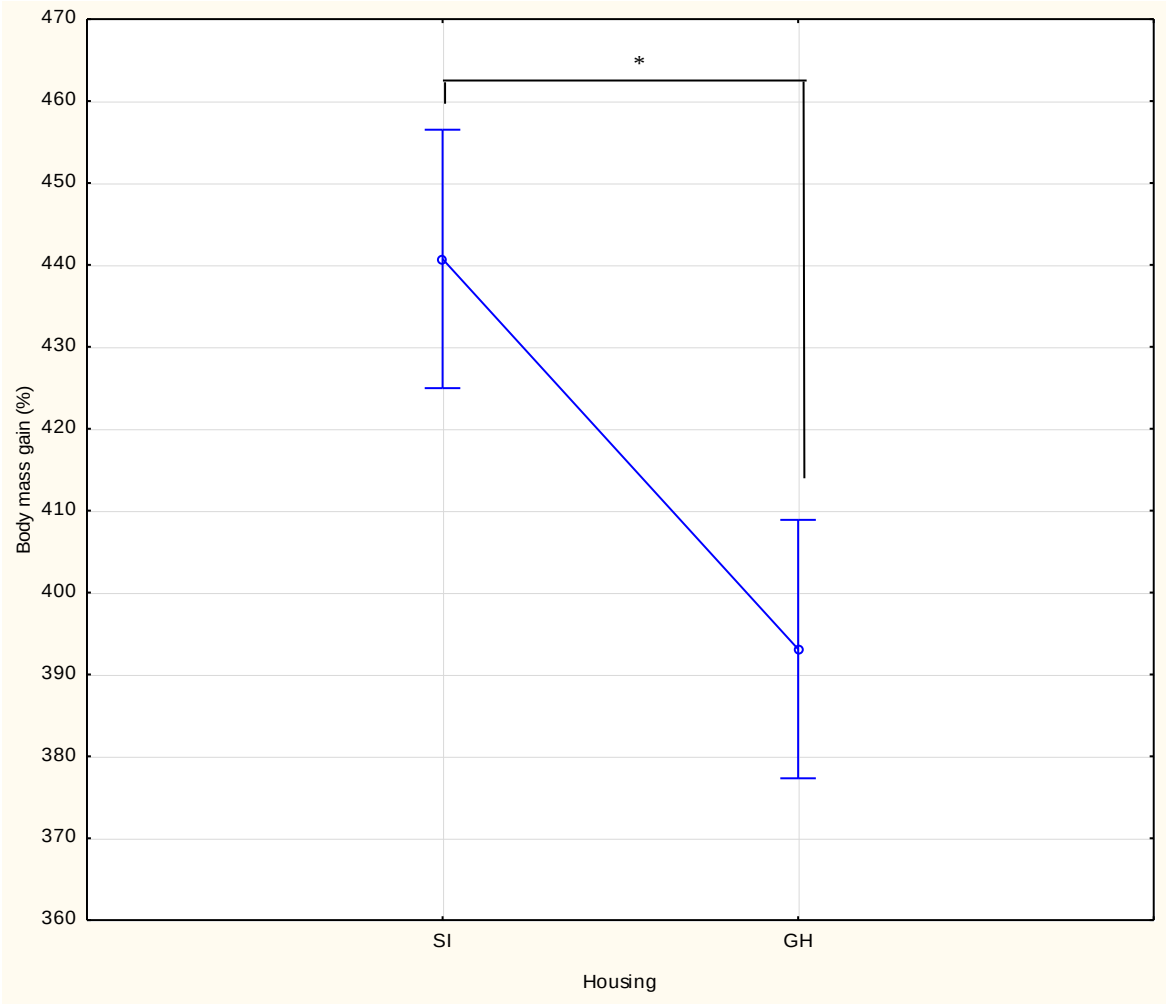


Figure 4.1.1: Effect of housing on the terminal body mass gain as a percentage increase from the initial body mass in female rats.

*p ≤0.05. SI= social isolation, GH= group housing. Data presented as mean ±SD; n=24 per group.

4.1.2 Effect of housing and foot-shock stress on visceral fat mass

Figure 4.1.2 shows the combined effects of housing and foot-shock stress on visceral fat mass as a percentage of terminal body mass in the female Sprague Dawley rats. A three-way ANOVA showed no significant interactions between early life stress, housing and foot-shock stress on visceral fat mass relative to body mass ($p>0.05$). However, a two-way ANOVA showed that rats that were socially isolated and not foot-shocked (SI x NFS) had significantly greater visceral fat mass than those that were housed in pairs and not foot-shocked (GH x NFS) ($p=0.003$).

The one-way ANOVA confirmed that animals that were socially isolated (SI) had greater visceral fat mass relative to body mass than rats that were housed in pairs (GH) ($p=0.001$) (Figure 4.1.2).

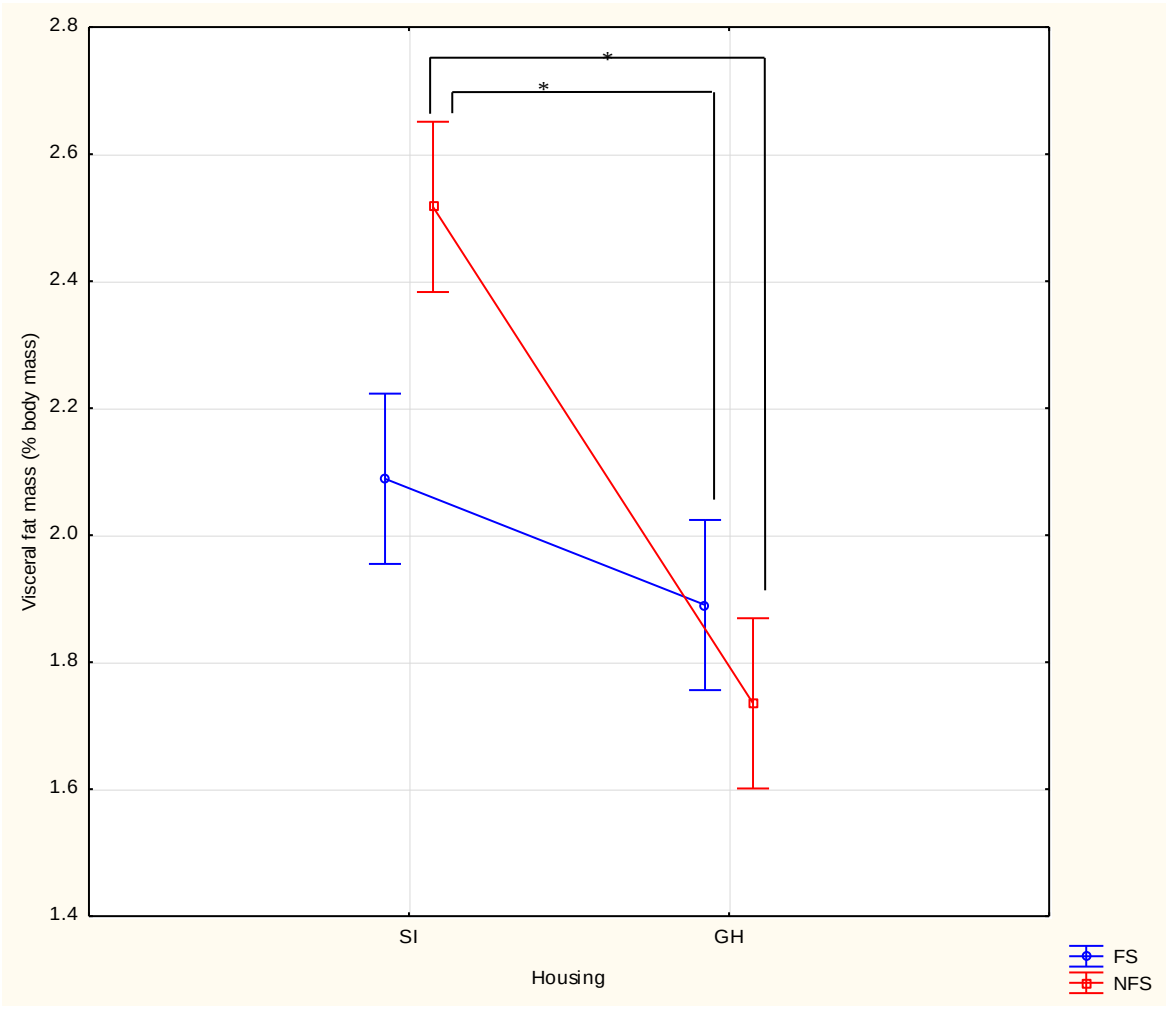


Figure 4.1.2: The combined effects of housing and foot-shock stress on visceral fat mass relative to body mass in female rats.

* p≤0.05. SI= social isolation; GH= group housing; FS = foot shock stress; NFS = non foot shock stress; GH x FS = group housing + foot-shock stress; GH x NFS = group housing + non foot-shock stress; SI x NFS= social isolation + non foot-shock stress. Data presented as mean ±SD; n=12 per group.

4.1.3 Effect of early life stress, housing and foot-shock stress on feed conversion ratio

The feed conversion ratio data for experimental phase two are shown in table 4.1.1A. Feed conversion ratio was similar in all the groups between PND21 and PND28. However, between PND28 and PND35, the socially isolated animals (SI) had significantly greater feed conversion ratio compared to animals that were housed in pairs (GH) ($p<0.001$). In addition, feed conversion ratio was similar in all the groups between PND35 and PND42.

The feed conversion ratio data for experimental phase three are shown in table 4.1.1B. Foot-shock stress was introduced on PND42. Between PND42 and PND49, animals that had been maternally separated (MS) had significantly lower feed conversion ratio compared to animals that were not maternally separated (NMS) ($p=0.007$). Furthermore, during the same period, animals that were maternally separated and foot-shocked (MS x FS) had significantly lower feed conversion ratio than those that were not maternally separated and foot-shocked (NMS x FS) ($p=0.014$). During the second week of foot-shock stress (PND49 to PND56), animals that were socially isolated (SI) had significantly greater feed conversion ratio compared to animals that were housed in pairs (GH) ($p=0.007$). Foot-shock stress was terminated on PND52. Animals that were not maternally separated and foot-shocked (NMS x FS) had significantly greater feed conversion ratio compared to animals that were not maternally separated and not foot-shocked (NMS x NFS) between PND56 and PND63 ($p=0.021$). Between PND63 and PND 69, animals that were socially isolated (SI) had a greater feed conversion ratio compared to those that were housed in pairs (GH) ($p=0.033$). Furthermore, in animals that were not maternally separated and socially isolated, foot-shock stress resulted in significantly greater feed conversion ratio compared to those that were not foot-shocked ($p=0.047$).

Table 4.1.1A: Effects of early life stress and housing on feed conversion ratio between PND21 and PND42 in female rats.

	PND21-PND28	PND28-PND35	PND35-PND42
Early life stress			
MS (n=24)	1.23±0.02	1.18±0.03	1.21±0.05
NMS (n=24)	1.33±0.04	1.16±0.03	1.20±0.05
Housing			
SI (n=24)	1.31±0.03	1.24±0.02[a]	1.24±0.04
GH (n=24)	1.23±0.03	1.04±0.03[b]	1.16±0.06
Early life stress x Housing			
MS x SI (n=12)	1.23±0.03	1.25±0.02	1.26±0.06
MS x GH (n=12)	1.23±0.02	1.05±0.03	1.13±0.09
NMS x SI (n=12)	1.38±0.04	1.23±0.03	1.21±0.07
NMS x GH (n=12)	1.23±0.06	1.03±0.04	1.18±0.08

Different superscripts [a,b], within the same column indicate significant differences ($p \leq 0.05$). NMS = non maternal separation; SI= social isolation; GH= group housing; FS = foot shock stress; NFS = non foot shock stress; MS x SI= maternal separation + social isolation; MS x GH= maternal separation + group housing; NMS x SI= non-maternal separation + social isolation; NMS x GH= non-maternal separation + group housing. Data presented as mean ±SD.

Table 4.1.1B: Effects of early life stress, housing and foot-shock stress on feed conversion ratio between PND 42 and PND69 in female rats.

	PND 42-PND49	PND49-PND56	PND56-PND63	PND63-PND69
Early life stress				
MS (n=24)	0.99±0.03[a]	0.94±0.03	0.81±0.04	0.64±0.04
NMS (n=24)	1.13±0.04[b]	0.96±0.03	0.86±0.06	0.67±0.05
Housing				
SI (n=24)	1.06±0.03	0.99±0.03[a]	0.85±0.05	0.69±0.04[a]
GH (n=24)	1.08±0.05	0.86±0.03[b]	0.81±0.04	0.57±0.03[b]
Stress				
FS (n=24)	1.06±0.05	0.93±0.04	0.89±0.06	0.70±0.04
NFS (n=24)	1.06±0.03	0.96±0.02	0.77±0.03	0.60±0.04
Early life stress x Housing				
MS x SI (n=12)	1.00±0.04	0.97±0.03	0.83±0.05	0.68±0.04
MS x GH (n=12)	0.98±0.07	0.84±0.06	0.77±0.06	0.55±0.05
NMS x SI (n=12)	1.11±0.05	1.01±0.05	0.87±0.08	0.69±0.06
NMS x GH (n=12)	1.17±0.05	0.88±0.02	0.84±0.05	0.60±0.03
Early life stress x Stress				
MS x FS (n=12)	0.94±0.05[c]	0.91±0.04	0.78±0.07	0.63±0.04
MS x NFS (n=12)	1.05±0.05	0.96±0.04	0.83±0.04	0.65±0.06
NMS x FS (n=12)	1.19±0.06[d]	0.97±0.07	1.00±0.09[a]	0.76±0.06

NMS x NFS (n=12)	1.08±0.04	0.96±0.02	0.72±0.03[b]	0.55±0.06
Housing x Stress				
SI x FS (n=12)	1.06±0.06	1.00±0.05	0.93±0.08	0.75±0.05
SI x NFS (n=12)	1.05±0.03	0.98±0.02	0.76±0.04	0.62±0.06
GH x FS (n=12)	1.06±0.09	0.82±0.02	0.82±0.06	0.59±0.04
GH x NFS (n=12)	1.09±0.06	0.92±0.05	0.79±0.05	0.56±0.05
Early life stress x Housing x foot-shock Stress				
MS x SI x FS (n=6)	0.97±0.07	0.97±0.03	0.80±0.09	0.64±0.06
MS x SI x NFS (n=6)	1.03±0.05	0.84±0.06	0.86±0.05	0.73±0.07
MS x GH x FS (n=6)	0.88±0.04	0.98±0.04	0.76±0.11	0.59±0.07
MS x GH x NFS (n=6)	1.08±0.12	0.97±0.04	0.77±0.07	0.51±0.07
NMS x SI x FS (n=6)	1.16±0.08	0.78±0.02	1.07±0.12	0.86±0.05[c]
NMS x SI x NFS (n=6)	1.07±0.05	0.94±0.16	0.67±0.01	0.53±0.07[d]
NMS x GH x FS (n=6)	1.25±0.05	1.05±0.12	0.87±0.07	0.58±0.03
NMS x GH x NFS (n=6)	1.09±0.08	0.99±0.03	0.82±0.07	0.63±0.05

Different superscripts [a,b], within the same column indicate significant differences ($p \leq 0.05$). Different superscripts [c,d], within the same column indicate significant differences ($p \leq 0.05$). NMS = non maternal separation; SI= social isolation; GH= group housing; FS = foot shock stress; NFS = non foot shock stress; MS x SI= maternal separation + social isolation; MS x GH= maternal separation + group housing; NMS x SI= non-maternal separation + social isolation; NMS x GH= non-maternal separation + group housing; MS x FS= maternal separation + foot-shock; MS x NFS= maternal separation + non foot-shock; NMS x FS= non-maternal separation + foot-shock; NMS x NFS= non-maternal separation + non foot-shock; SI x FS= social isolation + foot-shock; SI x NFS= social isolation + non foot-shock;

GH x FS= group housing + foot-shock; GH x NFS= social isolation + non foot-shock; MS x SI x FS= maternal separation + social isolation + foot-shock; MS x GH x FS= maternal separation + group housing + foot-shock; MS x SI x NFS = maternal separation + social isolation + non foot-shock; MS x GH x NFS = maternal separation + group housing + non foot-shock; NMS x SI x FS = non-maternal separation + social isolation + foot-shock; NMS x GH x FS = non-maternal separation + group housing + foot-shock; NMS x SI x NFS = non-maternal separation + social isolation + non foot-shock; NMS x SI x NFS = non-maternal separation + group housing + non foot-shock. Data presented as mean ±SD.

4.1.4 Effect of early life stress, housing and foot-shock stress on plasma glucose concentrations.

Figure 4.1.3 shows the effect of early life stress, housing and foot-shock stress on glucose concentrations in female rats. A three-way ANOVA showed a significant interaction between early life stress, housing and foot-shock stress on the plasma glucose concentration ($p=0.008$). For rats that were maternally separated and foot-shocked, social isolation had a tendency to decrease the plasma concentration of glucose while housing in pairs had a tendency to increase the plasma concentration of glucose. In contrast, for rats that were not maternally separated and foot-shocked, social isolation had a tendency to increase the plasma concentration of glucose while housing in pairs had a tendency to decrease plasma concentration of glucose. However, the plasma glucose concentrations were not significantly different between the groups ($p>0.05$).

Additionally, for animals that were maternally separated and not foot-shocked, social isolation had a tendency to increase the concentration of plasma glucose and housing in pairs had a tendency to decrease the plasma concentration of glucose. In contrast, for animals that were not maternally separated and not foot-shocked, social isolation had a tendency to decrease plasma concentration of glucose while housing in pairs had a tendency to increase the plasma concentration of glucose. However, the plasma glucose concentrations were not significantly different between the groups ($p>0.05$).

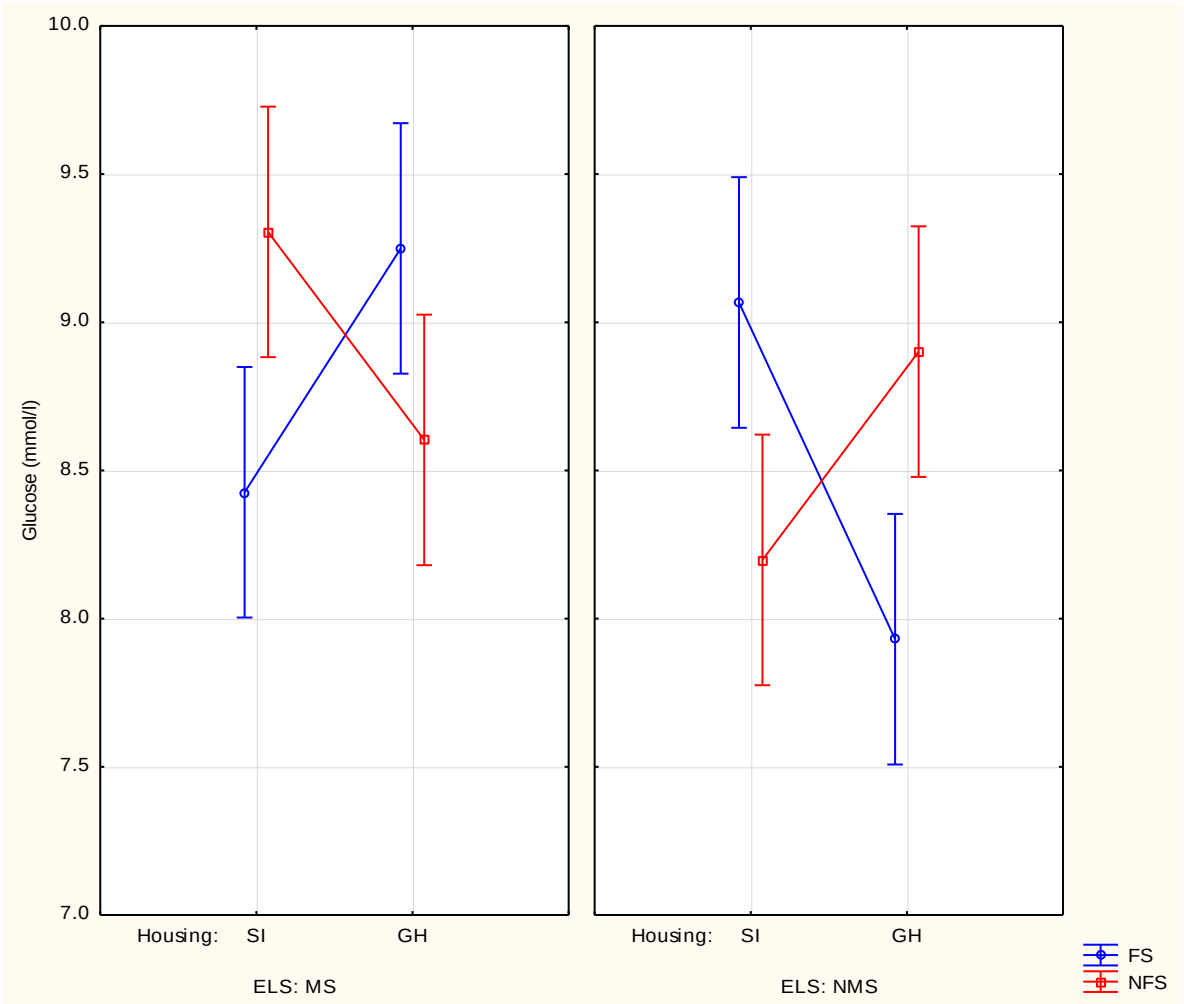


Figure 4.1.3: Effects of early life stress, housing and foot-shock stress on glucose concentrations in female rats.

There was a significant ($p \leq 0.05$) interaction between early life stress, housing and foot-shock stress on the plasma glucose concentration. MS = maternal separation; NMS = non maternal separation; SI= social isolation; GH= group housing; FS = foot shock stress; NFS = non foot shock stress. Data presented as mean ±SD; n=6 per group.

4.1.5 Effect of housing on serum insulin concentration

Figure 4.1.4 shows the effect of housing on serum insulin concentration in female rats. A Three way ANOVA showed no significant interactions between early life stress, housing and foot-shock stress on the serum insulin concentrations ($p>0.05$). However, rats that were socially isolated (SI) had significantly greater serum insulin concentrations compared to those that were housed in pairs (GH) ($p=0.046$).

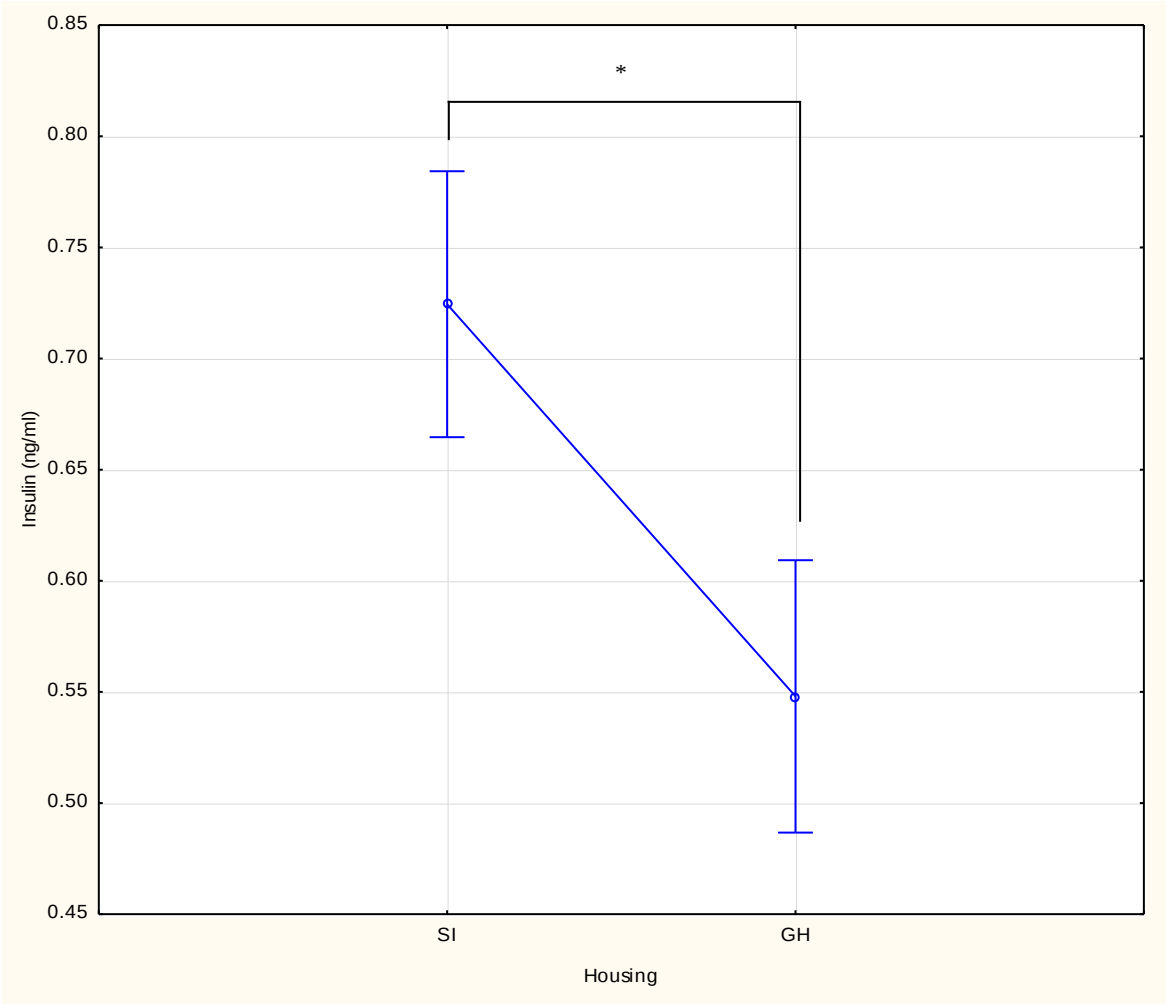


Figure 4.1.4: Effect of housing on serum insulin concentration in female rats.

*p ≤0.05. SI= social isolation, GH= group housing. Data presented as mean ±SD; n=24 per group.

4.1.6 Effect of early life stress, housing and foot-shock stress on HOMA-IR index.

Figure 4.1.5 shows the effect of early life stress, housing and foot-shock stress on HOMA-IR index. A three-way ANOVA showed no significant interactions between early life stress, housing and foot-shock stress on the HOMA-IR index ($p>0.05$). The HOMA-IR were similar in all the groups ($p>0.05$).

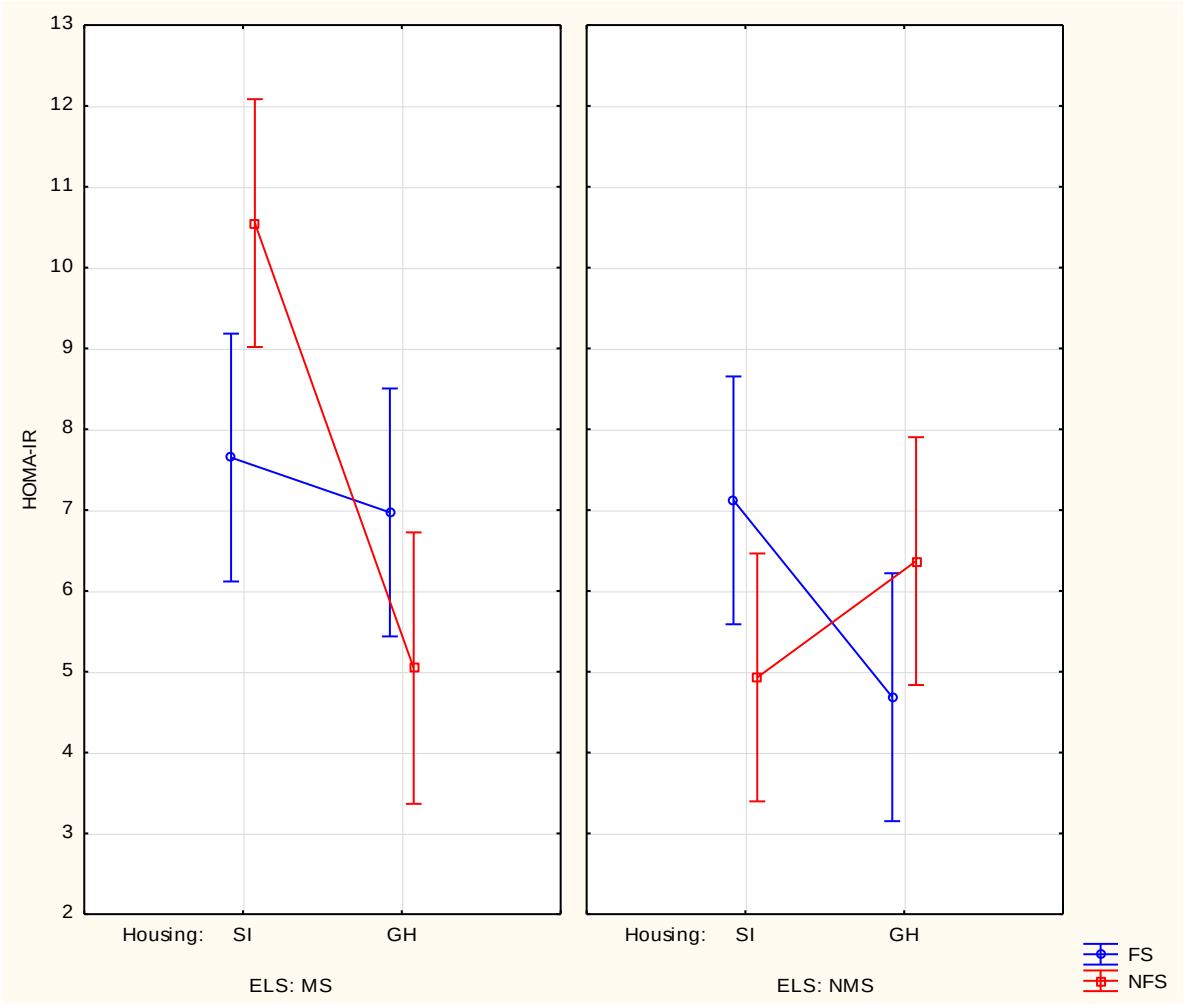


Figure 4.1.5: Effects of early life stress, housing and foot-shock stress on HOMA-IR index in female rats.

HOMA-IR index was not significantly different between the groups ($p>0.05$). MS = maternal separation; NMS = non maternal separation; SI= social isolation; GH= group housing; FS = foot shock stress; NFS = non foot shock stress. Data presented as mean ±SD; n=6 per group.

4.1.7 Effect of early life stress, housing and foot-shock stress on the immune response

In bivariate analysis, ROS production was significantly associated with the number of white blood cells (p=0.0026) (figure 4.1.6).

Table 4.1.2 shows the effects that early life stress, housing and foot-shock stress have on the immune response in terms of WBC count and the production of reactive oxygen species. Animals that were maternally separated and socially isolated (MS x SI) had significantly lower production of ROS compared to those that were maternally separated and housed in pairs (MS x GH) (p=0.034) as well as those that were not maternally separated and socially isolated (NMS x SI) (p= 0.031). The number of white blood cells were similar across all the groups (p>0.050). In addition, rats that were maternally separated had significantly lower ROS: WBC ratios than those that were not maternally separated (p=0.015).

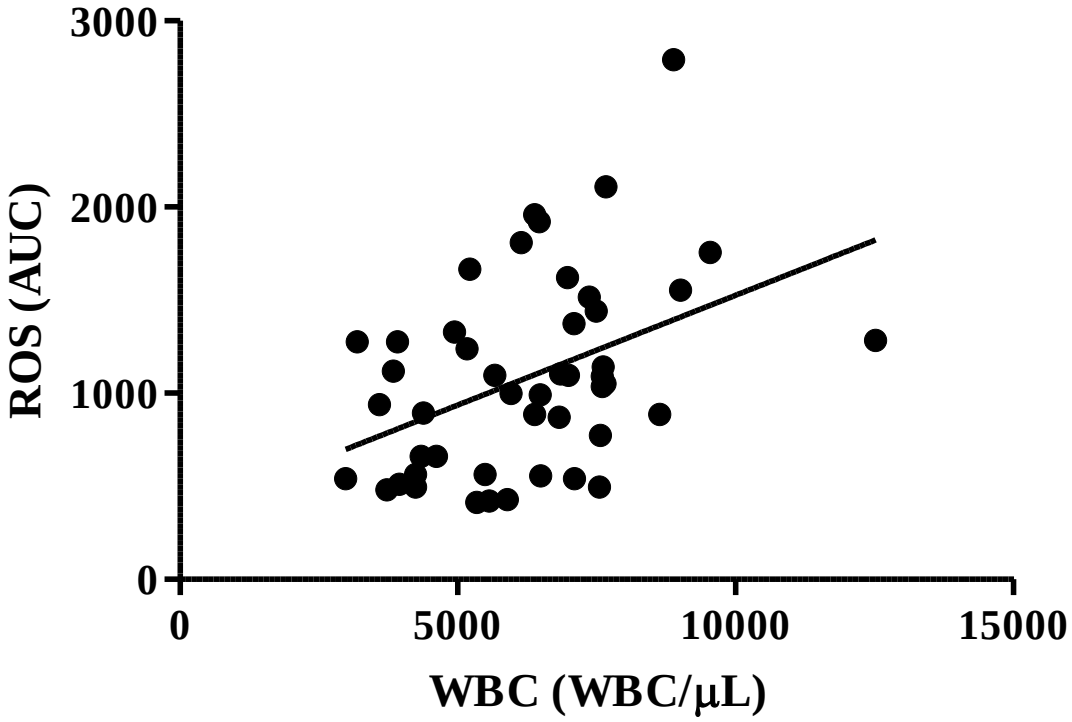


Figure 4.1.6: The bivariate relationship between the production of reactive oxygen species and the number of white blood cells in female rats.

$p<0.01$; $r=0.42461$. The production of ROS was associated with the number of white blood cells for all the animals.

Table 4.1.2: Effect of early life stress, housing and foot-shock stress on ROS production, WBC and ROS: WBC ratio in female rats.

	ROS	WBC (WBC/µl)	ROS/WBC
Early life stress			
MS (n=24)	954±120	6232 ±359	0.15±0.01[a]
NMS (n=24)	1200±88	6150 ±419	0.21±0.02[b]
Housing			
SI (n=24)	948±93	6059±253	0.16±0.02
GH (n=24)	1206±115	6322±488	0.20±0.02
Stress			
FS (n=24)	1040±98	5937±321	0.18±0.02
NFS (n=24)	1114±117	6444±442	0.18±0.02
Early life stress x Housing			
MS x SI (n=12)	643±71[a,x]	57621±373	0.12±0.02
MS x GH (n=12)	1266±194[b]	6702±599	0.18±0.02
NMS x SI (n=12)	1254±118[y]	6357±337	0.20±0.02
NMS x GH (n=12)	1146±133	5942±782	0.21±0.03
Early life stress x Stress			
MS x FS (n=12)	916±122	6352±493	0.15±0.02
MS x NFS (n=12)	992±213	6112±540	0.15±0.02
NMS x FS (n=12)	1164±151	5522±395	0.22±0.03
NMS x NFS (n=12)	1237±96	6777±710	0.20±0.02
Housing x Stress			

SI x FS (n=12)	876±133	5822±358	0.16±0.03
SI x NFS (n=12)	1020±132	6297±361	0.16±0.02
GH x FS (n=12)	1204±134	6052±548	0.21±0.02
GH x NFS (n=12)	1208±195	6592±826	0.19±0.02
Early life stress x Housing x Foot-shock stress			
MS x SI x FS (n=6)	638±107	5800±538	0.12±0.03
MS x SI x NFS (n=6)	648±103	5723±568	0.11±0.01
MS x GH x FS (n=6)	1195±151	6904±811	0.17±0.01
MS x GH x NFS (n=6)	1336±375	6500±950	0.19±0.03
NMS x SI x FS (n=6)	1115±208	5844±522	0.20±0.04
NMS x SI x NFS (n=6)	1393±103	6871±346	0.21±0.03
NMS x GH x FS(n=6)	1213±236	5200±610	0.24±0.04
NMS x GH x NFS (n=6)	1080±142	6683 ±1448	0.19±0.04

Different superscripts [a,b], within the same column indicate significant differences ($p \leq 0.05$). Different superscripts [x,y], within the same column indicate significant differences ($p \leq 0.05$). MS = maternal separation; NMS = non maternal separation; SI= social isolation; GH= group housing; FS = foot shock stress; NFS = non foot shock stress; GH x FS = group housing + foot-shock stress; GH x NFS = group housing + non foot-shock stress; SI x NFS= social isolation + non foot-shock stress. MS x SI x FS= maternal separation + social isolation + foot-shock; MS x GH x FS= maternal separation + group housing + foot-shock; MS x SI x NFS = maternal separation + social isolation + non foot-shock; MS x GH x NFS = maternal separation + group housing + non foot-shock; NMS x SI x FS = non-maternal separation + social isolation + foot-shock; NMS x GH x FS = non-maternal separation + group housing + foot-shock; NMS x SI x NFS = non-maternal separation + social isolation + non foot-shock; NMS x SI x NFS = non-maternal separation + group housing + non foot-shock. Data presented as mean ±SD.

4.1.8 Effect of housing and on plasma corticosterone concentrations.

Figure 4.1.7 shows the effect of housing on the plasma corticosterone concentration. A three-way ANOVA showed no significant interactions between early life stress, housing and foot-shock stress on the plasma corticosterone concentrations ($p>0.05$). However, rats that were socially isolated had significantly lower plasma corticosterone ($p=0.041$) concentrations compared to those that were housed in pairs (GH).

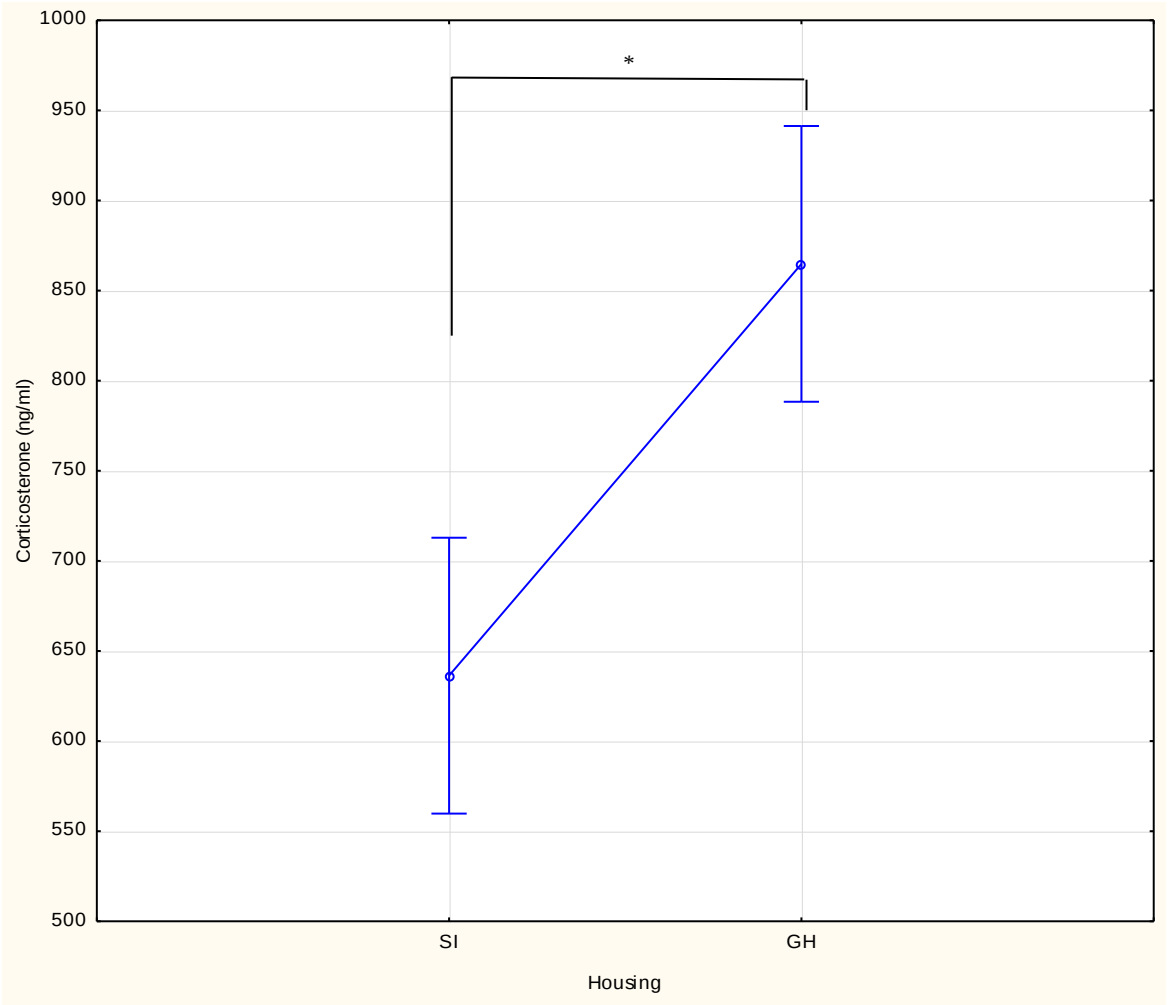


Figure 4.1.7: The effects of housing on glucocorticoid concentrations in female rats.

*p ≤0.05. SI= social isolation, GH= group housing. Data presented as mean ±SD; n=24 per group.

4.2: Results for experiments on male rats

Section 4.2 deals with the results for male rats.

4.2.1 Effect of early life stress, housing and foot-shock stress on body mass gain

Figure 4.2.1 shows the effect of early life stress, housing and foot-shock stress on body mass gain as a percentage increase from the initial body mass.

A three-way ANOVA showed no significant interactions between early life stress, housing and foot-shock stress on the change in body mass ($p \leq 0.05$). However, a two-way ANOVA showed that rats that were housed in pairs and not foot-shocked (GH x NFS) had significantly greater increase body mass compared to those that were socially isolated and foot-shocked (SI x FS) as well as those that were housed in pairs and foot-shocked (GH x FS) (Figure 4.2.1) ($p=0.049$; $p=0.004$ respectively). Rats that were maternally separated and foot-shocked (MS x FS) gained significantly less body mass compared to those that were maternally separated and not foot-shocked (MS x NFS), not maternally separated and foot-shocked (NMS x NFS) and not maternally separated and foot-shocked (NMS x FS) ($p=0.002$; $p=0.009$; $p=0.008$ respectively) (figure 4.2.1).

Rats that were foot-shocked (FS) gained significantly less body mass compared to those that were not foot-shocked (NFS) ($p=0.004$). In addition, rats that were maternally separated (MS) gained significantly less body mass compared to those that were not maternally separated (NMS) ($P=0.037$) (figure 4.2.1).

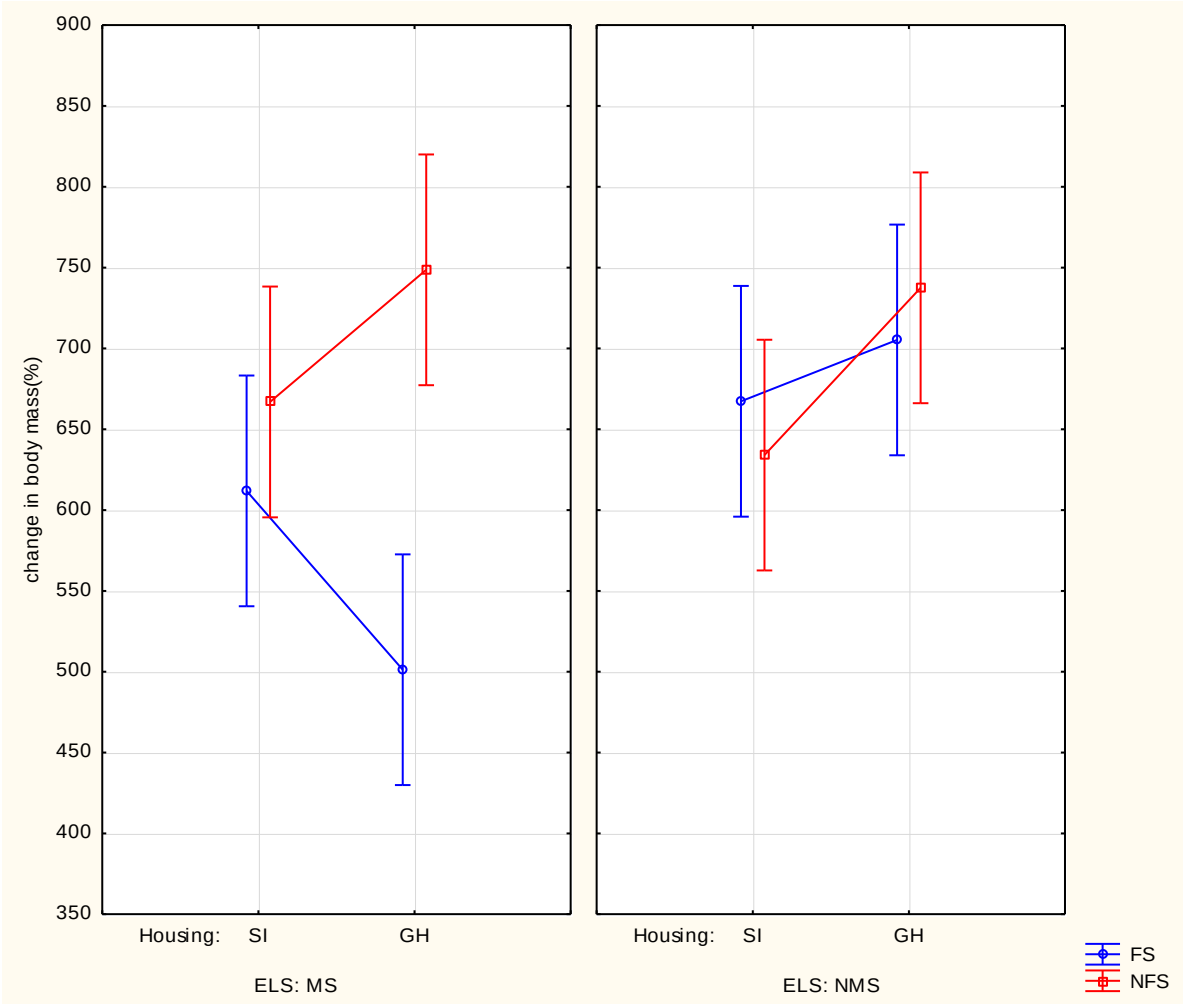


Figure 4.2 1: Effect of early life stress, housing and foot-shock stress on the terminal body mass gain as a percentage increase from the initial body mass in male rats.

Rats that were housed in pairs and not foot-shocked (GH x NFS) had significantly ($p \leq 0.05$) greater increase in body mass compare to rats that were socially isolated and SI x FS and those that were GH x FS. Rats that were MS x FS gained significantly ($p \leq 0.05$) less body mass compare to the rats that were MS x NFS, NMS x NFS and NMS x FS. Rats that were NFS had significantly ($p \leq 0.05$) greater increase in body mass compare to those that were FS. In addition, rats that were maternally separated (MS) gained significantly ($p \leq 0.05$) less body mass than those than those that were not maternally separated (NMS). MS = maternal separation; NMS = non maternal separation; SI= social isolation; GH= group housing; FS = foot shock stress; NFS = non foot shock stress. Data presented as mean ±SD; n=6 per group.

4.2.2 Effect of early life stress, housing and foot-shock stress on visceral fat mass

Figure 4.2.2 shows the effect of early life stress, housing and foot-shock stress on visceral fat mass relative to body mass.

A three-way ANOVA showed significant interactions between early life stress, housing and foot-shock stress on visceral fat mass (relative to body mass) ($p=0.035$). Rats that were maternally separated, housed in pairs and not foot-shocked (MS x GH x NFS) had significantly greater visceral fat mass (relative to body mass) compared to those that were not maternally separated, housed in pairs and not foot-shocked (NMS x GH x NFS) ($p=0.017$). Furthermore, rats that were maternally separated and housed in pairs (MS x GH) had greater visceral fat mass (relative to body mass) compared to those that were not maternally separated and housed in pairs (NMS x GH) ($p=0.01$).

Rats that were maternally separated (MS) had greater visceral fat mass (relative to body mass) compared to those that were not maternally separated NMS ($p=0.020$). In addition, rats that were not foot-shocked (NFS) had greater visceral fat mass, relative to body mass, compared to those that were foot-shocked (FS) ($p=0.007$).

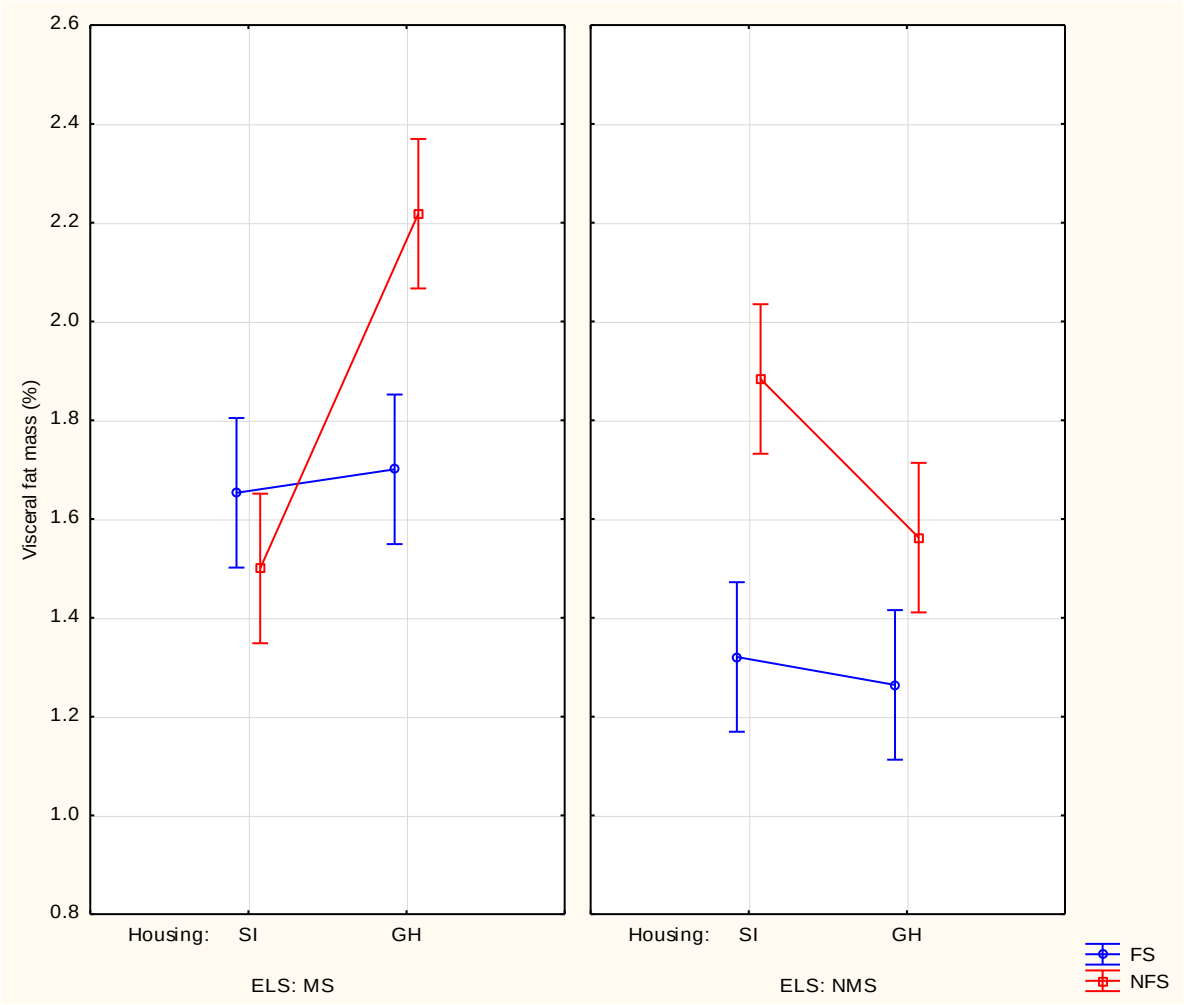


Figure 4.2.2: Effects of early life stress, housing and foot-shock stress on visceral fat mass in male rats.

There was a significant ($p \leq 0.05$) three way interaction between early life stress, housing and foot-shock stress s and visceral fat mass (relative to body mass). Rats that were maternally separated, housed in pairs and not foot-shocked (MS x GH x NFS) had significantly ($p \leq 0.05$) greater visceral fat mass (relative to body mass) compared to those that were not maternally separated, housed in pairs and not foot-shocked (NMS x GH x NFS). Rats that were maternally separated and housed in pairs (MS x GH) had significantly ($p \leq 0.05$) greater visceral fat mass (relative to body mass) compared to those that were not maternally separated and housed in pairs (NMS x GH). Rats that were maternally separated (MS) had significantly ($p \leq 0.05$) greater visceral fat mass (relative to

body mass) compared to those that were not maternally separated (NMS). Rats that were foot-shocked (FS) had significantly ($p \leq 0.05$) lower visceral fat mass (relative to body mass) compared to those that were not foot-shocked (NFS). MS = maternal separation; NMS = non maternal separation; SI= social isolation; GH= group housing; FS = foot shock stress; NFS = non foot shock stress. Data presented as mean ±SD; n=6 per group.

4.2.3 Effect of early life stress, social isolation and foot-shock stress on feed conversion ratio in male rats.

The feed conversion ratio data for experimental phase two are shown in table 4.2.1A. Feed conversion ratio was similar in all the groups between PND21 and PND28. However, between PND 28 and PND35, rats that were maternally separated and socially isolated (MS x SI) had significantly greater feed conversion ratio compared to those that were maternally separated and housed in pairs (MS x GH) ($p=0.001$). Furthermore, during the same period, animals that were not maternally separated and socially isolated (NMS x SI) had significantly greater feed conversion ratio compared to those that were not maternally separated and housed in pairs (NMS x GH) ($p=0.0001$). Housing had a significant effect on feed conversion ratio between PND 28 and PND35, socially isolated rats had greater feed conversion ratio compared to rats that were housed in pairs (GH) ($p<0.0001$). In addition, feed conversion ratio was similar in all the groups between PND35 and PND42 (table 4.2.1A).

The feed conversion ratio data for experimental phase three are shown in table 4.2.1B. Foot-shock stress was introduced on PND42. A two-way ANOVA showed that rats that were not maternally separated and socially isolated (NMS x SI) had significantly lower feed conversion ratio compared to those that were not maternally separated and housed in pairs (NMS x GH) ($p=0.034$), between PND42 and PND49. Rats that were socially isolated and foot-shocked (SI x FS) had lower feed conversion compared to those that were housed in pairs and foot-shocked (GH x FS) ($p=0.034$). Furthermore during the same period (PND42 to PND49), socially isolated (SI) rats had lower feed conversion ratio compared to rats that were housed in pairs (GH) ($p<0.001$).

Feed conversion ratio was similar in all the groups between PND49 and PND56, as well as between PND56 and PND63. Between PND63 and PND69, animals that were socially isolated and not foot-shocked (SI x NFS) had grater feeding efficiency compare to animals that were socially isolated and foot-shocked (SI x FS) ($p=0.046$) and compared to those that were housed in pairs and not foot-shocked (GH x NFS) ($p=0.020$). In addition, during the same period, animals that were socially isolated (SI) had significantly greater feeding efficiency compared to those that were housed in pairs (GH) ($p=0.005$) (4.2.1B).

Table 4.2.1A: Effects of early life stress and housing on feed conversion ratio between PND21 and PND42 in male rats.

	Pnd21-pnd28	Pnd28-pnd35	Pnd35-pnd42
Early life stress			
MS (n=24)	1.18±0.03	1.10±0.04	1.15±0.04
NMS (n=24)	1.31±0.04	1.16±0.04	1.20±0.06
Housing			
SI (n=24)	1.24±0.03	1.21±0.02[a]	1.12±0.04
GH (n=24)	1.26±0.03	0.97±0.04[b]	1.29±0.05
Early life stress x Housing			
MS x SI (n=12)	1.17±0.04	1.18±0.03[c]	1.09±0.04
MS x GH (n=12)	1.21±0.03	0.92±0.07[d]	1.27±0.04
NMS x SI (n=12)	1.31±0.05	1.23±0.03[t]	1.15±0.07
NMS x GH (n=12)	1.32±0.05	1.02±0.05[u]	1.30±0.09

Different superscripts [a,b] within the same column indicate significant differences. Different superscripts [c,d] within the same column indicate significant differences. Different superscripts [t,u] within the same column indicate significant differences. NMS = non maternal separation; SI= social isolation; GH= group housing; FS = foot shock stress; NFS = non foot shock stress; MS x SI= maternal separation + social isolation; MS x GH= maternal separation + group housing; NMS x SI= non-maternal separation + social isolation; NMS x GH= non-maternal separation + group housing. Data presented as mean ±SD.

Table 4.2.1B: Effect of early life stress, housing and foot-shock stress on feed conversion ratio between PND 42 and PND69 in male rats.

	PND42-PND49	PND49-PND56	PND56-PND63	PND63-PND69
Early life stress				
MS (n=24)	1.02±0.05	0.88±0.03	0.78±0.06	0.60±0.03
NMS (n=24)	1.05±0.06	0.90±0.02	0.89±0.03	0.62±0.02
Housing				
SI (n=24)	0.90±0.04[a]	0.87±0.02	0.83±0.03	0.64±0.02[a]
GH (n=24)	1.17±0.06[b]	0.93±0.02	0.86±0.09	0.55±0.02[b]
Stress				
FS (n=24)	0.98±0.05	0.87±0.02	0.83±0.03	0.57±0.02
NFS (n=24)	1.09±0.06	0.92±0.03	0.85±0.07	0.65±0.03
Early life stress x Housing				
MS x SI (n=12)	0.90±0.06	0.86±0.04	0.79±0.03	0.63±0.04
MS x GH (n=12)	1.14±0.08	0.91±0.04	0.77±0.16	0.55±0.03
NMS x SI (n=12)	0.89±0.06[e]	0.87±0.02	0.86±0.04	0.65±0.02
NMS x GH (n=12)	1.21±0.09[f]	0.95±0.03	0.95±0.05	0.54±0.04
Early life stress x Stress				
MS x FS (n=12)	0.89±0.07	0.83±0.02	0.79±0.03	0.54±0.02
MS x NFS (n=12)	1.15±0.07	0.94±0.05	0.78±0.14	0.66±0.05
NMS x FS (n=12)	1.07±0.07	0.89±0.03	0.87±0.04	0.60±0.01
NMS x NFS (n=12)	1.03±0.08	0.90±0.03	0.90±0.05	0.64±0.04

Housing x Stress				
SI x FS (n=12)	0.82±0.06[g]	0.84±0.02	0.79±0.03	0.58±0.02[e]
SI x NFS (n=12)	0.97±0.06[x]	0.89±0.03	0.87±0.05	0.69±0.03[f,x]
GH x FS (n=12)	1.14±0.08[h]	0.91±0.03	0.92±0.05	0.55±0.02
GH x NFS (n=12)	1.21±0.09[y]	0.95±0.03	0.81±0.17	0.54±0.04[y]
Early life stress x Housing x Foot-shock stress				
MS x SI x FS (n=6)	0.84±0.09	0.83±0.04	0.77±0.04	0.56±0.04
MS x SI x NFS (n=6)	0.95±0.08	0.90±0.08	0.83±0.05	0.69±0.06
MS x GH x FS (n=6)	0.94±0.12	0.84±0.01	0.83±0.03	0.51±0.01
MS x GH x NFS (n=6)	1.34±0.12	0.98±0.05	0.71±0.35	0.58±0.04
NMS x SI x FS (n=6)	0.79±0.08	0.85±0.03	0.81±0.04	0.60±0.02
NMS x SI x NFS (n=6)	1.00±0.09	0.89±0.04	0.90±0.07	0.70±0.03
NMS x GH x FS (n=6)	1.34±0.12	0.98±0.02	1.01±0.05	0.60±0.03
NMS x GH x NFS (n=6)	1.07±0.14	0.93±0.05	0.90±0.08	0.46±0.05

Different superscripts [a,b], within the same column indicate significant differences ($p \leq 0.05$).
Different superscripts [e,f], within the same column indicate significant differences ($p \leq 0.05$).
Different superscripts [g,h], within the same column indicate significant differences ($p \leq 0.05$).
Different superscripts [x,y], within the same column indicate significant differences ($p \leq 0.05$).
NMS = non maternal separation; SI= social isolation; GH= group housing; FS = foot shock stress; NFS = non foot shock stress; MS x SI= maternal separation + social isolation; MS x GH= maternal separation + group housing; NMS x SI= non-maternal separation + social isolation; NMS x GH= non-maternal separation + group housing; MS x FS= maternal separation + foot-shock; MS x NFS= maternal separation + non foot-shock; NMS x FS=

non-maternal separation + foot-shock; NMS x NFS= non-maternal separation + non foot-shock; SI x FS= social isolation + foot-shock; SI x NFS= social isolation + non foot-shock; GH x FS= group housing + foot-shock; GH x NFS= social isolation + non foot-shock; MS x SI x FS= maternal separation + social isolation + foot-shock; MS x GH x FS= maternal separation + group housing + foot-shock; MS x SI x NFS = maternal separation + social isolation + non foot-shock; MS x GH x NFS = maternal separation + group housing + non foot-shock; NMS x SI x FS = non-maternal separation + social isolation + foot-shock; NMS x GH x FS = non-maternal separation + group housing + foot-shock; NMS x SI x NFS = non-maternal separation + social isolation + non foot-shock; NMS x SI x NFS = non-maternal separation + group housing + non foot-shock. Data presented as mean ±SD.

4.2.4 Effect of early life stress, housing and foot-shock stress on plasma glucose concentrations.

Figure 4.2.3 shows the effect of early life stress, housing and foot-shock stress on glucose concentrations in male rats.

A three-way ANOVA showed no significant interactions between early life stress, housing and foot-shock stress on plasma glucose concentrations ($p>0.05$). However, a two-way ANOVA showed that rats that were housed in pairs and foot-shocked (GH x FS) had significantly lower plasma glucose concentrations compared to rats that were socially isolated and foot-shocked (SI x FS) and those that were housed in pairs and not foot-shocked (GH x NFS) ($p=0.002$; $p=0.0003$ respectively). Furthermore, rats that were maternally separated and foot-shocked (MS x FS) had significantly lower plasma glucose concentrations compared to those that were maternally separated and not foot-shocked (MS x NFS) ($p=0.009$) (figure 4.2.3).

In addition the rats that were foot-shocked (FS) had significantly lower plasma glucose concentrations compared to rats that were not foot-shocked (NFS) ($p=0.030$) (figure 4.2.3).

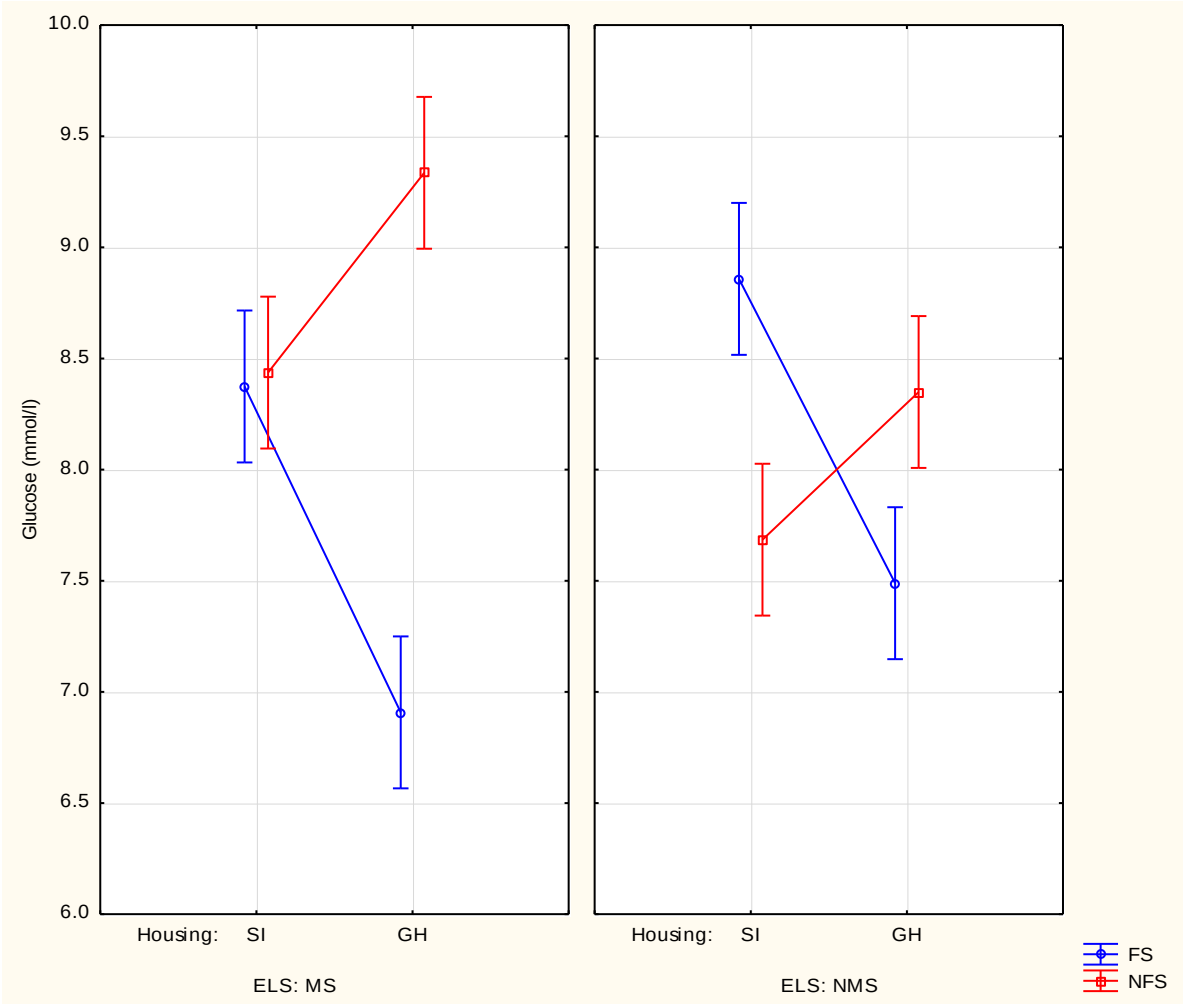


Figure 4.2.3: Effects of early life stress, housing and foot-shock stress on plasma glucose concentrations in male rats.

Rats that were housed in pairs and foot-shocked (GH x FS) had significantly ($p \leq 0.05$) lower glucose concentrations compared to those that were socially isolated and foot-shocked (SI x FS) and those that were housed in pairs and not foot-shocked (GH x NFS). Rats that were maternally separated and foot-shocked (MS x FS) had significantly ($p \leq 0.05$) lower glucose concentrations compared to those that were maternally separated and not foot-shocked (MS x NFS). Rats that were foot-shocked (FS) had significantly ($p \leq 0.05$) lower glucose concentrations than those that were not foot-shocked (NFS). NMS = non maternal separation; SI= social isolation; GH= group housing; FS = foot shock stress; NFS = non foot shock stress; MS x FS= maternal separation + foot-shock; MS x NFS= maternal separation + non foot-shock; SI x FS= social isolation + foot-shock; GH x FS= group

housing + foot-shock; GH x NFS= social isolation + non foot-shock. Data presented as mean ±SD; n=6 per group.

4.2.5 Effect of early life stress, housing and foot-shock stress on serum insulin concentrations

Figure 4.2.4 shows the effect of early life stress, housing and foot-shock stress on serum insulin concentrations. A three-way ANOVA showed significant three way interactions between early life stress, housing and foot-shock stress on serum insulin concentrations ($p=0.021$). In rats that were housed in pairs and not foot-shocked (GH x NFS), maternal separation (MS) had a tendency to increase serum insulin concentrations while non-maternal separation (NMS) had a tendency to lower serum insulin concentrations.

Rats that were maternally separated and housed in pairs (MS x GH) had greater serum concentrations of insulin compared to those that were not maternally separated and housed in pairs (NMS x GH) ($p =0.033$) (figure 4.2.4).

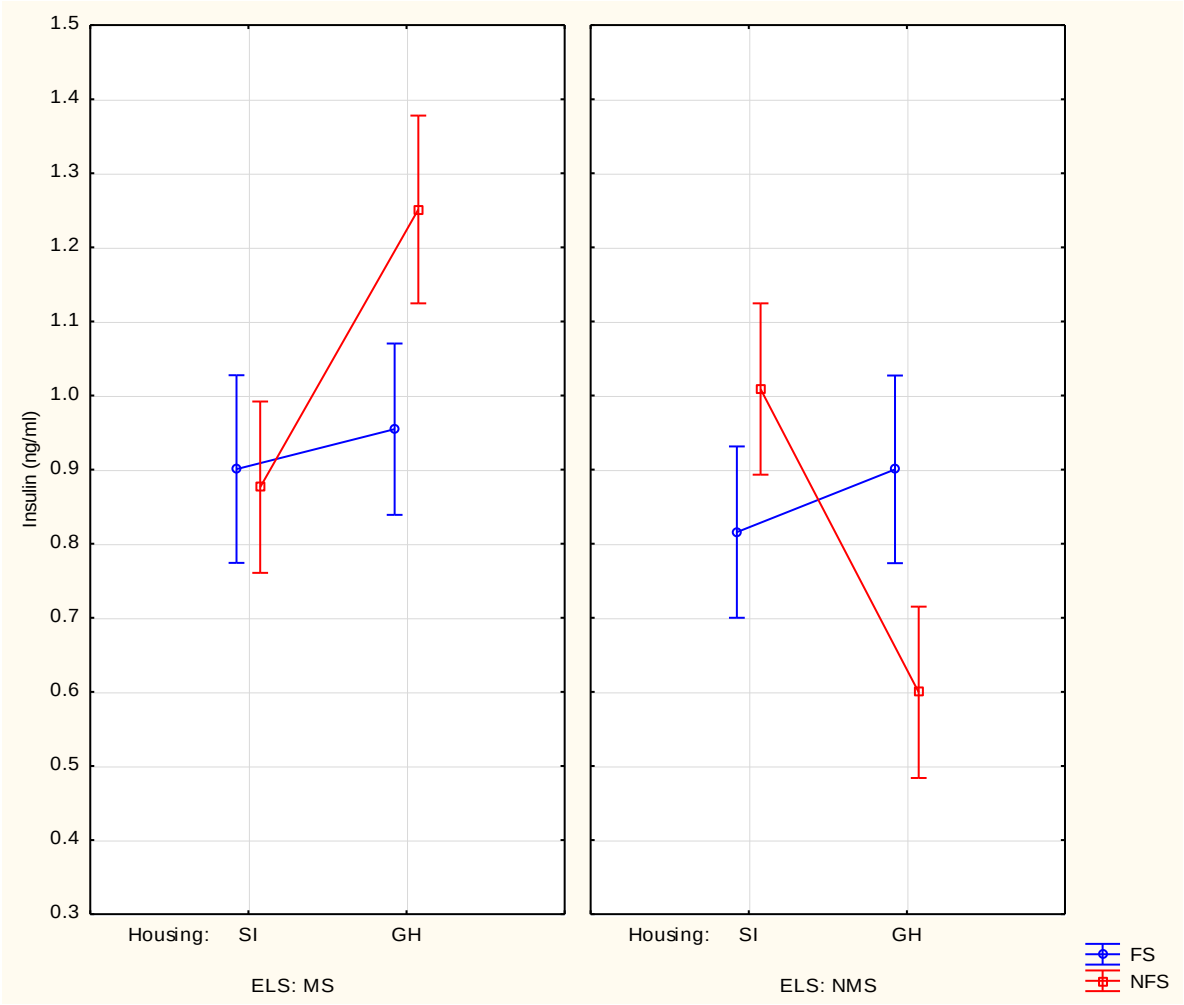


Figure 4.2.4: Effects of early life stress, housing and foot-shock stress on serum insulin concentrations in male rats

Rats that were maternally separated and housed in pairs (MS x GH) had significantly ($p \leq 0.05$) greater concentrations of insulin compared to those that were not maternally separated and housed in pairs (NMS x GH). MS = maternal separation; NMS = non maternal separation; SI= social isolation; FS = foot shock stress; NFS = non foot shock stress; GH= group housing; MS x GH= maternal separation + group housing; NMS x GH= non-maternal separation + group housing. Data presented as mean ±SD; n=6 per group.

4.2.6 Effect of early life stress, housing and foot-shock stress on HOMA-IR index.

Figure 4.2.5 shows the effect of early life stress, housing and foot-shock stress on HOMA-IR index.

A three-way ANOVA showed significant interactions between early life stress, housing and foot-shock stress on HOMA-IR index ($p=0.17$). In rats that were housed in pairs and foot-shocked (GH x NFS), maternally separation (MS) had a tendency to increase HOMA-IR index while non-maternally separation (NMS) had a tendency to lower HOMA-IR index. Rats that were maternally separated, housed in pairs and not foot-shocked (MS x GH x NFS) had greater HOMA-IR index compared to those that were not maternally separated, housed in pairs and not foot-shocked (NMS x GH x NFS) ($p=0.026$) (figure 4.2.5).

Rats that were maternally separated and not foot-shocked (MS x NFS) had significantly greater HOMA-IR index compared to those that were not maternally separated and not foot-shocked (NMS x NFS) ($p=0.035$). In addition, rats that were maternally separated and housed in pairs (MS x GH) had significantly greater HOMA-IR index compared to those that were not maternally separated and housed in pairs (NMS x GH) ($p=0.049$) (figure 4.2.5).

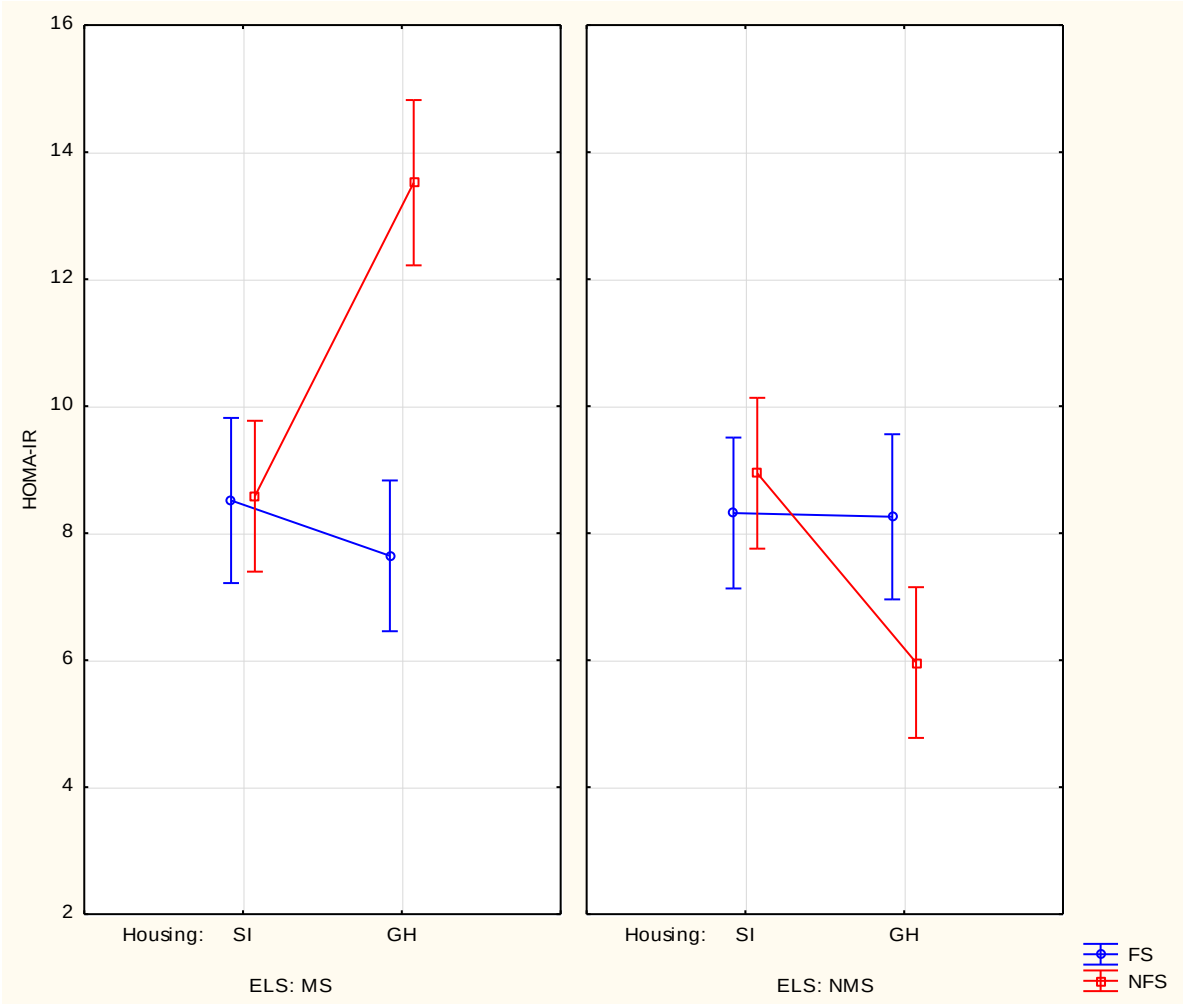


Figure 4.2.5: Effects of early life stress, housing and foot-shock stress on HOMA-IR index in male rats.

There was a significant ($p \leq 0.05$) three way interaction between early life stress x housing x foot-shock stress and HOMA-IR index. Rats that were maternally separated, housed in pairs and not foot-shocked (MS x GH x NFS) had significantly ($p \leq 0.05$) greater HOMA-IR index compared to those that were not maternally separated, housed in pairs and not foot-shocked (NMS x GH x NFS). Rats that were maternally separated and not foot-shocked (MS x NFS) had significantly ($p \leq 0.05$) greater HOMA-IR index compared to those that were not maternally separated and not foot-shocked (NMS x NFS). Rats that were maternally separated and housed in pairs (MS x GH) had significantly ($p \leq 0.05$) greater

HOMA-IR index compared to those that were not maternally separated and housed in pairs (NMS x GH). MS = maternal separation; NMS = non maternal separation; SI= social isolation; GH= group housing; FS = foot shock stress; NFS = non foot shock stress; MS x GH= maternal separation + group housing; NMS x GH= non-maternal separation + group housing; MS x NFS= maternal separation + non foot-shock; NMS x NFS= non-maternal separation + non foot-shock; MS x GH x NFS = maternal separation + group housing + non foot-shock; non-maternal separation + social isolation + non foot-shock. Data presented as mean ±SD; n=6 per group.

4.2.7 Effect of early life stress, housing and foot-shock stress on the immune response

In bivariate analysis, ROS production was significantly associated with the number of white blood cells (p=0.0002) (figure 4.2.6).

Table 4.2.2 shows the effects that early life stress x housing x foot-shock stress have on the immune response in terms of WBC count and the production of reactive oxygen species. Rats that were foot-shocked (FS) had significantly greater production of ROS compared to those that were not foot-shocked (NFS) (p=0.043). In addition, rats that were foot-shocked (FS) had greater ROS: WBC ratio compared to those that were not foot-shocked (NFS) (p=0.026) (table4.2.2). There were no significant differences in the WBC between all the groups (p>0.05).

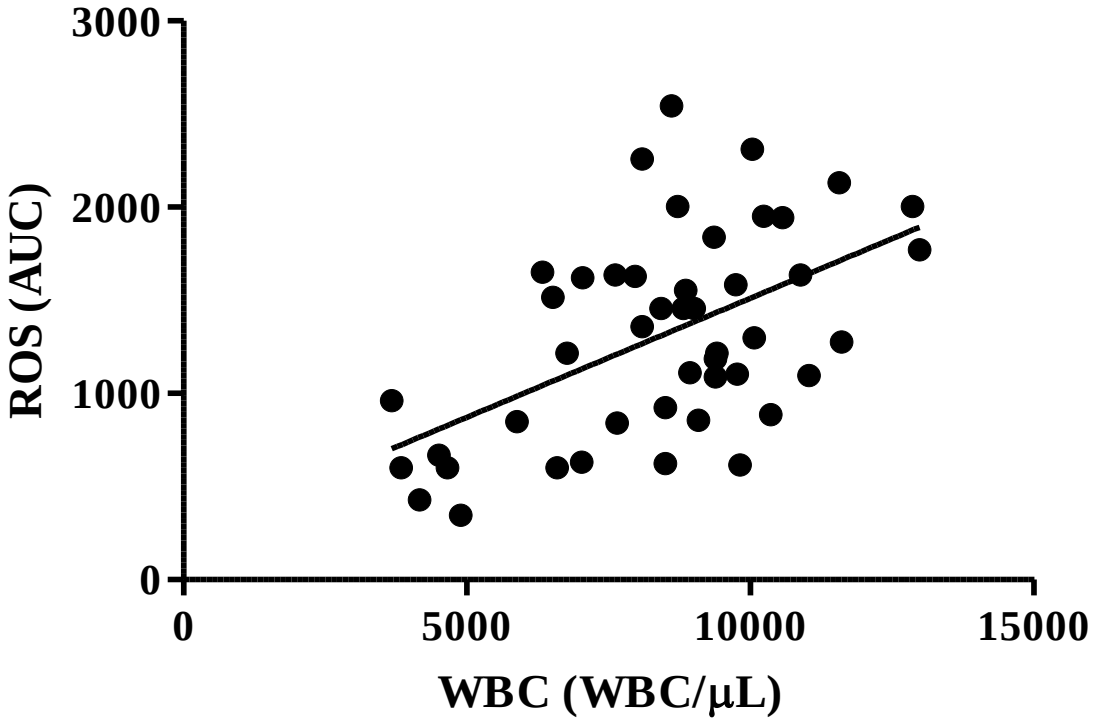


Figure 4.2.6: The bivariate relationship between the production of reactive oxygen species and the number of white blood cells in male rats.

$p<0.01$; $r=0.5286$. The production of ROS was associated with the number of white blood cells for all the animals.

Table 4.2.2: Effect of early life stress, housing and foot-shock stress on ROS production, WBC and ROS: WBC ratio in male rats.

	ROS	WBC	ROS/WBC
Early life stress			
MS (n=24)	1155±105	8514±518	0.14±0.01
NMS (n=24)	1411±124	8344 ±439	0.17±0.01
Housing			
SI (n=24)	1245±127	8496±405	0.15±0.01
GH (n=24)	1328±107	8355±540	0.16±0.01
Stress			
FS (n=24)	1445±107[a]	8353±458	0.18±0.01[a]
NFS (n=24)	1120±119[b]	8504±498	0.14±0.01[b]
Early life stress x Housing			
MS x SI (n=12)	1061±144	8725±641	0.14±0.02
MS x GH (n=12)	1257±154	8303±841	0.15±0.01
NMS x SI (n=12)	1430±201	8285±526	0.17±0.02
NMS x GH (n=12)	1393±155	8402±725	0.17±0.02
Early life stress x Stress			
MS x FS (n=12)	1240±139	8179±665	0.16±0.02
MS x NFS (n=12)	1063±160	8916±835	0.13±0.01
NMS x FS (n=12)	1651±146	8527±655	0.2±0.01
NMS x NFS (n=12)	1172±181	8160±608	0.15±0.02
Housing x Stress			
SI x FS (n=12)	1557±169	8781±490	0.18±0.02

SI x NFS (n=12)	934±144	8184±668	0.12±0.01
GH x FS (n=12)	1333±131	7925±777	0.17±0.01
GH x NFS (n=12)	1323±181	8824±758	0.15±0.02
Early life stress x Housing x Foot-shock stress			
MS x SI x FS (n=6)	1240±221	8496±657	0.15±0.03
MS x SI x NFS (n=6)	882±171	9000±1255	0.12±0.01
MS x GH x FS (n=6)	1239±189	7863±1214	0.16±0.02
MS x GH x NFS (n=6)	1280±276	8833±1247	0.14±0.02
NMS x SI x FS (n=6)	1874±193	9067±769	0.21±0.03
NMS x SI x NFS (n=6)	985±248	7504±619	0.13±0.03
NMS x GH x FS (n=6)	1428±191	7988±1085	0.18±0.01
NMS x GH x NFS (n=6)	1359±262	8817±1035	0.16±0.03

Different superscripts [a,b], within the same column indicate significant differences ($p \leq 0.05$). NMS = non maternal separation; SI= social isolation; GH= group housing; FS = foot shock stress; NFS = non foot shock stress; MS x SI= maternal separation + social isolation; MS x GH= maternal separation + group housing; NMS x SI= non-maternal separation + social isolation; NMS x GH= non-maternal separation + group housing; MS x FS= maternal separation + foot-shock; MS x NFS= maternal separation + non foot-shock; NMS x FS= non-maternal separation + foot-shock; NMS x NFS= non-maternal separation + non foot-shock; SI x FS= social isolation + foot-shock; SI x NFS= social isolation + non foot-shock; GH x FS= group housing + foot-shock; GH x NFS= social isolation + non foot-shock; MS x SI x FS= maternal separation + social isolation + foot-shock; MS x GH x FS= maternal separation + group housing + foot-shock; MS x SI x NFS = maternal separation + social isolation + non foot-shock; MS x GH x NFS = maternal separation + group housing + non foot-shock; NMS x SI x FS = non-maternal separation + social isolation + foot-shock; NMS x GH x FS = non-maternal separation + group housing + foot-shock; NMS x SI x

NFS = non-maternal separation + social isolation + non foot-shock; NMS x SI x NFS = non-maternal separation + group housing + non foot-shock. Data presented as mean ±SD.

4.2.8 Effect of early life stress on plasma corticosterone concentrations.

Figure 4.2.7 shows the effect of early life stress on plasma corticosterone concentrations in male rats. A three way ANOVA showed no significant interactions between early life stess, housing and foot-shock foot-shock stress on the plasma corticosterone concentrations ($p>0.05$). However, rats that were maternally separated (MS) had greater plasma corticosterone concentrations compared to those that were not maternally separated NMS ($p=0.001$).

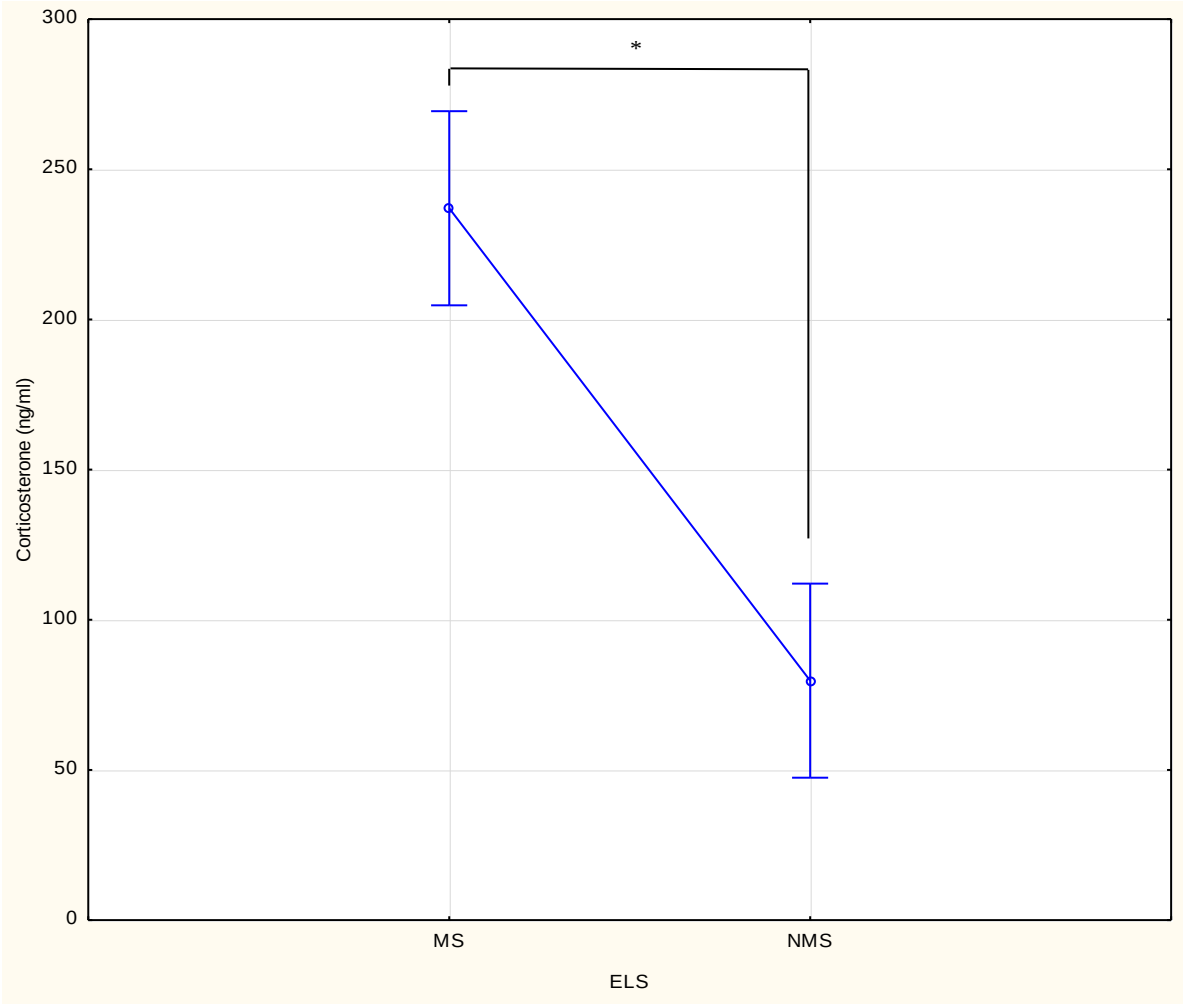


Figure 4.2 .7: Effects of early life stress on corticosterone concentrations in male rats.

*p ≤0.05. Rats that were maternally separated (MS) had significantly (p≤0.05) greater plasma corticosterone concentrations compared to those that were not maternally separated (NMS). MS = maternal separation; NMS = non maternal separation. Data presented as mean ±SD; n=24 per group.

CHAPTER 5: DISCUSSION

The current study investigated the effects of early life stress, followed by further significant stressors, on growth and metabolism of Sprague Dawley rats. Early life stress was invoked by periods of maternal separation in early postnatal life before weaning. Further stressors following weaning included social isolation (as opposed to group housing) and a situational stress imposed by brief, daily foot shocks administered in a custom plexiglass container. The effects of this stress protocol on growth and metabolism were determined by evaluating body mass gain, visceral fat mass, feed conversion ratio, plasma glucose, serum insulin, HOMA-IR index, the innate immune response and plasma corticosterone in female and male Sprague Dawley rats.

5.1 Feed conversion ratio

Feed conversion ratio measure the effectiveness of the rats in converting chow into body mass. During the second week of differential housing, socially isolated female rats had significantly increased feed conversion ratio compared to the female rats that were housed in pairs. Similarly in male rats, social isolation resulted in increased feed conversion ratio compared to housing in pairs. Similar results were reported by Nakhate *et al.*, (2011) who concluded that social isolation may result in increased food intake and body weight.

During the first week of foot-shock, maternally separated female rats has decreased feed conversion ratio compared to female rats that were not maternally separated. Moreover, for all female rats that were foot-shocked, maternal separation resulted in significantly decreased feed conversion ratio. However, this was not the case in male rats. Socially isolated male rats had decreased feed conversion ratio compared to male rats housed in pairs. During the second week of foot-shock, feed conversion ratio in male rats was similar in all the groups, while feed conversion ratio in socially isolated females was increased compared to female rats housed in pairs. In response to the foot-shock stress in the first week of foot-shock, female rats reduced feed conversion ratio.

Following the termination of the foot-shock stress, feed conversion ratio in males was similar between the groups. However, foot-shock stress resulted in significantly increased feed conversion ratio in female rats that were not maternally separated; meaning that even after foot-shock, there was a tendency to increase body mass relative to food intake in females in female rats that were not maternally separated. Between PND 63 and PND69, only social isolation persisted in increased feed conversion in both male and female rats compared to rats housed in groups. In a similar study, social isolation was found to result

in hyperphagia in female Sprague Dawley rats (Jahng *et al.,* 2012). They further reported that social isolation causes variable effects in feed intake in male rats.

5.2 Body mass gain

By the end of the study socially isolated female rats had gained significantly more body mass compared to female rats housed in pairs. This result is not surprising since feed conversion ratio was also significantly increased in socially isolated female rats compared to the group housed animals (see 5.1). Since body mass gain is dependent on energy intake and energy expenditure, it suggests that decreased energy expenditure did not contribute to the increase body mass gain, but rather increased food consumption accelerated body mass gain in socially isolated female rats. This implies that social isolation in females has significant effects on factors that contribute to the development of obesity in female Sprague Dawley rats when compared to grouped housed animals. Our results concur with previous findings that social isolation resulted in increased body mass gain in KKmice and C57BL/6J mice (Nonogaki *et al.,* 2007; Sakakibara *et al.,* 2012).

Unlike in female rats our study shows that social isolation had no effect on body mass gain of male rats. However, both maternal separation and foot shock in the juvenile phase independently resulted in significantly decreased body mass gain in socially isolated male rats. Similar results showing a decrease in body mass following maternal separation in male Sprague Dawley rats were reported by McIntosh *et al*., (1999). Increased concentrations of thyroid hormones, resulting from hypothermia during the maternal separation has been proposed as a plausible explanation for the decrease in body mass in maternal separated rats (d'Amore *et al.,* 1993). Although we did not measure thyroid hormones, it is unlikely that this explanation would explain our findings since maternally

separated males had significantly increased visceral fat mass relative to body mass and the room temperature in our setting was well regulated.

In addition, male rats that were foot-shocked gained significantly less body mass compared to male rats that were not foot-shocked (figure 5.1). Results from the current study are in agreement with findings reported by Farias-Silva *et al.,* (1999) and Marin *et al.,* (2007) who reported decreased body masses in male rats that were foot-shocked. Considering that foot-shock stress also resulted in decreased visceral fat and decreased glucose concentrations, it is possible that there was activation of the lipolytic pathway.

5.3 Visceral fat mass as a percentage of body mass

Increased visceral fat is associated with the development of metabolic syndrome in humans (Okauchi *et al.*, 2007; Matsuzawa *et al.*, 2011). Female rats that were socially isolated had significantly increased visceral fat mass as a percentage of total body mass, compared to female rats that were housed in pairs. Social isolation is a stressor in social species (Jahng *et al.,* 2012) and activates the HPA axis and increases glucocorticoids release. Elevated glucocorticoids can cause the accumulation of visceral adipose tissue. Bjorntorp (2001) argued that in the presence of high insulin concentrations, cortisol inhibits lipid mobilisation resulting in lipid accumulation in visceral adipose tissue. Although the plasma corticosterone of the socially isolated females was significantly lower at termination than group housed females, it is possible that chronic activation of the HPA axis resulted in adrenal fatigue (Adžić *et al.,* 2009). However, adrenal fatigue was not evaluated in the current study.

Moreover, social isolation in male rats was not associated with increased visceral fat mass relative to body mass. However, regardless of the other stressors, male rats that were

maternally separated had significantly increased visceral fat mass as a percentage of total body mass compared to male rats that were not maternally separated. Interestingly, body mass gain in maternally separated male rats was significantly lower compared to male rats that were not maternally separated. Karagiannides *et al.*, (2014), reported that chronic unpredictable stress in male Fisher rats results in the decreased lean body mass and increased body fat mass. This finding suggest that there is an accumulation of visceral fat mass rather than an increase in lean body mass in maternally separated male rats. In addition, both serum insulin and plasma corticosterone were significantly increased in maternally separated males, further supporting the notion by Bjorntorp (2001) that lipid accumulation in visceral tissue is driven by glucocorticoid and insulin concentrations.

In addition, male rats that were foot-shocked had significantly decreased visceral fat mass as a percentage of total body mass compared to rats that were not foot-shocked. This is not surprising considering that foot-shocked rats had significantly lower body mass gain compared to non foot-shocked rats. As previously mentioned, we hypothesize that this may be a result of the activation of the lipolytic pathway; however lypolisis was not evaluated in our study.

5.4 Plasma glucose

Plasma glucose concentrations were similar in female rats. Our results suggest that early life stress, housing and foot-shock stress did not have a significant effect on glucose concentration in female rats.

However, male rats housed in pairs and foot-shocked had significantly lower plasma glucose concentrations compared to male rats that were socially isolated and foot shocked

and compared to male rats that were housed in pairs and not foot-shocked. This suggests that the combination of group housing and foot-shock has a lowering effect on plasma glucose concentrations in male rats. It was previously shown that the group housing offers social support and helps overcome stressful events (Liu *et al.,* 2013). Perhaps the support of a cage mate helped to alleviate the effect of the foot-shock stress on glucose concentrations in our study.

Although, several studies have reported increased glucose concentrations in acutely foot-shocked rats (Farias-Silva *et al.,* 2002; Sim *et al.,* 2010), we found that foot-shocked male rats had significantly reduced plasma glucose concentrations at termination. Foot-shock stress is a physical stressor, thus resulting in increased energy demand and expenditure. The low glucose levels in chronically foot-shocked rats could be due to increased glucose utilisation resulting from increased energy expenditure. The hypoglycemic effect of mild chronic foot-shock has not been noted before (Si metal., 2010; Kohno and Ohkubo, 1998). Interestingly, foot-shocked rats had decreased body mass gain and decreased visceral fat mass at termination, which could suggest that hypoglycaemia in these rats, was chronic.

5.5 Serum insulin and HOMA-IR

Socially isolated female rats had significantly higher serum insulin concentrations compared to female rats housed in pairs. However, the HOMA-IR index ratios were similar for all the groups. Although the use of HOMA-IR index has limitation in clinical applications, it is a widely used way of assessing insulin resistance in research (Qu *et al.,* 2011). Our study shows that socially isolated female rats had a tendency to increase serum

insulin concentrations without causing insulin resistance, because plasma glucose concentrations are within the normal ranges.

Moreover, male rats that were maternally separated and housed in pairs had significantly higher serum insulin concentrations compare to male rats that were not maternally separated and housed in pairs. This finding suggests that maternal separation in males housed in pairs increases the serum insulin concentration. Additionally, for male rats that were housed in pairs and foot-shocked, maternal separation increased the HOMA-IR index compared to animals that were not maternally separated.

In male rats that were not foot-shocked, maternal separation had an increasing effect on HOMA-IR index compared to non maternal separation. In addition, in male rats that were housed in pairs, maternal separation had an increasing effect on HOMA-IR index compared to non maternal separation. These results suggest that insulin resistance was developing in maternally separated male rats. Manian *et al.*, (2014) argues that maladaptation to early life stress, resulting in increased corticosterone, has the potential to lead to insulin resistance.

5.6 Innate immune response

In both male rats and female rats the innate immune response, as measured by production of ROS, was positively correlated with the number of white blood cells. This linear relationship between ROS production and WBC suggests that there was no priming of white blood cells in response to the stressors. For the purpose of the current study, ROS: WBC ratio was used to determine ROS released following PMA stimulation of white blood cells.

Our study shows that maternally separated female rats had significantly lower ROS: WBC ratios compared to female rats that were not maternally separated. Dimatelis *et al.*, (2012) found that eighty-four key cytokine gene expression levels were significantly down regulated in maternally separated Wistar rats. Additionally, in non-human primates, maternal separation was found to be associated with immunosuppression (Coe *et al.*, 1989). Our findings are consistent with previous findings that maternal separation has suppressive effects on the immune response, however the mechanism by which maternal separation suppressed the innate immune response requires further investigation.

Although previous studies have reported a decrease in ROS production following foot-shock stress (Oishi *et al.*, 2003 and (Li *et al.*, 2005), the current study demonstrates that foot-shocked male rats had significantly increased ROS production upon stimulation of WBC by PMA compared to non foot-shocked males. The increase in the ROS produced per WBC suggests that there was activation priming due to foot-shock in male rats only. The over production of ROS can lead to oxidative stress resulting in tissue damage and the physiological disturbances associated with obesity and lipidemia (Coe *et al.*, 1988; Halliwell *et al.*, 1992).

5.7 Plasma corticosterone

The secretion of corticosterone in rats is regulated by the stress response. Our study shows that socially isolated female rats had decreased plasma corticosterone concentrations compared to female rats housed in pairs. Similar results have been reported by Serra and Biggio (2014) who demonstrated that social isolation decreased plasma corticosterone levels compared to group housed rats. Although plasma corticosterone was not increased at termination in socially isolated females, we hypothesize that there was probably an initial increase in corticosterone due to the stress of social isolation (Yarnell *et al.,* 2015). Prolonged activation of the HPA axis also results in HPA axis hypoactivity and hence a decrease in plasma corticosterone (Hermes *et al.,* 2009; Adžić *et al.,* 2009), which may be accompanied by adrenal hypertrophy in chronically isolated animals (Adžić *et al.,* 2009; Ruis *et al.,* 1999). However, adrenal gland mass was not evaluated in the current study. It is also possible that the group housed animals had an increased reactivity to the stressor of euthanasia, as animals were transported individually to the termination room. The novelty of sudden isolation could have caused an increased stress reactivity and hence an increase in plasma corticosterone.

Given that socially isolated females had increased feed conversion ratio as a result of increased food intake, it is further plausible that the increase in food intake was a stress coping mechanism that resulted in blunting of corticosterone in socially isolated females. Jahng *et al.,* (2012) noted that socially isolated female Sprague Dawley rats that ate more food had significantly blunted stress induced corticosterone levels, particularly when highly palatable food was introduced.

In addition, our study shows that maternally separated male rats had increased plasma corticosterone concentrations compared to male rats that were not maternally separated. Increased corticosterone concentrations have been previously reported in maternally separated male rats (Marais *et al.,* 2008; Veenema and Neumann, 2009 and Roque *et al.,* 2014). These findings suggest that early life stress in the form of maternal separation results in alterations in HPA axis functioning. Since the rats in the current study were terminated at PND69, it is reasonable to assume that the alterations to HPA axis functioning extend well beyond the initial stressor and may be permanent. The period from PND 7 to weaning, in rats is of great significance for the maturation of the HPA axis, thus stress exposure during this period may result in lasting effects on HPA axis (Schmidt *et al.,* 2011 and Roque *et al.,* 2014).

CHAPTER 6: CONCLUSION AND RECOMENDATIONS

Investigating the effects of stress in animal studies has often proved to be complicated and inconsistent, consequently resulting in contradictory outcomes. The outcomes of such studies are dependent on several factors including the animals (rats or mice), strain and the protocol used.

The findings of the current study showed that gender had a significant influence on the response to early life stress, adolescent social isolation and foot-shock stress in late adolescent/early adulthood. This draws attention to the fact that male and females respond differently to specific stressors. The mechanisms underlying these variations in gender related stress responses remain unknown, but it is suggested that reproductive hormones play a role (Kelly *et al*., 2007).

In female rats, the stress of social isolation appears to be a more significant stressor than maternal separation and foot-shock stress, although maternal separation did result in suppression of the innate immune response. Social isolation resulted in increased feed conversion ratio, an increase in body mass gain, increased visceral adiposity and hyperinsulinemia. Hence females are more sensitive to interpersonal stress than their male counterparts (Kelly *et al*., 2007).

Our findings showed that male rats are more affected by early life stress and by foot-shock stress than they are by the stress of isolation. Stress in early life caused visceral adiposity, hyperinsulinemia and increased corticosterone in males, while foot-shock stress decreased body mass gain, visceral fat mass, plasma glucose concentrations and activated the innate immune response.

These finding suggest that housing (SI vs GH) has significant effects on female rats, and therefore should be carefully considered in experimental design of animal studies. Further studies are required to define the neurochemical mechanism by which adolescence social isolation increases food intake and weight gain in female rats. This could look into the peptide that drive satiety as well as the hormones that drive feeding behaviour. In addition our results are in contrast to the classical hypothesis of cumulative stress, which states that cumulative stress results in cumulative effects on the body. Further investigation into the effects of cumulative stressor needs to be done to better explain our findings.

Models of early life stress need to reflect the human condition upon which they are based, which brings the need for valid controls. The systems that govern early life stress and metabolic disease are complex and require validated animal experimental models. Species and strain differences must be considered and these animals must be valid models for the stress conditions they are mimicking in humans.

LIMITATIONS

Our protocol did not include naïve controls. Future studies should consider including naïve controls when investigating similar protocols. Additionally, our study investigated the long term effects of the stressors and the animals were only sacrificed at the end of the protocol. Using protocols that only evaluate the effects of a single stressor at a time should be considered in future studies as this may provide insights into the effects of the single stressor without other interactions.

CHAPTER 7: REFERENCES

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APPENDIX I

AESC3

STRICTLY CONFIDENTIAL

ANIMAL ETHICS SCREENING COMMITTEE (AESC)

CLEARANCE CERTIFICATE NO. 2013/04/05

APPLICANT: Dr N Pitts

SCHOOL: Physiology
DEPARTMENT:
LOCATION:

PROJECT TITLE: Does early life stress, in the form of maternal deprivation, followed by differential housing after weaning, impact on body mass and nociceptive sensitivity in rats?

Number and Species

144 Sprague-Dawley rats

Approval was given for to the use of animals for the project described above at an AESC meeting held on 20130219. This approval remains valid until 20150218.

The use of these animals is subject to AESC guidelines for the use and care of animals, is limited to the procedures described in the application form and to the following additional conditions:

1. **Professor K Erlwanger to be added as co-worker**
2. **Dr DM Eide to be added as a co-worker**
3. **Professor P Kamerman to be added as a co-worker (not a co-applicant)**
4. **Anaesthetic to be used must be stated**

Signed: _____ (Chairperson, AESC) Date: 6/3/13.

I am satisfied that the persons listed in this application are competent to perform the procedures therein, in terms of Section 23 (1) (c) of the Veterinary and Para-Veterinary Professions Act (19 of 1982)

Signed: _____ (Registered Veterinarian) Date: 6/3/13

cc: Supervisor: N/A
Director: CAS

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