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THE EFFECTS OF HOUSING, DIETARY CHANGES AND CHRONIC RESTRAINT STRESS ON BODY WEIGHT AND METABOLIC PARAMETERS IN THE MALE WISTAR RAT

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A dissertation submitted to the Faculty of Science, University of the Witwatersrand, in fulfillment of the requirements for the degree of Master of Science

Johannesburg, 2012

DECLARATION

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

_____

_____ day of _____ 20_____

ABSTRACT

This study examined the effect of prolonged differential housing and/or diet following exposure to chronic restraint stress on body weight, body fat weight, body fat composition and corticosterone, glucose, insulin and leptin levels. To this end, male Wistar rats were individually-housed (IH) or pair-housed (PH) and fed a condensed milk diet (CD) or a mushy rat chow diet (MD) for twelve weeks. Subsequently, all rats underwent chronic restraint stress (CRS) for seven hours/day for four weeks. During CRS, only PH rats fed MD continued to gain weight, while growth of the other groups was stunted. Housing and diet impacted on body fat weights, where PH caused increased retrorenal fat (P<0.001) in rats fed MD, while in rats fed CD, PH gave rise to less visceral (P<0.01) and more interscapular (P<0.05) and retrorenal fat (P<0.001). The CD resulted in more retrorenal (P<0.001) and interscapular fat (P<0.05) in PH rats, with more visceral (P<0.001) and retrorenal fat (P<0.01) in IH rats. Housing influenced only the fatty acid profiles of the liver and subcutaneous fat in rats fed CD. The CD caused differing fatty acid profiles of the liver, subcutaneous fat, visceral fat, interscapular fat and muscle in PH rats, while altering the fatty acid profiles of the liver, subcutaneous fat, interscapular fat and muscle in IH rats. Housing and diet did not result in differences in corticosterone, insulin and glucose concentrations, while both resulted in significantly elevated leptin levels in PH rats fed CD. Therefore the types of housing and diet have various effects on body weight and glucose and fat metabolism following chronic stress.

This dissertation is dedicated to

My dear parents, Jeff and Avril Ackerman, for their on-going support

My loving husband, Brandon, for his encouragement,

assistance and patience

My children, Elazar Tzvi, Sara Esther, Yaakov Yehuda

and Rossi Bear

who have been my stress alleviators

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ABBREVIATIONS

α-MSH – α-melanocyte-stimulating hormone

ACTH – Adrenocorticotropin

AGRP – Agouti-related peptide

ANS – Autonomic nervous system

CART – Cocaine and amphetamine-related transcript

CD – Condensed milk diet

CRF – Corticotropin-releasing factor

GABA – γ-amino-butyric acid

HPA – Hypothalamic-pituitary-adrenocortical

IH – Individually-Housed

LC/NE – Locus ceruleus-norepinephrine

MD – Mushy rat chow diet

MUFA – Mono-unsaturated Fatty Acids

n3-PUFA – Omega 3 Polyunsaturated Fatty Acids

n6-PUFA – Omega 6 Polyunsaturated Fatty Acids

NE - Norepinephrine

NPY – Neuropeptide Y

PUFA – Polyunsaturated Fatty Acids

PH – Pair-housed

RCF/G – Relative centrifugal force

SFA – Saturated Fatty Acids

SNS – Sympathetic nervous system

TAG – Triacylglycerides

CHAPTER ONE

Introduction

1.1 General Introduction

An animal can experience stress when actual or perceived challenges either in the external or internal environment, known as stressors, disturb an animal's biological state of equilibrium (Institute for Laboratory Animal Research, 1992). If a stressor persists, it can result in chronic stress. The chronic stress response and consequent negative health effects of chronic stress have been examined in animal models of stress.

In animal models of chronic stress, laboratory animals, usually rodents, are primarily exposed to environmentally-induced stressors (Institute for Laboratory Animal Research, 1992, Ottenweller, 2000). A frequently used stressor is restraint stress, in which a rodent's free movement is limited. Alterations in the stress response depend on the type of restraint stress employed as well as the duration and frequency of the restraint periods (Paré and Glavin, 1986, Servatius *et al*. 2000). However, prior to exposure to restraint stress and between restraint sessions, a rodent may be housed either individually, in pairs or in groups. If the type of housing is stressful, it may impact on how a rodent copes with restraint stress.

Seeing that rodents are social animals, individual-housing may be a potential stressor. The functioning of the hypothalamic-pituitary-adrenal (HPA) axis and various aspects of metabolism in chronic differential housing depend on how a rodent is housed (Westenbroek *et al*. 2003). In most cases individual-housing has been found to be stressful, whereas as

social support, provided by pair- or group-housing, alleviates stress (Baldwin *et al*. 1995, Gavrilovic and Dronjak, 2005, Perelló *et al*. 2006, Ruis *et al*. 1999, Seeman and McEwen, 1996, Serra *et al*. 2005, Sharp *et al*. 2002, Westenbroek *et al*. 2005). In terms of exposure to stressors, there is evidence to suggest that chronically individually-housed rats are hyperresponsive to environmental stimuli and to other stressors (Perelló *et al*. 2006). In contrast, social support can modulate the response to stressors (Baldwin *et al*. 1995, Brown and Grunberg, 1995, Ehlers *et al*. 1993, Perelló *et al*. 2006, Ruis *et al*. 1999, Sachser *et al*. 1998, Seeman and McEwen, 1996, Serra *et al*. 2005, Sharp *et al*. 2002, Westenbroek *et al*. 2005). To my knowledge, the impact of chronic differential housing on the subsequent exposure to chronic restraint stress has not been explored.

Apart from its role in the stress response, the HPA axis and related systems also play roles in energy homeostasis, influencing food intake, body weight and carbohydrate and fat metabolism (Laugero 2001, Laugero 2008, Mercer and Archer, 2005). In addition, appetite is affected by leptin, a hormone produced by adipose tissue which acts in the hypothalamus (Ahima, 2006, Jéquier, 2002). Since chronic stress leads to modifications in HPA axis activity; body weight, food intake and metabolism can be affected (Laugero, 2008). It has been suggested that the ingestion of certain foods, such as carbohydrates, can modulate the stress response (Laugero, 2001). At present, there is no study in rodents

that investigates the effect of long-term consumption of a high-energy diet while experiencing different housing conditions and how this impacts on various measures of carbohydrate and fat metabolism. Furthermore, it has not been established whether the consumption of a high-energy diet may impact on the ability of a rodent to cope with exposure to a subsequent stressor, like restraint stress.

Therefore in this dissertation, male Wistar rats were housed for 12 weeks either individually or in pairs and fed either a normal rat chow diet or a condensed milk diet to determine 1) the effect of prolonged housing on body weight by individually- or pair-housing rats, consuming a similar diet, and 2) the effect of diet on body weight when male Wistar rats were housed under similar conditions. Rats were then exposed to chronic restraint stress for seven hours a day for four weeks to determine 3) the metabolic effect of chronic restraint stress on rats exposed to differing housing and diet conditions on carbohydrate and fat metabolism by looking at body fat distribution and composition and serum concentrations of corticosterone, insulin, glucose and leptin and 4) if prolonged housing and/or dietary conditions influence the rats' ability to cope with a subsequent stressor i.e. chronic restraint stress.

This dissertation is divided into five chapters.

The first chapter is the introduction which is comprised of this general introduction and the literature review. The literature review focuses on the

chronic stress, the chronic stress response and the various metabolic effects of chronic stress specifically on carbohydrate and fat metabolism, including effects on body fat distribution and fatty acid profiles. Particular attention is paid to restraint stress and its metabolic effects, chronic housing and its metabolic effects and chronic housing followed by exposure to a new stressor. The combination of chronic stress and a high carbohydrate diet is then addressed and lastly the interaction of the condensed milk diet and obesity in rodent models is explored. The chapter ends off with the objectives of this study.

Chapter two - Materials and methods. This chapter describes the experimental protocol, post-mortem procedure, sample collections and analyses as well as how the data was analysed. The experimental protocol has been divided into two parts. Part one: Male Wistar rats were exposed to a 12-week housing and diet regimen, where rats were either individually-housed or pair-housed in plexiglass rat cages and fed either a normal rat chow diet or a condensed milk diet. During the 12 weeks, body weight measurements were collected. Part two: Following the 12-week housing and diet regimen, all rats were exposed to chronic restraint stress, where rats were removed from their overnight cages and placed individually in plexiglass cylinders for seven hours a day for four weeks. When the rats were not restrained, they were maintained under pre-restraint housing and dietary conditions. Each day prior to restraint, body weight was determined. At the end of the chronic restraint stress protocol

the rats were sacrificed. Blood was collected by cardiac puncture and used to determine the serum concentrations of corticosterone, insulin, glucose and leptin. The liver plus samples of various fat sites and muscle were harvested for fatty acid determination.

Chapter three – Results. The results section has been divided into two sections. Section A looks at the impact of housing, where rats consuming similar diets but housed either individually or in pairs were compared. Section B investigates the impact of diet by comparing rats housed under the same conditions and consuming a normal rat chow diet or a condensed milk diet. Each section is subdivided into two parts; body weight and metabolic parameters. In the first part, changes in body weight a) for the 12-week housing and diet regimen and b) for the four weeks of chronic restraint stress are reported. The second part focuses on metabolic parameters including; a) changes in body fat weight, b) body fat composition by looking at fatty acid profiles and c) serum concentrations of corticosterone, insulin, glucose and leptin.

Chapter four - General discussion. The discussion begins with a summary of the significant results relating to the impact of housing and the impact of diet on body weight during the 12-week housing and diet regimen and chronic restraint stress as well as the various metabolic parameters assessed post-chronic restraint stress. This is followed by a section which highlights the limitations of this study. The final part of the discussion

explores how prolonged housing and/or dietary conditions impact on the ability to cope with chronic restraint stress firstly in rats fed the mushy rat chow diet and then in rats fed the condensed milk diet. The effect of housing and diet on the serum concentrations of corticosterone, insulin, glucose and leptin as well as fat weights following chronic restraint stress is addressed. Finally, I examine the impact of housing and diet on body fat composition following chronic restraint stress.

The dissertation ends with Chapter five, the conclusion. In this chapter I give the conclusion from my findings as well as make recommendations for future studies.

Following the conclusion are the references used in this dissertation and Appendix 1, which gives detailed explanations of fatty acid analysis and analyses of serum corticosterone, insulin, glucose and leptin concentrations.

1.2 Literature Review

1.2.1 Introduction to Literature Review

The literature review will focus on chronic stress in male rodents, specifically the neuroendocrine response, where I have concentrated on the sympathico-adrenomedullary system and the hypothalamic-pituitary-adrenal (HPA) axis. I have referred to the concepts associated with acute stress where appropriate. Although the response to stress involves many components, the metabolic effects of chronic stress, including changes in hormone levels, body weight, glucose and fat metabolism are of particular interest. I have also included a section on energy balance, feeding and body weight.

Considering that I wanted to find out if prolonged housing conditions influence the rats' ability to cope with chronic restraint stress, the literature review focused on restraint stress and housing. The metabolic effects of restraint stress and housing particularly on the HPA axis, leptin, body weight and carbohydrate metabolism were reported. For restraint stress, I only incorporated studies that used loose restraint. Although many studies looking at individual-housing have been conducted in rodents that were weaned early, my interest was in long-term housing of adult rodents.

I then addressed the concept of chronic housing followed by exposure to a new stressor. I also included a section on chronic stress and dietary

changes as the effects of ingesting a high carbohydrate diet while being exposed to chronic stress are of particular interest.

1.2.2 Acute Stress versus Chronic Stress

An animal can experience stress in response to an actual or perceived threat, known as a stressor, which causes a disturbance in an animal's biological state of equilibrium (Institute for Laboratory Animal Research, 1992, McEwen, 2000b). In response to a stressor, allostasis needs to occur. Allostasis is the ability of an organism to adapt or return to a state of homeostasis in order to ensure survival (McEwen 2000a). Allostasis occurs during and shortly after exposure to the stressor and involves modifications in physiological processes including neural, neuroendocrine and neuroendocrine-immune mechanisms, which may be accompanied by changes in behaviour. This is known as the allostatic response. The main allostatic response involves two systems namely; the sympathico-adrenomedullary system, and the hypothalamic-pituitary-adrenal (HPA) axis (McEwen, 1998, McEwen, 2000a, Pacak and McCarty, 2000).

The physiological mediators of the allostatic response are catecholamines and glucocorticoids, which are the end products resulting from the stimulation of the sympathico-adrenomedullary system and HPA axis respectively. The nature of the allostatic response is that it should be stimulated for the time that it is exposed to a stressor, therefore

catecholamines and glucocorticoids are released in appropriate amounts to meet the needs of the challenge. When the stressor has passed and reasonable adaptation has occurred, the allostatic systems should be switched off, resulting in the return of the mediators of allostasis to baseline levels (McEwen, 2000a). This successfully occurs in the case of acute stress. Therefore, the allostatic response is advantageous to an animal's well-being, as the response allows for adaptation, maintenance of homeostasis and ultimately survival (McEwen and Stellar, 1993). However, as stated by Bartolomucci *et al.* (2005), "the problem with the stress response is that it is adaptive in the short-term but it can become highly maladaptive in the long-term." This occurs in the case of chronic stress.

Chronic stress leads to changes in physiology and behaviour that continue for days or weeks during exposure to repeated stressors or after the exposure to a stressor or stressors (Ottenweller, 2000). The cost of "the cumulative strain on the body produced by repeated ups and downs of physiologic response, as well as by the elevated activity of physiologic systems under challenge..." (McEwen and Stellar, 1993, p. 2094) is known as allostatic load. There are four main conditions that result in allostatic load namely; 1) frequent stimulation of the allostatic systems resulting in exposure to elevated levels of stress hormones, 2) failure to turn off allostatic activity following stress, 3) poor allostatic response which leads to increased activity of other allostatic systems and 4) inadequate

adaptation to the exposure of repeated stressors (McEwen, 1998, McEwen, 2000a, McEwen and Stellar, 1993). Allostatic load and therefore chronic stress can result in long-term changes that have been implicated in pathophysiological conditions such as cardiovascular disease, diabetes, hyperlipidemia, hypertension and reduced immune function (McEwen, 2000a, Pacak and McCarty, 2000).

Progression to a diseased state depends on the way each animal deals with stress, which is associated with the type of species, age, sex, previous experience, genetic endowment and the animal's physiological and psychological condition when exposed to the stressor (Institute for Laboratory Animal Research, 1992, Ottenweller, 2000). With these individual differences in reaction to a stressor, a unique pattern of allostatic response occurs but the final pattern also depends on the nature, duration and intensity of the stressful experience (McEwen, 2000b, Steptoe, 2000). Thus research using one stressor and one species may not be applicable to other species. Despite this, there are some common effects of chronic stress.

Characteristic effects of chronic stress have been observed in animal models of stress, where persistent behavioural, immunological and neuroendocrine modifications occur (Ottenweller, 2000).

Frequently reported behavioural changes include hyperarousal, fear of novelty and altered cognitive ability, specifically an impairment of spatial memory (Kleen *et al.* 2006, Ottenweller, 2000).

With regards to immune function, although most studies report suppression of the immune system, which may be due in part to involution of the thymus (Martí *et al.* 1994), some claim that there is an increase in immune function (Ottenweller, 2000, Tsigos and Chrousos, 2002).

Neuroendocrine effects include; continuous activation of the autonomic nervous system (ANS) and the hypothalamic pituitary adrenal (HPA) axis, inhibition of the hypothalamic-pituitary-thyroid (thyroid) and the hypothalamic-pituitary-gonadal (reproductive) axes, growth hormone secretion is reduced and prolactin release in activated (Ottenweller, 2000, Tsigos and Chrousos, 2002).
In the field of the metabolic effects of stress, it is the neuroendocrine response to chronic stress which is the most important.

1.2.3 Neuroendocrine response to Chronic Stress

The neuroendocrine response to stress involves the central and peripheral components of two major systems- the sympathico-adrenomedullary system (a component of the autonomic nervous system) and the hypothalamic-pituitary-adrenal (HPA) axis. The final peripheral effectors of

these systems are catecholamines and glucocorticoids respectively, which influence numerous bodily systems (Chrousos, 2009, Sapolsky *et al.* 2005, Tsigos and Chrousos, 2002).

1.2.3.1 Sympathico-adrenomedullary system

The sympathico-adrenomedullary system responds to stress via two different pathways, which work concurrently. The first pathway involves the stimulation of the central catecholaminergic neurons of the locus ceruleus - norepinephrine (LC/ NE) system located in the brain stem, which causes the autonomic nervous system (ANS) to spring into immediate action. Both the parasympathetic and sympathetic nervous systems are stimulated. Activation of the parasympathetic component of the ANS aims to restore homeostatic balance by preserving energy. However, during stress, the sympathetic nervous system (SNS) is primarily activated, resulting in the rapid release of the catecholamines, epinephrine and norepinephrine, from postsynaptic neurons (Chrousos and Gold, 1992, Tsigos and Chrousos, 2002).

The second pathway comprises the direct neural pathway from the hypothalamus to the adrenal medulla (via the sympathetic preganglionic neurons), which results in the release of catecholamines into the blood, mainly epinephrine and to a lesser extent norepinephrine (Bartolomucci *et al*. 2005, Chrousos and Gold, 1992).

The catecholamines from both pathways bind to adrenergic receptors on peripheral tissues resulting in metabolic, cardiovascular and visceral effects, otherwise known as the “fight-or-flight” response (Chrousos and Gold, 1992, Chrousos, 2009).

There is an increase in arousal and alertness and a suppression of appetite and reproductive behaviour (Chrousos, 2009, Elenkov *et al.* 2000, Sapolsky *et al.* 2000). Physiologically, oxygen and nutrients are redirected to the central nervous system and stress-activated tissues (Chrousos, 2009, Sapolsky *et al.* 2000). There is an increase in blood pressure, heart rate, respiratory rate, gluconeogenesis, glycogenolysis and lipolysis as well as inhibition of the reproductive and digestive systems (Chrousos, 2009, Pacak and McCarty, 2000, Sapolsky *et al.* 2000).

In response to chronic stress, the autonomic nervous system becomes hyperactive due to continuous activation of the autonomic nervous system and excessive exposure to catecholamines, which often leads to hypertension and cardiovascular disease (McEwen, 1998, McEwen, 2000a, McEwen and Stellar, 1993). Moreover, with chronic exposure to the same stressor, various adaptive alterations take place which include increased production and storage of catecholamines, elevated basal concentrations of circulating catecholamines and reduced secretion of catecholamines following exposure to the same stressor (McCarty *et al*. 1988).

In addition to the secretion of catecholamines as part of the allostatic response, glucocorticoid release occurs, which is the final peripheral effector of HPA axis stimulation.

1.2.3.2 The Hypothalamic-Pituitary-Adrenal (HPA) axis

Like the sympathico-adrenomedullary system, stimulation of the HPA axis begins in the hypothalamus, where stimulatory inputs from various brain regions come together, resulting in activation of the parvocellular neurons in the paraventricular nucleus (Laugero, 2001, Tsigos and Chrousos, 2002). These neurons synthesize and secrete corticotropin-releasing factor (CRF) and arginine vasopressin (AVP), into the hypophyseal portal system, which stimulate the secretion of adrenocorticotropin (ACTH) from the anterior pituitary gland. ACTH is released into the blood where it in turn acts on the adrenal cortex resulting in the production and release of glucocorticoids (cortisol in humans and corticosterone in rodents) and mineralcorticoids (e.g. aldosterone) into the bloodstream (Laugero, 2001, Tsigos and Chrousos, 2002).

During acute stress, the primary hormones which are released are glucocorticoids. Glucocorticoids are capable of regulating their synthesis and secretion at a number of levels via negative feedback mechanisms; glucocorticoids regulate their own production and release within the adrenal gland, they inhibit both the secretion of CRF and production and release of ACTH via central glucocorticoid receptors (Pacak and McCarty,

2000). In the short term, the acute effects of glucocorticoids affect adaptive responses such as the regulation of blood pressure, water and salt balance, immune function and energy metabolism (Macfarlane *et al.* 2008, Sapolsky *et al.* 2005).

In contrast to acute stress, during chronic stress, the normal mechanism by which glucocorticoids inhibit ACTH secretion is considerably altered (Dallman *et al.* 2004) resulting in significantly elevated circulating ACTH and glucocorticoids and dysregulation of the HPA axis (Dallman *et al.* 2004). In addition, the negative feedback response to exogenous glucocorticoids is disrupted, as can be seen by the failure of dexamethasone (a synthetic glucocorticoid) to suppress the release of endogenous glucocorticoids during chronic stress (Bartolomucci *et al.* 2005, Mizoguchi *et al.* 2001). Other common observations include hypersecretion of basal cortisol and adrenal hypertrophy (Mizoguchi *et al.* 2001)

A further example of dysregulation of the HPA axis has been seen in chronically stressed rats that have undergone an adrenalectomy. When these rats were given corticosterone replacements after chronic stress, they required higher concentrations of corticosterone in order to facilitate an ACTH response. Moreover, basal levels of ACTH were elevated and CRF increased with the infusion of corticosterone intracerebroventricularly. Thus, when exposed to chronic stressors,

corticosterone causes excitation rather than inhibition in the brain (Dallman *et al.* 2004).

1.2.3.3 Links between the sympathico-adrenomedullary system and HPA axis

Reciprocal neural connections exist between CRF-secreting neurons of the hypothalamus and the central catecholaminergic neurons of the LC/NE system, where CRF and norepinephrine (through α_1-noradrenergic receptors) stimulate each other (Tsigos and Chrousos, 2002). Central CRF is essential for activation of central noradrenergic pathways and therefore regulates the response of the sympathico-adrenomedullary system to stress. This is evident from the inhibition of plasma catecholamine release by blockade of central CRF receptor (Jezova *et al.* 1999). In the paraventricular nucleus of the hypothalamus, central noradrenergic neurons terminate and synapse on CRF-secreting neurons, releasing norepinephrine. Norepinephrine is believed to be a powerful promoter of CRF production (Pacak and McCarty, 2000). Furthermore, ACTH can directly influence the transcription and expression of the genes encoding for norepinephrine biosynthetic enzymes (Serova *et al.* 2008).

Together glucocorticoids and catecholamines influence arousal, behaviour, growth, reproductive and thyroid hormone axes, wake-sleep centers, gastrointestinal, cardiovascular, respiratory and immune systems. Most importantly, glucocorticoids together with catecholamines influence

carbohydrate, protein and fat metabolism (Chrousos, 2009, Sapolsky *et al.* 2005).

1.2.4 Metabolic effects of Chronic Stress

Carbohydrate metabolism

With increases in blood glucose levels following feeding, insulin stimulates glucose uptake by muscle and adipose tissue. Therefore, insulin has hypoglycemic actions as it results in the lowering of blood glucose levels.

In contrast, the immediate response to stress involves the production and release of catecholamines, which function together with growth hormone and glucagon, to cause a rapid rise in blood glucose levels. This is achieved by mobilizing existing energy stores (glycogen and adipose tissue). These hormones also prevent the uptake of glucose by muscle and adipose tissue by downregulating glucose transporters in the peripheral tissues and thus further inhibit energy storage by insulin resistance. Catecholamines, glucagon and growth hormone are fast-acting, whereas glucocorticoids act gradually to enhance and prolong the increase in blood glucose levels (Sapolsky *et al.* 2000).

After the acute response, glucocorticoids maintain elevated glucose levels by increasing gluconeogenesis in the liver. Substrates for gluconeogenesis, gluconeogenic amino acids and glycerol, are derived

from the catabolic effects of glucocorticoids on 1) protein in peripheral tissues e.g. muscle and connective tissue (proteolysis) and 2) triacylglycerides (TAGs) stored in adipose tissue (lipolysis) respectively. Peripheral glucose transport and utilization are also inhibited by glucocorticoids (Macfarlane *et al.* 2008, Sapolsky *et al.* 2000). Therefore, the main metabolic effect of both catecholamines and glucocorticoids is to mobilize energy stores, resulting in an elevation of blood glucose levels to meet the higher metabolic demands produced by acute stress.

Once the stressor has passed, insulin's hypoglycemic effects return to reverse the metabolic actions of the other hormones, to re-establish energy reserves through glycogen formation and lipogenesis (accumulation of fat) (Sapolsky *et al.* 2000).

In contrast to acute stress, in the presence of elevated glucocorticoids, carbohydrate metabolism is significantly altered. The suppressive effect of glucocorticoids on insulin action is lost during chronic stress. Sustained glucocorticoid levels cause increases in plasma glucose which is followed by a compensatory and sustained rise in plasma insulin levels after a delay of a few hours (Dallman *et al.* 2004, Sapolsky *et al.* 2000). Thus, chronic stress presents with raised glucose due to impaired glucose uptake in the periphery and elevated insulin concentrations (hyperinsulinaemia), as a result of hepatic insulin resistance (Macfarlane *et al.* 2008).

Fat Metabolism

Stress has important acute and long term effects on fatty acid metabolism. As mentioned above, during stress, energy mobilization is necessary. Therefore, in the presence of elevated glucocorticoids, catecholamines and other lipolytic counter-regulatory hormones such as glucagon and growth hormone, the net effect on fatty acid metabolism is lipolysis of TAGs in adipose tissue (Macfarlane *et al*. 2008).

Lipolysis refers to the breakdown of TAGs into glycerol and free fatty acids by a multi-enzyme complex, hormone sensitive lipase. Both glycerol and the free fatty acids are released into the circulation and are used for energy generation. TAGs are an efficient source of energy and can yield twice as much energy per gram compared to glycogen and protein (Hillgartner *et al.* 1995). If feeding should occur during times of stress, insulin-stimulated storage of TAGs in the adipose tissue is inhibited so that the net release of free fatty acids is maintained (Macfarlane *et al.* 2008).

The effects of chronic glucocorticoid excess on fatty acid metabolism are not well understood, but are believed to cause abnormalities in the metabolism of fatty acids (Macfarlane *et al.* 2008). From human studies, chronic glucocorticoid levels, as seen in Cushing's syndrome, prevail in the presence of normal or low counter-regulatory lipolytic hormones and sustained hyperinsulinaemia, resulting in the net release of free fatty acids from subcutaneous adipose tissue and storage of TAGs in visceral

adipose tissue. Thus, redistribution of adipose tissue depots occurs in Cushing syndrome sufferers (Hillgartner *et al.* 1995). In times of chronic stress, perhaps like humans, redistribution of body fat may occur in rodents.

However, rather than gaining weight in response to chronic glucocorticoids, animals tend to lose body weight and fat (Elliott *et al.* 1971). The effects of glucocorticoids on appetite, insulin resistance and depot-specific free fatty acid turnover are more than likely responsible for a pattern of fatty acid metabolism comparable to that which occurs during acute stress. Thus lipolysis primarily occurs in rodents, with decreases in adipose tissue mass, body weights in turn may decrease (Macfarlane *et al.* 2008).

With the mobilization of TAGs during lipolysis, fatty acids are released, which in turn leads to the alterations in the fatty acid composition of adipose tissue. In the adipose tissue, certain fatty acids predominate; saturated fatty acids (SFAs) such as myristic acid, palmitic acid and stearic acid, monounsaturated fatty acids (MUFAs) such as palmitoleic acid and oleic acid and omega 6 polyunsaturated fatty acids (n6-PUFAs) for example linoleic acid (Albright and Stern, 1998, Body, 1988). With lipolysis, there is a selective release of fatty acids (Raclot and Oudhart, 1999).

Mobilization of saturated fatty acids (SFAs) in adipose tissue occurs the least, followed by intermediate mobilization of monounsaturated fatty acids (MUFAs) and the greatest mobilization of polyunsaturated fatty acids (PUFAs). Mobilization of fatty acids is based on the molecular structure of the fatty acid i.e. the degree of unsaturation and chain length, rather than on their proportion in adipose tissue depots. Therefore the greater the degree of unsaturation and the shorter the chain length, the greater the chance that the fatty acid will be mobilized i.e. PUFAs first, SFAs last. Furthermore, seeing that SFAs and MUFAs are incorporated in the cell membrane, it would make sense that they are not mobilized as readily as PUFAs (Connor *et al.* 1996, Hollenberg and Angel, 1963, Raclot and Oudhart, 1999).

Considering that fat metabolism is significantly influenced by stress, it comes as no surprise that leptin levels will in turn be affected.

Leptin

Leptin, an *ob* gene product, is a hormone produced by adipocytes that signals to the brain the amount of energy stored in the adipose tissue (Ahima, 2006, Jéquier, 2002). Leptin promotes sympathetic outflow and attenuates the HPA axis response to stress (Heiman *et al.* 1997). Conversely, the SNS appears to inhibit the secretion of leptin while glucocorticoids stimulate leptin release (Ahima and Osei, 2004). Therefore seeing that both the SNS and HPA axis are activated during stress,

adipocytes may be receiving opposing signals. Depending on the type, duration and intensity of the stressor, leptin secretion may be stimulated or inhibited during chronic stress (Sandoval and Davis, 2003).

During chronic stress, reductions in leptin levels appear to result from more prolonged and/or higher intensity stressors. In order for a reduction to take place, the inhibitory effects of the SNS would need to override the stimulatory effects of the HPA axis. In most cases leptin levels have been found to decrease (Sandoval and Davis, 2003).

In addition, it has been suggested that a major role of leptin is to switch fuel utilization from carbohydrates to fat. Therefore, leptin enhances the oxidation of fatty acids, while decreasing glucose levels. However, during stress when glucose requirements increase, it would make sense for leptin levels to decrease as faster energy generation is achieved through carbohydrate metabolism (Sandoval and Davis, 2003). Furthermore, depletion of fat stores, due to the catabolic effects of glucocorticoids and catecholamines during chronic stress, is consistent with a decrease in the amount of circulating leptin (Jéquier, 2002).

Metabolic-brain feedback system

Laugero (2001, 2008) proposed that the metabolic effects of glucocorticoids and catecholamines on energy availability in the periphery feed back to the brain through a metabolic-brain feedback system, modulating central CRF as well as central NE activity. In times of acute

stress, when glucose requirements have been met, the metabolic-brain feedback system is inhibited resulting in no further release of central CRF and NE (Laugero 2008).

In the case of chronic stress, elevations in CRF and NE activity result in raised glucocorticoid and catecholamine levels (McEwen, 2000a). Energy reserves are significantly depleted due to the overall catabolic effect of glucocorticoids and catecholamines, resulting in disinhibition of the metabolic-brain feedback system. The disinhibition of the metabolic-brain feedback system and/or positive feedback from glucocorticoids sustains further increases in CRF and NE (Laugero, 2001, Laugero, 2008).

Therefore, the overall metabolic effects of chronic stress result in increased CRF, catecholamine, glucocorticoid, glucose and insulin levels and reduced leptin secretion and changes in fatty acid profiles of adipose tissue and possibly changes in fat distribution.

1.2.5 Energy balance, feeding and body weight

Over and above its role in the stress response, the hypothalamus plays an important role in regulating energy homeostasis (Bhatnagar *et al.* 2006, Mercer and Archer, 2005). Energy homeostasis is the delicate balance between energy intake and energy expenditure. Considering that stress leads to alterations in HPA axis activity and in the metabolic-brain

feedback system (Laugero, 2008), energy homeostasis will also be influenced. An imbalance between energy intake and energy expenditure, can have adverse effects in terms of body weight, resulting in obesity or anorexia (Kretschmer *et al*. 2005).

The hypothalamus plays a role in energy homeostasis by influencing normal food intake and body weight. Both CRF and glucocorticoids control food intake. CRF acts as an anorexigenic/anorectic neuropeptide in the central nervous system, where it inhibits the drive to eat, possibly by suppressing the appetite stimulant neuropeptide Y (NPY) in the hypothalamus (Laugero, 2001). In contrast to CRF, feeding is enhanced by glucocorticoids due to its effects, either directly or indirectly, on the secretion of NPY. Glucocorticoids are also essential for maintaining normal feeding, as seen in adrenalectomized animals, where the absence of glucocorticoids inhibits feeding (Laugero, 2001, Newman *et al*. 2007). In addition, glucocorticoids stimulate appetite possibly by counteracting the effects of leptin, an appetite suppressant (Zakrzewska *et al.*, 1997).

In rodents, leptin could also act as a satiety factor thereby controlling food intake. In the hypothalamus, leptin binds to leptin receptors, which are located on two types of hypothalamic neurons. One type of neuron produces and secretes two orexigenic neuropeptides namely; neuropeptide Y (NPY) and agouti-related peptide (AGRP). Leptin inhibits the expression of both NPY and AGRP. The other type of neuron

produces anorexigenic neuropeptides including α-melanocyte-stimulating hormone (α-MSH) and cocaine-and-amphetamine-related transcript (CART). Leptin stimulates the synthesis and secretion of α-MSH and CART. Therefore, by both the inhibition of orexigenic and the stimulation of anorexigenic neuropeptides, leptin reduces appetite and in turn food intake, resulting in a reduction in body weight (Ahima, 2006, Jéquier, 2002).

The role of leptin appears to be at multiple levels as a feedback regulatory loop consisting of three stages has been clearly established in rodents: stage 1- a sensor (leptin) detects the amount of adipose tissue, stage 2- leptin receptors in the hypothalamus obtain and integrate the incoming leptin signal and stage 3- effector systems, which includes the sympathetic nervous system, maintain energy balance by controlling energy intake and expenditure (Jéquier, 2002, Zakrzewska *et al.* 1997). Seeing that weight gain is followed by an increased production of leptin and weight loss by a decrease in leptin (Jéquier, 2002, Zakrzewska *et al.* 1997), it would appear that this feedback regulatory loop ensures that a constant body weight is maintained.

CRF, glucocorticoid and leptin levels interact with many other factors to control food intake. During stress, alterations in CRF, glucocorticoid and leptin levels occur, which could influence food intake. Changes in food intake may be accompanied by changes in body weight.

During acute stress, the release of CRF at the initiation of the HPA axis response to stress has inhibitory effects on food intake. Although basal levels of glucocorticoids stimulate food intake, during stress raised concentrations of glucocorticoids reduce food intake (Sapolsky *et al.* 2000).

During chronic stress, the actions of CRF are reinforced by the excess secretion of glucocorticoids, causing a reduction in food intake (Dallman *et al.* 2006).

Body weight

Chronic stress is usually associated with a drop in body weight or a reduced gain in body weight (Bhatnagar *et al.* 2006), which can be due to a reduction in food intake or an increase in energy expenditure or a combination of both. With the maintained elevated CRF levels during chronic stress, the decreased food intake may be accompanied by a reduction in body weight (Marin *et al.* 2007, Martí *et al*., 1994).

The observed decrements in body weight may be accounted for by a reduction in the amount of adipose tissue as well as lean tissue mass. During chronic stress when the action of glucocorticoids and catecholamines is prolonged, a reduction of fat stores occurs together with loss of muscle, bone, connective tissue and skin mass, i.e. body protein,

which results in a decrease in lean tissue (Harris *et al*. 2002, Harris *et al*. 2006).

At some point during a period of stress, the appetite stimulatory effects of glucocorticoids come into play. It has been proposed by Laugero (2008) that glucocorticoids help to re-establish energy stores that were depleted by stress. When leptin was injected intracerebroventricularly into adrenalectomized rats, it produced a significant decrease in body weight and food intake compared to normal rats. However, administration of glucocorticoids to the same adrenalectomized rats suppressed the “potent effects” of leptin i.e. the effects that leptin have on food intake, body weight and adipose tissue mass were dampened (Solano and Jacobson, 1999, Zakrzewska, 1997).

However, others have found that high but physiological plasma corticosterone levels reduced only the anorectic effects of leptin and that the central infusion of leptin influences body weight, accumulation and transport of lipids, and adipose and muscle lipoprotein lipase activity, independent of corticosterone status (Arvaniti *et al.* 2001). Therefore under stressful conditions, when glucocorticoid secretion is elevated, the weight- and food intake- reducing effects of leptin may be inhibited, resulting in increased food intake and body weight.

As mentioned previously, leptin has been identified as a hormone produced by the adipose tissue and signals the amount of energy stored in adipose tissue. In addition, leptin plays a role in determining fat distribution. This can be seen from rats that were administered with leptin chronically and the amount of visceral fat decreased (Cases and Barzilai, 2000). Thus, leptin inhibits visceral adiposity as it promotes fatty acid oxidation (Jéquier, 2002).

Leading on from this, during chronic stress in the presence of excess glucocorticoids, an individual may consume substantially more food resulting in increased glucose and insulin levels and a greater possibility that the energy consumed will be deposited as fat, specifically in the abdominal area/ visceral region where there is an increased concentration of glucocorticoid receptors (Laugero, 2001, Newman *et al*., 2007). Therefore, chronic exposure to some stressors may play a factor in the gain of body weight and the development of obesity.

Therefore, in addition to the other metabolic effects of chronic stress mentioned previously, food intake is inhibited and reductions in body weight occur. However, over time, glucocorticoids help to re-establish energy stores through appetite-stimulating effects and also by suppressing the activity of leptin, which may possibly lead to the development of obesity if there is an over-indulgence in food.

Whether changes in body weight will occur, also depends on the severity of the stressor. Repeated stress can stimulate alterations in energy balance pathways resulting in regular decreases in body weight and changes in food intake (Bhatnagar *et al*. 2006). Although different chronic stress paradigms can result in a reduction in body weight or reduced gain in body weight, the decrease is not always due to a reduction in food intake, as an increase in food consumption has been observed. Therefore, the relationship between body weight and food intake is not similar for all stress procedures. It is plausible that the resulting body weight decrement may be caused by the metabolic costs of a particular stressor (Vallès *et al.* 2000).

1.2.6. Environmentally-induced Stressors

The neuroendocrine and subsequent metabolic effects of the chronic stress response have been examined in animal models of stress (Ottenweller, 2000). The use of these stress models has been fundamental in our understanding of how the human body reacts to stress and the extent to which stress affects our health. Furthermore, animal models have been helpful in developing therapies to cope with stress. Rodents are often the animals of choice for models of stress seeing that they are genetically similar to humans and are convenient to work with. In these models of stress, laboratory rodents are commonly exposed to

environmentally-induced stressors (Institute for Laboratory Animal Research, 1992, Ottenweller, 2000).

Environmentally-induced stressors in the captive environment are probably the most persistent type of stressor to which laboratory animals are exposed and can be grouped into one of six categories namely; 1) relationships with animals of the same species (conspecifics), 2) predator-prey relationships, 3) shelter, 4) spatial architecture, 5) feeding and foraging and 6) environmental events (Institute for Laboratory Animal Research, 1992). Environmental events can consist of stressors in the immediate environment such as noise, odour and temperature but can also relate to animal husbandry routines, blood collection, handling, and experimental procedures which regularly involve exposure to various acute and chronic environmentally-induced stressors (Balcombe *et al*. 2004, Institute for Laboratory Animal Research, 1992). An example of an environmentally-induced stressor that is frequently used in animal models of stress is restraint stress.

1.2.6.1 Restraint stress

Restraint stress refers to the method employed to limit a rat's free movement (Servatius *et al*. 2000). Hans Selye initiated groundwork on restraint stress, where he observed that restraint resulted in a stress response in the rat (Selye, 1936). The use of restraint stress in experimental protocols was instigated in the 1950s by Serge Bonfilo of

France and reviewed in 1971 by David Brodie of the United States (Brodie, 1971). Restraint stress was initially used to produce gastric lesions in rats. In later years, restraint stress was primarily used to investigate the biochemical and physiological reactions that occur in response to stress. Currently, restraint stress is used to examine the effects of various drugs as well in the fields of physiology, pharmacology, immunology and behavioural neurobiology (Paré and Glavin, 1986, Servatius *et al*. 2000).

An advantage of restraint stress is that the protocol is very flexible, as a researcher can vary the time a rodent spends in restraint (duration), the amount of restraint periods (frequency), the time interval between restraint periods or the amount of food consumed prior to restraint (Glavin *et al*. 1994, Servatius *et al*. 2000). Restraint stress protocols can produce a number of stress-related alterations which include; increased plasma corticosterone, fatty acids and glucose concentrations, adrenal hypertrophy, atrophy of the thymus and spleen, gastric ulcers and elevated noradrenaline and dopamine use in a variety of areas in the brain (Paré and Glavin, 1986).

Restraint stress is often referred to as an acute stressor; however it can be acute or chronic depending on the duration and frequency/number of the restraint periods. In general, the longer the duration of a restraint stress period, the greater the chance of developing stress-related pathologies. Conversely, it has been observed in less intense restraint types, such as

loose restraint, that an increase in duration of episodes may be linked to reduced responsiveness indicating some degree of habituation (Paré and Glavin, 1986, Servatius *et al*. 2000).

With an increase in the frequency of restraint stress periods, there is an elevation in associated pathologies. However, similar to duration, an increased frequency of sessions may result in decreased physiological responsiveness and magnitude of the stress response. There is evidence to suggest that there is adaptation/sensitization to repeated restraint periods. Adaptation is somewhat dependent on the ratio of stress-free periods to restraint periods, and occurs when the duration of the stress-free period is longer relative to the restraint period (Glavin *et al*. 1994, Paré and Glavin, 1986). Furthermore, a number of factors can influence the response to restraint stress as well as the extent to which rats habituate to the restraint stress protocol. These factors include; age, gender, intensity of the restraint stress, strain of rat, time of day, season and pre-restraint food deprivation period (Servatius *et al*. 2000)

Restraint stress has been widely utilized either on its own or together with other stressors to investigate the effects. For the initial restraint protocol, the rat's legs were tied together and the rat was wrapped tightly in a towel (Paré and Glavin, 1986). Currently, there are various ways in which rats can be restrained. These include loose restraint, tight restraint, supine

restraint and a combination of restraint stress with other stressors (Servatius *et al*. 2000).

Loose restraint involves restraint to limit free movement; however, some movement is feasible. Loose restraint entails restricting rats to a small chamber such as a cylindrical plastic tube or a wire mesh container for periods ranging from 5 minutes to several hours. Loose restraint is not only used while researching stress but may also be used when performing basic laboratory procedures (Paré and Glavin, 1986, Servatius *et al*. 2000).

In tight restraint, a rat's movement is entirely restricted and is also known as immobilization stress. This type of stressor is more intense than loose restraint. In most cases, immobilization stress, as developed by Kvetňansky and associates, involves the taping of rats' limbs, while in the prone position, to mounts attached to a metal frame, while motion of the head is restricted by a pair of metal loops connected to the metal frame. A single exposure to immobilization may last from 5 to 120 min or more. To produce chronic stress, rats may be immobilized daily for weeks or even months (Kvetňansky and McCarty, 2000).

Other methods of tight restraint include a jacket or a harness restraint. Supine restraint, a form of tight restraint, is where a rat is tied down while in the supine position. Lastly, restraint stress may be combined with another stressor. For example; water immersion, heat, cold or tail shock (Servatius *et al*. 2000).

Loose restraint is of particular interest and Table 1.1 provides a summary of studies that have used different types of loose restraint.

The data from Table 1.1 indicates that various types of restrainers, with varying dimensions, have been used and that the duration and frequency of restraint is variable. Furthermore, since different types of stressors influence behaviour and stress profiles in various ways (Glavin *et al*. 1994), the type of restrainer used may play an integral part in how rats interpret the restraint procedure. In terms of the response to restraint stress, various metabolic effects have been observed.

Table 1.1: Summary of studies using different types of loose restraint

Study	Rat Strain	Type of restrainer	Dimensions	Time in restraint
Martí *et al.* 1994	Sprague-Dawley	Plastic tube with several bored holes	Not clearly described	1 h/day for 27 days
Baldwin *et al.* 1997	Sprague-Dawley	Polypropylene restraining tubes, Baxter Scientific Products, McGaw Park, IL	Not clearly described	4h/day for 6 weeks
Harris *et al.* 2002	Wistar	Plastic tube, Plas Labs,MI	L = 21.6 cm ID = 6.4 cm V = 695 ml	3h/day for 10 days or two bouts of 3h/day for 3 days separated by 7 days
Retana-Márquez *et al.* 2003	Wistar	Plexiglass cylinder	L=16.0 cm ID = 5.0 cm V = 98 ml	2 h or 6 h once or for either 4, 12 or 20 days
Harris *et al.* 2006	Sprague-Dawley	Plastic tube, Plas Labs,MI	L = 21.6cm ID = 6.4 cm V = 695 ml	3 h/day for 3 days
Kleen *et al.* 2006	Sprague-Dawley	Cylindrical wire mesh restraint	L=25.0cm ID =8.0cm V = 1257 ml	6 h/day for 21 days
Zardooz *et al.* 2006	Wistar	1. Plexy glass box with lid 2. Polyvinyl chloride tube	1. L= 15.0 cm B= 5.0 cm V= 375 ml 2. L=15.0 cm ID = 4.5 cm V = 239 ml	1 h twice daily for 15 or 30 days
Marin *et al.* 2007	Wistar	Opaque plastic cylinder	L = 18.0cm ID = 5.5 cm V = 131 ml	1 h/day for 10 days
McLaughlin *et al.* 2007	Sprague-Dawley	Wire mesh restraints, Flynn and Enslow Inc, San Francisco, CA	L=24.0cm ID =5.7cm V = 613 ml Or L=28.0cm ID = 7.3cm V = 1172 ml	2 h/day or 6 h/day for 10 or 21 days

L- length, B- breadth, ID- internal diameter, V-volume.

1.2.6.2 Metabolic effects of chronic restraint stress

HPA axis

Restraint stress (1h/day for 14 days) has resulted in increased corticosterone concentrations (Araujo *et al.* 2003), with evidence of adrenal hypertrophy (6h/day for 21 days-Kleen *et al*. 2006). Furthermore, when rats were restrained for and 2h and 6h/day for 4, 12 and 20 days, a significant increase in corticosterone concentrations occurred the longer the rats were exposed to restraint, but only when restraint occurred at the onset of the light phase (Retana-Márquez *et al*. 2003).

One study showed that significant increases in serum corticosterone levels occurred initially, followed by a steady decrease in corticosterone release on subsequent days of restraint (3h/day for 10 days). Thus, rats appeared to adapt to the repeated restraint stress protocol (Harris *et al*. 2002). Adaptation to restraint stress was also observed in another study where restraint for 1h/day twice daily for 15 or 30 days resulted in higher fasting plasma corticosterone levels than unrestrained rats on day 15 of restraint, but by day 30, levels had recovered to values similar to those prior to stress exposure (Zardooz *et al.* 2006).

Some studies have found that plasma corticosterone concentrations at the end of restraint were similar to unrestrained rats (1h/day for 10 days, Marin *et al*. 2007 and 4h/day for 6 weeks, Baldwin *et al.* 1997). Either the rats may have required increased exposure to restraint stress in order to

produce changes in corticosterone levels or they may have adapted to the restraint stress protocol. The latter was more than likely the case and may have been verified had corticosterone measurements been determined during the restraint protocol.

Carbohydrate metabolism

With respect to carbohydrate metabolism, a marginal but significant increase in fasting plasma glucose levels occurred in restrained rats (1h/day twice daily for 15 or 30 days) on day 15 of restraint, however these levels returned to control values by day 30. Furthermore, on both days 15 and 30 plasma glucose levels were elevated.

Fasting plasma insulin levels were significantly reduced on days 15 and 30 and a decreased plasma insulin concentration occurred on day 15.

The above glucose and insulin results are quite fascinating, as one would expect insulin levels to be raised if glucose levels are elevated. This may indicate that restraint stress induces a degree of insulin resistance, while impairing carbohydrate metabolism (Zardooz *et al.* 2006).

Leptin

In restrained rats, leptin levels took several days to begin to decline following the onset of the stressor (3h/day for 10 days) and remained lower than in controls, even a week after the stressor had ended (Harris *et al*. 2002).

With the infusion of leptin during restraint stress, the release of CRH was reduced. This observation implicates leptin in the control of corticosterone secretion (Heiman *et al.* 1997).

Body weight

It has been commonly reported in restraint stress studies that a loss of body weight occurred in restrained rats compared to unrestrained rats (3h/day for 3 days-Harris *et al*. 2006, 6h/day for 21 days-Kleen *et al*. 2006, 2h and 6h/day for 10 and 21 days-McLaughlin *et al*. 2007 and 1h/day twice daily for 15 or 30 days-Zardooz *et al*. 2006). Furthermore, McLaughlin *et al*. (2007) noticed that rats restrained for 6h/day weighed significantly less than those restrained for 2h/day.

Harris *et al*. (2006) found that although restraint stress (3h/day for 3 days) resulted in a reduction in body weight, body weights did not recover to that of the non-stressed individuals, even for an extended period of time after the stressor had ended. The initial loss of body weight was primarily due to a reduction in lean tissue. However, the sustained reduction in body weight that occurred in restrained rats following restraint stress was due to the loss, although not significant, of both fat and lean tissue (Harris *et al*. 2002).

In addition, a further reduction in body weight occurred when rats were exposed to a second session of restraint (3h/day for 3 days), but not when the restraint stress protocol was extended (3h/day for 10 days) (Harris *et*

al. 2002). In contrast, Kleen *et al*. (2006) observed that when the restraint protocol persisted (6h/day for 21 days), rats exhibited reductions in body weight. Furthermore, it was found that these stressed rats required more food to maintain their body weight as the stress regime continued and were thus provided with more food.

Some studies have reported that the gain in body weight in restrained rats was less than that of unrestrained rats (4h/day for 6 weeks-Baldwin *et al.* 1997). Similarly, Retana-Márquez *et al*. (2003) identified that rats restrained for 2h or 6h/day for 20 days gain less weight than controls, when measured at the onset of the light or dark phase. In addition, similar body weights have been observed in restrained and unrestrained rats (1h/day for 10 days, Marin *et al.*, 2007).

In conclusion, from the above studies, restraint stress significantly influences various metabolic parameters, with significant alterations occurring even from the first day of restraint. Limited studies have examined the effect of restraint stress on carbohydrate metabolism and leptin levels.

Although Harris *et al*. (2002) show that restraint stress can reduce general fat content, the effect of restraint stress on body fat distribution and fatty acid profiles of various adipose tissue depots has not been investigated sufficiently.

In terms of the effect of restraint stress on HPA axis activity and body weight, a range of effects were noted, however, the findings were inconsistent and may be due to the variations in the restraint stress protocols and the different strains of rats used. McLaughlin *et al*. (2007) suggest that comparisons should not be made between restraint stress studies as restraint stress protocols differ and each strain of rat produces a specific response to stress.

Perhaps the differing results that occurred during restraint stress could be explained by previous unnoticed stressors for example the type of housing?

1.2.6.3 Housing

In this review the term “housing” will be used to refer to the number of rodents per standard cage. Therefore if rodents are housed one per cage, that would be referred to as individual-housing, two per cage is pair-housing and more than two, is group-housing.

In experimental settings, rodents may be individually-, pair- or group-housed as part of an experimental protocol. The type of housing conditions may pose a problem, as the housing, in itself, may contribute to stress, depending on whether a rodent prefers to be alone or enjoys the company of others (Institute for Laboratory Animal Research, 1992). It has become common practice for investigators to house rodents individually, in order to measure an individual’s response to a certain treatment.

According to Bartolomucci *et al*. (2003), individually-housed rodents cannot be used as an appropriate control group in experimental settings as “individual housing is not equivalent to living in stable social groups for rodents.” Considering that rodents are social animals, individual-housing of rodents may be a potential stressor.

According to Brown and Grunberg (1995), housing rodents in different conditions are models of social and/or psychological stressors, as they represent situations that people may come across in their everyday lives. Other stressors used in animal models of stress, such as uncontrollable foot shock or forced swimming are not comparable with real-life encounters. Differential housing of rodents has resulted in various changes observed including alterations in behaviour (Sharp *et al*. 2002, Thorsell *et al*. 2006, Westenbroek *et al*. 2003), body weight (Perelló *et al*. 2006, Thorsell *et al*. 2005), functioning of the HPA axis (Baldwin *et al*. 1995, Brown and Grunberg, 1995, Ehlers *et al*. 1993, Perelló *et al*. 2006, Seeman and McEwen, 1996, Serra *et al*. 2005, Westenbroek *et al*. 2003), immunity (Baldwin *et al*. 1995) and neurochemistry (Hall, 1998, Serra *et al*. 2007, Thorsell *et al*. 2006, Westenbroek *et al*. 2004). These changes may depend on a number of factors.

The response to individual-housing depends on the age at which isolation occurs, the period of isolation, gender, species and strain of rodent, and test condition (Ehlers *et al*. 1993, Faraday, 2002, Hall, 1998, Starkey *et al*.

2007). Significant ages or developmental stages have been used to examine the effects of individual-housing in rodents. These are preweaning/neonatal, postweaning/adolescent and adult. Isolation during each of these developmental stages produces specific behavioural and neurochemical effects (Hall, 1998). Long-term individual-housing of adult rodents has resulted in a number of effects including; increased corticosterone secretion (Baldwin *et a*l. 1995), altered circadian pattern of corticosterone and leptin secretion (Perelló *et al*. 2006), dysregulation of the HPA axis negative feedback system (Serra *et al*. 2005) and loss of body weight (Perelló *et al*. 2006, Thorsell *et al*. 2005).

In contrast to individual-housing, social support, in most cases, contributes to the minimization of life stress and assists in protecting an individual from disease and coping with stress, by promoting the improvement of health and well-being (Sachser *et al*. 1998, Seeman and McEwen, 1996). It would appear that social support, achieved through pair- or group-housing of rodents, can limit the adverse physiological effects and can attenuate the long-term effects of stress generated by individual-housing (Baldwin *et al*. 1995, Perelló *et al*. 2006, Ruis *et al*. 1999, Seeman and McEwen, 1996, Serra *et al*. 2005, Sharp *et al*. 2002, Westenbroek *et al*. 2005). However, some studies have found that social housing may be stressful (Brown and Grunberg, 1995, Westenbroek *et al*. 2003) and there are even differences between pair- and group-housing. For example, although all male rats display stress-like responses to common husbandry and

experimental procedures, group-housed rats are significantly less stressed than individually- and pair-housed rats (Sharp *et al*. 2002).

An important outcome from Brown and Grunberg's study on differential housing (1995) was "the profound impact housing conditions have on baseline biochemical levels before any treatment manipulations are performed (p. 1088)." Leading on from this, it is evident that in all experiments, even those that do not focus on housing, researchers cannot ignore the effect that housing may have on their results.

Therefore, since housing is a possible stressor, it is crucial to understand how the type of housing may influence the allostatic response, as it could provide useful information to researchers on how best to house rodents to minimize this response.

1.2.6.4 Metabolic effects of chronic housing stress

Even before exposure to new stressors, chronic individual- and pair-housing of adult male rodents has produced marked effects with regards to the mediators of the allostatic response and various metabolic parameters.

HPA axis

Serra *et al.* (2005) observed that chronic individual-housing increases the sensitivity of the pituitary to CRF, decreases plasma ACTH concentrations

and impairs the HPA axis negative feedback mechanism. In addition, Ehlers *et al*. (1993) found that a reduced number of corticotrophin releasing factor (CRF) receptors occurred in several cortical areas in individually-housed rodents.

Prolonged individual-housing of rats has an apparent effect on adrenocortical function, as it enhances the activity of the adrenal cortex, resulting in an increased secretion of corticosterone, as well as altering the circadian pattern of corticosterone release (Baldwin *et al.* 1995, Gavrilovic and Dronjak, 2005, Perelló *et al*. 2006). Furthermore, Perelló *et al*. (2006) found that the significant correlations of plasma corticosterone levels with plasma ACTH and adrenal gland corticosterone concentration that occurred in group-housed rats, were no longer apparent in individually-housed rats. Gavrilovic and Dronjak (2005) observed an increase in basal plasma ACTH in response to long-term individual-housing.

Long-term social-housing (pair- or group-housing) has produced reductions in HPA axis activity (Seeman and McEwen, 1996) and does not impair the HPA axis negative feedback mechanism, as seen from the inhibition of endogenous glucocorticoid release by the dexamethasone suppression test (Serra *et al*. 2005). Perelló *et al*. (2006) showed that the circadian release of ACTH and corticosterone was maintained during group-housing and the daily pattern of adrenal corticosterone release was similar to that of the blood. Although, the patterns of ACTH release as well as plasma ACTH concentrations were similar to individually-housed rats,

plasma corticosterone was substantially lower compared to individually-housed rats (Baldwin *et al*. 1995, Perelló *et al*. 2006).

When rats were injected with CRF intracerebroventricularly, an increase in plasma corticosterone occurred in both individually- and group-housed rats. However, this effect was significantly reduced in the group-housed rats, possibly illustrating that the sensitivity of the pituitary to CRF is less in group-housed rats (Serra *et al*. 2005).

Only one study has claimed that group-housing was stressful, as indicated by increased basal corticosterone levels in group-housed rats (Brown and Grunberg, 1995).

Carbohydrate metabolism

A significant increase in blood glucose levels occurred in individually-housed rats in comparison to socially-housed rats (Baldwin *et al*. 1995, Perelló *et al*. 2006). Similarly, when rats were group-housed, individually-housed and then returned to group-housing, blood glucose were higher when rats were isolated (Pérez *et al.* 1997). Thus, chronic individual-housing may lead to hyperglycemia.

The effects on insulin levels were not determined in any of these studies. Furthermore, although many social isolation studies look at HPA axis activity, they do not extend their research to include the effects on carbohydrate metabolism. One could make possible deductions but without the actual data, these may be inaccurate.

Leptin

Individual-housing has produced hyperleptinemia and a modified circadian pattern of plasma leptin secretion, whereas, the circadian release of leptin was maintained during group-housing and plasma leptin levels were substantially lower compared to individually-housed rats (Perelló *et al*., 2006).

Body weight

In terms of body weight, Perelló *et al*. (2006) claim that despite no change in food intake, a significant loss of body weight occurred in individually-housed rats. However, no data is shown to support this claim.

Although some studies have found that long-term individual-housing results in a reduction of body weight (Thorsell *et al*. 2005), other studies have observed similar changes in body weight compared to pair-housed rats (Kretschmer *et al.* 2005, Thorsell *et al*. 2006).

In conclusion, based on the above studies, chronic individual-, pair- and group- housing produces varying and sometimes conflicting responses. Limited research into the effect of chronic housing on carbohydrate metabolism, leptin levels and body weight has been conducted, while the impact of chronic housing on fat metabolism, including body fat weights and fatty acid profiles of various adipose tissue stores, has not yet been explored. It would appear in most cases that chronic individual-housing is

stressful and results in chronic stress, while pair- or group-housing is not stressful.

If a rodent is chronically individually-housed prior to exposure to a new stressor, the chronic stress previously experienced may impact on a rodent's ability to cope with the new stressor. According to McLaughlin *et al*. (2007), housing manipulations can interfere with new stress paradigms and therefore it is important to consider the type of housing prior to and during a stressor, as sociability can vary between strains and conspecifics (members of the same species), resulting in stress relief or stress accumulation.

1.2.6.5 Chronic housing followed by exposure to a new stressor

According to Westenbroek *et al.* (2004), the type of housing during exposure to a stressor can affect the way an animal copes with that stressor. This depends on whether the type of housing is stressful for the rodent. Evidence indicates that when rats are chronically individually-housed they are hyperresponsive to environmental stimuli and to other stressors (Perelló *et al*. 2006). In contrast, the social support derived from living in a familiar social environment can modulate the response to stressors. Thus, an animal that has social support, achieved through pair- or group-housing, may be less stressed and therefore better equipped to deal with a new stressor (Ruis *et al*. 1999, Sachser *et al.*1998, Seeman and McEwen, 1996).

It has been observed that when chronically stressed rodents were exposed to a novel stressor, an exaggerated response of the sympathico-adrenomedullary system ensued (McCarty *et al.* 1988). Furthermore, chronic stress can also induce changes in the HPA axis response to subsequent novel and familiar stressors. Dallman *et al.* (2004) have shown that chronically stressed rats display a facilitated ACTH response to acute novel stressors. Similarly, when rats were exposed to a novel environment after exposure to chronic restraint stress, the rats had a higher corticosterone response than when exposed to a challenge restraint (Marin *et al.* 2007). Therefore a novel stressor elicits a greater HPA axis response than a familiar stressor. It is possible that the familiar stressor results in desensitization of the HPA response, whereas sensitization of HPA axis may occur if a challenge by a novel stressor follows chronic stress exposure (Bhatnagar *et al.* 2006, Marin *et al.* 2007, Mizoguchi *et al.* 2001).

A number of studies have examined the effect of different types of housing, specifically individual-housing, on rodents' responses to additional acute and chronic stressors. Examples of stressors used include; common husbandry procedures (Sharp *et al*. 2002), inescapable footshock (Westenbroek *et al*. 2003, Westenbroek *et al*. 2004, Westenbroek *et al*. 2005), elevated plus maze test for anxiety (Starkey *et al*. 2007, Thorsell *et al*. 2006), novel environment (Baldwin *et al*. 1995), raised platform (Leshem and Sherman, 2006), open field (Leshem and

Sherman, 2006, Thorsell *et al*. 2006, Westenbroek *et al*. 2003, Westenbroek *et al*. 2005), forced swim (Baldwin *et al*. 1995), ethanol consumption (Thorsell *et al*. 2005), social interaction test (Starkey *et al*. 2007) and social defeat (Ruis *et al*. 1999). To my knowledge, the impact of chronic differential housing on the subsequent exposure to chronic restraint stress has not been explored.

Although the McLaughlin study emphasizes the extent to which housing matters prior to and during exposure to a subsequent stressor, surprisingly they do not specify the length of time that their rats were pair-housed before restraint was implemented. Furthermore, no other studies infer that housing influences restraint stress. Therefore, there is a lack of studies that investigate the impact of differential housing on the subsequent exposure to chronic restraint stress.

In addition, it has not been established whether prolonged consumption of a different diet may impact on the ability of a rodent to cope with exposure to a subsequent stressor, like restraint stress.

1.2.7 The interaction between chronic stress and diet

Depending on the stress paradigm, repeated stress can produce alterations in the hypothalamic energy balance systems. Diminished weight gain is consistently observed, which is often associated with

changes in food consumption (Bhatnagar *et al.* 2006, Morley, 2000). On the one hand, stress can influence the type and amount of food ingested (Newman *et al*. 2007), while on the other, the ingestion of certain foods can modulate the stress response (Laugero, 2001).

To recap briefly, during stress, feeding is inhibited (due to increased CRF activity) but at the same time there is an increased demand for glucose. Therefore, it is imperative that endogenous energy stores are mobilized to ensure that energy requirements are met. This is achieved by an increase in HPA axis activity, where the consequent release of glucocorticoids results in an elevation in the endogenous production of glucose. As stated previously, glucocorticoids affect energy balance in the periphery and the resulting metabolic effects feed back to the brain modulating HPA axis activity (metabolic-brain feedback system) (Laugero, 2008).

Leading on from this, if anything induces changes in energy balance, which may occur before, during or after exposure to a stressor, there is a high probability that it will alter HPA axis activity via this pathway. Therefore the type of diet an animal consumes could interact with the energy balance systems and could impose changes in neural, endocrine and/or metabolic responses.

Increased ingestion of carbohydrates can modify basal and neuroendocrine, metabolic and behavioural responses in rodents to a

variety of stressors (Laugero, 2001). In addition, high-energy diets, particularly high in fat, act as a form of chronic stress resulting in raised glucocorticoid concentrations (Tannenbaum *et al.* 1997). Since glucose requirements are increased during stress, ingesting carbohydrates such as sucrose, could normalize energy balance, by overcoming the CRF-induced appetite suppression. In support of this, it has been observed that consumption of a high calorie diet and/or carbohydrate diet prevents significant loss of energy, maintains increased activity of the metabolic-brain feedback system and dampens or switches off the HPA axis response to stress (Laugero, 2008, Levin *et al.* 2000).

With continuous exposure to stressors i.e. chronic stress, the appetite stimulating effects of glucocorticoids accompanied by consumption of a high carbohydrate diet may give rise to obesity and obesity-related pathologies. Obesity is strongly linked to dysregulation of the HPA axis (Laugero, 2001). Obesity in rodents has been relieved with the removal of the adrenal glands and has been restored with supplementation of glucocorticoids. This finding suggests that the glucocorticoids secreted in response to stress, play a factor in the development of obesity. Since glucocorticoids also promote the secretion of insulin, the combination of insulin secretion and increased food consumption increases the chances that the excess energy consumed will be stored as fat specifically in the central, visceral areas, where the density of glucocorticoid receptors is relatively high. These receptors, when activated, signal lipid accumulation

in the adipose tissue (Laugero, 2001, Levin *et al.* 2000, Newman *et al*. 2007).

With an elevation in fat deposition, there is an increase in leptin production in the adipose tissue. Accumulation of fat in the visceral areas may also be explained by the rising leptin levels. As mentioned previously, under normal conditions leptin inhibits visceral adiposity (Jéquier, 2002). However, despite the high levels of circulating leptin associated obesity, leptin is ineffective in preventing visceral adiposity, as well as its own expression. Therefore, leptin fails to regulate fat distribution in obesity due to the development of leptin resistance this leads to increases in visceral fat deposition (Cases and Barzilai, 2000).

The combination of chronic stress and ingestion of a high carbohydrate diet could result in an overproduction of glucocorticoids and elevated leptin levels, which in turn could lead to hyperleptinemia, leptin resistance and a decrease in satiety, overeating, hyperinsulinemia, insulin resistance and resultant obesity (Laugero, 2001, Levin *et al.* 2000, Newman *et al*. 2007).

1.2.7.1 The condensed milk diet in rodent models

Various types of diets have been administered to rodents such as; high-fat diets, high-carbohydrate diets, high-energy diets (high fat and high carbohydrate), palatable liquid diets, highly palatable high-energy diet, cafeteria diets and yoyo diets (Dourmashkin *et al.* 2005, Harrold *et al.*

2000, Kretschmer *et al.* 2005, Mercer and Archer, 2005). A highly palatable high- carbohydrate diet that is often used is a condensed milk diet, which consists of powdered chow, condensed milk, sucrose and water (Elliott *et al.* 2004, Harrold *et al.* 2000, Triscari *et al.* 1985). This diet, together with many of the abovementioned diets are employed to produce diet-induced obesity in rodents, where the diet composition results in a specific form of obesity (Dourmashkin *et al.* 2005, Harrold *et al.* 2000, Kretschmer *et al.* 2005, Mercer and Archer, 2005).

Obesity is not just defined by total body weight but also depends on the quantity of adipose tissue present. In rodents, there are eight major adipose depots, four of which are within the abdominal cavity: 1) The paired gonadal depots are attached to the uterus and ovaries in females and the epididymis and testes in males, 2) the paired retroperitoneal depots are found along the dorsal wall of the abdomen, surrounding the kidney, and may extend into the pelvis. 3) the mesenteric depot supports the intestines, and 4) the omental depot, which originates near the stomach and spleen, and, when massive, extends into the ventral abdomen. The two superficial depots are 5) the paired inguinal depots, which are found anterior to the upper segment of the hind limbs (underneath the skin) and 6) the subscapular depots, paired medial mixtures of brown adipose tissue adjacent to regions of white adipose tissue, which are found under the skin between the dorsal crests of the scapulae. The layer of brown adipose tissue in this depot is often covered

by a thin layer of white adipose tissue; sometimes these two types of fat (brown and white) are hard to distinguish. Minor depots include 7) the pericardial, which surrounds the heart and 8) the paired popliteal depots, between the major muscles behind the knees, each containing one large lymph node (Body, 1988). Dietary fatty acids are also stored to a small extent in the liver and muscle as TAGs (Abbott *et al.* 2012, Igarashi *et al.* 2009).

Imbalances in energy intake and expenditure lead to changes in adipose tissue mass. For example, consumption of a high carbohydrate diet results in increases in circulating glucose. In response to raised glucose levels, insulin is secreted which favours the synthesis of body fat via *de novo* lipogenesis. This is the process whereby ingested carbohydrates (and protein) are converted into non-esterified fatty acids/ free fatty acids primarily in the liver (and also in adipose tissue). These free fatty acids are then transported to adipose tissue, where they are converted into triacylglycerides (TAGs) for storage. In rodents, various dietary and hormonal factors have been implicated in *de novo* lipogenesis. In general, insulin stimulates *de novo* lipogenesis, whereas adrenaline, glucagon and free fatty acids inhibit the process (Hillgartner *et al.* 1995).

Apart from the free fatty acids generated from *de novo* lipogenesis, an additional source of fatty acids is from the diet. Dietary fatty acids are the predominant source of fatty acids for lipogenesis and fat deposition.

Following a meal, insulin is the major hormone involved in lipogenesis, where insulin enhances fat storage and inhibits the mobilization and oxidation of fatty acids. Lower metabolic costs are incurred when dietary TAGs are deposited in adipose tissue compared to conversion of carbohydrate to fat for storage (Macfarlane *et al.* 2008).

Furthermore, with an increase in carbohydrate ingestion, glucocorticoid release is also stimulated, which either acts on its own or synergises with insulin, further promoting lipogenesis. In addition, the expression of obesity-promoting peptides- neuropeptide Y (NPY) and agouti-related protein (AgRP) is elevated. The overall effect of consuming a high carbohydrate diet for prolonged periods of time is accrual of body fat, which can lead to obesity (Dourmashkin *et al*. 2005).

In addition to influencing fat deposition and therefore the size of fat stores, dietary fatty acids can also alter both the composition and quantity of various fatty acids in the adipose tissue (Lin *et al.* 1993). Similar to the mobilization of fatty acids during lipolysis, the degree of deposition of fatty acids is related to the structure of the fatty acid i.e. degree of unsaturation and chain length, rather than on the amount in the diet (Lin *et al.* 1993). By varying the fatty acid composition of the diet i.e. the amount of dietary SFAs, MUFAs and PUFAs, the fatty acid profile of adipose tissue can be significantly influenced (Abbott *et al.* 2012, Igarashi *et al.* 2009). For example, when rats were fed the cafeteria diet to promote diet-induced

obesity and then returned to rat chow, the changes that affected certain PUFAs were long-lasting, which were possibly associated with the obesity (Lladó *et al.* 1996).

Certain dietary fatty acids have been implicated in the development of obesity (specifically visceral obesity): SFAs and trans fatty acids. While PUFAs like linoleic acid have been shown to improve insulin resistance and down regulate the genes involved in lipogenesis in adipose tissue (Vara Pasad *et al.* 2010). The enzyme, 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) converts inactive glucocorticoids into their active form and thereby stimulates intracellular glucocorticoid action, In adipose tissue in mice, overexpression of 11β-HSD1 has resulted in visceral adiposity and insulin resistance. A diet rich in trans fatty acids upregulates the expression of 11β-HSD1 in the retroperitoneal white adipose tissue, more so than a diet rich in SFAs and even more so than a diet rich in PUFAs. Therefore diets enriched with SFAs and trans fatty acids enhance the conversion of inactive to active glucocorticoids in adipose tissue, which may be responsible for promoting weight gain (Vara Pasad *et al.* 2010).

In rodent models of diet-induced obesity, rodents are housed individually, in pairs or in groups and are fed a specific diet, which is meant to induce obesity. After two weeks on the highly palatable high-energy condensed milk diet, no significant differences in body weight, fat-pad mass or plasma

insulin, glucose or free fatty acids occurred compared to rats fed a pelleted diet. However, diet-fed rats displayed significantly elevated leptin levels. When rats were maintained on the diet for approximately 8-10 weeks, diet-fed rats had variable hyperphagia, which resulted in the development of moderate to severe obesity. Thus, some rats were more susceptible to developing obesity while others were somewhat resistant. Rats had increased fat-pad and brown adipose tissue mass, were hyperleptinemic and hyperinsulinemic and had an increased rate of fatty acid synthesis (Elliott *et al.* 2004, Harrold *et al.* 2000, Triscari *et al.* 1985). The hyperleptinemia and hyperinsulinemia which develop appear to be linked to the rise in adiposity but not related to changes in circulating metabolites i.e. glucose and lipids (Dourmashkin *et al*. 2005, Harrold *et al*. 2000).

Furthermore, visceral fat is very dynamic as it performs various complex metabolic and endocrine activities. This can be seen in obese rats, with the surgical removal of visceral fat, plasma leptin levels decreased as well as leptin gene expression in adipose tissue was diminished. Furthermore, with removal of certain visceral fat, there was a significant improvement in vivo insulin action on hepatic glucose production. Thus, visceral fat accumulation in obesity influences plasma leptin levels and hepatic insulin activity (Cases and Barzilai, 2000).

Therefore, when rats are fed a high-energy diet they develop diet-induced obesity as well as many of the characteristic abnormalities of the metabolic

syndrome (e.g. hyperleptinemia, hyperinsulinemia) as seen in humans. The diet leads to increases in adipose tissue as well as various determinants of TAG metabolism. Furthermore, it is apparent from the above studies that rats need to remain on the diet for a long time in order for the diet to result in a significant body weight gain and to cause alterations in fatty acid metabolism.

In rats fed a diet (rat chow) that maintains a low lipid flux i.e. low energy diet, the impact of corticosterone on TAG metabolism is minimal. However, TAG metabolism is greatly influenced by corticosterone in rats fed a diet that increases lipid flux (Mantha *et al.* 1999). However, when consumption of a high-energy diet is restricted, corticosterone affects TAG metabolism only to a small extent. Therefore, in the long-term determinants of TAG metabolism are significantly affected through the central modulation of energy intake by glucocorticoids rather than by their direct peripheral actions on TAG metabolism (Mantha and Deshaies, 2000).

Furthermore, in the presence of low lipid flux, corticosterone maintains adipose tissue stores/fat reserves. When corticosterone is absent i.e. adrenalectomy, subcutaneous and visceral fat depots were smaller in adrenalectomised rats than intact rats. This effect was dampened by consumption of a high energy diet (Mantha *et al.* 1999).

As mentioned above, the combination of chronic stress and a high carbohydrate diet has many metabolic ramifications. However, limited investigations into stress in diet-induced obese rodents have been done. In one study, chronic unpredictable stress was examined and rats with diet-induced obesity were found to be hyporesponsive to chronic stress. Levin *et al.* (2000) suggested that this blunted stress response was possibly due to the action of increased leptin in the rats with diet-induced obesity. Leptin may block the release of ACTH and corticosterone, through inhibition of CRF release, resulting in a diminished response (Heiman *et al.* 1997). Furthermore, perhaps the diet itself was stressful and therefore impacted on the ability of the rodents to cope with the new stressor. This requires further investigation.

In conclusion, since diet-induced obesity influences hypothalamic energy-balance systems, the effects of diet manipulation, changes in body weight and metabolic parameters over longer time intervals can be studied. However, there appears to be a deficiency of studies a) that compare animals housed in different conditions and b) that investigate the effects of chronic stress in rodents with previous diet-induced obesity.

1.3 Objectives

Therefore the aim of this study was to examine metabolic effects of chronic differential housing, prolonged consumption of a high carbohydrate diet and chronic restraint stress in rats that were housed for 12 weeks either individually or in pairs and fed either a normal rat chow diet or a condensed milk diet and then exposed to chronic restraint stress for four weeks.

1. To determine the effect of prolonged housing on body weight by individually- or pair-housing rats, consuming a similar diet.

2. To determine the effect of diet on body weight when male Wistar rats were housed under similar conditions.

3. To determine the metabolic effect of chronic restraint stress on rats exposed to differing housing and diet conditions i.e. various measurements of carbohydrate or fat metabolism.

4. To determine if prolonged housing and/or dietary conditions influence the rats' ability to cope with a subsequent stressor i.e. chronic restraint stress.

CHAPTER TWO

Materials and Methods

2. Materials and Methods

2.1 Animals

Thirty three male Wistar rats (200-250g) were used in the study. Before allocation to the study, these rats came from a colony of rats that were group-housed and fed standard rat chow. Rats were housed in a temperature controlled room (T_a ~ 20-25°C) on a 12-hour light/dark schedule (lights on at 07:00h). Both food and water were available *ad libitum*. All experimental procedures were approved by the Animal Ethics Committee of the University of the Witwatersrand (Clearance No. 2005/60/3).

2.2 Experimental Protocol

The experimental protocol was divided into two parts. Part one: rats were housed either individually or in pairs and fed one of two diets (a condensed milk diet or a mushy rat chow diet) for 12 weeks. During the 12 weeks, body weight measurements were collected.

Part two: Following the 12-week housing and diet regimen, all rats were exposed to chronic restraint stress for seven hours a day for four weeks. When the rats were not restrained, they were maintained under pre-

restraint housing and dietary conditions. Each day prior to restraint, rat body weight was determined.

At the end of the chronic restraint stress protocol the rats were sacrificed. Blood was collected by cardiac puncture and used to determine the serum concentrations of corticosterone, insulin, glucose and leptin. The liver plus samples of various fat sites and muscle were harvested for fatty acid determination.

2.2.1 Part one- Housing and diet regimen

a. Housing

Rats were either individually-housed (IH) (n=15) or pair-housed (PH) (n=18) in conventional Type 3 plexiglass rat cages for housing rats. It is unclear if littermates were allocated to the pair-housed groups. Rats were housed in a temperature controlled room (T_a ~ 20-25 °C) on a 12-hour light/dark schedule (lights on at 07:00h). Both food and water were available *ad libitum*. Rats were weighed at least once within each three-day period (Snowrex Electronic Scale, Clover Scales, South Africa. max=30 kg, min=50 g).

b. Diet

Both the individually-housed and pair-housed groups were further divided into two groups and fed either a mushy rat chow diet (MD) or a condensed milk diet (CD) for approximately 12 weeks.

The mushy rat chow diet consisted of 1.32 kg crushed standard rat chow (Epol, Pinegowrie, South Africa) to which 812 ml boiling water was added, resulting in a softened mixture (Nutritional Information per 100 g: Energy = 18.734 J/g (as determined by bomb calorimetry), Protein = 11%, Fat = 3%, Carbohydrate = 40%, Moisture content = 43.83% (results are expressed on a wet basis, as determined by ARC-Irene Analytical Services). A mushy rat chow diet was used instead of standard rat chow in order to avoid the mushiness of condensed milk diet being an additional factor.

The condensed milk diet was prepared as follows; 280 g of brown sugar (Selati, Mpumalanga, South Africa, Nutritional Information per 100 g serving: Energy = 1600 kJ, Carbohydrates = 100 g) was dissolved in 812 ml of boiling water and was added to 1.32 kg crushed standard rat chow (Epol, Pinegowrie, South Africa). Four cans (each can contains 385 g of condensed milk) of condensed milk (Nestlé, Nutritional Information per 100 g: Energy = 350 kJ, Protein = 6.8 g, Fat = 8 g, Carbohydrate = 55.7 g, Fibre = 0 g) was then added to the above mixture. The final nutritional values per 100 g of the condensed milk diet were: Energy= 18.757 J/g (as determined by bomb calorimetry), Protein= 9%, Fat = 4%, Carbohydrate =

48%, Moisture content = 34.40% (results are expressed on a wet basis as determined by ARC-Irene Analytical Services).

The experiment therefore consisted of the following four groups namely; pair-housed rats that received a mushy rat chow diet (PH-MD, n=6), individually-housed rats that received a mushy rat chow diet (IH-MD, n=9), pair-housed rats that received a condensed milk diet (PH-CD, n=12) and individually-housed rats that received a condensed milk diet (IH-CD, n=6).

2.2.2 Part two- Exposure to chronic restraint stress

Following part one, all four groups of rats (PH-MD, IH-MD, PH-CD and IH-CD) were exposed to chronic restraint stress for a continuous period of four weeks. To achieve chronic restraint stress, rats were removed from their overnight cages and placed individually in plexiglass cylinders (length 300 mm, diameter 90 mm, volume 1.56 l) for seven hours a day for four weeks. The cylinders had a gas inlet port at one end and an outlet port at the other end. Compressed room air (Sullivan III Nasal CPAP System, ResCare Limited, Sydney, Australia) was continually flowing into the cylinders at a low flow rate (0.03 l/min), so as to provide the rats with oxygen and flush out carbon dioxide. Steel wool was placed at the intake end of the cylinder to dissipate the air stream so that the rats were not disturbed (adapted from the procedure used by Romain, 2006). One day

prior to exposure to the above procedure, rats were acclimatised to the plexiglass cylinders for seven hours.

The chronic restraint stress was applied during the twelve-hour light phase, when the rats would ordinarily be inactive. Between 08:00-10:00 each morning, the rats were weighed (Snowrex Electronic Scale, Clover Scales, South Africa. max=30 kg, min=50 g) prior to being placed in the plexiglass cylinder, which were maintained at room temperature. The rats' diet and housing conditions (from part one) were not changed during this period, however while the rats were in the plexiglass cylinders, they had no access to food or water. Following the daily seven-hour exposure period, rats were removed from the plexiglass cylinders and returned to their home cages.

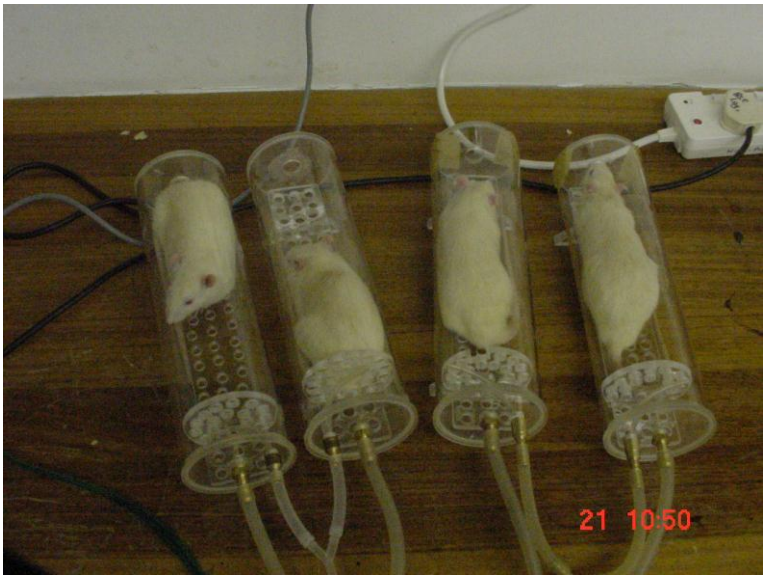


Figure 2.1: Male Wistar rats in plexiglass cylinders.

2.3 Post-mortem procedure, sample collections and analyses

At the end of the four weeks, all rats were deeply anaesthetised with Ketamine (Anaket-V 80 mg/kg, Centaur Labs, South Africa) and Xylazine (Chanazine 4 mg/kg, Centaur Labs, South Africa) in a 4:1 ratio and a dosage of 0.1 ml/100 g rat intramuscularly (i.m.). Approximately 4-8 ml of blood was collected by cardiac puncture. The rats were euthanased thereafter with Eutha-naze (1 ml/kg rat) (Centaur Labs, South Africa). This procedure was performed at approximately noon on each day.

2.3.1 Fat sample collection and analyses

a. Fat sample collection

Rats were dissected immediately post-mortem and the liver, as well as samples of muscle and fat were removed. The whole liver as well as all interscapular, retrorenal and visceral fat (refers to abdominal fat and includes omental and mesenteric fat depots) were removed. Representative samples were taken from subcutaneous fat (beneath the skin in the abdominal region and/or in the dorsal lumbar region) and muscle of the thigh. All samples were weighed and stored at -20 °C until fatty acid determination.

b. Analysis of fat samples

The fatty acid composition of each of the samples was determined using a standard method for fatty acid analysis (Bligh and Dyer, 1959) (see Appendix 1 for a detailed explanation of the fatty acid analysis procedure).

2.3.2 Blood sample collection and serum analyses

a. Blood sample collection

All blood samples were collected in red-top BD Vacutainers (BD Vacutainer Systems, Plymouth, UK) and immediately put onto ice. Samples were centrifuged (1876.9 rcf or 1876.9 g) for 15 minutes at 4 °C (Sorvall RT 6000B, Dupont, USA). Serum was removed and stored at -70 °C until assayed.

b. Analysis of serum corticosterone concentrations

Serum corticosterone concentration was determined using ImmuChem™ Double Antibody Corticosterone ^{125}I RIA Kit (MP Biomedicals, LLC, New York, USA). The minimum level of rat serum corticosterone concentration that could be detected by this assay kit was 7.7 ng/ml. The concentration (ng/ml) of corticosterone in the serum samples was calculated using a calibration curve derived from the samples with known corticosterone concentrations (standards). The assay was performed according to the procedure outlined in the manual that accompanied the kit (MP

Biomedicals, LLC, New York, USA). A detailed summary of the assay appears in Appendix 1.

c. Analysis of serum insulin concentrations

The levels of serum insulin were determined using a radioimmunoassay kit specific for rat insulin (Linco Research, Missouri, USA). The lowest level of rat serum insulin concentration that could be detected by this assay kit was 0.1 ng/ml when using 100 µl sample of serum. The concentration (ng/ml) of rat insulin in the unknown samples was calculated from the calibration curve derived from the samples with known insulin concentrations (standards). The assay was performed according to the procedure outlined in the manual that accompanied the kit (Linco Research, Missouri, USA). A detailed summary of the assay appears in Appendix 1.

d. Analysis of serum glucose concentrations

To determine the quantitative levels of serum glucose, the Reflotron® (Roche Diagnostics Ltd, UK) was used. The measuring range of the Reflotron ® is 0.56-33.3 mmol/l. Details of the procedure are outlined in Appendix 1 and are according to the method prescribed by the manual that accompanies the Reflotron® and the information sheet that accompanies the Reflotron® test strip (Roche Diagnostics Ltd, UK).

e. Analysis of serum leptin concentrations

Serum leptin concentrations were analysed using a radioimmunoassay kit specific for determining rat serum leptin levels (Linco Research, Missouri, USA). The lowest level of rat serum leptin concentration that could be detected by this assay kit was 0.5 ng/ml when using 100 µl sample of serum. The concentration (ng/ml) of rat leptin in the unknown samples was calculated from the calibration curve derived from the samples with known leptin concentrations (standards). The assay was performed according to the procedure outlined in the manual that accompanied the kit (Linco Research, Missouri, USA). A detailed summary of the assay appears in Appendix 1.

2.4 Data Analysis

For the housing and diet regimen (part one), the body weights for each group of rats are reflected as a mean change in body weight from baseline (mean + standard deviation). To calculate the mean change in body weight from baseline on a particular day, the change in body weight from baseline was first determined for each rat by subtracting the body weight at the start of the experiment from the actual body weight (actual body weight – initial body weight = change in body weight from baseline). Then, the mean change in body weight from baseline was calculated by averaging the change in body weight from baseline for all the rats on a particular day. Data for 2, 4, 6, 8, 10 and 12 weeks were used.

Generally for nutritional studies when animals are housed in pairs or groups, the statistical unit is the cage and not the number of animals in each cage. However, this was not done in this study as the sample size would have been significantly reduced.

A Students t-test was used to compare the mean change in body weight from baseline at the end of the 12-week housing and diet regimen between two groups for each of the four comparisons made. In addition, an area under the curve analysis, with a Tukey *post hoc* test for multiple comparisons was performed to compare the mean changes in body weight between all four groups over the 12-week housing and diet regimen.

After part one, for the chronic restraint stress, body weight data were first normalised. The calculated change in body weight from baseline was averaged for each week of the four weeks of exposure to chronic restraint stress. Body weight data are expressed as mean + standard deviation. A repeated measures ANOVA, with a Tukey-Kramer *post hoc* test for multiple comparisons, was performed on the data for the four weeks of chronic restraint stress to indicate whether within each group the weekly gains in body weight were significantly different from the week before and from baseline.

In addition, an area under the curve analysis, with a Tukey *post hoc* test for multiple comparisons, was used to compare the mean changes in body weight from baseline between all four groups over the four weeks.

The total weight of the liver, visceral fat, retrorenal fat and interscapular fat as a percentage of body weight are expressed as mean ± standard deviation. For each of the above fat samples, a one-way analysis of variance (ANOVA) with a Tukey *post hoc* test for multiple comparisons was used to compare the four groups.

For the fatty acid profiles, the amount of a particular fatty acid is represented as a percentage of the total fatty acids present in the sample. Data are expressed as mean ± standard deviation. For each fat sample; liver, subcutaneous fat, visceral fat, retrorenal fat, interscapular fat and muscle, a one-way analysis of variance (ANOVA) with a Tukey *post hoc* test for multiple comparisons was used to compare the amount of each type of fatty acid present between the four groups.

The serum concentrations of corticosterone, insulin, glucose and leptin, are expressed as mean ± standard deviation. For each hormone and glucose, a one-way analysis of variance (ANOVA), with a Tukey *post hoc* test for multiple comparisons, was used to compare hormone and glucose levels between all four groups.

All statistical tests were performed using GraphPad Prism Version 4.00 for Windows, GraphPad, San Diego California, USA, www.graphpad.com and significance was set at $p < 0.05$.

CHAPTER THREE

Results

3. Results

There were four groups of rats in the study:

1. Pair-housed rats that consumed a mushy rat chow diet (PH-MD) (n=6)
2. Individually-housed rats that consumed a mushy rat chow diet (IH-MD) (n=9)
3. Pair-housed rats that consumed a condensed milk diet (PH-CD) (n=12)
4. Individually-housed rats that consumed a condensed milk diet (IH-CD) (n=6)

The mushy rat chow diet was considered to be the normal diet.

The results are divided into two sections.

Section A - Impact of housing, compared rats consuming similar diets and housed either individually or in pairs, in order to assess if any variables were influenced by the type of housing. Therefore the PH-MD group was compared to the IH-MD group and the PH-CD group was compared to the IH-CD group.

In Section B - Impact of diet, comparisons were made in rats housed under the same conditions and consuming either a normal diet or a condensed milk diet, in order to assess if any variables were influenced by the type of diet. Thus, the PH-MD group was compared to the PH-CD group and the IH-MD group was compared to the IH-CD group.

Each section is subdivided into two parts; body weight and metabolic parameters.

With the division of the project into two parts (Part one- Housing and diet regimen and Part two- Exposure to Chronic Restraint Stress) and the results into two sections (Section A- Impact of Housing and Section B- Impact of Diet), the overall changes in body weight for all four groups may not be obvious. Figure 3.1 represents all changes in body weight for both parts of the study and for all dietary and housing groups, while Figure 3.2 shows the changes in body weights for the four experimental groups that occurred during the four weeks chronic restraint stress.

3.1 Section A – Impact of housing

In this section, the PH-MD rats were compared to the IH-MD rats and the PH-CD rats were compared to the IH-CD rats. Thus keeping the diet constant and changing only the housing. Considering that in experimental conditions laboratory rats are commonly housed individually, therefore the individually-housed rats were assigned to be the control group for both of the above comparisons.

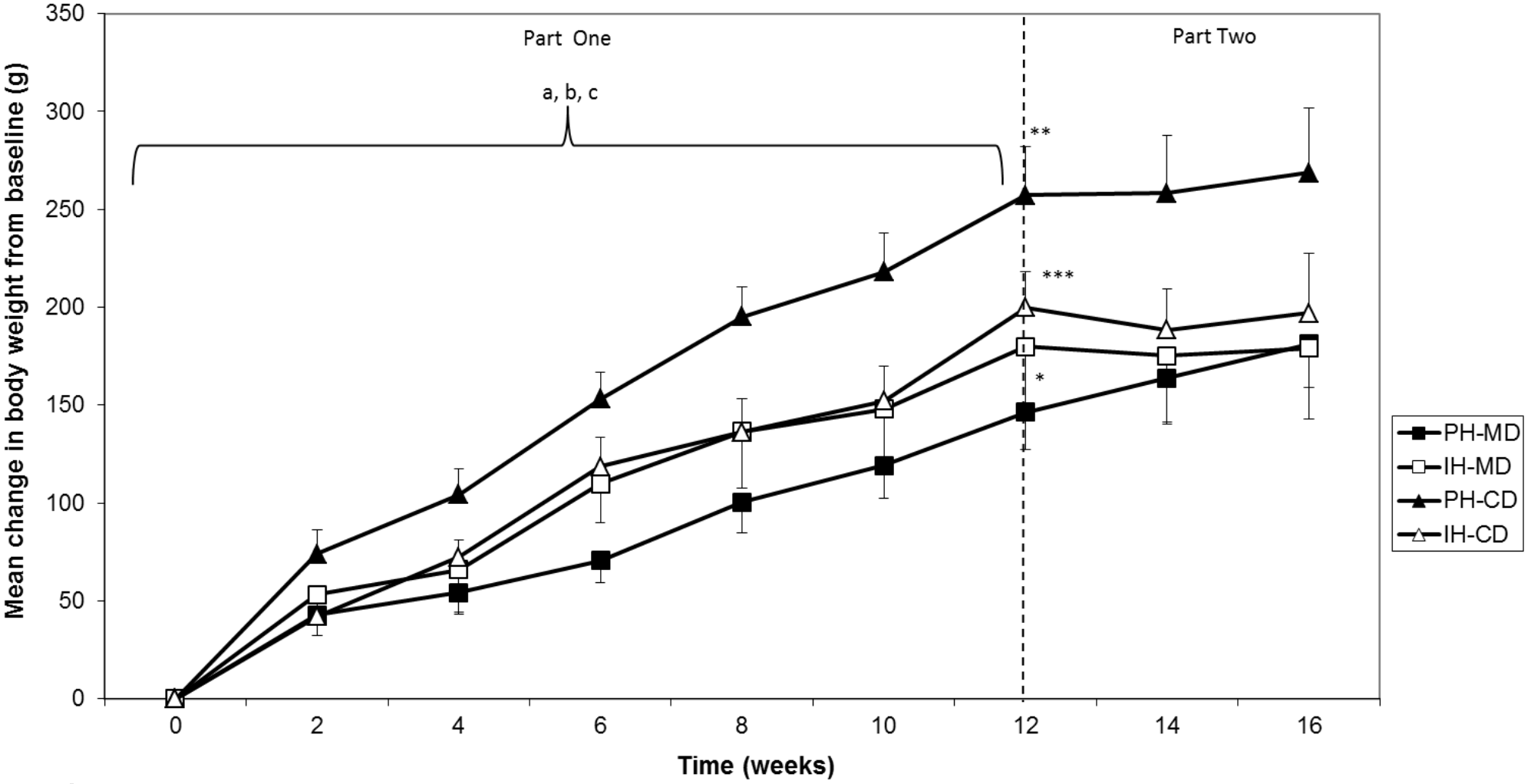

Part One
Part Two
a, b, c
**
***
*
Mean change in body weight from baseline (g)
Time (weeks)
0
50
100
150
200
250
300
350
0
2
4
6
8
10
12
14
16
PH-MD
IH-MD
PH-CD
IH-CD

Figure 3.1: Mean change in body weight from baseline in male Wistar rats that were housed either individually or in pairs and fed either a mushy rat chow diet or a condensed milk diet over the whole experimental period. Data expressed as mean values + standard deviation and are averaged for each two week period. Part one indicated on the left of the dotted line represents the 12-week housing and diet regimen. Part two indicated on the right of the dotted line which represents the beginning of the intervention with chronic restraint stress. PH-MD: pair-housed rats and a mushy rat chow diet (n=6), IH-MD: individually-housed rats and a mushy rat chow diet (n=9), PH-CD: pair-housed rats and a condensed milk diet (n=12), IH-CD: individually-housed rats and a condensed milk diet (n=6).[*] $P<0.05$ – end mean change in body weight from baseline, PH-MD vs IH-MD (Students t-test). [**] $P<0.001$ – end mean change in body weight from baseline, PH-CD vs IH-CD (Students t-test). *** $P<0.0001$ – end mean change in body weight from baseline, PH-CD vs PH-MD (Students t-test). [a] $P<0.05$ – PH-MD vs IH-MD (area under the curve). [b] $P<0.01$ – PH-CD vs PH-MD (area under the curve). [c] $P<0.001$, PH-CD vs IH-CD (area under the curve).

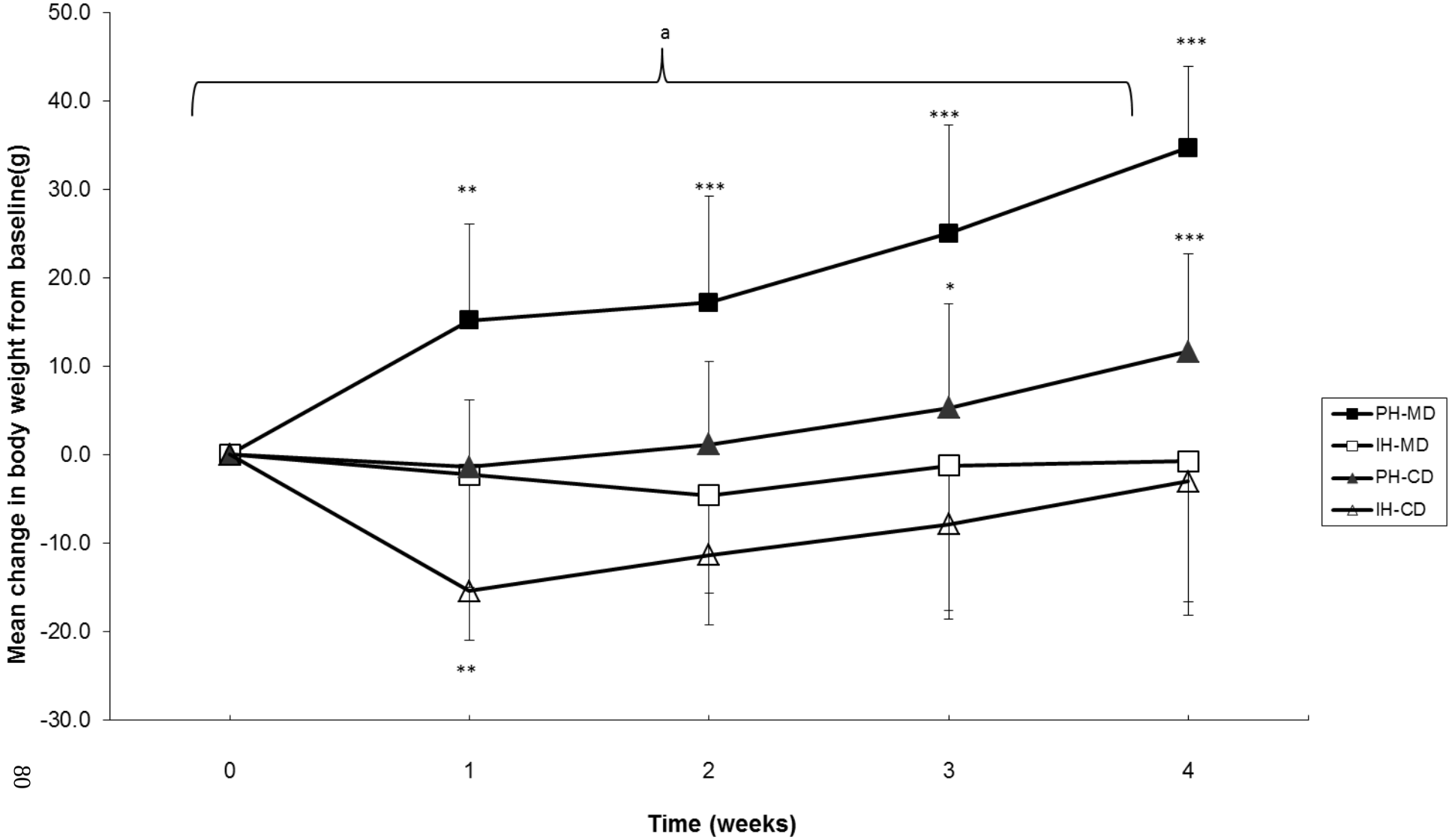

50.0
40.0
30.0
20.0
10.0
0.0
-10.0
-20.0
-30.0
Mean change in body weight from baseline(g)
a
***
***
**
***
*
***
**
0
1
2
3
4
Time (weeks)
PH-MD
IH-MD
PH-CD
IH-CD

Figure 3.2: Mean change in normalised body weight over four weeks of chronic restraint stress in individually- and pair-housed male Wistar rats that were consuming either a mushy rat chow diet or a condensed milk diet. Data were normalised at the start of the four-week period and are expressed as mean+SD. Body weights were averaged for each week. PH-MD: pair-housed rats consuming a mushy rat chow diet (n=6), IH-MD: individually-housed rats consuming a mushy rat chow diet (n=9), PH-CD: pair-housed rats consuming a condensed milk diet (n=12), IH-CD: individually-housed rats consuming a condensed milk diet (n=6). Comparisons were done with repeated measures ANOVA and Tukey-Kramer *post-hoc* test. * $P<0.05$, for PH-CD, week three vs. week four. ** $P<0.01$, for PH-MD, week one vs. baseline and for IH-CD, week one vs. baseline. *** $P<0.001$, for PH-MD, week two, three, four vs. baseline, and for PH-CD, week four vs. baseline. [a] $P<0.01$, PH-MD vs. IH-MD and PH-MD vs. PH-CD (area under the curve).

3.1.1 Body weight

a. Housing and diet regimen

Firstly, it was investigated if the rats displayed differences in the mean change in body weight from baseline at the end of the 12 weeks, which is referred to as end mean change in body weight from baseline. Secondly, it was determined if there was a significant difference in the mean change in body weight from baseline over the 12-week period by using the area under the curve analysis (Figure 3.1).

After 12 weeks, the pair-housed rats eating the mushy diet had a significantly lower end mean change in body weight from baseline than the individually-housed rats (control) (Figure 3.1, Students t-test, $P<0.05$). Furthermore, an area under curve analysis confirmed that the pair-housed rats had a significantly lower mean change in body weight from baseline than the control rats (Figure 3.1, $P<0.05$) over the 12-week housing and diet regimen

The pair-housed rats consuming the condensed milk diet had a significantly higher end mean change in body weight from baseline than the individually-housed rats (control) (Figure 3.1, Students t-test, $P<0.001$). An area under the curve analysis verified that the pair-housed rats had a greater mean change in body weight from baseline than the

control rats (Figure 3.1, $P<0.001$) over the 12-week housing and diet regimen.

b. Exposure to chronic restraint stress

Following the twelve weeks of the housing and diet regimen, change in mean body weight data were normalized for the four groups for the four weeks of chronic restraint stress and is represented in Figure 3.2. A repeated measures ANOVA was first conducted to determine whether the intragroup weekly gains in body weight were significantly different from the week before and from baseline. Then, an area under the curve analysis was used to determine if there was a significant difference in the mean change in body weight from baseline over the four-week period of chronic restraint stress.

The pair-housed rats consuming the mushy rat chow diet had a significant increase in body weight in the first week of chronic restraint stress (Figure 3.2, repeated measures ANOVA, $P<0.01$), which was maintained up to week four (Figure 3.2, repeated measures ANOVA, $P<0.001$).

In the individually-housed rats consuming the mushy rat chow diet, there were no significant changes in body weight over the four weeks of chronic restraint stress (Figure 3.2, repeated measures ANOVA, $P = 0.7363$) .

The pair-housed rats consuming the condensed milk diet showed a significant increase in body weight at week four compared to week three (Figure 3.2, repeated measures ANOVA, $P<0.05$) and was significantly

different from baseline at week four (Figure 3.2, repeated measures ANOVA, $P<0.001$).

In the individually-housed rats consuming the condensed milk diet, a significant drop in body weight occurred in the first week of restraint (Figure 3.2, repeated measures ANOVA, $P<0.01$), which then returned to normal and was not significantly different to baseline after four weeks.

When comparing between the groups, over the four weeks of chronic restraint stress, in the rats consuming the mushy rat chow diet, the pair-housed group had a greater mean change in body weight from baseline than the control group (IH-MD) and therefore gained significantly more weight than the controls (Figure 3.2, area under the curve, $P<0.01$).

In the rats consuming the condensed milk diet, the mean change in body weights did not differ significantly between the pair-housed group (area under the curve, 130.9±33.0) and the control (IH-CD) (area under the curve, 83.9±25.0).

3.1.2 Metabolic Parameters

a. Liver weight and body fat weight

The average liver weight, visceral fat, retrorenal fat and interscapular fat weights as a percentage of body weight for all groups are shown in Table 3.1.

Housing did not influence liver weights in any of the groups, as liver weights, as a percentage of body weight, were similar across all four groups.

Housing did not affect visceral fat weight or interscapular fat weight in rats consuming the normal diet. However, retrorenal fat weight was significantly greater in the pair-housed group (one-way ANOVA, $P<0.001$) compared with the individually-housed group (IH-MD, control).

Pair-housing of rats, consuming a condensed milk diet, resulted in a decrease of visceral fat weight (one-way ANOVA, $P<0.01$) and an increase in both interscapular fat weight (one-way ANOVA, $P<0.05$) and retrorenal fat weight (one-way ANOVA, $P<0.001$) compared to the individually-housed group (IH-CD, control).

b. Fatty Acid Profiles

For retrorenal fat, there were no significant differences between the groups. Levels of saturated fatty acids (SFA) ranged from 31-35 %, while levels of mono-unsaturated fatty acids (MUFA) ranged from 64-68 % in the four groups.

In rats consuming the normal diet, housing had no impact on the fatty acid profiles of the liver (Figure 3.3), fat (Figures 3.4, 3.5, 3.6) and muscle samples (Figure 3.7).

Table 3.1: The effects of housing and diet on average liver weight and fat weights as a percentage of body weight in male Wistar rats after 12 weeks of differing diets and housing and four weeks of chronic restraint stress

Sample	IH-MD (n=9) (%)	PH-MD (n=6) (%)	IH-CD (n=6) (%)	PH-CD (n=12) (%)
Liver	3.5 ± 0.3	3.3 ± 0.1	3.6 ± 0.2	3.4 ± 0.3
Visceral Fat	1.4 ± 0.3	1.5 ± 0.2	2.2 ± 0.1 ***	1.8 ± 0.2 **
Retrorenal Fat	0.9 ± 0.3	1.4 ± 0.1 ***	1.3 ± 0.2 **	2.0 ± 0.2 ***
Interscapular Fat	0.17 ± 0.04	0.21 ± 0.02	0.20 ± 0.06	0.27 ± 0.04 *

Values shown as mean ± standard deviation. PH - pair-housed, IH - individually-housed, MD - mushy rat chow diet, CD - condensed milk diet. * P <0.05- PH-CD vs. IH-CD and PH-MD for interscapular fat (one-way ANOVA, Tukey post hoc test), ** P <0.01- PH-CD vs. IH-CD for visceral fat and IH-CD vs. IH-MD for retrorenal fat (one-way ANOVA, Tukey post hoc test), *** P < 0.001- IH-CD vs. IH-MD for visceral fat and PH-MD vs. IH-MD and PH-CD vs. IH-CD and PH-MD for retrorenal fat (one-way ANOVA, Tukey post hoc test).

In rats consuming the condensed milk diet, housing had no effect on the fatty acid profiles of the visceral fat (Figure 3.5), retrorenal fat, interscapular fat (Figure 3.6) and muscle (Figure 3.7).

However, pair housing affected the fatty acid profile of both the liver (Figure 3.3) and subcutaneous fat (Figure 3.4). Compared to the individually-housed group (IH-CD – control), the pair-housed rats displayed the following fatty acid profiles. SFA were reduced in both the liver (P<0.001) and subcutaneous fat (P<0.001). MUFA were increased in the liver (P<0.001), but similar in the subcutaneous fat. Omega-6 polyunsaturated fatty acids (n6-PUFA) were reduced in the liver (P<0.001) but were elevated in the subcutaneous fat (P<0.001), while reduced amounts of omega-3 polyunsaturated fatty acids (n3-PUFA) occurred in the liver (P<0.01).

c. Serum concentrations

In rats consuming the normal diet, housing had no impact on serum concentrations of corticosterone, insulin, glucose and leptin (Table 3.2).

In rats consuming the condensed milk diet, housing did not influence the serum concentrations of corticosterone, insulin and glucose. However, the rats that were pair-housed had a significantly higher serum leptin concentration than the individually-housed rats (one-way ANOVA, Tukey *post hoc* test, P<0.001).

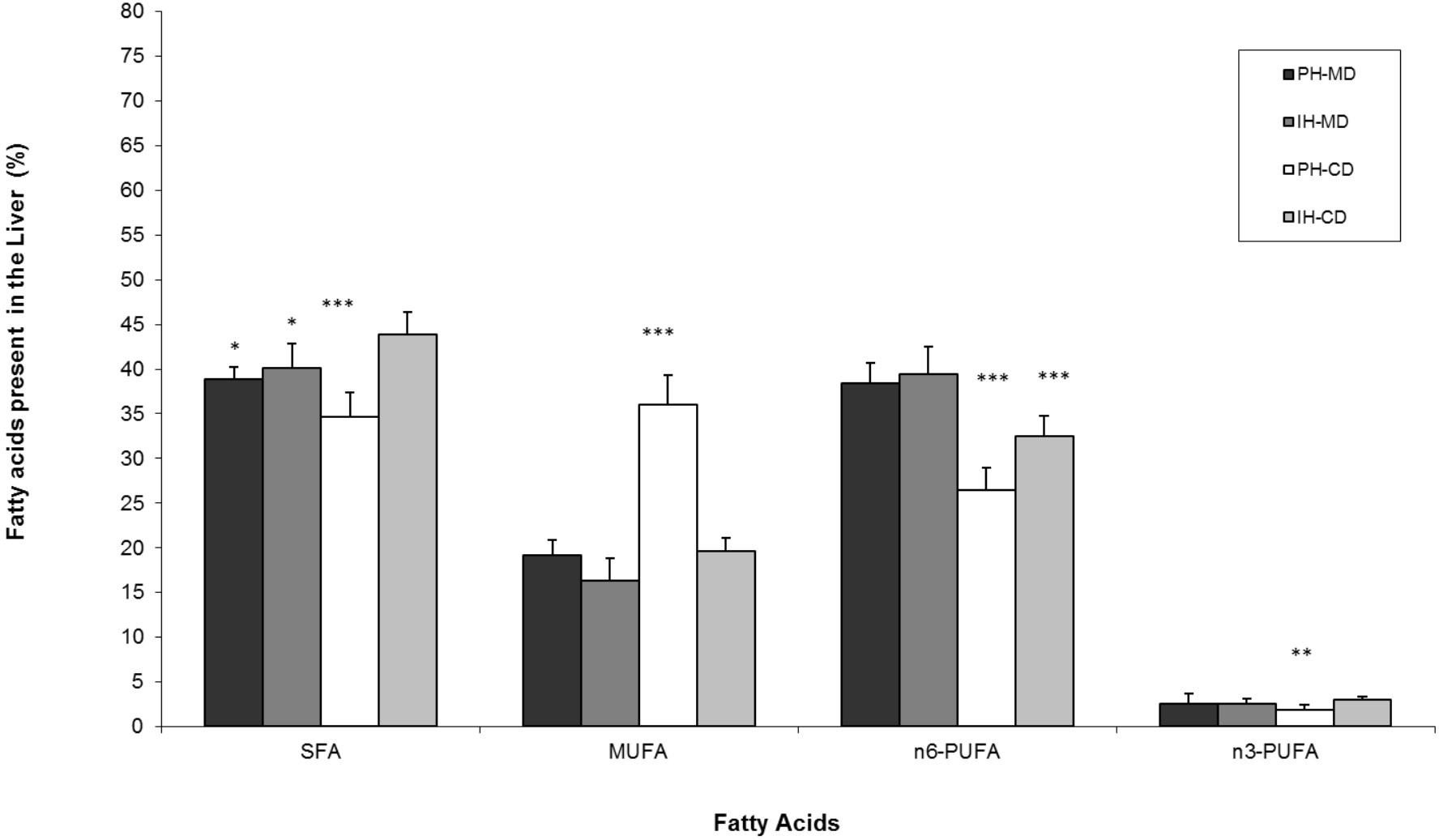

Fatty acids present in the Liver (%)
80
75
70
65
60
55
50
45
40
35
30
25
20
15
10
5
0
PH-MD
IH-MD
PH-CD
IH-CD
*
*
***
***
***
***
**
SFA
MUFA
n6-PUFA
n3-PUFA
Fatty Acids

Figure 3.3: The effects of housing and diet on the fatty acid profiles of male Wistar rat liver after 12 weeks of differing diets and housing and four weeks of chronic restraint stress. Fatty acids represented as a percentage of the total fatty acids present in the sample. Data expressed as mean ± standard deviation. To compare the four groups, a one-way ANOVA with Tukey *post hoc* test for multiple comparisons set at one significance level was used. SFA: saturated fatty acids, MUFA: monounsaturated fatty acids, n6-PUFA: omega-6 polyunsaturated fatty acids, n3-PUFA: omega-3 polyunsaturated fatty acids. PH-MD: pair-housed rats and a mushy rat chow diet (n=6), IH-MD: individually-housed rats and a mushy rat chow diet (n=9), PH-CD: pair-housed rats and a condensed milk diet (n=12), IH-CD: individually-housed rats and a condensed milk diet (n=6). **SFA**: * $P<0.05$, PH-MD vs. PH-CD and IH-MD vs. IH-CD, *** $P<0.001$, IH-CD vs. PH-CD. **MUFA**: *** $P<0.001$, IH-CD vs. PH-CD and PH-MD vs. PH-CD. **n6-PUFA**: *** $P<0.001$, IH-CD vs. PH-CD, PH-MD vs. PH-CD and IH-MD vs. IH-CD. **n3-PUFA**: ** $P<0.01$, IH-CD vs. PH-CD.

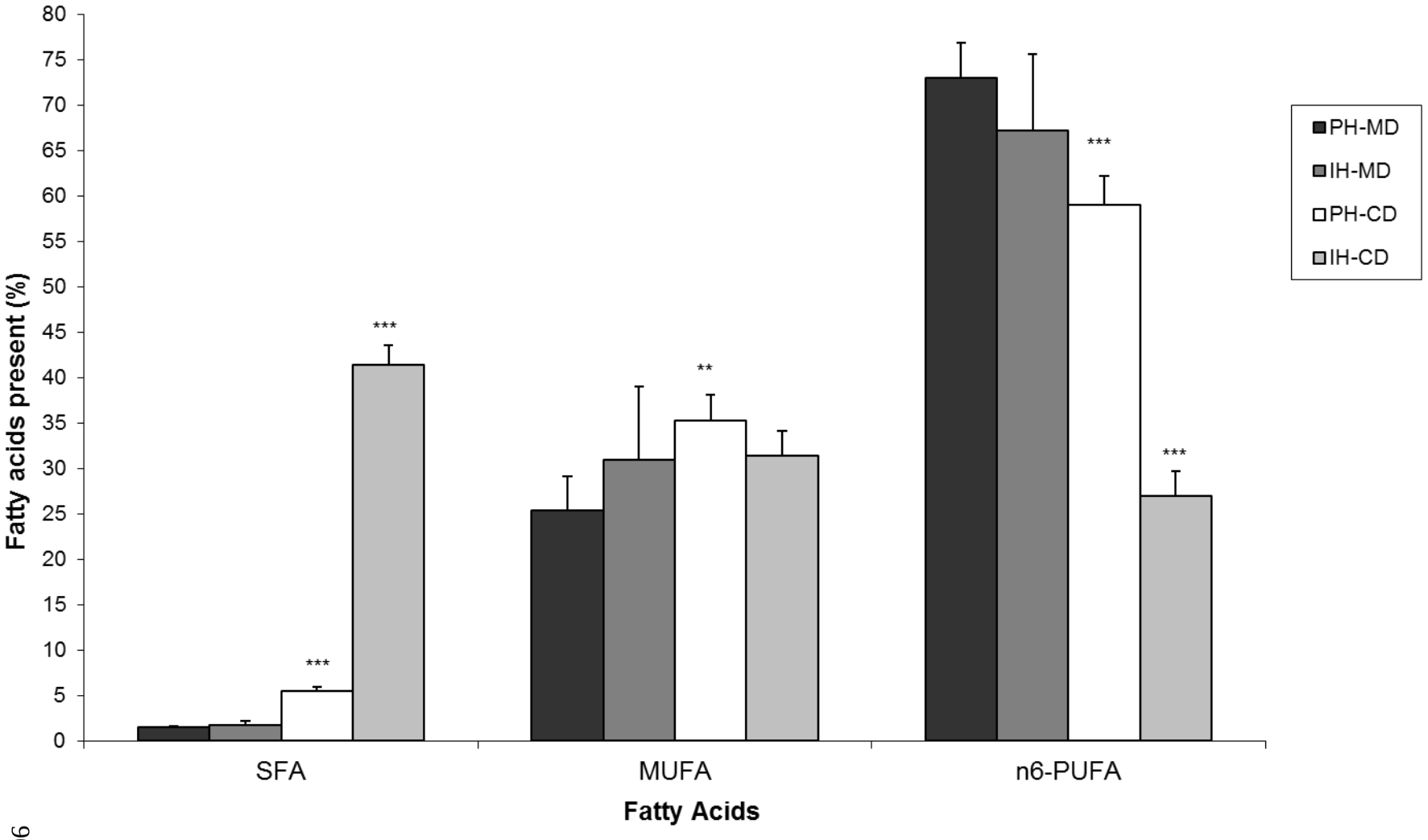

80
75
70
65
60
55
50
45
40
35
30
25
20
15
10
5
0
Fatty acids present (%)
***
**
***
***
PH-MD
IH-MD
PH-CD
IH-CD
SFA
MUFA
n6-PUFA
Fatty Acids

Figure 3.4: The effects of housing and diet on the fatty acid profiles of the subcutaneous fat of male Wistar rats after 12 weeks of differing diets and housing and four weeks of chronic restraint stress. Fatty acids represented as a percentage of the total fatty acids present in the sample. Data expressed as mean ± standard deviation. To compare the four groups, a one-way ANOVA with Tukey *post hoc* test for multiple comparisons set at one significance level was used. SFA: saturated fatty acids, MUFA: monounsaturated fatty acids, n6-PUFA: omega 6 polyunsaturated fatty acids. PH-MD: pair-housed rats and a mushy rat chow diet (n=6), IH-MD: individually-housed rats and a mushy rat chow diet (n=9), PH-CD: pair-housed rats and a condensed milk diet (n=12), IH-CD: individually-housed rats and a condensed milk diet (n=6). **SFA** and **n6-PUFA**: *** $P<0.001$, IH-CD vs. PH-CD, PH-MD vs. PH-CD and IH-MD vs. IH-CD. **MUFA**: ** $P<0.01$, PH-MD vs. PH-CD.

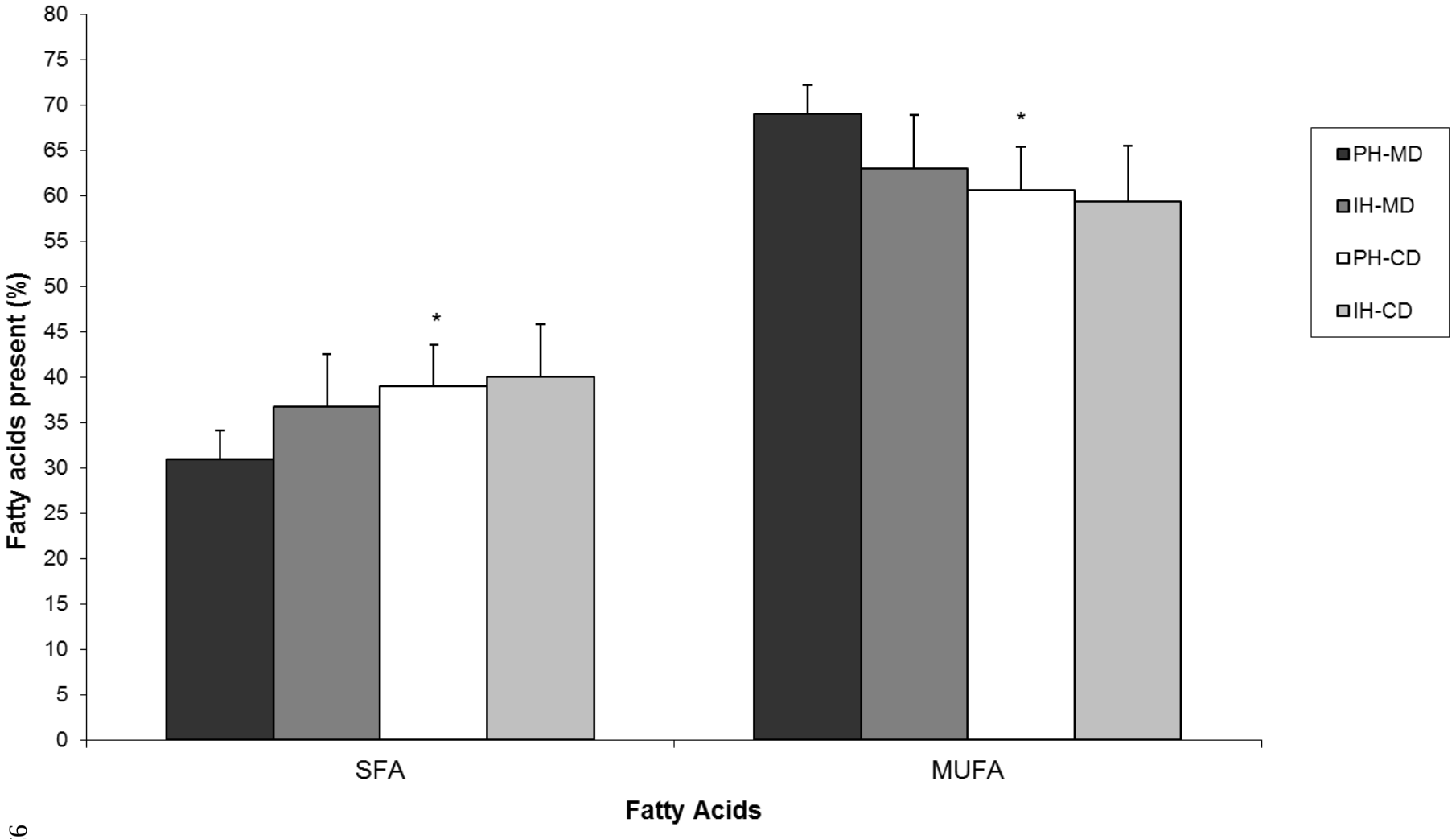

80
75
70
65
60
55
50
45
40
35
30
25
20
15
10
5
0
Fatty acids present (%)
*
*
SFA
MUFA
Fatty Acids
PH-MD
IH-MD
PH-CD
IH-CD

Figure 3.5: The effects of housing and diet on the fatty acid profiles of visceral fat of male Wistar rats after 12 weeks of differing diets and housing and four weeks of chronic restraint stress. Fatty acids represented as a percentage of the total fatty acids present in the sample. Data expressed as mean ± standard deviation. To compare the four groups, a one-way ANOVA with Tukey *post hoc* test for multiple comparisons set at one significance level was used. SFA: saturated fatty acids, MUFA: monounsaturated fatty acids. PH-MD: pair-housed rats and a mushy rat chow diet (n=6), IH-MD: individually-housed rats and a mushy rat chow diet (n=9), PH-CD: pair-housed rats and a condensed milk diet (n=12), IH-CD: individually-housed rats and a condensed milk diet (n=6). **SFA** and **MUFA**: * $P<0.05$, PH-MD vs. PH-CD.

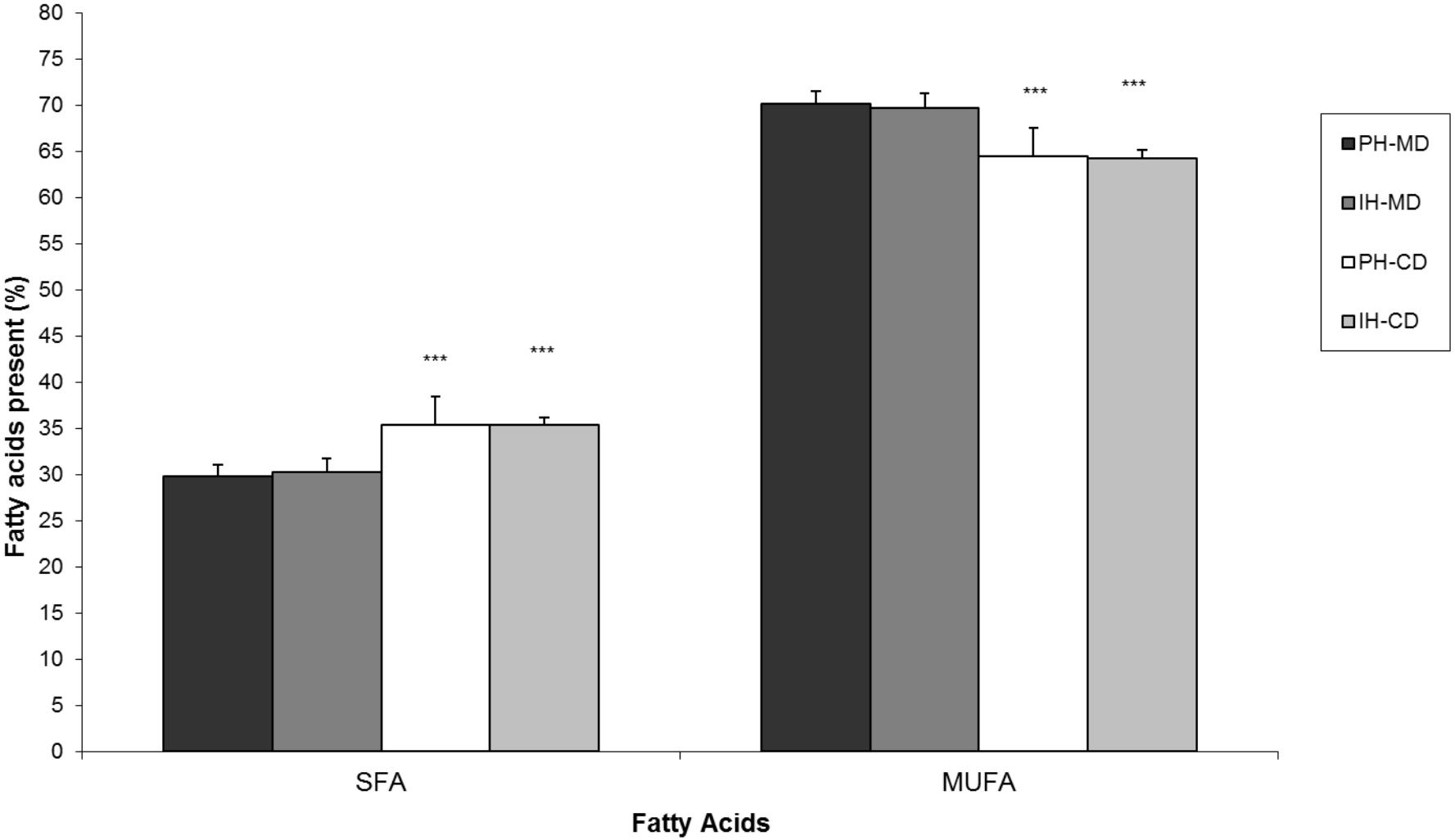

80
75
70
65
60
55
50
45
40
35
30
25
20
15
10
5
0
Fatty acids present (%)
***
***
***
***
SFA
MUFA
Fatty Acids
PH-MD
IH-MD
PH-CD
IH-CD

Figure 3.6: The effects of housing and diet on the fatty acid profiles of the interscapular fat of male Wistar rats after 12 weeks of differing diets and housing and four weeks of chronic restraint stress. Fatty acids represented as a percentage of the total fatty acids present in the sample. Data expressed as mean ± standard deviation. To compare the four groups, a one-way ANOVA with Tukey *post hoc* test for multiple comparisons set at one significance level was used. SFA: saturated fatty acids, MUFA: monounsaturated fatty acids. PH-MD: pair-housed rats and a mushy rat chow diet (n=6), IH-MD: individually-housed rats and a mushy rat chow diet (n=9), PH-CD: pair-housed rats and a condensed milk diet (n=12), IH-CD: individually-housed rats and a condensed milk diet (n=6). **SFA** and **MUFA**: *** $P<0.001$, PH-MD vs. PH-CD and IH-MD vs. IH-CD.

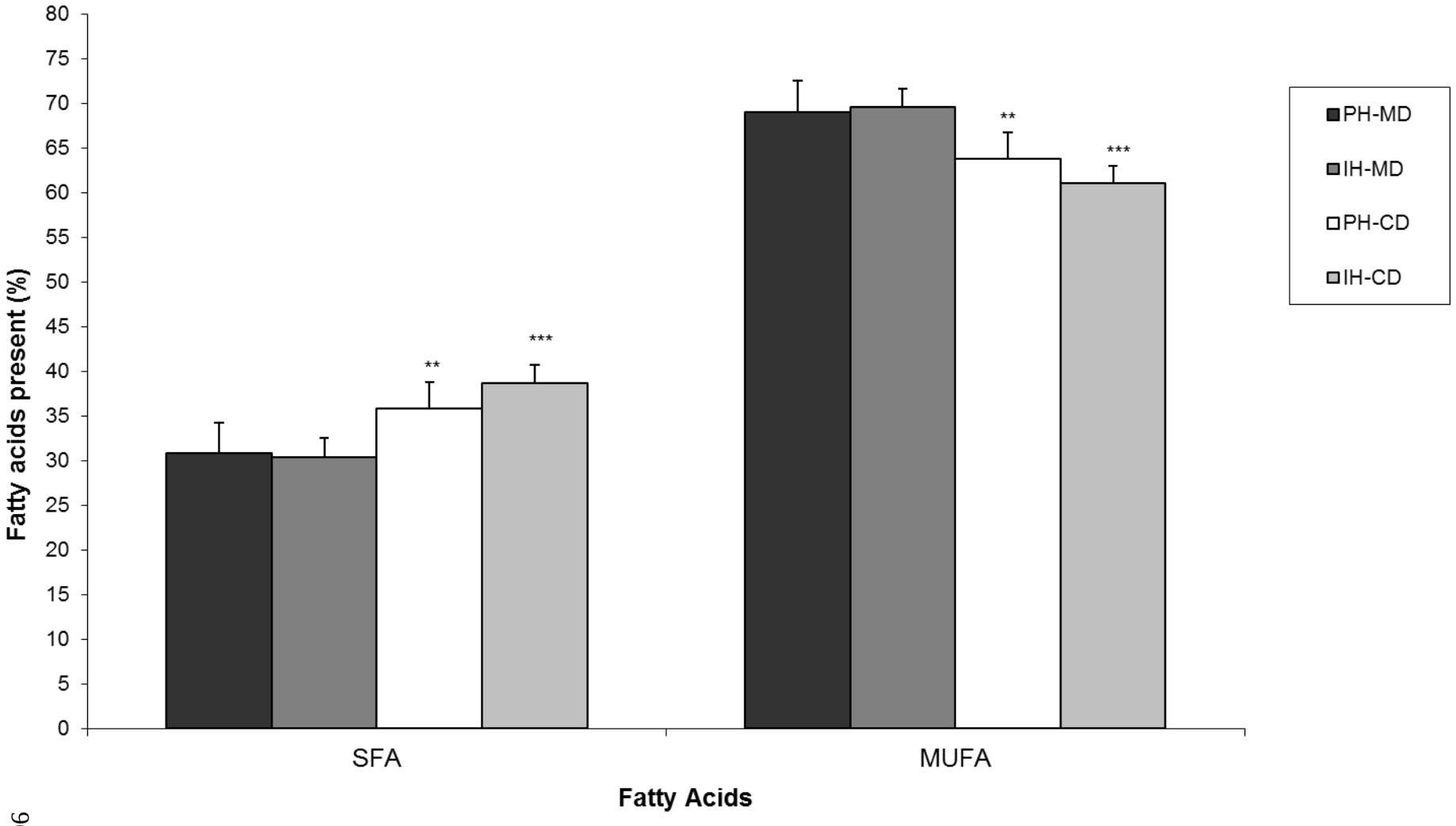

80
75
70
65
60
55
50
45
40
35
30
25
20
15
10
5
0
Fatty acids present (%)
**
***
**
***
SFA
MUFA
Fatty Acids
PH-MD
IH-MD
PH-CD
IH-CD

Figure 3.7: The effects of housing and diet on muscle fatty acid profiles of male Wistar rats after 12 weeks of differing diets and housing and four weeks of chronic restraint stress. Fatty acids represented as a percentage of the total fatty acids present in the sample. Data expressed as mean ± standard deviation. To compare the four groups, a one-way ANOVA with Tukey *post hoc* test for multiple comparisons set at one significance level was used. SFA: saturated fatty acids, MUFA: monounsaturated fatty acids. PH-MD: pair-housed rats and a mushy rat chow diet (n=6), IH-MD: individually-housed rats and a mushy rat chow diet (n=9), PH-CD: pair-housed rats and a condensed milk diet (n=12), IH-CD: individually-housed rats and a condensed milk diet (n=6). **SFA** and **MUFA**: ** $P<0.01$, PH-MD vs. PH-CD, *** $P<0.001$, IH-MD vs. IH-CD.

Table 3.2: The effects of housing and diet on serum concentrations in male Wistar rats after 12 weeks of differing diets and housing and four weeks of chronic restraint stress

Serum Concentrations	IH-MD (n=9)	PH-MD (n=6)	IH-CD (n=6)	PH-CD (n=12)	Normal values
Corticosterone (ng/ml)	220 ± 170	360 ± 130	340 ± 70	250 ± 190	0 - 330
Insulin (ng/ml)	0.4 ± 0.1	0.5 ± 0.2	0.4 ± 0.2	0.6 ± 0.2	0.5 - 2.0
Glucose (mmol/l)	15.0 ± 6.0	17.0 ± 4.0	13.0 ± 3.0	14.0 ± 5.0	4.2 - 6.1
Leptin (ng/ml)	4.7 ± 0.9	4.6 ± 1.4	6.5 ± 1.8	11.6 ± 2.9 ***	1.3 - 3.1

Values shown as mean ± standard deviation. PH - pair-housed, IH - individually-housed, MD - mushy rat chow diet, CD - condensed milk diet, *** $P < 0.001$, PH-CD vs. IH-CD and PH-CD vs. PH-MD (one-way ANOVA with Tukey post-hoc test).

3.2 Section B – Impact of diet

For these results, the PH-MD rats were compared to the PH-CD rats and the IH-MD rats were compared to IH-CD rats. Therefore the housing was kept constant while only the diet was changed. Considering that rat chow would be the standard type of diet given to laboratory rodents, the control group for both of the above comparisons are the rats consuming the mushy rat chow diet.

3.2.1 Body weight

a. Housing and diet regimen

After 12 weeks, in the pair-housed groups, the rats consuming the condensed milk diet had a significantly elevated end mean change in body weight from baseline compared to the rats consuming the mushy rat chow diet (control) (Figure 3.1, Students t-test, $P<0.0001$). Furthermore, an area under curve analysis confirmed that the rats consuming the condensed milk diet had a significantly greater mean change in body weight from baseline than the control rats (Figure 3.1, $P<0.01$).

In individually-housed rats, diet did not impact on weight gain, as both the IH-MD and IH-CD groups showed similar changes in body weight (end mean change in body weight from baseline; IH-MD: 179.7±18.5g, IH-CD: 199.9±32.4g, area under the curve; IH-MD: 1915.1±346.1, IH-CD: 2016.8±235.0).

b. Exposure to chronic restraint stress

During the four weeks of chronic restraint stress, in the pair-housed groups, the rats consuming the condensed milk diet had a smaller mean change in body weight from baseline than the rats consuming the mushy rat chow diet (control) and therefore gained significantly less weight than the controls (Figure 3.2, area under the curve, $P<0.01$).

The type of diet did not result in significant differences in mean change in body weight from baseline in the individually-housed rats (area under the curve; IH-MD: 111.6±52.8, IH-CD: 83.9±25.0).

3.2.2 Metabolic Parameters

a. Liver weight and fat weight

The average liver weight, visceral fat, retrorenal fat and interscapular fat weights as a percentage of body weight for all groups are shown in Table 3.1.

Diet did not influence liver weights in any of the groups. Concerning the rats that were pair-housed, consumption of the condensed milk diet resulted in an increase in retrorenal (one-way ANOVA, $P<0.001$) and interscaspular fat (one-way ANOVA, $P<0.05$) weights but no change in visceral fat weight compared to the rats fed the mushy rat chow diet (PH-MD).

With regards to the rats that were individually-housed, consumption of the condensed milk diet resulted in an increase in visceral (one-way ANOVA, $P<0.001$) and retrorenal fat weights (one-way ANOVA, $P<0.01$) with no alteration to the interscapular fat weight compared with the rats fed the mushy rat chow diet.

b. Fatty Acid Profiles

In pair-housed rats, the type of diet did not impact on the fatty acid profile of the retrorenal fat. However, diet was responsible for the differing fatty acid profiles of the liver (Figure 3.3), subcutaneous fat (Figure 3.4), visceral fat (Figure 3.5), interscapular fat (Figure 3.6) and muscle (Figure 3.7). Compared to the rats consuming the mushy rat chow diet (PH-MD, control), rats consuming the condensed milk diet (PH-CD) showed the following fatty acid profiles. SFA were increased in subcutaneous fat ($P<0.001$), visceral fat ($P<0.05$), interscapular fat ($P<0.001$) and muscle ($P<0.01$), but reduced in the liver ($P<0.05$). MUFA were only elevated liver ($P<0.001$) and subcutaneous fat ($P<0.01$), but reduced in the visceral fat ($P<0.05$), interscapular fat ($P<0.001$) and muscle ($P<0.01$). n6-PUFA occurred only in the liver ($P<0.001$) and subcutaneous fat ($P<0.001$), where they occurred in reduced amounts. Similar levels of n3-PUFA occurred but were only present in the liver.

In the individually housed groups, diet did not affect the fatty acid profile of the visceral fat (Figure 3.5) and retrorenal fat.

Differences in the fatty acid profiles of the liver (Figure 3.3), subcutaneous fat (Figure 3.4), interscapular fat (Figure 3.6) and muscle (Figure 3.7) were due to the diet consumed. In comparison to rats consuming the mushy rat chow diet (IH-MD, control), rats consuming the condensed milk diet showed the following fatty acid profiles. SFA were elevated in the liver ($P<0.05$), subcutaneous fat ($P<0.001$), interscapular fat ($P<0.001$) and muscle ($P<0.001$). MUFA were lower in the interscapular fat ($P<0.001$) and muscle ($P<0.001$), while similar amounts occurred in the liver and subcutaneous fat. n6-PUFA levels were significantly reduced in both the liver ($P<0.001$) and subcutaneous fat ($P<0.001$), while similar levels of n3-PUFA occurred in the liver.

c. Serum concentrations

In rats housed in pairs, diet had no impact on serum concentrations of corticosterone, insulin and glucose (Table 3.2). However, the condensed milk diet resulted in elevated serum leptin concentrations (one-way ANOVA, $P<0.001$) compared to the rats consuming the mushy rat chow diet.

In rats that were individually-housed, diet did not influence the serum concentrations of corticosterone, insulin, glucose and leptin.

CHAPTER FOUR

General Discussion

4. Discussion

To determine the impact of housing, male Wistar rats that were fed the same diet, but were housed either individually or in pairs were compared. The results show that the type of housing influences weight gain whether rats consume a mushy rat chow diet or a condensed milk diet for 12 weeks. However, the effect that housing had on body weight was not the same and depended on the diet consumed. Pair-housed rats weighed less than individually-housed when fed the mushy rat chow diet, whereas pair-housed rats gained more weight than individually-housed rats when fed the condensed milk diet.

Following the 12-week housing and diet regimen, rats were chronically restrained for seven hours a day for four weeks. Rats were maintained in the same housing conditions and were fed the same diet when not in restraint. Pair-housed rats consuming the mushy rat chow diet continued to gain weight during restraint. Body weights of the individually-housed rats consuming the mushy rat chow diet stayed constant over the four weeks of chronic restraint stress.

When the rats consuming the mushy rat chow diet were compared to each other, the results indicate that during chronic restraint stress, only the pair-housed rats gained significant weight.

In rats consuming the condensed milk diet, the pair-housed rats did not gain or lose weight for the first three weeks of chronic restraint stress. A gain in body weight occurred in the fourth week only. The individually-

housed rats lost weight in the first week of restraint. Body weights returned to values at the start of restraint by the third week.

However, when compared to each other, housing did not have an effect on the body weights of both groups of rats fed the condensed milk diet.

Housing produced differences only in retrorenal fat weight in rats consuming the mushy rat chow diet, with pair-housed rats having increased retrorenal fat. Liver, interscapular fat and visceral fat were not affected by the type of housing. Differences in retrorenal, interscapular and visceral fat weights occurred in rats consuming the condensed milk diet, with pair-housed showing increased interscapular and retrorenal fat and reduced visceral fat. No difference in liver weight occurred.

In rats consuming the condensed milk diet, the type of housing affected fatty acid profiles of the liver and subcutaneous fat but not of the visceral fat, interscapular fat or muscle. In addition, pair-housing of these rats resulted in elevated serum leptin levels. Housing did not cause significant changes in the serum insulin concentration. Although housing did not influence serum corticosterone and glucose levels, it must be noted that both of these levels were particularly high.

To determine the impact of diet, comparisons were made in rats that share the same housing conditions but consume different diets. It is evident from the results that for both the 12-week housing and diet regimen and chronic restraint stress that the type of diet produced differences in body weight

only when rats were pair-housed. During the 12 weeks, the condensed milk diet-fed rats gained more weight than rats fed the mushy rat chow diet, with the opposite occurring during chronic restraint stress, i.e. the pair-housed rats fed the mushy rat chow diet gained more weight than the rats fed the condensed milk diet. Although the body weights of the individually-housed groups were similar both prior to and during restraint, the rats fed the condensed milk diet lost weight during the first week of restraint with body weights returning to pre-restraint values by week three of restraint. The body weight of the rats fed the mushy rat chow diet remained constant during restraint.

In pair-housed rats, diet resulted in elevated retrorenal and interscapular fat weights in rats fed the condensed milk diet, with no differences in liver and visceral fat. In individually-housed rats, increased visceral and retrorenal fat weights occurred in rats fed the condensed milk diet, with no differences in liver and interscapular fat.

Diet was responsible for differing fatty acid profiles of the liver, visceral fat, interscapular fat and subcutaneous fat in pair-housed rats, while fatty acid profiles of the liver, subcutaneous fat, interscapular fat and muscle were influenced in individually-housed rats.

In the pair-housed rats, only serum leptin levels were affected by the type of diet consumed, where elevated levels occurred in rats fed the condensed milk diet. Diet did not impact on serum levels of corticosterone, insulin and glucose.

At the outset of this study, I did not anticipate the marked effect that housing would have, until the data was analysed. Considering that body weight is influenced by an animal's energy intake and energy expenditure, measurements of food intake, metabolic rate and activity may have accounted for the differences in body weights observed during the housing and diet regimen as well as during restraint stress.

Furthermore, considering that stress affects body weight and metabolism, it would have been useful to collect frequent blood samples during the housing and diet regimen and restraint stress to determine serum concentrations of corticosterone, glucose, insulin and leptin and to produce a profile of the response of these levels.

I acknowledge that it would have been valuable to determine the effect of both housing and diet on body fat weight, body fat composition and serum concentrations of corticosterone, glucose, insulin and leptin at the end of the housing and diet regimen, thus rats should have been sacrificed before restraint stress as well. This would have required an increased sample size. Although the increased sample size may have allowed us to prove significance for results that were otherwise not significant, it may have not been feasible due to space constraints in the Central Animal Services (the place where the rats were housed).

Leading on from this, ordinarily in statistical analysis where there are multiple animals in a cage, the cage is considered to be the sample unit and not the number of animals in the cage. However, this was not done in this study as the sample size would be significantly reduced.

Since there was a lack in pre-chronic restraint stress values, it was very difficult to pinpoint the actual effect of chronic restraint stress. Furthermore, I recognize that groups of both individually- and pair-housed rats that were not exposed to chronic restraint stress to act as the non-stressed unrestrained controls were required.

However, food intake may be difficult to obtain in pair-housed rats, as one cannot know with certainty how much each rat would be eating. To overcome this issue, the cage should be considered as the sample unit and not the number of animals in the unit. Therefore the average food intake of the animals in the cage should be determined and the value used as a single data point. This could not be done in this study due to space constraints.

Body weight loss or reduced weight gain is often used in animal models of chronic stress as an indication of stress (Bhatnagar *et al.* 2006). Furthermore, weight loss is a common response to chronic stress (Marin *et al.* 2007, Martí *et al*., 1994). In this study, when rats were exposed to chronic restraint stress, only the pair-housed group consuming the mushy rat chow diet continued to gain weight during restraint, whereas growth

was stunted in the individually-housed group consuming the same diet. Therefore, I would have concluded that perhaps chronic restraint stress was not stressful for the pair-housed rats but the individually-housed rats found it stressful. However, when other restraint stress studies were examined, it was observed that although pair-housed restrained rats continued to grow while in restraint, they displayed a reduced gain in weight compared to unrestrained rats (Baldwin *et al.* 1997, McLaughlin *et al*. 2007, Zardooz *et al*. 2006).

When investigated further, I discovered that individually-housed restrained rats not only suffered a reduced weight gain but even a loss of body weight, which persisted for the time the rats were restrained (Harris *et al*. 2002, Harris *et al*. 2006, Kleen *et al*. 2006). Even though it is not ideal to compare across restraint stress studies, as the way in which certain strains deal with stress differ and different restrainers and restraint stress protocols have been used (McLaughlin *et al*. 2007), I would like to suggest that when individually-housed rats are restrained, they face increased stress as indicated by weight loss/ constant body weight. Pair-housed rats are less stressed as they show a reduced gain in body weight and unrestrained rats experience no stress.

Therefore my revised conclusion is that the pair-housed rats in this study were less stressed, as they continued to grow during restraint, whereas

the individually-housed rats were more stressed, as their growth was stunted/ their body weights remained the same during restraint stress.

What is it then about the combination of pair-housing and the mushy rat chow diet that protected these rats from the severe effects of chronic stress? It is likely that the conditions prior to restraint stress influenced how the rats responded to the stressor. Based on previous observations that chronically individually-housed rats are hyperresponsive to other stressors (Perelló *et al*. 2006) and that social support can reduce the response to stressors (Ruis *et al*. 1999, Sachser *et al.*1998, Seeman and McEwen, 1996), it is my contention that two social animals eating a wholesome diet, are not as stressed and will therefore cope better with a new stressor than a social animal by himself. Loneliness for a sociable animal may stress the animal and this stress will then compromise the animal's ability to cope with a new stressor.

According to the body weight profiles prior to restraint, the pair-housed rats eating the mushy rat chow diet had a lower body weight than the individually-housed rats. This may imply that in pre-restraint conditions, the pair-housed rats were stressed as they were not gaining as much weight. However, I do not think that this is the case, as the lack of weight gain is not necessarily by itself an indication of whether or not an animal is stressed. Rather, as stated previously, it is how the animal copes with a new stressor that indicates whether or not the animal was initially stressed.

It needs to be borne in mind that, although they did not gain as much weight as the individually-housed rats on the same diet, the weight gain of the pair-housed rats was still normal. The lack of weight gain relative to the individually-housed rats could very well relate to increased energy expenditure in the pair-housed rats due to the social nature of the rats. This would require further investigation.

Based on the results, pair-housed rats consuming a normal rat chow diet coped better with restraint stress; therefore they were less stressed initially. However, it is the individually-housed rats that were more stressed initially, due to their stunted growth observed during restraint stress.

Although the individually-housed rats were stressed, their gain in weight prior to restraint may be explained by either an increase in food intake to cope with their stress or reduced energy expenditure as they lacked company and social interaction. In contrast to this study, other studies have found that pair-housed rats have showed a tendency to gain more weight than individually-housed rats (Thorsell *et al.* 2005, Thorsell *et al*. 2006). The only notable difference between my study and the studies mentioned above was the presentation of the diet; I used a mushy rat chow diet, while rat chow pellets were used in the other studies. Another study found that individually-housed rats had a reduced gain of body weight with unchanged daily food intake compared to group-housed rats (Perelló *et al*. 2006).

Based on my findings, I would agree with the statement made by Brown and Grunberg (1995) regarding “the profound impact housing conditions have … before any treatment manipulations are performed (p. 1088).” Therefore, when experiments are conducted on laboratory rats, the type of housing is an important variable to consider and researchers need to mention how animals are housed, even when rats are fed their regular diet. I would like to suggest that in order to minimize stress; rats consuming a normal diet should be pair-housed, as results may be compromised by the stress induced by individual-housing. In addition, seeing that conditions prior to restraint stress impact on how rats respond to a new stressor, long-term pair-housing and consumption of the mushy rat chow diet are a good combination for coping with subsequent stressors.

With regards to the condensed milk diet, when the pair- and individually-housed were restrained, there was no significant difference in their body weights when compared to each other. However, when examined in isolation, the pair-housed group gained weight only in the fourth week of restraint, while the individually-housed rats lost weight in the first week of restraint, with a recovery to pre-restraint values by the third week. Furthermore, although pair-housed groups no matter what diet gain weight during restraint, the pair-housed rats consuming the condensed milk diet showed significantly lower body weights than pair-housed rats consuming the mushy rat chow diet. Based on my previous assumption that pair-

housed rats consuming the mushy rat chow diet were less stressed during restraint, one could deduce that both pair- and individually-housed rats fed the condensed milk diet showed signs of added stress when restrained, where the pair-housed rats appeared to be less stressed (gained weight) then the individually-housed rats (lost weight).

From the literature, I was unable to find previous studies that describe how different types of housing influence body weight, in rats exposed to chronic restraint stress when fed the condensed milk diet. It has been reported, however, that despite no differences in food intake, when rats fed the condensed milk diet were stressed, they were predisposed to gaining less weight than unstressed rats fed a similar diet (Levin *et al.* 2000). Although, stressed and unstressed rats were not compared in this study, the condensed milk diet as well as the combination of the condensed milk diet and individual-housing appeared to be responsible for reduced weight gain in these rats when they were chronically restrained. Similar to rats fed the mushy rat chow diet, the way in which these rats responded to restraint stress may very well indicate that the conditions prior to restraint were perhaps also stressful.

In pre-restraint conditions, the pair-housed group consuming the condensed milk diet gained substantially more weight than the pair-housed rats fed the mushy rat chow diet. However, it was the rats eating the mushy diet that coped better with restraint. Taken together, these

results further support my previous statement that the lack of weight gain prior to restraint is not necessarily a sign that rats are experiencing more stress or visa versa. In addition, one could infer that it is the substitution of the mushy rat chow diet for the condensed milk diet that was an added stress.

If you take into account that diet interacts with the energy balance systems resulting in alterations in neural, neuroendocrine and metabolic responses, thus impacting on HPA axis activity (Archer and Mercer, 2007, Mercer and Archer, 2005). One could understand how diet could be a source of stress. This is supported by Tannenbaum *et al.* (1997) who suggest that high-energy diets, particularly high in fat, act as a form of chronic stress resulting in raised glucocorticoid concentrations. With the increased ingestion of a high carbohydrate diet, modifications in the stress response can occur. Evidence shows that prolonged consumption of the condensed milk diet has lead to changes in hypothalamic energy balance systems and can result in dysregulation of the HPA axis (Laugero, 2001).

If consumption of the condensed milk diet is stressful, then why is it that the pair-housed rats fed the condensed milk diet gained more weight than pair-housed rats fed the mushy rat chow diet prior to restraint? The answer lies in the fact that the condensed milk diet has been used in rat models of diet-induced obesity. Rats with diet-induced obesity weigh more than rats fed a standard rat chow diet, regardless of housing (Elliott *et al.* 2004,

Harrold *et al.* 2000, Kretschmer *et al.* 2005, Triscari *et al.* 1985). Based on this, one would have expected that like the pair-housed rats, the individually-housed rats consuming the condensed milk diet in this study would have significant weight gain. However, this did not occur as both groups of individually-housed rats had similar body weights prior to restraint.

Earlier studies on diet-induced obesity do not indicate that a certain type of housing is necessary to produce diet-induced obesity, as rats in different types of housing have been studied independently. From examining some of these studies, it was observed that pair-housed rats consuming the condensed milk diet gain between 300-500 g (Elliott *et al.* 2004, Harrold *et al.* 2000), while individually-housed rats gain approximately 200 g (Triscari *et al.* 1985) compared to rats eating a normal diet and housed similarly. Although, both individually- and pair-housed rats become obese, it would appear that the obesity is more pronounced in the pair-housed rats. However, in my study, the condensed milk diet promoted significant weight gain only in the pair-housed but not in the individually-housed rats, which is in contrast to previous studies.

However obesity is not limited to body mass but should also take into account the amount of fat stores in the animal (Cases and Barzilai, 2000). In comparison to the individually-housed rats fed the mushy rat chow diet, the individually-housed rats on the condensed milk diet were shown to

have significantly increased visceral fat and retrorenal fat. However, the increases were only a small percentage, ±0.8 % and ±0.4 % respectively, which in a small animal may be clinically significant and may hint at slight obesity.

Previously, I concluded that individual-housing when fed a mushy rat chow diet was stressful. Albeit the individually-housed rats in my study were fed a diet that promoted weight gain, it was the combination of the condensed milk diet and individual-housing that acted as a double stressor and their combined effect is evident in that the body weight response of this group of rats was the most severe during chronic restraint stress (Figure 3.2). Therefore, in comparison to pair-housed rats fed the mushy rat chow diet, both the condensed milk diet and the combination of the condensed milk diet and individual-housing are stressful and although both result in a reduced gain in body weight in response to a subsequent stressor, a change in diet alone appeared to be not as stressful as a change in both diet and housing.

In terms of models of diet-induced obesity, I would like to argue that the type of housing is extremely important in these models. According to my findings, I would strongly recommend that rats should be pair-housed, as they gain substantially more weight than individually-housed rats fed the condensed milk diet. Furthermore, the condensed milk diet was not able to

produce significant diet-induced obesity in individually-housed rats, which is in contrast to that which has been reported.

Apart from a reduced gain in body weight or loss of body weight, chronic stress is also characterized by changes in HPA axis activity and glucose and fat metabolism. Perhaps the differences in body weight that occurred during chronic restraint stress could be further explained by the serum concentrations of corticosterone, insulin, glucose and leptin.

To recap, body weight measurements were used above to predict the stressfulness of chronic restraint stress. Seeing that only the pair-housed group consuming the mushy rat chow diet gained significant weight during restraint, I would propose that chronic restraint stress is least stressful for these rats, but is more stressful for the other three groups, as indicated by their stunted growth.

Following on from this, one would expect corticosterone levels to be the lowest in the pair-housed group consuming the mushy rat chow diet, with higher levels occurring in the other three experimental groups, but this was not the case, as similar corticosterone levels were observed across all four groups at the end of restraint.

Similar corticosterone measurements may be indicative of adaptation to the restraint stress protocol, which could possibly indicate that restraint

stress was too long. In support of this theory of adaptation, two other studies have shown that although corticosterone levels increase initially in restrained rats, values were similar to control rats when measured at the end of restraint. Thus, it seems that these rats were adapting to repeated exposure to restraint stress (Harris *et al*. 2002, Zardooz *et al*. 2006).

In yet another study, plasma corticosterone concentration was determined at the end of restraint and was found to be similar to unrestrained rats (Baldwin *et al.* 1997). This possibly hints again to adaptation. In many instances, regardless of housing prior to or during restraint, rats adapt/habituate to chronic restraint paradigms quite easily depending on the duration and frequency of the restraint periods, the intensity of the restraint protocol as well as a number of other factors (Glavin *et al*. 1994, Paré and Glavin, 1986, Servatius *et al*. 2000). Although the corticosterone concentrations are similar, they are particularly high compared to normal levels.

Considering that rats are nocturnal and corticosterone release follows a diurnal pattern, the highest serum concentration occurs in the late afternoon and the lowest concentrations in the early morning (Retana-Márquez *et al.* 2003). In male Wistar rats, corticosterone concentrations can range from negligible amounts (in early morning) to ± 330 ng/ml (late afternoon) over 24 hours. Blood sampling in this study occurred at approximately noon. This time would correspond to when corticosterone

values be on the rise (between 0 - 330 ng/ml). The levels in this study were relatively high compared to values at a similar time of day. As mentioned previously, chronic stress can result in significantly elevated circulating glucocorticoids and dysregulation of the HPA axis (Dallman *et al.* 2004). Therefore it is possible that that housing, diet and chronic restraint stress (chronic stress) have resulted in significant changes to the regulation of corticosterone release.

Based on the elevated corticosterone levels, it may be concluded that all groups of rats were stressed, but this does not tie in with the observed body weight differences. A possible explanation for the raised glucocorticoid levels could be as a result of the anesthesia and cardiac puncture, which are known to be very stressful. Furthermore, it has been observed that a challenge of a novel stressor, in this case anesthesia, following exposure to chronic stress elicits a greater HPA axis response than a familiar stressor (Bhatnagar *et al.* 2006, Marin *et al.* 2007, Mizoguchi *et al.* 2001).

Therefore, corticosterone concentrations were unable to explain the body weight findings. However, similar levels could hint at adaptation to chronic stress, while similar elevated levels could be due to the effects of a novel stressor (anesthesia) following chronic stress.

Serum concentrations of insulin and glucose were not significantly different between the four groups at the end of restraint. However, in comparison to normal values for insulin (0.5 – 2.0 ng/ml), the values obtained were on the lower end of the normal range, while glucose levels were extremely high compared to the normal range for glucose is ± 4.22 - 6.11 mmol/l. A similar result was observed in response to restraint stress, where lower plasma insulin values occurred despite an increase in plasma glucose levels (Zardooz *et al*. 2006). However, one may have anticipated that both glucose and insulin levels would be elevated in the pair-housed rats fed the condensed milk diet and in both individually-housed groups.

Both chronic stress and diet-induced obesity from consumption of the condensed milk diet often result in elevated glucose levels, hyperinsulinemia and possibly even insulin resistance (Dourmashkin *et al*. 2005, Elliott *et al.* 2004, Harrold *et al.* 2000, Triscari *et al.* 1985). However, taking into account that restraint stress can alter glucose metabolism, restraint stress may have negated the effect that diet-induced obesity had on insulin and glucose levels. It would be difficult to conclude that restraint stress altered carbohydrate metabolism in my study, as pre-restraint values and values during restraint were not determined.

In one study, although initially fasting plasma glucose increased significantly during restraint stress, concentrations returned to values prior to restraint, thus reflecting possible adaptation to restraint stress (Zardooz

et al. 2006). Therefore together with this observation, I would like to suggest that similar to corticosterone, the similar levels of insulin and glucose that occurred in my study could be attributed to adaptation to restraint stress.

Another point to consider is that high glucose levels could be due to the postprandial effect. However, this is unlikely to be the case as the levels are especially high. Like corticosterone, the increased glucose levels are more than likely due to the method of blood sampling, whereby the rats were anesthetized and then blood collected by cardiac puncture. The process of euthanasia is in itself very stressful resulting in an acute hyperglycemia. It may have been more ideal for this type of study, where stress hormone measurement and determination of blood glucose levels were required, that the rats should have been fasted and killed by decapitation.

Therefore the similar levels of glucose and insulin cannot be used to explain the differences in body weight, but may possibly indicate adaptation to the chronic stress protocol. On the other hand, elevated glucose levels may have occurred in response to euthanasia and thus may have masked the actual effect of chronic restraint stress.

Possibly leptin levels could explain the discrepancies in body weight. The normal range for leptin is 1.3 – 3.1 ng/ml. Therefore in this study, leptin

levels were high in all the groups, but especially elevated in pair-housed rats fed the condensed milk diet. Although leptin levels have not been examined in many restraint stress studies, in contrast to my study, one study found that restraint stress influenced leptin levels, such that levels remained lower in restrained rats than in unrestrained rats, even a week after the stressor had ended (Harris *et al.* 2002). Similar to the above study, reduced leptin levels are commonly reported in response to chronic stress (Sandoval and Davis, 2003).

Seeing that the results of this study are contrary to previous findings relating to chronic stress, I would like to suggest that the increased leptin levels observed in the pair-housed rats consuming the condensed milk diet may not be a function of restraint stress but rather it could be attributed to diet-induced obesity. Furthermore, the increased leptin levels that were found post-restraint, possibly indicates that diet-induced obesity has long-term effects with regards to leptin, with the maintenance of elevated levels

In diet-induced obesity, prolonged consumption of a high carbohydrate diet like the condensed milk diet favours the accrual of body fat leading to excessive weight gain. Apart from an increased body weight, hyperleptinemia is also evident in rats with diet-induced obesity caused by a high-energy diet (Harrold *et al.* 2000). Hyperleptinemia results from the rise in adiposity (Dourmashkin *et al*. 2005, Harrold *et al*. 2000).

In conclusion, the serum concentrations of corticosterone, insulin, glucose and leptin could not account for the differences in body weight observed during restraint stress in rats consuming the mushy rat chow diet and the condensed milk diet. Similar corticosterone, insulin and glucose levels may indicate adaptation to the restraint stress protocol as well as possible dysregulation of the HPA axis. However, the significantly elevated corticosterone and glucose levels may be due to the anesthesia.

In terms of leptin, the hyperleptinemic effects of diet-induced obesity were not lost when rats were exposed to chronic restraint stress. The raised leptin levels in pair-housed rats consuming the condensed milk diet appear to be related to pre-restraint weight-gain rather than weight changes during restraint. I would like to emphasize that serum concentrations were only determined at the end of the restraint procedure, so any increases that may have occurred, would have gone undetected as frequent sampling was not performed.

Lean tissue mass, mass of the fat depots (adipose tissue), organ weights and excess fluids contribute to body weight. Adipose tissue, however, is the main factor influencing body weight. Seeing that the serum concentrations were unable to explain the body weight differences in restrained rats, perhaps fat weights could shed some light on the matter.

This is the first study to examine fat weights and body fat distribution in differentially housed rats consuming different diets following chronic restraint stress.

Pair-housed rats consuming the condensed milk diet did not gain as much weight during restraint as pair-housed rats fed the mushy rat chow diet, yet their retrorenal and interscapular fat weights were greater. Possibly the increased fat weights, like leptin, may be as a result of the conditions prior to restraint i.e. diet-induced obesity. In rodent models of diet-induced obesity, there is evidence for increases in various fat weights (before any stressor is applied) (Harrold *et al.* 2000, Triscari *et al.* 1985). Furthermore, the elevated leptin levels may be as a result of the increased interscapular and retrorenal fat weights.

Raised leptin levels reduce adiposity (Jéquier, 2002), however in diet-induced obesity, hyperleptinemia and subsequent resistance to the raised leptin levels alters the effect of leptin on adiposity i.e. increased deposition of fat (Cases and Barzilai, 2000). The pair-housed rats consuming the condensed milk diet had an increased body weight before they were exposed to chronic restraint, which may have significantly altered the body fat distribution.

Even though individually housed rats showed similar body weights during restraint, there were differences in the fat weights due to consumption of different diets, with higher visceral and retrorenal fat in the rats fed the condensed milk diet. The consumption of a high-energy diet favours the deposition of fat in the visceral area (Laugero, 2001, Levin *et al.* 2000,

Newman *et al*. 2007). This appears to hold true only for the individually-housed rats.

Although housing and diet impacted on various fat masses, it is important to note that fat accounted for at most 2.5 % of body weight, while the differences in body weight between the groups were much greater than that. Therefore, the fat weights were unable to account for the differences in body weight that were observed. It would be recommended for future studies that lean body mass should also be determined. To do this linear growth measurements (e.g. tibial length) would be required.

Chronic stress leads to alterations in glucose and fat metabolism (Sapolsky *et al.* 2000). However, the effect of chronic restraint stress on the body fat composition has not been determined. Similar to the fat weights, it is possible that the fatty acid profiles were affected by the pre-restraint housing conditions, but this would require further investigation. This study, however, would be the first to examine fatty acid profiles under differential housing and dietary conditions following chronic restraint.

I concluded above that the individually-housed rats were more stressed than the pair-housed rats fed mushy rat chow diet, based on the differences in body weights. Nevertheless, the fatty acid profiles of the liver, fat and muscle samples from rats fed the mushy rat chow diet were not influenced by the type of housing. Considering that insulin and

corticosterone influence fat metabolism (Macfarlane *et al.* 2008) and these were found to be similar at end of restraint, this could explain the similar fatty acid profiles.

Furthermore, although the body weights of rats consuming the condensed milk diet were not significantly different from each other at the end of restraint, the individually-housed rats were more stressed than the pair-housed rats. The type of housing resulted in distinctive fatty acid profiles for the liver and subcutaneous fat, while similar fatty acid profiles occurred in the visceral fat, retrorenal fat, interscapular fat and muscle regardless of housing. Based on this observation, when rats are fed the condensed milk diet, it appears that the liver and subcutaneous fat are far more prone to the effects of a stressor, while the integrities of the other sites are maintained or perhaps they adapt far quicker to challenges.

From the fatty acid profiles of the visceral fat, retrorenal fat, interscapular fat and muscle, only SFAs and MUFAs occur, with lower levels of SFAs and higher levels of MUFAs in these sites. This profile is constant across all groups and therefore is not significantly affected by housing, diet and chronic restraint stress.

However, the type of diet affects the amount of fatty acids deposited. This is evident in the visceral fat, interscapular fat and muscle, where the condensed milk diet-fed rats have more SFAs and less MUFAs than the

rats fed the mushy rat chow diet. Although not significant, a similar trend occurs in the visceral fat and retrorenal fat.

As mentioned previously, the fatty acid composition of the diet influences the fatty acid composition of the fat stores (Lin *et al.* 1993). Therefore, the condensed milk diet is responsible for more SFAs and less MUFAs being deposited in these sites.

SFAs and MUFAs, if not used immediately for energy are stored in adipose tissue for later use. They are incorporated in the cell membrane and thus serve a structural purpose (Hollenberg and Angel, 1963). They are not as readily mobilized as n3-PUFAs and n6-PUFAs (Connor *et al.* 1996). Seeing that n3-PUFAs and n6-PUFAs do not occur in these sites and the SFAs and MUFAs profile is stable, possibly illustrates that these sites are not metabolically active to a significant extent. Even if they were metabolically active, there appears to be maintenance of the proportion of the SFAs and MUFAs.

In all groups, the livers contain SFAs, MUFAs, n6-PUFAs and n3-PUFAs, while the subcutaneous fat contains SFAs, MUFAs and n6-PUFAs.

Thus, regardless of housing and diet, similar fatty acids were present. However, the amounts of the relative fatty acids differed depending on the diet and housing.

In rats fed the mushy rat chow diet, the type of housing did not affect the amount of these fatty acids in either the liver or the subcutaneous fat.

With a change of diet, one may have expected the fatty acid profiles to be similar regardless of housing, just like the rats fed the mushy rat chow diet. However, in rats fed the condensed milk diet, the type of housing significantly impacted on the amounts of the various fatty acids in the liver and subcutaneous fat. Seeing that the pair-housed rats fed the condensed milk diet develop diet-induced obesity and the individually-housed rats do not, this finding could be explained as diet-induced obesity affects fat metabolism and therefore will influence the quantity of fatty acids deposited. Consumption of the mushy rat chow diet on the other hand appears to make it easier to adapt to challenges.

In terms of the effect of diet on the liver, the only effect that appears to be similar between the individually-housed and pair-housed groups is that the n6-PUFAs are lower in rats fed the condensed milk diet. Otherwise, diet had various effects on the other fatty acids present.

In the subcutaneous fat, both the individually- and pair-housed groups, rats fed the condensed milk diet had increased SFAs and reduced n6-PUFAs compared to rats consuming the mushy rat chow diet and similarly housed.

Like the visceral fat, interscapular fat and muscle, the subcutaneous fat was also influenced by the type of diet, where the condensed milk diet was

responsible for producing a particular fatty acid profile which was different to the profile of rats fed the mushy rat chow diet. Even though the amounts were different, the effect was the same. Thus, whether diet or housing were changed, each of the fatty acid profiles of the fat storage sites appear to impacted in a similar way. The liver, in contrast, is not a principal storage site for fatty acids; it is primarily involved in the trafficking of fatty acids. Therefore, it is more metabolically active and is more susceptible to vast changes in its fatty acid profile.

The liver fatty acid profiles in the rats fed the normal diet were not affected by the type of housing, however, substantial changes occurred when either the diet was changed or the diet and housing were altered.

Polyunsaturated fatty acids are essential fatty acids and can only be obtained from the diet. From the fatty acid profiles of the liver and subcutaneous fat, it is evident that both diets were higher in n6-PUFAs than n3-PUFAs, thus the ratio of n6:n3 was elevated. Although rats fed the condensed milk diet had lower amounts of n6-PUFAs in their livers and subcutaneous fat than rats fed the mushy rat chow diet, the ratio of n6:n3 was still elevated.

The ratio of n6-PUFAs to n3-PUFAs is of important consequence. Both n6-PUFAs and n3-PUFAs are precursors to eicosanoids, which are signaling molecules in the body. The eicosanoids derived from n6-PUFAs are potent and are known to cause inflammation, while eicosanoids derived from n3-PUFAs are anti-inflammatory. The higher the n6 to n3 ratio, the greater the

chance of developing chronic diseases such as cardiovascular problems, autoimmune diseases and inflammatory disorders (Igarashi *et al.* 2009).

In summary, changes in diet and housing did not influence the types of fatty acids that occurred in the various samples. The fatty acid profiles of the samples of adipose tissues examined were sensitive to changes in diet, where the amounts of the various fatty acids in the samples varied depending on the diet consumed. This finding supports previous observations that diet influences the fatty acid composition of adipose tissue (Lin *et al.* 1993). Despite differences in the actual amounts of fatty acids present, by changing the diet consistent effects were observed in some of the samples. Liver as well as muscle fatty acid profiles were also affected by the type of diet consumed, where the muscle fatty acid profile was similar to some of the other adipose tissue sites. The type of housing also altered fatty acid profiles but only in rats consuming the condensed milk diet, where differences occurred in the fatty acid profiles of the liver and subcutaneous fat only. A possible interpretation of this is that the condensed milk diet alters the way in which the liver and subcutaneous fat deal with stress.

CHAPTER FIVE

Conclusion

5. Conclusion

In this study, male Wistar rats were housed either individually or in pairs for 12 weeks while being fed either a mushy rat chow diet or a condensed milk diet to determine the impact of housing and diet on body weight. Rats were then exposed to chronic restraint stress for seven hours a day for four weeks to evaluate the impact of housing, diet and chronic stress exposure on body weight and measures of glucose and fat metabolism including; serum concentrations of corticosterone, insulin, glucose and leptin, body fat weights and body fat composition. This study was also done to determine if prolonged housing and/or dietary conditions influence the rats' ability to cope with a subsequent stressor i.e. chronic restraint stress.

From my study, it is evident that housing and dietary conditions can interact to create a confusing picture. An important outcome from this study is the impact that housing and diet can have just on body weights before any other stressor is applied. In addition, the type of housing i.e. pair-housing significantly impacts on the obesity that is the produced when rats are fed the condensed milk diet, which has implications for models of diet-induced obesity. Therefore, future studies that manipulate diets must indicate the type of housing and vice versa, as well as for any other measurements and any other variables that changed due to either factor.

I analysed my results according to the following premise that animals that are less stressed will respond well to a new stressor. Based on this premise, I would have assumed for example that the pair-housed rats consuming the condensed milk diet would have responded better to chronic restraint stress. However, they did not respond well and this lead me to believe that they were initially stressed, despite their significant gain in weight prior to restraint stress. In animal models of stress, a good gain in weight is used as indication that animal is less stressed, however I posit that weight gain in and of itself is not a good indication that an animal is not stressed. Therefore I have concluded that the condensed milk diet induced stress. Furthermore, based on the same premise described above, I propose that individual-housing and the combination of individual-housing and the condensed milk diet are also sources of stress.

Serum concentrations of corticosterone, insulin, glucose and leptin were unable to account for the differences in body weight during restraint stress. Similar corticosterone, insulin and glucose levels may indicate adaptation to the restraint stress protocol and perhaps dysregulation of the HPA axis. However, the significantly elevated corticosterone and glucose levels may have been due to the anesthesia. In terms of leptin, the raised leptin levels in pair-housed rats consuming the condensed milk diet appear to be related to pre-restraint weight-gain rather than weight changes during restraint. Thus the effects of leptin are long-lasting. Future studies need to include frequent sampling of the serum concentrations of corticosterone,

insulin, glucose and leptin during restraint stress which would possibly indicate a) if at any point restraint stress was stressful and when and if adaptation occurred and b) the effect of restraint stress on glucose and fat metabolism.

Regarding body fat weights, seeing that fat weights constituted a small percentage of body weights and therefore could not account for the greater differences in body weights, it would be recommended that linear growth measurements should be made in future studies.

For body fat composition, housing and diet did not affect the types of fatty acids present i.e. saturated fatty acids, monounsaturated fatty acids and polyunsaturated fatty acids, in each of the samples. Housing did not result in differences in fatty acid profiles in rats consuming the mushy rat chow diet. However, the type of housing was only relevant in rats fed the condensed milk diet, where only the fatty acid profiles of the liver and subcutaneous fat were significantly affected. Consumption of the condensed milk diet influenced the quantities of fatty acids present in the various samples examined. In addition, whether rats consumed the mushy rat chow diet or the condensed milk diet the adipose tissue sites and muscle appear to be affected in a similar way.

Future work needs to include measurements of food intake and activity of rats during the housing and diet regimen, which may then explain differences in body weight, as body weight measurements should not be taken in isolation. However, food intake may be difficult to obtain in pair-

housed rats, as one cannot know with certainty how much each rat would be eating. To overcome this issue, the cage should be considered as the sample unit and not the number of animals in the unit. Therefore the average food intake of the animals in the cage should be determined and the value used as a single data point. This could not be done in this study due to space constraints. In addition, hormone profiles, body fat weights and body fat composition need to be determined, in order to assess the effect of the changes in housing and diet alone. In turn, this will determine if individual-housing and/or the condensed milk diet is actually stressful in itself as well as to what extent restraint stress is responsible for any differences that were observed in the variables measured.

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APPENDIX 1

a. Fatty acid analysis

Fatty acid analysis is divided into three steps namely; extraction, purification and determination.

Extraction: Between 5 g and 20 g of sample was weighed out (Precisa Instruments AG, Switzerland. Max=310.000 g), depending on the expected lipid concentration. Fat from the samples was extracted overnight at 4°C in 250 ml flat-bottomed flasks (Schott Duran, Germany) with 50 ml of chloroform:methanol (2:1) (both reagents, Merck Chemicals, South Africa). The following day, the resultant extraction was filtered through filter paper (Albet 502, EEC) into a 250 ml separating funnel (Pyrex, USA). The solid components that remained on the filter paper were left to dry, following which they were reweighed to calculate the lipid dry weight. 0.9% saline (Sabax Pour Saline™ 0.9%, Adcock Ingram, South Africa) was added (30 ml) into the separating funnels. The solution was mixed and left overnight at 4°C. The following day, the bottom phase was removed into a round-bottomed flask (Schott Duran, Germany) and reduced to dryness under a vacuum (Büchi Laboratoriums Technik AG, Switzerland) at 37°C (water bath, Charles Hearson & Company Ltd, England). The extract was then made up to 20 ml with chloroform (Merck Chemicals, South Africa) in a 20 ml glass vial (Packard, USA) and stored at -20°C.

Purification: For lipid dry weight determination, 1 ml of extract was placed into a dried, pre-weighed vial (PerkinElmer, USA) and redried at 50°C for

30 minutes. The vial was cooled and reweighed. Fatty acid methyl esters (FAME) were prepared by placing a volume of extract, to give between 10-20 mg of lipid, into a clean vial. Petroleum ether (distillation range 40°-60°C, Merck Chemicals, South Africa) was added (100 µl) to the vial followed by 1 ml of 10% acetylchloride (AcetylCl) (Sigma-Aldrich Chemicals, Germany) in methanol (Merck Chemicals, South Africa). The vial was closed and left overnight at 50°C. The following day, the vial was cooled and 2 ml of 5% sodium chloride (NaCl) (Merck Chemicals, South Africa) was added to the vial, followed by 5 ml petroleum ether. The vial was closed, vortexed (Vortex Mixer VM18, Chiltern Scientific Enterprises, UK) for 10-15 seconds and 4 ml of the top phase was removed with a micropipette into a further vial. A further 5 ml of petroleum ether was added into the original vial and a second 4 ml removed resulting in a total extract of 8 ml.

Determination: The vial content was dried under a gas stream (GAST Manufacturing Corporation, Michigan, USA) and made up to 200 µl with petroleum ether and 1 µl of the resulting fatty acid methyl solution was injected (injector temperature -220°C) into the gas chromatograph (GC) column (Varian 3400 Gas Chromatograph with SP4270 Integrator, FID Detector (220°C) and 10% SP-2330 column on 100/120 Chromosorb WAW, 195°C. Helium-carrier gas). The percentage of the fatty acid methyl esters present in the samples was determined.

b. Analysis of serum corticosterone concentration

Serum corticosterone concentration was determined using ImmuChem™ Double Antibody Corticosterone ^{125}I RIA Kit (MP Biomedicals, LLC, New York, USA). Glass tubes were used for the assay. Each step of the assay procedure was performed in duplicate to ensure accuracy of measurement.

Rat serum was diluted with steroid diluent (MP Biomedicals, LLC, New York, USA), by taking 10 µl of sample to 2 ml of steroid diluent (1:200). Steroid diluent was added into tubes labelled “non specific binding” (0.3 ml) and “blank” tubes (0.1 ml). Of the sample tubes seven contained 0.1 ml of a corticosterone calibrator of known corticosterone concentration ranging from 25 ng/ml to 1000 ng/ml and two tubes contained 0.1 ml of reconstituted quality control solutions. The remainder of the sample tubes contained 0.1 ml of serum with unknown corticosterone concentration. Corticosterone ^{125}I (0.2 ml) was added to all tubes. A rat-specific corticosterone antibody (0.2 ml) was added to all the tubes except the tubes labelled “non specific binding”. All assay tubes were vortexed (Heidolph, Germany) and allowed to incubate at room temperature (22°-25°C) for two hours. After incubation, 0.5 ml of precipitant solution was added to all the tubes after which all the tubes were vortexed thoroughly and centrifuged (Sorvall® Instruments, Dupont, USA) at a speed of 1167xg for 15 minutes at 4°C. The supernatant from all the tubes was decanted and the rims of the test tubes were blotted with absorbent paper. The precipitate from each tube was counted in a gamma counter for one

minute. The concentration (ng/ml) of corticosterone in the serum samples was calculated using a calibration curve derived from the samples with known corticosterone concentrations.

c. Analysis of serum insulin concentration

The levels of serum insulin were determined using a radioimmunoassay kit specific for rat insulin (Linco Research, Missouri, USA). In all the assay procedures we used Nunc-Immuno™ tubes (Nunc Intermed, Denmark). Each step was performed in duplicate to ensure accuracy of measurement.

Briefly, assay buffer was pipetted into tubes labelled for “non-specific binding” (200 µl) and reference/total binding tubes (100 µl). Of the sample tubes, seven tubes contained 100 µl of standard insulin solutions (purified rat insulin in insulin standard buffer) with concentrations ranging from 0.1 ng/ml to 10.0 ng/ml and two tubes contained 100 µl of quality control solutions (purified rat insulin in assay buffer). The remainder of the sample tubes contained 100 µl of serum. Hydrated ^{125}I-Insulin tracer (100µl) was then pipetted into all tubes, including two tubes labelled “total counts”. A rat insulin antibody (100 µl) was pipetted into all tubes except the tubes labelled “total counts” and “non-specific binding”. All the tubes were vortexed for 30 seconds each, using a single tube vortex (Heidolph, Germany). The tubes were covered and allowed to incubate overnight at 4°C.

The next morning 1ml of cold (4°C) precipitating reagent was added to all the tubes except the tubes labelled “total counts”. All the tubes were vortexed and allowed to incubate at 4°C for 20 minutes. All the tubes except the tubes labelled “total counts” were then centrifuged (Sorvall® Instruments, Dupont, USA) at 4°C for 20 minutes at a speed of approximately 4005 rpm (2000-3000xg). Immediately thereafter the supernatant of all the tubes, except the tubes labelled total counts were decanted. Each tube was drained for at least 20 seconds and excess liquid was blotted from the lip of the tubes. The contents of all the tubes were counted in a gamma counter for one minute. The concentration (ng/ml) of rat insulin in the unknown samples was then calculated from the calibration curve derived from the samples with known insulin concentrations.

d. Analysis of serum glucose concentration

To determine the quantitative levels of serum glucose, the Reflotron® (Roche Diagnostics Ltd, UK) was used.

Using the Reflotron® Pipette (Roche Diagnostics Ltd, UK), 30 µl of rat serum was drawn into the pipette and applied to the centre of the application zone on the Reflotron® test strip (Roche Diagnostics Ltd, UK). The test strip was then placed on to the guide within 15 seconds and was slid forward horizontally until it locked into place.

The glucose concentration was calculated automatically from the readings taken using a function and conversion factors that were entered into the

instrument via the magnetic strip on the underside of each test strip. The glucose concentration was displayed in mmol/l. The test strip was removed from the Reflotron® and disposed of.

Test principle: Following application to the test strip, the sample is absorbed into the reaction zone. D-glucose is oxidised to δ-D-gluconolactone by atmospheric oxygen in the presence of glucose oxidase. The resulting hydrogen peroxide oxidises an indicator in the presence of peroxidase. The amount of dye formed in the above manner is proportional to the glucose concentration of the sample.

e. Analysis of serum leptin concentration

Serum leptin concentrations were analysed using a radioimmunoassay kit specific for determining rat serum leptin levels (Linco Research, Missouri, USA). In all the assay procedures Nunc-Immuno™ tubes (Nunc Intermed, Denmark) were used. Each step of the assay procedure was performed in duplicate to ensure accuracy of measurement.

Assay buffer was pipetted into tubes labelled for “non-specific binding” (300 µl), reference/total binding tubes (200 µl) and all sample tubes (100 µl). Of the sample tubes, seven tubes contained 100 µl of standard leptin solutions (purified recombinant rat leptin in assay buffer) with concentrations ranging from 0.5 ng/ml to 50.0 ng/ml and two tubes contained 100 µl of quality control solutions (purified recombinant rat leptin in assay buffer). The remainder of the sample tubes contained 100 µl of

serum sample. A rat leptin antibody (100 µl) was pipetted into all the tubes except the tubes labelled “total counts” and “non-specific binding”. All the tubes were vortexed for 30 seconds each, using a single tube vortex (Heidolph, Germany). The tubes were covered and allowed to incubate overnight at room temperature.

The next morning the ^{125}I-Rat Leptin was prepared by hydrating the entire contents with label hydrating buffer. The solution was left at room temperature for 30 minutes, with occasional gentle mixing. The prepared ^{125}I-Rat Leptin (100 µl) was added to all the tubes. All tubes were vortexed and covered and allowed to incubate overnight at room temperature.

The following morning 1.0 ml of cold (4°C) precipitating reagent was added to all tubes except tubes labelled total count. All the tubes were vortexed and allowed to incubate at 4°C for 20 minutes. All the tubes were then centrifuged (Sorvall® Instruments, Dupont, USA) at 4°C for 20 minutes at a speed of approximately 4005 rpm (2000-3000xg) and immediately thereafter the supernatant of all tubes was decanted, except the tubes labelled total counts. Each tube was drained for at least 20 seconds and excess liquid was blotted from the lip of the tubes. The content of all the tubes was counted in a gamma counter for one minute. The concentration (ng/ml) of rat leptin in the unknown samples was calculated from the calibration curve derived from the samples with known leptin concentrations.