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Evidence for cardiovascular autonomic dysfunction in foetal programming of hypertension following a maternal high fat or high sucrose diet

by

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DECLARATION

This thesis is of my own composition and to the best of my knowledge, it contains no material previously published or written by another person, except where due reference is made. Surgeries, staining and sectioning were performed with the supervision and assistance of Dr. Jaimie Polson.

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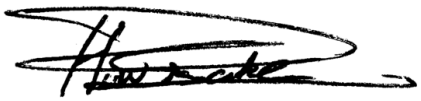

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ABBREVIATIONS

ACTH	adrenocorticotropic hormone
ANS	autonomic nervous system
Ang-II	Angiotensin-II
BP	blood pressure
BPM	beats per minute
BRS	baroreceptor sensitivity
CO	cardiac output
CRH	corticotropin releasing hormone
DBP	diastolic blood pressure
DMH	dorsomedial hypothalamus
HFD	high fat
HF	high frequency
HPA	hypothalamic-pituitary-adrenal
HR	heart rate
HRV	heart rate variability
IUGR	intrauterine growth restriction
LF	low frequency
min	minutes
mmHg	millimeter of mercury
mt	mammillothalamic tract
MAP	mean arterial pressure
n	number of replicates
p	probability
PeF	perifornical area
PI	pulse interval
PP	pulse pressure
PVN	paraventricular hypothalamic nucleus
RAAS	renin-angiotensin aldosterone system
SBP	systolic blood pressure
sBRS	spontaneous baroreflex sensitivity
SNA	sympathetic nerve activity
SV	stroke volume
TPR	total peripheral resistance

ABSTRACT

The increasing prevalence of obesity in the community has major implications for cardiovascular disease. One of the major sequelae of obesity is hypertension; data suggest that obesity is implicated as a contributory factor in 60 to 70% of cases of essential hypertension. It is thought that a maternal high fat (HF) diet, high sucrose (HSU) diet or obesity during pregnancy may cause adverse changes to the foetus during development and predispose the offspring to develop obesity and/or hypertension. However to date there is little understanding of exactly which facets of a HF diet, HSU diet or obesity are responsible for these programming effects in the offspring. In particular, there is uncertainty as to whether established obesity or simply a maternal diet high in fat or sucrose during pregnancy is the stimulus for programming. Furthermore, the pathophysiological mechanisms underlying the development of maternal high fat-programmed hypertension are not clear. It is thought that dysregulation of the autonomic nervous system (ANS) may be pivotal in the development of obesity related, HSU or high fat –related hypertension. Therefore in this study we tested the effects of a maternal HF diet and HSU diet without development of obesity, on cardiovascular and autonomic function during rest and in response to physiological and psychological stressors.

Rat dams were placed on either a HF (34% fat) or HSU (10% w/v) diet for one month before mating, until parturition. Control dams were fed a standard chow (4.8%) fat, and protein levels were maintained at 21% in all groups. At birth, litters were reduced to 8 pups to control for postnatal nutrition. At six months of age, the offspring were implanted with a telemetry blood pressure (BP) transmitter in the abdominal aorta. After a recovery period of two weeks, BP waveform was recorded (5min/hr, 24hr/day) over a 9-day protocol. This protocol comprised of three days baseline, three days of water deprivation and three days of recovery. Following a three-week recovery period from the 9-day dehydration protocol, the rats were subjected to an air jet stress (psychological stressor). The air jet protocol comprised of a series of air puffs (pressurised air) directed to the head of the rat. Various cardiovascular (systolic blood pressure (SBP), diastolic blood pressure (DPB) and heart rate (HR)), and autonomic (spontaneous baroreflex sensitivity (sBRS), baroreflex effectiveness index (BEI), dP/dt_{max}, heart rate variability (HRV) and blood pressure variability (BPV)) indices were determined. Comparisons between high fat, high sucrose

and control offspring's were made at rest and in response to the psychological (air jet stress) and the physiological (dehydration) stressor.

Our data showed that a maternal high fat diet during pregnancy results in hypertension at rest, demonstrating that the risk of developing hypertension is increased in these offspring when compared to offspring from mothers given a control diet. In response to a psychological or physiological stressor, all animals showed an increase in cardiovascular variables. However despite the high fat rats having a higher blood pressure to start, the magnitude of increase in cardiovascular variables were similar between groups in both male and female rats. There were no obvious differences in autonomic responses to either air jet stress or dehydration between high fat and control animals, indicating that any increased susceptibility to hypertension in high fat programmed rats is unlikely to be due to heightened sympathetic responses to stress. Further studies are necessary to elucidate the pathophysiological mechanisms that underlie hypertension in our model of high fat programming.

In our second model, a maternal high sucrose diet failed to program hypertension in the offspring at rest or in response to either a psychological or physiological stressor. There were also no differences in autonomic function between groups at rest or in response to a psychological or physiological stressor. Therefore this model failed to detect any detrimental effects of a maternal high sucrose intake on the cardiovascular or autonomic function in the offspring.

Our results therefore indicate that a maternal diet high in fat during pregnancy, even in the absence of developed obesity, increases the risk of hypertension in adult offspring, although autonomic control of blood pressure does not appear to be significantly compromised. In contrast, a diet high in sugar during pregnancy does not appear to have a major effect on blood pressure or blood pressure control in the offspring.

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CHAPTER 1: INTRODUCTION

1.1 OVERVIEW

Hypertension, a disease of blood pressure control, is a major risk factor for potentially fatal cardiovascular and cerebrovascular disease. Due its high prevalence, it is one of the leading causes of death worldwide (WHO, 2012), and a major public health concern. Despite decades of research, the underlying causes of hypertension remain unclear. A major consequence of this is a failure of the treatment strategies currently used in the management of hypertension, whereby approximately half of all patients on anti-hypertensive medications fail to adequately control their blood pressure (Ausdiab report, 2012).

While the etiology of hypertension is yet to be elucidated, the major risk factors are becoming increasingly evident. Traditionally, these risk factors are divided into environmental and genetic, while more recently the importance of epigenetic risk factors has come to light (Chmurzynsk, 2010; Reik *et al.,* 2001). It is likely that complex interactions between these factors result in hypertension. The concept of developmentally programmed hypertension encompasses all of these risk factors. Programming in this context, is a process in which intrauterine factors permanently affect the developing foetus, including changes in gene expression, in a manner that predisposes it to develop disease as an adult.

Epidemiological studies indicate that a significant proportion of cases of primary hypertension may have a programmed or developmental origin (Barker *et al.,* 1986; Woods *et al.,* 2004). However, the *in-utero* insults that program a hypertensive phenotype are not fully understood and appear varied. The two most well studied animal models of programmed hypertension involve maternal undernutrition (including low calorie and low protein diet) and raised foetal exposure to glucocorticoids (Pladys *et al.,* 2004; Vehaskari *et al.,* 2001; Woods *et al.,* 2001; Igosheva *et al.,* 2004; Celsi *et al.,* 1998; Ortiz *et al.,* 2001). Many reports indicate that raised levels of glucocorticoids may represent a common overarching causative factor in many animal models of programming. For example, there is evidence that maternal undernutrition increases the production and circulation of maternal glucocorticoids (Lesage *et al.,* 2001), as well as increasing foetal

access of maternal glucorticoids (Lesage *et al.,* 2001ref), which results in foetal glucocorticoid overexposure and altered development.

More recently, the rising severity of the worldwide obesity epidemic has led to an increasing interest in the impact of maternal overnutrition on the developing foetus and the subsequent long-term consequences (Poston, 2012). Epidemiological evidence has highlighted that adults whose mothers were obese during pregnancy have increased incidence of metabolic syndrome, including hypertension (Gamborg *et al.,* 2007) with a noteworthy report highlighting increased mortality rates from cardiovascular events and hypertension (Reynolds *et al.,* 2013). Animal studies have shown that maternal obesity and/or diets high in fat produce offspring with metabolic dysfunction, including hyperphagia, adiposity and insulin resistance (Nivoit *et al.,* 2009; Samuelsson *et al.,* 2008), impaired blood pressure regulation (Khan *et al.,* 2003; Samuelsson *et al.,* 2008; 2010), vascular problems (Khan *et al.,* 2003; 2004) and disrupted circadian rhythm (Borengasser *et al* 2014; Lemmer, 2006).

1.2 HYPERTENSION

1.2.1 A global public health concern

Hypertension refers to a disease in which arterial pressure is chronically elevated. Clinically, it is defined as a blood pressure greater than or equal to 140/90 mmHg (WHO, 2013). It is a highly prevalent disease, affecting approximately 40% of the human population (WHO, 2013). In Australia, hypertension is the greatest attributor to cardiovascular diseases such as stroke and heart attack, accounting for 42.1% of all cardiovascular disease burden (National Heart Foundation of Australia, 2012). Alarmingly, the prevalence of hypertension and the secondary complications associated with it appear to be increasing; over the past decade, age-adjusted rates of stroke have risen, while the incidence of end-stage renal disease and the prevalence of heart failure have also increased (Carretero and Oparil, 2000). This may be attributed to population growth, aging, and environmental risk factors such as obesity, stress and alcohol consumption (WHO, 2013; Hajjar *et al.,* 2006). However, a major contributor is also the inadequate control of blood pressure in the hypertensive population. It has been estimated that almost 70% of hypertensive patients in Australia (Ausdiab report 2012, National Heart Foundation of Australia, 2012), and worldwide

(Roger *et al.,* 2012) fail to adequately control their blood pressure, despite treatment with multiple anti-hypertensive medications. This outcome may be explained in part, by a poor understanding of the etiology of hypertension and therefore, sub-optimal treatment targets.

1.2.2 Current treatment strategies

The initial approach to the management and treatment of hypertension involves changing lifestyle factors, including adjustment to the individual's diet, exercise regime, alcohol consumption and tobacco use. In fact, a clinical trial on the effects of dietary patterns on blood pressure revealed that a diet rich in fruits, vegetables, low-fat dairy foods with reduced saturated and total fat, along with abstinence of alcohol and tobacco can be successful in lowering blood pressure by as much as 11 mmHg in hypertensive individuals (Appel *et al.,* 1997). However, due to poor patient compliance to strict diets, and the complex, multi-factorial nature of hypertension, a pharmaceutical approach is often necessary to reduce blood pressure in hypertensive people.

Current anti-hypertensive medications have had limited success. In a global survey on the management of hypertension, the proportion of patients on treatment regimes achieving a blood-pressure target below 140/90 mmHg ranged from a maximum of 27% in the USA to a minimum of less than 3% in Zaire (Mancia and Grassi, 1999). Further exacerbating this problem is the occurrence of resistant hypertension, a condition that describes the presence of hypertension despite the concurrent use of three anti-hypertensive agents from different classes (Calhoun *et al.,* 2008). Rates of resistant hypertension appear to be increasing, and is currently estimated to affect approximately 30% of hypertensive patients (Hajjar and Kotchen, 2003).

Since the current approach for the treatment of hypertension involves dietary, lifestyle and the use of multiple drugs, the problem of patient adherence inevitably arises. One study evaluating patient compliance to prescribed diets reported that only 13% of the patients were able to reduce their dietary fat intake as recommended (Hamalainen *et al.,* 2000). It has also been reported that approximately 40% of patients prescribed anti-hypertensive medications will discontinue their treatments within the first year of the prescription, due to reasons such as inconvenience and intentional "drug holidays" (Speckman *et al.*, 1999). These issues may be rectified with the development of novel anti-hypertensive agents, which entail more specific targets. Of course, this can only occur once the pathophysiology of hypertension has been better elucidated.

1.3 BLOOD PRESSURE REGULATION

Blood pressure is tightly regulated around two major requirements: blood pressure must remain high enough to maintain constant perfusion of various tissues and meet metabolic demands, while remaining low enough to avoid structural damage to the tissues (Boron and Boulpaep, 2012). Such regulation is achieved by interdependent adjustments of heart rate (HR), stroke volume (SV), and the total peripheral resistance of the vasculature (TPR). The mean arterial pressure (MAP) represents the average force propelling blood flow, and is defined mathematically as the product of cardiac output (CO- the product of HRxSV) and TPR. Homeostatic mechanisms regulate MAP around an optimal set point, which is largely dependent on levels of physical activity. For example, postural changes induce changes in MAP, which are buffered by reflex mechanisms. Short-term regulation involves the baroreceptor and chemoreceptor reflexes. The most important effector mechanisms are the parasympathetic and sympathetic divisions of the autonomic nervous system (Boron and Boulpaep, 2012).

1.3.1 Role of the autonomic nervous system

The sympathetic nervous system is central to the control of blood pressure via direct adjustments to CO and TPR, and indirect adjustments to BV via changes in renal function. A neurogenic component to hypertension is becoming increasingly realised, as several lines of evidence in animal models and humans show convincingly that sympathetic over-activity plays a major role in the etiology of the disease.

Studies in the Spontaneously Hypertensive Rat (SHR) have been important in establishing the link between sympathetic over-activity and hypertension. In one study, sympathetic nerve activity and blood pressure were shown to increase rapidly as SHR's aged, and by 5 weeks of age, both blood pressure and sympathetic nerve activity were significantly higher than values observed in normotensive strains of rat (Judy *et al.,* 1976). When sympathetic ganglionic transmission was reduced via hexamethonium administration in SHRs, both sympathetic nerve activity and mean arterial pressure were reduced to a level comparable with normotensive controls (Judy *et al.,* 1976). This evidence highlights the role of elevated sympathetic activity in producing hypertension in rats genetically predisposed to hypertension. Of interest, it has also been demonstrated that

transplantation of hypothalamus, a key region of blood pressure control, from embryonic SHRs into the hypothalamus of a normotensive strains of rat induces hypertension in the recipient (Eilam *et al.,* 1991). Although a rise in sympathetic nerve activity was not confirmed in these studies, they show convincingly that alteration in the function of the hypothalamus in the SHR is important for the development of hypertension in this model. Related to this, blockade of glutamate or angiotensin II receptors in the rostral ventrolateral medulla reduces blood pressure to a much greater extent in the SHR than control rat (Sved *et al.,* 2003; Ito *et al.,* 2003) and this is believed to be due to increased excitatory drive from the hypothalamus to the rostral ventrolateral medulla in the SHR (Guyenet, 2006). These data support the hypothesis that the pathophysiology of hypertension has a strong neurogenic component.

Data from human studies provides further evidence to support a major role for autonomic dysfunction in hypertension. Firstly, children with a familial history of hypertension, but who are normotensive according to clinical definition, have been shown to have an elevated blood pressure, heart rate, and plasma noradrenaline concentrations (Lopes et al 2010). These children also exhibit decreased baroreflex sensitivity (Lopes *et al.,* 2000). This may underlie an increased risk of developing hypertension in adult life. Furthermore, there is convincing evidence from studies measuring noradrenaline spill over and from direct neural recordings in hypertensive patients that sympathetic over-activity is present in both overt hypertension and in borderline hypertension (Anderson *et al.,* 1989; Esler, 2000; 2011; Grassi *et al*. 1998; Lambert *et al.,* 2007; Smith *et al.,* 2004). Indeed, such “neurogenic hypertension” has been reported to account for at least 50% of all cases of high blood pressure (Esler *et al.* 2010). Taken together, these data indicate that sympathetic dysfunction plays a major role in the pathophysiology of hypertension.

1.3.2 Baroreceptor reflex

The arterial baroreceptor reflex is a rapid, homeostatic reflex which buffers acute fluctuations in blood pressure that occur during every day behaviors such as postural changes, stress and physical activity (Guyenet , 2006; Heusser *et al.,* 2005). Such buffering is crucial for maintaining regulation of blood flow and facilitating gas and nutrient exchange with the tissues. Changes in blood pressure are detected by specialized mechanoreceptors known as baroreceptors located in the carotid sinus and aortic arch. The reflex acts via adjustments to the level of sympathetic and

parasympathetic nerve activity in response to signals from the baroreceptors. For example, when blood pressure rises, vascular distension is sensed by the baroreceptors and the resulting baroreceptor afferent fibre activation triggers reflex parasympathetic activation and sympathetic inhibition, resulting in a reduction in cardiac output and vascular resistance, buffering the increase in blood pressure.

Although clearly of major importance in the short term regulation of blood pressure, the role of the baroreflex in the long term regulation of blood pressure remains controversial. The consensus view is that the baroreceptors only play a role in the short-term regulation of blood pressure. This stems from the work of Cowley in the 1970's, who showed that denervation of the baroreceptors increased blood pressure lability but did not affect mean blood pressure over a period of days (Cowley, 1992). This is believed to be due to the re-setting of the baroreceptors to a newly established blood pressure over a period of hours to days (Guyenet, 2006; Cowley, 1992). However, there is evidence that the baroreceptors can exert long-term control of blood pressure. In dogs, unloading of the intact baroreceptors produces a sustained rise in blood pressure over a period of five weeks (Thrasher, 2004). The difference in these results to those of Cowley's may be due to neural remodeling effects following denervation (Schreihofer and Sved, 1992). In addition, in the rabbit, an increase in blood pressure from infusions of angiotensin II produces a sustained fall in renal sympathetic nerve activity over a period of 7 days (Barrett *et al.,* 2003), while chronic stimulation of the carotid baroreceptors reduces blood pressure for at least 7 days (Lohmeier and Iliescu, 2011). Thus, there is evidence that the baroreceptors are capable of exerting a long-term effect on blood pressure, at least in the medium term. However, the question arises as to whether inhibition of the baroreceptors, or a decrease in baroreceptor reflex sensitivity may play a role in the etiology of hypertension.

1.3.3 Baroreflex function in hypertension

Hypertensive individuals have often been shown to have normal, or frequently, an elevated heart rate (Heusser *et al.,* 2005). This suggests that the baroreflex has either adapted to a higher blood pressure and has reset to operate around a higher set point, or reflects a primary dysfunction (Bristow *et al.,* 1969). One study calculated baroreflex sensitivity in hypertensive individuals via sudden intravenous injections of angiotensin and phenylephrine (to increase blood pressure)

(Bristow *et al.,* 1969). The corresponding reflex changes in heart rate following these injections were determined and plotted against blood pressure. This revealed diminished baroreflex sensitivity in hypertensive patients compared to normotensive individuals, such that higher blood pressures were required to produce corresponding changes in heart rate in hypertensive patients (Bristow *et al.,* 1969). This suggests that baroreflex dysfunction, such as a reduced sensitivity of the reflex, may play a permissive role in hypertension.

However, some recent evidence suggests that the baroreflex plays a more central role in hypertension. A study by Heusser *et al.,* 2005 showed that two previously normotensive patients that presented with neck trauma and subsequent damage to carotid arteries became severely hypertensive after the trauma. Compensatory baroreflex-mediated changes in heart rate were severely reduced in these patients, most likely attributed to the loss of function of baroreceptors in the carotid sinus. Thus, since damage to the baroreceptors may result in extreme, chronic hypertension, it is likely that the reflex plays a crucial role in producing a hypertensive phenotype.

1.3.4 Chemoreceptor reflex

Another short-term feedback system aimed at regulating blood pressure is the chemoreceptor reflex. Chemoreceptors are specialized receptors in the carotid and aortic bodies, which respond primarily to decreases in the partial pressure of oxygen (Dampney *et al.,* 2001). The arterial chemoreceptor reflex serves an important regulatory role in the control of alveolar ventilation, but also exert a powerful influence on cardiovascular function and blood pressure control directly (by interacting with medullary vasomotor centers) or indirectly (via altered pulmonary stretch receptor activity) (Schultz, 2007). Stimulation of the chemoreceptors reflex evokes increases in the rate of respiration, and sympathetically mediated vasoconstriction. Both of these mechanisms are aimed at increasing and conserving oxygen, however, the increase in sympathetic vasoconstriction results in an increase in blood pressure (Dampney *et al,* 2001).

1.4 RISK FACTORS FOR HYPERTENSION

Over 95% of all cases of hypertension are of unknown etiology (WHO, 2013) and termed essential or primary hypertension. Essential hypertension will be the sole focus of this thesis. Although the underlying causes of hypertension are yet to be elucidated, there are known risk factors. These risk factors can be classified into several categories: environmental, genetic, and epigenetic.

1.4.1 Environmental factors

The modification of environmental risk factors is usually the first step in the prevention and management of hypertension. Common environmental risk factors include poor diet and psychological stress. Modern diets are commonly high in fat, sugar, salt and alcohol. Salt and alcohol are independent risk factors for hypertension (Carretero and Oparil, 2000; Kotsis *et al.,* 2010) while increased consumption of fat and sugar can lead to obesity and insulin resistance. Of particular relevance to this thesis is obesity and the associated high fat and high sugar diets, which account for over 30% of the morbidity and mortality due to hypertension (WHO 2013).

The relationship between obesity and hypertension is well established in both humans (Hsueh and Buchanan, 1994; Nasser *et al.,* 1999; Faloia *et al.,* 2000) and animal models (Kamal *et al.,* 2005; Kurtz *et al.,* 1989; Dobrian *et al.,* 2001), although the mechanisms behind this relationship are still unclear. However, there is good evidence that obesity-related hypertension has a strong neurogenic component. Evidence for this hypothesis has been provided from direct, microneurographic measurements of sympathetic activity in obese patients, which reveal elevated muscle sympathetic nerve activity (Grassi *et al.,* 1995). Similarly, diets high in fat have been shown to result in the stimulation of peripheral α1- and β-adrenergic receptors, resulting in an increase in sympathetic activity and subsequent hypertensive responses (Rocchini *et al*., 2004; Kotsis *et al.,* 2010). The concept of sympathetic over-activity playing a central role in hypertension will be revisited later in the thesis.

In more recent studies, psychosocial stress has been highlighted as a major risk factor for hypertension. Chronic job strain, defined by high work demands, decision latitude and low reward, is a widely studied model of psychosocial stress in humans. Studies have shown that subjects who identified as having a high level of job strain are at greater risk of having sustained, elevated blood

pressure (Schwartz *et al*., 1996; Vrijkotte *et al*., 2000; Spruill, 2010). More recently, investigators reported 15.9% of a patient sample developed hypertension in response to laboratory-induced mental stressors over a 3-year follow-up period (Hamer *et al*., 2012). The notion of a relationship between a heightened cardiovascular response to stress and hypertension will be discussed later in the thesis.

1.4.2 Genetic factors

It is widely accepted that individuals differ in their susceptibility to hypertension, and that this can be attributed, in part, to various genetic determinants. For example, meta-analyses have shown that polymorphisms in the gene encoding angiotensinogen (Jeunemaitre *et al.,* 1992; Sethi *et al.,* 2003) and the epithelial sodium channel γ subunit (Hansson 1995; Young*,* 2007) are associated with an increased risk of hypertension. Furthermore, familial aggregation studies highlight that these predispositions are inheritable (Fuentes *et al.,* 2000; Fermino *et al.,* 2009). Although evidence from these studies have produced strong associations, the attributable risk of these polymorphisms has been described as minor, and only accounts for a fraction of actual occurrence (Gluckman *et al.,* 2010). Epigenetic changes have been proposed as an explanation for this 'missing heritability.

1.4.3 Epigenetic factors

The most plausible explanation for the predilections to hypertension that have been reported in the literature is that a complex interaction of both genetic and environmental factors produces the disease (Millis, 2010). Epigenetics refers to gene-environment interactions, which ultimately result in changes in gene expression. These changes are stable and long-term, and occur via DNA methylation or acetylation (Millis, 2010). If these processes occur during foetal development, the maturation of tissue types may be altered, resulting in an increased risk of developing chronic disease in adulthood (Thornburg *et al*., 2010). This concept is known as developmental programming. In the context of programmed hypertension, certain genes related to blood pressure regulation may be switched on or off via *in utero* environmental cues.

1.4.4 The Barker Hypothesis and programmed hypertension

The *in utero* environment encountered in foetal life has a profound influence on foetal development. The Barker hypothesis states that sub-optimal *in utero* conditions may permanently alter the growth and maturation of foetal tissues, changing their structure and physiological function. These changes become harmful during adulthood when the insult is no longer present, increasing the risk of certain diseases in adult life (Jones *et al.,* 2012; Langley Evans, 2006).

This concept first arose after Professor David Barker's epidemiological studies in the 1980's, which revealed that the geographical distribution of infant mortality in England and Wales closely matched the distribution of death rates from cardiovascular disease decades later (Barker and Osmond, 1986). From this observation, Barker and colleagues deduced that in regions of high infant mortality, those babies that survived appeared to be at increase risk of developing cardiovascular disease in their adult lives. They also observed that the mean birth weights in these disadvantaged regions were significantly lower, highlighting that an inverse relationship existed between birth weight and cardiovascular disease (Barker and Osmond, 1986; Barker *et al.,* 1989).

A low birth weight (<2.5 kg) can result from pre-term birth or intrauterine growth restriction (IUGR). IUGR is the failure of the foetus to reach its growth potential, due to placental abnormalities or factors related to maternal nutrition, disease and stress (Resnik, 2002). Barker and colleagues noted that disadvantaged regions had higher rates of maternal undernutrition, particularly in regards to protein and total caloric intake, as well as increased psychological stress during pregnancy. They hypothesized that these factors exerted a major influence on foetal development and were a cause of the low birth weights observed. However, it is unlikely that low birth weight itself is the underlying cause of cardiovascular disease such as hypertension decades later, but rather is a surrogate observation reflecting foetal exposure to intrauterine stressors (Davis and Sandman, 2010).

1.5 ANIMAL MODELS OF PROGRAMMED HYPERTENSION

Beginning with David Barker's theory of foetal origins of adult disease, epidemiological studies have shown that an adverse intrauterine environment may lead to adult hypertension in the offspring (Barker DJP, 1989; Denton *et al.,* 2006; Langley-Evans, 2009; Nuyt, 2008). However,

there are a number of limitations with such epidemiological studies. For example, not all epidemiological reports show an association between low birth weight and hypertension (Stanner *et al.,* 1997). Moreover, most epidemiological studies have been criticized on the basis that they do not account for all confounding variables (Huxley *et al.,* 2002), which is particularly significant in the field of programming due to the long time interval between the stimulus (during gestation) and the effect twenty to fifty years later (Langley-Evans, 2009). Finally, criticism may be applied on the basis that human epidemiological studies do not provide a cause–effect relationship. Thus, it is very hard to provide robust evidence for *in utero* programming in the human. Properly designed prospective cohort studies would require 50-60 years to assess the impact of gestational stressors on adult blood pressure and cardiovascular disease, which is impractical to say the least (Langley Evans, 2009). In an effort to overcome these issues, and develop a "proof of principle" foundation, animal models have been developed. Animal models serve two purposes; first, they eliminate the variation in genetic background and thereby other potential risk factors for hypertension. Second, animal models hold the key to unravelling the mechanisms involved in programmed hypertension due to the ability to investigate the physiology in a more invasive and more detailed manner (Vehaskari *et al.,* 2005).

Most experimental models have aimed to duplicate foetal growth impairment because original reports of prenatally programmed hypertension were linked to intrauterine growth restriction (Alexander, 2006; Denton *et al.,* 2006; Langley-Evans, 2009). Therefore prenatal manipulations can be divided into three categories; (1) Maternal under nutrition; (2) maternal glucocorticoid exposure; and, (3) obtrusion of placental function (Vehaskari *et al.,* 2005). For the purposes of this thesis we will focus on maternal diet. Most animal models have used a variety of nutritional manipulations during gestation, these include; calorie-restricted diet, protein restricted diet, cafeteria-like diet, iron and other micronutrient-restricted diets; all of which have been shown to program hypertension in rats, pigs, sheep and guinea pigs (Nuyt *et al.,* 2009).

1.5.1 Animal models of programmed hypertension using altered maternal diet

Global food restriction: Studies of programming of hypertension by maternal nutritional manipulation have been conducted since the early 1990's. Woodall and colleagues (1996) showed in rats that severe food restriction, equivalent to only 30% of their normal food intake during

pregnancy programmed the offspring for hypertension. These results have been confirmed and extended such that in rats it has been shown that hypertension is induced in offspring with a 20-30% reduction in maternal food intake during the latter part of pregnancy (Woods *et al.,* 2005). In sheep, restricting maternal dietary intake by 15% during the first half of gestation increased the mean arterial pressure in offspring by approximately 10mmHg (Hawkins *et al.,* 2000). Thus, it is evident that food restriction in the mother for all or part of gestation leads to increased blood pressure in the offspring, although the exact timing of the restriction varies, and is possibly species dependant. However, what is not clear from these experiments is if overall reduction in calories or a reduction in a specific nutrient is the important factor. One of the major nutrients that have been the focus of much attention in the field of programmed hypertension is protein.

Protein Restriction: a maternal low protein diet while maintaining a normal total calorie intake has been widely studied in the rat. The normal dietary intake of protein is approximately 19% (21% casein) of total calorie intake, with restricted levels ranging from 12% (mild) to 9% (modest) to 5% (severe) (Langley-Evans *et al.,* 1994; 1996; 1999; Pladys *et al.,* 2004; Vehaskari *et al.,* 2001; Woods *et al.,* 2001). It has been shown that arterial blood pressure in the offspring in adulthood is increased at all three levels of restriction (Langley *et al.,* 1994). In the pig, mean arterial pressure of adult offspring increased by 10-25mmHg when dams were fed a diet consisting of 1.0% protein (Bagby *et al.,* 2001). In all the models described above, a normal protein diet is given after birth, highlighting the likelihood that it is low protein during foetal development that programmes hypertension

Manipulation of other maternal dietary factors: Supplementing a maternal low protein diet with glycine protects the offspring from the development of high blood pressure (Jackson *et al.,* 2002), suggesting that the programming effects of nutrient restriction may be due to specific deficits. Glycine, in particular is important in the metabolism of methionine and homocysteine, which in turn play a crucial role in DNA methylation; thus suggesting a mechanism whereby maternal low protein diet may engender epigenetic changes (Rees, 2002). Another study by Langley-Evans *et al.,* (2000), compared the programming effects of two alternative maternal low protein diets: one with more fat and starch, the other with more sugar. Interestingly, only the low protein diet with high fat/starch produced increased systolic blood pressure in the offspring (compared to a control diet),

while low protein and high sugar failed to program for hypertension in the offspring (Langley-Evans *et al.,* 2000). Therefore differences in the specific nutrient content of a diet, and not only the protein content may be important in programming of hypertension, and can potentially lead to confounding results when interpreting the results of different studies.

Recently there has been emerging evidence that numerous other dietary manipulations may program offspring for hypertension in later life. These include both high and low sodium intake (Woods *et al.,* 2001; Battista *et al.,* 2002), water deprivation (Ross *et al.,* 2005), iron deficiency (Lisle *et al.,* 2003), and obesogenic diets, such as the high fat diet (Khan IY, 2003). The overwhelming conclusion is that the foetus may be highly susceptible to maternal dietary influences that appear to alter development in such a way as to affect blood pressure homeostasis in later life. Of these examples listed above, obesogenic diets, or diets high in fat are particularly interesting in light of the changes in diet in society, with greater intake of fat and sugars.

Evidence of maternal obesity or high fat diet-related hypertension in offspring: As described above, initial studies directed their focus on maternal/foetal under-nutrition and low birth weights. However, in light of the obesity epidemic, more attention has recently been directed towards maternal over-nutrition in programming of hypertension. There is now accumulating evidence from numerous animal studies supporting the hypothesis that maternal overweight, obesity or high fat dietary intake is associated with obesity and hypertension in the offspring (Khan *et al.,* 2003; Elahi *et al.,* 2009; White *et al.,* 2010; Samuelsson *et al.,* 2010).

There has been some controversy as to whether maternal high fat diet/obesity programmed hypertension is secondary to obesity or whether obesity and hypertension exist separately in programmed offspring. Sprague Dawley rats fed a lard rich diet 10 days prior to mating throughout pregnancy and during the suckling period produced offspring that became obese in adulthood, although only the female progeny became hypertensive (Khan *et al.,* 2003). However, recently Samuelsson *et al.,* (2010) showed that offspring from dams fed a highly palatable diet rich in saturated fat and sugar for 5 weeks prior to mating developed hypertension, had increased systolic blood pressure compared to control groups as early as at 3 months of age, hence prior to their development of obesity (Samuelsson *et al.,* 2010). Consistent with this, in mice male offspring of fat fed dams had increased systolic blood pressure without elevated body fat, however litter

matched females developed obesity and hypertension (Elahi *et al.,* 2009). Thus, it is apparent that, at least in males, maternal high fat diet programming of hypertension can occur independently of obesity, although the two frequently do occur together.

1.5.2 Programming vectors responsible for maternal obesity or maternal high fat diet-related hypertension in offspring

There are many facets of obesity (hyperleptinaemia, dyslipidaemia, hyperinsulinaemia, or hyperglycaemia) that could be responsible for programming of maternal obesity/high fat diet-related hypertension in offspring. Indeed, it is unclear as to whether a high fat/hypercaloric diet per se, or established obesity is the stimulus for programming of hypertension. The identification of vectors that cross the placenta is of significant importance as it provides a target for potential therapeutic treatment or behavioural modification.

Maternal dietary imbalance, obesity or overweight; which is more important? Studies done on Japanese macaques suggest that a maternal diet high in fat is sufficient to establish programming effects and that maternal obesity is not required. Monkeys resistant to diet induced obesity were placed on a high fat diet during pregnancy, and the foetus was found to develop a fatty liver phenotype identical to monkeys that were obese (McCurdy *et al.,* 2009). However other studies indicate a prerequisite of maternal obesity for programming; this was shown in rats fed a diet rich in fats during pregnancy and suckling, where the offspring developed hypertension only if their mothers were obese (White *et al.,* 2010). Interestingly, a high fat obesogenic maternal diet programmed offspring for hypertension at rest (Samuelsson *et al.,* 2010), while a high fat – nonobesogenic maternal diet did not produce hypertension at rest, but did evoke a hypertensive response to stress (Rudyk *et al.,* 2011). These results suggest that there may be a dose interaction between maternal high fat diet and obesity in programming the offspring phenotype, such that although hypertension may not be programmed at rest with maternal high fat diet alone, it may nevertheless increase the risk of developing hypertension when subjected to other environmental factors. Other studies done in sheep suggest that the mothers' body condition before pregnancy may have programming effects irrespective of maternal condition during pregnancy (Rattanatray *et al.,* 2010). To date there is no clear consensus as to whether maternal dietary imbalance, obesity or overweight is most likely to program hypertension in the offspring. A clearer understanding of

the differences in these factors for risk of programming is crucial to better understand the risk of programmed cardiovascular disease in society, considering that overweight and obesity is now present in 34% of the Australian obstetric population (Callaway *et al.,* 2006).

Importance of the type of fatty acid intake in pregnancy: Many studies have looked at manipulating saturated, polyunsaturated and omega-3 polyunsaturated fatty acid content during pregnancy. Of these, it appears that maternal saturated fatty acid intake is deleterious to the health of offspring, while omega-3 polyunsaturated fatty acid consumption may be beneficial for offspring health. Offspring of dams fed a diet rich in omega-3 fatty acids throughout pregnancy, suckling and 6 weeks post weaning had normal blood pressure despite the total fat content of the diet being high (10%), (Weisinger *et al.,* 2001). Furthermore, it was described by Weisinger *et al.,* (2010), that a diet low in omega-3 fatty acids programmes hypertension and increased sodium appetite; and this is thought to be due to persistent changes in fatty acid composition of the hypothalamus (Weisinger *et al.,* 2006). These data suggest that the type of fatty acid consumed during pregnancy may have profound implications for offspring heath, with a maternal diet high in saturated fatty acids rather than polyunsaturated fatty acids increasing the risk for programmed obesity or hypertension in offspring.

Is the amount of placental lipid transfer important? Lipid transfer from the mother to the foetus is vital, providing a crucial source of energy for the developing foetus. Placental lipases aid in the transportation of lipids from the maternal circulation to the foetus by liberating non-esterified fatty acids from triglycerides. These non-esterified fatty acids are then transferred to the synctiotrophoblast via fatty acid binding proteins and fatty acid translocase. Studies suggest that there is an association between excessive foetal growth rates and increased maternal plasma triglyceride and diglyceride concentration (Son *et al.,* 2010), and that this may contribute to accelerated development of atherosclerosis in offspring of hypercholesterolaemic rabbits (Napoli *et al.,* 2000). Studies done in sheep show overfeeding results in increased foetal plasma triglyceride concentration, and that this in turn causes an upregulation of fatty acid translocase expression allowing more fatty acid transport to the foetus (Zhu *et al.,* 2010). The amount of fatty acids that transport to the foetus or neonate can be quantified using a fatty acid with a stable ^{13}C isotope (Gil-

Sanchez *et al., 2010),* however to date, it is unclear as to whether the kinetics would increase due to a maternal high fat diet, obesity or overweight.

Increases in inflammation and placental cytokines: Placental cytokines are undoubtedly important for normal conception, placentation, foetal growth, parturition, and immune cell activity (Szukiewicz, 2012). Maternal obesity during pregnancy is linked to an exaggerated inflammatory response (Henry *et al.,* 2012). However, it is unclear what the effects are of excess maternal inflammation on foetal development. It is known that women who are obese and have gestational diabetes during pregnancy release higher concentrations of leptin from the placentae, however it remains unclear as to how leptin may affect the foetus (Henry *et al.,* 2012). Furthermore, macrophage recruitment to the placenta is increased during maternal obesity, which results in increased cytokine production and upregution of pro-inflammatory adipokines and cytokines such as leptin, interleukin-6 and tumour necrosis factor (Challier *et al.,* 2008). Of interest, leptin increases sympathetic activity in the adult (Haynes *et al.,* 1997), however the direct effect of leptin on autonomic function in the foetus is unclear. A gestational diet high in saturated fat produces hypertriglyceridemia and hyperleptinemia in both mothers and foetuses (Mazzucco *et al.,* 2013). Moreover, the foetus exhibit increased body weight, increased leptin expression in the placenta and foetal liver and increased accumulation of lipds in the liver (Mazzucco *et al.,* 2013). This suggests the possibility that leptin may exert significant influence over the developing foetus.

Given the information above, it is likely that maternal obesity, over weight or a maternal diet rich in fats during pregnancy may results in obesity or high-fat related hypertension in offspring. There are many potential programming vectors described above. Identifying these vectors and understanding their potential programming effects on the developing foetus is of great importance in devising therapeutic and behavioural strategies to mitigate the development of programmed cardiovascular disease.

In addition to the increased prevalence of obesity and increased consumption of diets high in fat in the obstetric population, there is also a growing trend in the consumption of beverages high in sugar. Maternal exposure to high sucrose intake may also have profound implications to offspring health in later life. A cohort study by Englund-Ogge, *et al* (2012) showed an increased risk of mortality and morbidity during preterm delivery in offspring from mothers with a high intake of

sugary drinks during pregnancy. We will now examine the literature on high sucrose intake during pregnancy and putative programming effects that may predispose the offspring to hypertension in later life.

1.5.3 Evidence of a maternal high sucrose diet in programming hypertension

Diets high in simple sugars result in a high glycemic load (GI) and are therefore a major risk factor for obesity and metabolic syndrome and it's associated sequela (Brand-Miller *et al.,* 2002). An increased GI diet during pregnancy is associated with an increased risk for gestational diabetes mellitus and maternal obesity (Zhang *et al.,* 2006). Conversely, a low GI diet during pregnancy may reduce gestational weight gain and improve glucose homeostasis in the mother (Walsh *et al.,* 2012). Despite the growing interest of a high GI diet and its role in obesity, exposure to a high sucrose diet during pregnancy and its effects *in utero* has not been extensively investigated. It is unclear as to whether a maternal high sucrose diet causes hypertension as a result of obesity and its related sequela or if hypertension is a consequence of direct influence of a maternal diet high in sucrose. A study by Metzger *et al.,* (2008) found a strong linear relationship between maternal glucose intake and increased infant adiposity, based upon direct measurement of infant skin-fold thickness. Fructose given to pregnant rats in drinking water (10% w/v) resulted in hyperglycemia in both the mother and foetus (Flynn *et al.,* 2013). Moreover, such pups are found to have hyperinsulinaemia indicative of developing insulin resistance (Vickers *et al.,* 2012). In pregnant mice, obesity induced by feeding a diet rich in both animal fat and sugars resulted in hypertension and increased fat mass in the offspring, however it was uncertain whether the programming effects were due to the high fat or high sugar (Samuelsson *et al.,* 2008). These same investigators subsequently examined the effects of a gestational high sugar diet in isolation and found that a diet high in sugar (26% of total energy) but low in fat produced hypertension in both male and female offspring from the high sucrose group at three months of age (Sammuelsson *et al.,* 2013). Furthermore, both male and female offspring showed increased low:high frequency ratio in their HRV, suggesting that the hypertension may be due to increased sympathetic tone.

In summary, high sucrose diets during pregnancy cause changes to maternal metabolic parameters that in turn affect foetal metabolic parameters and foetal development, ultimately

increasing the risk of chronic disease in later life. However, the mechanisms behind these metabolic changes and increased disease risk are not well understood.

1.6 PROGRAMMED HYPERTENSION: PLAUSIBLE MECHANISMS

1.6.1 Kidney dysfunction

Since the kidney plays an important role in the control of blood pressure by regulating water and salt excretion (Hall *et al.,* 1991), it has been the subject of considerable investigation in studies aimed at determining the etiology of programmed hypertension. Extensive studies in the rat and sheep show windows of susceptibility to blood pressure programming that appear to occur during the early stages of kidney development (Vehaskari *et al.,* 2001). A study by Zeman *et al.,* (1968) reported that rats exposed to severely protein-restricted diets during thoughout gestation had 30-45% fewer glomeruli than controls. Since then, several laboratories have confirmed this finding (Woods *et al.,* 2001, Ortiz *et al.,* 2001). Furthermore, rats born with reduced nephron numbers were shown to develop hypertension by 3 months of age (Vehaskari *et al.,* 2005).

The mechanism behind the inverse relationship between low nephron number and blood pressure is thought to arise due to a decrease in the total kidney filtration surface area. This decrease in filtration surface area may not meet the demands of a growing animal, thus resulting in sodium retention (Mackenzie *et al.,* 1996; Brenner *et al.,* 1988). Furthermore, a greater workload is placed on each nephron, resulting in glomerular and arterial hypertension and subsequent hyperfiltration. This can result in glomerular sclerosis and greater loss of nephron function (Mackenzie *et al.,* 1996; Brenner *et al.,* 1988). Other studies of programmed hypertension including uterine artery ligation, food restriction, iron deficiency, glucocorticoid exposure and low protein diet have also observed reduced nephron numbers in programmed animals (Kett *et al.,* 2004). It is therefore believed that a reduction in nephron number may play a role in prenatally programmed hypertension (Brenner *et al.,* 1998).

However, there is conflicting evidence regarding the importance of a reduction in nephron number in producing a hypertensive phenotype. Studies has shown, when given a low protein diet with a combination of dietary supplements prevented any reduction in nephron number without affecting

the development of hypertension (Langley-Evans *et al.,* 2003; Jackson *et al.,* 2002). Furthermore, studies show hypertension in the absence of reduced nephron number (da Silva *et al.,* 2003) and reduced nephron number with no apparent hypertension (Zimanyi *et al.,* 2002). Although it is physiologically plausible that abnormalities in the kidney may be of importance to the etiology of programmed hypertension, the data indicate that while nephron deficit may play a permissive role, it is not the primary cause of programmed hypertension.

Another mechanism that may cause adult hypertension via increase sodium retention is thought to be due to programming of epithelial sodium co-transporters located in the renal tubles. Sodium co-transport are located throughout the kidney from the proximal tubule to the collecting duct (Su and Menon, 2001). However, most of the sodium reabsorption occurs in the proximal tubule of the nephron while the fine control of reabsorption occurs in the collecting duct of the distal nephron (Schnermann, 2001; Schnermann, 2000). Only a few studies have looked at the direct effect of renal sodium transporter function in programming models. A study by Manning et al*.,* 2002, looking at 4 week old offspring rats from mothers fed a low protein diet during the second half of gestation reported an increase in (mRNA and protein levels) of sodium co-transporters located in the thick ascending limb (302% compared to controls) and in the distal convoluted tubule (160% compared to controls). These changes in the kidney was manifested before the onset of hypertension, therefore already the foetal kidney was programmed inappropriately to retain sodium (Manning *et al.,* 2002). Furthermore, at 8 weeks of age, sodium transporters in these rats were not down regulated with the onset of hypertension at 8 weeks of age (Manning *et al.,* 2002). These findings are of significance, as down-regulation of sodium co-transporters in the distal convoluted tubule is known to be an important part of the normal pressure natriuresis response (Wang *et al.,* 2001). In spite of the large number of programming studies on the kidney, looking at both a decrease nephron number and prenatal programming of renal sodium co-transporters, to date there is no evidence of reduced nephron number or prenatal programming of renal sodium co-transporters and hypertension reported in offspring due to a maternal obesogenic or high fat diet.

1.6.2 Autonomic dysfunction

Recent studies on humans and animal models have suggested that abnormalities in autonomic control may be pivotal in the development of obesity or high-fat related hypertension.

Measurements of noradrenaline spill over from sympathetic nerves and direct sympathetic nerve recordings show obese humans have increased sympathetic outflow to the blood vessels in skeletal muscles and increased sympathetic outflow to the kidneys (Esler *et al.,* 2006). Animal models of diet-induced obesity also show a net increase in sympathetic activity. This has been demonstrated via adrenergic blockade, which attenuates elevated blood pressure in obese rabbits and dogs (Antic *et al.,* 2000). Furthermore, a study by Samuelsson et al (2010), showed hypertension in juvenile offspring (30 days of age) and as young adults (90 days) from mothers fed an obesogenic diet during pregnancy. The increased blood pressure was accompanied by evidence of increased sympathetic activity, including an increase in the LF:HF ratio of HRV, indicative of increase sympathetic activity. Moreover, administration of α- and β-adrenergic blockers reduced blood pressure to levels comparable to control rats. Therefore, these results support the hypothesis that sympathetic overactivity may play a role in programmed hypertension.

It has been hypothesised that hormones such as leptin and insulin are responsible for the chronic stimulation of the sympathetic nervous system in obesity-related and programmed hypertension. These hormones play a crucial role in peripheral signalling involved in energy homeostasis control. Leptin is primarily produced by white adipose tissue, and is often found to be elevated in people who are obese (Elmquist *et al.,* 2004) and obverweight (Zhang *et al.* 1994). Leptin has been shown to increase blood pressure by increasing renal sympathetic nerve activity (Marsh *et al.,* 2003 and Mark *et al.,* 2009). A study by Franco *et al.,* (2012) showed that offspring from mothers fed a high fat diet during pregnancy developed hyperleptinaemia and subsequent leptin resistance. It has been shown that leptin resistance appears to affect only the appetite-inhibitory aspects of leptin signalling, while the sympatho-excitatory aspects remain unchanged. This may result in inappropriately high appetite and persistent over activation of sympathetic nervous system, thus leading to the hypertension observed (Esler *et al.,* 2008).

Baroreflex dysfunction has also been studied in animal models of programmed hypertension. For example, sheep exposed to dexamethasone (a synthetic glucocorticoid) *in utero* display altered baroreflex function prior to hypertension (Segar *et al.,* 2006). The baroreflex curve, relating changes in HR to MAP, was found to be shifted toward a higher set-point in these animals. Similarly, rats programmed by either maternal protein restriction or maternal high fat diet have

displayed altered baroreflex responses that are pro-hypertensive (Pladys *et al.*, 2004; Samuelsson *et al.,* 2010). These data suggest that the baroreflex may indeed play an important role in long-term blood pressure control, and play a permissive role in programmed hypertension.

1.6.3 Heart and vasculature

Changes to the cardiovascular system are associated with the development and maintenance of hypertension. Increased resistance in arteries and changes to myocardial contractility can impact cardiac output and total peripheral resistance. However there are limited studies available describing the effects of programming on the cardiovascular system.

Heart: intrauterine insults of hypoxemia (Murotsuki *et al.,* 1997) and anemia (Broberg *et al.,* 2003) are shown to have a significant effect on the foetal heart. A model of perinatal anemia in sheep showed remodelling of coronary vasculature whereby coronary reserve and conductance increased, resulting in a physiological advantage (Davis *et al.,* 2003). However, maternal administration of dexamethasone resulted in increased cardiac output and hypertension in the offspring (Dodic *et al.,* 2001). There have been no studies done investigating diet-induced programming changes in the offspring heart.

Vasculature: one of the primary vascular defects known to date is impaired endothelium-dependent relaxation. This has been shown in offspring programmed by maternal protein restriction (Brawley *et al.,* 2003), high fat intake (Koukkou *et al.,* 1998) under nutrition (Franco *et al.,* 2002) and placental insufficiency (Payne *et al.,* 2003). It is unclear to as what is the underlying cause of reduced endothelium-dependent dilation; a potential mechanism might be impaired response in the vascular smooth muscle cells to nitric oxide (Lamireau *et al.,* 2002) or impaired synthesis of nitric oxide (Payne *et al.,* 2003). Another possibility could be increased responsiveness to vasoconstrictors (Ozaki *et al.,* 2001).

There is some evidence for vascular dysfunction in offspring from mothers fed an obesogenic diet. Isolated femoral arteries of 15 day old rat pups from mothers fed a high fat diet (30% wt/wt) showed blunted responses to endothelium depended relaxation to acetylcholine. Furthermore, 60-day-old offspring showed increased constrictor responses to norepinephrine (Koukkou *et al.,* 1998).

1.6.4 Epigenetic changes in programmed hypertension: evidence from animal models

Very little is known about the epigenetic changes which orchestrate programmed hypertension. Recent studies on rats have shown that maternal protein restriction during gestation decreases methylation of the angiotensin II type 1β gene (Bogdarina *et al.,* 2007) in the offspring. This may result in stable, elevated AT1β gene expression in the brain, and may cause hyper-responsiveness to angiotensin, a vasoconstrictor, in the offspring. Likewise, it has been observed that hypertensive offspring exposed to low protein diets during gestation have excessive brain angiotensin type 1 (AT1) receptor binding (Pladys *et al*., 2004). Importantly, blockade of central AT1 receptors reduced blood pressure in the low protein group, but not controls (Pladys *et al.,* 2004), indicating that increased expression of AT1 receptors in the brain may play a role in producing a programmed-hypertensive phenotype. Further studies into the epigenetic changes that precede a programmed hypertensive phenotype are required.

1.7 SEX DIFFERENCES ASSOCIATED WITH DEVELOPMENTAL ORIGINS OF CARDIOVASCULAR DISEASE

It is widely known that men tend to have higher blood pressures than age matched women (Li *et al.,* 2010). However, aging decreases these sex differences (Kotsis *et al.,* 2006) and increases the risk of cardiovascular diseases in women (Jousilahti *et al.,* 1999). These sex differences are thought to be due to differences in sex steroids. There have been numerous programming studies that show sex differences, whereby males are worse off due to programming than age matched females. In a model of intrauterine growth restriction (IUGR) in the rat, hypertension in the male offspring was associated with a two-fold increase in circulating testosterone (Ojeda *et al.,* 2007). In this model the importance of testosterone in the etiology of IUGR induced hypertension was shown by castration, whereby hypertension was abolished (Ojeda *et al.,* 2007). In addition, the male IUGR rats show greater increases in blood pressure to acute Ang II injection than control rats, an effect that is also abolished by castration. Therefore these studies strongly indicate that testosterone acts as a pro-hypertensive factor in the male IUGR rat models (Ojeda *et al.,* 2010). In comparison, female IUGR offspring are found to be normotensive after puberty (Alexander *et al.,* 2003). Ovariectomy induces hypertension in these rats, and replacement of estradiol reverses the

increased blood pressure (Ojeda *et al.,* 2007). Thus, loss of ovarian hormones in IUGR rats is associated with increases in blood pressure. Furthermore, ovariectomized IUGR females also show increased blood pressure sensitivity to Ang II in comparison to control females who have also been ovarectomized (Ojeda *et al.,* 2011). The studies above suggest differences in sex hormones may be associated with differences in offspring health outcomes due to gestational stress. Consistent with this, protein restriction in the pregnant dam (9% vs 20%) elicits programming of hypertension in the male offspring (Woods *et al.,* 2001) but not in the female (Woods *et al.,* 2005).

In summary, many models of developmental programming show sex differences in blood pressure during baseline conditions.. An increased risk of high blood pressure is more often seen in male offspring compared to controls regardless of the method of insult (undernutrition, obesogentic diet, glucocorticoid exposure or protein restriction) or timing of insult (prenatal vs postnatal) (Alexander *et al.,* 2014). However, it should be noted that not all studies indicate a role of sex hormones in the etiology of sex differences seen in foetal programming of hypertension (Alexander *et al.,* 2014).

1.7.1 Sex differences implicating the renin-angiotensin-aldosterone system (RAAS)

The RAAS plays a major role in blood pressure and volume homeostasis. Recent studies indicate that the vasodilatory arm of RAS is heightened in females compared to males (Sampson *et al.,* 2012) and that the RAAS is modulated in a different manner via sex steroids, which may contribute to sex differences in blood pressure control (Harrison-Bernard *et al.,* 2003; Hinojosa-Laborde., 2004). There are a number of differences in the RAS between male and females. Firstly, there is increased receptor density of renal angiotensin type 1 receptor (AT_1R) in the male compared to female rats (Sandberg *et al.,* 2003). Conversely receptor density of angiotensin type 2 receptor (AT_2R) which opposes vasoconstrictor activity (non classical pathway), is increased in females compared to male rats (Sampson *et al.,* 2008). Secondly, sex hormones have been implicated in the modulation of components of the RAAS. There is evidence that the classical vasoconstrictor arm of RAAS is augmented in males in way that is testosterone dependent (Yanes *et al.,* 2009), while estrogen increases production of angiotensin-(1-7) peptide associated with the regulation of the non classical dilator pathway, acting as an anti-hypertensive hormones in females (Brosnihan *et al.,* 1997). Therefore these studies suggest that the RAAS is implicated in sex specific developmental programming of hypertension.

The RAAS is also implicated in sex specific developmental programming of hypertension. Programming studies using a variety of maternal insults show female offspring in young adulthood are more protected from increases in blood pressure than their male counterparts during baseline conditions and in response to acute Ang II administration (Loria *et al.,* 2013; Ojeda *et al.,* 2007; Ojeda *et al.,* 2011, Woods *et al.,* 2005; Xiao *et al.,* 2008). Moreover, placental insufficiency in the rat programmes marked increases in renal angiotensin converting enzyme (ACE) activity in the offspring (Grigore *et al.,* 2007), indicating that endogenous levels of Ang II may be raised in these animals. However, the females in this model show a marked increase in renal ACE2 expression that is reduced by overiectomy (Ojeda *et al.,* 2007). Ovariectomy also induces hypertension in the female IUGR rat (Ojeda *et al.,* 2007). Consistent with this, bilateral uterine ligation in the rat causes up-regulation of renal AT_1R in the male, but not female offspring (Moritz *et al.,* 2009; Wlodek *et al.,* 2007), while moderate protein restriction shows increased renal AT_2R expression in female, but not male offspring (McMullen *et al.,* 2004; McMullen *et al.,* 2005). Finally, severe gestational protein restriction also causes an increase in expression of vascular AT_1R in the male offspring by three months of age, whereas the in females, this is delayed until 6 months of age (Sathishkumar *et al.,* 2012). In summary, programmed hypertension in response to a number of different *in utero* insults is implicated with the up regulation of the vasoconstrictor arm of RAAS in male offspring, and up regulation of the vasodilatory arm of RAAS in female offspring. This appears to play a significant role in delaying the development of programmed hypertension in the female.

Clearly sex differences are associated with developmental origins of cardiovascular diseases. Differences in the RAAS, renal nerve and the influence of sex steroids, contribute to differences in blood pressures of male and female offspring during young adulthood.

1.7.2 Sex difference implicating renal nerves in programmed models

There has been a wide range of studies that show programmed hypertension is associated with changes to renal nerves and there is evidence for sex-specific differences associated with these changes (Alexander *et al.,* 2014). In particular, programming changes in renal nerves appear to be present from birth in the male offspring, whereas in the female, a secondary insult may be needed to uncover programming of hypertension (Alexander *et al.,* 2014). In IUGR, female offspring are normotensive during young adulthood but develop age dependent hypertension associated with

increased visceral fat and increased leptin levels (Alexander *et al.,* 2003; Intapad *et al.,* 2013). Renal denervation abolishes hypertension in these rats (Intapad *et al.,* 2013). This suggests that IUGR programmed hypertension in females is dependent on a leptin-mediated activation of the renal sympathetic nerve (DiBona *et al.,* 2002). In contrast, in the male offspring, hypertension is not associated with increased fat mass and circulating leptin levels (Alexander *et al.,* 2014). Thus, although both male and female offspring from programming models show hypertension in adulthood, the mechanism by which the renal nerves are activated in developmental programming of hypertension might be sex specific.

1.8 STRESS AND SYSTEMIC RESPONSE TO STRESS

1.8.1 What is stress?

Stress can be broadly defined as a state of disharmony or threatened homeostasis; the stressor inflicted can be of biological (physiological) or psychological origin (Martinez- Lavin 2007). A Physiological stressor such as a pain, haemorrhage or cold cause direct harm to the body as the homeostatic regulation is challenged. Psychological stressors on the other hand do not cause direct harm to the body, but are perceived as a potential harmful insult by an individual. Such stressors include feeling threatened, fear, confrontation and startle. Stress can also manifest due to unpleasant memories of stressors. However, both physiological and psychological stress cause marked cardiovascular and autonomic changes, preparing an organism to respond or escape from the challenge (Carrive, 2011).

1.8.2 Psychological stress

In the context of humans, there is concern over the adverse effects of increased psychological stress on health, in particular the risk of cardiovascular disease. An on going problem in today's society is work related stress, (psychosocial stress). Such chronic stress is an ongoing stimulus for the stress response system. A prospective cohort study has shown that employees with increased job strain (defined by low salary, few career opportunities and lack of social approval) had a 2.2 fold increased risk of cardiovascular mortality compared to employees with a low job strain (Kivimaki *et al.,* 2002).

Acute psychological stressors have also been shown to increase risk of cardiovascular events, an example of this was shown in a study of the 1998 football world cup (Carroll *et al.,* 2002). There was a 25% increase in admission for acute myocardial infarction on 30th June 1998 (when England lost to Argentina in a penalty shoot out) and for the two days following the loss (Carroll *et al.,* 2002). Patients who survive myocardial infarction usually report a triggering activity, that is most commonly identified as physical exertion or being emotionally upset (Tofler *et al.,* 1990). Further evidence for health risks of acute stress comes from studies investigating adverse health outcomes surrounding catastrophic environmental events. An earthquake in 1994 at Northridge, southern California, saw a 35% increased admissions for myocardial infarction for a week following the earthquake compared to before the earthquake, and the proportion of hospitals reporting increased admissions were closer to the epicentre (Lear *et al.,* 1996).

Other studies have shown significant increases in sympathetic nerve activity following catastrophic environmental events. For example, hospitalised patients showed increased heart rate and heart rate variability (HRV) following an earthquake in Taiwan (Huang *et al.,* 2001). The increase in the low-frequency:high-frequency ratio in HRV is indicative of increased sympathetic nerve activity, and the increased cardiovascular risk associated with acute psychological stress may in part be due to increased activity of the sympathetic nervous system (Huang *et al.,* 2001). With regard to developmental programming, an increased sympathetic response to stress may play a significant role in the pathogenesis of cardiovascular diseases and may be responsible for the increased propensity to develop hypertension (O'Regan *et al.,* 2010).

1.8.3 Physiological stress

Physiological stress refers to the homeostatic responses to various environmental stressors such as predation, infection, injury, fasting, water loss and temperature extremes (Girod and Brotman, 2004; Hindmarch *et al.,* 2011). The responses to a physiological stressor include specific responses particular to the stress stimulus and a more general response common to psychological stress, described below (Nesse and Young , 2000; Ulrich-Lai and Herman, 2009). It is important to note, however, that although there are very similar responses between physiological and psychological stressors, or even between different physiological stressors, the central pathways mediating the responses are not necessarily the same (Dampney *et al.,* 2008).

The physiological stress used in the present study was dehydration. Extended periods of water deprivation increase plasma osmolality and decrease blood volume (Toney and Stocker, 2010). This response is highly similar to that of high salt consumption (Toney and Stocker, 2010). Despite the hypovolemia, blood pressure usually increases in the conscious rat due to hormonal and sympathetic activation (Veitenheimer *et al.,* 2012). There is accumulating evidence that exaggerated sympathetic responses to increased plasma osmolarity may contribute to salt sensitive hypertension (Adams, 2004; Brooks et al. 2005; O'Donaughy et al. 2006), although the mechanisms by which this occurs are not well elucidated (Toney and Stocker *et al.,* 2010).

1.8.4 The stress response system

When an organism experiences a psychological or physiological stressor, information related to the stressor from all sensory systems is conveyed to the brain, in response, the neural and neuroendocrine systems are activated (Ulrich-Lai and Herman, 2009). The autonomic nervous system (ANS) is the most immediate response system to the stressor; activation of the ANS, particularly the sympathetic branch provides rapid changes to the physiological state (Iversen *et al.,* 2000). The activation of the hypothalamic pituitary-adrenal (HPA) axis, which is a neuroendocrine cascade, results in increased circulating glucocorticoids; it has been found that glucocorticoids levels peak 10 minutes after the initiation of a stressor (Droste *et al.,* 2008). When activated by a stressor, these two systems together cause stereotypical changes such as piloerection, pupillary dilatation, sweating, increased blood pressure and heart and changes in metabolism. The brain responds to stress in proportion to the nature of the stressor.

During a psychological stressor, the limbic and thalamic nuclei are triggered in response to a cognitive interpretation of the stressor from the cortical and amygdaloid regions of the brain. This results in the activation of the ANS and HPA-axis via neuronal projections to certain structures in the hypothalamus (Ulrich-Lai and Herman, 2009). Physiological stressors such as dehydration also activate the ANS and HPA-axis via structures in the hypothalamus (Toney and Stocker, 2010). Thus the hypothalamus appears to be a key structure in mediating the stress responses to both psychological and physiological stressors.

1.8.5 The autonomic response to stress

The autonomic nervous system serves as the most immediate responder to stress, resulting in rapid changes to the physiological state by neural activation of targeted organs such as the sweat glands, pupils, gut, heart and vasculature beds via its sympathetic and parasympathetic branches (Critchley *et al.,* 2000; Carrive and Gorissen, 2008; Ulrich-Lai and Herman, 2009; Harrison *et al*., 2010). For the purposes of this thesis, we will focus on stress-mediated changes in autonomic control of cardiovascular function. Animal models have shown, acute psychological stress such as air jet stress increases arterial pressure and heart rate, and has varied affects on vascular beds, including vasodilatation to skeletal muscles and vasoconstriction to skin. These responses are typically a result of sympathetic activation (Alves *et al.,* 2010; Brotman *et al.,* 2007; Blessing *et al.,* 2003; and Schadt and Hasser, 1998), however this is not always the case. There is evidence that the skeletal muscle vasodilatation may be due to stress-induced inhibition of sympathetic vasoconstrictor fibres to the skeletal muscle (Gebber *et al.,* 2000). These coordinated responses help to mobilize the animal to respond appropriately to the environmental conditions that have evoked the stress. However, if the autonomic nervous system responds inappropriately, or if the animal is unable to remove itself from the stress, a non-homeostatic or pathological response may develop.

Stress may have adverse effects on health and disease when there are exaggerated, sympathetic responses to a stressor or in response to long term exposure to a stressor, and this may lead to overt hypertension (Grassi *et al*., 2010; Razanski *et al*., 2005). For example, a recent study reported that patients treated for chronic psychosocial stress had increased blood pressure as well as increased low frequency: high frequency heart rate variability, increased low frequency systolic blood pressure variability and decreased baroreflex sensitivity compared to control patients (Lucini *et al.,* 2014). All of these signs of autonomic dysfunction are commonly associated with hypertension (Grassi *et al.,* 2010). Animal studies have also shown changes in blood pressure in response to chronic stress. Henry and coworkers (1993) showed psychosocial competitive behavior between group-housed aggressive male Long Evans rats increased basal blood pressure by approximately 20 mmHg (Henry *et al.,* 1993), while chronic stress evoked by repeated foot shock in rats increased basal blood pressure and heart rate after only two weeks (Xiao *et al.,* 2013). With regard to physiological stress, the most common example is high salt diet, which is a

well-known risk factor for hypertension (Weinberger, 1996). Studies show that increased consumption of salt raises sympathetic nerve activity in rats (Toney and Stocker, 2010), and that preventing the increase in sympathetic activity also prevents the development of hypertension (Brody, 1988). These studies demonstrate a strong association between chronic stress and hypertension, however evidence for more mild stress eliciting hypertension via a sympathetic pathway is less robust.

Air jet stress is considered a mild psychological stress (Dampney *et al.,* 2008). A study in the borderline hypertensive rat, exposed to daily air jet stress for 8 weeks showed increased heart rate and basal arterial pressure, compared to controls (Mansi and Drolet, 1997). This study suggests that chronic mild stress may sensitise the sympathetic system and lead to hypertension. It should be noted that the borderline hypertensive rat has a genetic predisposition to develop hypertension when exposed to a stressor. Thus, some individuals may have increased sensitivity to chronic stress due to genetic and/or epigenetic factors, predisposing them to developing hypertension.

In the context of foetal programming, studies have shown heightened anxiety-like behavior from offspring of mothers exposed to a stressor such as raised glucocorticoids during pregnancy (Welberg *et al.,* 2001; Welberg and Seckl, 2001). A study by Igosheva *et al.,* (2004) showed, offspring rats exposed to gestational restraint stress had increased blood pressure when they themselves were exposed to restraint stress at 6 months of age. Furthermore, blood pressure remained elevated during the recovery phase in these rats, indicating that programming both increased the magnitude and extended the duration of the stress response (Igosheva *et al.,* 2004; O'Regan *et al.,* 2008). Similar results were obtained in an obesogenic high fat diet model of programming (Samuelsson *et al.,* 2010). Arterial pressure increased more and remained elevated for longer in the high fat offspring in response to restraint stressed (Samuelsson *et al.,* 2010). These programming studies add to the complexity of understanding the development of hypertension, furthermore, they provide evidence that stressors or unfavorable environments experienced in early life may in fact predispose offspring to develop hypertension.

1.8.6 The endocrine response to stress

Glucocorticoids (GC) are one of the main effectors of the endocrine response to stress, being released from the adrenal cortex after the hypothalamic-pituitary-adrenal-axis (HPA) is activated (De Kloet *et al.,* 1998) (**Figure 1.1**) .The HPA-axis consists of three major endocrine glands; the hypothalamus, pituitary gland and the adrenal gland. These interact with each other to produce a complex network of feedback that controls the amount of glucocorticoid release in response to stress (O'Connor *et al.,* 2000). Activation of the HPA-axis begins with hypothalamus-stimulated release of corticotropin-releasing hormone from the paraventricular nucleus (PVN) (Bornstein *et al.,* 2008). This results in the release of adrenocorticotropic hormone (ACTH) from the anterior pituitary, which then enters the circulation and acts on the adrenal cortex to stimulate the production of GCs (Bornstein *et al.* 2008). GCs in the circulation go on to act on a wide range of target organs, producing an array of biological responses, including glycogenesis and gluconeogenesis in the liver, lipolysis and proteolysis in adipose, muscle, lymphoid and connective tissue (Smith *et al.*, 2006). These biological responses all act to provide adequate substrate to overcome a challenging situation.

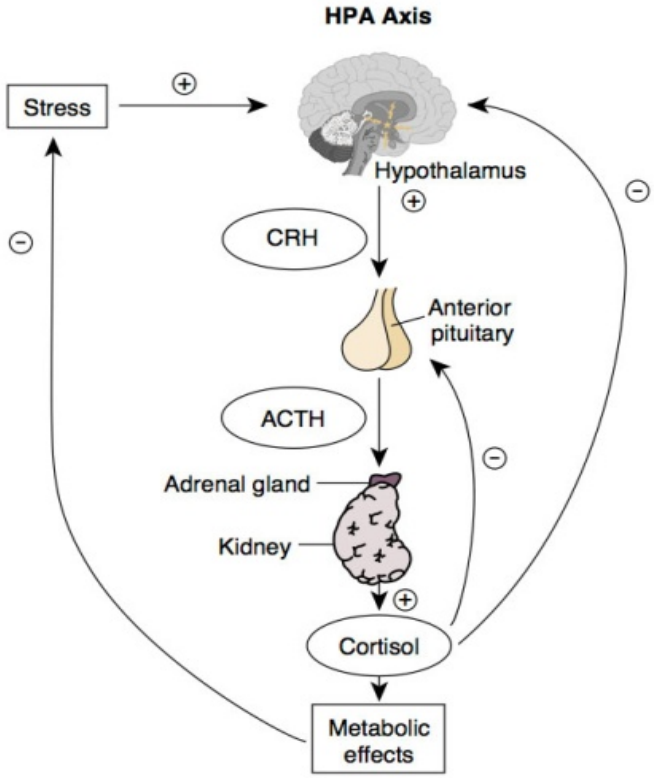

Figure: 1.1 ***Schematic representation of feedback mechanism of HPA axis***, "+" represents positive feedback loop whilst "– " represents negative feedback loop; corticotropin releasing hormone (CRH), adrenocorticotropic hormone (ACTH). Figure adapted from Hiller-Sturmhöfel and Bartke, 1998.

1.8.7 Central circulatory mediating control of psychological stress

The integrated central pathway of the autonomic nervous system that responds to stress is highly complex and remains poorly understood, however there is convincing evidence that suggests that

this response may be orchestrated within the hypothalamus. This became evident 70 years ago from studies done by Hess and Brugger. Their experiments showed electrical stimulation of the hypothalamus in a conscious cat produced behavioural and autonomic responses that were identical to those of a cat exposed to a threatening stimulus. This response is known as the "defense reaction" described by hissing, growling and piloerection behaviours observed (Hess and Brugger, 1943). Although these studies provided good evidence implicating the hypothalamus, it was impossible to delineate the precise regions of the hypothalamus or identify pathways associated with the defense reaction due to technical limitations of the day. However, more recent studies that have used more precise and localised methodology have enabled the identification of key regions within the hypothalamus, these include the lateral areas surrounding the fornix as well as the ventromedial and dorsal areas of the hypothalamus, the collection of these areas is often termed the hypothalamic defense area (Hilton, 1965).

1.8.8 Key hypothalamic nuclei involved in stress response

Studies with improved and more sophisticated techniques have confirmed previous experiments indicating the role of the hypothalamus in mediating autonomic responses to stress. These studies have been able to identify regions *within* the hypothalamic defense area that are important in producing cardiovascular and behavioral responses to stress. The three main regions identified include; the perifornical nucleus (PeF), the dorsomedial hypothalamus (DMH) and the paraventricular nucleus (PVN). **Figure 1.2** shows a summary of these structures that mediate the cardiovascular responses to stress.

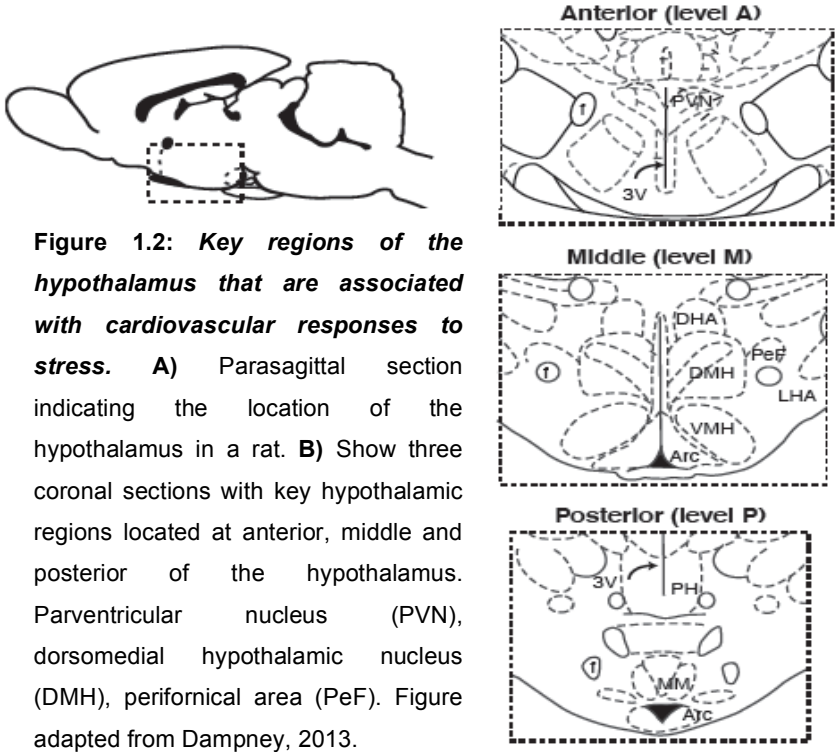

Figure 1.2: ***Key regions of the hypothalamus that are associated with cardiovascular responses to stress.*** **A)** Parasagittal section indicating the location of the hypothalamus in a rat. **B)** Show three coronal sections with key hypothalamic regions located at anterior, middle and posterior of the hypothalamus. Parventricular nucleus (PVN), dorsomedial hypothalamic nucleus (DMH), perifornical area (PeF). Figure adapted from Dampney, 2013.

The PeF: is located in the middle of the hypothalamus, surrounding the fornix. Stimulation of this area evokes defense reactions as described by Hess and Brugger, 1943, (Nakao, 1958; Smith *et al.,* 1990). Further, studies by Dampney and coworker (2008); suggest that the PeF is important in producing cardiovascular and autonomic responses to contextual stressors. Contextual stressors are defined as a stimulus that is perceived by the animal as threatening due to prior experiences, whereas non-contextual stressors are unconditioned fear, they do not depend upon prior experience and so are intrinsically threatening, such as a loud noise (Dampney *et al.,* 2008). Studies by Furlong and Carrive (2007) showed that lesions made in the PeF reduced cardiovascular and autonomic responses to an environment in which the rat had been conditioned to fear (i.e. a contextual stress), but no changes were seen in cardiovascular and autonomic responses to restraint stress (a non-contextual stress). This result indicates that the PeF may play an important role in mediating the cardiovascular responses to contextual stressors. In contrast, the DMH is thought to play a role in mediating responses to non-contextual stressors (Palmer and Printz, 2002; Dampney *et al.*, 2008).

The DMH: regulates cardiovascular and autonomic responses to acute stress (Ulrich-Lai and Herman, 2009, Dampney *et al.,* 2002; 2008) (**Figure 1.3**). Discrete stimulation of the DMH increases blood pressure, heart rate, respiratory rate and increases HPA-axis responses to a psychological stressor (Bailey and Dimicco, 2001; Ulrich-Lai and Herman, 2009; Dampney *et al.*, 2008). In contrast, inhibition of the DMH by injection of neuroinhibitory compounds decreases the increase in blood pressure and heart rate following an acute psychological stress such as air restraint or jet stress (Morin *et al*., 2001; Stotz-Potter *et al.,* 1996; Ulrich-Lai and Herman 2009).

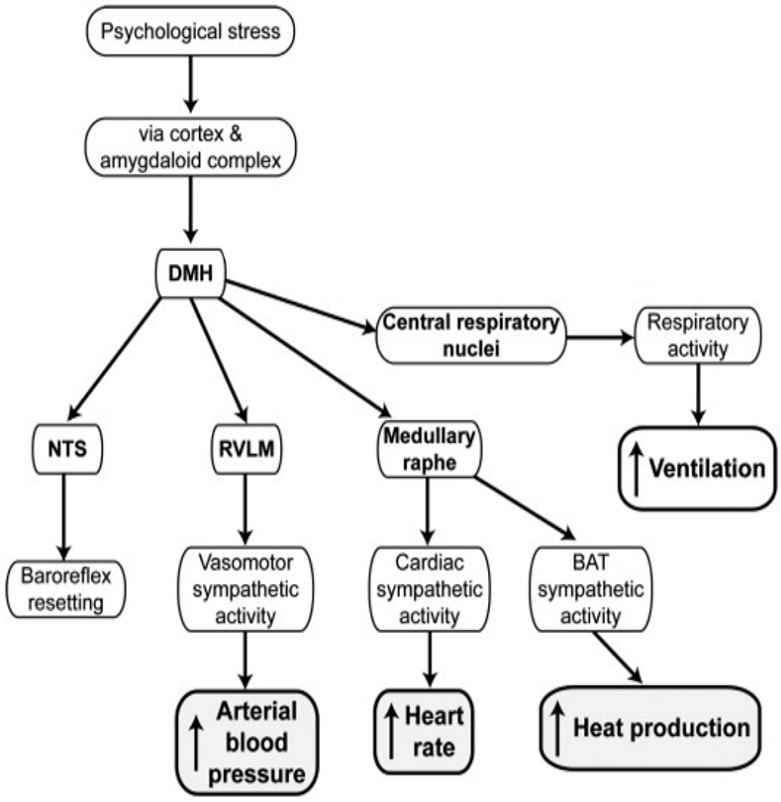

Figure 1.3: ***Flow diagram showing proposed central pathways subserving the autonomic, neuroendocrine and respiratory responses to psychological stress.*** DMH, dorsomedial hypothalamus; NTS, nucleus of the solitary tract; RVLM, rostral ventrolateral medulla, BAT, brown adipose tissue. Figure adapted from Dampney *et al.,* 2008.

Afferent projections from other hypothalamic, cortical and subcortical regions input into the DMH, these projections are known to be associated with the perception of stress (Stotz-Potter *et al.,* 1996). The afferent inputs that initiate cardiovascular responses to a psychological stress in the DMH, however, are currently unknown (Dampney *et al.,* 2008). The efferent projections from the DMH are defined more clearly. With the activation of the DMH, a modulation of the baroreflex is observed (McDowall *et al.,* 2006). This probably arises via a direct input from the DMH to the nucleus tractus solitaries (NTS), which is a site at which primary baroreceptor afferents terminate (Thompson *et al.,* 1996) and well known to be an important site of baroreflex modulation (Boscan *et al.,* 2002). Direct projections from the DMH to the raphe pallidus have also been identified and thought to play an important role in regulating heart rate in response to a stressor (Sarker *et al.,*

2007; Samuels *et al.,* 2002). Furthermore, studies have shown a functional projection from the DMH to the sympathetic premotor neurons located in RVLM (Fontes *et al.,* 2001), and these projections may be responsible for changes in sympathetic vasomotor activity and therefore increases in blood pressure associated with a psychological stressor (Horiuchi *et al.,* 2004, Dampney *et al.,* 2002). However this remains controversial (Furlong *et al.,* 2014). The descending pathways that mediate the stress evoked respiratory changes have not yet been defined (Dampney *et al.,* 2008). Finally, there is evidence that the DMH is involved in gating PVN activation during a psychogenic stressor, which may play a role in both sympathetic and glucocorticoid responses to stress (DiMicco *et al.,* 2002).

The PVN: Inputs from other hypothalamic regions converge at the PVN, which plays a crucial role in regulating numerous homeostatic function, including temperature, appetite and fluid regulation. In addition, the PVN is a principle integrator of stress signals to autonomic and HPA axis (Ulrich-Lai and Herman, 2009; Guyenet, 2006). The PVN is highly involved in the regulation of HPA-axis; the medial parvocellular subdivision of the PVN contains neuroendocrine neurons that synthesise and release corticotropin-releasing hormone (CRH) via the pituitary gland (Ulrich-Lai and Herman, 2009). CRH then stimulates the secretion of adrenocorticotropic hormone (ATCH) producing glucocorticoid hormones from the adrenals (**Figure 1.4**). In addition to their role in mobilizing glucose and fatty acids, glucocorticoids also play a major role in regulating the stress response, via negative feedback cycle inhibition of

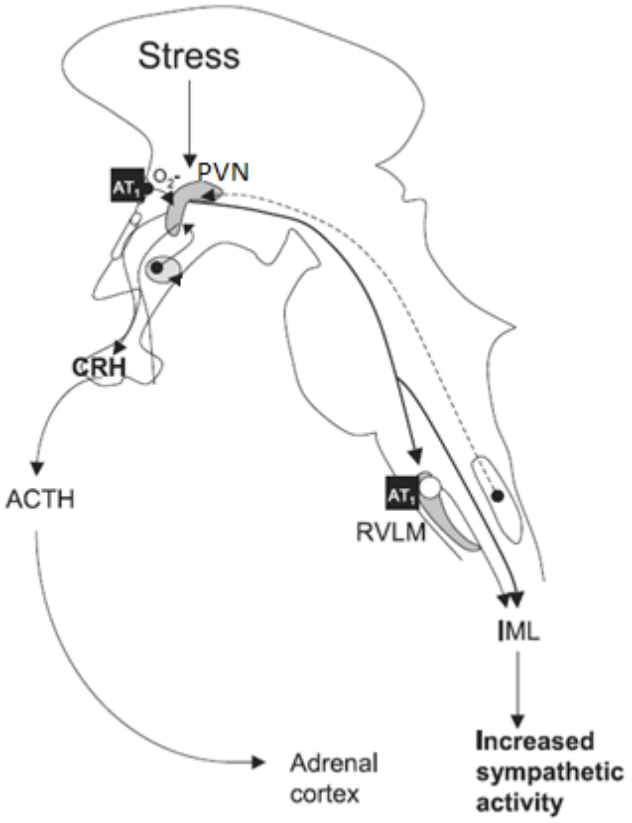

Figure 1.4: ***role of paraventricular nucleus (PVN) in response to stress***, showing major projections from the PVN in modulating HPA-axis and sympathetic activity. Adapted from Benarroch, 2005.

the HPA-axis (Sawchenko *et al.,* 2000; Ulrich-Lai and Herman, 2009).

The posterior subdivision of the PVN projects directly to autonomic nuclei in the brain stem and spinal cord, including the spinal intermediolateral cell column (IML) and the rostral ventrolateral medulla (RVLM). These pathways are thought to mediate part of the autonomic responses to stress (Shafton *et al.,* 1998; Coote *et al.,* 1998; Dampney *et al.,* 2002) (**Figure 1.4**).

The PVN also plays a crucial role in mediating sympathetic and endocrine responses to dehydration- and osmotic stress (Brooks *et al.,* 2005; Guyenet, 2006). Inhibition of PVN neurons significantly attenuates the sympathetic activation in response to acute hyperosmolality (Antunes et al. 2006; Chen and Toney, 2001). The increase in sympathetic nerve activity is also mediated by activation of presympathetic RVLM neurons (Brooks *et al.,* 2005), suggesting that the pathway fro the PVN to the RVLM plays a crucial role in osmotic and possible volume-dependent increases in sympathetic activity.

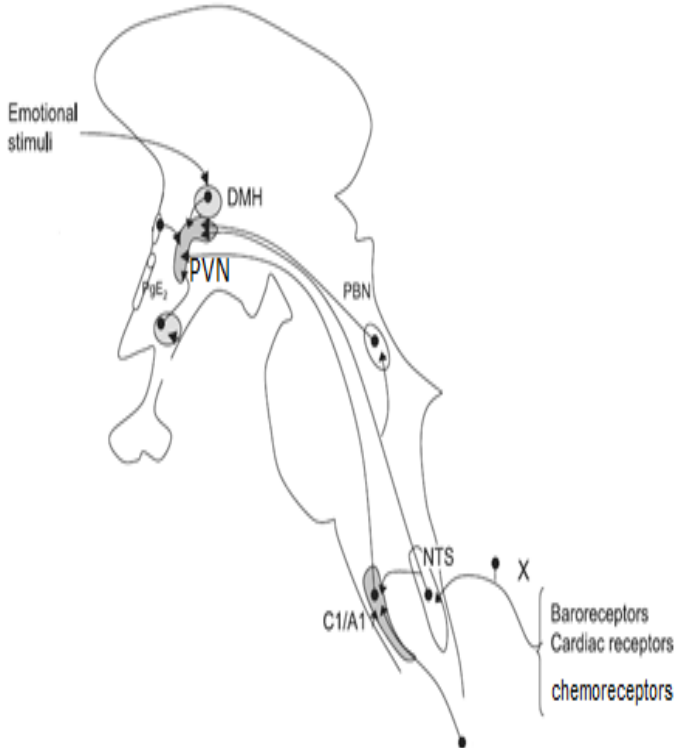

Figure 1.5: ***inputs to the parventricular nucleus (PVN).*** The PVN receive visceral and nociceptive inputs via the nucleus of the solitary tract (NTS), either directly or via a relay in the C1/A1 groups of catecholaminergic nerurons of the ventrolateral medulla or the parabrachial nucleus (PBN). Adapted from Benarroch, 2005.

The PVN receives both direct and indirect inputs from key cardiovascular regulatory sites such as the NTS and A1 neurons in the caudal ventrolateral medulla. The exact pathways are not fully elucidated but do include a synapse in the pontine parabrachial nucleus (PBN) (Benarroch, 2005) (**Figure 1.5**). These inputs provide information on bood pressure, blood volume and oxygen saturation (Karim *et al.,* 1972, Clement *et al.,* 1972; Lovick and Coote, 1988; Reddy *et al.,* 2005) and may play an important role in modulating sympathetic homeostatic responses such as during

dehydration.

Finally, studies indicate that the PVN may play a crucial role in initiating the increase sympathetic activity associated with heart failure (Felder *et al.,* 2003) and hypertension (Ito *et al.,* 2002; Allen, 2002; Guyenet, 2006; Toney and Stocker, 2010). To date the role of the PVN in programmed hypertension has not been investigated.

1.9 NON-INVASIVE MEASUREMENT OF AUTONOMIC FUNCTION

Traditionally, the assessment of autonomic function requires the use of invasive techniques, such as direct recording of sympathetic nerve activity from electrodes placed on nerve fibers. These measurements of autonomic function cannot be done easily in a conscious freely moving animal and usually require conducting experiments under anestheisa. This makes it extremely difficult, if not impossible to determine the response to psychological stressors. Similarly, in humans the assessment of autonomic function is ethically complicated by the use of invasive techniques. Therefore, several non-invasive techniques have been developed to measure indices of autonomic function without causing undue stress to the subject. In this thesis, autonomic function was assessed by the following techniques: (i) spontaneous baroreflex sensitivity (sBRS), (ii) baroreflex effectiveness index (BEI), (iii) frequency analysis of heart rate variability (HRV), (iv) frequency analysis of systolic blood pressure variability (BPV) and (v) determination of maximum rate of change of aortic pressure during ventricular systole (dP/dt_{max}). These measurements are all conducted post-hoc from the blood pressure waveform, without the need to manipulate cardiovascular variables, and therefore minimise the disturbance to the animal.

1.9.1 Spontaneous baroreceptor reflex sensitivity (sBRS)

sBRS is a method of determining cardiac baroreflex function; this method has been used both clinically and in animal studies to identify problems in blood pressure control (La Rovere *et al.,* 1998; Polson *et al.,* 2006; Tank *et al.,* 2000; Waki *et al.,* 2006). sBRS measures the relationship between pulse interval (or R-R interval from electrocardiogram) and systolic blood pressure during spontaneous fluctuations in blood pressure. Traditionally, baroreflex sensitivity is determined by injecting vasoactive drugs into the animal resulting in changes in blood pressure. The resultant heart rate or pulse interval response is then plotted against blood pressure; this relationship

provides a measure of the baroreflex sensitivity. This method is known as the Oxford technique (Smyth *et al.,* 1969). This technique has been used to show decreased baroreflex sensitivity in hypertensive patients and in animal models of hypertension (Bristow *et al.,* 1969; Casto and Phillips, 1986; Pladys *et al.,* 2004).

More recently, analysis of sBRS has been done using computer-based techniques without the use of vasoactive drugs. These computer based techniques scan the blood pressure waveform using an algorithm that identifies beat-to-beat fluctuations in blood pressure and heart rate. These techniques provide a low cost, noninvasive and simple way of measuring sBRS. An advantage of this technique is that it allows for an assessment of the baroreflex function at its functional operating point and how it's modulated in daily life (Parati *et al.,* 1995). There are two basic approaches commonly used: the sequence method and the spectral method. The sequence method incorporates the identification of sequences of consecutive beats in which progressive increases in SBP (pressor ramps) are followed by a progressive lengthening of pulse interval or progressive decreases in SBP (depressor ramps) are followed by progressive shortening of pulse interval. For each ramp, these values (SBP and PI) are plotted and fitted with a linear regression line, the slope of the regression between SBP and PI values in each sequence are indicative of baroreflex sensitivity (Di Rienzo *et al.,* 2001; Waki *et al.,* 2006). This technique has two major advantages; firstly measurement variability is decreased due to the automatic and standardized way of computing the data, secondly, different measurements are taken for increasing and decreasing blood pressure values, these account for any asymmetry of baroreceptor response (La Rovere *et al.,* 2008). The sequence method was used for the analysis of baroreflex in our experiments.

Spontaneous BRS can also be determined by investigating SBP and pulse interval variability (or heart rate variability (HRV), see below) in the frequency domain, and is based on the assumption that a component of the HRV in a certain frequency range is dependent upon the baroreflex, and where HRV and BPV will display a high coherence (Persson *et al.,* 2001). In other words, where the oscillations in HR and SBP are linearly related. The gain of the transfer function between SBP and HRV provides a measure of the BRS. In practice, this can be determined as the square root of ratio of HRV and BPV powers in the low frequency and high frequency ranges, respectively (La

Rovere *et al.,* 2008). Both the sequence method and the spectral method have been validated using a number of tests, including denervation of baroreceptors producing a loss of gain and correlation with the gain as determined by the Oxford technique (Persson *et al.,* 2001; La Rovere *et al.,* 2008).

1.9.2 Baroreflex effectiveness index (BEI)

In healthy individuals the baroreflex is not always activated following every fluctuation in blood pressure, and this phenomenon can be quantified as the baroreflex effectiveness index. It is the ratio between the number of SBP ramps followed by the respective PI reflex response and the total number of SBP ramps observed for a given time period (Di Rienzo *et al.,* 2001), calculated using the formula below:

$$BEI = \frac{total\ number\ of\ PI/SBP\ sequences}{total\ number\ of\ SBP\ ramps}$$

1.9.3 Heart rate variability (HRV)

Measurements examine quantitatively how heart rate varies over time. The measures are usually performed over both long and short term and are an important quantitative marker of autonomic activity. The association of HRV as a function of autonomic activity is seen in experimental evidence as an inclination to lethal arrhythmias and either increased sympathetic or reduced vagal activity. Such evidence has therefore allowed the development of quantitative markers and HRV represents a promising marker of autonomic activity (Heart rate variability, 1996). There are a variety of techniques to measure quantitatively how heart rate varies over time

The frequency domain measures of HRV are determined by performing fast fourier transform (FFT) over a heart rate waveform that has previously been created from the raw blood pressure waveform or ECG trace. This enables the separation of the fluctuations into three pre-determined frequency components, very low frequency component (0-0.25 Hz), low- (0.25-0.75 Hz) and high frequency (0.75-3.3 Hz). These frequency ranges in the rat have been previously determined (Waki *et al.,* 2006). Two of the frequency bands have been shown to be related to autonomic function: low frequency reflects the level of sympathetic modulation and high frequency reflects the

parasympathetic modulation. Furthermore, there is evidence that the very low frequency component is associated with hormonal influences (Malik *et al.,* 1996).

1.9.4 Blood pressure variability (BPV)

Blood pressure can differ significantly in an individual at different times, for example during night and day, changes in the beat to beat blood pressure variation during sleep and wakefulness and in response to physiological or psychological stimuli. Evidence suggests that if such representation of BPV is augmented, there is an increased cardiovascular risk (Floras, 2013). Similar to HRV, spectral analysis of the beat-to-beat fluctuations in SBP in the low frequency band corresponds to levels of sympathetic vasomotor activity (Waki *et al*., 2006).

1.10 THE C-FOS TECHNIQUE FOR FUNCTIONAL-ANATOMICAL IDENTIFICATION OF NEURONAL POPULATIONS ACTIVATED BY A SPECIFIC STIMULUS

1.10.1 What is c-fos?

C-fos is an immediate early gene, the mammalian homologue to the osteosarcoma-causing viral oncogene v-*fos* (Sagar *et al.,* 1988; Curran et al., 1983). These genes are the first to be activated following virus integration into an infected cell and are responsible for transcriptional reprogramming of the host to promote virus replication (Milde-Langosch, 2005). C-*fos* is a part of the Fos family of transcription factors, including FosB, Fra-1 and Fra-2. It encodes for the protein product Fos, which forms heterodimers with the protein product from another immediate early gene c-*jun* to form a protein complex known as Activator Protein-1 (AP-1). AP-1 is a transcription factor: it binds DNA at AP-1 specific binding sites at the promoter and enhancer regions of target genes, thereby altering gene expression of other genes (**Figure 1.6**) (Rylski and Leszek Kaczmarek, 2004). Although AP-1 is involved in many aspects of the brain physiology, only a few downstream target genes have been identified. Some of these include genes encoding for neurotransmitters or neuromodulators, such as tyrosine hydroxylase, corticotrophin releasing hormone and arginine vasopressin, while other encode for transmembrane protein and neurotrophine and cytokines (Hoffman et al., 1993; Icard-Liepklans et al., 1993; Rylski and Leszek Kaczmarek, 2004).

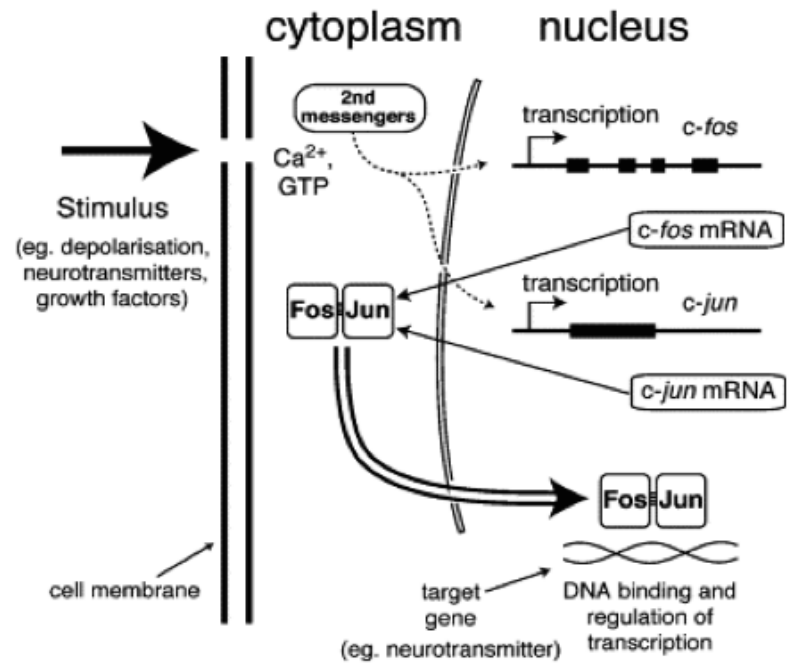

Figure 1.6: ***illustration of intracellular pathways leading to Fos expression.***
Adapted from Dampney et al., 2003

1.10.2 Stimulus for c-fos expression in neurons

Fos expression was first identified in brain neurons in the late 1980s and the expression was shown to be increased by generalised seizure, noxious stimulus or electrical stimulation (Sagar *et al.,* 1988; Dragunow and Faull, 1989; Dragunow and Robertson, 1988; Herrera and Robertson, 1996). In particular, the activation of c-*fos* appears to be dependent on increasing intracellular calcium concentrations, such as occurs during depolarisation-activated voltage dependent calcium channels (Dragunow and Faull, 1989; Haby et al., 1994; Herrera and Robertson, 1996; Sheng and Greenberg, 1990). Thus, a major stimulus for c-*fos* expression is neuronal activation.

Following a stimulus, c-*fos* is expressed rapidly, within a few minutes, reaching peak mRNA levels within 30 minutes (Kovacs, 2008) and maximum protein expression by approximately 60-120 minutes (Sheng and Greenberg, 1990; Kovacs, 2008). Protein expression persists for 2-5 hours (Kovacs, 2008; Morgan et al., 1987). Basal c-*fos* expression in most neurons is very low (Dampney and Horiuchi, 2003), indeed usually a strong and sustained stimulus is required before c-*fos* expression can be reliably induced (Dampney and Horiuchi, 2003; Dragunow and Faull,

1989). These simple expression characteristics have made the identification of c-*fos* mRNA levels using in situ hybridisation or Fos protein levels using immunohistochemistry one of the most powerful and reliable techniques for identification and mapping of neuronal populations that are activated by a specific stimulus (Dampney and Horiuchi, 2003; Ferrera and Robertson, 1996). Since these early studies, there have been hundreds of reports that have investigated c-*fos* expression in the central nervous system following specific stimuli (Dampney and Horiuchi, 2003; Ferrera and Robertson, 1996). It is important, however, to be aware of the limitation of the c-*fos* technique when used as a tool for functional neuroanatomical identification.

1.10.3 The c-fos Technique as a tool for functional mapping of neuronal activity

Identification of brain regions or neuronal populations responsible for a specific function is of crucial importance in neuroscience. Earliest techniques involved lesioning or electrical stimulation of brain regions of interest and observation of the evoked responses. Clearly these techniques were coarse in their nature and could not discriminate between effects on cell bodies or axons of passage (Dampney and Horiuchi, 2003; Dragunow and Faull, 1989). Electrophysiological recording of individual or small groups of neurons to identify neurons activated by a stimulus allowed for greater discrimination within brain regions, but at the expense of better understanding of more global effects. Such experiments could not identify more than a few neurons at any given time, and so were unable to provide useful information about populations of neurons that are activated by a stimulus (Dampney et a, 1995; Dampney and Horiuchi, 2003).

To obtain a more comprehensive picture of the distribution of neurons that are activated by a specific stimulus, it is necessary to use a functional mapping technique. An early method was the 2-deoxyglucose method, which uses injected radioactive deoxyglucose to identify brain regions whose metabolic activity is altered as a consequence of the stimulus (i.e. because of increased neural activity) (Dampney et al., 1995; Dampney and Horiuchi, 2003). The disadvantage of this technique is its lack of resolution. The incorporated radioactive glucose in the tissue is simply exposed onto photographic plates and so there is no microscopic magnification of the area under observation. Therefore, it is not possible to identify individual neurons, or even whether the radioactivity is in neuronal cell bodies or terminals. Moreover, the basal level of metabolic activity is fairly high, meaning there is often a low signal to noise ratio that can make it difficult to identify

some activated regions (Dampney et al., 1995; Dampney and Horiuchi, 2003; Greenberg *et al.,* 1981). The c-*fos* technique is a more advanced functional mapping technique that overcomes many of the problems described above.

Fos protein can be stained for immunohistochemically, staining the nucleus of the neuron, and allowing the tissue to be examined under the microscope. Therefore, the c-*fos* technique has the dual advantage of being able to identify activated (by the presence of Fos) *populations* of neurons at the cellular level. Moreover, Fos labelling can be combined with other neuroanatomical techniques, such as double labelling for retrograde tracers or neurotransmitters, allowing more information to be evaluated (Dampney et al., 1995; Dampney and Horiuchi, 2003).

Limitations: There are a number of limitations to the c-*fos* technique that need to be considered. First, neurons differ in their capacity to produce Fos and the time course over which Fos is produced is not consistent between different cell populations. Therefore, absence of Fos expression does not necessarily mean that the neurons were not activated. For example, following a raised blood pressure stimulus, no Fos was found in the nucleus ambiguous, the site of cardiac vagal preganglionic neurons, despite it being well reported that these neurons are activated by raised blood pressure (Dampney et al., 1995). A second limitation is that Fos expression is affected by anaesthesia (Dragunow and Faull, 1989), making it complicated to carry out studies in anaesthetised animals (Dampney et al., 1995). For example, barbiturates have been reported to inhibit Fos expression, while urethane increases baseline expression (Dragunow and Faull, 1989; Dampney et al., 1995). Finally, because the expression of Fos is linked to neuronal activation, any stimulus that increases neuronal activation will likely cause Fos expression. Therefore, it is important to control the local environment in which the experiments are taking place: for example noise levels and smells may elicit Fos expression (Beckett et al., 1997).

1.10.4 The c-fos technique in psychological stress

There are numerous reports describing neural activation in response to acute (Ceccatelli et al., 1989; Furlong et al., 2014; Palmer and Printz, 1999; Porter and Hayward, 2011; Spencer and Day, 2004; Spencer et al., 2005), and contextual (Carrive and Gorissen, 2008; Furlong et al., 2009) stress. Many of the regions reported to be activated include regions known to be involved in mediating sympathoexcitatory responses to stress, including the rostral ventrolateral medulla,

midbrain periaqueductal grey and several structures in the hypothalamus (Carrive and Gorissen, 2008; Furlong et al., 2009; 2014; Palmer and Printz, 1999; Porter and Hayward, 2011; Spencer and Day, 2004; Spencer et al., 2005).

Air jet stress, used in this study, is considered an acute stress of moderate intensity (Dampney et al., 2008). Rats exposed to an air jet stress show significant increases in Fos expression in certain hypothalamic regions, including the DMH, PVN, and PeF, (Furlong et al., 2014; Palmer and Printz, 1999; Spencer et al., 2005; Spencer and Day, 2004). As described in **Section 1.8.9**, these nuclei are crucial for the normal expression of sympathoexcitatory responses to stress. There are no reports describing stress-evoked Fos expression in the hypothalamus in programmed hypertension, however increases in stress-evoked Fos expression in the hypothalamus have been described in the spontaneously hypertensive rat (Imaki et al., 1998; Palmer and Printz, 1999). Therefore, the exaggerated cardiovascular responses to stress in the spontaneously hypertensive rat (Palmer and Printz, 1999) may be due to increased activity in these hypothalamic regions. Investigation of Fos expression following acute stress in programmed hypertension is crucial to better understand the mechanisms underlying hypertension in this model.

1.11 HYPOTHESIS AND AIMS

1.11.1 Hypothesis

1. Programmed rats (offspring of dams exposed to a high fat (HF) or high sucrose diet (HSU) during pregnancy) exhibit a hypertensive phenotype and altered autonomic function at rest
2. Programmed rats exhibit altered cardiovascular and autonomic responses to physiological (dehydration) and psychological (air jet) stress
3. Programmed rats have an increased number of activated neurons (as determined by c-*fos* expression_ in response to air jets stress

1.11.2 Aims

1. To compare blood pressure and derived cardiovascular and autonomic parameters in offspring at 6 months of age of dams fed a high fat (34% fat) or control (4.8% fat) diet non-invasively using radiotelemetry. The cardiovascular parameters include systolic and diastolic blood pressure, pulse pressure, heart rate and pulsue interval. The autonomic parameters include heart rate and systolic blood pressure variability, spontaneous baroreflex sensitivity, baroreflex effectiveness index and maximum rate of change of the rise in aortic pressure during systole (dP/dt_{max}, as an index of cardiac contractility). Parameters will be measured in the absence stress and during exposure to a physiological (dehydration) or psychological (air jet) stressor.
2. To compare blood pressure and derived cardiovascular and autonomic parameters in offspring at 6 months of age of dams fed a high sucrose (10% sucrose) or control (0% sucrose) diet using radiotelemetry. Parameters will be measured in the absence of stress and during exposure to a physiological (dehydration) or psychological (air jet) stressor.
3. To determine whether the offspring of dams fed a high sucrose diet exhibit differences in Fos expression (as a marker of neuronal activation) in key hypothalamic structures (dorsomedial hypothalamus, perifornical area and paraventricular nuscleus) following a psychological (air jet) stress.

CHAPTER 2: METHODOLOGY

2.1 OVERVIEW

These experiments were designed to investigate the propensity for hypertension in the offspring of rat dams fed a high fat (HF) or high carbohydrate (sucrose, HSU) diet during pregnancy. Pregnant rats were fed either a standard chow (6% fat), high fat chow (34% fat) or high carbohydrate (10% sucrose solution in water) diet for a period of time (discussed in detail below). Protein levels were maintained similar at 20-26% energy in all feed, a difference that is unlikely to impact on foetal programming. The offspring were kept for 6 months before commencement of experiments.

Adult offspring rats (6 months old) were implanted with a radio telemetry probe under general anaesthesia, for blood pressure measurement in the awake, freely moving animal. Following a recovery period of approximately 7 days, blood pressure was recorded in the rat over a nine-day period, during which time they were subjected to dehydration (a physiological stressor) by removing access to water for three days. Following recovery, the rats were also subjected to an air jet stress (psychological stressor), which consisted of 15 minutes of repeated exposure to puffs of air. The rat was then perfused using paraformaldehyde to fix the brain. The brain was removed and prepared for c-fos staining of neurons using an immunohistochemical procedure.

The Animal Care and Ethics Committee at The University of Sydney approved all experimental protocols and all experiments were done in accordance with the NHMRC Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (2004). Experiments were conducted in *Sprague Dawley (SD*) rats for all the experiments. A total of 23 males and 20 female SD rats were used for HF experiments and a total of 16 male SD rats were used for HSU experiments.

2.2 HIGH FAT AND HIGH SUCROSE MODELS OF PROGRAMMED HYPERTENSION

2.2.1 High fat diet

Female Sprague-Dawley rats (age 5 weeks; approximate weight 157-173g) were maintained under light and temperature-controlled conditions and fed either a HF diet (34% fat omega-6 PUFA, 26% protein, energy 22.3 KJ/g) or control diet (6% fat, 23% protein, energy 13.9 KJ/g), by Specialty Feeds Services, Western Australia, commencing four weeks prior to mating. After mating, female pairs were established for the period of gestation. Dietary interventions were maintained throughout gestation and switched to a standard diet on day one of lactation.

2.2.2 High sucrose diet

A second group of rats (age 5 weeks; approximate weight (g) 249 -267.) were also maintained under controlled conditions and given either a high sucrose diet or a control diet. The high sucrose diet rats consumed a standard chow (4.8% fat, 20% protein, energy 14.0 MJ/Kg digestible energy, Specialty Feeds), but in addition to being provided with normal drinking water, they were also provided with drinking water that contained sucrose at 10% wt/vol (Coles white sugar; Victoria, Australia) concentration (**Figure 2.1**). The average daily amount of drink intake of sucrose drink was 112.5±17.3 mL and water was 29.9±6.8mL for the sucrose group (**Figure 1 in Appendix**). The control rats were fed the same control diet and normal drinking water only. The dietary interventions were the same as for HF rats: established four weeks prior to mating, one week during mating (where they were co-housed with a male rat), and subsequently for the 3 weeks of gestation. For both high fat and high sucrose protocols, female rats were housed separately and mated at night by introducing a male rat into the cage. The following morning vaginal swabs were performed to confirm mating by the presence of sperm on the swab. If sperm was identified, this was taken as confirmation of pregnancy and as embryonic day zero. All rats were housed in The University of Sydney animal houses throughout gestation and lactation.

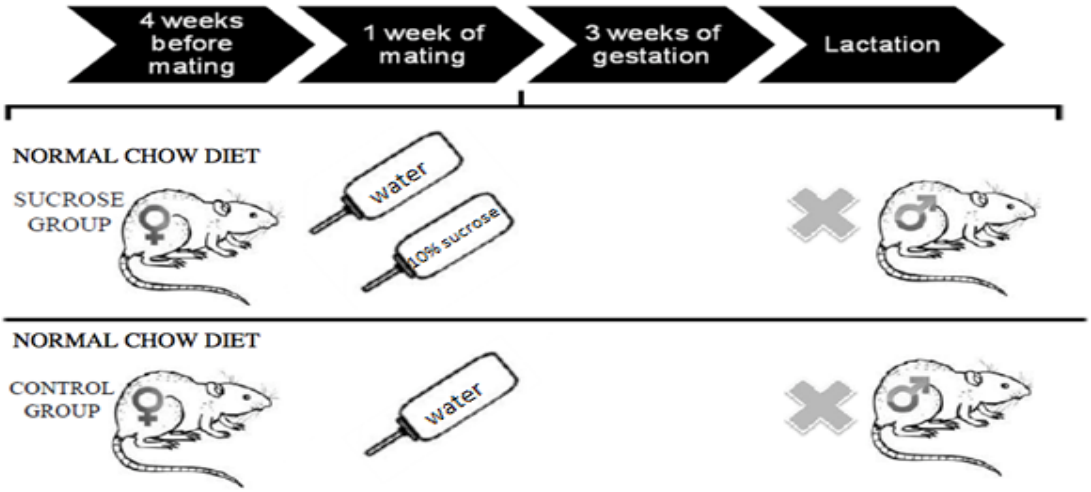

Figure 2.1: ***Experimental design for high sucrose model***

Both maternal control and sucrose groups were provided with a chow diet and water. In adition to this the sucrose group was provided with a 10% sucrose solution (w/v). The dietary intervention period was implemented 4 weeks before mating, 1 week during mating and for thee weeks of gestation. Figure adapted from Ekayanti, 2013.

On the day of parturition, litters were culled to 8 pups per dam to ensure equal nutrition within the offspring. At this stage, all dams were provided with a standard diet and pups remained with the mother until weaning at an age of three weeks. Post weaning, the pups were housed in-group cages of males and females until the commencement of experiments. Prior to commencement of experiments rats were brought up to the laboratory and housed in an approved animal holding room within the laboratory. The room in which the rats were held was temperature controlled (approximately 25°C) with a 12:12 light: dark cycle and all rats had free access to water and chow.

2.3 MEASUREMENT OF CARDIOVASCULAR PARAMETERS IN THE OFFSPRING

2.3.1 Surgical implantation of radio telemetry probes

At approximately 6 months of age the rats underwent a surgical procedure to implant a telemetry blood pressure transmitter (PAC11-40, Data Science International, St, Paul, MN, USA). Blood pressure was measured using a pressure-sensing catheter that was inserted in the upstream direction into the abdominal aorta. The catheter is connected to a pressure transducer that transmits the pressure signal via radio frequency signals. This radio frequency signal can be detected and recorded when the rat is placed on a receiver, which is connected to a computer. The

pulsatile arterial pressure signal was recorded at a sampling rate of 1000 Hz using DATAQUEST software. Cardiovascular variable (heart rate (HR) pulse interval (PI), systolic blood pressure (SBP), diastolic blood pressure (DBP) and pulse pressure (PP)) and autonomic indices (spontaneous baroreflex sensitivity (sBRS), baroreflex effectiveness index (BEI), heart rate variability (HRV), systolic blood pressure variability (BPV) and maximum rate of change of blood pressure (dP/dt_{max})) were calculated post-hoc using Spike2 software (CED, Cambridge, UK). This method of recording blood pressure is considered a gold standard method whereby blood pressure waveforms can be measured in the conscious rat in its home cage without disturbing it or causing any unintended stress (Huetteman and Bogie, 2009).

2.3.2 Anesthesia

The rat was anaesthetized via a two-step process; anaesthesia was first induced by inhalation of isoflurane (5%, Isoflo, Abbott Laboratories Inc.) by placing the animal in a box connected to a ventilator that pumped an isoflurane-air mixture into the box. Anesthesia was maintained by intraperitoneal injection of medetomidine hydrochloride (300-500 µg/kg) and ketamine hydrochloride (60-100 mg/kg). To ensure adequate anaesthesia, pinching of the hind paw was used to test withdrawal reflexes. The level of anesthesia was tested throughout the surgical procedure to ensure that the animal felt no pain and was not arousing from anaesthesia. Supplementary doses of medetomadine and ketamine were administered if the animal showed any withdrawal reflexes during the surgical procedure. Throughout the procedure, body temperature of the rat was monitored and maintained at approximately 36°C by a heating blanket placed under the animal.

2.3.3 Implantation procedure

The surgical procedure was performed under aseptic conditions: all surgical instruments were washed overnight using a medical instrument detergent (Pyroneg, Suma) and water. Blood pressure transmitters were also sterilized in a 2% Glutaraldehyde solution H_2O for up to three hours and soaked in saline overnight.

After appropriate anesthesia was acquired the animal was placed in a supine position and the abdomen was shaved and cleaned with saline solution. A midline incision was made through the skin and abdominal muscles; the intestines were carefully removed from the abdominal cavity to

expose the descending aorta at the level between the renal arteries and bifurcation of the iliac arteries. The intestines were kept moist by covering them with gauze soaked in saline.

A section of the aorta was cleared of overlaying connective tissue and fat using cotton tip applicators to allow good visualization of the aorta. A section of the aorta was isolated vascularly by temporarily ligating lateral vessels arising from the aorta using a suture. Vascular clamps were placed proximally and distally on the aorta from the site of catheter insertion. Using a 19G needle a small hole was made in the aorta to allow the insertion of the catheter. The catheter was inserted 1cm into the abdominal aorta, in the direction of the heart (**Figure 2.2**), and glued in place using few drops of tissue adhesive (3M Vetbond), which also facilitated haemostasis. A small sheet of cellulose patch (Data Science International) was placed over the aorta at the site of cannula insertion to help tissue growth and to promote healing. Following successful implantation the vascular clamps were removed and intestines repositioned. The body of the transmitter was sutured to the abdominal muscles and the abdominal muscles sutured closed (**Figure 2.3**). The skin incision was closed using autoclips. The entire procedure took approximately one hour.

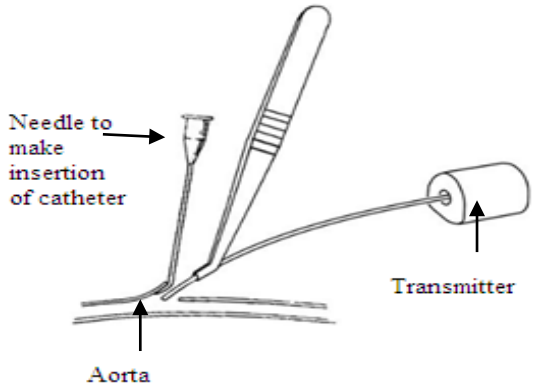

Figure 2.2: ***Vessel cannulation technique***

A small hole is made in the abdominal aorta using a needle and the catheter is inserted 1 cm into the aorta in the direction of the heart. Adapted from Huetteman and Bogi (2009).

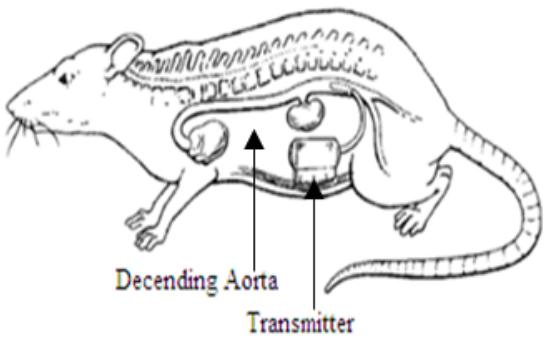

Figure 2.3: ***illustration of a rat implanted with a blood pressure telemetry device***

The catheter is glued in place with tissue adhesive. The body of the transmitter is sutured to the abdominal wall. Adapted from Huetteman and Bogi (2009).

2.3.4 Postoperative care

Following the surgical procedure the rat was given fluids (5% glucose, subcutaneously), antibiotics (procaine penicillin, 30 mg/kg, subcutaneously), non-steroidal anti-inflammatory (Carprofen, 4mg/kg subcutaneously) and an alpha-2 receptor antagonist (atipamazole, 1mg/kg) to reverse the actions of medetomidine. The animal was then placed in its home cage in a quiet room with a heating lamp and monitored until awake. Following recovery from anaesthesia, the animal was monitored twice daily and weights checked to ensure no post-surgery complications. This comprised checking for hind limb weakness, signs of pain or distress and hydration status by following guidelines on animal monitoring forms. If the weight of the rat was found to have reduced below 15% of the pre-operative weight, the rat was euthanized. Postoperatively, the rats were housed separately and left to recover for at least 7 days before commencements of experiments.

2.4 TEST PROTOCOLS (PHYSIOLOGICAL AND PSYCHOLOGICAL STRESSORS)

2.4.1 Physiological stressor (dehydration protocol)

Dehydration produces hypovolaemia and hyperosmolarity, both of which activate the sympathetic nervous system, increase vasopressin release and increase blood pressure (Collister *et al.,* 2014). There is evidence that the physiological response to dehydration is similar to that of a high salt diet, a known risk factor for hypertension (Toney and Stocker, 2010). Therefore this physiological test was performed both to increase sympathetic activity and according to the rationale that dehydration may mimic the effects of a high salt diet over a shorter time course.

Following the post-surgery recovery period, the rat was transferred in its home cage to the telemetry recording room. The telemetry room was arranged to allow recordings to take place with minimal external disturbances during the experimental protocol. The test room was temperature controlled (approximately 25 degrees) with a 12:12 light: dark cycle.

The dehydration protocol comprised three days of baseline recording, followed by three days of dehydration, where the animal was deprived of water, and then three days recovery, where access to water was returned. Therefore the dehydration protocol comprised a total of 9 days (baseline, dehydration and recovery). During this entire time recordings of blood pressure were made for 5

minutes each hour at a sampling rate of 1000 Hz. The animal was monitored throughout the protocol and body weight measured daily to ensure the dehydration was being managed adequately: dehydration was ceased and water returned to the rat if its body weight fell below 15% during the dehydration period. This did not occur in any of the animals studied.

2.4.2 Psychological stressor (air jet stress protocol)

After a period of recovery from the dehydration protocol, the animal was subject to a psychological stressor. The air jet stress is commonly used and has been well characterized in studies done previously in rats (Spencer *et al.,* 2005; Furlong *et al.,* 2014). The animal was left in its home cage in the telemetry room for baseline recording for 30 minutes before the air jet stress was performed.

The air jet protocol consisted of a series of air puffs (approximately 500 kPa pressure) blown towards the head of an unrestrained animal at a distance of approximately 10 cm. The air puffs were delivered via a metal nozzle trigger connected to a cylinder of medical oxygen via a plastic tube. The air jet stress consists of air puffs in blocks; each block consisted of three 2-second air puffs, each separated by a 10 second gap. In total 9 blocks of air puffs were administered to the rat with a rest period of 1 minute between each block. The total duration of the air jet stress protocol was approximately 15minutes (**Figure 2.4**). Following the air jet stress, the animal was left quietly for 2hours for further recording and to allow for c-fos expression before the animal was perfused with 4% paraformaldehyde solution (see below).

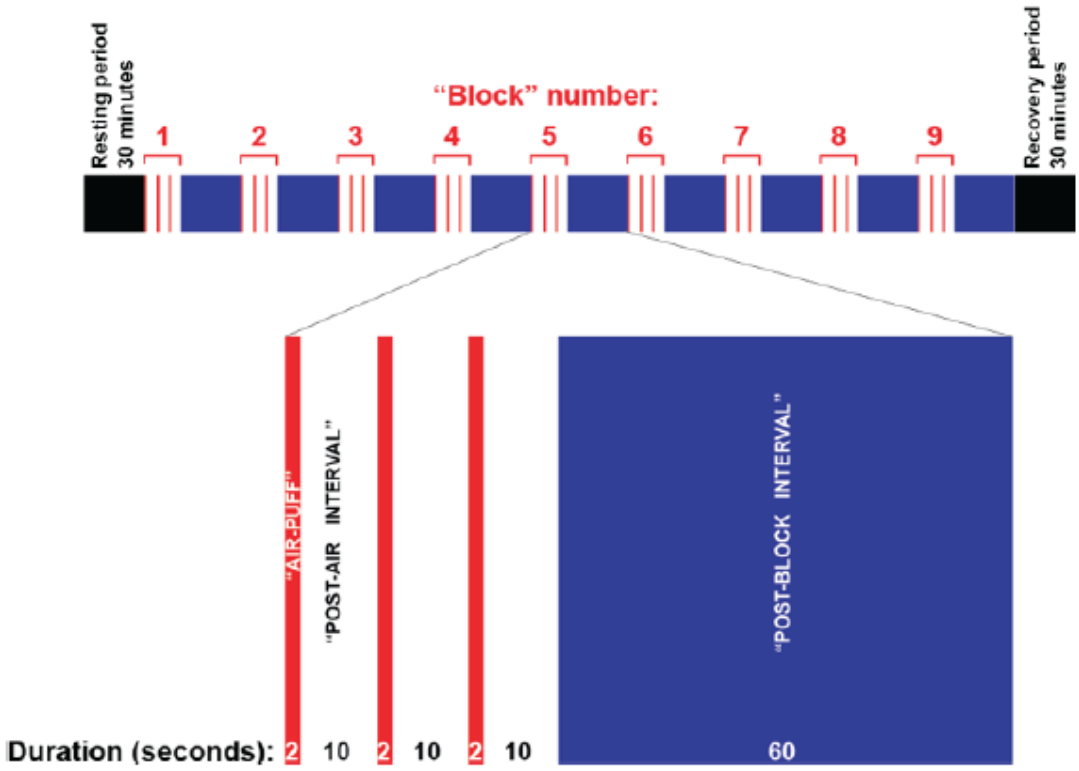

Figure 2.4: ***Diagram representing the air jet protocol***

Data were recorded for 30 minutes before the commencement of air jet stress. The air jet stress consists of air puffs delivered in blocks with a total of 9 blocks. Each block consisted of three puffs, 10 seconds apart. Each block was 60 seconds apart. Each air puff was delivered for 2 seconds. At the end of the air jet stress, data were recorded for 30 minutes as the recovery period. Figure adapted from McDowall (2007).

2.5 HISTOLOGICAL PROCESSING

2.5.1 Perfusion

Rats were deeply anaesthetised using pentobarbital sodium (120 mg/kg, delivered intraperitoneally). A deep level of anaesthesia was confirmed by the total absence of any withdrawal response to a strong pinch of the hind paw and observation of a depressed respiratory rate. After adequate anaesthesia was established the animal was placed in a supine position and a large midline incision was made through the skin and abdominal muscles. The incision was extended up to the thorax and the diaphragm was exposed. Using scissors the ribs were cut to expose the heart and an injection of 500 units of heparin was made into the circulation, via the left ventricle. The perfusion of the rat was performed transcardially by inserting a 14 gauge needle into the ascending aorta via the apex the left ventricle. The needle was clamped in place using a

hemostat. Silicone tubing was connected to the needle and perfusion solutions were pumped through using a peristaltic pump (Masterflex model 7553-75 Cole Parmer Instruments Company Ltd) at a rate of 25ml/min. Initially, warmed 0.9% saline solution was pumped into the systemic circulation to wash away the blood. A small cut was made on the right atrium to allow for blood and saline to drain from the body. Whilst the saline was pumped through the body, the transmitter was removed and using a hemostat the aorta was clamped downstream.

When the fluid from the right atrium drained clear, the rat was perfused with cold, 4% paraformaldehyde (Sigma-Aldric Inc.) in phosphate buffer (0.1 M, pH 7.4) for approximately 20 minutes. The brain was then carefully removed and post-fixed in 4% paraformaldehyde solution for up to 45minutes at 4°C, before the solution was replaced with 20% sucrose in phosphate buffer solution (PBS) and refrigerated at 4°C for a minimum of 24 hours.

2.5.2 Sectioning

Using fine forceps any remaining pia mater surround the brain and brainstem was carefully removed, this allowed the smooth sectioning. The brain was divided into three separate blocks, cut transversely for sectioning. The first transverse cut was made at approximately the level of the colliculus and the other at the level of the optic chiasm. This region included most of the midbrain and the hypothalamus. Each block was carefully mounted on the stage of a carbon dioxide-freezing microtome (model 1320, Leica) using 20% sucrose-PBS solution (0.1 M pH 7.4) and sections were cut at a 40 µm thickness. To ensure minimal damage to the brain whilst cutting, firstly a solid ice base was formed on the microtome stage using sucrose solution. The brain was carefully placed on the solidified solution base and a protective barrier was formed around the brain by freezing the sucrose solution, this allowed stable and protective layer during cutting. Five sequential series of brain sections were collected into sample containers with PBS (0.1M, pH 7.4) and refrigerated. The brain sections in series one were transferred into separate wells on a well tray (ProSciTech, HCE11) to maintain correct order of sections. Series two was used for immunohistochemical staining for Fos.

2.6 IMMUNOHISTOCHEMICAL STAINING

2.6.1 c-Fos immunohistochemistry

c-Fos expression is used as a marker for neuronal activity throughout the neuraxis following peripheral stimulation (Bullitt). Fos is the protein product of the gene c-fos; Fos protein can be stained to allow for visualisation in a semi-quantitative manner by using methods of immunohistochemistry. The formation of a black precipitate in the nucleus of a neuron is a marker for Fos and is indicative of an activated neuron (Dragunow and Faull, 1989). There is evidence that Fos expression is greatest approximately two hours after a stimulus is applied (Nestler, 2001), therefore rats were perfused two hours after the air jet stress test to allow for maximum Fos expression.

One of the five series of sections were used for Fos staining; these sections were carefully washed and incubated three times with 50% ethanol in water for 10 minutes on an orbital mixer. Incubating the tissue in ethanol allows the lipid membrane to become more permeable, thereby facilitating the penetration of antibodies into the cell. To block non-specific antigen binding, sections were incubated with 20% normal horse serum (NHS) in PBS for 30-60 minutes using the orbital mixer. Sections were then incubated in the primary antibody at 4°C for 36-48 hours. The primary antibody used was rabbit polyclonal anti-Fos IgG (sc-52, Santa Cruz Biotechnology Inc.) at a dilution of 1:1000 in a solution containing 20% NHS in 0.1M PBS.

After 48-36 hours sections were washed three times with 0.1M PBS for 10 minutes per wash on an orbital mixer. Following the three washes, sections were incubated in the secondary antibody, biotinylated donkey anti-rabbit IgG (1:400 dilution in PBS, GE Healthcare Australia Pty. Ltd.) overnight at 4°C.

Sections were washed again for 10 minutes (3 X 10min) with 0.1M PBS, and incubated in ExtrAvidin peroxidase conjugate (diluted 1:1000 with PBS, Sigma) at room temperature for one hour. At the end of the ExtrAvidin peroxidase incubation the sections were washed (3 X 10 min) with 0.1M Tris buffer and placed in a solution of 0.1 M Tris buffer containing: 3,3' diaminobenzidinetetra-hydrochloride (DAB, 25μg/ml), D-glucose (0.5%), ammonium chloride (0.04%) and nickel sulphate (1%) for five minutes. Expression of Fos was then revealed by

reacting the tissue by adding 10μl of glucose oxidase to produce a black reaction product. The reaction was then stopped by washing in 0.1M PBS.

2.6.2 Mounting

Following immunohistochemical staining, sections were placed in rostrocaudal order. Sections were mounted onto gelatinized slides (0.5% gelatin) and allowed to dry in room temperature for 24 hours. Sections were dehydrated by dipping the slides in 100% alcohol, cleared in histolene and coverslipped.

2.7 DATA ANALYSIS

2.7.1 Telemetry data

Blood pressure waveform recordings were obtained using acquisition software (Data Sciences International Inc.). A sample rate of 1000 samples per second was used to allow for a high fidelity of construction of the digitized blood pressure waveform. Other measurements such as HR, SBP, DBP, PP, heart rate variability (HRV) and spontaneous baroreceptor reflex sensitivity (sBRS) were derived from the blood pressure waveform using a script within Spike2 software (Cambridge Electronic Design Ltd.). Any further statistical analysis was performed using Excel or Graphpad Prism version 6.

2.7.2 Data grouping

The dehydration protocol consisted of scheduled recordings of blood pressure every hour for 5 minutes. Each five-minute period was grouped into 12-hour blocks, consisting of 12-hour day and 12-hour night cycles for each of the nine days. Each of these 12 hour blocks were then averaged and cardiovascular parameters were obtained.

The air jet protocol consisted of continuous recordings of blood pressure. Cardiovascular parameters were calculated for two 15 minutes periods immediately before the commencement of the air puff stress test to determine the baseline values, and for two 15 minute periods after air puff to determine the post-stress recovery values. The data during the stress test were analysed during the one minute wait-period between each block of air puffs. It was not possible to measure blood pressure during the air puff because of the movement artifacts caused by the rat's running. Each

one-minute period was averaged to obtain cardiovascular parameters for the stress period, a total of nine, one-minute measurements were made.

2.7.3 Spontaneous baroreceptor reflex (sBRS)

In the rat, for a given change in SBP there is a time delay before the reflex response in PI is initiated, this baroreflex delay has been calculated previously as a delay of 3-5 beats (Oosting et al. 1997). To summarise, a sBRS sequence was accepted if there was a minimum of three beats of consecutive increasing or decreasing SBP (four SBP-PI pairs), the linear regression was positive and the correlation (r^2) coefficient greater than 0.85 for each time delay (3, 4 and 5 beats). If these criteria were fulfilled, then the sBRS for each sequence was calculated as the average of the slopes for each time delay.

Baroreflex effectiveness index (BEI) is the ratio between the number of sBRS sequences (SBP ramps followed by the appropriate PI reflex response) and the total number of SBP ramps observed for a given time period (day/night or baseline, stress, recovery) (Di Rienzo *et al.,* 2001). BEI is therefore an index of how frequently the baroreceptor reflex is initiated in response to a change in SBP.

2.7.4 Spectral analysis of heart rate and systolic blood pressure variability

HRV and BPV were determined in spike2 software (CED) using customized scripts. A power spectral analysis was done by performing a fast Fourier transformation (FFT) on heart rate and SBP waveforms, which provides information on the distribution of power (variance) in different frequency components.

HRV: Measurements examine quantitatively how heart rate varies rhythmically over time (Task Force of The European Society of Cardiology and The North American Society of Pacing and Electrophysiology 1996).

BPV: The analysis of the variation in beat-to-beat systolic blood pressure is very similar to that of HRV. A systolic BP waveform is created at 10 Hz using a spline interpolation protocol and an FFT performed as described above. The frequency ranges are identical to that of HRV. The VLF (0-0.25 Hz) and the LF (0.25-0.75 Hz) components similarly represent hormonal influences on total

peripheral resistance and sympathetic vasomotor modulation, respectively (Waki *et al.,* 2006). The HF component of BPV is unlikely provide any information about parasympathetic influence because there is no significant parasympathetic innervation of the blood vessels.

2.7.5 dP/dt_{max}

In this study, dP/dt_{max} was used as an estimated index of left ventricular contractility; it is the maximum rate of left ventricular pressure rise during systole and is determined by the first differential of the ventricular contractility. This differential provides an estimate of the sympathetic inotropic state of the left ventricle

2.7.6 Statistical analysis

All data were expressed as mean ± SEM. Birth weights, cardiovascular and autonomic parameters at rest and Fos-labelled neurons between groups were analysed by an unpaired student t-test. Cardiovascular and autonomic parameters during dehydration and air jet stress were statistically determined using two-way analysis of variance (ANOVA) with or without repeated measures, as necessary, and a Bonferroni's correction for multiple comparisons (GraphPad Prism 6, GraphPad software).

2.8 IDENTIFICATION AND QUANTIFICATION OF FOS-LABELLED NEURONS

Microscopy and image analyses were carried out in the Bosch Advanced Microscopy Facility. A comparison of the number of Fos-labelled neurons in response to air jet stress between control and high sucrose programmed rats was made in 3 key hypothalamic structures: the dorsomedial hypothalamic area (DMH), the perifornical area (PeF) and the paraventricular nucleus (PVN). Hypothalamic sections of interest were selected according to the location of these nuclei identified by their cytoarchitectural features, according to an atlas of the rat brain (Paxinos and Watson, 2007). The major identifying features were the shape and location of the optic tract, the distance between the fornix and the mammillothalamic tract and the third ventricle. Sections were examined microscopically and photographed using an Olympus stereology microscope with a motorised xyz-stage. A high-resolution image montage at 200x magnification ("virtual slice") was

created using Stereo Investigator software (MBF Bioscience, VT, USA) for 2D anatomical mapping. The image montage was analysed using Metamorph (Molecular Devices Inc, CA, USA) to create a “mask” image that identified Fos-labelled nuclei according to the criteria of colour intensity and shape area. Each region (DMH, PeF and PVN) was then selected and cell counts extracted via an automated process. This automated procedure for Fos identification minimises subjective bias as to the identification criteria for Fos labelling. Counts were done bilaterally for each area and then halved. Therefore an estimate of Fos-labelled neurons was determined for one side of the hypothalamus.

CHAPTER 3: RESULTS

3.1 OVERVIEW

To investigate the propensity for programming of hypertension following a high fat diet (high fat model) or sucrose diet (high sucrose model), eight pregnant dams were given a high fat diet (HFD) and 12 dams were given a high sucrose diet (SUD). To compare these dietary interventions, 12 pregnant dams were given a control diet (CD) of standard chow. The dietary intervention was established for eight weeks; first for four weeks prior to mating, one week during mating, and subsequently for three weeks of gestation. On the day of parturition, litters were culled to eight pups per dam to ensure equal nutrition within the offspring and all litters were treated identically - at this stage mothers were given a control diet. For the high fat model, male and female offspring were investigated while for the high sucrose model only male offspring were investigated. The HFD and SUD rats were kindly provided to us by Dr. Kieron Rooney from the Faculty of Health Sciences and Prof. Robert Boakes from the Faculty of Science, respectively.

Our hypothesis was that offspring rats exposed to a HFD or a SUD during pregnancy may exhibit elevated blood pressure, caused at least partly by altered autonomic function resulting in elevated cardiovascular sympathetic activity. We also hypothesised that these effects may be exaggerated when animals are exposed to certain physiological stressors (such as dehydration) or psychological stressors (such as air jet stress).

To investigate these hypotheses, we used the method of radio telemetry to measure blood pressure in the awake, freely moving animal. From the blood pressure waveform, we derived a number of indices of autonomic function, including spontaneous baroreflex sensitivity (sBRS), baroreflex effectiveness index (BEI), heart rate variability (HRV), blood pressure variability (BPV) and maximum rate of change of aortic blood pressure during systole (dP/dt_{max}).

For the high fat model, both male and female offspring were investigated. Blood pressure probes were implanted in 12 control male offspring and 11 high fat (HFD) male offspring at six months of age. One control rat died during the implant whilst a further five HFD and four control rats did not recover adequately from surgery. Furthermore, the computer failed to record data on another two

HFD and two control rats. In addition, one control rat was excluded when a tumour was identified in the abdomen. Subsequently, data could only be obtained on four high fat and four control rats. Blood pressure probes were also implanted in 12 control and eight HFD female offspring at six months of age. Of these, four HFD and eight control rats did not recover adequately from surgery, leaving four control and four HFD female offspring from which data were obtained.

We are unclear as to why such a large number of rats did not recover well from the surgical procedure in the high fat model. Many of the rats exhibited rasping like breathing sounds pre-surgically. This is indicative of mucous secretion in their airways and suggests the possibility of infections present in our rat colony. We believe this may have significantly increased the risk of postoperative complications such as wound healing and nerve damage in these rats. In addition, It was noticed that there was a large number of obese rats in the female control group, with an average weight of 513g ± 4.0g, with large amounts of abdominal fat. The high failure rate in this surgical procedure will be discussed in more detail in **Section 4.2.1** of the discussion.

3.2 HIGH FAT MODEL

3.2.1 Offspring body weight

Gestation lasted for 21-22 days and all pups were born approximately 22 days post conception. Total of 54 males were born, of these 32 were from mothers fed a standard chow diet and 22 from mothers fed a high fat diet. A total of 34 females were born, of these 25 were born from mothers fed a standard chow and 9 from mothers fed a high fat diet.

Birth weights of males and females were similar between HFD and control dams (**Figure 3.2.1A**). At day 24-weaning, females had reduced body weight compared to their litter matched male offspring: this was statistically significant only in the HFD rats (P=0.02), while approaching statistical significance in control rats (P=0.06) (**Figure 3.2.1B**). At this age, there was no difference in body weight between HFD and controls within each sex (**Figure 3.2.1B**). At 94 days, females remained lighter than their litter matched male siblings. This was observed in both HFD and control rats ($P<0.01$ for both groups). At this age, male HFD rats had a lower body weight than male controls (P=0.02) (**Figure 3.2.1C**), although no difference was observed in the female rats (P=0.15).

Finally, at six months of age (time of implantation of telemetry transmitter) male offspring were approximately 143g heavier, on average, than females in both control and HFD rats (P<0.01 for both groups) (**Figure 3.2.1D**). Males no longer indicated a statistically significant difference in weight between groups (P=0.26), while in females there was a tendency for HFD rats to be lighter (P=0.07).

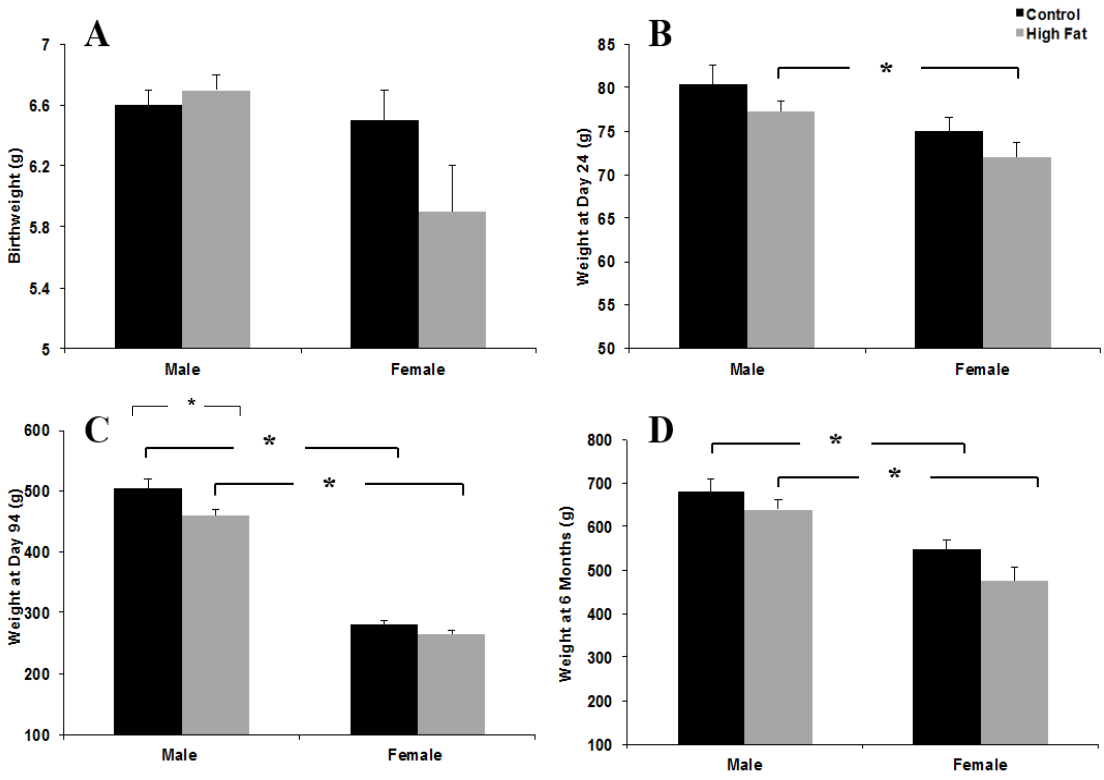

Figure 3.2.1: ***Body weights of male and female offspring in HFD and control groups at birth, day 24, day 94 and at six months of age (mean ± SEM) (unpaired t-test)***

(A) *No significant difference in weight between male and female rats at birth. Further, there was no significant difference between HFD and control rats within each sex.* ***(B)*** *At day 24 there was a significant difference between male and female HFD rats (P=0.02).* ***(C)*** *At day 94 females rats had a significantly lower body weight compared to males, this was observed in both HFD (P<0.01) and control (P<0.01) rats. Furthermore, male, HFD rats had a significantly reduced body weight compared to controls.* ***(D)*** *At six months of age there were significant differences observed between sexes in HFD (P<0.01) and control (P<0.01) rats, however no differences were observed between groups within sexes.*

3.2.2 Data acquisition protocol for measurement of cardiovascular and autonomic parameters in the awake, freely moving rat

Following a minimum of one week recovery from transmitter implantation surgery, blood pressure waveform was recorded at rest for each rat. Rats remained in their own home cage, which was transferred to the telemetry recording room. Recordings of blood pressure waveform (sampling rate of 1,000 Hz) were made for five minutes per hour, 24 hours a day. This ensured that the rats acclimatised to the recording room, which allowed for any diurnal differences in blood pressure or other cardiovascular variables to be examined carefully. From the blood pressure waveform signal, several cardiovascular and autonomic variables were derived. These included, SBP, DBP, HR, PP, sBRS, BEI, HRV, BPV and dP/dt_{max}. In general, the blood pressure trace was satisfactory; however any major movement artifacts or signal dropouts that occurred during this period were excluded from the analysis. The blood pressure waveform was deemed satisfactory if BP and PP measures were similar to other recorded rats with no major signal dropouts or major movement artefact occurring for long durations.

Figure 3.2.2 illustrates an example of a blood pressure waveform recording and derived parameters over 24 hours in a control rat. Note the discontinuity in the recorded data with sudden increases or decrease in the blood pressure trace. This is indicative of variations in the blood pressure between recording periods at different hours throughout day and night. There was considerable variability observed, depending on the level of activity of the rat. These fluctuations appeared to be greatest at night during the active phase of a rat. At times, large increases were seen in the blood pressure signal that occurred as a result of sudden movements caused by the rat. These values pertaining to the sudden movements were identified as movement artefacts and therefore were not representative of the true blood pressure. As such, these areas of the trace were excluded from analysis. The hourly recordings of blood pressure measurements were averaged into a night phase (1900 to 0600) and a day phase (0700 to 1800) for comparison between groups.

Figure 3.2.2: ***Blood pressure waveform recording and derived parameters over 24 hours in a control rat***

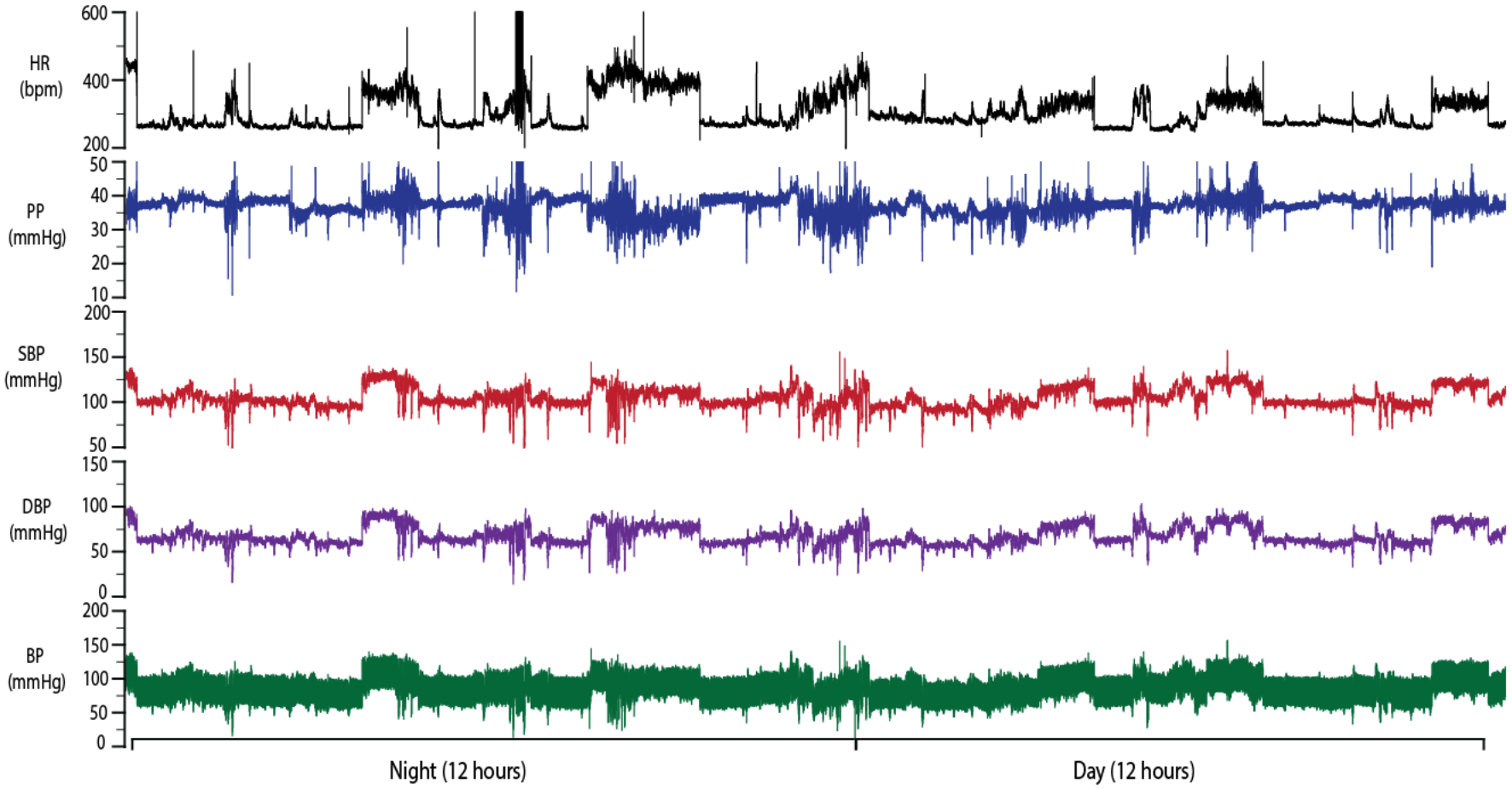

Figure 3.2.2: ***Blood pressure waveform recording and derived parameters over 24 hours in a control rat***

SBP: systolic blood pressure, DBP: diastolic blood pressure, HR: heart rate, PP: pulse pressure, BP: blood pressure. Note: some animals showed signs of diurnal rhythm, but that this was not consistently observed in all animals and the grouped data did not demonstrate diurnal rhythm.

3.2.3 Comparison of cardiovascular parameters in male and female control rats at rest

To determine if it was feasible to combine data from males and females, a comparison of cardiovascular parameters (SBP, DBP, PP and HR) was made from litter-matched male and female offspring, from mothers given a control diet.

There was no difference in resting DBP or PP between male and female rats (**Figure 3.2.3**). However, there were significant differences in resting SBP and HR between male and female rats during both night and day. It was also noted that neither male nor female rats exhibited a diurnal rhythm in their cardiovascular measures. This was evident as there were no significant differences in any of the cardiovascular variables between night and day in either males or females (**Figure 3.2.3**). Based on the differences observed in SBP between sexes in the control rats, it was decided to separate males and females for further analysis when comparing control and HFD offspring.

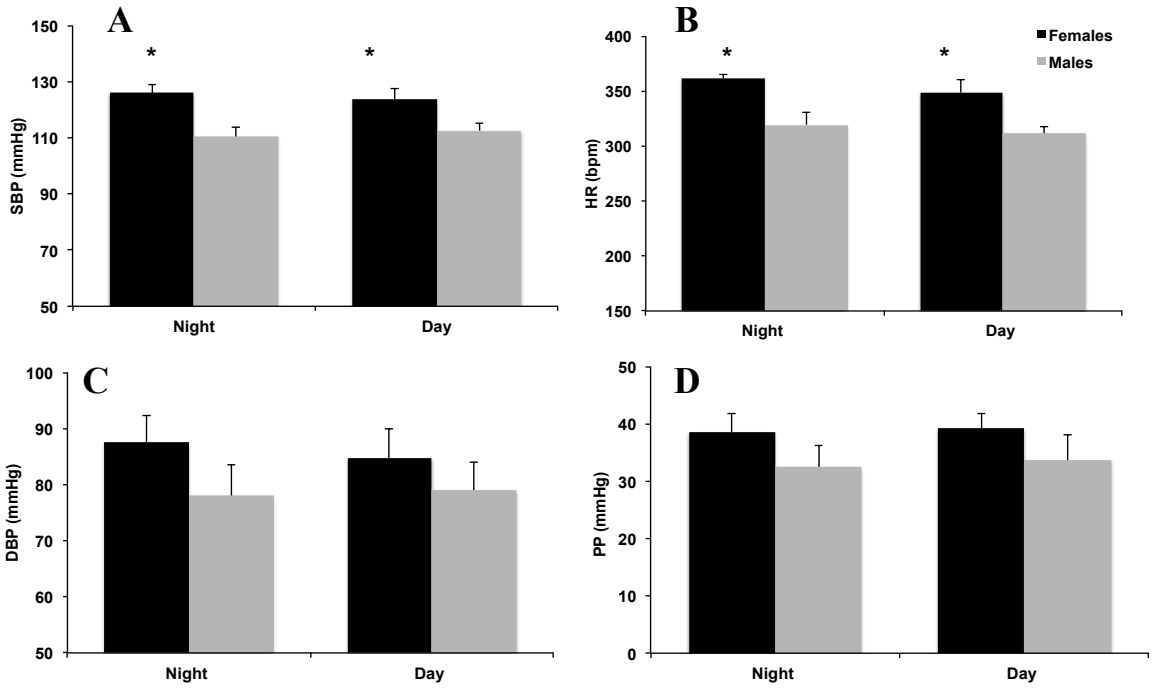

Figure 3.2.3: ***Resting baseline values for SBP, DBP, HR and PP in male and female rats (mean ± SEM) (unpaired t-test)***

A) *SBP was significantly higher in female offspring compared to their litter-matched male offspring during both night (P=0.01) and day (P=0.04).* ***B)*** *HR was significantly higher in female offspring compared to their litter matched male offspring during both night (P=0.01,) and day (P=0.03).* ***C)*** *There was no difference in DBP observed between male and female rats during the night (P=0.24) or day (P=0.47) phases.* ***D)*** *There was no difference in PP observed between male and female rats during the night (P=0.26) or day (P=0.31) phases.*

3.2.4 Comparison of cardiovascular and autonomic parameters between high fat and control rats at rest

Cardiovascular parameters: Male and female data were separated in order to isolate the gender differences in cardiovascular and autonomic parameters. In males, SBP was approximately 23 mmHg higher, on average, in the HFD group than controls during night and day (**Figure 3.2.4A**). DBP was also higher in the HFD group during the night and close to significant during the day (**Figure 3.2.4B**), while HR was higher in the HFD group only during the night, by approximately 40 beats/min, on average (**Figure 3.2.4C**). There was no difference in PP between groups either during the night or day (**Figure 3.2.4D**).

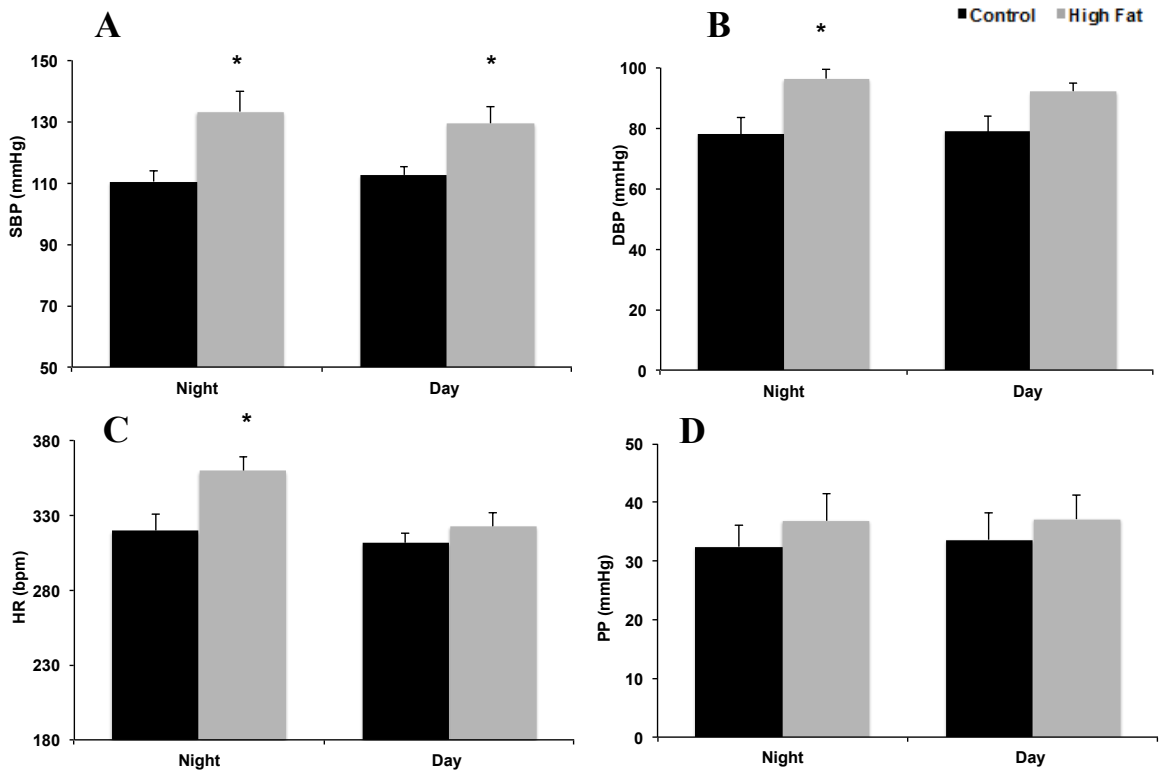

Figure 3.2.4: ***Comparison of cardiovascular parameters in HFD and control male rats during night and day (mean ± SEM) (unpaired t-test)***

A) *There was a significant difference observed in male SBP between groups during night (P=0.02) and day (P=0.03).* ***B)*** *There was a significant difference in DBP observed between groups only during the night (P=0.03) and approaching statistical significance during the day (P=0.06).* ***C)*** *There was a significant difference in male HR observed between groups only during the night (P=0.04) and not during the day (P=0.35).* ***D)*** *There was no significant difference in PP between group either during the night (P=0.48) or day (P=0.59).*

In females, SBP was higher in the HFD group than controls during both night and day, by approximately 10-15 mmHg (**Figure 3.2.5A**). However, other cardiovascular variables: DBP, HR. and PP did not indicate a significant difference at rest between the two groups (**Figures 3.2.5B-D**).

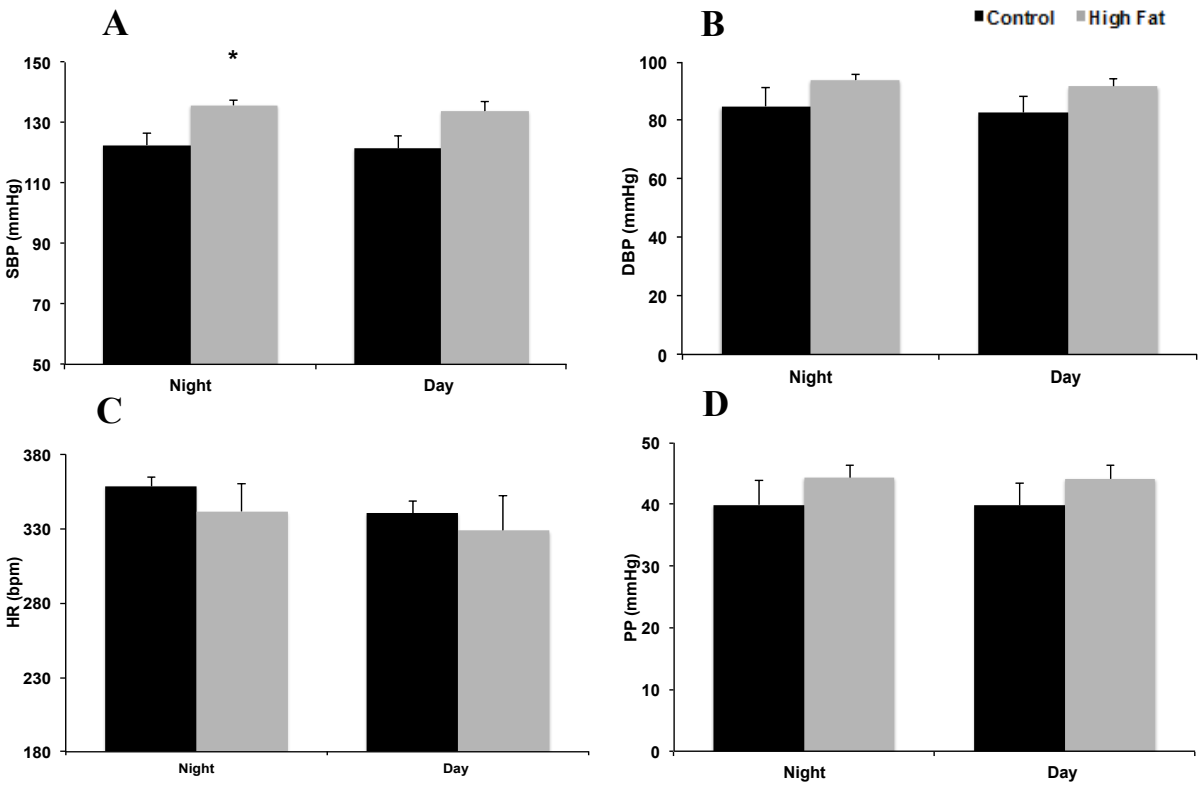

Figure 3.2.5: ***Comparison of cardiovascular parameters in HFD and control female rats during night and day (mean ± SEM) (unpaired t-test)***

A) *There was a significant difference observed in SBP between groups at night (P=0.05) and approaching significance during the day (P=0.08).* ***B)*** *There was no significant difference observed in DBP between groups during the night (P=0.29) or day (P=0.26).* ***C)*** *There was no significant difference observed in HR between groups during the night (P=0.63) or day (P=0.38).* ***D)*** *There was no significant difference in PP between group either during the night (P=0.37) or day (P=0.38).*

Autonomic parameters: Measurement of autonomic function of a rat is quite complex and generally requires invasive techniques to be used. To measure autonomic function in the conscious, freely moving rat, we used a non-invasive methodology. This methodology provides indices of autonomic function by examination of certain components of the blood pressure waveform and their relationship to heart rate. In particular, power spectral analysis on HR and SBP waveforms provide information on cardiac and vasomotor autonomic function. In addition, we used a sequence technique for measurement of spontaneous baroreflex sensitivity (sBRS) and baroreflex effectiveness index (BEI), a

measure of how often the baroreflex is activated in response to changes in blood pressure. Finally, we measured dP/dtmax, which has been reported to provide an estimate of cardiac contractility (Brington et al., 1997) and therefore represents the level of sympathetic activity to the ventricular myocardium.

sBRS and BEI: There was no significant difference in resting sBRS (**Figure 3.2.6A**) or BEI (**Figure 3.2.6B**) between control and HF offspring in either males (**Figure 3.2.6**) or females (**Figure 3.2.7**). It was also noted that neither HF nor control rats exhibited a diurnal rhythm in the baroreflex measures in the males or the females.

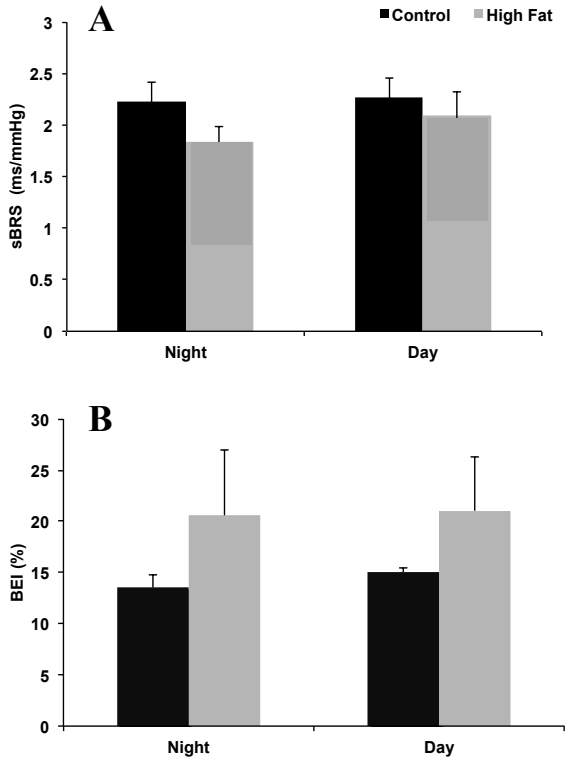

Figure 3.2.6: ***Comparison of sBRS and BEI at rest in HFD and control male rats (mean ± SEM) (unpaired t-test)***

A) *There was no difference in sBRS observed between groups during night (P=0.16) or day (P=0.60).* ***B)*** *There was no difference in BEI observed between groups during night (P=0.26) or day (P=0.23).*

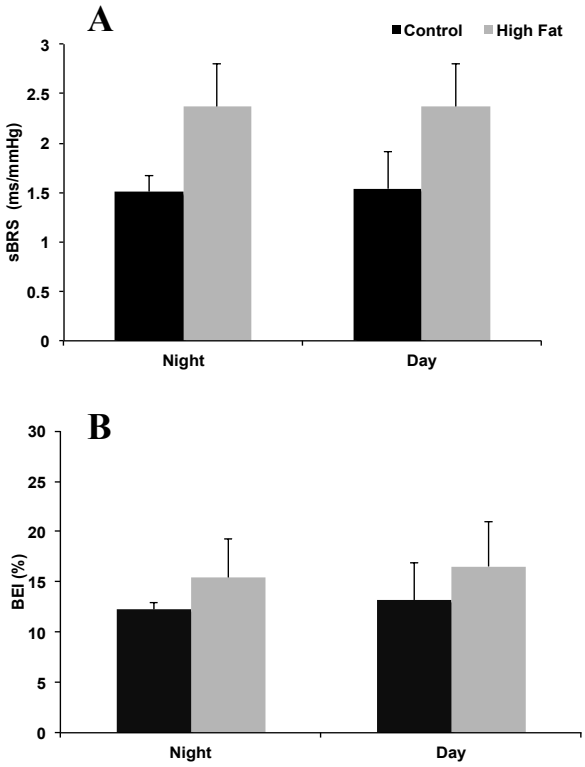

Figure 3.2.7: ***Comparison of sBRS and BEI at rest in HFD and control female rats (mean ± SEM) (unpaired t-test)***

A) *There was no difference in sBRS observed between groups during night (P=0.23) or day (P=0.28).* ***B)*** *There was no difference in BEI observed between groups during night (P=0.57) or day (P=0.63).*

HRV and BPV: Frequency domain analysis of HRV and BPV at rest did not indicate any differences between the groups at rest in either male (**Table 3.2.1**) or females (**Table 3.2.2**). Furthermore, neither HFD nor control rats exhibited a diurnal rhythm in any of the frequency ranges of HRV or BPV in the males or the females.

Table 3.2.1: Comparison of *HRV at rest in male and female HFD and control rats*

	LF (bpm^2)	LF%	LF:HF	HF (bpm^2)	HF%	TPWR (bpm^2)
Males						
Control (Day)	***0.22 ± 0.0***	***0.25 ± 0.03***	0.34 ± 0.05	***0.70 ± 0.15***	***0.75 ± 0.03***	***2.94 ± 0.34***
Control (Night)	0.37 ± 0.09	***0.25 ± 0.03***	0.33 ± 0.05	1.31 ± 0.28	***0.75 ± 0.03***	***5.17 ± 0.89***
HFD (Day)	0.26 ± 0.09	0.26 ± 0.01	0.37 ± 0.01	0.70+ ± 0.20	0.74 ± 0.01	4.19 ± 0.67
HFD(Night)	0.37 ± 0.09	0.24 ± 0.02	0.32 ± 0.02	1.03 ± 0.18	0.76 ± 0.02	5.15 ± 1.05
Females						
Control (Day)	***0.65 ± 0.21†***	***0.40 ± 0.03†***	0.31 ± 0.02	***1.94 ± 0.43†***	***0.603 ± 0.028†***	***8.13 ± 2.05†***
Control (Night)	0.70 ± 0.24	***0.37 ± 0.02†***	0.36 ± 0.01	1.96 ± 0.72	***0.633 ± 0.015†***	***8.57 ± 0.86†***
HFD (Day)	0.66 ± 0.20	0.25 ± 0.02	0.57 ± 0.10	1.17 ± 0.19	0.755 ± 0.016	5.73 ± 1.11
HFD(Night)	0.80 ± 0.20	0.27 ± 0.01	0.55 ± 0.06	1.74 ± 0.52	0.727 ± 0.013	6.23 ± 1.44

Note: † denotes difference between control male and female rats during either night or day.

There were no observed diffrences between HFD and controls within each sex. However, differences in HRV were observed between male and female offspring from the control group during night and day, these gender differences were lost in the programmed rats. (mean ± SEM) (unpaired t-test).

Table 3.2.2: ***Comparisons of BPV at rest in male and female HFD and control rats***

	LF ($mmHg^2$)	LF%	HF ($mmHg^2$)	HF%	TPWR ($mmHg^2$)
Males					
Control (Day)	**0.02 ± 0.00**	**0.56 ± 0.06**	0.01 ± 0.00	0.34 ± 0.05	**0.19 ± 0.01**
Control (Night)	**0.02 ± 0.00**	**0.58 ± 0.08**	0.02 ± 0.00	0.33 ± 0.05	0.23 ± 0.02
HFD (Day)	0.03 ± 0.00	0.60 ± 0.03	0.02 ± 0.00	0.37 ± 0.01	0.19 ± 0.02
HFD(Night)	0.04 ± 0.01	0.61 ± 0.04	0.02 ± 0.00	0.32 ± 0.02	0.21 ± 0.04
Females					
Control (Day)	**0.04 ± 0.00**†	**3.52 ± 0.30**†	0.02 ± 0.00	0.64 ± 0.01	**0.27 ± 0.05**†
Control (Night)	**0.04 ± 0.01**†	**4.61 ± 0.90**†	0.02 ± 0.00	0.63 ± 0.01	0.26 ± 0.04
HFD (Day)	0.04 ± 0.01	4.59 ± 1.51	0.03 ± 0.00	0.56 ± 0.06	0.21 ± 0.04
HFD (Night)	0.05 ± 0.01	4.37 ± 1.11	0.03 ± 0.00	0.61 ± 0.02	0.20 ± 0.03

Note: † ***denotes difference between control male and female rats during either night or day where significant.***
Differences in BPV were observed between male and female offspring from the control group, these gender differences were lost in the programmed rats. Furthermore, there were no observed differences between HFD and controls within males or females. (mean ± SEM) (unpaired t-test).

dP/dT$_{max}$: In this study, dP/dt_{max} was used as an estimated index of left ventricular contractility; it is the maximum rate of aortic pressure rise during systole and is determined by the first differential of the aortic blood pressure signal. This differential provides an estimate of the sympathetic inotropic state of the left ventricle (see **Figure 3.2.8** for a graphical representation; Salo et al., 2009). There was no difference in dP/dt_{max} at rest between HFD and controls rats in either male or female offspring (**Figure 3.2.9A**, **B**).

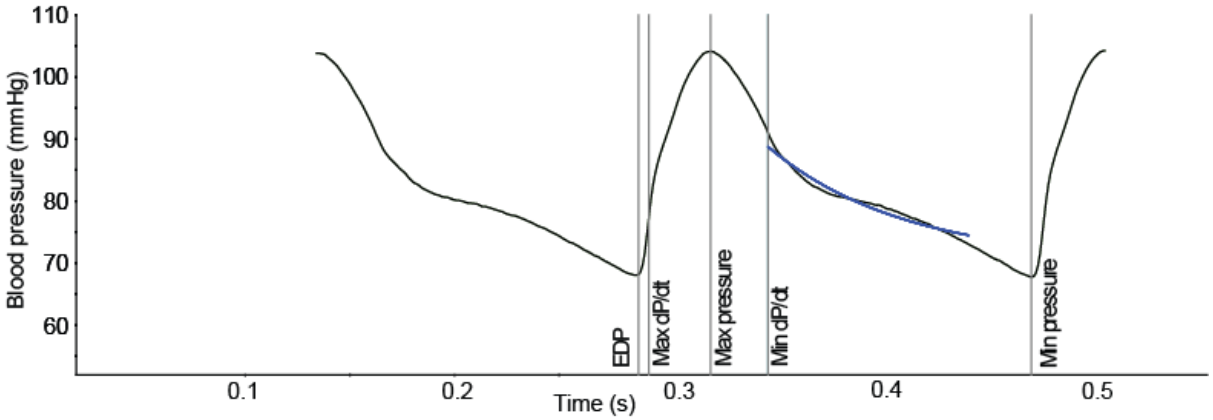

Figure 3.2.8: ***dP/dtmax during a single cardiac cycle***

EDP: end of diastole, dP/dtmax: maximum rate of change of rise in pressure difference over time during systole, dP/dtmin: maximum rate of fall in pressure difference over time during diastole. Figure adapted from Salo et al., 2009.

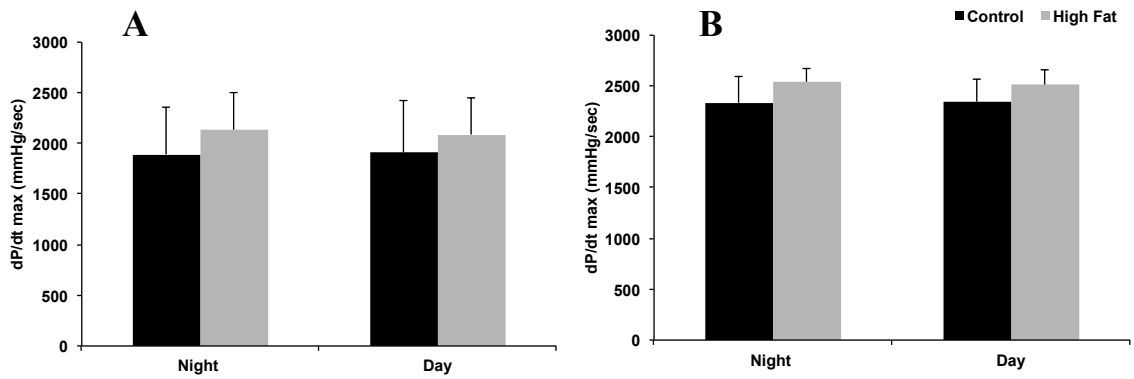

Figure 3.2.9: Comparison of *dP/dt$_{max}$ between control and high fat rats during night and day (mean ± SEM) (unpaired t-test)*

A) *Male, dP/dt$_{max,}$ there was no difference observed between groups during night (P=0.70) or day (P=0.78).* ***B)*** *Female, dP/dt$_{max,}$ there was no difference observed between groups during night (P=0.57) or day (P=0.57).*

3.2.5 Comparison of cardiovascular and autonomic parameters between high fat and control rats during dehydration

Following a baseline recording of three days, the rats were subjected to three days of dehydration (physiological stressor). Water was removed from the cage and the rats were deprived of water for a period of three days, after which free access to water was re-established for a three-day recovery period. Thus, the dehydration protocol consisted of a total of nine days; three days baseline, three days dehydration and three days recovery. For purposes of statistical analysis of dehydration data, we have focused on the data acquired during the night phase, when rats are most active. This period was chosen, as there were no significant differences between night and day for cardiovascular and autonomic parameters. However, the night phase of the rats showed the greatest differences in cardiovascular and autonomic parameters between groups (HFD and controls).

Cardiovascular parameters: In males, SBP and DBP were consistently higher in HFD rats compared to controls throughout the entire protocol (SBP: P=0.02) and (DBP: P=0.05; **Figure 3.2.10A, B**). HR and PP indicated a trend towards being higher in the HFD rats. However, this was not statistically significant (HR: P=0.08 and PP: P=0.54; **Figure 3.2.10C, D**).

With the initiation of dehydration, both groups indicated a trend towards an increase in SBP and DBP of approximately 10 mmHg. However, this did not reach statistical significance (**Figure 3.2.10A, B**). During the recovery period SBP and DBP appeared to drop below baseline levels in both groups, although again this was not statistically significant (**Figure 3.2.10A, B**). HR and PP did not differ

significantly to baseline levels throughout the dehydration and recovery periods in either group (**Figure 3.2.10C, D).** However, SBP and DBP were consistently higher in the HFD rats throughout the dehydration protocol. HFD rats responded to dehydration with a similar magnitude as the control rats (**Figure 3.2.11**).

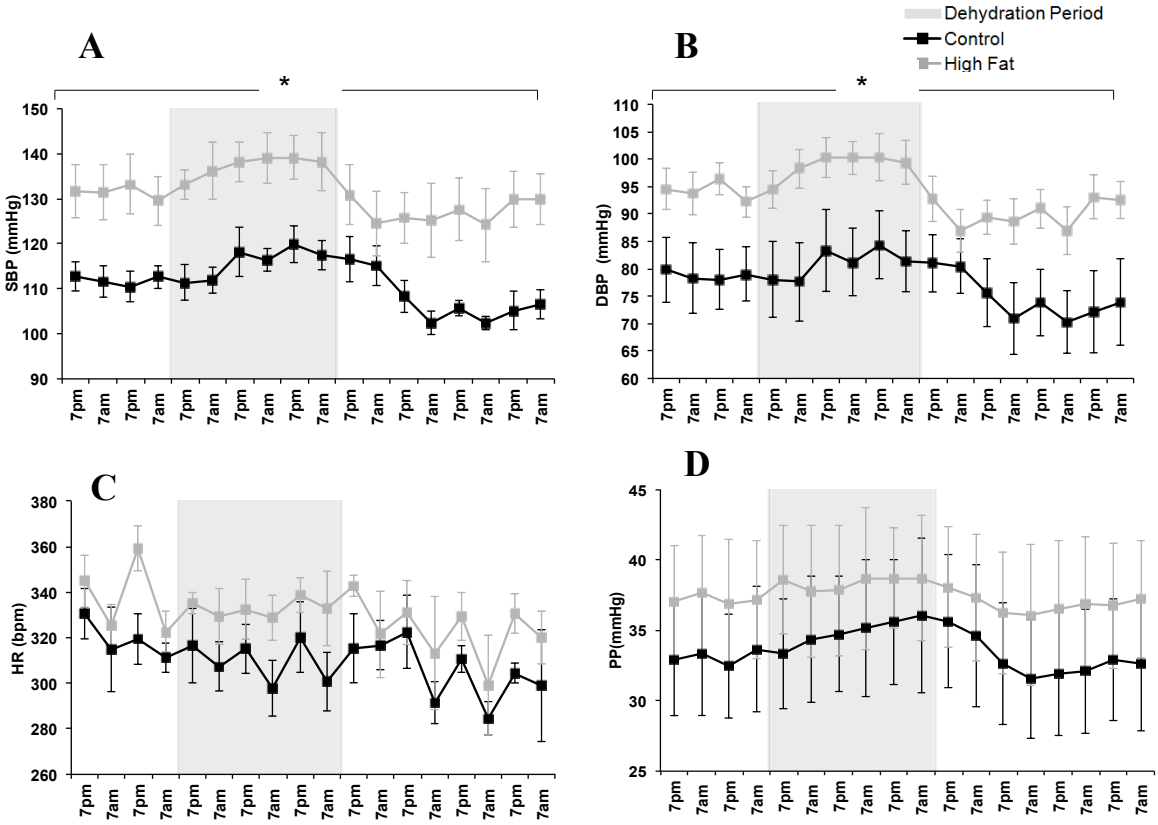

Figure 3.2.10: ***Cardiovascular parameters during dehydration protocol in the male rat (2-way ANOVA)***

The dehydration protocol is divided into three blocks, Baseline, Dehydration and recovery, the shaded area reflects the duration of dehydration and each data point signifies 12 hours of day or night. ***A)*** *There was a significant difference in SBP between groups (SBP: P=0.02), SBP remained higher in HFD rats throughout the dehydration protocol.* ***B)*** *There was a significant difference in DBP between groups (P=0.05) throughout the dehydration protocol.* ***C)*** *There was a trend for HR to by higher in the HFD group compared to controls throughout the protocol, however this was not statistically significant (P=0.08).* ***D)*** *There was no significant difference in PP between groups (P=0.54).*

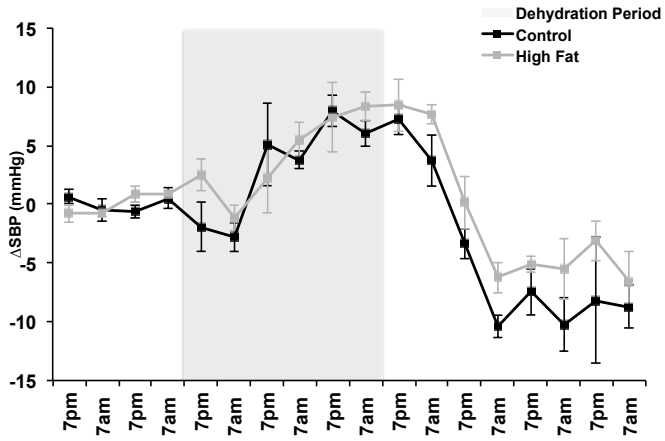

Figure 3.2.11: ***Change in SBP during dehydration protocol in the male rat (2-way ANOVA)***

There was no significant difference in the change in SBP between groups in response to dehydration (P=0.68).

In females, SBP was higher in HFD rats compared to controls throughout the entire protocol (P=0.04). However there was no difference in DBP, HR or PP between groups (**Figure 3.2.12**). With the initiation of dehydration there was an increase in SBP of approximately 8 mmHg in both groups (HFD: P=0.02, control: P=0.06; **Figure 3.2.12A**). There was no significant change in DBP in response to dehydration (HFD: P=0.61, control: P>0.99; **Figure 3.2.12B**).

In contrast to males, dehydration caused a significant increase in PP from baseline in both groups of approximately 7 mmHg (control: P<0.01, HFD: P=0.01), although the magnitude of increase was similar between groups. There was no significant increase in HR in response to dehydration in either HFD or control rats (**Figure 3.2.12C**). Following re-introduction of water, SBP and DBP dropped below baseline levels by approximately 8 mmHg (HFD, SBP: P=0.01, DBP: P=0.01 and control, SBP: P<0.01, DBP: P<0.01; **Figure 3.2.12A, B**), this drop in SBP and DBP was similar between groups. There were no observable difference in HR or PP in either HFD or control rats from baseline (**Figure 3.2.12C, D**).

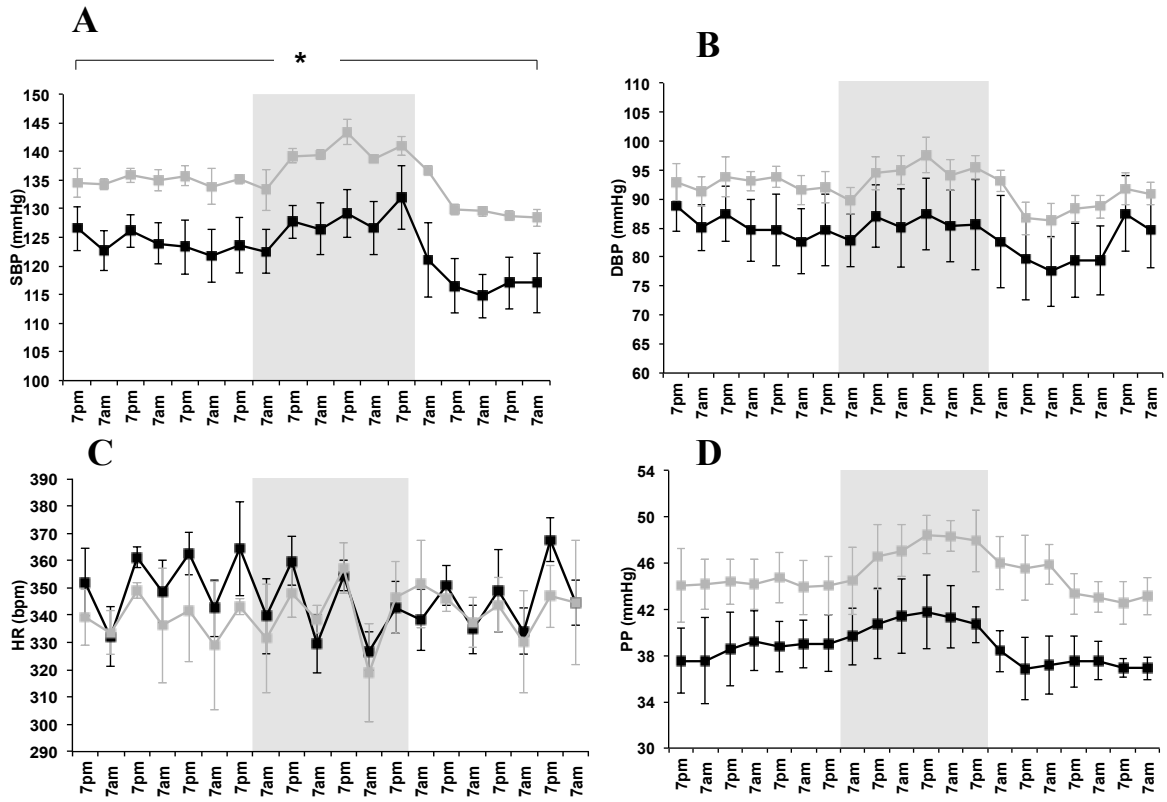

Figure 3.2.12: ***Cardiovascular parameters during dehydration protocol*** *in the female rat* ***(2-way ANOVA)***

The dehydration protocol is divided into three blocks, Baseline, Dehydration and recovery, the shaded area reflects the duration of dehydration and each data point signifies 12 hours of day or night. ***A)*** *There was a significant difference in SBP between groups (P=0.04), SBP remained elevated in HFD rats throughout the dehydration protocol.* ***B****) There was a trend for DBP to be higher in the HFD group however this was not statistically significant (P=0.31).* ***C)*** *There was no significant difference in HR between groups during the protocol (P=0.40).* ***D)*** *There was a trend for PP to by higher in the HFD group compared to controls throughout the protocol although this was not statistically significant (P=0.14)*

Autonomic parameters

sBRS and BEI: In males, there was no significant difference in sBRS or BEI throughout the dehydration protocol (P=0.09 and 0.14, respectively). Furthermore, there was no significant difference in sBRS in response to dehydration in either group (control: P=0.90, HFD: P=0.10). Similarly, there was no difference in BEI in response to dehydration in either group (control: P=0.99, HFD: P=0.14).

In female HFD rats, sBRS was significantly higher throughout the dehydration protocol (P=0.05; **Figure 3.2.13A**). There was no change in sBRS in response to dehydration in control rats. However, in HFD rats, sBRS increased by approximately 40% (P=0.03). Furthermore, during recovery, sBRS remained unchanged in control rats (P=0.31), but remained elevated in HFD rats by approximately 20% above baseline levels (P=0.03; **Figure 3.2.13A**). There was no significant difference in BEI

between groups throughout the dehydration protocol (P=0.65; **Figure 3.2.13B**). There was no significant difference in BEI in response to dehydration in either group (control: P>0.99, HFD: P=0.65; **Figure 3.2.13B**).

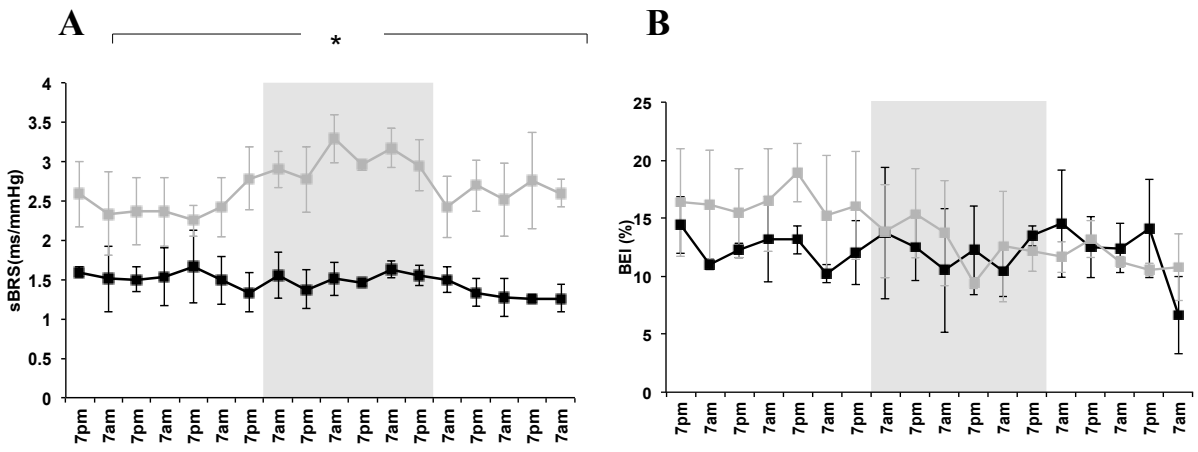

Figure 3.2.13: ***sBRS and BEI during the dehydration protocol in females (2-way ANOVA)***

A) sBRS *was higher in the HFD rats throughout the protocol (P=0.05).* ***B)*** *There was no significant difference in BEI between groups (P=0.65) throughout the protocol).*

HRV: In males, dehydration produced no significant change in HRV in the VLF, LF and HF frequency ranges in either group (**Table 3.2.3**). The LF:HF ratio increased above baseline (resting) levels by approximately 45% in control rats (P=0.03), but not in HFD rats (P=0.35), despite this, there was no significant difference in LF:HF ratio between groups (P=0.47). During recovery, the LF:HF ratio returned to baseline levels, for both groups, upon re-introduction of water.

Table 3.2.3: ***HRV in males during dehydration protocol (2-way ANOVA)***

	Night/Day Cycle	VLF (bpm^2)	LF (bpm^2)	LF:HF	HF (bpm^2)
Control					
Baseline	7pm	3.50 ± 0.53	0.37 ± 0.09	**0.33 ± 0.05***	1.31 ± 0.28
	7am	1.95 ± 0.26	0.22 ± 0.06	0.34 ± 0.05	0.70 ± 0.15
Dehydration	7pm	3.54 ± 0.47	0.44 ± 0.13	**0.48 ± 0.07**	1.16 ± 0.35
	7am	2.37 ± 0.13	0.20 ± 0.04	0.48 ± 0.05	0.53 ± 0.06
Recovery	7am	2.09 ± 0.22	0.29 ± 0.15	0.31 ± 0.05	1.26 ± 0.74
	7pm	2.52 ± 0.29	0.52 ± 0.26	0.31 ± 0.06	2.57 ± 1.69
High Fat					
Baseline	7pm	3.02 ± 0.46	0.37 ± 0.09	0.32 ± 0.02	1.03 ± 0.18
	7am	3.12 ± 0.45	0.26 ± 0.09	0.37 ± 0.01	0.76 ± 0.20
Dehydration	7pm	4.69 ± 1.01	0.55 ± 0.17	0.40 ± 0.02	1.43 ± 0.34
	7am	5.11 ± 1.30	0.58 ± 0.28	0.50 ± 0.10	1.83 ± 0.92
Recovery	7am	3.59 ± 1.26	0.41 ± 0.19	0.33 ± 0.01	1.34 ± 0.59
	7pm	3.95 ± 1.31	0.41 ± 0.21	0.29 ± 0.02	1.34 ± 0.49

** Denotes comparison within groups where significant.*

In females, there was no significant difference in LF and HF frequency ranges observed throughout the dehydration protocol (**Table 3.2.4**). However, the LF:HF ratio was significantly higher in the HFD rats (P=0.03; **Figure 3.2.14**) throughout baseline, stress and recovery.

In addition, VLF increased by approximately 35% (P=0.01) in the HFD rats, with no difference observed in control rats (P=0.58). Upon replacement of water VLF returned to baseline levels.

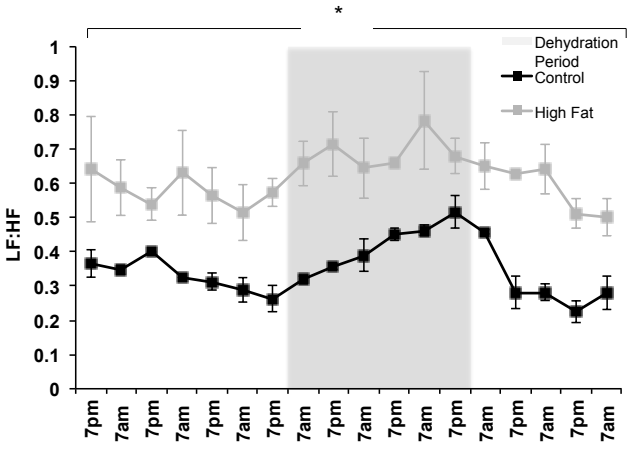

Figure 3.2.14: Female *LF: HF ratio component of HRV during dehydration protocol (2-way ANOVA)*

The LF: HF ratio was higher in high fat than controls thoughout the protocol (P=0.03).

Table 3.2.4: ***HRV in females during dehydration protocol (2-way ANOVA)***

	Night/Day Cycle	VLF (bpm^2)	LF (bpm^2)	LF:HF	HF (bpm^2)
Control					
Baseline	7pm	3.37 ± 1.35	0.70 ± 0.24	**0.33 ± 0.05**	1.96 ± 0.72
	7am	2.69 ± 0.85	0.65 ± 0.21	0.31 ± 0.02	1.94 ± 0.43
Dehydration	7pm	3.87 ± 1.41	1.04 ± 0.54	**0.41 ± 0.01**	2.33 ± 1.03
	7am	3.07 ± 1.03	0.91 ± 0.42	0.43 ± 0.02	2.16 ± 0.88
Recovery	7am	3.35 ± 0.63	0.56 ± 0.28	0.25 ± 0.04	2.01 ± 0.43
	7pm	3.12 ± 0.34	0.47 ± 0.15	**0.28 ± 0.04**	1.72 ± 0.15
High Fat					
Baseline	7pm	**4.37 ± 1.11**	0.80 ± 0.20	**0.55 ± 0.06**†	1.74 ± 0.52
	7am	4.59 ± 1.51	0.66 ± 0.20	0.57 ± 0.10	1.17 ± 0.19
Dehydration	7pm	**5.88 ± 0.82***	0.97 ± 0.16	**0.69 ± 0.04**†	1.41 ± 0.08
	7am	5.47 ± 1.37	1.07 ± 0.42	0.71 ± 0.11	1.38 ± 0.38
Recovery	7am	5.30 ± 0.54	0.79 ± 0.15	0.57 ± 0.02	1.32 ± 0.15
	7pm	4.29 ± 0.82	0.73 ± 0.21	**0.57 ± 0.06**†	1.39 ± 0.39

**Denotes comparison within groups where significant and † between groups (HFD and control) where significant*

BPV: In males, there was no difference in VLF, LF and HF at any point during the dehydration protocol. In response to dehydration, there were no significant differences observed in BPV in any of the frequency ranges (VLF, LF and HF) in either high fat or control rats. Furthermore there were no significant differences observed during recovery, all parameters remained unchanged from baseline levels. (**Table 3.2.5**).

Table 3.2.5: ***BPV in males during dehydration protocol***

	Night/Day Cycle	VLF ($mmHg^2$)	LF ($mmHg^2$)	HF ($mmHg^2$)
Control				
Baseline	7pm	0.19 ± 0.01	0.02 ± 0.00	0.02 ± 0.00
	7am	0.16 ± 0.01	0.02 ± 0.00	0.01 ± 0.00
Dehydration	7pm	0.19 ± 0.06	0.04 ± 0.01	0.02 ± 0.00
	7am	0.13 ± 0.01	0.02 ± 0.00	0.01 ± 0.00
Recovery	7am	0.29 ± 0.13	0.02 ± 0.01	0.02 ± 0.01
	7pm	0.29 ± 0.12	0.03 ± 0.01	0.02 ± 0.01
High Fat				
Baseline	7pm	0.15 ± 0.03	0.03 ± 0.01	0.02 ± 0.00
	7am	0.13 ± 0.02	0.03 ± 0.00	0.02 ± 0.00
Dehydration	7pm	2.04 ± 1.89	0.30 ± 0.25	0.63 ± 0.60
	7am	2.26 ± 2.14	0.38 ± 0.34	1.14 ± 1.12
Recovery	7am	0.16 ± 0.03	0.03 ± 0.01	0.02 ± 0.00
	7pm	0.16 ± 0.02	0.03 ± 0.01	0.02 ± 0.00

In females, dehydration produced no significant change in BPV in the VLF or HF frequency ranges in either group, while LF increased by 75% in control rats only (P=0.01). On recovery, LF returned to baseline levels in controls while all other BPV variables remained unchanged from baseline (**Table 3.2.6**).

Table 3.2.6: ***BPV in females during dehydration protocol***

	Night/Day Cycle	VLF ($mmHg^2$)	LF ($mmHg^2$)	HF ($mmHg^2$)
Control				
Baseline	7pm	0.15 ± 0.04	**0.04 ± 0.01**	0.02 ± 0.00
	7am	0.15 ± 0.07	0.04 ± 0.00	0.02 ± 0.00
Dehydration	7pm	0.13 ± 0.02	**0.07 ± 0.01***	0.02 ± 0.00
	7am	0.12 ± 0.01	0.06 ± 0.00	0.03 ± 0.00
Recovery	7am	0.13 ± 0.03	0.03 ± 0.00	0.02 ± 0.01
	7pm	0.15 ± 0.06	0.03 ± 0.00	0.02 ± 0.01
High Fat				
Baseline	7pm	0.16 ± 0.03	0.05 ± 0.01	0.03 ± 0.00
	7am	0.16 ± 0.03	0.04 ± 0.01	0.03 ± 0.00
Dehydration	7pm	0.16 ± 0.02	0.06 ± 0.00	0.03 ± 0.01
	7am	0.12 ± 0.01	0.05 ± 0.01	0.03 ± 0.00
Recovery	7am	0.20 ± 0.02	0.04 ± 0.00	0.03 ± 0.00
	7pm	0.20 ± 0.04	0.04 ± 0.01	0.03 ± 0.00

** Denotes comparison within groups where significant*

dP/dT $_{max}$: In both males and females, there was no significant difference in dP/dT_{max} between HFD and control groups throughout the entire dehydration protocol (P=0.30 and 0.87, respectively).

3.2.6 Comparison of cardiovascular and autonomic parameters between high fat and control rats during air-jet stress

Figure 3.2.15 illustrates an example of a recording of blood pressure and derived parameters during the air jet stress protocol. During each burst of air, rats showed behavioural changes. These included jumping or running away from the jet of air or freezing. Consistent with this, measured cardiovascular parameters such as BP and HR, were increased indicating clear physiological changes during air jet stress.

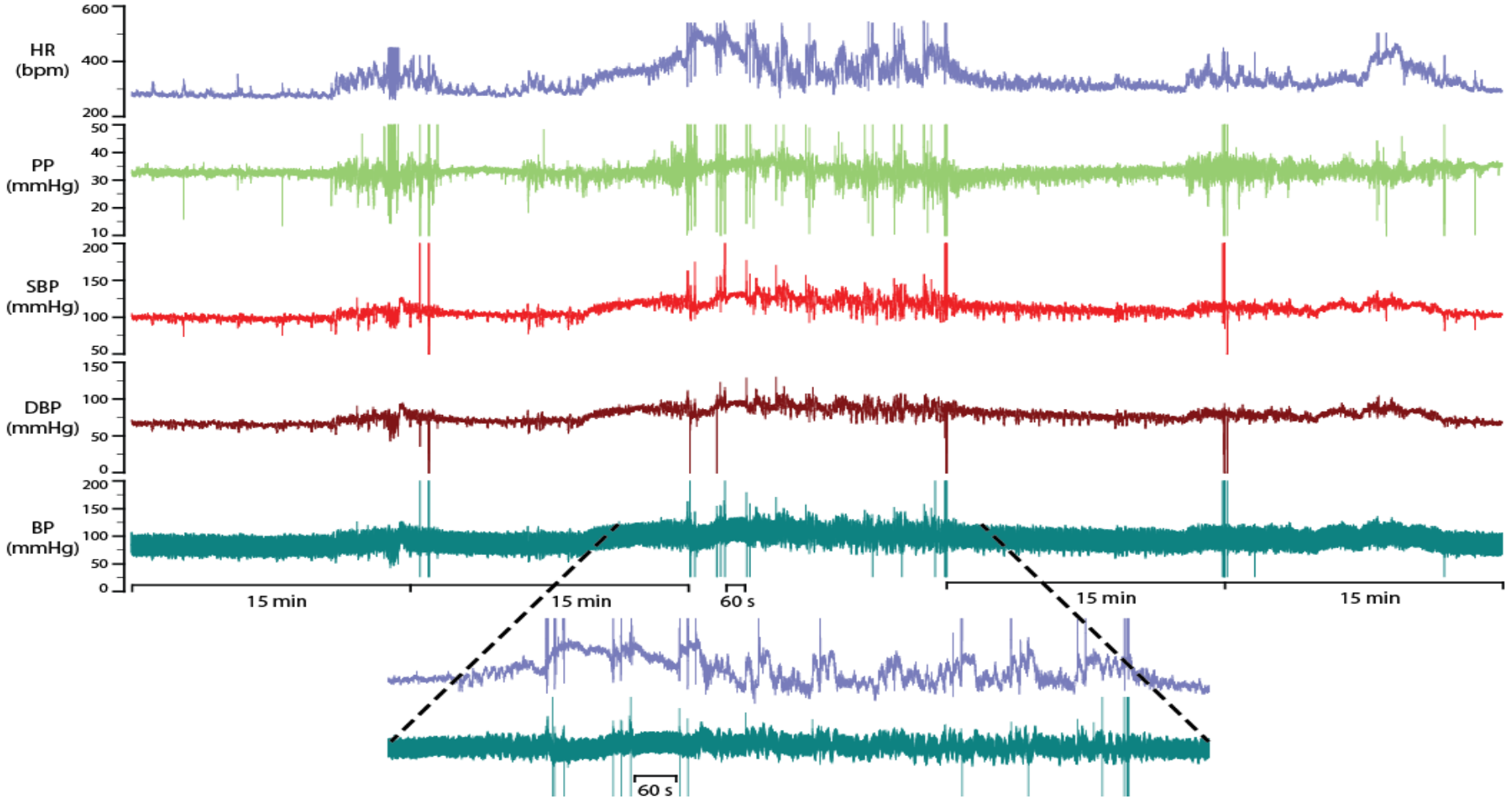

Figure 3.2.15: *Cardiovascular responses due to air jet protocol*

Raw data recording of BP and derived cardiovascular parameters during 30 minutes of baseline recording, 15 minutes of air jet stress and 30 minutes of recovery. Cardiovascular and autonomic measures during the stress period were taking during the 60-second intervals as shown on the illustration. SBP: systolic blood pressure, DBP: diastolic blood pressure, HR: heart rate, PP: pulse pressure, BP: blood pressure.

Cardiovascular parameters: In males, during the baseline period, SBP was higher in HFD rats than controls (P=0.04), while no difference was observed in DBP (P=0.13), HR (P=0.40) or PP (P=0.07) between groups (**Figure 3.2.16**).

With the initiation of the first series of air puffs, SBP, DBP, and HR all increased significantly from resting levels in all rats, while PP increased only in the HFD group, by approximately 10 mmHg (**Figure 3.2.16**). During the air jet protocol, SBP remained significantly higher in the HFD group than controls (P=0.02), although no difference was observed between groups in the change in SBP from baseline (P=0.77; **Figure 3.2.16**). There was no difference between groups in either the increase or absolute values of DBP and HR (**Figure 3.2.16**), while a higher observed PP of approximately 35% in HFD rats approached significance (P=0.06; **Figure 3.2.16**).

During the first 15 minutes of the recovery period, SBP remained significantly elevated ($P<0.01$) in both groups (**Figure 3.2.16A**), while PP returned to baseline (**Figure 3.2.16D**). At the end of recovery, HR returned to resting levels in both groups ($P>0.99$), DBP appeared to be elevated in both HFD and control rats, this was statistically significant only in the control group (P=0.04; **Figure 3.2.16B**). In addition, no difference was observed between groups in the change in SBP from baseline to the initiation of the first air jet (P=0.77; **Figure 3.2.17**).

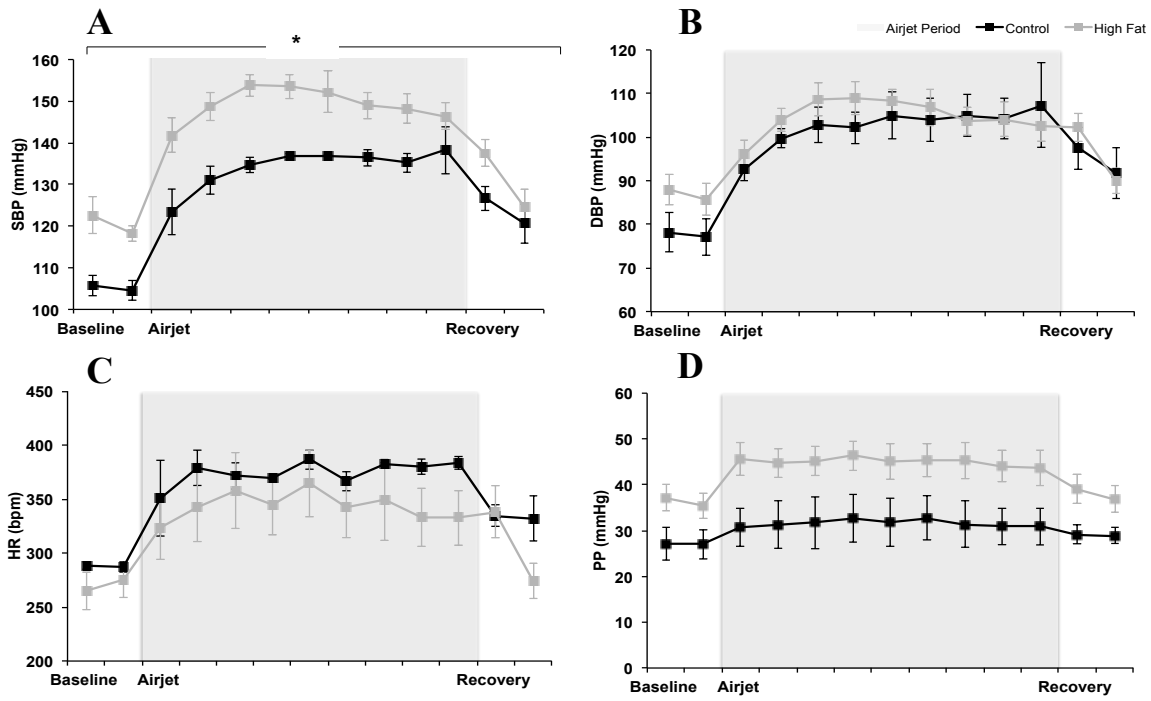

Figure 3.2.16: ***Male, cardiovascular parameters during air jet protocol (2-way ANOVA)***

The first two points indicate baseline values with two 15-minute averages. Each 9 points in the stress period are averages of each 60-second period following an air puff. The recovery period consists of two points, which are two 15 minute averages. ***A)*** *Overall SBP remained significantly higher in the HF rats throughout the stress protocol (P=0.02).* ***B)*** *There was no difference in DBP between groups at any stage of the air jet protocol (P=0.29)* **C)** *There was no difference in HR between groups at any stage of the air jet protocol (P=0.79).* ***D)*** *There was a trend for PP to be elevated in the HF rats throughout the air jet protocol, although this was not statistically significant (P=0.06).*

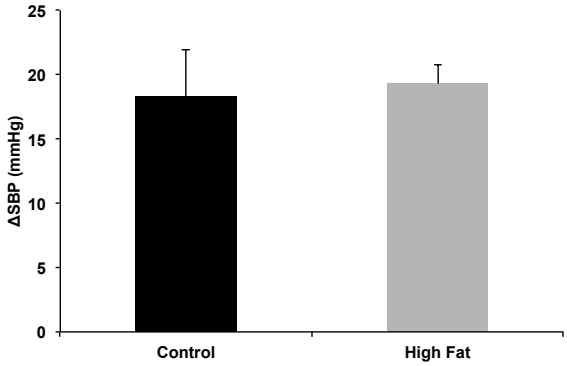

Figure 3.2.17: ***The initial change from baseline in systolic blood pressure immediately following the first air puff (mean ± SEM) (unpaired t-test)***

There was no significant difference observed in the change in SBP between groups (P=0.77).

In females, SBP (P=0.64), DBP (P=0.78) and HR (P=0.34) were not significantly different between HFD and control rats during baseline, while PP was approximately 20% higher in the HFD group (P=0.05; **Figure 3.2.18**). With the initiation of the first series of air puffs, SBP, DBP and PP all increased significantly from baseline (resting) levels in both groups, with similar magnitudes (**Figure 3.2.18**). HR did not increase significantly from resting levels in the control groups but did so in the HFD rats by approximately 40 beats/min (P=0.02).

During the recovery period SBP, DBP, HR and PP all returned towards baseline levels in both groups (**Figure 3.2.18**). In addition no difference was observed between groups in the change in SBP from baseline to the initiation of the first air jet (P=0.34; **Figure 3.2.19**).

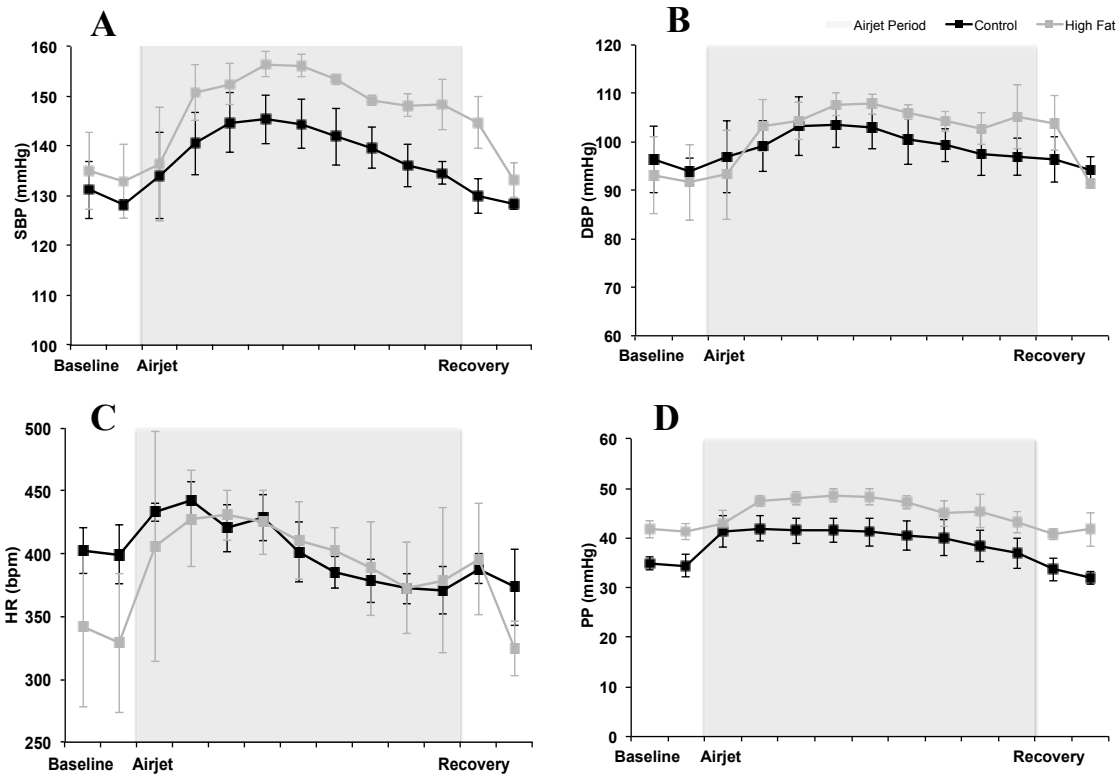

Figure 3.2.18*: Female cardiovascular parameters during air jet protocol (2-way ANOVA)*

The first two points indicate baseline values with two 15-minute averages. Each 9 points in the stress period are averages of each 60-second period following an air puff. The recovery period consists of two points, which are two 15 minute averages. ***A)*** *There was trend for SBP to be elevated in HF rats throughout the air jet protocol, however this was not statistically significant (P=0.23)* ***B)*** *There was no statistical difference in DBP between groups at any stage of the air jet protocol (P=0.76).* ***C)*** *There was no difference in HR between groups at any stage of the air jet protocol (P=0.70).* ***D)*** *There was a trend for PP to be elevated in the HF rats throughout the air jet protocol, although this was not statistically significant (P=0.06).*

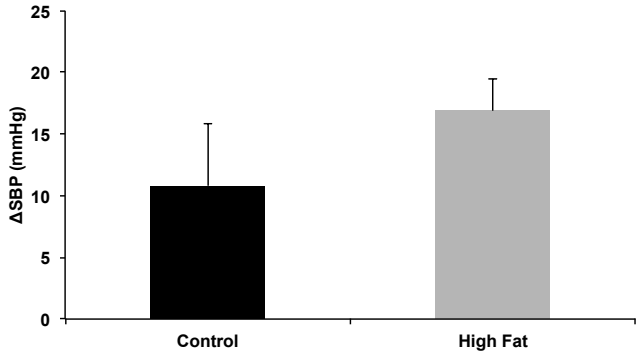

Figure 3.2.19: *The initial change from baseline in systolic blood pressure immediately following the first air puff (mean ± SEM) (unpaired t-test)*

There was no significant difference observed in the change in SBP between groups (P=0.57).

Autonomic parameters

sBRS and BEI: in the male, in response to air jet stress sBRS decreased significantly in both groups (P<0.05) with no significant difference between groups (P=0.40). During recovery sBRS remained decreased and did not return to resting levels in either group (P<0.05; **Figure 3.2.20A**). BEI appear to decrease in response to stress in both groups, although this was not statistically significant, with no significant difference between groups (P=0.53). BEI returned to resting levels during the recovery period in both groups (**Figure 3.2.20B**).

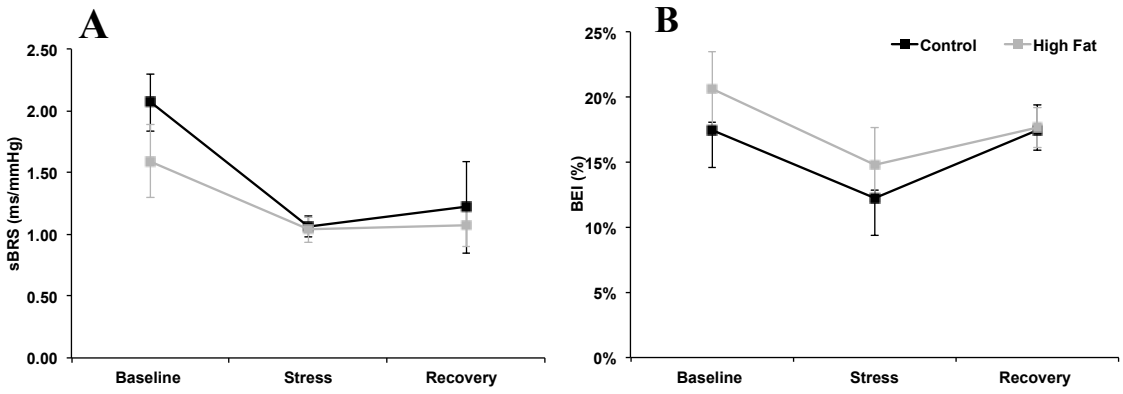

Figure 3.2.20: ***sBRS and BEI during the air jet protocol in males, each data point is indicative of averages for baseline, stress and recovery (2-way ANOVA)***

(A)*There was a significant decrease in sBRS in HFD (P=0.04) and control (P=0.01) rats in response to stress, with no significant difference observed between groups (P=0.40).* ***B)*** *There was no significant difference in BEI between groups (P=0.53) in response to stress.*

In the female, in response to stress, sBRS did not change significantly from resting levels in either HFD (P=0.78) or controls (P=0.56), with no significant difference observed between groups (P=0.90). Furthermore, sBRS remained unchanged during recovery (**Figure 3.2.21A**). In response to stress BEI did not change significantly from resting levels in either group (P=0.10 for both HFD and control rats), with no significant difference observed between groups (P=0.70) and remained constant during recovery (**Figure 3.2.21B**).

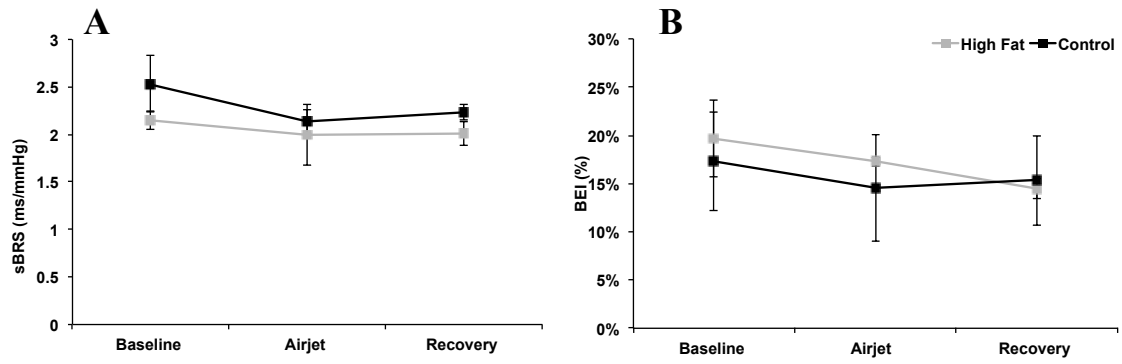

Figure 3.2.21: ***sBRS and BEI during the air jet protocol in females, each data point is indicative of averages for baseline, stress and recovery (2-way ANOVA)'***

A) *sBRS did not change significantly from resting levels in response to stress in either HFD (P=0.78) or control (P=0.56) groups with no observed difference between groups during stress (P=0.87).* ***B)*** *BEI did not change significantly from resting levels in response to stress in either HFD (P=0.10) or control (P=0.10) groups with no observed difference between groups during stress (P=0.53).*

HRV: In response to stress, there was no significant difference observed in HF, LF or the LF: HF ratio in either HFD or control rats (**Table. 3.2.7**). However VLF decreased from resting levels by approximately 86% in the HFD rats (P<0.01) but not in the control rats. Similarly, total power decreased by approximately 90% in the HFD rats (P<0.01) but no change was observed in the control rats.

There was no significant difference observed during recovery. Total power and LF:HF returned to resting levels in HFD rats. All other frequency ranges (VLF, LF and HF for both groups and LF:HF ratio and total power for control rats) remained similar to baseline levels.

Table 3.2.7: ***HRV in males during air jet protocol (2-way ANOVA)***

	VLF (bpm^2)	LF (bpm^2)	LF:HF	HF (bpm^2)	TPWR (bpm^2)
Control					
Baseline	1.16 ± 0.09	0.09 ± 0.01	0.31 ± 0.06	0.31 ± 0.08	1.64 ± 0.20
Air jet	0.45 ± 0.27	0.45 ± 0.27	0.45 ± 0.27	0.45 ± 0.27	0.45 ± 0.27
Recovery	1.86 ± 0.15	0.17 ± 0.04	0.38 ± 0.14	0.57 ± 0.20	2.73 ± 0.29
High Fat					
Baseline	**1.60 ± 0.37**	0.13 ± 0.04	0.29 ± 0.06	0.36 ± 0.06	**2.12 ± 0.48**
Air jet	**0.22 ± 0.07***	0.22 ± 0.07	0.22 ± 0.07	0.22 ± 0.07	**0.22 ± 0.07***
Recovery	1.91 ± 0.23	0.15 ± 0.04	0.23 ± 0.05	0.68 ± 0.11	2.82 ± 0.33

** Denotes comparison within groups where significant*

In females, stress elicited a decrease in HRV in all frequency ranges in control rats. Total power was increased by 74% (P<0.01), VLF by 74% (P=0.01), LF by 87% (P=0.02) and HF by 73% (P<0.01). There were no significant differences in these variables observed in the HF rats. There was a significant decrease in LF:HF ratio in both control and HFD rats to a similar degree (control: P=0.04, HF: P<0.01). During recovery all HRV variables returned to resting levels, except for HFD in control rats, which decreased below resting levels by approximately 50% (P<0.01; **Table. 3.2.8**).

Table 3.2.8: ***HRV in females during air jet protocol (2-way ANOVA).***

	VLF (bpm^2)	LF (bpm^2)	LF:HF	HF (bpm^2)	TPWR (bpm^2)
Control					
Baseline	**4.84 ± 1.30**	**1.50 ± 0.69**	**0.48 ± 0.09**	**3.94 ± 0.98**	**10.48 ± 3.16**
Air jet	**1.24 ± 0.20***	**0.20 ± 0.09***	**0.31 ± 0.07***	**1.08 ± 0.28***	**2.72 ± 0.66***
Recovery	4.16 ± 1.11	0.76 ± 0.28	0.53 ± 0.09	**1.98 ± 0.43#**	7.08 ± 1.54
High Fat					
Baseline	3.38 ± 0.98	0.54 ± 0.04	**1.01 ± 0.29**	0.72 ± 0.21	6.15 ± 2.01
Air jet	1.44 ± 0.24	0.33 ± 0.07	**0.46 ± 0.15***	1.04 ± 0.48	3.07 ± 0.30
Recovery	3.45 ± 0.62	0.74 ± 0.17	0.76 ± 0.19	1.02 ± 0.29	5.94 ± 0.92

** #denotes comparison within groups where significant.*

BPV: In response to stress, VLF and total power remained unchanged in both HFD and control rats. HF and LF increased in response to the stress in the control group (by 2150% and 1400%, respectively; P=0.03 for both), no difference was observed in HFD rats. These BPV variables returned to resting levels during the recovery period. There was no significant difference observed in BPV variables during the recovery period in comparison to resting values in either HFD or control rats.

Table 3.2.9: BP*V in males during air jet protocol (2-way ANOVA).*

	VLF (bpm^2)	LF (bpm^2)	HF (bpm^2)	TPWR (bpm^2)
Control				
Baseline	0.12 ± 0.01	**0.03 ± 0.00**	**0.02 ± 0.00**	0.17 ± 0.00
Air jet	0.45 ± 0.27	**0.45 ± 0.27***	**0.45 ± 0.27***	0.45 ± 0.27
Recovery	0.42 ± 0.08	0.09 ± 0.01	0.02 ± 0.01	0.55 ± 0.10
High Fat				
Baseline	0.15 ± 0.02	0.04 ± 0.00	0.02 ± 0.00	0.22 ± 0.03
Air jet	0.22 ± 0.07	0.22 ± 0.07	0.22 ± 0.07	0.22 ± 0.07
Recovery	0.31 ± 0.06	0.11 ± 0.02	0.04 ± 0.01	0.46 ± 0.08

****** denotes comparison within groups where significant.*

In females, in response to stress, total power decreased significantly in HFD rats only, by approximately 61% (P=0.03). VLF remained unchanged in both groups, while LF decreased significantly in control rats by approximately 63% (P<0.01), but was unchanged in HFD rats (P>0.99). Finally, HF increased in control groups by approximately 50% (P<0.01) and in HF rats by approximately by 43% (P<0.01). Stress-induced changed in BPV all returned to resting levels during the recovery period, so that there were no significant difference observed in and frequency range during the recovery period compared to resting values in either HFD or control rats (**Table. 3.2.10**).

Table 3.2.10: BP*V in females during air jet protocol (2-way ANOVA).*

	VLF ($mmHg^2$)	LF ($mmHg^2$)	HF ($mmHg^2$)	TPWR ($mmHg^2$)
Control				
Baseline	0.29 ± 0.10	**0.16 ± 0.05**	**0.04 ± 0.01**	0.52 ± 0.18
Air jet	0.20 ± 0.08	**0.06 ± 0.02***	**0.02 ± 0.01***	0.27 ± 0.10
Recovery	0.18 ± 0.03	0.10 ± 0.03	0.03 ± 0.01	0.32 ± 0.06
High Fat				
Baseline	1.01 ± 0.23	0.13 ± 0.02	**1.01 ± 0.23**	**0.61 ± 0.17**
Air jet	1.44 ± 0.25	0.04 ± 0.01	**1.44 ± 0.25***	**0.24 ± 0.09***
Recovery	0.76 ± 0.15	0.12 ± 0.01	0.76 ± 0.15	0.39 ± 0.02

** denotes comparison within groups where significant*

dP/dT_{max}: In males, there was a trend towards an increase in dP/dT_{max} immediately upon commencement of air jet, however the size of the increase between HFD and control rats was similar (P=0.77; **Figure 3.2.20A**). Furthermore there was no difference in dP/dT_{max} between groups at any time point during the air jet protocol (P=0.30; **Figure 3.2.20A**).

In females, there was a trend towards increase in dP/dT_{max} immediately upon commencement of air jet in the HFD group but not in controls, however the size of the increase between HFD and control rats was similar (P=0.32; **Figure 3.2.20B**). Similarly there was no difference in dP/dT_{max} between groups at any time point during the air jet protocol (P=0.87; **Figure 3.2.20B**).

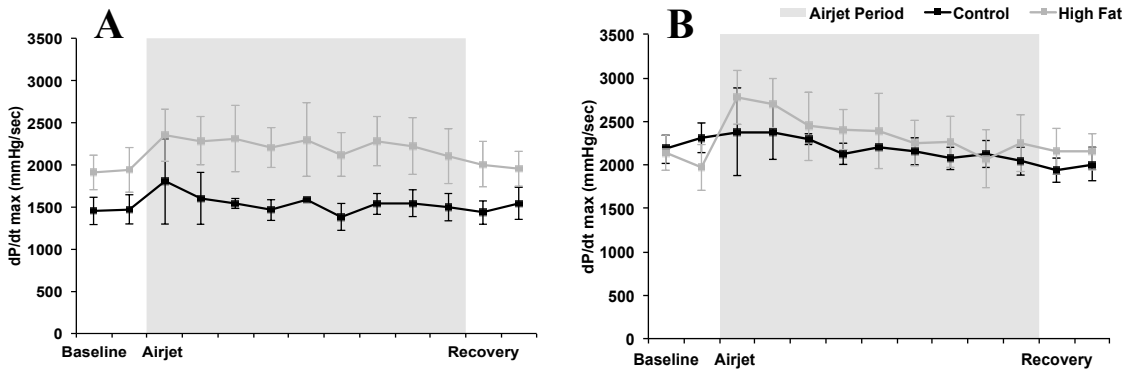

Figure 3.2.22: *dP/dT$_{max}$ between HF and control rats during air jet protocol (2-way ANOVA)*

A) *In males, there was no difference in dP/dT$_{max}$ between groups at any time point during the air jet protocol (P=0.30).* ***B)*** *In females, there was no difference in dP/dT$_{max}$ between groups at any time point during the air jet protocol (P=0.87).*

3.3 HIGH SUCROSE MODEL

For the high sucrose model (HSU), we had access to only male offspring, thus only males were investigated. Blood pressure probes were implanted in eight control offspring (six months of age) and eight sucrose offspring (six months of age). Of these, one sucrose rat and one-control rat did not recover adequately from surgery, leaving a total of seven control and seven high sucrose offspring from which data were recorded.

3.3.1 Offspring body weight

As with HFD rats, gestation period of HSU rats lasted for 21-22 days with all pups born approximately after 22 days of conception. There was no significant difference in birth weights between control and sucrose rats (P=0.80; **Figure 3.3.1A**). Also, there was no difference in weights at day 10 between control and sucrose rats (P=0.72; **Figure 3.3.1B**). At time of implantation (approximately six months age), body weight did not differ significantly between groups (control: 625g ± 19g vs HSU: 605g ± 28g, P=0.50; **Figure 3.3.1C**).

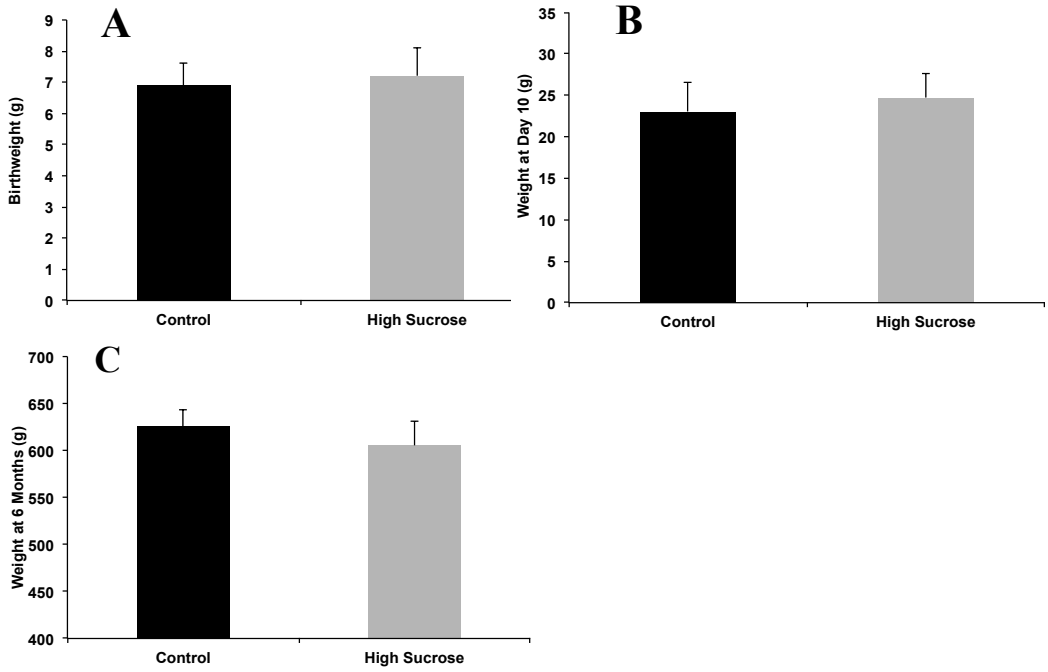

Figure 3.3.1: ***Birth weights of male offspring in high sucrose and control groups (mean ± SEM) (unpaired t-test)***

A) *There was no significant difference in weight between high sucrose and control groups at birth (P=0.80).* ***B)*** *There was no significant difference in body weight at day 10 between groups.* ***C)*** *There was no significant difference in weights at six months of age between groups (P=0.50).*

3.3.2 Comparison of cardiovascular and autonomic parameters between high sucrose and control rats at rest

Cardiovascular parameters: There were few differences observed in baseline cardiovascular parameters between control and HSU offspring. Blood pressure (SBP, DBP and PP) was not different between groups during either night or day. Similarly, HR was not different during the day, although at night (during the active phase of the rat) HR was slightly higher (by approximately 20 bpm) in the HSU rat than controls (P<0.03; **Figure 3.3.2**). Surprisingly, the only evidence of diurnal rhythm in these rats (both HSU and controls) was observed in the HR, where both exhibited higher HR of approximately 50 bpm at night (P<0.01 for both groups). Systolic BP, DBP and PP did not show significant differences between night and day within groups (**Figure 3.3.2**).

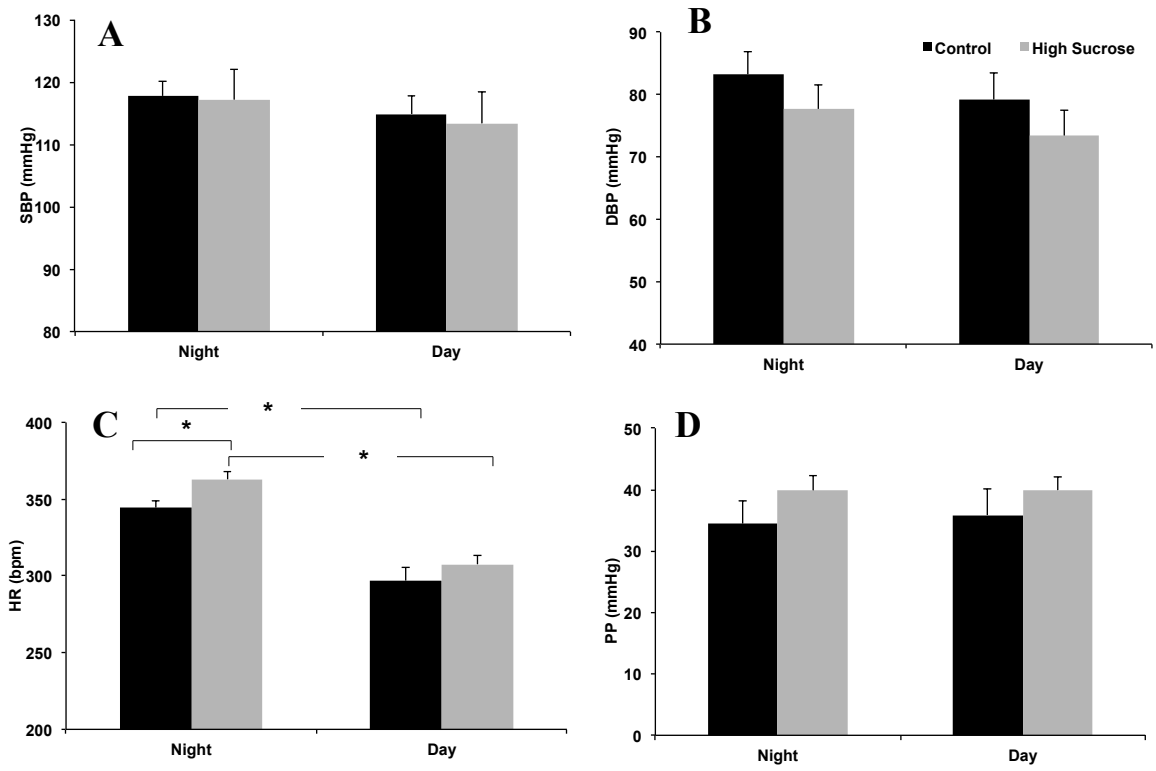

Figure 3.3.2: ***Comparison of cardiovascular parameters at rest in high sucrose and control rats (mean ± SEM) (unpaired t-test)***

A) *There was no significant difference observed in SBP between groups during night (P=0.93) or day (P=0.81).* ***B)*** *There was no significant difference in DBP between groups during night (P=0.32) or day (P=0.36).* ***C)*** *There was a significant difference in HR observed between groups only during the night (P=0.03) and not during the day (P=0.34).* ***D)*** *There was no significant difference in PP between groups during night (P=0.26) or day (P=0.42).*

Autonomic parameters

sBRS and BEI: There was no significant difference in resting sBRS between groups during night (P=0.91) or day (P=0.84). Similarly there was no difference in resting BEI between groups during night (P=0.42) or day (P=0.52). Neither HSU nor control rats exhibited a diurnal rhythm in these autonomic measures: there were no significant differences in sBRS or BEI during the night and day within each group (**Figure 3.3.3**).

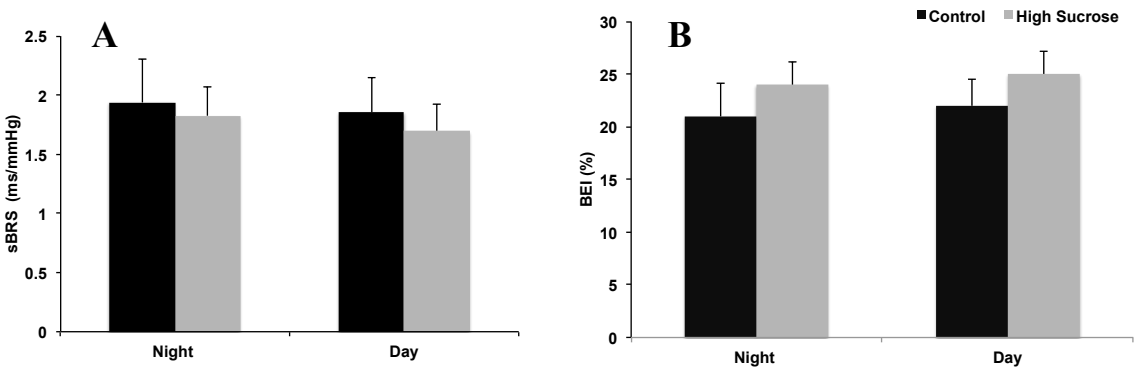

Figure 3.3.3: ***Resting values for sBRS and BEI of high sucrose and control groups (mean ± SEM) (unpaired t-test) (mean ± SEM)***

A) *There was no difference in sBRS observed between groups during night (P=0.91) or day (P=0.84).* ***B)*** *There was no difference in BEI observed between groups during night (P=0.42) or day (P=0.52).*

HRV and BPV: Frequency domain analysis measurements of HRV and BPV at rest did not show any differences between the groups. There were also no differences observed between night and day within each group (**Table 3.3.1** and **Table 3.3.2**).

Table 3.3.1: ***HRV in control and HSU rats at rest (unpaired t-test) (mean ± SEM)***

	Night/Day Cycle	VLF (bpm^2)	LF (bpm^2)	LF:HF	HF (bpm^2)
Control					
Baseline	7pm	3.26 ± 0.32	0.64 ± 0.19	0.33 ± 0.05	1.85 ± 0.43
	7am	2.92 ± 0.16	0.44 ± 0.09	0.40 ± 0.04	1.39 ± 0.19
High Sucrose					
Baseline	7pm	5.33 ± 1.10	0.87 ± 0.34	0.32 ± 0.05	2.80 ± 0.82
	7am	4.41 ± 1.02	0.64 ± 0.13	0.33 ± 0.05	2.82 ± 1.06

Table 3.3.2: BP*V in control and HSU rats at rest*

	Night/Day Cycle	VLF ($mmHg^2$)	LF ($mmHg^2$)	HF ($mmHg^2$)
Control				
Baseline	7pm	0.34 ± 0.04	0.04 ± 0.00	0.03 ± 0.00
	7am	0.31 ± 0.04	0.03 ± 0.00	0.03 ± 0.00
High Sucrose				
Baseline	7pm	0.37 ± 0.04	0.10 ± 0.04	0.05 ± 0.01
	7am	0.42 ± 0.02	0.09 ± 0.03	0.05 ± 0.01

dP/dT$_{max}$: There was no difference observed in baseline dP/dt$_{max}$ between control and HSU rats (**Figure 3.3.4**). Similar to other measures, there was no observed difference between day and night values of dP/dt$_{max}$.

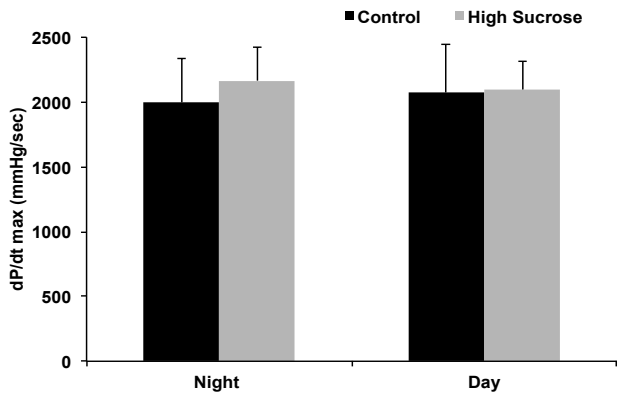

Figure 3.3.4: *dP/dt$_{max}$ between control and high sucrose rats during night and day (mean ± SEM) (unpaired t-test) (mean ± SEM)*

There was no observed difference in dP/dt$_{max,}$ between groups during night (P=0.70) or day (P=0.95).

3.3.3 Comparison of cardiovascular and autonomic parameters between high sucrose and control rats during dehydration

The dehydration protocol used here was identical to that used for the study on high fat-programmed rats described above (**Section 3.2.5**). For purposes of statistical analysis of the dehydration data, we have only considered data acquired during the night phase. This was because there were no significant differences between night and day for cardiovascular and autonomic parameters.

Cardiovascular parameters: With the initiation of dehydration there was a small, but consistent increase in SBP and DBP of approximately 5-10 mmHg (SBP: P<0.01 both groups, DPB: P<0.01 for HSU and P=0.03 for control rats). The increase in SBP and DBP in response to dehydration was similar between groups (**Figure 3.3.5A, B**). Dehydration produced no significant difference in HR or PP in either group in response to dehydration (PP: P=0.22 or HR: P=0.37), consistent with the males offspring in the HFD study described above (**Figure 3.3.5C, D**).

During the recovery period SBP fell below baseline levels by approximately 5 mmHg in both HSU (P=0.01) and control (P=0.02) rats. In contrast, DBP fell below baseline only in the HSU rats (P=0.02), whereas in the control rat it returned to baseline levels (comparison of baseline to recovery in control, P=0.60). The changes in SBP during dehydration and recovery were similar between groups (**Figure 3.3.5A, B**).

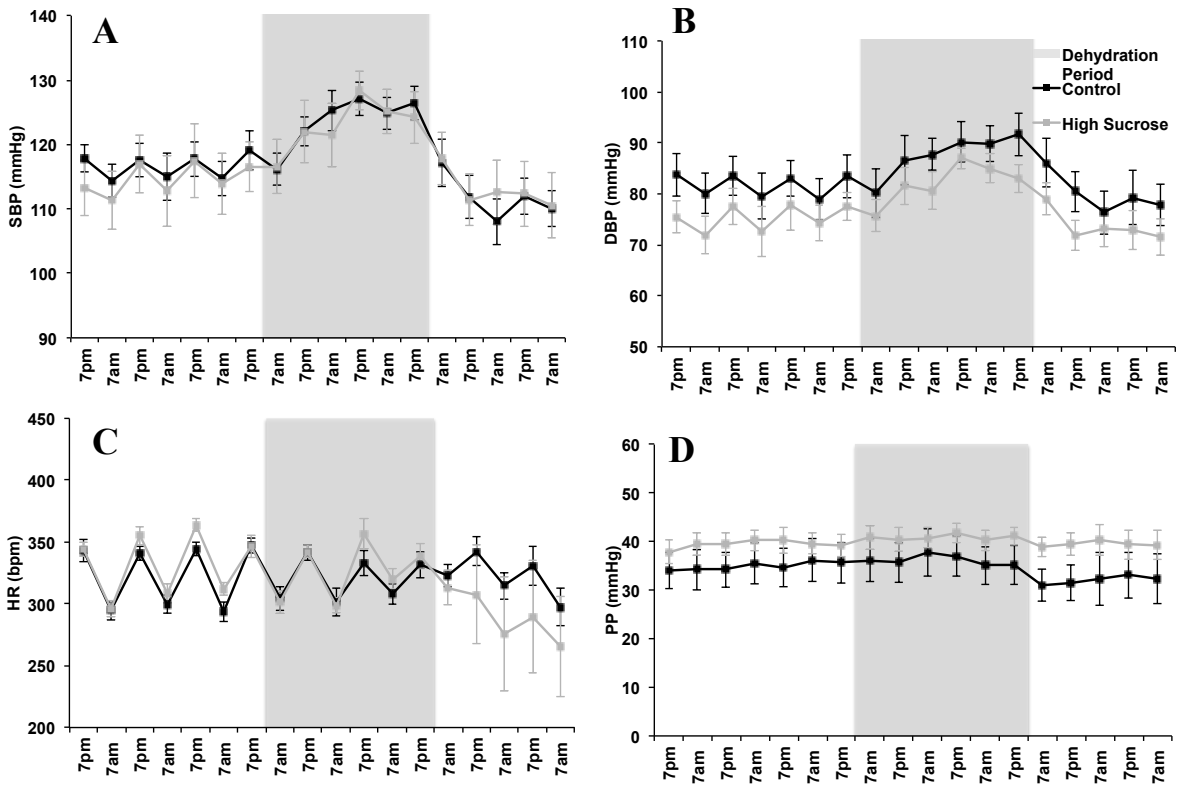

Figure 3.3.5: ***Cardiovascular parameters during dehydration protocol (2-way ANOVA)***

The dehydration protocol is divided into three blocks, Baseline, Dehydration and recovery, the shaded area reflects the duration of dehydration and each data point signifies 12 hours of day or night. ***A)*** *There was no significant difference in SBP between groups during the dehydration protocol (P=0.93),* ***B)*** *There was no significant difference in DBP between groups during the dehydration protocol (P=0.25).* ***C)*** *There no significant difference in HR between groups during the dehydration protocol (P=0.37).* ***D)*** *There was no significant difference in PP between groups throughout the dehydration protocol (P=0.22).*

Autonomic parameters

sBRS and BEI: Consistent with the results in the HFD study, dehydration produced no effect on sBRS or BEI in either group (P>0.99 for both parameters and both groups;). Similarly, there was no change in these parameters during the recovery phase, when water was returned to the cages. Finally, there were no differences in the effects of dehydration and recovery on sBRS and BEI between groups (sBRS: P=0.84; BEI: P=0.65).

HRV: In response to dehydration, there was no difference observed in HRV in all frequency ranges (VLF, LF and HF) in either high sucrose or control rats. However, the LF:HF ratio did increase significantly from baseline levels in response to dehydration in control rats (P=0.04), no difference was seen in the high sucrose rats (**Table 3.3.3**). Despite this, overall, there was no significant difference observed in HRV between HSU and control rats throughout the dehydration protocol in any of the frequency ranges (VLF, LF or HF or LF:HF ratio; **Table. 3.3.3**). Similarly, there was no significant difference observed in HRV between baseline and recovery in either HSU or control rats.

Table 3.3.3: ***HRV during dehydration protocol (2-way ANOVA)***

	Night/Day Cycle	VLF (bpm^2)	LF (bpm^2)	LF:HF	HF (bpm^2)
Control					
Baseline	7pm	3.26 ± 0.32	0.64 ± 0.19	**0.33 ± 0.05**	1.85 ± 0.43
	7am	2.92 ± 0.16	0.44 ± 0.09	0.40 ± 0.04	1.39 ± 0.19
Dehydration	7pm	5.31 ± 1.01	1.34 ± 0.67	**0.40 ± 0.03***	3.34 ± 1.24
	7am	3.97 ± 0.50	0.54 ± 0.05	0.37 ± 0.02	1.82 ± 0.17
Recovery	7am	4.76 ± 0.95	0.88 ± 0.22	0.38 ± 0.05	2.86 ± 0.75
	7pm	4.01 ± 0.67	0.71 ± 0.12	0.37 ± 0.02	2.57 ± 0.54
High Sucrose					
Baseline	7pm	5.33 ± 1.10	0.87 ± 0.34	0.32 ± 0.05	2.80 ± 0.82
	7am	4.41 ± 1.02	0.64 ± 0.13	0.33 ± 0.05	2.82 ± 1.06
Dehydration	7pm	7.03 ± 1.41	1.31 ± 0.24	0.34 ± 0.05	4.62 ± 1.16
	7am	5.99 ± 1.16	1.11 ± 0.20	0.34 ± 0.05	4.17 ± 1.07
Recovery	7am	6.26 ± 1.18	0.79 ± 0.08	0.31 ± 0.05	3.22 ± 0.51
	7pm	4.98 ± 0.84	0.81 ± 0.19	0.34 ± 0.05	3.90 ± 1.35

** denotes comparison within groups where significant*

BPV: In response to dehydration, there were no significant differences observed in BPV in any of the frequency ranges (VLF, LF, and HF) in either HSU or control rats (**Table 3.3.4**). Furthermore there were no significant differences observed during recovery, all parameters remained unchanged from baseline levels. There was also no difference between HSU and control rats throughout the dehydration protocol in any of the frequency ranges (VLF, LF or HF; **Table 3.3.4**).

Table 3.3.4: ***BPV during dehydration protocol (2-way ANOVA)***

	Night/Day Cycle	VLF ($mmHg^2$)	LF ($mmHg^2$)	HF ($mmHg^2$)
Control				
Baseline	7pm	0.34 ± 0.04	0.04 ± 0.00	0.03 ± 0.00
	7am	0.31 ± 0.04	0.03 ± 0.00	0.03 ± 0.00
Dehydration	7pm	0.32 ± 0.03	0.04 ± 0.00	0.04 ± 0.01
	7am	0.32 ± 0.03	0.06 ± 0.01	0.06 ± 0.01
Recovery	7am	0.33 ± 0.04	0.04 ± 0.00	0.03 ± 0.00
	7pm	0.34 ± 0.04	0.05 ± 0.01	0.04 ± 0.01
High Sucrose				
Baseline	7pm	0.37 ± 0.04	0.10 ± 0.04	0.05 ± 0.01
	7am	0.42 ± 0.02	0.09 ± 0.03	0.05 ± 0.01
Dehydration	7pm	0.33 ± 0.04	0.09 ± 0.03	0.05 ± 0.02
	7am	0.42 ± 0.09	0.08 ± 0.02	0.06 ± 0.02
Recovery	7am	0.40 ± 0.06	0.05 ± 0.01	0.05 ± 0.01
	7pm	0.37 ± 0.01	0.04 ± 0.00	0.05 ± 0.01

dP/dt_{max}: There was no significant difference in dP/dT_{max} between groups throughout the entire dehydration protocol (P=0.98).

3.3.4 Comparison of cardiovascular and autonomic parameters between high sucrose and control rats during air-jet stress

The air jet protocol used here was identical to that used for the study on high fat-programmed rats described above (**Figure 3.2.2**).

Cardiovascular parameters: There was no difference in cardiovascular parameters (SBP: P=0.47; DBP: P=0.40; HR: P=0.57; PP: P=0.61) between groups throughout the air jet protocol (**Figure 3.3.7**). Baseline levels in all cardiovascular parameters were similar in control and HSU rats. With the initiation of the first series of air puffs, SBP, DBP and HR all increased significantly except for PP from baseline levels in both groups, to a similar level (**Figure 3.3.7**).

Similarly, during the recovery period SBP, DBP, PP and HR all returned towards baseline levels in both groups. In addition, no difference was observed between groups in the change in SBP from baseline to the initiation of the first air jet (P=0.58; **Figure 3.3.8**).

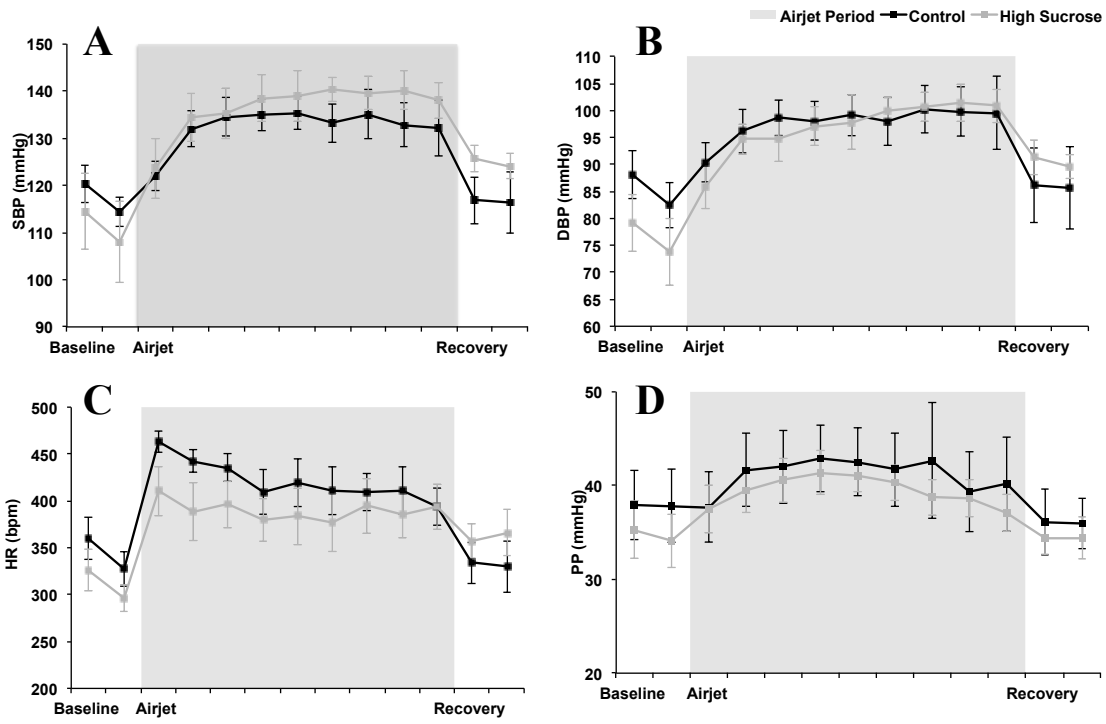

Figure 3.3.7: ***Cardiovascular parameters during air jet protocol (2-way ANOVA)***

The first two points indicate baseline values with two 15-minute averages. Each 9 points in the stress period are averages of each 60-second period following an air puff. The recovery period consists of two points, which are two 15mintues averages. ***A)*** *There was no significant difference in SBP between groups (P=0.47) during the air jet protocol.* ***B)*** *There was no difference in DBP between groups at any stage of the air jet protocol (P=0.40).* **C)** *There was no difference in HR between groups at any stage of the air jet protocol (P=0.57).* ***D)*** *There was no difference in PP between groups at any stage of the air jet protocol (P=0.61).*

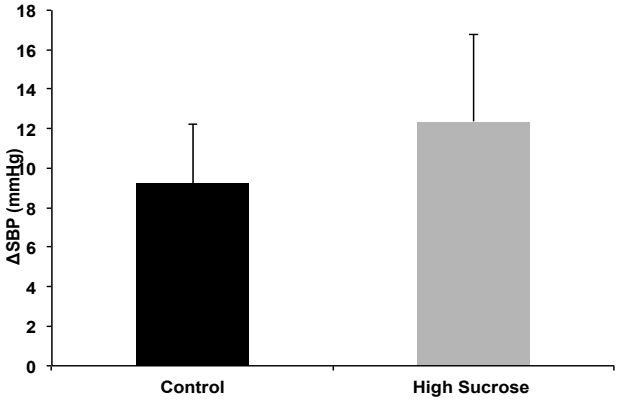

Figure 3.3.8: ***The initial change from baseline in systolic blood pressure immediately following the first air puff (unpaired t-test) (mean ± SEM)***

There was no significant difference observed in the change in SBP between groups (P=0.76).

Autonomic parameters

sBRS and BEI: In response to stress, sBRS remained unchanged in both control (P>0.99) and HSU rats (P>0.99; **Figure 3.3.9A**) with no significant difference between groups (P=0.93). Furthermore, sBRS during recovery was similar to baseline levels in controls (P>0.99) and HSU (P=0.79) rats (**Figure 3.3.9A**).

In response to stress there was a significant fall (approximately 45%) in BEI in the HSU group (P=0.03) with no difference seen in control rats (P>0.99). During the recovery period, BEI returned to baseline levels in both control and HSU rats (**Figure 3.3.9B**).

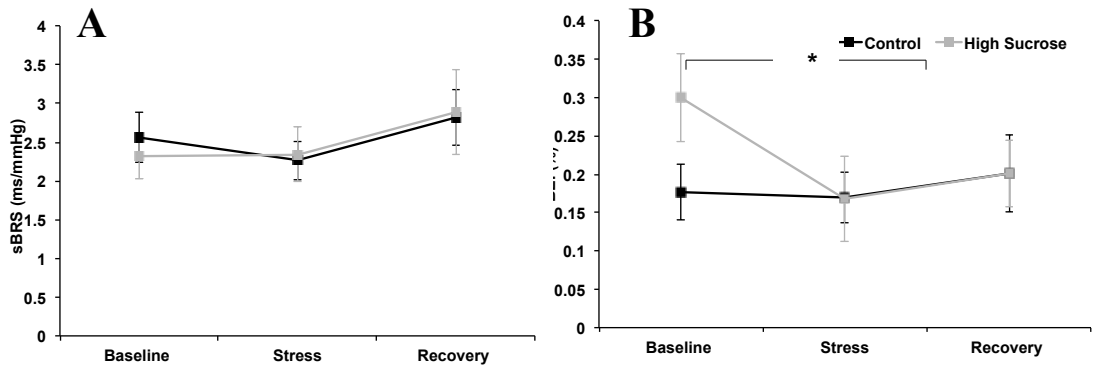

Figure 3.3.9: ***sBRS and BEI during the air jet protocol, each data point is indicative of averages for baseline, stress and recovery (2-way ANOVA)***

A) *There was no significant difference in sBRS between groups in response to stress (P=0.93).* ***B)*** *In response to stress there was a significant decrease in BEI in the high sucrose rats (P=0.03) with no significant difference between groups (P=0.48)*

HRV: There was no difference in HRV between HSU and controls throughout the air jet protocol in any of the frequency ranges (VLF, LF, HF or LF:HF **Table 3.3.5**). Air jet stress produced no change in HRV in any frequency range in either control or HSU rats (**Table 3.3.5**). Likewise, there was no effect on HRV observed during recovery, all frequency ranges remained similar to baseline levels in both HF and control rats.

Table 3.3.5: ***HRV during air jet protocol (2-way ANOVA)***

	VLF ($mmHg^2$)	LF ($mmHg^2$)	LF:HF	HF ($mmHg^2$)
Control				
Baseline	2.46 ± 0.55	0.36 ± 0.12	0.34 ± 0.04	1.33 ± 0.47
Air jet	6.78 ± 2.68	1.77 ± 1.32	0.26 ± 0.06	5.82 ± 3.69
Recovery	1.78 ± 0.26	0.76 ± 0.51	0.32 ± 0.06	4.31 ± 3.61
High Sucrose				
Baseline	2.26 ± 0.56	0.17 ± 0.05	0.32 ± 0.04	0.48 ± 0.13
Air jet	4.63 ± 1.77	1.78 ± 1.21	0.52 ± 0.26	2.18 ± 1.03
Recovery	3.44 ± 0.36	0.55 ± 0.14	0.46 ± 0.12	1.44 ± 0.45

BPV: As with HRV, BPV was not affected by air jet stress in either group of rats (**Figure 3.3.6**). In response to stress, VLF, LF and HF remained unchanged in both high sucrose and control rats. There was no significant difference observed in VLF, HF or LF during the recovery period in comparison to baseline values in either high sucrose or control rats.

Table 3.3.5: ***BPV during air jet protocol (2-way ANOVA)***

	VLF ($mmHg^2$)	LF ($mmHg^2$)	HF ($mmHg^2$)
Control			
Baseline	0.27 ± 0.04	0.05 ± 0.01	0.03 ± 0.00
Air jet	0.87 ± 0.32	0.21 ± 0.15	0.08 ± 0.05
Recovery	0.36 ± 0.08	0.05 ± 0.01	0.03 ± 0.01
High Sucrose			
Baseline	0.22 ± 0.05	0.03 ± 0.01	0.02 ± 0.00
Air jet	0.68 ± 0.28	0.14 ± 0.07	0.04 ± 0.01
Recovery	0.68 ± 0.24	0.08 ± 0.02	0.04 ± 0.00

3.3.5 Fos immunohistochemistry

Effects of air jet stress on Fos expression were examined in the hypothalamus using the DAB protocol. This process formed a black precipitate in the nucleus of Fos-positive neurons; these neurons could be distinguished from neurons that did not have Fos due to an absence of a black precipitate (**Figure 3.3.10**). Fos-positive neurons were identified in hypothalamic regions previously identified as containing Fos-positive labelling following air jet stress (Furlong *et al.,* 2014), such as the DMH (**Figure 3.3.11**) PeF and PVN (**Figure 3.3.12**) in response to air jet stress (**Figure 3.3.10**). However, we did not find a difference in the number of Fos-positive neurons between high sucrose and control rats in any of these regions (**Figure 3.3.10).**

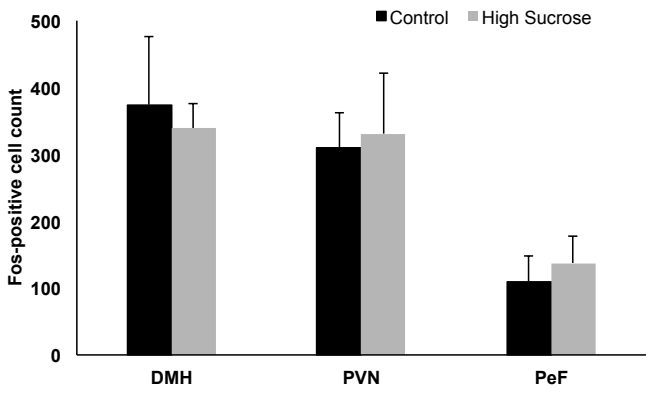

Figure 3.3.10*: Fos labelling in different regions of the hypothalamus including DMH, PVN and PeF. (unpaired t-test) (mean ± SEM)* The number of Fos positive cells located in the DMH (P=0.80), PVN (P=0.88) and PeF (P=0.67) was not different between groups.

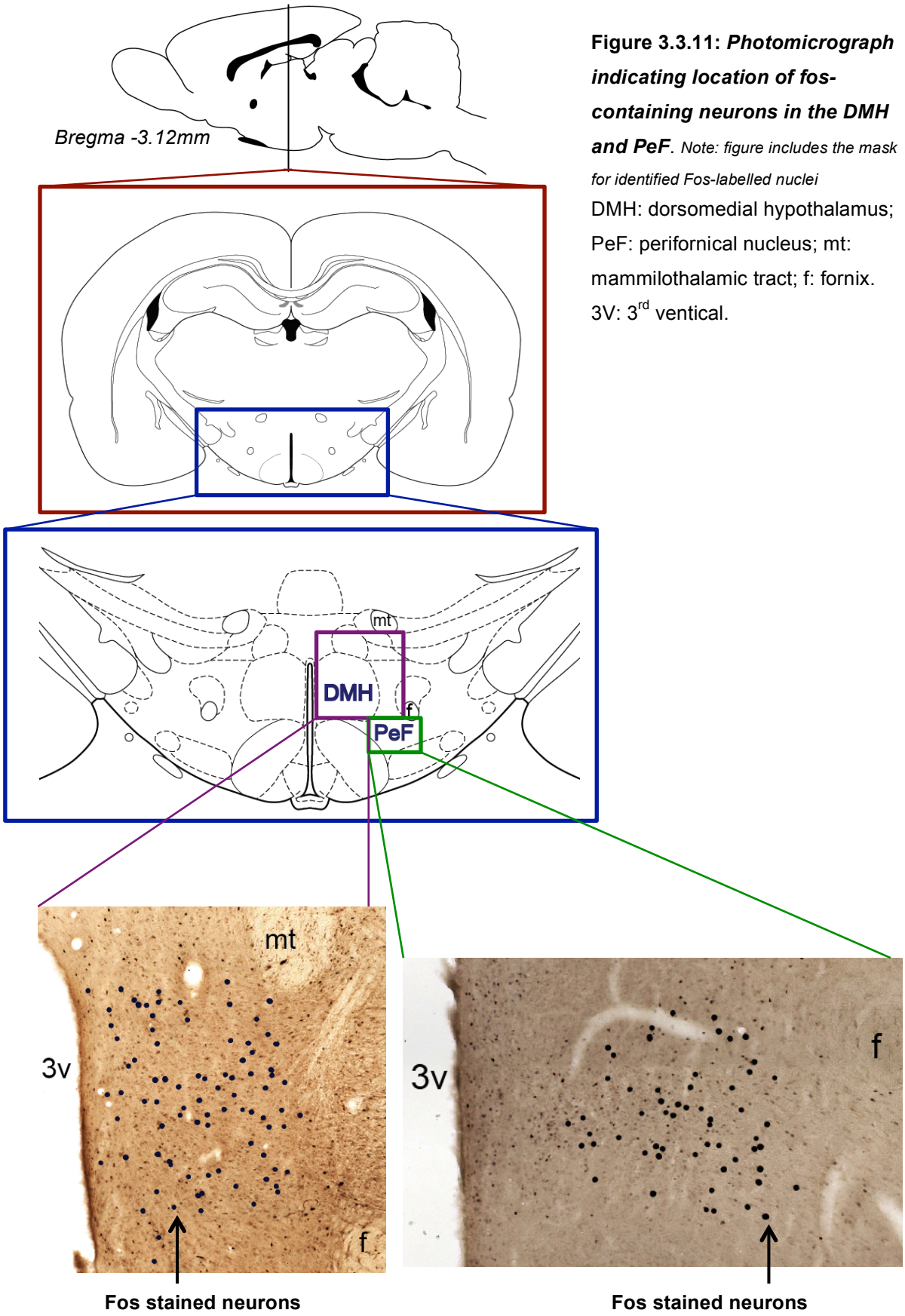

Figure 3.3.11: ***Photomicrograph indicating location of fos-containing neurons in the DMH and PeF***. *Note: figure includes the mask for identified Fos-labelled nuclei*
DMH: dorsomedial hypothalamus; PeF: perifornical nucleus; mt: mammilothalamic tract; f: fornix. 3V: 3rd ventical.

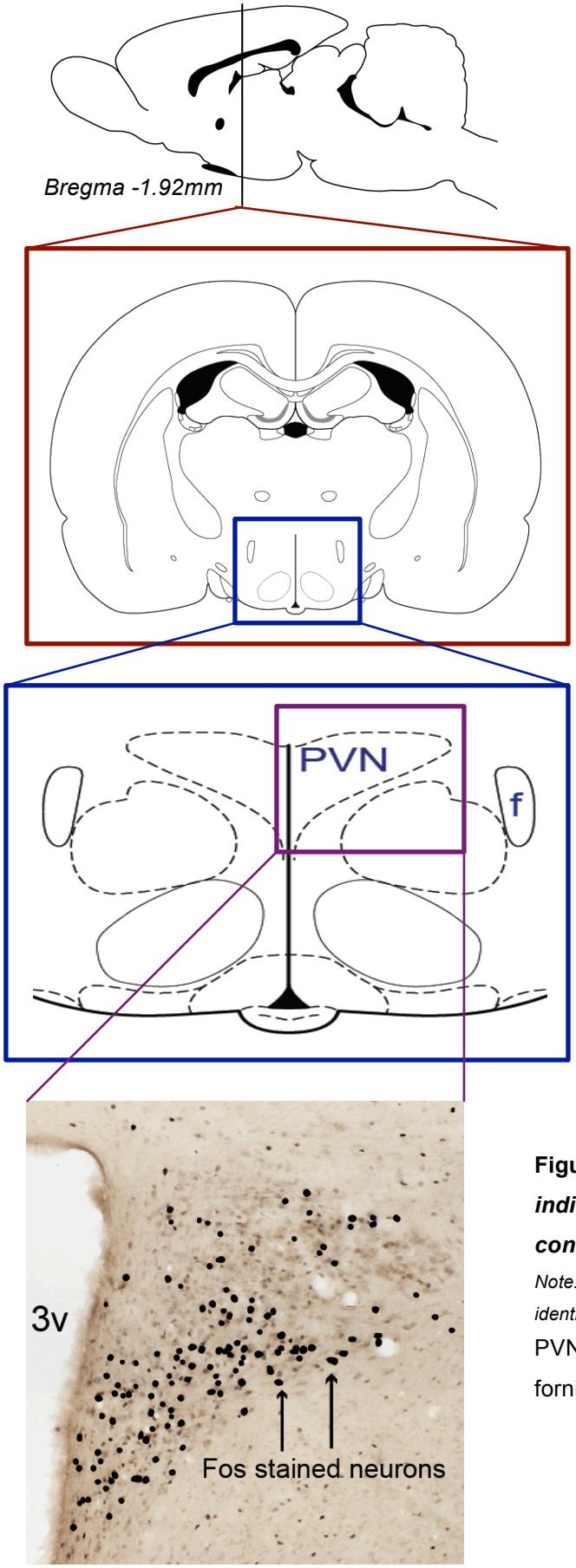

Figure 3.3.12: ***Photomicrograph indicating location of fos-containing neurons in PVN***

Note: figure includes the mask for identified Fos-labelled nuclei

PVN: paraventricular nucleus; f: fornix.

CHAPTER 4: DISCUSSION

The broad aims of this study were to compare cardiovascular and autonomic function in rats exposed to either a high fat or high sucrose diet during gestation with control rats. Variables were compared at rest and in response to an unconditioned psychological stress (air jet stress) and a physiological stressor (dehydration).

Blood pressure was recorded using radio telemetry and measurements were made for 5 minutes each hour over a 9-day protocol. This protocol consisted of three days of baseline recording, three days of water deprivation and three days of recovery. Following completion of this protocol, rats remained in their home cage for three weeks, before the acute air jet stress procedures were performed. For the air jet procedure, blood pressure was recorded continuously for 75 minutes, this included a 30 minute baseline period, a 15 minute stress period and a 30 minute recover period.

The primary findings were as follows. Firstly, in the high fat model, SBP was significantly higher than controls during baseline conditions and during dehydration in both male and female rats. Differences in SBP in response to air jet stress was seen only in the male rats with no difference between groups in the female rats. All animals showed an increased cardiovascular response during exposure to physiological and psychological stressors. However, despite the hypertension observed in the high fat programmed rats, the magnitude of the increase in blood pressure and heart rate from baseline to stress was similar between groups in both male and female rats.

The sustained increased blood pressure observed in high fat programmed females was accompanied by a two fold increase in the low:high frequency ratio of heart rate variability. This ratio represents sympatho-vagal balance, and may be indicative of increased sympathetic modulation, or decreased vagal activity to the heart. This may partly explain the hypertension observed in high fat programmed females. However, there were no obvious differences in autonomic responses to either air jet stress or dehydration between high fat and control animals, indicating that any increased susceptibility to hypertension in high fat programmed rats is unlikely to be due to heightened sympathetic responses to stress.

Second, in the high sucrose model, there was no difference in blood pressure at rest compared to controls. Moreover, there were also no differences in cardiovascular or autonomic variables observed between the two groups in response to either stressor, with both groups showing similar increases in cardiovascular variables in response to the stressors. Therefore the high sucrose model in our studies failed to detect any detrimental effects of maternal high sucrose intake on the cardiovascular or autonomic function in the offspring.

4.1 LOW BIRTH WEIGHT PHENOTYPE

In programming studies, low birth weight is often used as a convenient, albeit simplistic marker for intrauterine adversity. However, in this study, birth weights did not differ significantly between rats programmed by either maternal high fat or high sucrose diets and controls. It is important to note that although birth weight reflects systemic growth restriction *in utero*, it does not provide any information on more subtle, tissue specific growth restriction.

The focus on low birth weight is historical: the original reports on programmed hypertension were human epidemiological studies that relied on birth weight as the major indicator for adverse foetal development (Barker, 1998). Subsequently, the animal models developed to investigate the mechanisms underlying programming also focussed on low birth weight as a defining phenotypic trait of programmed animals (Persson and Jansson, 1992; Langley-Evans *et al.,* 1998). However, accumulating evidence highlights there are many adverse events that affect the development of the foetus that have no effect on birth size (Gluckman and Hanson, 2004; Hanson, 2002; Oliver *et al*., 2002; Pladys *et al*., 2004; Tamashiro *et al*., 2009).

With regard to the effect of high fat and high sucrose maternal diets on birth weight, the data are inconclusive: some studies affirm our results, with no difference in the birth weight of offspring from a maternal high fat or high sucrose diet (Ainge *et al.,* 2011; Ferezou-Viala *et al.,* 2011), while others report reduced birth weight compared to controls (Couvreur *et al.,* 2011; Howie *et al.,* 2009; Khan *et al.,* 2003). Consistent with our results, in sheep a maternal obesogenic diet (high calorie rather than high fat) also has no impact on birth weight (Long *et al.,* 2010; Ford *et al.,* 2009). Thus, possible

programming effects following a maternal high fat or high sucrose diet appear to be independent of birth weight

4.2 METHODOLOGICAL CONSIDERATIONS

Firstly, the methodologies used in this project will be examined before discussing cardiovascular and autonomic results in high fat and high sucrose programmed rats at rest and in response to a stressor.

4.2.1 Low statistical power in the high fat model

It is imperative to indicate that there was a low statistical power in our high fat model. The final total numbers of rats used in our high fat model for statistical analysis included 4 control and 4 high fat rats in the male and 4 control and 3 high fat rats in the female. Therefore the interpretation of the data of the high fat model must be made with caution, as it may not reflect a true effect.

The reason for the small *n* values was because of high mortality during the surgical implantation of the radio telemetry probe in our high fat study. Because we had obtained the rats through a collaboration with another faculty, it was not possible for us to obtain more rats. The success rate in this study was only 40%, in comparison to the success rate of our high sucrose model, which was 88%. We are uncertain as to the exact cause of the low success rate in the high fat study, although there could be a number of possibilities that may have contributed to it

One of the most obvious post surgical complications observed was a lower limb neurapraxia. If the rats persisted to drag their hind limbs for longer than 24-36 hours without signs of improvement, they were euthanized for ethical reasons. Similar incidence was observed in both male and female rats as well as between groups (HFD and control). Previous experiences by Dr Jaimie Polson and by our laboratory have indicated a strong correlation between the duration of occlusion of the aorta during the insertion of the catheter and incidence of neurapraxia. Based on cumulative experience, we believe that clamping of the aorta for 2-3 minutes results in minimal risk of apraxia. The aortic clamp period during the surgical implants in the high fat study was approximately 4-5 minutes, and this slightly extended time period may have contributed to post surgical complications. However, we do not believe that this was a significant factor, as we have had instances in the laboratory where the aorta was clamped for as long as 10 minutes with no major postsurgical complications. Indeed, we observed

good recovery in our high sucrose model in some rats when the occlusion of the aorta lasted 7 minutes. For this reason, other factors must also be considered.

Another factor that may have impacted on post surgical survival was that many of the female rats were obese; surprisingly this was seen in our control group and not in the high fat group (**Figure 4.1**). Post surgical recovery was diminished in these rats. Complications most commonly observed post surgically in these rats were that they were slow to rouse following administration of the alpha-2 antagonist (taking approximately 1-2 hours compared to a normal period to waken of approximately 15 minutes), remained sluggish for an extended period after waking, showed hind limb neurapraxia as described above, and exhibited porphyrin secretion from the nose and eyes. It is widely known that obesity is a major surgical risk factor and is associated with a number of post surgical complications, however the exact mechanism by which this occurs is not clear cut (Yvonne *et al.,* 2014). Therefore, the adverse health outcomes seen in our obese rats post-surgically may be attributed to obesity related surgical complications, such as retarded wound healing and nerve damage, similar to that reported in humans (Yvonne *et al.,* 2014).

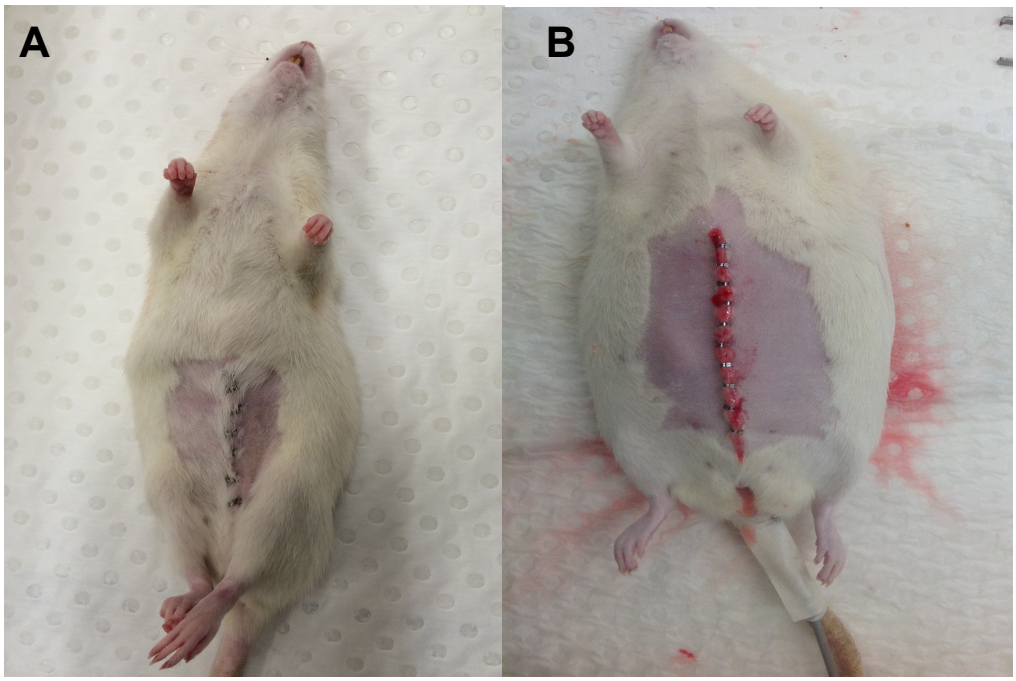

Figure 4.1: ***Non-obese versus an obese rat following telemetry implant from our high fat rat colony. Post-operative mortality was greatly increased in obese rats. A)*** *Non-obese, female, control rat (11 months old) following telemetry implant.* ***B)*** *Obese, female, control rat (6 months) following telemetry implant.*

A third factor in poor post-surgical recovery in the high fat study was the possible presence of bacterial or viral infection of the rat colony at the time. It was noted that many of the rats exhibited rasping-like breath sounds pre-surgically, indicative of mucous secretion in their airways. The likelihood of the rat colony being exposed to an infectious agent is highly plausible as these rats were raised by another investigator in another animal house before being moved to one of the University of Sydney main campus animal houses for our experiments. Therefore this move may have increased exposure to a potential infectious agent. Furthermore, it is well documented that immunity is altered under obese conditions (Macia *et al.,* 2006; Meade *et al.,* 1978). Therefore the combination of increased adiposity together with a potential infectious agent may explain the adverse post surgical consequences in these rats.

Finally, ascites was often observed in a number of these rats. It is unclear as to why such large amount of fluid accumulation was present in the abdominal cavity of these rats, although this commonly occurs with hepatic or cardiac disease. Therefore, there is a possibility that some of these animals may have been suffering from underlying disease.

Therefore, although we are uncertain as to the exact reason, there are a number of possible explanations as to why we experienced low survival rates following surgery in the high fat study. This was particularly problematic because we were unable to obtain additional rats and therefore had low *n* values in the study and subsequently low statistical power. Two power calculations were performed on DBP in female rats at night. The first calculation was done using: the population mean of controls for mu(0), the mean of our sample (high fat) for mu(1) and the known standard deviation for the population for sigma. The power calculation was based on two-sided criteria with 0.05 for the alpha value and 0.80 for power of the test. According to this power calculation a sample size of 16 is needed to be certain of the effect seen. Our second power calculation was done using; the population mean of controls for mu(0), the averages taken from the two highest DBP values for mu(1) and the known standard deviation for the population for sigma. Similar to the first power calculation, the power calculation was based on a two-sided criteria with 0.05 for the alpha value and 0.80 for power of the test. According to this power calculation a sample size of 12 is needed to be certain of the effect seen. Therefore a minimum sample size of 12 is required to be certain of the effect seen. Hence as

discussed in the thesis we had a low statistical power in the female high fat model. In order to be more confident with the results, it will be necessary to breed animals and raise offspring according to the original protocol so that we can increase the *n* values.

4.2.2 The programming models used

Maternal malnutrition during pregnancy can be defined as either a nutrient deficiency or overnutrition, with both types of maternal malnutrition during pregnancy associated with foetal programming and adverse consequence to the health of the offspring in later life. To investigate the effect of increased maternal intake of high fat or high sucrose diet on the offspring, we have developed two rodent models; a high fat model and high sucrose model.

Programming model one - high fat: A number of studies support the hypothesis that maternal overnutrition and or obesity is associated with obesity and hypertension in the offspring (Armitage *et al.,* 2008, 2005; Samuelsson *et al.,* 2007; Khan *et al.,* 2003; Elahi *et al.,* 2009). In most rodent models of maternal overnutrition, the females are maintained on an obesogenic diet, consisting of high carbohydrate and fat or high fat alone until they become significantly heavier than control animals. These diets are implemented throughout gestation and lactation. In these studies, the offspring are exposed to maternal obesity as well as maternal overnutrition during pregnancy and lactation; therefore, the effects of maternal obesity alone cannot be distinguished from maternal over nutrition. Hence, it is unclear if facets of obesity in the mother such as dyslipidaemia, hypeleptinaemia and hyperinsulinaemia may have on programming of hypertension in the offspring. Furthermore, what is yet to be established is whether programming of hypertension is a consequence of established obesity in the offspring (i.e. a secondary consequence of obesity) or if hypertension and obesity exist separately in programmed offspring. Given the increased prevalence of maternal overweight, without obesity, it is surprising to see only a few studies have investigated the programming effects of maternal overweight in the absence of obesity.

The high fat programming model in our study consisted of a diet composed of 34% fat from omega-6 PUFA, 26% protein and with energy intake of 22.3 KJ/g, note, protein levels were maintained at 20-26%, a difference that is unlikely to impact on foetal programming. The dietary interventions were established four week prior to mating, one week during mating (whilst co-housed with a male rat) and subsequently for three weeks of gestation.

It should be noted that in our study, the high fat mothers did not develop obesity at any stage of the dietary intervention, therefore our data support to reject the hypothesis that maternal obesity and its associated facets, such as hyperleptinemia, hyperinsulinemia and hyperglycaemia, as maternal factors that pre-dispose offspring to develop hypertension (**Table 1 in Appendix**). In addition, plasma leptin levels can be used as a surrogate to measure adiposity due to its association with fat mass (Hajer *et al.,* 2008). In our study, although plasma leptin levels were significantly different between maternal groups pre-gestation, circulating leptin levels were found to be similar at gestation day 19 (**Table 1 in Appendix**), hence indicating similar levels of maternal adiposity during gestation. Furthermore there was no significant difference in maternal weight pre-gestation or during gestation **(Table 1 in Appendix)**.

At 6 months of age both control and high fat offspring had similar weights and frank obesity was not noted in the high fat offspring. However, without adequate measures of body composition, it is difficult to conclude if there was, in fact, a difference between offspring body composition at 6 months (time of blood pressure recording). Therefore it is impossible to determine if hypertension arises due to obesity, and its associated facets, in the offspring. To investigate if hypertension arises due to a direct consequence of maternal high fat diet *in utero*, experiments in the younger rats at 3 months of age should be done, before the onset of increased adiposity or obesity hyperleptinemia as seen in the adult rat.

The specific fats that make up a diet commonly used in programming studies of maternal overnutrition include; high saturated, monounsaturated and polyunsaturated fats (Khan *et al.,* 2003; Menegon *et al.,* 2008; Armitage *et al.,* 2008, 2005; Kirk *et al.,* 2009) and since these diets are most often obesogenic, maternal obesity is induced. The high fat diet (34%) used in our study was purely composed of omega-6 PUFA and a non-obesogenic diet. However, this diet is characteristic of a commonly consumed diet in today's society with an overall decrease in omega-3 PUFA intake (Simopoulos, 2011). The consumption of the right ratio of omega-6: omega-3 fats has been thought to be important. An optimum ratio of omega-6: omega-3 is thought to be 1-4 : 1, however in a commonly consumed Western diet, this ratio has changed dramatically to range within 10: 1 (Olivier *et al.,* 2011). Coincidently, there is also an increase in the prevalence of inflammatory processes such as obesity, cardiovascular disease, neurodegenerative and psychiatric illness (Corsinovi *et al.,* 2011) seen with

the consumption of these diets. Therefore a diet high in omega-6 PUFA and low in omega-3 PUFA is thought to shift the physiological state, toward proinflammatory and prothrombotic with increased blood viscosity, vasoconstriction and vasopasam, resulting in increased incidence of developing diseases (Patterson *et al.,* 2012). Furthermore, a dietary imbalance of this ratio results in increased metabolic products with increased omega-6 PUFA. These metabolites include; thromboxanes, prostaglandins, leukotrienes, lipoxins and hydroxyl fatty acid forming in larger quantities, the net effect of this is an increased proinflammatory profile with increased lipids, inflammatory mediators and cytokines which may contribute to the development of inflammatory diseases (Simpopulos, 2002). As described in the introduction, excess maternal inflammation, increased placental cytokines and increased lipid transfer is thought to play a role in foetal programming (Mazzucco *et al.,* 2013; Challier *et al.,* 2008), however further studies need to be done to investigate this. In our studies, it can be concluded that this high fat model does program for hypertension in the offspring at rest (**section 4.4**) but not in response to stress. The failure of a hypertensive response to a stressor is discussed in **sections 4.5** and **4.6** of the discussion. It is also inviting to speculate the effects increased pro-inflammatory and lipid profiles (due to this high fat diet) may have on programming.

Programming model two - high sucrose: in addition to high consumption of a high fat or obesogenic diet in the western world, there has also been increased consumption of sugar sweetened beverages (SSB) (Ismali *et al.,* 1997). Like many dietary insults to the intrauterine environment that alter foetal development, it is thought that maternal access to increased levels of sucrose may also alter foetal development and predispose them for diseases in later life (Metzger *et al.,* 2008; Flynn *et al.,* 2013; Vickers *et al.,* 2012: Sammuelsson *et al.,* 2008). However, exposure to a high sucrose diet during pregnancy and its effects *in utero* has not been extensively investigated. Similarly to our discussions above, it is unclear as to whether a maternal high sucrose diet causes hypertension as a result of obesity and its related sequela or if hypertension is a consequence of the direct influence of a maternal diet high in sucrose.

In our sucrose model, both control and sucrose rats received a standard chow (4.8% fat, 20% protein, energy 14.0 MJ/Kg digestible energy) and water. In addition, the sucrose group was provided with drinking water that contained sucrose at 10% wt/vol. This dietary intervention was applied in the same

manner as the high fat model; established four weeks prior to mating, one week during mating and subsequently for the 3 weeks of gestation.

In most rodent models looking at a high sucrose diet during pregnancy, females are maintained on either a diet high in fructose (Vickers et al 2011), a high sucrose diet alone (Sammuelsson *et al.,* 2008) or a diet high in fructose (10%) and fat (45%), (Flynn *et al.,* 2013). In all these studies there have been observed changes in the offspring compared to controls, these changes include; hypertension and increased fat mass (Sammuelsson *et al.,* 2008), increased plasma leptin, fructose and blood glucose levels in female foetuses (Vickers *et al.,* 2011) and hyperglycemia (Flynn *et al.,* 2013). The latter study by Flynn and colleagues (2013) may be confounding as the diet consisted of both a high fat and high sucrose component, therefore it is unclear as to what the effect of sucrose alone was on offspring health. Furthermore, it was noted that most of these studies do not keep fat and protein levels consistent between control and experimental (sucrose) groups, in most cases the control groups contained higher protein and lower fat levels whilst experimental rats had lower protein and higher fat percentages. This may be confounding as there is ample evidence to show that a low protein diet programs for disease in offspring (Langley-Evans *et al.,* 1994; 1996; 1999; Pladys *et al.,* 2004; Vehaskari *et al.,* 2001; Woods et al., 2001), hence it is unclear as to which component of the diet is having effects on the mother and foetus.

Although a person who consumes a high fat diet is likely to consume a high sucrose diet, differentiating the effect of a high fat diet relative to a high sucrose diet is important as this allows the dietary components that may or may not be detrimental to health outcomes to be teased out. Furthermore, a better phenotypic profile of altered physiological states due to different diets may be developed; allowing investigation of potential over-arching "vectors" (described in introduction **section 1.5.2**) common to each dietary group that might be responsible for programming. This will also allow the investigation of how these vectors may be influencing foetal programming, and therefore assist in identifying the possible pathophysiological mechanisms underlying programmed hypertension. In addition, the vectors common to maternal overnutrition studies may be compared to those in other models of programmed hypertension, such as low protein diet or increased maternal glucocorticoids (Denton *et al.,* 2006; Langley-Evans, 2009)..

The sucrose model in our study consisted of high sucrose (10%) in the absence of high fat; levels of fat, protein and other macronutrients were consistent between controls and sucrose rats. Although some studies described above use a high fructose solution, our model did not. Sucrose is a disaccharide composed of two monosaccharides, glucose and fructose (Kanarek and Orthen-Gambil, 1982). Most ingested food and sugar sweetened beverages are in the form of sucrose, which is later broken down into glucose and fructose during digestion, therefore the sucrose diet used in this model is more reflective of a commonly consumed diet. As described above increased plasma leptin levels, hyperglycaemia, hyperinsulimia and higher blood triglycerides are reported in most of the high sugar model studies (Vickers *et al.,* 2011; Flynn *et al.,* 2013; Rooney *et al.,* 2013; Sammuelsson *et al.,* 2008). In our study it was found that dams exposed to high sucrose showed higher fasting blood glucose levels associated with glucose intolerance (**Figure 2 in Appendix**) and higher blood triglycerides (**Figure 3 in Appendix**), however maternal body weights between groups were similar (**Figure 4 in Appendix**).

In addition the studies mentioned above had different intervention periods for the diet; these included a diet implemented six weeks before mating (Flynn et al 2013), day one of pregnancy to postnatal day 10 (Vickers *et al.,* 2011), six weeks before conception and throughout pregnancy and suckling (Sammuelsson *et al.,* 2008). The dietary intervention period for our study was for a total of 8 weeks; four weeks prior to mating, one week during mating and subsequently for the three weeks of gestation. Foetal programming is thought to be susceptible at different periods during gestation (Entringer *et al.,* 2012; Fisher *et al.,* 2012;) and the effects of the diet on the mother are dependent on the intervention period. Therefore due to differences in the period of dietary intervention, interpreting results from different studies may confound each other.

Overall, the high sucrose model failed to detect any detrimental effects of a maternal high sucrose diet in the offspring. Therefore from this model, it is concluded that a diet consisting of high sucrose alone, in the absence of high fat does not cause adverse effects in the offspring.

4.2.3 Radiotelemetry

Radiotelemetry is now considered the preferred method of blood pressure measurement in the rat. Earlier techniques of blood pressure measurement used methods of arterial cannulation and tail cuff to obtain blood pressure measurements. The use of radiotelemetry provides a number of advantages

over these earlier techniques. Firstly, blood pressure recordings can be obtained in the conscious, freely moving rat. Earlier experiments such as the use of arterial cannulation method had to place the rat under anaesthesia; it is widely known that anaesthesia can affect blood pressure and autonomic regulation, which may confound results and impact on interpretation of data (Dampney and Horiuchi, 2003). Furthermore when investigating the effects of either physiological or psychological stress, it is imperative to use unanesthetised, unrestrained animals; this ensures that proper behavioral and physiological responses are evoked in response to the stress.

The tail cuff method, does provide a technique that does not involve the use of anaesthesia, however this method only allows for accurate measurement of systolic blood pressure rather than measurement of a blood pressure waveform. Therefore detailed analysis of cardiovascular function (including heart rate derived from the pulse interval) is not possible. In particular, is it not possible to make calculations of sBRS, HRV, BPV or dP/dt_{max} with tail cuff. Further, tail cuff is likely to elicit additional stress in the animal that may affect the results (Kubota *et al.,* 2006; Denton *et al.,* 2006). This might be of particular importance in studies looking at programmed hypertension, where blood pressure may not be altered during rest but may change between groups in response to a stressor. For example, a study by Ortiz *et al.,* (2001) using the tail cuff method, showed hypertension at rest in dexamethasone-programmed rats. However, a study by O'Regan and colleagues (2008) repeated the experiments 7 years later, using radio telemetry, and showed that blood pressure in dexamethasone programmed rats did not differ from controls at rest but showed an exaggerated response to stressors such as restraint stress. Studies done by Prestipino *et al.,* (2014) have confirmed the observations made by O'Regan and colleagues (2008), that blood pressure measurements in dexamethasone programmed rats at rest are no different to controls but show an exaggerated response to air jet stress.

The technique of tail cuff recording of blood pressure essentially mimics restraint stress. This is because, in order to measure blood pressure, the rat is essentially restrained in a box in an almost identical manner to that used to evoke restraint stress (Igosheva *et al.,* 2004). Chronic indwelling catheters have also been used to measure blood pressure in the conscious programmed rat (Pladys *et al.,* 2004; Woods *et al.,* 2001; 2004). Although use of this technique will provide the investigator with measurement of blood pressure waveform, the animal is still likely to be experiencing greater

levels of stress simply due to the presence of the externalised catheters running from its body. Therefore the use of telemetry may uncover important differences in blood pressure control that was masked by the use of earlier techniques.

Secondly, the use of telemetry allows for long-term recording of blood pressure without the use of any additional manipulation. After the implantation of the radiotelemetry probe, a rat can be placed on a receiver and blood pressure recordings can be made for extended periods of time without disturbing the animal. With regard to our study, this has been key for blood pressure measurements during the 9-day dehydration protocol. Use of radioteleletry allowed measurement of differences in blood pressure in response to a three-day period of dehydration and diurnal fluctuations in blood pressure. Furthermore, the acquisition program used to record blood pressure, allows up to 8 animals to be recorded at a time, enabling collection of blood pressure data from 8 animals at a time over a 9-day protocol. Therefore, telemetry provides a very powerful tool for long-term recording of blood pressure, as well as other indices of cardiovascular and autonomic function in a very efficient manner.

However, further refinement to this technique is possible. The transmitters used in our studies were rat (PA-C40 DSI) transmitters, which are designed for use in rats weighing above 170g. It was noted during the implantation of the probe that the rat transmitter appeared to be quite big when sutured to the wall of the abdomen in some rats. Therefore the use of another smaller and lighter mouse transmitter (PA-C10, DSI) may be a possibility, this would reduce any discomfort in the rat that may be associated with the placement of the transmitter, and allow for better and faster recovery from surgical implantation of the probe, thus reducing any stresses caused. This may be of particular significance in our high fat study, where a high mortality rate following surgery was experienced. Studies using mouse transmitters have shown blood pressure data to be of same stability and quality as the larger rat transmitter (Polson *et al.,* 2010; Braga and Prabhakar, 2009; Samuelsson *et al.,* 2010). It has also been thought that there may be physiological benefits for using a smaller transmitter, Prabhakar *et al.,* (1985) showed evidence of altered breathing patterns with the addition of extra mass to the abdominal cavity. Therefore using a smaller transmitter might reduce physiological disruptions due to the presence of a transmitter thus improving animal welfare. Furthermore, the use of a smaller mouse transmitter would enable us to obtain blood pressure recordings in younger rats (as young as three weeks of age, Samuelsson *et al.,* 2010), allowing us to investigate changes in blood pressure

regulation at a younger age and hence before the onset of any obesity or age related changes to blood pressure regulation (Samuelsson *et al.,* 2010; Simms *et al.,* 2009). On the other hand, the main disadvantage of using a mouse transmitter is the greater cost of purchase and reduced battery life in these smaller transmitters; this will in turn increase costs associated with refurbishment of transmitters.

4.2.4 The use of non invasive indices of autonomic function

The use of non-invasive measures of autonomic function has a major advantage in the fact that they can be carried out under normal physiological behaviors, i.e in an unrestrained, freely moving animal. However, a major disadvantage of these measures is that they are all indirect measures of autonomic function and are subjected to over interpretation and potential errors. The indices used to describe autonomic function in our study include dP/dt_{max}, HRV, BPV, sBRS and BEI; these indices have all been validated and used in previous studies and have been useful in the estimation of autonomic function provided care is taken with interpretation (Waki *et al.,* 2006; Dowell and Houdi, 1997; Malpas, 2002).

$dP/d_{max}t$: dP/dt_{max} of the aortic blood pressure waveform was used in our study as an index of left ventricular contractility. Traditional techniques used to determine ventricular dP/dt_{max} are quite invasive, these techniques require the insertion of a catheter into the left ventricle to detect pressure (de Roest *et al.,* 2009). However, studies show aortic pressure can be used as a surrogate for ventricular pressure when determining ventricular contractility. For example, a study by Dowell and Houdi (1997) showed that injections of positive or negative cardiac inotropic agents have significant effects on aortic pressure and flow; these effects are directionally appropriate and produce similar data to use of cardiac catheterisation. Further supporting this theory, Brington et al (1997), found a good correlation (r=0.87) between data from peripheral dP/dt_{max} calculations and invasive catheterised ventricular recordings of blood pressure. However, it is important to note that there are situations in which dP/dt_{max} calculations from aortic blood pressure measurements are imperfect and become an incorrect predictor of the inotropic state. In particular, vessel stiffness in the abdominal aorta is an important factor to consider when making dP/dt_{max} calculations from the aorta. This is because a decrease in arterial vessel elasticity produces increased pulse pressures amongst an array of other pathophysiological changes (Quinn *et al.,* 2012), and this would produce a higher dP/dt_{max} despite unaltered ventricular contractility. We did not investigate arterial stiffness, and so this caveat remains

open to interpretation. However, none of the rats in any cohort showed unusually high pulse pressures. Therefore, although the possibility cannot be excluded, it is unlikely that arterial stiffness was altered in this study. Calculations of dP/dt_{max} are also influenced by changing load conditions on the ventricle (preload and afterload), which affect intrinsic myocardial contractility (Hamlin and del Rio, 2012). Because it was not possible to measure ventricular pressures, the finding that there was no difference in cardiac contractility between groups is dependent on the assumption that intrinsic myocardiac tension-length relationship and cardiac filling conditions are also the same.

HRV and BPV: Beat to beat fluctuations in heart rate and blood pressure are present in all investigated mammals including humans (Malpas, 2002). There are a number of factors that underlie this observed variability including differences in the inherent biophysical properties in the sino-aortic pacemaker myocytes and haemodynamic properties of the circulation (Malpas, 2002). In addition, there is good evidence that variability is influenced by temperature regulation, vasoactive hormone levels and sympathetic and parasympathetic nerve activity (Malik, 1996; Stauss, 2003). The importance of the autonomic nervous system in heart rate variability is clear in patients following cardiac transplantation, where heart rate variability is markedly reduced (Toledo *et al.,* 2002). On the basis of the different frequency response characteristics of sympathetic and parasympathetic modulation of heart rate, frequency analysis of heart rate variability can identify a low frequency component that is correlated to sympathetic modulation and a high frequency component that is correlated to parasympathetic modulation of heart rate (see below). However, despite the strong autonomic modulation of heart rate the LF and HF frequency components of HRV may not always be very reliable markers for cardiac sympathetic and parasympathetic activity (Malpas, 2002). Specifically, while the LF component is influenced by the sympathetic nervous system, there are many examples where known increases in sympathetic nerve activity are not associated with changes in low frequency variability. For example, there is a decrease in the LF HRV with myocardial infarction despite increased levels of sympathetic activity (Houle and Billman, 1999). Similarly, there is a decrease in the LF HRV during exercise, while sympathetic activity is increased (Arai *et al.,* 1989).

Spectral analysis using a fast fourier transform (FFT) algorithm is one of the most commonly used methods of investigating HRV and BPV in the frequency domain (Malpas, 2002; Cerutti *et al.,* 1991), as used in our studies. The FFT algorithm deconstructs the HR and SBP waveforms into different

components depending on frequency. The most common analysis divides the frequency range into three distinct frequency bands; very low frequency (VLF), low frequency (LF) and high frequency (HF). Spectral analysis provides information on how much variance or "power" there is in each frequency band (VLF, LF and HF), (Jenkins and Watts, 1968; Kay and Marple, 1981; Malik *et al.,* 1996). The VLF band comprises a frequency range of HR and BP oscillations between 0-0.27 Hz in the rat. Variability in the VLF component is thought to reflect changes associated with levels of vasoactive hormones (Cerutti *et al.*, 1991; Askelrod *et al.,* 1981). The LF component consists of frequencies in the range of 0.27-0.75 Hz in the rat. Although the LF component is more complex, it is often thought to be a dominant indicator of sympathetic activity, or the level of sympathetic modulation in both HRV and BPV (Berntson *et al.,* 1997; Billman, 2011; Malpas, 2002). This association with BPV has been shown by the use of ganglionic blockade, which abolishes these oscillations in the BPV component (Cerutti *et al.,* 1994). The HF band is associated with respiratory sinus arrhythmia and the parasympathetic influences on HR, consisting of a frequency range between 0.75-3.3 Hz in the rat (Waki *et al.,* 2006). With regard to BPV, the HF component is not related to autonomic function, but is associated with respiratory changes in the intrathoracic pressure perturbing venous return and cardiac output. The HF component in HRV is indicative of parasympathetic activity, and has been demonstrated by using parasympathetic blockers such as atropine, which substantially diminishes the HF component in HRV, but this is not seen in BPV (Malpas, 2002; Malik *et al.,* 1996).

Like any technique there are negative aspects involved in the use of a FFT algorithm in spectral analysis. The FFT takes into account all the data available from the selected signal when producing the power spectrum; therefore it includes the entire signal variance regardless of whether the frequency components emerge as specific spectral peaks or non-peaks (Parati *et al.,* 1995). Furthermore, the recorded signal may frequently contain signal artifacts, and as the FFT takes into account the entire recorded signal, these artifacts, if large in amplitude, may have a substantial influence on the signal variance, thereby affecting the power spectrum. It is important to consider these factors in our study, especially when analysing the dehydration protocol. During our protocol, blood pressure was recorded for five minutes per hour over the entire 9 days. One technique to reduce effects of signal variance is to carefully select short periods of time when there is minimal variance. However, a disadvantage of this is that the resulting power spectrum may not be representative of the true variability over the course of the study. Therefore, to both minimise this

signal variance and yet maintain an accurate estimate of true variability, the analysis was performed over every 5 minute period separately and the powers averaged for night and day. By way of comparison, the study of Rudyk et al. (2011) made estimates of HRV in their programmed rats based upon a single 5 minute recording performed between 9-10 am. It is apparent that measurements made over such a small time interval will not be representative of the overall variability in a 24 hour period, but very dependent upon events that have occurred at the time point where the measurements were made.

An alternate method to overcome these artifact- associated influences is the use of another technique, known as autoregressive (AR) modeling. The AR method identifies a best fitting model in the raw data from which the final spectrum is derived, and any component of the recorded signal that does not fit the model is regarded as noise and is either partially or totally removed (Box and Jenkins, 1970, Kay, 1988).

However, in our study, FFTs done over a recorded signal consisting of moderate artifacts did not appear to alter the HRV and BPV analysis, although regions with major artifacts were removed manually. This was mainly done during the air jet protocol; the 2 second time period during which the stream of air was directed at the rat. The sudden puff of air provoked escape behavior (running and jumping), which often elicited substantial movement artifacts in the blood pressure waveform. For this reason, this period was excluded, and data was sourced from the 1 minute intermittent resting phase between sets of puffs. This period was used to describe the stress period and its associated changes in HRV and BPV.

4.2.5 Fos immunohistochemistry

As described in the introduction, the method of immunohistochemical labelling of fos as a marker for neuronal activity has a number of advantages over older traditional techniques such as identification of changes in metabolic activity or invasive electrophysiological recordings (Greenberg *et al.,* 1981). Specific characteristics of c-*fos* transcription and the protein product Fos make it an ideal tool for use as a marker of neurons recently activated. For example, basal Fos levels are almost always negligible in neurons, but is rapidly activated in response to a stimulus (Dampney and Horiuchi, 2003). Therefore any Fos expression observed is likely to be from recently activated neurons. This characteristic of Fos has served well for the specific detection, quantification and mapping of activated neurons after the air jet protocol in our studies. In addition, Fos labelling can be performed in conjunction with other techniques to allow for a more detailed analysis, such as double-labelling of neurotransmitters/neuromodulators and the use of retrograde/anterograde tracing (Furlong *et al.,* 2014; Dampney and Horiuchi, 2003). More importantly, the technique of using Fos as a marker of neuronal activity can be used in the conscious freely moving animal, negating any confounding influences of surgical stress or anaesthesia on neuronal activation (Dampney and Horiuchi, 2003).

The use of this method has some possible drawbacks. Firstly, although this technique allows studies to be carried out in the conscious animal, the animal may be susceptible to other environmental stimuli such as noise and smell, which may arouse and stress the animal (Li and Dampney, 1994). Any noise or smell in the lab could therefore cause activation of neurons thereby increasing Fos expression. To minimise these effects, we allow the animal to acclimatise to the lab environment for two weeks before the commencement of the experiment. By this time, the animal should have been well adapted for the laboratory environment and any noise or smell should no longer act as a significant stimulus to evoke Fos expression in the animal. Possible false positive labelling from environmental stimuli would have been accounted for with the use of control experiments (i.e. without exposure to air jest stress), in which basal levels of Fos were quantified. Such a control can serve as a baseline for comparison of the level of Fos expression in rats at rest and of those exposed to the air jet stress. These basal measures of Fos expression could not be performed in the present study due to the limited number of animal available and time constraints. However, it should be acknowledged that these measures would provide valuable information about basal Fos expression in high sucrose and high fat programming.

Another drawback of the Fos technique is that Fos expression is not seen in all neurons when activated. For example, neurons activated in the substantia nigra do not express Fos, regardless of the stimulus (Dragunow and Faull, 1989), while in cardiac vagal preganglionic neurons Fos expression is delayed by up to 8 hours before it is detectable (Dampney and Horiuchi, 2003). Therefore, in such studies, it is not possible to label Fos in both cardiac vagal preganglionic neurons and other brainstem neurons due to the transient nature of the Fos expression in other neurons. It is therefore necessary when interpreting results to be aware factors such as these that would elicit false negative data.

The use of Metamorph to discriminate labelled neurons was used, the accuracy of this semi-automated counting program has been validated in our laboratory previously by performing manual counts on random selection of sections (Iigya et al., 2012)

4.2.6 Receptor sensitisation and desensitisation

To investigate the stress response in programming, rats were subject to two types of stressors, a physiological (dehydration) and a psychological (air jet) stress. Rats were exposed to both stressors; firstly the dehydration protocol was performed, followed by a three-week rest period before the air jet protocol was implemented. A potential confounder in the data obtained from the air jet stress protocol is receptor sensitisation or desensitisation due to prior dehydration. Sensitisation is a process whereby the repeated administration of a stimulus causes an amplification of the process (Shettleworth, 2010). Conversely, receptor desensitisation is a process where by the receptor has a diminished response to a stimulus due to prolonged exposure (Fehmann *et al.,* 1991). It is unlikely that sensitisation or desensitisation due to dehydration would have impacted the blood pressure data obtained in the air jet study. This is because dehydration has been a persistent challenge to homeostasis throughout evolution (Debora *et al.,* 2012) and therefore plasma osmolality and blood volume are robustly defended and controlled tightly in the mammal via homeostatic processes (McKinley *et al.,* 2004; Autunes-Rodrigues *et al.,* 2004). Hence, It is very unlikely that these evolutionarily homeostatic processes would result in any receptor sensitisation or desensitisation, particularly because of the inclusion of a three week resting period, between dehydration and air jet stress. Furthermore, receptor sensitisation is mostly characterised in the central sensitisation of nociceptive neurons, processes of long-term potentiation (learning), drug sensitisation and allergic sensitisation (Collingridge *et al.,* 2004; Robinson *et al.,* 1993; Kohno *et al.,* 2003; Janeway *et al.,* 2001). Therefore, it is unlikely that a stimulus such as dehydration would cause receptor sensitisation under these categories of

sensitisation. Similarly, receptor desensitisation is often associated with increased agonist exposure to a receptor, which decreases its sensitivity. Although hormones such as antidiuretic hormone are in action during dehydration (Autunes-Rodrigues *et al.,* 2004; Burbach *et al.,* 2001) there are no known pathways whereby sensitising or desensitising these hormonal receptors may impact blood pressure data following psychological stress. Ideally, control experiments should have been performed to determine if rats exposed to dehydration respond differently to psychological stress compared to rats that did not experience dehydration. However, due to the limited number of available rats, this was not practicable.

4.3 SEX SPECIFIC DIFFERENCES

There are three main factors that give rise to sexual dimorphism and consequently sexual dimorphism in developmental programming. These three factors include; differences in developmental patterns (genetic, morphological and transcriptional), differences in the timing of development, and the effect of steroid hormone exposure during *in utero* and postnatal life (Aiken and Ozanne, 2012). These factors together give rise to mature adult organisms that are sexually dimorphic in their physiology, anatomy, behaviour and reproductive capacity (Aiken and Ozanne, 2012). Therefore, it was important to consider both male and female offspring in our study to investigating how sex-specific differences may influence blood pressure in our high fat programmed model.

In reviewing the literature, it is evident that male offspring appear to be more susceptible to developing programmed hypertension due to an adverse *in utero* stimulus compared to females. In the rat, there are reports in models of maternal gestational dexamethasone exposure (Alexander, 2003; Ortiz *et al.,* 2003), maternal low protein diet (Woods *et al.,* 2005: Langley-Evans *et al.,* 1996) and in studies of uterine artery hypoperfusion (Alexander, 2003) that describe raised blood pressure in male, but not female offspring. With regard to programming following maternal high fat diet, there are conflicting data. An early study by Langley-Evans (1996) showed mothers fed a diet high in saturated fat results in hypertension in the male offspring but not in females. In contrast, Khan et al (2003) showed a maternal obesogenic high fat diet programmed hypertension in female offspring only, while another paper reported that an obesogenic high fat diet programmed hypertension in both male and female offspring (Samuelsson *et al.,* 2010). Similar conflicting results have been reported in mice, with both

high fat programming producing hypertension in both male and female offspring (Samuelsson *et al.,* 2008) and in females only (Elahi *et al.,* 2008). Thus, it appears that there is not a strong sex-specific difference in high fat programming of hypertension.

In our study, both male and female rats programmed by a maternal high fat diet developed hypertension to a similar degree. The LF:HF ratio, an index of cardiac sympathetic modulation, was elevated in the HRV spectra in females. This response was exacerbated when the females were exposed to dehydration. This suggests that maternal high fat programming may alter cardiac autonomic function in females, which in turn, may increase the risk of developing hypertension under certain conditions. In males, there was no evidence of autonomic dysfunction either at rest or in response to physiological or psychological stressors. Therefore, the hypertensive phenotype observed in programmed males does not appear to have an autonomic origin.

The studies of maternal overnutrition described above indicate that female offspring may have a greater tendency to develop hypertension than the males. The reason for this remains elusive, however, a clue may lie in the observed difference of growth rates in the male and female foetuses *in utero* (Gilbert and Nijland., 2008). Faster growing male foetuses may differ in their susceptibility to nutritional insults in comparison with female foetuses. There is also evidence that there may be sex-specific differences in the mechanisms that produce programmed hypertension. For example, a maternal low protein diet during gestation has been reported to program hypertension in males via a glucocorticoid-mediated mechanism. Conversely, in females it has been shown to be mediated via changes in angiotensin receptor expression (McMullen and Langley-Evans, 2005). It is therefore possible that similar sex specific differences are present in high fat programming, however, this remains to be investigated.

4.4 CARDIOVASCULAR AND AUTONOMIC PARAMETERS AT REST

Cardiovascular parameters at rest*:* Similar to previous studies of high fat programmed hypertension in Sprague Dawley rats (Khan *et al.,* 2003; Samuelsson *et al.,* 2009), our high fat model programmed for hypertension at rest in both male and female offspring. Although there are clear differences in the high fat diets used and the duration of the intervention period implemented across most of the studies of maternal overnutrition, including ours ,, all studies show hypertension in offspring of over-nourished

mothers compared to offspring from mothers given a control diet (Khan *et al.,* 2003; Samuelsson *et al.,* 2009). However, an important difference is, our study appears to be the first to demonstrate programmed hypertension using the high fat diet model in the absence of maternal obesity and offspring obesity. In contrast, Rudyk et al. (2011) found that a non-obesogenic maternal high fat diet (23.6% lard) did not produce raised blood pressure at rest in the offspring. It is possible that the difference in programming effect is due to the timing of exposure to the high fat diet or the amount of fat in the diet. In the study by Rudyk and colleagues, the dams were exposed to the high fat diet for only 10 days prior to conception, compared to one month in our study. This early pre-conception stage is crucial in determining the quality of the oocyte (Minge *et al.,* 2008) and extended exposure to an unfavourable diet may produce early developmental effects. In addition, there was a difference in the amount and type of fat used between our study and that of Rudyk et al (2011), which may impact on possible developmental or epigenetic changes. Nevertheless, in our model, hypertension can arise purely due to gestational dietary high fat intake. This is an important consideration in the interpretation of possible causative mechanisms because it shows that obesity and the changes associated with obesity, such as raised leptin levels (Taylor *et al.,* 2014), are not necessary to produce changes in blood pressure regulation.

In the high sucrose model, raised blood pressure was not observed at rest. There are only two reports in the literature that have investigated the programming effects of gestational high sucrose diet on blood pressure (Samuelsson *et al.,* 2008, 2013). The first of these was a study in mice using radio telemetry and demonstrated programming of raised blood pressure. However, that study used a gestational diet consisting of both high fat (16%) and high sucrose (33%), and importantly, the diet was obesogenic. This raises the possibility that the reported hypertension was due to raised fat or obesity, rather than high sucrose. The same investigators recently reported results of a study in mice using a maternal high sucrose diet in the absence of high fat (Samuelsson *et al.,* 2013). In contrast to the results of the current study, they reported raised blood pressure in the high sucrose rat during the active phase (night). The increased SBP observed by Samuelsson and colleagues, but not seen in our high sucrose model, is difficult to compare as Samuelsson and colleagues used a mouse model and there may be species differences in programming effects of high sucrose on blood pressure control.

An explanation for the difference in programming effect on blood pressure following gestational high sucrose may be due to the shorter dietary intervention period in our study compared to that of Samuelsson and colleagues (2013). Dams were maintained on the high sucrose diet for 4 weeks prior to mating, one week during mating and three weeks of gestation. In contrast, Samuelsson and colleagues maintained their dams on a high sucrose diet for 6 weeks prior to mating, throughout gestation and suckling. In other programming studies of maternal obesity, it has been found that oocyte quality is determined before fertilisation even occurs (Minge *et al.,* 2008), thus the longer dietary intervention period prior to mating may prime adverse changes in the oocyte associated with impaired embryo development. In addition, the early postnatal exposure to high sucrose may also have a significant impact on developmental changes, further priming the offspring to develop hypertension (Myrie *et al.,* 2012). Therefore, it is unclear from the study of Samuelsson et al. (2013) whether the effect that they describe on blood pressure is due to a programming effect during the antenatal or postnatal period. Finally, the sucrose content was much higher (55% greater in simple sugars) compared to our study (10% sucrose), which may impact programming differently. A 10% sucrose solution was used in our model as this is based on the standard energy density of a popular sugar sweetened beverage (1.7kJ/ml), therefore, the sugar content in our study is more applicable to today's society than previous studies. However, further investigation is necessary to develop a better picture of the effects of high sucrose during development of blood pressure regulation.

Autonomic parameters at rest*:* There was no indication of baroreflex dysfunction at rest in either male or female offspring of the high fat or the high sucrose models according to BEI and sBRS indices. This is not in agreement with other programming studies utilising maternal high fat diet (Rudyk *et al.,* 2011), maternal obesogenic diet (Samuelsson *et al.,* 2010), maternal dexamethasone exposure in sheep (Segar *et al.,* 2006; Dodic *et al.,* 1999) and maternal protein restriction (Pladys *et al.,* 2004). It is important to note that in these previous studies the Oxford technique was used to measure baroreflex. The Oxford technique allows for a detailed description of baroreflex function, as comparisons of the relationship between blood pressure and heart rate are made over a wide range of blood pressures. Therefore, changes associated with baroreflex function can be shown in the absence of changes to baroreflex sensitivity (Pladys *et al.,* 2004). However, a negative aspect of the Oxford technique is that there is an element of stress involved in the protocol, which may confound the results obtained. It is widely known that stress causes changes in baroreflex function (Dampney *et al.,* 2008).

Therefore, the differences in baroreflex function seen in previous studies may be attributed to the way the programmed animals reacted to the stress of exteriorised catheter implantation. Consequently, the spontaneous technique used in our study may reflect a more accurate measure of baroreflex function at rest.

Baseline measurements of $dP/dt_{max,}$ LF and LF:HF component of HRV and the LF component of BPV are indicative of sympathetic and vasomotor modulation. In our study there was no indication of increased cardiovascular sympathetic activity at rest in the male or female offspring of the high fat or the high sucrose models. This is surprising, as previous studies in the rat using similar techniques commonly show increases in sympathetic cardiac and vasomotor activity during rest in programming models using maternal obesogenic diet (Samuelsson *et al.,* 2010) and protein restriction (Pladys *et al.,* 2004). A possibility for this difference may be attributed to differences in these programming models, which may result in different autonomic function. For example, the study by Samuelsson et al (2010) used an obesogenic high fat diet, whereby obesity is induced in the mother (not the case in our study). This means that the offspring are exposed to both maternal obesity and maternal overnutrition during pregnancy and lactation; hence effects of maternal overnutriiton per se cannot be distinguished from maternal obesity. These are potentially important differences because increased adiposity produces a number of changes that may affect central nervous system regulation of sympathetic function, such as increased leptin concentration or insulin production (Head *et al.,* 2014). Therefore the differences in sympathetic and vasomotor modulation seen in our study to that of other programming studies may be attributed to difference in programming models. Consistent with this, no differences in HRV or BPV were found at rest in a similar model to the one employed in this study, where pregnant dams were exposed to a non-obesogenic high fat diet (Rudyk *et al.,* 2011), although, as mentioned above, in that study there was no difference in blood pressure reported between groups. In addition, results of previous studies and our study should be considered with caution, as sympathetic activity is not always reflected by changes in HRV or BPV if the modulatory effects are changed (Stauss *et al.,* 1995; Eisenhofer *et al.,* 1996). This is seen most clearly in heart failure, where it is reported that the LF component of HRV is reduced despite clear evidence for increased cardiac sympathetic activity (Van de Borne *et al.,* 1997). This reduced LF band has been explained as indicative of a raised, but invariant level of sympathetic activity in heart failure.

There was no observed difference in other frequency components of HRV or BPV between groups, in either male or female offspring, thus supporting the conclusion that cardiac autonomic function is not altered in either the high fat or high sucrose models.

4.5 CARDIOVASCULAR AND AUTONOMIC PARAMETERS DURING PSYCHOLOGICAL STRESS

Cardiovascular parameters during psychological stress: In our study, the high fat programmed rats (male and female) had sustained higher blood pressure throughout the air jet protocol (baseline, stress and recovery). However there was no observed difference in the magnitude of the blood pressure and heart rate response to the air jet stress. Therefore, it was concluded that the high fat rats did not respond to the air jet stress differently to controls with respect to their hypertensive response. Cardiovascular changes in response to stress between our study and other studies (Samuelsson *et al.,* 2010; Rudyk *et al.,* 2011) are difficult to compare, as the type of stressors used are different, and different psychological stressors may elicit both qualitatively and quantitatively different cardiovascular and sympathoexcitatory responses (Furlong *et al.,* 2009). The choice of psychological stressor used in our experiments was the air jet protocol, which is regarded as a mild to moderate psychological stressor (Dampney *et al.,* 2008). This stressor has been shown previously to evoke hypertensive, tachycardic and putative sympathoexcitatory responses in the naive (non-programmed) rat (Furlong *et al.,* 2014). Although both control and high fat rats did respond to the stressor, the blood pressure data in our study are not consistent with the view that a maternal high fat diet during gestation causes offspring to respond to a stressor in a hypertensive manner, in contrast to previous reports (Samuelsson *et al.,* 2010; Rudyk *et al.,* 2011). The differences observed in the stress responses may be attributed to the type of stress used. Previous studies have used restraint stress by placing the rat in a confined space for 20 minutes (Samuelsson *et al.,* 2010; Rudyk *et al.,* 2011). This is regarded as a significantly more severe form of stress. Studies by McDougall *et al.,* (2006) showed that air jet stress and restraint stress elicited clear differences in their cardiovascular responses in rats, with restraint evoking a much greater increase in blood pressure and heart rate than the air jet protocol. It is therefore thought that a gating mechanism is operative in the central nervous system that consequently determines the amount of cardiovascular activation depending on the degree of the

perceived threat (McDougall *et al.,* 2006). It is possible that in our study the more mild form of stress failed to evoke a difference in cardiovascular or sympathetic responses between the control and high fat programmed rats. As mentioned above, there were differences in maternal diet and/or duration of the intervention period of the diet in these previous studies,. It is possible that these differences may also underlie differences in programming effects and subsequent cardiovascular responses to stress. In this context it is worth noting that the study of Samuelsson et al. (2010), which showed a greater difference in cardiovascular response to restraint stress between control and programmed rats, used an obesogenic high fat diet that is likely to exert greater programming effects than a non-obesogenic high fat diet. In contrast, the high fat diet of Rudyk and colleagues (2011) was not obesogenic, although it did extend into the postnatal lactation period. That study reported a difference in cardiovascular responses to restraint stress between groups in male, but not female offspring, suggesting that the responses are graded and dependent on the severity of the programming insult. These dietary differences would need to be examined more carefully to determine the exact effect that they may have on programming cardiovascular responses to psychological stress

The second model, ustilising high sucrose programming, did not show any differences compared to controls in blood pressure or heart rate responses to air jet stress at any time point throughout the protocol (baseline, stress and recovery). At the time of writing, there has been no study done previously that has examined the cardiovascular responses to a psychological stress in a high sucrose programmed model. Although preliminary, the results of the current study suggest that gestational exposure to high dietary sucrose does not program an altered cardiovascular response to psychological stress in the offspring.

A number of studies using other models of programming have reported enhanced cardiovascular responses to psychological stress, including gestational high glucocorticoid exposure (O'Regan *et al.,* 2008; 2010), stress in pregnancy (Igosheva *et al.,* 2004); maternal hypoxia (Peyronnet *et al.,* 2002) and maternal high salt diet (Porter *et al.,* 2009). In addition, an exaggerated cardiovascular response to exposure to ammonia has been demonstrated in offspring following maternal low protein diet (Tonkiss *et al.,* 1998). Thus there is considerable diversity in the type of programming insult that can lead to enhanced responses to stress, suggesting that this is a common alteration in phenotype. It is possible that there may be an underlying disturbance common to these different programming models.

There is good evidence that many of these foetal insults either directly or indirectly produce a down regulation of glucocorticoid receptor levels in the hypothalamus (Seckl, 2004; Gonzalez-Rodriguez *et al.,* 2014), and that this may in turn increase the hypothalamic-pituitary-adrenal axis response to stress in the offspring, producing exaggerated cardiovascular and autonomic responses to stress (Seckl, 2004). Although there is no evidence for prenatal high salt diet producing an increase in foetal glucocorticoid, increases in levels of corticotropin-releasing hormone mRNA in the hypothalamus have been reported (Porter *et al.,* 2009) and this may produce a similar exaggeration of stress responses. It is interesting to note that a maternal obesogenic diet in rats also increases systemic corticosterone levels in the dam, and produces an increase in glucocorticoid receptor gene expression in the amygdala of the offspring (Sasaki et a., 2013), that is associated with exaggerated behavioural stress responses. It is not known whether a maternal high fat diet that is not obesogenic, or a maternal high sucrose diet, such as those used in the present study, also produce altered glucocorticoid responses in the hypothalamic pituitary adrenal axis or amygdala. It is possible that the failure to observe a difference in the cardiovascular responses to stress in these models may be reflected by a lack of alteration in glucocorticoid receptor levels within these areas of the brain.

Autonomic parameters during psychological stress: In the high fat study, there was no difference in sBRS or BEI between groups during rest or in response to stress in either male or female rats. However, there were differences observed between sexes in response to stress. In males, both control and high fat showed a decrease in sBRS to a similar degree in response to stress. Both groups also showed a similar trend towards a decrease in BEI in response to stress, but this was not statistically significant in either group. In contrast, female rats did not show a significant change in either sBRS or BEI in response to stress.

Similarly in our high sucrose model there was no difference in sBRS or BEI between groups at rest or in response to stress. However, in contrast to the high fat study (males), there was no change in sBRS in response stress in either sucrose model or control rats, while BEI decreased in the high sucrose rats but remained unaffected in the controls.

The original hypothesis was that the programmed rats may show a depression of their baroreflex function (both sBRS and BEI) either at rest or in response to stress, based on numerous reports that decreased baroreflex sensitivity is associated with hypertension or increased risk of hypertension

(Dodic *et al.,* 1999; La Rovere *et al.,* 1998; Leotta *et al.,* 2007; Pladys *et al.,* 2004). A depressed baroreflex function in response to stress may help to explain the observation of an augmented hypertensive response to stress reported previously in high fat/obesogenic diet programmed hypertension (Samuelsson et al, 2010; Rudyk *et al.,* 2011), although these studies did not investigate the effects of stress on baroreflex function. Unlike these previous studies, our study did not observe an augmented cardiovascular response in response to stress in the programmed animals, and therefore lack of an effect on baroreflex function between groups is consistent with the similarity in cardiovascular response between groups.

It is noteworthy that a difference was found in the effect of stress on sBRS in the male *control groups* of our high fat (decreased) and high sucrose (unchanged) studies. It is not possible to explain this discrepancy without further investigation. A recent study by Bajic et al. (2010) in the Wistar rat examined baroreflex responses to an air jet protocol similar to the one used in our study. It reported that stress evoked no change in sBRS consistent with the controls in the current high sucrose study. It is possible that the differences in sBRS that were observed in the two control groups may indicate a technical error in the analysis of sBRS in the high fat study, however the results were carefullly examined and this is not considered to be the case. Identical procedures were used for the analysis of sBRS in the high fat- and high sucrose- studies. One possibility is that the low *n* values, due to the high mortality linked to the telemetry surgery, may have generated a type I error (false positive), However, due to the inability to access more animals, this could not be investigated further.

A review of the literature indicates that there may be variability in the effect of stress on baroreceptor function, possibly dependant on the type of stressor, or other variations in how the stress is evoked. Early literature supported the idea that stress elicited a depression of baroreflex function, based on studies of the hypothalamic defence area (Hilton, 1982). Stimulation of this region has been shown to inhibit the baroreflex via GABAergic inhibition of baroreceptor-sensitive neurons in the nucleus of the solitary tract (Silva- Carvalho et al. 1995; Mifflin *et al.,* 1988). However, more recent studies report that baroreceptor reflex function during a stressor, or in response to activation of the stress “centre” in the dorsomedial hypothalamus, shifts the operating range of the reflex to a higher blood pressure without having an effect on sensitivity (Kanbar *et al.,* 2007; Dampney *et al.,* 2008). Thus there is inconsistency in the literature, and it is sensible to conclude that the effects of stress on baroreflex

function may be highly variable, and dependant on a number of factors, including state of arousal and use of anaesthetics.

We also found inconsistencies in our data on stress-evoked changes in BEI. A decrease in BEI observed in the high fat control is consistent with the Bajic et al (2010) study. A decrease in BEI would indicate that although the sensitivity of the reflex is not diminished during air jet stress, it is no longer triggered as frequently by fluctuations in blood pressure. Thus, the effect of a decrease in BEI is to reduce the strength of the baroreflex at a given pressure, and this may explain the right shift in the baroreflex function curve to a higher blood pressure that is described by Dampney *et al.,* (2008). Presumably this assists in a permissive manner to allow for stress-evoked increases in sympathetic nerve activity, heart rate and blood pressure.

There were only a few changes in HRV and BPV observed in response to stress, with no significant differences seen in sympathetic indices (LF or LF:HF ratio) between groups in either the high fat or high sucrose rats. This is consistent with our blood pressure data obtained in our study, which also showed no differences between groups in response to air jet stress. Our study is the first to examine HRV and BPV in response to stress in maternal high fat programming, and thus it is not possible to make any comparisons with previous studies. The possible reasons for the lack of an augmented response to air jet stress in the programmed rats have been discussed above: such differences may lie in the dietary models or intervention period used.

In summary, we found few differences in the cardiovascular and autonomic responses to air jet stress between control and high fat- and high sugar-programmed rats. This is surprising because a number of other studies of programmed hypertension (O'Regan *et al.,* 2008; 2010; Igosheva *et al.,* 2004; Peyronnet *et al.,* 2002; Porter *et al.,* 2009), including models of maternal high fat diet programmed hypertension (Samuelsson *et al.,* 2010; Rudyk *et al.,* 2011), have reported increases in stress-related cardiovascular function. Different gestational stressors are likely to elicit a different pattern of developmental changes in the offspring, which can explain in part the lack of effect that was observed. However an explanation of the differences between our study and that of Rudyk et al. (2011) is more complicated because both studies used a non-obesogenic high fat diet for the programming model. There are differences in the protocol of high fat exposure: our study provided a 34% fat diet to the dam

for one month prior to mating and throughout gestation, whereas Rudyk and colleagues provided a 23.6% fat diet for two weeks prior to conception, throughout gestation and also throughout lactation. The continuation of the high fat diet during the neonatal phase means that these investigators were studying the effects of *peri*-natal rather than prenatal programming and this may impact on the results. The perinatal period is a time of increased plasticity, especially for the stress system, and is therefore particularly sensitive to alterations in environment. Thus handling during the neonatal period affect behaviour in the adult, including exaggerated responses to stress (Meaney *et al.,* 1998). Thus postnatal high fat diet may have an added and substantial effect on the offspring response to stress (Samuelsson *et al.,* 2010; Rudyk *et al.,* 2011). Therefore, although we cannot suggest a mechanism to explain the differences in our results, it is possible that the different methodologies for high fat-programming may underlie these differences.

4.6 CARDIOVASCULAR AND AUTONOMIC PARAMETERS DURING DEHYDRATION

Cardiovascular parameters during physiological stress (dehydration): Disturbances in the control of fluid balance and electrolyte homeostasis are associated with hypertension (Morris, 1982). A common osmotic challenge in today's society is one that is imposed by a high salt (NaCl) diet. There are a growing number of studies that show increased prevalence of salt sensitive cardiovascular diseases such as hypertension (Adams, 2004; Weinberger, 1996; DiBona and Sawin, 1991). Moreover, a number of studies have highlighted a salt-sensitive component to programmed hypertension (Rudyk *et al.,* 2011; Sander *et al.,* 2005; Tang *et al.,* 2011; Woods *et al.,* 2001; 2004), indicating that programming may not only increase the risk of hypertension due to psychological stressors, but also physiological stressors such as osmotic challenge. To investigate the effect of an osmotic challenge we chose to use a dehydration protocol (described in **Section 2.2**). Similar to a high salt diet, dehydration causes a change in the fluid balance and electrolyte homeostasis resulting in a hyperosmotic extracellular fluid environment (Toney and Stocker, 2010), and an increase in sympathetic nerve activity (Osborn *et al.,* 2007; Toney and Stocker, 2010). Thus, dehydration may partly mimic the effect of high salt diet over a shorter time course.

In our study, the high fat programmed rats (male and female) had sustained higher blood pressure throughout the dehydration protocol, including the baseline period, the period of water deprivation and the recovery period. However, in response to dehydration both high fat and control rats showed a similar increase in SBP, suggesting that acute dehydration, resulting in a reduced, hyperosmotic extracellular fluid volume, produced similar effects in both groups. To our knowledge, there are no reports in the literature of cardiovascular responses to dehydration in programmed animals. However a study by Rudyk and colleagues (2011) showed that high fat diet programmed rats had a significant increase in blood pressure in response to salt loading, which, as discussed above evokes a similar change in plasma osmolarity. Surprisingly, in contrast to our study, there was no difference in blood pressure at rest between groups. It is hard to explain these differences between our study and that of Rudyk et al. (2011). However, there are differences in the two models, as described in **section 4.5** of air jet stress. Our study used a 34% fat diet commencing 4 weeks prior to mating and terminating at parturition, while the other study used a 23.6% fat diet and commenced 2 weeks prior to mating and continued until after weaning. Therefore the study of Rudyk et al, 2011 exposed the offspring to raised fat both prenatally and postnatally. This suggests the possibility that the early neonatal environment is a crucial period in the rat for the programming of altered cardiovascular autonomic regulation. The postnatal period is of crucial importance in the rat as it is developmentally quite immature at birth and therefore processes of maturation continue during the immediate postnatal period (Patel and Srinivasan 2010). This period is characteristic of great plasticity, and thus developmental programming can occur during this time similar to in the gestation period (Ostadalova and Babickey, 2012). It is unclear as to when the developmental regulation of cardiac and autonomic responsiveness start developing in the rat. However some studies offer some clues. A study in the rat show parasympathetic innervation to the heart is first detected before birth (Marvin *et al.,* 1980). Conversely, there is no indication of sympathetic innervation to the rat ventricle during the first week after birth (Robinson, 1996). Furthermore studies have shown developmental expression of α-1 adrenergic, β-adrenergic and muscarinic receptors in the heart appear to peak during the first and second week after birth, this period is thought to be a time of rapid maturation of innervation to the heart (Kojima *et al.,* 1990). In addition, at the time of birth in a rat, a period of potential autonomic imbalance has been found, whereby parasympathetic innervation is already established to the heart but not sympathetic innervation (Robinson, 1996). This indicates that the postnatal period is of crucial

importance for autonomic development and any adverse insult may have negative impacts on autonomic homeostatic regulation. The extended postnatal exposure of a high fat diet in the study by Rudyk et al, 2011 may have influenced cardiac and autonomic development adversely, thus this may have lead to the increased pressor response seen to a physiological stressor, in contrast to our observations. Therefore, the phenomenon of postnatal plasticity and/or postnatal programming is important to consider when making comparisons between studies.

Our second model of high sucrose programming rats did not show any difference in blood pressure or heart rate between groups at any time point during the protocol (baseline, stress, recovery). Similar to our male high fat programmed rats, in response to dehydration both high sucrose and controls showed an increase in SBP and DBP, and this increase was similar between groups. To our knowledge, there has been no study done previously that has examined the cardiovascular responses to a physiological osmotic stress in a high sucrose programmed model. Although preliminary, our results suggest that gestational exposure to high dietary sucrose does not program an altered cardiovascular response at rest or in response to either psychological (air jet) or physiological (dehydration) stressors in the offspring.

In addition to the study on high fat programming mentioned above (Rudyk *et al.,* 2011), a number of studies using other models of programming have reported enhanced cardiovascular responses to a physiological osmotic stress, although in these cases the osmotic stress was produced by high salt diet. These include, maternal protein restriction (Wood *et al.,* 2004), gestational diabetes (Nehiri *et al.,* 2008), uterine artery ligation (Sanders *et al.,* 2005), and raised maternal glucocorticoids (Tang *et al.,* 2011). Thus, a range of maternal insults can program an exaggerated response to an osmotic stress, suggesting that there may be a common alteration in phenotype throughout a variety of different programming models. However, our results indicate that not all prenatal stressors evoke this common altered phenotype. It may be that the lack of a difference in response to dehydration between groups is because dehydration is a stronger stimulus than high salt, activating both osmoreceptors and volume receptors (due to the low blood volume). The stronger stimulus may saturate the hypertensive response, and thus mask any difference between groups. However, consistent with our results, there are a number of reports in models of programmed hypertension that failed to elicit further increases in blood pressure in response to a high salt diet (Langley-Evans *et al.,* 1996; Moritz *et al.,* 2011; Zimanyi

et al., 2004). Thus it seems that the programming paradigm does not consistently elicit a hypertensive or exaggerated response to either a psychological or physiological stressor. Further investigation is necessary to better elucidate what circumstances lead to these hypertensive responses, particularly if they pose a heightened risk for the development of overt hypertension in the long term.

The increase in blood pressure in response to dehydration is consistent with previous studies, and is believed to be due to increased sympathetic activity (Colombari *et al.,* 2011; Gardiner and Bennet, 1985; Woods and Johnston, 1983; Burnier *et al.,* 1983). Therefore, based upon the observed similarity in pressor response, we would argue that dehydration evoked a similar degree of sympathoexcitation in high fat, high sucrose and control rats. We examined non-invasive indices of autonomic function, as described in the methods, to investigate this further.

Autonomic parameters during physiological stress (dehydration): In the high fat study, there was no difference in sBRS or BEI between groups during rest or in response to dehydration in males. However, in females, although BEI was not different between groups, there was a significant difference in sBRS throughout the entire protocol (baseline, dehydration and recovery), with HFD females having a higher sBRS. Furthermore, in response to dehydration sBRS increased in HFD, but not control rats. This result is surprising in light of the HFD rats having a raised blood pressure, which is usually associated with a decrease in sBRS (Lantelme *et al.,* 1998; Waki *et al.,* 2006). Indeed, previous studies using an obesogenic high fat diet to programme hypertension also found reduced baroreflex sensitivity at rest in offspring of mothers fed an obesogenic diet (Samuelsson *et al.,* 2009). As discussed already, the protocol in this study was significantly different to ours because the pregnant dams developed obesity and also the diet was maintained during the weaning period. We are uncertain as to why there is increased sBRS, however one possibility is related to the high polyunsaturated fatty component of the high fat diet used in our study, as high polyunsaturated fatty acids have been shown to increase baroreflex sensitivity in patients following myocardial infarct (Radaelli *et al.,* 2005).

There were no significant changes in HRV and BPV between groups in the male high fat model. Interestingly female HFD rats showed an increased LF:HF ratio throughout the dehydration protocol, with a significant increase in the LF:HF ratio in response to dehydration. This increased LF:HF ratio in

HFD rats is thought to reflect a shift towards a sympathetic dominance in cardiac autonomic function (Billman, 2013). It is possible, therefore, that the increase in LF:HF ratio in the HFD females may relate to their higher blood pressure. If this were true, it would suggest that the mechanism producing raised blood pressure in the male HFD rats is different to that of the females. Such gender differences have been described previously (**see section 4.3**).

In summary, we found little difference in the cardiovascular and autonomic responses to dehydration stress between control and high fat- and high sugar-programmed rats. This is surprising as a recent study by Rudyk and colleagues (2011) has shown alterations in high salt-related function. Difference in duration of the diet by Rudyk and colleagues may partly explain the lack of effect we observed. These differences have been described extensively in **Section 4.5**.

4.7 COMPARISON OF FOS LABELLING IN HIGH SUCROSE AND CONTROL RATS

We compared Fos expression in the hypothalamus in high sucrose and control rats following air jet stress in order to determine whether there were differences in neuronal activation in response to the stress between groups. Three main regions of the hypothalamus are thought to be involved in mediating cardiovascular response to acute stressors, including the PVN, DMH and PeF. Similar to other studies that have used air jet stress (Furlong *et al.,* 2014; Palmer and Printz, 1999), our studies showed Fos expression in these three regions. There were no differences in the number of Fos-labelled nuclei between control and high sucrose rats. Our initial hypothesis was that high sucrose programmed rats would display a greater hypertensive response to air jet stress, and that such a response may lead to increased Fos expression in the hypothalamus. Considering that we did not see any major differences in cardiovascular or autonomic responses between groups during the air jet stress, it is not surprising that we did not see differences in Fos expression. We are unable to compare the Fos labelling in our high sucrose model with other studies of high sucrose programming as to our knowledge there have been no other study that have looked at Fos expression in rats following air jet stress. Indeed, no studies to date have examined Fos expression in any model of programming. However, genetic models of hypertension have found differences in Fos expression compared to normotensive controls. For example, following stress, the number of Fos-positive neurons in these

hypothalamic regions is increased in the SHR (Palmer and Printz, 1999) and in the Schlager generically hypertensive mouse (Davern *et al.,* 2010), consistent with the increased cardiovascular responses to stress in these animals. Utilization of the c-*fos* technique in other models of programming will help to identify those regions of the brain that exhibit alterations in the central processing of stress responses, such as described following gestational raised glucocorticoids (O'Regan *et al.,* 2008) or maternal hypoxia (Peyronnet *et al.,* 2002).

CHAPTER 5: CONCLUSIONS

In summary, the data showed that a maternal high fat diet during pregnancy results in hypertension at rest, demonstrating that the risk of developing hypertension is increased in these offspring when compared to offspring from mothers given a control diet. In response to a psychological or physiological stressor, all animals showed an increase in cardiovascular variables, however despite the high fat rats having a higher blood pressure during baseline, the magnitude of increase in cardiovascular variables were similar between groups in both male and female rats.

There is limited evidence of altered autonomic function in the high fat programmed rats in our study, despite their higher blood pressure. Evidence of autonomic differences were seen in the females, where a sustained higher blood pressure throughout the dehydration protocol was accompanied by a large increase in the low:high frequency ratio of heart rate variability. This is indicative of increased sympathetic activity. A similar change in the low:high frequency ratio of HRV was not seen in males. This suggests the possibility that at least in the female high fat programmed rats, the increase in blood pressure may be due in part to enhanced sympathetic activity. However, there were no obvious differences in autonomic responses to either air jet stress or dehydration between high fat and control animals, indicating that any increased susceptibility to hypertension in high fat programmed rats is unlikely to be due to heightened sympathetic responses to stress. Further studies are necessary to elucidate the pathophysiological mechanisms that underlie hypertension in our model of high fat programming.

In our second model, a maternal high sucrose diet failed to program hypertension in the offspring at rest or in response to either a psychological or physiological stressor. There were also no differences in autonomic function between groups at rest or in response to a psychological or physiological stressor. Therefore, this model failed to detect any detrimental effects of a maternal high sucrose intake on the cardiovascular or autonomic function in the offspring.

In relevance to humans, programming may play a pivotal role in sensitising an individual to developing hypertension as a consequence of nutrition-related factors during pregnancy. The nature of the signal that instigates foetal programming from the mother to the fetus has not been delineated. The remarkable similarity of phenotype in offspring from studies of nutritional manipulation suggests that

there is an over arching signal influencing programming in these studies. However, the mechanisms that link nutritional factors in development of hypertension remain elusive. Our studies are not strongly indicative of autonomic dysfunction in the high fat programmed hypertensive rats. Further investigations into pathways that mediate blood pressure homeostasis are required. An area that requires further research is renal ontogeny and epigenetic mechanisms. In addition, it is important to consider details of specific dietary components and methodological differences between groups when making conclusions.

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APPENDIX

Dr Kieron Rooney and Prof. Robert Boakes provided the supplementary graphs and tables stated in the appendix, based on the biochemical data and blood analysis from the animals used in this thesis. This material was not a part of the thesis.

Table 1: Maternal out comes in the high fat model

	Chow Fed (n=4)	**Fat Fed (n=8)**
Body Weight (g)		
Starting weights	173.7 ± 3.1	157.6 ± 2.5
Pre-gestation	256.1 ± 9.4	234.7 ± 7.3
Gestation day 19	347.0 ± 10.4	312.2 ± 16.4
PND2	325.7 ± 8.3	303.3 ± 11.4
Blood Glucose (mM)		
Pre-gestation	5.0 ± 0.3	**5.8 ± 0.2***
Gestation day 19	4.1 ± 0.4	4.1 ± 0.3
Plasma Insulin (pM)		
Pre-gestation	38.8 ± 11.0	20.2 ± 5.0
Gestation day 19	179.4 ± 61.2	143.8 ± 63.6
Plasma Leptin (pg/mL)		
Pre-gestation	511.2 ± 108.9	**996.4 ± 170.2***
Gestation day 19	768.8 ± 74.5	925.4 ± 92.1

Table 1: ***Maternal summary data high fat model. * denotes significance between groups.***
Maternal body weight: *There were no significant differences in maternal weight at any point.*
Blood glucose levels: *were significantly higher in the fat fed mother ($P<0.05$), pre gestation (four weeks prior to mating). However at gestation day 19, blood glucose levels were similar between maternal groups*
Plasma insulin: *there were no significant differences in maternal plasma insulin levels pre-gestation or at gestation day 19.*
Plasma leptin: *levels were significantly higher in the fat fed mother mother ($P<0.05$), pre gestation (four weeks prior to mating). However at gestation day 19, plasma leptin levels were similar between maternal groups.*

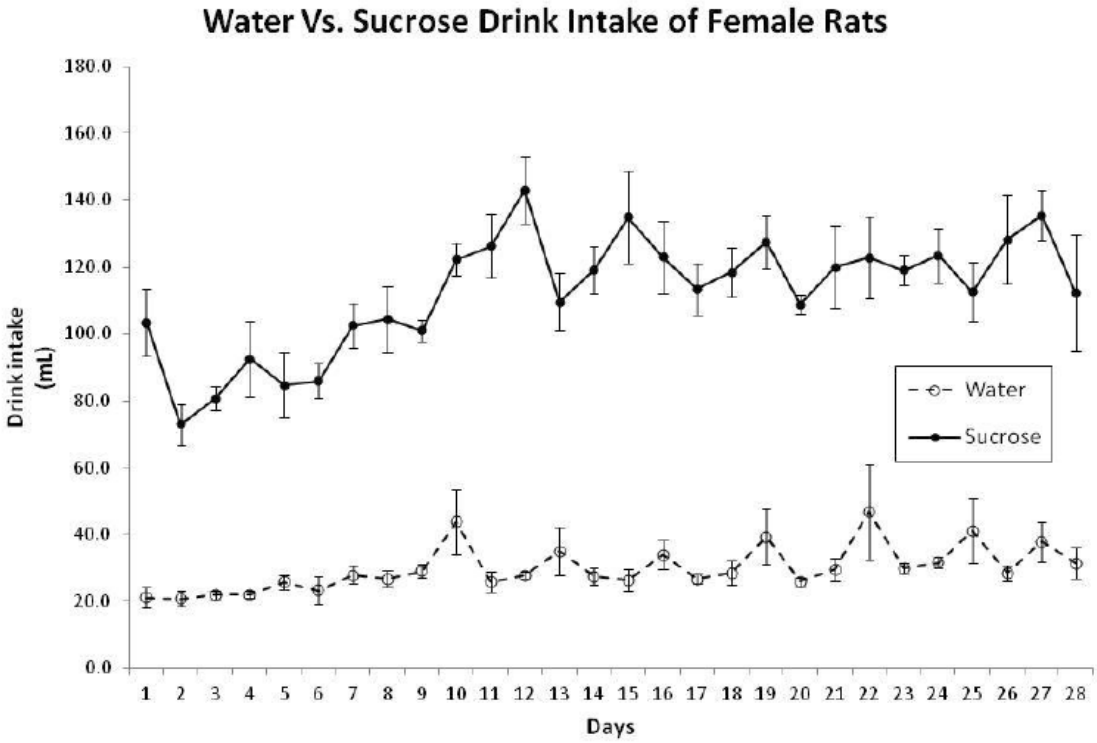

Figure 1: ***water and sucrose solution intake in female rats means ± SD***. Average daily intake of sucrose water (sucrose group) was 112.5±17.3 mL and water (control group) was 29.9±6.8 mL

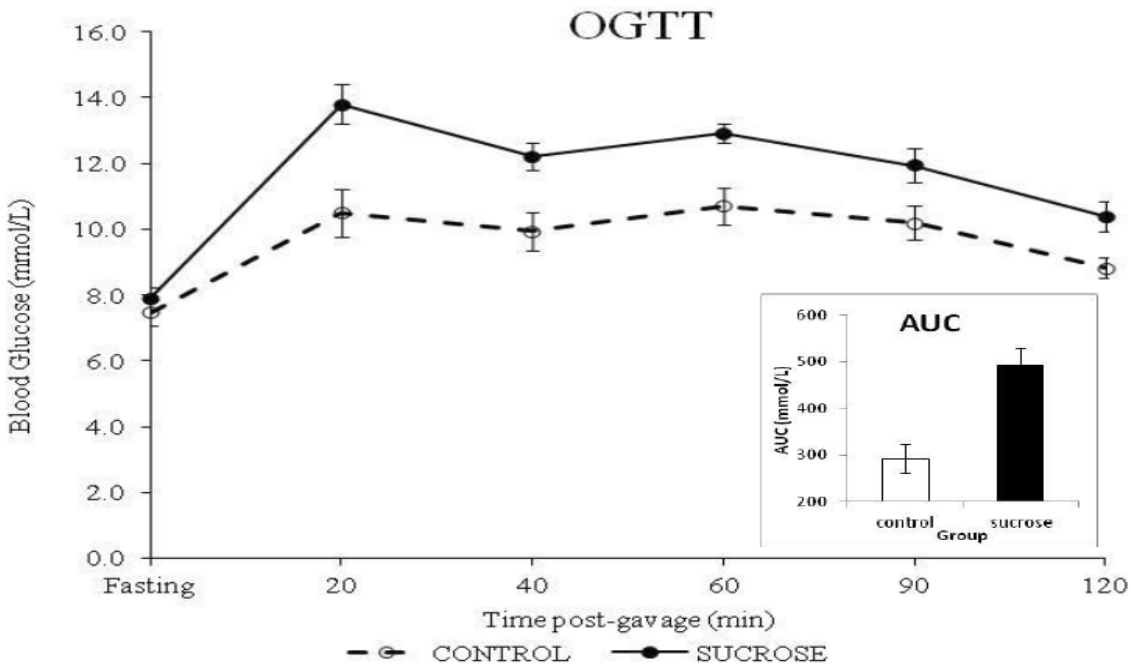

Figure 2: ***oral glucose tolerance test in control and sucrose mothers.*** *Blood glucose levels were higher in sucrose rats throughout the test (P<0.01). Total area under the curve (figure inset) also showed a significant higher blood glucose levels in sucrose rats (P<0.01).*

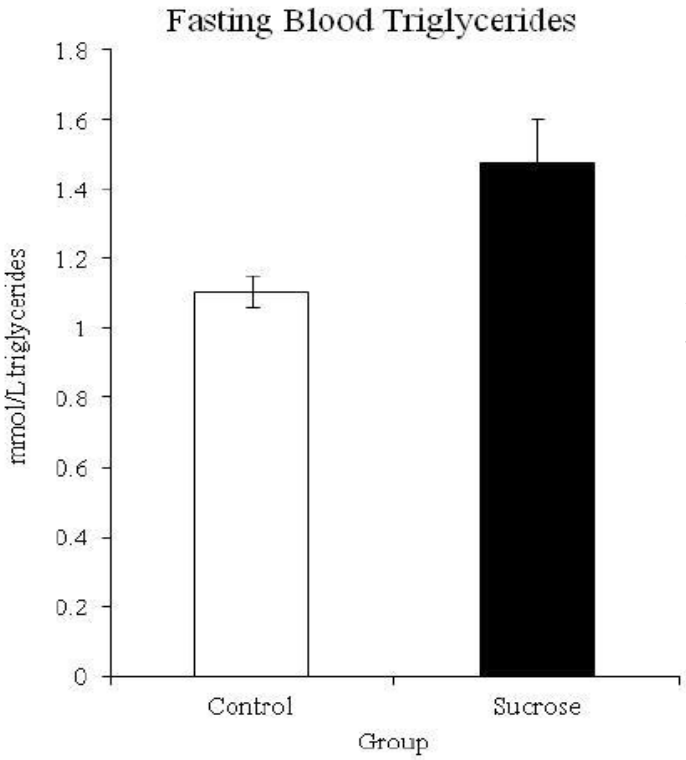

Figure 3: ***fasting blood triglycerides of control and sucrose mother following 28 days of dietary intervention.*** *Average fasting blood triglycerides were higher in the sucrose group compared to controls (P=0.015).*

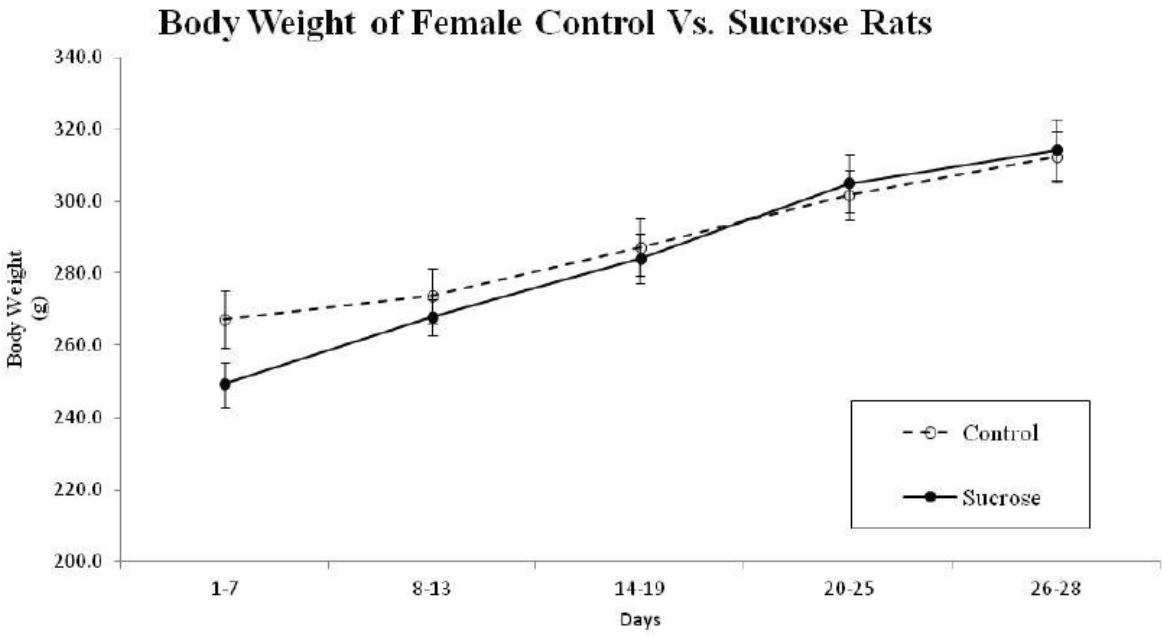

Figure 4: ***Body weight of female control versus sucrose rats throughout intervention:*** *There was no significant difference in body weight between maternal high sucrose or control groups.*