ABSTRACT

Vitamins are readily available in the modern diet due to liberalized fortification and supplementation policies. This research tested the hypothesis that high multi-vitamin intake by Wistar rats during pregnancy leads to the development of obesity and characteristics of the metabolic syndrome in the offspring. Pregnant Wistar rats were fed the AIN-93G diet containing either the recommended (RV) or 10-fold increase (HV) in vitamin content. Pups were weaned to the RV diet (Study 1), an obesogenic liquid diet (Ob, Study 2), low vitamin diets (1/3RV or 1/6RV, Study 3), or a nutrient selection paradigm (NSP) with 10% and 60% casein diets (Study 4). Body weight (BW), food intake (FI), glucose and insulin responses, appetite hormones, abdominal fat pad mass (FPM) and systolic blood pressure (SBP) was measured. Expressions of mRNA for hypothalamic serotonin (5-HT) receptors and proopiomelanocortin (POMC) were measured in Study 4. Males, but not females, born to HV dams had higher post-weaning BW and FI when weaned to the RV or 1/3RV diet, and exhibited components of metabolic syndrome, including higher FPM, hyperglycemia, insulin resistance and elevated SBP compared with those born to RV dams. The Ob diet led to exaggerated weight gain and expressions of components of metabolic syndrome in both sexes born to dams fed the HV diet. Female pups on the 1/6RV diet from HV dams had two-fold higher glucose response and lower insulin response, but no difference in post-weaning BW and daily FI compared to those from RV dams. In contrast to the pups born to HV dams and fed a single diet, those from the HV dams and on the NSP gained less weight and ate less, and had lower hypothalamic mRNA expressions of 5-HT receptors and POMC. In conclusion, high multi-vitamin intake during pregnancy may lead to obesity, and result in a higher risk of developing characteristics of metabolic syndrome in the offspring. However, sex, weaning diet composition, and the presence of diet choice alter the outcomes.
ACKNOWLEDGEMENTS

I would like to thank Dr. G. Harvey Anderson for his guidance in research and in life. I truly appreciate your “Come See Me. – Harv.” notes on my sub-par writings, and most importantly, during my personal downtimes. I know from time to time I have disappointed you for not focusing on my research, but you also taught me “You’ve gotta do what you gotta do, and move on.” I deeply owe you for your teachings on thinking critically, seeing the big pictures, and translating research into useful knowledge for the good of everyone.

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Also, I would like to thank my PhD committee members, Dr. Ahmed El-Sohemy and Dr. Wendy Ward, for their invaluable inputs toward the degree. Also, I thank Louisa Kung for your timely check-ups on my [slow] progress of PhD completion. I am sure I forget to thank a lot of people, especially the numerous undergraduate summer students that contributed tremendously to the studies (Nobuhiko Okubo, Sharon Zhang and Daniel Cho), and every member of the big Anderson Lab and the Department of Nutritional Sciences.

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<tr>
<td>11-β-HSD2</td>
<td>11 Beta-Hydroxysteroid Dehydrogenase Type 2</td>
</tr>
<tr>
<td>1/3RV</td>
<td>One-third of the Recommended Vitamin Content</td>
</tr>
<tr>
<td>1/6RV</td>
<td>One-sixth of the Recommended Vitamin Content</td>
</tr>
<tr>
<td>5-HT</td>
<td>5-Hydroxytryptamine (Serotonin)</td>
</tr>
<tr>
<td>ACC</td>
<td>Acetyl Coenzyme A Carboxylase</td>
</tr>
<tr>
<td>AgRP</td>
<td>Agouti-Related Peptide</td>
</tr>
<tr>
<td>AIN-93</td>
<td>American Institute of Nutrition (1993)</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
</tr>
<tr>
<td>ARC</td>
<td>Arcuate Nucleus (in Hypothalamus)</td>
</tr>
<tr>
<td>A&lt;sup&gt;vy&lt;/sup&gt;</td>
<td>Viable Yellow Agouti Mice</td>
</tr>
<tr>
<td>BMI</td>
<td>Body Mass Index</td>
</tr>
<tr>
<td>BW</td>
<td>Body Weight</td>
</tr>
<tr>
<td>CART</td>
<td>Cocaine-Amphetamine-Regulated Transcript</td>
</tr>
<tr>
<td>CCK</td>
<td>Cholecystokinin</td>
</tr>
<tr>
<td>CNS</td>
<td>Central Nervous System</td>
</tr>
<tr>
<td>d</td>
<td>Day</td>
</tr>
<tr>
<td>Δ</td>
<td>Delta (Change in food intake)</td>
</tr>
<tr>
<td>DBP</td>
<td>Diastolic Blood Pressure</td>
</tr>
<tr>
<td>DMN</td>
<td>Dorsomedial Nuclei</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>Dnmt</td>
<td>DNA (cytosine-5)-Methyltransferase</td>
</tr>
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<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>DPP-IV</td>
<td>Dipeptyl-Peptidase IV</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylene Diamine Tetraacetic Acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-Linked Immunosorbent Assay</td>
</tr>
<tr>
<td>FAS</td>
<td>Fatty Acid Synthase</td>
</tr>
<tr>
<td>FFA</td>
<td>Free Fatty Acid</td>
</tr>
<tr>
<td>FI</td>
<td>Food Intake</td>
</tr>
<tr>
<td>FPM</td>
<td>Fat Pad Mass</td>
</tr>
<tr>
<td>g</td>
<td>Grams</td>
</tr>
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<td>GD</td>
<td>Gestational Diet</td>
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<td>GHS-R</td>
<td>Growth-Hormone-Secretagogue Receptor</td>
</tr>
<tr>
<td>GI</td>
<td>Gastrointestinal</td>
</tr>
<tr>
<td>GLP-1</td>
<td>Glucagon-Like Peptide-1</td>
</tr>
<tr>
<td>GLUT4</td>
<td>Glucose Transporter Type 4</td>
</tr>
<tr>
<td>GNMT</td>
<td>Glycine N-methyltransferase</td>
</tr>
<tr>
<td>GR</td>
<td>Glucocorticoid Receptor</td>
</tr>
<tr>
<td>GTG</td>
<td>Gold Thioglucose</td>
</tr>
<tr>
<td>h</td>
<td>Hour</td>
</tr>
<tr>
<td>HPA</td>
<td>Hypothalamic-Pituitary-Adrenal (Axis)</td>
</tr>
<tr>
<td>HV</td>
<td>High Vitamin Diet</td>
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<tr>
<td>iAUC</td>
<td>Incremental Area Under the Curve</td>
</tr>
<tr>
<td>kg</td>
<td>Kilograms</td>
</tr>
<tr>
<td>KIU</td>
<td>Kallikrein Inhibitor Units</td>
</tr>
<tr>
<td>LepR</td>
<td>Leptin Receptor</td>
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<tr>
<td>Acronym</td>
<td>Full Form</td>
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<td>-----------</td>
<td>------------------------------------------------</td>
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<tr>
<td>LHA</td>
<td>Lateral Hypothalamic Area</td>
</tr>
<tr>
<td>LNAA</td>
<td>Large Neutral Amino Acid</td>
</tr>
<tr>
<td>M</td>
<td>Moles</td>
</tr>
<tr>
<td>mCPP</td>
<td>1-(3-Chlorophenyl)piperazine hydrochloride</td>
</tr>
<tr>
<td>MD</td>
<td>Maternal Diet (Diet during Pregnancy + Lactation)</td>
</tr>
<tr>
<td>MLP</td>
<td>Maternal Low Protein (Diet)</td>
</tr>
<tr>
<td>MTHFR</td>
<td>Methylene Tetrahydrofolate Reductase</td>
</tr>
<tr>
<td>min</td>
<td>Minutes</td>
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<tr>
<td>ml</td>
<td>Millilitres</td>
</tr>
<tr>
<td>mM</td>
<td>Millimoles</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>ng</td>
<td>Nanograms</td>
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<td>NSP</td>
<td>Nutrient Selection Paradigm</td>
</tr>
<tr>
<td>NTS</td>
<td>Nucleus of the Tractus Solitaries</td>
</tr>
<tr>
<td>NPY</td>
<td>Neuropeptide Y</td>
</tr>
<tr>
<td>NS</td>
<td>Not significant</td>
</tr>
<tr>
<td>Ob</td>
<td>Obesity-inducing Palatable Liquid Diet</td>
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<td>OGTT</td>
<td>Oral Glucose Tolerance Test</td>
</tr>
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<td>PCR</td>
<td>Polymerase Chain Reaction</td>
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<td>PD</td>
<td>Pup Diet</td>
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<td>PEPCK</td>
<td>Phosphoenolpyruvate Carboxykinase</td>
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<tr>
<td>pM</td>
<td>Picomoles</td>
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<td>POMC</td>
<td>Pro-opiomelanocortin</td>
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<td>PPAR</td>
<td>Peroxisomal Proliferation-Activated Receptors</td>
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<td>PVN</td>
<td>Paraventricular Nuclei</td>
</tr>
<tr>
<td>PYY</td>
<td>Peptide YY</td>
</tr>
<tr>
<td>RDA</td>
<td>Recommended Dietary Allowance</td>
</tr>
<tr>
<td>RIA</td>
<td>Radioimmunoassay</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic Acid</td>
</tr>
<tr>
<td>RV</td>
<td>Recommended Vitamin Diet</td>
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<tr>
<td>SAH</td>
<td>S-adenosylhomocysteine</td>
</tr>
<tr>
<td>SAM</td>
<td>S-adenosylmethionine</td>
</tr>
<tr>
<td>SBP</td>
<td>Systolic Blood Pressure</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard Error of the Mean</td>
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<td>VMN</td>
<td>Ventromedial Nuclei</td>
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LIST OF PUBLICATIONS ARISING FROM THESIS

Peer-Reviewed Publications (4):


Commentary (1):


Submitted Publications (4):


Jahan-mihan, A., **Szeto, I.M.,** Luhovvy, B.L., Huot, P.S., and Anderson, G.H.  The effect of protein source in protein complete maternal diets on development of characteristics of the
metabolic syndrome in the Wistar rat dams and offspring. (Br J Nutr, In Revision: Jan 18, 2011. Manuscript #: BJN-2010-015977-R1) (Collaboration)


Prospective Publications (3):

Szeto, I.M. and Anderson, G.H. Overnutrition of macro and micronutrients during pregnancy on offspring health. (Submitting to Nutr Rev in Apr 2011)


CHAPTER 1.

INTRODUCTION
CHAPTER 1. INTRODUCTION

The prevalence of obesity and metabolic syndrome has escalated in both developing and developed countries over the past three decades [1-4]. This health problem is likely generated by the interaction between genetic and environmental factors [2, 5-7]. However, significant changes in the human genome are highly unlikely to occur in this relatively short period of time, suggesting that environment and nutrition are playing major roles in the progression of obesity [8, 9]. Associated with the increase in obesity are several changes in dietary patterns, including increased intakes of energy, polyunsaturated and trans-fatty acids, sugars and rapidly digested carbohydrates, and minerals and vitamins through supplementation and fortification of foods over the last 30 years [10, 11]. These changes may be causally related because diet has a significant impact on the intrauterine environment of the fetus during pregnancy. Too much or too little of any of the required nutrients can lead to detrimental development in the fetus, both for immediate survival and long term health in adulthood. The consequence of the in utero environment on chronic disease in adulthood has been ascribed to fetal programming. Fetal programming is the term used to describe the effect of manipulating the in utero environment on the development of metabolic regulation in the offspring, and on the risk of chronic diseases, such as insulin resistance, adiposity, and abnormal eating behaviour [12, 13].

The focus of this research is on the health outcome of offspring of Wistar rats supplemented with vitamins during pregnancy. The rationale for the studies was three-fold. First, the availability of vitamin-fortified foods and dietary supplements in the consumer environment has increased substantially along with the rise in obesity and chronic disease. In
the United States, the percentage of adults, in all demographic and lifestyle groups, consuming multivitamin supplements daily increased from 17.4% in 1987 to 27.9% in 2000, and women consume more than men, regardless of age or ethnicity [11]. During pregnancy, there is a higher requirement for various vitamins, such as folic acid, vitamin B₆ and vitamin E, because inadequate intakes will lead to fetal damage and growth retardation [14]. For example, a deficiency in folic acid during early pregnancy leads to a higher risk of developing neural tube defects in human offspring [15]. To prevent deficiency, pregnant women are advised to take vitamin supplements to minimize adverse fetal outcomes. However, there is currently no report of randomized placebo-controlled study looking at the effect of long-term vitamin supplementation during pregnancy on the health of the offspring.

Second, multi-vitamin supplementation is often recommended to pregnant women as insurance against an inadequate diet [16]. However, a significant portion of these multi-vitamin supplements contain 100% or more of the daily need for pregnancy in one or more of the vitamins, and a chronic high total vitamin intake may occur [17].

Third, vitamins play a crucial role in fetal development; they contribute to all phases of cell growth and differentiation, and are key components of enzyme and cell structures. Many vitamins regulate the development of genes involved in growth, proliferation and in the functional characteristics of specific organs [18]. One of the most significant roles of vitamins (folate, vitamin B₆ and B₁₂) is to facilitate the methylation of certain genes on the DNA, and subsequently lead to higher or lower gene expression and altered phenotype. These susceptible genes can include genes that regulate offspring adiposity, appetite and metabolic regulation. The pattern of DNA methylation can also be passed onto the next generation, which means during pregnancy, a high intake of the vitamins involved in methyl
metabolism can lead to elevated DNA methylation in the offspring, and hence alter their phenotype.

Therefore, the hypothesis of this thesis was that vitamin intakes above requirement during pregnancy results in fetal programming for increased risk of chronic disease development in Wistar rat offspring.
CHAPTER 2.
LITERATURE REVIEW
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2.1 INTRODUCTION

This literature review provides a summary of the limited number of original studies relevant to the current research conducted. The first section introduces the concepts of fetal programming and the Predictive Adaptive Responses (PARs) hypothesis. The second section examines the effects of gestational malnutrition in human and animal models on the health of the offspring in adulthood along with the suggested mechanisms responsible for their altered phenotypes. The final section reviews the relevance of high vitamin intake prior to and during human pregnancy and its possible link to the recent increase in the development of obesity and metabolic syndrome, followed by selection of an appropriate animal model.

2.2 CONCEPT OF FETAL PROGRAMMING

The idea that the well-being of the mother before and during pregnancy may have crucial impact for the future health of the offspring is not new, and social and geographical inequalities in population health have been the topic of debate since Victorian times [19]. In 1977, the first investigator that showed a causal link between early life environmental factors and offspring disease in an epidemiological study was Anders Forsdahl in Norway [20]. In 1986, David Barker and Clive Osmond suggested that poverty, poor nutrition and health of the mothers contributed to high rates of infant mortality and a long-term risk of coronary heart disease [21].

To date, a number of theoretical and epidemiological models have been proposed in an effort to explain the cause-and-effect of fetal programming, with emphasis on the effect
maternal under-nutrition. The term “fetal programming” describes the process whereby a nutritional or environmental stimulus occurring at a critical period of early life development leads to a permanent change in the offspring physiology [22]. In the 1990s, the Fetal Origin Hypothesis (FOH) was proposed describing the fetal programming phenomenon. FOH stated that intrauterine undernutrition affects the development of organs and hormonal systems of the fetus, leading to adult metabolic diseases, including type 2 diabetes, hypertension and CVD [23, 24]. Intrauterine undernutrition by pregnant mothers as defined by the FOH includes either a low intake of protein or a low intake of calories, or combined. However, the FOH does not include the effects of overnutrition during pregnancy on the health of the offspring, nor does it account for developmental stages outside of pregnancy (pre-conception and lactation).

In the early 2000s, a more thorough concept known as the Developmental Origins of Health and Disease (DOHaD) emerged [25, 26]. DOHaD acknowledges the role of both over- and under-nutrition during the developmental period that spans from pre-conception to early childhood, and the subsequent detrimental programming effects of adult diseases in the offspring. The current thesis is based on the DOHaD concept, where an over-nutrition of multi-vitamins during pregnancy in Wistar rats is hypothesized to lead to the development of obesity and components of metabolic syndrome in offspring. The following will summarize a number of fetal programming hypotheses that led to the conceptualization and application of the DOHaD concept.
2.2.1 Developmental Origins of Health and Disease

A number of retrospective cohort studies have showed that undernutrition during pregnancy leads to newborn infants with low birth weight, and increases the likelihood of developing diseases such as type 2 diabetes, hypertension, and metabolic syndrome later [22, 26-28]. Several notable theories have been proposed to explain these relationships, including the Thrifty Phenotype Hypothesis [29], the Fetal Salvage Hypothesis [30], the Fetal Insulin Hypothesis [31], and the Predictive Adaptive Responses (PARs) hypothesis [32].

The Thrifty Phenotype Hypothesis was proposed in 1992 by Hales and Barker to explain the epidemiological associations between maternal malnutrition and adulthood insulin resistance, decreased beta-cell mass, and type 2 diabetes [29]. They hypothesized that in response to poor *in utero* nutrition, the fetus sacrifices the development of non-essential visceral organs, such as skeletal muscle, liver, spleen and pancreas, and divert nutrients to crucial organs, such as the brain and lungs, in order to promote fetal survival. However, these physiological consequences may not be suitable if the offspring is exposed to an environment of adequate or over-nutrition, and thus lead to metabolic disorders later in life.

The Fetal Salvage Hypothesis proposed that the redistribution of nutrients, mainly glucose, to crucial organs occurs because the fetal-malnourished offspring are programmed to have peripheral insulin resistance, therefore reducing glucose utilization in the periphery by lowering the amount or function of skeletal glucose transporter (GLUT4) [30]. This also stimulates the beta-cell to have a higher insulin production for more compromised glycemic control, which eventually leads to beta-cell exhaustion. In contrast, the Fetal Insulin Hypothesis was formulated by Hattersley *et al* through the observation of the concurrent existence of low birth weight and type 2 diabetes in rat offspring exposed to low protein diet
during gestation [31]. This hypothesis suggested that genetically determined insulin resistance leads to both fetal growth retardation and type 2 diabetes in adulthood. However, to date, this hypothesis was not highly regarded because there are no common diabetes susceptibility genes identified across genome-wide scans [33].

Drake and Walker proposed an intergenerational programming hypothesis to address the possibility that the effect of programming can be passed onto subsequent generations [34]. Initially, the pregnant mother (F0) is exposed to an adverse environmental stimulus, and permanently programs the offspring with altered cardiovascular and metabolic function. This leads to higher CVD risk, blood pressure, insulin resistance, and altered hypothalamic-pituitary-adrenal (HPA) axis function in the F1 generation [35, 36]. These symptoms will affect the following generation (F2) upon pregnancy due to higher maternal cortisol, insulin and blood pressure in the F1 generation. This cascade reaction will continue in a cyclic fashion until an intervention is in place. Despite the clear logics of this hypothesis, it has received minimal attention because the primary research effort to date is to explore the mechanism of the DOHaD observations in the immediate F1 generation.

Recently, the Predictive Adaptive Responses (PARs) hypothesis was proposed by Gluckman and Hanson as extension of the DOHaD concept to refine the prediction of the metabolic disease risk in adulthood [32]. The PARs hypothesis states that upon changes or stimuli in the in utero environment, such as undernutrition, the fetus will predict that the postnatal environment is also nutrient-scarce, and adapt physiologically based on the prediction. When this predictive adaptive response is correct, the offspring phenotype into adulthood will be normal. However, when there is a mismatch between the predicted and actual postnatal environment, the risk of developing chronic metabolic diseases increases.
In Study 3 of this thesis (Chapter 6), the PARs hypothesis is tested to determine if lowering the vitamin content of the post-weaning diet would lead to exaggerated expression of obesity and components of the metabolic syndrome in offspring born to dams fed the multi-vitamin supplemented diet during pregnancy.

### 2.2.2 Fetal Programming by Epigenetic Mechanisms

A number of investigators have explored the epigenetic mechanism to explain the DOHaD observations. The epigenetic mechanism is based on the effects of diet enrichments containing a generous amount of methyl donors, such as choline and betaine, or cofactors of methyl metabolism, such as folic acid, vitamin B6 and B12. Methyl groups can attach to the DNA sequence at cytosines in CpG dinucleotides, which can lead to changes in gene expression and function by DNA methylation or histone modification without changing the DNA sequence [37, 38].

DNA methylation has been proposed to play a role in maintaining genomic stability, silencing and regulation of gene function [39]. The genomic patterns of CpG methylation are primarily reprogrammed in the early embryo phase and maintained for the rest of the life [40, 41]. Therefore, if the maternal diet contains a high amount of methyl donor species or cofactors of methyl metabolism, such as folic acid, then it will epigenetically affect certain gene expression and possibly enhancing the susceptibility of chronic disease onset later in life.

Waterland et al conducted a study of epigenetic modification of the agouti gene expression in viable yellow agouti (A\textsuperscript{vy}) mice [42] by supplementing the dams with folate, vitamin B\textsubscript{12}, choline and betaine. There is an increase in CpG methylation at the A\textsuperscript{vy} locus in
the offspring from supplemented dams, which affects the coat colour expression. Also, epigenetic alteration can cause variation in glucose tolerance and adiposity [43, 44]. Therefore, maternal dietary supplementations, such as folate and vitamin B_{12}, can have unintended silencing or deleterious effect on the epigenetic gene regulation, leading to compromised metabolic phenotypes in adulthood.

The following sections will provide a review of the role of under-nutrition and overnutrition in fetal programming of adult diseases.

2.3 MATERNAL NUTRITION AND FETAL PROGRAMMING

Epidemiological and retrospective cohort studies dating as far back as the 1940s have focused on the effect of under-nutrition during pregnancy and lactation on disease risks for the offspring [45, 46]. More recently, agricultural technologies and food distributing logistics have enabled both developed and more developing countries to obtain an abundant amount of food throughout seasonal and political instabilities [47-49]. Therefore, over-nutrition during pregnancy is increasingly important to contribute to fetal programming of adult diseases. Because the majority of evidence that nutrition can lead to fetal programming comes from studies of under-nutrition, these are reviewed first, followed by a review of the effect of over-nutrition on fetal programming. Each section is divided into human and animal studies, and sub-divided into macro- and micro-nutrients.

2.3.1 Fetal Programming by Maternal Under-Nutrition

Most evidence relating to the concept of fetal programming is based on the observation that adverse stimuli, such as famine or low protein intake during the prenatal
period, results in a higher susceptibility to obesity and metabolic syndrome in adult life. Moreover, the programming outcomes are dependent on the specific macro and/or micro-nutrient(s), the timing of the insult during gestation, and the sex and postnatal diet of the offspring. Prospective clinical, epidemiological and experimental studies have consistently repeated this relationship, but the underlying mechanisms between prenatal adversity and health outcome after birth are largely unknown and speculative.

2.3.1.1 Human studies

A limited number of retrospective epidemiological and prospective observational studies have reported the programming effects of undernutrition during pregnancy on the human offspring. These studies have primarily focused on the effects of food deprivation, low protein or low vitamin intakes, such as folate and vitamin D, during pregnancy on the offspring health.

A. Energy restriction / food deprivation during pregnancy

Human offspring born to malnourished mothers during pregnancy have a higher risk of developing cardiovascular diseases and insulin resistance in adulthood. Two main studies formed the primary basis for the support of the Fetal Origin Hypothesis, which are the Dutch Famine [46] and the Siege of Leningrad [50] studies. Both studies showed that the outcomes are dependent on the timing of food deprivation during pregnancy and the nutritional environment of the offspring in the postnatal period. In addition, these two studies support the Predictive Adaptive Responses (PARs) hypothesis, which highlighted the negative effects of nutrition mismatch between prenatal and postnatal environments [32].
In the winter from 1944 to 1945, at the end of World War II, part of the Netherlands suffered an abrupt 5-month famine due to transportation interruption from war. During this famine, the available food provided less than 800 kilocalories a day, and pregnant mothers experienced a distinctive period of under-nutrition in both energy and macronutrients [46]. One of the most important findings of this famine study is that the effect of under-nutrition during pregnancy is dependent on the timing of the nutritional insult. Exposure to food deprivation in the middle and/or late stage of pregnancy (2nd and/or 3rd trimester) was associated to higher prevalence of impaired glucose tolerance, higher proinsulin secretion and 2-h postprandial circulating insulin concentrations in 50 year old males and female offspring [51-53]. Moreover, pregnant mothers that were exposed to food deprivation in the early stage of pregnancy (1st trimester) led to offspring with higher prevalence of obesity, higher body mass index (BMI), compromised atherogenic lipid profile, higher fibrinogen concentration, decreased plasma factor VII, which resulted in a greater risk of developing coronary heart disease [45]. In addition, pregnant mothers who were exposed to the food deprivation for a minimum of 10 weeks, independent of the insult timing, gave birth to offspring with a higher prevalence of increased systolic blood pressure (SBP) and hypertension [54].

In contrast, the data from the Siege of Leningrad (1941 – 1944) demonstrated that offspring exposed to intrauterine under-nutrition did not develop a higher risk of obesity, hyperglycemia, insulin resistance, hypertension nor compromised atherogenic lipid profile [50]. The main difference between the Siege of Leningrad and the Dutch Famine studies is that the offspring in the Dutch Famine experienced little to no postnatal under-nutrition due to the restored food supply, which caused the offspring to experience catch-up growth in early childhood. This catch-up growth was associated with high risk of obesity and metabolic
syndrome in adulthood [26, 50, 55]. However, offspring born to mothers during the Siege of
Leningrad continued to be exposed in an under-nutrition environment for a prolonged period
of time, and minimal catch-up growth was documented [50]. These offspring in the Siege of
Leningrad had likely developed PARs in utero, and because there was no mismatch between
pre- and postnatal nutrition (e.g. poor nutrition before and after pregnancy), no characteristics
of metabolic syndrome or obesity was observed in the offspring adulthood.

**B. Macronutrient under-nutrition during pregnancy**

Among the macronutrients of protein, fat and carbohydrate, under-nutrition of protein
during human pregnancy has received the most attention, although the number of studies is
very limited [46, 56, 57]. No studies have looked at the effects of under-nutrition of
carbohydrates during pregnancy on the later life health of the offspring.

In the Dutch Famine study, a low protein to carbohydrate ratio during late pregnancy
was more closely associated with increased risk of hypertension in adulthood than lower birth
weight or length [46]. In addition, two separate prospective observational studies in
Caucasian women in Adelaide, Australia [56] and Southampton, United Kingdom [57] found
a positive association between protein intake during pregnancy and body weight at birth.
Every 1% decrease in protein intake during early pregnancy was associated with a 16 grams
decrease in birth weight [56], and every 1 gram decrease of meat protein intake during late
gestation was associated with a 3 gram decrease in birth weight [57]. Also, pregnant women
who restricted milk consumption during pregnancy to less than 250 ml/day had lower daily
intakes of protein and vitamin D, and gave birth to babies with lower body weight [58].
Fat intake by pregnant mothers is also shown to alter offspring physiology. An inverse relationship was found between the percentage of energy from fat in the diet of pregnant Filipino mothers and the systolic and diastolic blood pressure in girls, but not in boys [59]. In addition, the fatty acid profile of the diet during pregnancy affects anthropometry at birth [60], where the maternal plasma docosahexaenoic acid (DHA) concentration during pregnancy was positively associated with birth weight and head circumference.

C. Micronutrient under-nutrition during pregnancy

Inadequate intakes of micronutrients (vitamins and/or minerals) are common in both developing and developed countries, and usually occur due to poor diets and absorption, and dependent on gene polymorphisms [61]. However, there are very few reports of the long-term effects of maternal under-nutrition of micronutrients on the offspring in later life. This is due to the fact that micronutrient undernutrition is unlikely to occur in isolation, and micronutrient deficiencies were not considered as the primary candidates for poor fetal outcomes [62].

A deficiency of vitamin D during pregnancy has been suggested to program the bone mineral content of the offspring. Pregnant mothers that delivered during the winter months (January to March) had babies with a higher bone mineral content, lower serum osteocalcin level, and higher serum calcium than babies born in the summer months (July to September) [63]. Seasonal timing of the sun exposure and its role on the endogenous vitamin D₃ production is the mechanism behind the findings, and this study is another example showing the importance of the timing of stimuli on the programming effect on the offspring.
However, this study did not follow the infants to adulthood, thus there is no evidence of fetal programming of adult diseases.

It is well known that folate deficiency during pregnancy increases the risk of premature delivery, low birth weight and neural tube defects in humans [64, 65]. Low folate intake of less than 240 ng per day leads to a 3-fold increase in premature delivery and low birth weight [66], after adjusting for maternal intake nutrients, such as fiber, zinc and vitamin B₁₂. Although low birth weight has been associated with a higher risk of obesity in adulthood, this study also did not follow-up on the infants over time. In addition, pregnant mothers with polymorphism in the methylene tetrahydrofolate reductase (MTHFR) gene have shown to have higher blood homocysteine if their intake of folate were low during pregnancy, which subsequently led to higher risk of birth defects [67].

### 2.3.1.2 Animal studies

The epidemiological evidence indicating in utero programming of major chronic human diseases has been heavily criticized because of inconsistency between cohorts, and the application of inadequate statistical methodologies [28, 68-70].

To thoroughly examine the DOHaD hypothesis, animal models have been applied, and programming effects of under-nutrition in energy, macro- and micronutrients have been reported. The effects of diet manipulation during pregnancy have been examined in animals with both short pregnancy period, such as rodents and guinea pigs (22 and 65 days, respectively), and long pregnancy period, such as sheep and pigs (150 and 115 days, respectively) [71]. Animal models allow a higher degree of design control to isolate the
dietary effects. Also, animal models can help to elucidate the mechanisms and confirm the biological principle underpinning the nutritional programming.

A. Energy restriction / food deprivation during pregnancy

Energy restriction at 30% of \textit{ad libitum} intake during pregnancy is a common intervention in animal studies [72-75]. In rats, maternal caloric restriction results in hyperphagia in the offspring and plays a major role in adult pathophysiology. Pups born to energy-restricted dams exhibited lower birth weight, but higher post-weaning weight gain due to catch-up growth, fasting plasma insulin and leptin concentrations, and higher systolic blood pressure later in life compared to those born to control dams [76]. Another study with the same maternal dietary intervention led to endothelial dysfunction in the pups, which is a key component to cardiovascular disease [77]. Overall, pups that have experienced \textit{in utero} energy restriction developed components of the metabolic syndrome over the long term. In addition to rodent models, guinea pig offspring also exhibit similar phenotypes if they were born to mothers fed a 30% energy-restricted diet during pregnancy [78, 79].

The programming effect of intake restriction during pregnancy is dependent on the sex of the offspring. A food intake restriction of 50% \textit{ad libitum} during pregnancy in Sprague Dawley dams led to normal body weight of the offspring at birth and weaning [80]. However, by 5 weeks of age, male offspring showed greater weight gain and hyperphagia compared to those born to \textit{ad libitum} dams, but no change in weight gain and food intake was observed in female offspring. Moreover, the postnatal diet further affected the phenotypes, where the male offspring weaned to a high-fat diet exhibited exaggerated weight gain and hyperphagia.
In contrast to rodents, sheep have a longer pregnancy (21 weeks), which makes it a suitable model to look at the effect of timing on the maternal nutritional intervention towards the offspring. Offspring born to ewes on a 15% reduction in energy requirement during early and mid stage of pregnancy (first 70 days of gestation) exhibited a blunted fetal growth trajectory and reduced activity of the hypothalamic-pituitary-adrenal (HPA) axis during fetal life [81, 82]. However, these offspring showed no difference in body weight at birth, but an increased insulin response to glucose load and HPA activity at 12 weeks after birth. Moreover, pregnant ewes that experienced a 30% reduction in energy intake during late gestation (day 100 to term) led to offspring with 18% lower body weight at birth, but no significant difference on HPA activity in adulthood at 18 months of age [83]. Overall, these two studies showed that a maternal nutritional insult, in this case an energy intake deficiency, leads to different compromised metabolic phenotypes in the offspring depending on the timing of the insult. This finding is similar to the findings of the Dutch Famine Study [46].

B. Macronutrient under-nutrition during pregnancy

Beside calorie and global nutrition restrictions, the use of an isocaloric low protein (MLP) diet is one of the most extensively utilized manipulations to induce programming effects on the offspring in animal models [84-88]. This model involves ad libitum feeding to pregnant rats of a low protein diet containing 5% to 8% (w/w) protein (casein), which is slightly under half the protein content but equal in energy of a control diet containing 18% to 20% (w/w) protein [84, 89]. Rat offspring born to dams fed low protein diet had approximately 15% to 20% lower body weight at birth [87]. Continuing the MLP diet throughout the lactation period enhanced the weight gain difference and permanently
restricted growth later in adulthood. In contrast, if the offspring were cross-fostered to dams fed a control diet during lactation, the rat offspring would exhibit rapid catch-up growth, obesity, and components of the metabolic syndrome in adulthood [87]. Also, these offspring had a shorter lifespan that was associated with accelerated loss of kidney telomeric DNA [90].

Glucose metabolism in the rat offspring is also altered by the MLP diet. In early adulthood, lower fasting plasma glucose and insulin levels were found in MLP offspring, suggesting better insulin sensitivity [91]. However, by 15 months of age, glucose intolerance was found, and males exhibited hyperinsulinemia while females exhibited hypoinsulinemia [91]. The mechanism of the MLP diet was associated with a reduction in beta cell proliferation, islet size and vascularity along with an increase in beta cell apoptosis [86]. Offspring from MLP dams also had significantly higher SBP at an early age in comparison to those from control dams [84]. However, MLP diet without methionine supplementation showed either no change or a slight decrease in SBP in the offspring [89], which indicated the importance of the balance of micronutrients in determining the long-term health effects of gestational nutrition.

To date, low intakes of total fat or carbohydrate in the gestational diet have not been investigated in animal models.

C. Micronutrient under-nutrition during pregnancy

There are very few reports of the effect of under-nutrition of micronutrients during pregnancy on the adult offspring. In one study, a 50% restriction of recommended vitamin intake prior to and during pregnancy led to Wistar rat offspring with a significantly higher
percentage of body fat and higher plasma triglycerides, lower lean body mass and fat-free mass, along with increased oxidative stress at 6 months of age [92]. However, these compromised phenotypes were only presented when the offspring were weaned to the same vitamin-restricted diet as the dams. In contrast, a maternal mineral restriction at 50% of the recommended amount resulted in the same compromised phenotypes as the ones born to vitamin-restricted dams [93]. Among the few studies that described single micronutrient deficiencies, the focus was mostly on vitamin A, folate and iron and their subsequent poor reproductive outcomes and fetal organ defects [94-98], which are not related to this thesis.

2.3.2 Fetal Programming by Maternal Over-Nutrition

In the past 30 years, only a few studies have examined the effect of gestational over-nutrition on the development of chronic disease in the offspring. However, in recent years, with the escalating prevalence of obesity in pregnancy and its association with gestational diabetes, there is a rising interest in the potentially harmful programming effects of over-nutrition during gestation and the risk of disease in childhood and adulthood [99-101].

2.3.2.1 Human studies

A. Excess energy intake and maternal obesity

The effects of excess caloric intake during human pregnancy have received little investigation. However, maternal obesity is known to associate with increased risk of obesity in the child [102]. Pre-pregnancy body composition alone is a crucial factor of determining the risk of obesity in the child, where a higher body fat [103] and BMI [104] were associated with the overweight phenotype in adulthood. Children born to obese women with gestational
diabetes have a higher risk of becoming obese and develop insulin resistance in adulthood [105, 106].

B. Macronutrient over-nutrition during pregnancy

A very recently published prospective study explored the possible associations between the energy and macronutrient intakes of the mothers in pregnancy and the energy and macronutrient intakes of their offspring at 10 years of age [107]. Higher intakes of protein, fat (adjusted for energy intake), and carbohydrate during pregnancy were associated with higher dietary intakes of the same nutrients in the children. However, macronutrient intake during pregnancy was not associated with offspring adiposity or lean mass [107], and thus the findings may only be due to the daily eating habits and home environment of the families, but not the effect of fetal programming.

Protein intake during pregnancy has been reported to affect the blood pressure of the offspring, but the effects are inconsistent. During 1952 to 1976, pregnant women were advised to consume a high-animal protein low-carbohydrate diet (1 pound of red meat per day) to prevent preeclampsia in Motherwell, Scotland [108]. High intakes of meat and fish during the second half of pregnancy were associated with higher blood pressure in the offspring at 30 years of age. However, this study cannot exclude maternal saturated fat or salt intakes as confounders [108], which suggests that metabolic stress can be imposed on the pregnant mothers by an unbalanced diet where high intakes of essential amino acids are not accompanied by the nutrients needed to utilize them. In contrast, a study in Philippines found that boys (age 16) born to mothers who had a higher percentage of energy intake derived from protein at 30 weeks of pregnancy had lower systolic blood pressure [59]. However, in
girls (age 15), the lower SBP observation was seen in those born to mothers who had a higher percentage of energy intake derived from fat. Overall, studies on macronutrient intakes during pregnancy in humans have many limitations and are difficult to interpret, especially when the data were derived from food recalls that are crude measures of diet and nutrition and consisted of relatively large sample losses to follow-up over the years.

C. Micronutrient over-nutrition during pregnancy

Only a few human studies suggest an effect of increased micronutrient intake on fetal programming. A prospective cohort study in the United States has revealed an inverse relationship between gestational calcium supplement level and blood pressure of infants at 6 months of age [109]. Two month old infants from mothers who received two grams of calcium per day starting between week 13 to 21 of pregnancy until term had a 2.2 mmHg lower systolic blood pressure than the control group, and 4.8 mmHg lower when they reached 2 years of age [110]. This prenatal calcium supplementation is thus suggested to induce fetal programming of blood pressure regulation in adulthood.

Micronutrient intake during pregnancy affects anthropometric indices at birth. A prospective study explored the relationship of the intake of 20 micronutrients during pregnancy in a cohort of 222 Caucasian women in Boston, USA with birth weight, placental weight, birth length and head circumference of the offspring [111]. The data showed that the upper third quartile of micronutrient intake of pregnant women was 2 to 7 times the recommended dietary allowances (RDAs) for 10 vitamins, and the lower first quartile of intake met or exceeded the RDAs for most of the micronutrients. Pantothenic acid, sodium and vitamin E were positively associated with all four birth parameters mentioned, and zinc
was negatively associated with the parameters. However, the mechanism behind these observed associations was unclear.

However, a number of vitamins consumed during pregnancy have been shown to have no effect on the metabolic regulation in adulthood. Cardiovascular risk factors were reported in men and women around 50 years old and born to mothers who took part in the Oxford Nutrition Survey (1942 – 1944), and maternal vitamin A, B1, C and phosphatase levels were measured [69]. No association was found among these maternal micronutrients and offspring blood pressure, glucose tolerance, or components of the lipid profile. It is presently unknown if other fat-soluble vitamins, such as vitamin D, E and K, consumed during pregnancy can affect the metabolic indices of the offspring later in life.

### 2.3.2.2 Animal studies

Compared to human studies, there are more studies performed in animal models investigating the effect of overnutrition during pregnancy. Excess intakes of energy [112], total fat and saturated fatty acids [113], protein [114], and vitamins (folic acid [115-117] and vitamin E [118]) result in programming of adverse metabolic effect in the offspring.

#### A. High caloric intake during pregnancy

Only a few studies have explored the long-term effects of a maternal high energy intake during pregnancy on the programming of obesity in the offspring [112]. The common methods of inducing hyperphagia in rodent models are either forced-feeding (intragastric feeding), or injection of gold thioglucose (GTG) that promotes overeating by damaging the ventromedial hypothalamus responsible for suppression of food intake [119]. However, the
application of these methods in the understanding of obesity development in humans is limited.

Overfeeding female rodents before pregnancy is associated with obesity and insulin resistance in their offspring. Female mice that received a single injection of GTG prior to pregnancy developed hyperphagia, rapid weight gain, and hyperglycemia 6 weeks after the GTG injection [119]. The offspring of these GTG-dams were obese, hyperphagic and glucose intolerant by 3 months of age. In contrast, feeding high-sugar liquid diets intragastrically to adult female Sprague-Dawley rats at 15% excess calories three weeks prior to pregnancy led to a significant increase in weight gain, adiposity, serum insulin, leptin, and insulin resistance in the dams before mating [120]. Subsequently, offspring fed a high-fat weaning diet and born to the overfed dams had 25% greater weight gain and two-fold the adiposity at 18 weeks of age compared to those born to control dams [120].

B. Macronutrient over-nutrition during pregnancy

To date, among the macronutrients, high fat intake during pregnancy comprises the majority of the studies investigating the effect of fetal over-nutrition on the long-term health of the offspring. High fat diet during pregnancy is a preferred intervention because of its relevance to the obesogenic and high fat environment of humans in developed and developing countries. In contrast, high protein or carbohydrate intake during pregnancy received very little attention.

High maternal intake of fat has been reported to cause obesity and components of the metabolic syndrome in the Wistar rat offspring. Feeding a 40% fat diet during pregnancy and lactation in Wistar rat dams led to offspring with higher insulin to glucose ratios at birth,
along with higher adiposity, liver weight, liver lipid content, blood glucose and triglyceride at weaning [121]. Similar studies have shown that Sprague-Dawley rat offspring born to dams fed a 24% fat diet exhibited higher weight gain and a two-fold increase in the visceral fat pad mass, along with higher systolic blood pressure, whole body insulin resistance and pancreatic beta-cell dysfunction at 6 months of age [121-123].

In addition to the programming of obesity and metabolic syndrome, high fat diets during pregnancy affect the bone structure of the offspring. Mice offspring born to C57BL/6 dams fed a high animal lard diet from weaning and through pregnancy and lactation exhibited elevated bone marrow adiposity in the distal femur and altered trabecular structure at 30 weeks of age [124]. However, the mechanism of the higher fat storage in the bone marrow is unknown at the moment.

The composition of dietary fat during pregnancy also plays a role in fetal programming in rodents. Several rat studies have shown that offspring from dams fed a diet high in saturated fats during pregnancy exhibited insulin resistance and elevated blood pressure later in life compared to those born to dams fed a diet similar in fat amount during pregnancy [113, 125, 126]. In contrast, rat offspring born to dams fed a diet enriched in unsaturated fatty acids prior to mating until the end of lactation exhibited lower postnatal weight gain and increased number of pancreatic islet cells without affecting glucose tolerance at 12 weeks of age [113], suggesting a protective effect. In addition, higher weight gain was also reported in offspring born to Sprague-Dawley dams fed 10 g olive oil per 100 g diet compared to those born to dams fed fish oil [127]; and in offspring born to sows on diet supplemented with 2 g conjugated linoleic acid per 100 g diet compared to those born to dams on diet supplemented with linoleic acid [128].
Unlike the high fat diet during pregnancy, a high protein diet during pregnancy has received very little attention on the long term health of animal offspring. One study has shown that embryos from female mice fed a high-protein diet (25%) one month before pregnancy compared to those from mice fed the control diet (14%) were 16% less likely to develop into fetuses (65% vs. 81%) [114]. Also, the embryos conceived by pre-pregnancy high-protein dams were more likely to spontaneously abort later in pregnancy (16% vs. 1%). The high-protein diet also led to three times more ammonium in the reproductive tract due to protein breakdown, and this may affect imprinting of genes by turning specific parental genes on or off. One growth-related gene, H19, was affected by the treatment, and potentially explains the poor growth exhibited by the embryos from the mothers on a high-protein diet during pre-pregnancy [114].

C. Micronutrient over-nutrition during pregnancy

Vitamins play a crucial role in fetal development and all phases of cell growth and differentiation, and are key components of enzyme and cell structures. Many vitamins regulate the development of genes involved in growth, proliferation and in the functional characteristics of organs [18]. However, to date, only a few studies have looked at the programming effect of high intake of micronutrients below the toxic level on the non-teratogenic phenotype development of the offspring.

Among all the vitamins, folic acid has received the most attention due to its widespread chronic supplementation during preconception and gestation, along with its short-term benefits on fetal growth [129]. Chronic high intake of folate at 20 times the recommendation during pregnancy leads to lower birth weight and body length compared
with those born to control dams [115, 116]. Low birth weight and intrauterine growth retardation are associated with higher risk of metabolic syndrome in adulthood, such as hypertension, cardiovascular disease, and obesity [26]. However, it is believed that these observations were due to the effect of toxicity from high folate intake during pregnancy.

A series of studies have shown that high intakes of methyl donors (choline and betaine) and methyl metabolism cofactors (vitamin B$_{12}$ and folate) during pregnancy can alter the phenotype of the offspring [42, 130, 131]. Supplementation of choline and betaine (3 – 9 fold), vitamin B$_{12}$ (20 – 60 fold), and folate (3 – 9 fold) in the diet of pregnant viable yellow Agouti (A$^v$) mice mothers led to an increase in the proportion of offspring with darker fur coat colour compared to those born to mothers on control diet. These offspring were also leaner, with normal insulin level and food intake, and lived longer, which suggests altered metabolic regulation by fetal programming. However, a study on the maternal supplementation of methyl metabolism cofactors alone (folate and vitamin B$_{12}$) has yet to be performed, and the implication on wild-type rodents is unknown.

Besides water soluble vitamins, fat soluble vitamin supplementation (vitamin A, D, E, K) during pregnancy are also prime candidates to program the health of offspring. Supplementation of vitamin D at 12 times the recommended intake (12000 IU / kg diet) in the diet of pregnant Sprague-Dawley rats during pregnancy and lactation led to offspring with lower aortic elastin content, number of elastic lamellae in the aorta and force generation in the aortic rings [132]. Also, a chronic high intake of vitamin E during pregnancy also leads to changes in the gene expression profile of a set of vitamin E sensitive genes in the developing rat fetal brain on day 17 of gestation [118]. An example of an up-regulated gene expression in the fetal brain due to vitamin E supplementation during pregnancy is hemeoxygenase-3.
(HO-3), which translates into a heat shock protein that takes part in cellular defense mechanism. On the other hand, an example of a down-regulated gene is apolipoprotein B (apoB), which takes part in lipoprotein metabolism. However, there is no study looking at fat soluble vitamin supplementation during pregnancy on the health of the offspring later in life.
TABLE 2.1

_Vitamins in the AIN-93G diet and their programming effects of adult phenotypes_ 

<table>
<thead>
<tr>
<th>Vitamin</th>
<th>Programming Effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin B₁</td>
<td>(thiamin-HCl)</td>
</tr>
<tr>
<td>Vitamin B₂</td>
<td>(riboflavin)</td>
</tr>
<tr>
<td>Vitamin B₃</td>
<td>(nicotinic acid)</td>
</tr>
<tr>
<td>Vitamin B₅</td>
<td>(Ca pantothenate)</td>
</tr>
<tr>
<td>Vitamin B₆</td>
<td>(pyridoxine-HCl)</td>
</tr>
<tr>
<td>Biotin</td>
<td></td>
</tr>
<tr>
<td>Folic acid</td>
<td>3 to 9-fold folate (with vitamin B₁₂, choline and betaine supplementation) in diet of pregnant viable yellow agouti (A⁹⁹) mice leads to an increase in proportion of offspring with darker fur coat colour, leaner and longer lifespan [42, 130].</td>
</tr>
<tr>
<td>Vitamin B₁₂</td>
<td>20 to 60-fold in diet of pregnant A⁹⁹ mice: See folic acid for description [42, 130]</td>
</tr>
<tr>
<td>Vitamin A</td>
<td></td>
</tr>
<tr>
<td>Vitamin D</td>
<td>12-fold in diet of pregnant Sprague-Dawley rats leads to lower aortic elastin content, # of elastic lamellae, and force generation in aortic ring in the offspring [132].</td>
</tr>
<tr>
<td>Vitamin E</td>
<td>5-fold in diet of pregnant Sprague-Dawley rats (200 nmol/g diet) leads to up-regulation HO-3 gene, and down-regulation of apoB and GADPH genes in fetal brain [118].</td>
</tr>
<tr>
<td>Vitamin K</td>
<td></td>
</tr>
</tbody>
</table>

¹ Programming effects of vitamin supplementation during pregnancy and/or lactation exclude the effects of chronic or acute vitamin intake toxicity.
2.4 POTENTIAL MECHANISMS OF FETAL PROGRAMMING FOR ADULT DISEASES

To date, the mechanisms of fetal programming of metabolic diseases are not well understood. Most of the suggested mechanisms originated from research in under-nutrition during pregnancy, with one exception stemmed from over-nutrition of methyl donors and cofactors in a genetically-modified mice model (Section 2.3.2.2-C). The following will discuss the factors inducing fetal programming of adult diseases, and the suggested mechanisms of altering the hypothalamic development and regulation of food intake by fetal programming.

2.4.1 Factors Inducing Fetal Programming

The three most common factors known to induce fetal programming effects without causing teratogenesis are glucocorticoid over-exposure [133-136], maternal stress [137, 138], and epigenetic regulations due to changes in maternal nutrition [22, 26].

2.4.1.1 Glucocorticoid over-exposure

It has been proposed that the primary mediator of fetal programming is the level of glucocorticoid exposure to the fetus during various stages of pregnancy [34, 55, 139, 140]. Glucocorticoids are a class of steroid hormones involved in the feedback mechanism of the immune system to suppress inflammation [141]. The primary evidence of the involvement of glucocorticoid in fetal programming arise from the effect of dexamethasone, a synthetic glucocorticoid that plays an important role in the maturation of fetal organs in the preparation for preterm birth, and is used to prevent respiratory distress syndrome in preterm babies with
immature lungs [142]. If the fetus is exposed to inappropriately high levels of either endogenous glucocorticoids or exogenous dexamethasone, fetal programming of chronic diseases would occur through changes in the development of the hypothalamic pituitary adrenal (HPA) axis [139, 143, 144]. Because maternal under-nutrition in energy or protein intake leads to lower expression of 11 beta-hydroxysteroid dehydrogenase type 2 (11β-HSD2) in the placenta [145], which increases the fetal exposure to glucocorticoid, this is seen as a common pathway of fetal programming.

2.4.1.2 Maternal stress

Both acute and chronic maternal stresses were shown to alter the development of the fetal HPA axis. Restraining the rat dams only once for a tail pricking for blood sample during late gestation leads to higher corticosterone concentration in the 7 wk old offspring [146]. In contrast, restraining the rat dams daily during the last week of gestation leads to offspring with lower stress-induced plasma corticosterone levels compared to those from control dams [147]. Lower spine density and basal dendrites of pyramidal neurons of hippocampus are found, perhaps reflecting the critical programming changes in the neuronal development [148].

Maternal stress is also known to disturb the fetal glucocorticoid environment and reduce body, adrenal and pancreas weight as well as plasma corticosterone and glucose levels at birth [149]. Maternal stress induced by cage changes 3 times a day during the last week of pregnancy led to hyperglycemia, glucose intolerance and decreased basal leptin levels in rats 24 months of age [137]. Also, these male rats exhibited increase in food intake after a 24-
hour fast, which suggests that maternal stress induces a life-long alteration in food intake regulation and probable dysfunctions related to type 2 diabetes.

Although maternal stress may arise from under-nutrition during pregnancy, it is not applicable to the current thesis because multi-vitamin supplementation at 10 times the recommended intake during pregnancy in rats has been shown to lead to no difference in birth weight, litter size, and fasting plasma corticosterone level in the pups [150].

2.4.1.3 Epigenetic regulation induced by maternal under-nutrition

Complex diseases, including obesity and the metabolic syndrome, are determined by the multi-dimensional interactions between genes and environmental factors. DNA methylation is an epigenetic mechanism that regulates gene expression and thus cell differentiation and organogenesis [151]. Because the establishment of the epigenotype during embryogenesis is a process that is susceptible to changes in the environmental conditions, the epigenetic regulation of gene expression is thus a crucial link between maternal nutrition, gene expression and the long-term health of the offspring [152].

Recently, a series of studies conducted by Karen Lillycrop and Graham Burdge in the United Kingdom have addressed the molecular and epigenetic mechanisms behind the effect of under-nutrition using the low protein diet model [153-155]. Offspring born to rat dams fed a protein-restricted diet during pregnancy showed tissue-specific alterations in the expression of transcription factors that regulate a wide range of developmental and metabolic processes, notably the glucocorticoid receptor (GR) [153, 156] and peroxisomal proliferation-activated receptors (PPAR-α) [157]. In particular, these offspring had lower levels of CpG methylation of the PPAR-α and GR genes in the liver than those from control dams, which also correlated
with higher expression of these genes [153]. Interestingly, folic acid supplementation of the
protein-restricted diet during pregnancy prevents the hypomethylation of PPAR-α and GR
genes [158], possibly due its capacity to methylate DNA during mitosis in fetal development
[155].

In addition, rat offspring from low-protein dams also exhibited altered expression of
genes involved with de novo lipogenesis, specifically acetyl coenzyme A carboxylase (ACC)
and fatty acid synthase (FAS) [159]. There was a significant increase in the expression of
hepatic ACC and FAS, along with an increase in plasma triacylglycerol and non-estrified
fatty acid concentrations [157]. This observation explains the higher adiposity in rat
offspring from protein-restricted dams as mentioned compared to those born to well-fed
dams.

Most importantly, Lillycrop et al [155] has proposed the mechanism behind the
compromised glucose homeostasis as seen in the offspring from protein-restricted dams
[160]. As mentioned above, the higher expression of GR due the hypomethylated GR
promoter was suggested to lead to higher phosphoenolpyruvate carboxykinase (PEPCK)
expression, which resulted in elevated hepatic gluconeogenesis in the offspring. This
mechanistic link may explain the hyperglycemia and insulin resistance exhibited in offspring
born to protein-restricted dams.

The suggested role of maternal vitamin supplementation, especially the methyl
metabolism cofactors (folate and vitamin B₁₂), on the epigenetic regulation of gene
expression and programming of adult diseases will be discussed in Section 2.5.2.
2.4.2 Role of Hypothalamus in the Fetal Programming of Food Intake Regulation and Appetite Control

The crucial role of the hypothalamus in the control of food intake is well documented. Central and peripheral signals relay information about the current state of energy balance to important areas in the brain, including the hypothalamus and brainstem [161]. Hunger and satiety represent complex but coordinated responses to these signals, which include neural and hormonal messages from the gastrointestinal tract (Figure 2.1). However, only recently it is recognized that the hypothalamus plays an important role in fetal programming of short-term appetite and long-term food intake dysregulation [162-167].

Rat offspring born to dams fed a high fat diet (55% margarine) during pregnancy exhibit an exaggerated short-term feeding response (two-fold in food intake) to injections of neuropeptide Y (NPY) into the lateral brain ventricle [168]. NPY is an orexigenic signal expressed by the arcuate nucleus (ARC) neurons in the hypothalamus [169, 170], and the observed hyperphagia after NPY injection reflects the possible alteration in the function and/or networking of neurons responsible for appetite regulation. Similarly, maternal over-nutrition (155% of energy requirement) in sheep led to an altered appetite regulation in the early postnatal period, with impaired signaling of the appetite-inhibiting cocaine- and amphetamine-regulated transcript (CART), and reduced expression of the leptin receptor in the hypothalamus [165, 171].

In contrast, long-term energy homeostasis determines the progression of obesity over time, and both insulin, and to a lesser extent leptin, are likely to represent as the long-term hormonal mediators for the fetal programming of long-term food intake. Insulin appears to elicit crucial influences on the development of circuits in the hypothalamus that regulate food
intake and energy homeostasis [169]. Maternal injections of insulin between day 15 and 20 of pregnancy in rats, which is an important period for hypothalamic development, led to a higher risk of obesity in the offspring [172]. Also, the metabolic abnormalities observed in the offspring born to insulin-injected dams were coupled with increased hypothalamic norepinephrine levels and higher density of norepinephrine-containing neuronal fibers innervating the paraventricular nuclei (PVN) [172, 173]. In parallel, maternal diabetes induced by streptozotocin injections led to hyperinsulinism associated with hypothalamic alterations, such as lower hypothalamic NPY mRNA and protein expression, and altered neuronal morphology in the arcuate nucleus in the fetus [174, 175]. In general, fluctuations in insulin levels, especially hyperinsulinism, during pregnancy can induce alterations in hypothalamic organization and thus program the metabolism of the offspring later in life.

A central role for leptin in hypothalamic programming has also been proposed. Subcutaneous administration of leptin day 3 to 13 after birth reversed excess weight gain and hyperphagia in adult rat offspring born to dams exposed to undernutrition during pregnancy [176]. In addition, leptin was found to promote neuronal outgrowth from the ARC to the PVN during the lactation period in rat offspring, thus possibly hardwired the hypothalamic appetite regulatory system [169]. Leptin was suggested to affect the two opposing pathways controlling energy intake in rats, favoring the development of appetite stimulatory NPY and agouti-related protein (AgRP) projections from the ARC to the PVN.
**Figure 2.1.** Gut hormones and energy homeostasis

Ghrelin, released from the empty stomach activates NPY and AgRP containing neurons in the ARC to stimulate food intake in the LHA, while inhibiting POMC and CART system responsible for the satiety. In contrast, leptin positively regulates POMC and CART neurons in ARC to activate satiety and to inhibit ghrelin-NPY/AgRP pathway. Peripheral neural (vagal) signals and different gut hormones act on hypothalamic centers to affect food intake.

This figure is modified from Konturek et al (2005) [177].
2.4.3 Hypothalamic Serotonergic Development and Food Intake Regulation

Maternal malnutrition, especially under-nutrition, has been consistently associated with metabolic programming of adult diseases in the offspring. However, the hypothalamic serotonergic function, which plays a critical role of catabolic component of energy homeostasis, has not been fully explored. The serotonin system involved in the regulation of food intake is a prime target of fetal programming.

In animal models, malnutrition during pregnancy and/or lactation permanently alters the development and activity of the serotonergic system. The synthesis of serotonin (5-HT) in the brain is accelerated starting in the fetal period of malnourished Wistar rats [178]. This elevated amount of 5-HT is directly associated with an increase in the concentration of both brain L-tryptophan and the activity of the rate limiting enzyme tryptophan hydroxylase, which suggests that an activation of the serotonergic system occurs during nutritional stress [179]. In a recent study, adult female rats exposed to intrauterine undernutrition were fatter and resistant to insulin-induced hyperphagia [180]. These female offspring failed to respond with hypophagia to an intracerebroventricular injection of serotonin. Also stimulation of extracellular levels of serotonin in medial hypothalamus by food ingestion was enhanced and lasted longer. The hypothalamic levels of 5-HT$_{2C}$ was lowered, while the 5-HT$_{1B}$ receptor and serotonin transporter proteins were elevated. These results suggest that compensatory adaptations for the functional serotonergic impairment were apparent, and it is likely that the serotonergic component of energy expenditure was compromised and contributed to the obese phenotype in these offspring [180].

Similarly, human babies that experienced intrauterine nutritional restriction had lower birth weight, higher free fraction of plasma L-tryptophan and lower bound fraction of L-
tryptophan and plasma proteins [181]. The elevation of the free fraction of plasma L-Trp suggests an elevated transport to the brain with a possible enhancement of the serotonin synthesis during the last trimester of pregnancy, which is a critical period of brain differentiation and neuronal development [182-184].

2.5 ROLE OF VITAMINS IN FETAL PROGRAMMING OF OBESITY AND COMPONENTS OF METABOLIC SYNDROME

The escalating prevalence of obesity and metabolic syndrome in the past 30 years has been coupled with an increase in use of multi-vitamin supplementation during pregnancy in the population [1, 4, 11], and it has become clear that maternal vitamin supplementation affects metabolism of offspring. To date, no study has looked at the novel relationship between gestational multi-vitamin supplementation and the development of obesity and components of the metabolic syndrome in the offspring. The following sections will briefly discuss the description of metabolic syndrome, and speculate on the two possible mechanisms between gestational multi-vitamin intake and programming of adult diseases later in life in the offspring, which are the epigenetic mechanism and fetal swallowing development.

2.5.1 Components of the Metabolic Syndrome

Obesity and overweight are linked with a series of metabolic and cardiovascular disorders that have been named the metabolic syndrome [185]. The primary criteria of metabolic syndrome are generally agreed to exhibit five key features, which are hyperglycemia, insulin resistance, dyslipidemia, central obesity and hypertension [186]. Many researchers suggested that insulin resistance is the primary pathological cause among
these features, while others argued that metabolic syndrome is the result of the complications originated from obesity [187]. The discrepancy of the pathological cause of metabolic syndrome stems from the fact that central obesity is strongly associated with insulin resistance, but only half of the obese subjects exhibit insulin resistance [188].

Insulin resistance and type 2 diabetes are rapidly emerging as major diseases in children and adolescents, and this appears to be linked to the escalating prevalence of obesity in the pediatric population [3, 189, 190]. Childhood metabolic syndrome prematurely promotes the development of atherosclerosis and increases the risk of cardiovascular disease as a young adult [191], which significantly contributes to the heightened morbidity in this age group. Therefore, recent research has focused on the exploration of the pathophysiology of the obesity development in children, in which in utero programming of hyperphagia due to imbalanced nutrition during pregnancy is the strongest candidate responsible for the origin of the obesity problem later in life [26, 192].

In the current research, components of metabolic syndrome are measured in rat offspring born to dams fed the multi-vitamin supplemented diet during pregnancy. These parameters include post-weaning weight gain, abdominal adiposity, short-term appetite and long-term food intake, glucose tolerance and insulin response after an oral glucose load, and systolic and diastolic blood pressures.

### 2.5.2 Epigenetic Regulation of Gene Expression Involved in Metabolic Regulation

A number of studies have found that the agouti gene is related to the development of obesity, and methylation of this gene due to a high concentration of methyl donors or methyl metabolism cofactors, such as folate, vitamin B₆ and B₁₂, in the diet during pregnancy can
lead to epigenetic variability. In fact, the agouti gene is the earliest gene that was cloned and is heavily studied; and it was believed to be the obesity-causing gene [193]. However, obesity is later regarded as a disease with polygenic origins, thus the agouti gene takes on a role that contributes to the obesity development [194].

The instability of the agouti gene causes a wide variation in adiposity and glucose tolerance in the A^vy mice [43]. Although the actual mechanism is still under investigation, it is certain that early methyl donor malnutrition, either over or under nutrition, can lead to premature “epigenetic aging” as suggested by Lamb et al [195], and thus increase the susceptibility to chronic disease later in life [38]. Also, the agouti protein are endogenous antagonists of melanocortin receptors, and the melanocortin system can activate the hypothalamic-pituitary-adrenal (HPA) axis [196, 197], and it is thus logical to speculate that the fetal HPA axis development can be indirectly disturbed by a change in maternal nutrients. Further investigation is necessary in order to understand the fetal-epigenetic mechanism, but the HPA axis must be the most crucial and common target if the above speculated mechanism is valid. In other words, if multi-vitamin supplementation during pregnancy leads to fetal programming of adult metabolic syndrome, the HPA axis would be the primary target, although it is unclear how the HPA axis would be manipulated.

Beside the B-complex vitamins, vitamin A can induce enzymatically active glycine N-methyltransferase (GNMT), which is a key hepatic cytosolic enzyme essential in optimizing methyl group supply in the folate-dependent one-carbon pool and in the regulation of S-adenosylmethionine (SAM) and the SAM to S-adenosylhomocysteine (SAH) ratio [198, 199]. A higher intake of vitamin A during pregnancy can potentially lead to a higher GNMT activity and a biologically crucial loss of methyl groups, and thus impairs the
transmethylation processes in the hepatic cells of offspring. This retinoid-mediated hypomethylation of hepatic DNA can affect a number of important processes, notably the gene expression and development [151, 200, 201].

Overall, vitamins that are involved in one-carbon metabolism, such as folate, vitamin B6 and B12, and vitamin A, are the primary candidates that contribute to the possible link between gestational multi-vitamin intakes and its metabolic programming effects on the offspring. However, other vitamins that are not involved in the methyl metabolism, are likely to play a role through different mechanisms.

2.5.3 Fetal Swallowing and the Appetite-Regulation System Development In-Utero

Another possible mechanism could be in the context of fetal swallowing and the sensitivity of the fetus to the taste of amniotic fluid [202]. In brief, fetal swallowing develops in utero and contributes to fetal gastrointestinal and appetite regulation development; and the normal fetal appetite development consist of both peripheral taste receptors and certain hypothalamic orexic centers maturations. A change in fetal swallowing can cause alterations in appetite regulation, and pathologically, cardiovascular status and obesity risk after birth. The taste of the amniotic fluid is assumed to be a contributor to determine the rate of fetal swallowing. A study showed that ovine fetuses respond to acid taste very early in gestation, and both bitter and salt taste slightly later [203, 204]. Also, in rat pups, behavioral changes were detected in response to acidic, sweet, and salty stimulus immediately after birth [205].

A study of oral supplementation of vitamin C and E during pregnancy showed that maternal plasma vitamin C and E levels were correlated with the amniotic fluid vitamin C and chorioamnion vitamin E levels [18]. Also, vitamin supplementation, especially the fat-
soluble ones, should affect the vitamin levels in the maternal-placental system. Therefore, maternal multi-vitamin supplementation may affect the taste of the amniotic fluid to either more bitter or acidic, or both. This could affect the fetal swallowing and appetite regulation development in the fetus, which may lead to dysfunctional appetite control and thus obesity in the adulthood.

2.6 USE OF DIETARY SUPPLEMENTS BY PREGNANT WOMEN

During pregnancy and lactation, nutritional requirements are increased to support fetal and infant growth and development, and changes in maternal tissues and metabolism [206, 207]. Recommended intakes for 14 of the 21 essential micronutrients increase during pregnancy, which are 7 vitamins, 6 minerals, and choline [62]. Therefore, it is crucial to increase the intake of foods containing these nutrients to avoid deficiencies, and the use of dietary supplements in pregnant women is recommended due to their potential benefits in pregnancy and birth outcomes [62, 208].

A number of studies have looked at the supplement intakes in the general population, where a steady increase has been observed in the past 20 years [11]. Data from the 1999 to 2000 National Health and Nutrition Examination Survey (NHANES) in the United States indicated the high usage of dietary supplement among Americans [17]. The NHANES found that 52% of adults took a dietary supplement within a month prior to the survey, and 67% of these adults took a multi-vitamin and multi-mineral supplement, which supported the high usage of supplement use among Americans as shown previously [209, 210]. Also, females had a higher frequency of taking dietary supplements, and of the women that took
supplements, 45% took 1 supplement per day, 22% took 2 supplements, and 32% took 3 or more per day [17].

In contrast, although most pregnant women are advised to take a multi-vitamin and multi-mineral supplement, only one epidemiological study in the past 20 years has reported such usage during pregnancy. A total of 344 self-reported low-income pregnant women were surveyed, and 86% reported a consumption of 4 or more prenatal vitamins per week [211]. Also, a more recent survey has estimated the vitamin intakes by pregnant women in Boston, MA, USA [111], in which the data showed vitamin intake of the pregnant women in the upper third quartile was between 2 to 7 times the recommended dietary allowances (RDAs) for 10 vitamins. In addition, many common multi-vitamin supplements recommended to pregnant women were shown to contain 2 to 5 times the RDAs for folate, vitamin B6 and B12 [16], which are known to play crucial roles in epigenetic regulation of gene expression involved in the development of chronic diseases [212-214].

To date, only a few studies have looked at the benefits of using multi-vitamin supplementation during pregnancy [65, 215]. The studies by Czeizel et al were conducted as randomized, double-blind, placebo-like controlled trials with over 4500 subjects. The results suggested that multi-vitamin supplementation lowered the risk of cardiovascular malformations, urinary tract abnormalities, and neural tube defects. Other observational studies also supported the potential beneficial effects of prenatal multivitamin use on the risk of preeclampsia, preterm birth, cleft palate, and other fetal malformations [216-219]. However, more controlled prospective studies are required to confirm these observations.
2.7 JUSTIFICATION OF USING A RAT MODEL

Animal models are crucial in demonstrating the biological mechanisms behind the associations of early life disturbance and adult disease risk observed in human populations, and they provide the proof of principle to the DOHaD concept. A variety of small (rats and mice) and large animal models (sheep and pigs) have significantly contributed to the DOHaD field, providing strong support of a casual relationship between various compromised developmental environments and the corresponding metabolic risk factors in the long term. Also, the use of animal models allows a strong degree of control over confounding factors that are embedded in the design of human studies.

A major advantage of working with small animals, such as rats and mice, is the short gestation period (22 days) and lifespan (2 years), and thus the higher cost-effectiveness of maintaining a large cohort [71]. These factors allow studies to be conducted across the entirety of the lifespan and into subsequent generations. Changes in gene expressions detected in early life are often transient, and resulting changes in gene expression and tissue function later in life contribute to better understanding of the life course of the pathology progress [40, 151, 194]. Another advantage of working with rodents is the feasibility of diet manipulation, which allows the controlled evaluation of specific changes in dietary composition that are relevant to the human population [71]. In the current research, the multi-vitamin mix (AIN-93-VX) content [220] was adjusted in the pregnancy and post-weaning diets in order to investigate the effects of exposure to high vitamin status \textit{in utero} and post-weaning vitamin deficiency. In future studies, the vitamin content and individual vitamin(s) can be customized in the diet to determine the dosage and specific vitamin(s) needed to induce the fetal programming effect.
For this research, rats are chosen over mice because rats are easier to handle for weight measurements, gavages and tail pricks on a frequent basis due to their calmness. More blood can be withdrawn for hormonal measurements (i.e. plasma glucose and insulin) on a regular interval in rats because of their larger size. Also, short- and long-term food intake measurements are more accurate in rats compared to mice because rats produce relatively less spillage than mice while consuming powdered diets, and data of food intake from individual rat are needed.

Wistar and Sprague-Dawley rats are the most popular and extensively used animal models in investigating the effects of fetal programming of adult diseases. These ideal outbred rat strains have similar growth rate, regulation of food intake, stress response, and metabolic parameters under normal conditions [221-224]. However, Wistar rat is a more ideal rat strain to study the effect of fetal programming of obesity because of its higher final body fat content over time, regardless of the types of diets (normal chow, high-fat or high-calorie diet) [225]. In terms of the metabolic syndrome, it has been a challenge to find an adequate animal model to study its pathology due to the multi-factorial nature. However, in an obesogenic environment, Wistar rats exhibit changes similar to those seen in human metabolic syndrome, such as abdominal obesity, hyperglycemia and hypertension [76, 226, 227]. Therefore, Wistar rat is the chosen rat model for this research.
2.8 SUMMARY AND RESEARCH RATIONALE

The escalating prevalence of obesity and metabolic syndrome is currently a major public health issue in both the developing and developed world. It is becoming increasingly apparent that, in addition to environmental and lifestyle risk factors, the susceptibility to obesity may have origins in early life. It has been demonstrated that the fetus, besides being a passive recipient of maternal nutrition, has the capacity to respond and physiologically and structurally adapt to higher or lower levels of nutrient supply.

Along with the escalating prevalence of obesity in the last 30 years, there has been a substantial increase in the fortification and supplementation of vitamins in the food supply. Pregnant women can unintentionally and intentionally consume a much higher than recommended intake of vitamins, and its effect on the long-term health of the offspring is little known.

Therefore, the current thesis will explore the programming effects of high multi-vitamin diet during pregnancy in Wistar rats on the risk of developing obesity and insulin resistance in the adult offspring exposed to a normal, obesogenic, low vitamins or nutrient-selection paradigm diets. The main focus of this thesis will be on the changes in the regulation of both long- and short-term food intake and weight gain, and glucose-insulin metabolism induced by the gestational multi-vitamin supplementation.
CHAPTER 3.

HYPOTHESES AND OBJECTIVES
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3.1 OVERALL HYPOTHESIS AND OBJECTIVE

**Hypothesis:** High multi-vitamin intake during pregnancy leads to the development of obesity and components of the metabolic syndrome in the rat offspring.

**Objective:** To determine the effects of multi-vitamin supplementation during gestation on components of the metabolic syndrome in the rat offspring. Four studies were designed to test this hypothesis; Studies 1 and 2 aimed at establishing the obese and insulin-resistant phenotype in offspring born to dams on the vitamin supplemented diet during pregnancy. Studies 3 and 4 aimed at manipulating the vitamin and protein content of the offspring diet after weaning and exploring the interaction between gestational and pup diets.

3.2 SPECIFIC HYPOTHESES AND OBJECTIVES

**Study 1:** High multi-vitamin intake during pregnancy results in increased food intake and components of the metabolic syndrome in male Wistar rat offspring.

**Hypothesis:** High vitamin intake by Wistar rats during pregnancy would affect the regulation of food intake, body weight, and metabolic phenotype of the offspring.

**Objective:** To determine the effects of high multi-vitamin intakes during pregnancy on the long-term weight gain, food intake, and metabolic control in the offspring later in life.

**Study 2:** Multi-vitamin supplementation during pregnancy accelerates the development of obesity in Wistar rat offspring fed an obesogenic diet.
**Hypothesis:** A liquid obesogenic diet post-weaning accentuates the effect of multivitamin supplementation of the gestational diet on the phenotypic expression of obesity in both male and female offspring.

**Objective:** To determine if the obesogenic diet would accelerate the development of obesity and components of the metabolic syndrome in male and female offspring born to dams on the high multi-vitamin diet during pregnancy.

**Study 3:** The effect of high vitamin diet during pregnancy on food intake and glucose metabolism in Wistar rat offspring fed low vitamin diets post-weaning.

**Hypothesis:** Increasing the mismatch in the gestational and pup diets created by feeding rat offspring a low or deficient vitamin diet at weaning would amplify the effect of high multi-vitamin intakes during pregnancy on the development of the characteristics of the metabolic syndrome in the offspring.

**Objective:** To determine the effect of high multi-vitamin intakes during pregnancy on the adaptive responses to the amplified nutrient mismatch by the post-weaning low vitamin diets of the offspring.

**Study 4:** Multi-vitamin supplementation during Pregnancy alters body weight and macronutrient selection in Wistar rat offspring.

**Hypothesis:** Compared to offspring born to dams fed the control AIN-93G diet, those born to dams fed the high vitamin diet during gestation have altered diet choice, as well as energy intake, in which their food intake behaviour could be associated with altered expressions of serotonin receptors and pro-opiomelanocortin (POMC) in the hypothalamus.
Objective: To examine the effect of feeding a high vitamin diet during pregnancy on food intake, the selection of protein and carbohydrate, and the expression of hypothalamic serotonergic receptors and POMC of the melanocortin system in the offspring.
CHAPTER 4.

STUDY 1: HIGH MULTI-VITAMIN INTAKE BY WISTAR RATS DURING PREGNANCY RESULTS IN INCREASED FOOD INTAKE AND COMPONENTS OF METABOLIC SYNDROME IN MALE OFFSPRING
CHAPTER 4. HIGH MULTI-VITAMIN INTAKE BY WISTAR RATS DURING PREGNANCY RESULTS IN INCREASED FOOD INTAKE AND COMPONENTS OF METABOLIC SYNDROME IN MALE OFFSPRING

Preface:

To address the effects of high multi-vitamin intakes during pregnancy on the long-term weight gain, food intake, and metabolic control in the offspring later in life.

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4.1 ABSTRACT

The effect of high multi-vitamin intake during pregnancy on the metabolic phenotype of rat offspring was investigated. Pregnant Wistar rats (n=10/group) were fed the AIN-93G diet with the recommended vitamin (RV) content or a ten-fold increase (high vitamin, HV). In Exp 1, male and female offspring were followed for 12 wk post-weaning, whereas in Exp 2, only males were followed for 28 wk. Body weight (BW) was measured weekly. Every 4 wk, after an overnight fast, food intake over 1-h was measured 30 min after a gavage of glucose or water. An oral glucose tolerance test (5g/kg) was performed every 3 to 5 wk. Post-weaning fasting glucose, insulin, ghrelin, GLP-1, and systolic blood pressure (SBP) were measured. No difference in BW at birth or litter size was observed. Males born to HV dams had higher food intake (P<0.05), and at 28 wk post-weaning, 8% higher BW (P<0.05) and 27% higher fat pad mass (P<0.05). Food intake reduction after the glucose preload was nearly two-fold less in males born to HV dams at 12 wk post-weaning (P<0.05). Fasting glucose, insulin and ghrelin were 11%, 62% and 41% higher in males from HV dams at 14 wk post-weaning (P<0.05). Blood glucose response was 46% higher at 23 wk post-weaning (P<0.01), and SBP was 16% higher at 28 wk post-weaning (P<0.05). Females born to HV dams exhibited no differences in the parameters measured. In conclusion, high multi-vitamin intake during pregnancy programmed the male offspring for the development of the components of metabolic syndrome in adulthood, possibly by its effects on central mechanisms of food intake control.
4.2 INTRODUCTION

Many nutrients are recommended prior to and during pregnancy to women for the prevention of deficiencies and for improving gestational outcomes in the offspring. Animal studies suggest that they may have long-lasting health impacts. For example, diets high in folate, vitamin B₁₂, choline and betaine (9 to 60 times the recommended level) fed to viable yellow Agouti (A<sup> vy </sup>) mice during pregnancy and lactation increased the proportion of offspring with brown coat colour and lean phenotype compared to yellow coat colour and an obese phenotype through epigenetic modification of gene expression [130, 214]. Similarly, gestational diets high in genistein, a bioactive isoflavone readily found in soybeans, results in similar alteration in the phenotype of the A<sup> vy </sup> mice offspring and in histone modification [228]. These effects are observed in a transgenic obese model, but nothing is known about the impact of micronutrient supplementation on phenotypic expression in a rodent model with no known genetic defects.

The relevance of these observations in rodents to humans is unclear, but bioactive compounds are consumed both intentionally and unintentionally. The importance of vitamin-adequate diets in the prevention of birth defects prior to and during pregnancy is well known, and therefore, multi-vitamins are recommended, leading to increased intake during pregnancy by the majority of women [11]. However, unintentional consumption of bioactive compounds also occurs. An example is glycyrrhetinic acid, an active constituent of liquorice that has been shown to lower the activity of 11-beta hydroxysteroid dehydrogenase type 2 (11β-HSD2) in the human placenta [229, 230]. As a result, the exposure of the fetus to gestational glucocorticoid is increased and in utero development of the hypothalamic-pituitary-adrenal (HPA) axis is altered [143, 231, 232]. Associated with chronic liquorice
consumption are a lower birth weight and a shorter gestation period along with hypertension and hyperglycemia in adult life of both rats and humans.

The possibility of unintentional consumption of excess intake of vitamins during pregnancy by women can be suggested for three reasons. First, multi-vitamins are the most popular supplement consumed in both developing and developed countries [11]. A recent survey of intakes by pregnant women in Boston found intakes by those in the upper third quartile to be between 2 to 7 times the RDA for 10 vitamins [111]. As a result, the intakes of some vitamins exceed the daily upper intake levels set by the National Academy of Sciences [233], and a chronic excess of intake may be occurring with long term consequences for the developing fetus [17]. Second, one of the consequences may be a predisposition of the children to obesity and the metabolic syndrome. Thus it is of interest that increased use of multi-vitamin and other supplements has occurred [11], concurrent with the increased prevalence of obesity in the last three decades [4]. Third, high intakes of vitamins involved in methyl group metabolism by pregnant viable yellow Agouti (A<sup>vy</sup>) mice alter expression of the hypothalamic orexigenic Agouti related protein in the offspring [214], suggesting that the development of food intake regulation was affected. Therefore, we hypothesized that high vitamin intake by the Wistar rat during pregnancy would affect the regulation of food intake, body weight and metabolic phenotype of the offspring.

4.3 MATERIALS AND METHOD

**Animals and Diets.** First-time pregnant Wistar rats were purchased from Charles River Inc., (Montreal, QC, Canada). Upon arrival, at 3 d of pregnancy, they were housed individually in ventilated plastic transparent cages with bedding in a 12:12 h light-dark cycle
(lights on at 0600h), at a temperature of 22 ± 1 °C. The pregnant rats had free access to water by an automated water system. The University of Toronto Animal Care Committee approved the protocols and maintenance of the animals conforming to the guidelines of the Canadian Council on Animal Care.

From 3 d of pregnancy to term, dams were fed the AIN-93G diet [220] containing either the recommended (RV, recommended vitamins) or 10 times higher vitamin (HV, high vitamin) content by addition of the AIN-93 vitamin mix. The composition (in g/kg) for the AIN-93G diet was casein (200), cornstarch (529.4), sucrose (100.1), soybean oil (70), cellulose (50), vitamin mixture (10), mineral mixture (35), choline bitartrate (2.5), and tertbutyl hydroquinone (0.014). Cornstarch, high-protein casein (87%), and cellulose were purchased from Harlan Teklad (Madison, WI). The vitamin mixture, mineral mixture, choline bitartarate, and tertbutyl hydroquinone were purchased from Dyets (Bethlehem, PA), whereas sucrose and soybean oil were purchased from local suppliers in Toronto, Canada (Allied Food Service and Loblaws, respectively). Each 10 grams of vitamin mix contains 9.75 grams of sucrose as a carrier. Therefore, in the HV diet, the added sucrose was reduced to 9.5 grams to adjust for the 97.5 grams coming from the vitamin mix. The vitamin additions used in the HV diet were well below that expected to have teratogenic effect and half the lowest intake of vitamins known to have an adverse effect in rats [116, 234].

During lactation, dams were fed the RV diet. At weaning, pups were housed individually in ventilated plastic transparent cages with bedding. Pups in both experiments were weaned to the RV diet. The powdered diet was provided in stainless steel cups with a mesh disk insert in each of them to reduce spillage. All gestational and pup diets were provided ad libitum in both Experiment 1 and 2.
**Design.** This study consisted of two experiments. In both experiments, pregnant female rats were fed either the RV or HV diets from d 3 of pregnancy until labour. At birth, each litter was randomly culled to 10 pups in order to minimize the difference in milk availability in each litter. Litters of less than 10 pups were excluded. In Experiment 2, two litters were excluded: one litter was from the control dams (N = 5 pups / litter), and one litter was from the high vitamin dams (N = 8 pups per litter). In both experiments, after culling, there were 10 litters from control dams and 10 litters from high vitamin dams. During lactation, the dams were fed only the RV diet. At weaning, male and female (Exp 1) and male (Exp 2) offspring from the RV and HV dams were fed the RV diet. Groups of ten (one pup / sex / dam) were formed for each of the dependent measures.

**Experiment 1: Effect of multi-vitamin supplementation during pregnancy on body weight, food intake and glucose tolerance of the offspring to 12 weeks post-weaning.** Body weight (BW) was measured weekly from weaning to 12 wk post-weaning. 24-h food intake was measured once every 3 wk. Fat pad mass (FPM, sum of abdominal, peri-renal and epididymal (males only) fat pads) was collected upon sacrifice at 12 weeks post-weaning.

Oral glucose tolerance test (OGTT) was performed at 0, 3, 6 and 9 wk post-weaning. Rats were fasted overnight for 10 hours before the OGTT. At the beginning of OGTT, a blood sample was withdrawn from the capillary bed of the tail tip and baseline glucose was immediately assayed using a commercial glucometer (MediSense Precision Xtra). The rats
were then gavaged (0.375 g glucose / ml, 5 grams of glucose / kg body weight), and blood glucose concentrations were determined 15, 30 and 60 minutes later.

**Experiment 2: Effect of multi-vitamin supplementation during pregnancy on the regulation of food intake and the appearance of components of the metabolic syndrome in male offspring to 28 weeks post-weaning.** The objective of the second experiment was threefold. First, we aimed at reproducing observations from Exp 1 showing that the male offspring from HV dams had higher food intake, body weight, and adipose tissue, and were developing glucose intolerance. Second, hormones (ghrelin, GLP-1 and insulin) that are known to regulate food intake [161] were measured at weaning and at 14 wk post-weaning in order to derive physiological evidence of altered regulation of food intake in offspring from HV dams. Third, body weight, insulin resistance and blood pressure were measured to 28 wks post-weaning to add further evidence that the rats had developed components of the metabolic syndrome [235, 236].

BW was measured at birth (1 d) (after culling to a litter size of 10), 7, 14 and 21 d. At weaning, 20 pups (10 pups per mother group) were sacrificed and 20 offspring from each gestational group were assigned to the RV diet. At 14 wk post-weaning, 10 rats from each gestational group were sacrificed. The remaining 10 rats per group were maintained to 28 wk post-weaning. Post-weaning BW was measured weekly, and 24-h food intake was measured every 3 wk to 28 wk post-weaning. OGTT was performed every 4 to 5 wk to 28 wk post-weaning.

A 1-h food intake assessment was conducted every 4 wk to 20 wk post-weaning. After a 10-h overnight fast, rats were randomly assigned to be gavaged with either a glucose preload (0.375 g glucose / ml, 5 grams of glucose / kg body weight) or a water preload. 30
min after the gavage, food intake was measured for 1 h. After a washout day, rats were again fasted for 10 h overnight and gavaged with the opposite preload, 30 min after the gavage, food intake was measured for 1 h.

Systolic (SBP) and diastolic blood pressure (DBP) (mmHg) were measured at 24 and 28 weeks post-weaning by a non-invasive, light-based indirect blood pressure monitor (The BP-2000 Series II blood pressure monitor, Visitech Systems Inc. Apex, NC) [237]. Rats were acclimatized daily to the device, beginning 5 days prior to the measurement. On the day of measurement, between 1000h to 1300h, 5 mock measurements preceded a series of 10 measurements, of which only the latter were averaged to produce the blood pressure values.

At weaning, 14 wk and 28 wk post-weaning, plasma samples were collected. Plasma glucose was measured from trunk blood obtained immediately via the neck opening upon decapitation using a plasma-compatible glucose oxidase kit (Bayers Ascensia Elite XL). Plasma insulin, ghrelin (total) and corticosterone were determined using radioimmunoassay kits (Catalogue # RI-13K and GHRT-89HK purchased from Linco Research, St. Charles, MO; and Catalogue # 07-120103 purchased from MP Biomedicals, Orangeburg, NY). Active GLP-1 concentrations in plasma was determined using an enzyme-linked immunosorbent assay (Catalogue # EGLP-35K purchased from Linco Research, St. Charles, MO).

**Statistical analysis.** To analyze the individual times of measurement, cumulative food intake was calculated as the sum of the five 24-h food intake measurements. For the 1-h food intake assessments, the food intake after the water and glucose preload was analyzed, as well as the difference (delta) in food intake after preloads. For the OGTT, blood glucose response was calculated as the net incremental area under the curve (iAUC) of the blood
glucose concentration from 0 (fasting) to 60 min after the glucose gavage. Insulin resistance index was calculated as fasting glucose multiplied by fasting insulin [238]. In Experiment 2, weaning to 14 wk post-weaning, two male pups from the same dam were included in each group for a total of 20 pups per group. However, the BW of pups born to the same dam were averaged to obtain one value for the litter as recommended [239].

The treatment effects on the BW, 24-h and short term food intake, glucose response, SBP and DBP in the offspring were analyzed using the PROC MIXED MODEL procedure in SAS (Version 9.1, SAS Institute Inc., Carey, NC, USA) with gestational diets and age (post-weaning period) as main factors in both experiments. In addition, the unpaired t-test was used to compare the means for the dependent measures at each of the post-weaning time points. Cumulative 24-h food intake, fasting glucose, insulin, ghrelin, corticosterone, GLP-1, and FPM between the groups were analyzed by unpaired t-test. Significance of difference was considered if P < 0.05. All data are expressed as means ± SEM.

4.4 RESULTS

Experiment 1: Effect of multi-vitamin supplementation during pregnancy on body weight, food intake and glucose tolerance of the offspring to 12 weeks post-weaning. Gestational diet did not affect litter size at birth (RV = 13.2 ± 0.5 vs. HV = 13.0 ± 0.5 pups per litter), but at weaning, males from the HV dams had 4% lower BW (69.5 ± 1.0 vs. 72.1 ± 0.9 g; P < 0.05) compared to those from the RV dams. Similarly, at weaning, females from the HV dams had 4% lower BW (63.9 ± 1.0 vs. 66.8 ± 1.3 g; P < 0.05). Gestational diet (P < 0.05) and age (P < 0.0001) affected post-weaning BW in male offspring,
along with a significant gestational diet x age interaction (P < 0.01) (Figure 4.1a). Beginning at 10 wk post-weaning, BW of males from HV dams was higher (P < 0.05), and at 12 wk post-weaning, they weighed 6% more compared to those from the RV dams (559.7 ± 13.9 vs. 530.6 ± 7.4 g; P < 0.05). Age (P < 0.0001), but not gestational diet, affected post-weaning BW in female offspring (Figure 4.1b).

Gestational diet (P < 0.05) and age (P < 0.0005) affected 24-h food intake in male offspring, but there was no gestational diet x age interaction (Figure 4.2a). 24-h food intake was higher in males from dams fed the HV diet at 1 wk (16.7 ± 0.9 vs. 13.3 ± 0.5 g; P < 0.05) and 12 wk (31.4 ± 1.1 vs. 27.7 ± 1.3 g; P < 0.05) post-weaning. Males from HV dams had a 10% higher cumulative 24-h food intake from weaning to 12 wk post-weaning (138.1 ± 3.1 vs. 125.5 ± 3.7 g; P < 0.05). Age (P < 0.0001), but not gestational diet, was a significant factor in 24-h food intake in female offspring (Figure 4.2b). Cumulative 24-h food intake from weaning to 12 wk post-weaning was not statistically different in females from RV or HV dams (117.2 ± 4.8 vs. 121.3 ± 3.7 g).

A significant interaction between gestational diet and age was observed for post-weaning blood glucose response (P < 0.01) (Table 4.1) in the male offspring, but not female offspring. At 9 weeks post-weaning, blood glucose response (iAUC) was 69% higher during the OGTT in males from HV dams compared to those from RV dams (P < 0.05), and at 12 wk post-weaning, FPM was 18% heavier in the males from HV dams compared to those from RV dams (P < 0.05) (Table 4.2).

Experiment 2: Effect of multi-vitamin supplementation during pregnancy on the regulation of food intake and the appearance of components of the metabolic syndrome
in male offspring to 28 weeks post-weaning. Gestational diet did not affect litter size (RV = 13.0 ± 0.6 vs. HV = 13.5 ± 0.5 pups per litter) or BW at birth of the offspring from RV and HV dams (8.3 ± 0.3 vs. 8.3 ± 0.2 g, N = 10 means / dam group). Gestational diet (P < 0.01) and age (P < 0.0001) affected post-weaning BW in male offspring, along with a significant gestational diet x age interaction (P < 0.05) (Figure 4.1c). BW of the male pups from HV dams was 5% lower compared to those from RV dams at weaning (68.2 ± 1.0 vs. 72.2 ± 1.6 g; P < 0.05) and 2 wk (168.7 ± 2.0 vs. 181.0 ± 2.1 g; P < 0.01) post-weaning. Beginning at 11 wk post-weaning, BW of the offspring from the dams fed the HV diet was higher than that from dams fed the RV diet (P < 0.05), and at 28 wk post-weaning, they weighed 8% more (782.4 ± 14.0 vs. 725.0 ± 19.7 g; P < 0.05) (Figure 4.1c).

Gestational diet (P < 0.05) and age (P < 0.0005) affected 24-h food intake in male offspring, but there was no gestational diet x age interaction (Figure 4.2c). 24-h food intake was higher at 1 wk (15.4 ± 0.4 vs. 13.4 ± 0.4 g; P < 0.01), 12 wk (32.3 ± 1.1 vs. 30.0 ± 0.8 g; P = 0.07), 18 wk (35.2 ± 1.1 vs. 31.7 ± 0.9 g; P < 0.05), and 28 wk (30.8 ± 1.0 vs. 28.2 ± 1.1 g; P < 0.05) post-weaning in the male offspring from HV dams compared to those from RV dams. Males from HV dams had a 7% higher cumulative 24-h food intake from weaning to 28 wk post-weaning (292.1 ± 5.0 vs. 272.3 ± 4.8 g; P < 0.01).

Both gestational diet (P < 0.01) and age (P < 0.05) affected 1-h food intake after glucose preloads, but there was no interaction (P = 0.08) (Table 4.3). Rats from the HV dams ate more after the glucose preloads at 4 wk (P < 0.05) and 12 wk (P < 0.05) post-weaning. Although the response to the glucose preload, as shown by the delta, was not affected by gestational diet nor age, and there was no interaction, post-hoc analysis at 20 weeks post-weaning by unpaired t-test indicated a greater response to the glucose load,
perhaps due to the higher intake in the males from HV dams after the water preload. The 1-h food intake after the glucose preload was lower than after the water preload in both groups (P < 0.005).

Gestational diet did not affect the fasting glucose and insulin concentration at weaning (Table 4.4). However, fasting ghrelin and GLP-1 of the offspring from HV dams were 27% (P < 0.05) and 32% (P < 0.05) higher, respectively. At 14 wk post-weaning, FPM (Table 2), fasting glucose, insulin, insulin resistance index and ghrelin were 27% (P < 0.05), 11% (P < 0.05), 62% (P = 0.07), 80% (P < 0.05) and 41% (P < 0.005) higher (Table 4.4). At 28 wk post-weaning, fasting ghrelin of the offspring from HV dams was 32% (P < 0.005) lower. No difference was observed for fasting corticosterone level at 0, 14 and 28 wk post-weaning, and no difference for fasting insulin and insulin resistance index at 28 wk post-weaning.

Gestational diet (P < 0.01) and post-weaning age (P < 0.001) interacted (P < 0.01) in their effect on the blood glucose response during the OGTT (Table 4.1). Response decreased with age in the males from RV dams, but not from the HV dams. At 23 and 28 wk post-weaning, blood glucose response (iAUC) of the male offspring from HV dams was 46% (P < 0.05) and 47% (P < 0.05) higher (Table 4.1). Maternal diet (P < 0.05), but not age, affected systolic blood pressure (Table 4.5). At 24 and 28 wk post-weaning, SBP of the male offspring from HV dams was 5% (P < 0.05) and 8% higher (P < 0.05) respectively.

4.5 DISCUSSION

The results of this study show that high multi-vitamin intakes during pregnancy by Wistar rats resulted in increased food intake, body weight, fat pad mass, insulin resistance and elevated blood pressure in the male offspring fed a diet that is not known to be
obesogenic. Fetal programming of obesity and metabolic disease during gestational nutrient deprivation is well recognized [73, 76, 240, 241], but these results are the first to show that vitamins alone, albeit at high levels during pregnancy, potentially program the offspring for increased food intake, obesity and metabolic disease. Furthermore, it can be suggested that the phenotype was driven by \textit{in utero} programming of the regulation of intake control, predisposing the offspring to eat excess food.

The cause of the increase in body weight and the expression of components of the metabolic syndrome may be due to the effect of the high vitamin diet on the early development of intake regulatory mechanisms in the central nervous system (CNS). High multi-vitamin intake during pregnancy produced male rats that were hyperphagic in early life as shown by the higher 24-h food intake at 1 wk post-weaning in both experiments. Furthermore, higher fasting ghrelin and GLP-1 levels were found at weaning. Ghrelin is an orexigenic hormone [161] and its higher concentration in blood at weaning suggests that offspring from high vitamin dams may be programmed to increase food intake from an early age. Fasting concentrations of GLP-1, an anorexigenic hormone [161], were also higher in the offspring of dams fed the high vitamin diet at weaning.

The effect of age on the fasting plasma concentration of ghrelin and GLP-1 suggests that compensatory mechanisms were occurring that would prevent further hyperphagia. The concept of compensatory adaptation favoring reduced energy intake in hyperinsulinemia has been observed. Like the offspring from the high vitamin mothers, men with elevated insulin and body mass index reduced food intake more after a glucose preload than normal insulinemic controls [242]. Thus the development of insulin resistance in the high vitamin
offspring may have been a factor in their stronger response to the glucose preload (Table 4.3),
the normalization of GLP-1 and reduction of ghrelin at 28 wk post-weaning (Table 4.4).

The effect of the high vitamin diet on the phenotype of the offspring is unlikely to be
attributable to a stress response of the dams. First, there was no difference in litter size and
birth weight of the offspring. We chose not to involve close monitoring of the dams,
including taking blood for measurement of plasma corticosterone, because this would add
stress to the dams and unborn fetus. Restraining the dams only once during late pregnancy
and tail pricking for a blood sample has been shown to elevate corticosterone concentration in
the offspring [146]. However, we have recorded the body weight of the dams one day before
delivery and one day after delivery in subsequent experiments, and have found no evidence
that they are avoiding or are stressed by the high vitamin diet. The body weight of the dams
on the control diet and high vitamin diets are not different one day before delivery (369.3 ±
8.7 vs. 367.7 ± 11.1 g) nor one day after delivery (287.5 ± 7.9 vs. 289.7 ± 8.8 g).

The additions of 8 of the 12 required vitamins in the high vitamin diet were on
average many times lower than that reported to cause adverse effects on the fetus, although it
must be recognized that the amounts have not been fully quantified [220, 234]. Folate at 10
times the recommended diet was estimated to be closest to an adverse effect level [234].
Folate addition at 20 times that in the AIN-93G diet (2 mg/kg) and fed during pregnancy to
Wistar rats led to lower birth weight but not litter size [116]. Moreover, the high vitamin diet
was much lower in vitamins involved in methyl group metabolism than those used in
previous studies showing their epigenetic effects in the viable yellow Agouti mouse. Folate,
vitamin B_{12}, and choline fed at 9, 60, and 9 times the control diet during pregnancy and
lactation were not reported to lead to affects on litter size or weaning weights [42, 130].
An explanation for the lower body weight of the pups at weaning cannot be provided at present, but the similar fasting plasma corticosterone of the two groups at weaning suggests that stress, either arising from the gestational diet or nursing behavior of the mother, was not a factor. Dams that are stressed provide less nursing or access to the milk to the pups. Pups that receive less grooming, licking, and arched-back nursing from the dams during lactation have excess hypothalamic-pituitary-adrenal responses to stress as early as 7 days after birth [243]. However, it is possible that milk volume or composition was in some way affected, or that suckling and intake control of the offspring was a factor.

The increased post-weaning food intake may support the predictive adaptive response hypothesis, which assumes that in utero adaptive responses prepare the offspring for survival in a nutrient environment similar to the mothers [32, 244]. Based on the predictive adaptive response hypothesis [25, 32], it would be predicted that the high multi-vitamin gestational diet would program the offspring to require a high vitamin postnatal environment. There is evidence that rats have nutrient selective appetite that leads to increased food intake of the diets with marginal nutrient [245]. If this is an explanation then it would be predicted that by allowing the offspring access to a high vitamin diet similar in vitamin content to their mothers, the overeating and development of the components of the metabolic syndrome would be ameliorated.

The absence of effect of gestational diet on the body weight and food intake of female offspring is consistent with other studies showing that the effect of gestational diet is dependent on the gender of the offspring [246]. Male offspring are more susceptible to changes in gestational nutrition [145, 247], possibly due to their faster growth rate compared to females, and thus, more critical nutrient needs [22]. Due to the absence of phenotypic
changes in Experiment 1, the focus was on male offspring in Experiment 2. Because females have been shown to develop insulin resistance later in life compared to males [248], and a previously formulated obesogenic diet has been proved to accelerate the development of insulin resistance [249, 250], the effect of weaning the female offspring to the obesogenic diet needs to be explored in future studies.

The mechanism by which the high multi-vitamin gestational diet modified the phenotype has not been defined. However, placental transfer of vitamins would be expected to result in elevated concentrations in the developing fetus, and result in epigenetic modification of gene expression. Increased cytosine methylation of the A^v^ gene has been found after pregnant mice were fed high dietary levels of methyl donors (betaine and choline) or methyl-metabolism cofactors (folate and Vitamin B_{12}) [214], which led to a change in the coat colour of the offspring [42, 130]. In addition, the higher intake of fat-soluble vitamins not known to be involved in gene methylation process may also contribute to the observations. Both vitamin A and D during pregnancy affect gene expression in the offspring [251, 252]. Vitamin A, in the form of 9-cis retinoic acid, binds to retinoic X receptors, which allows for interaction with retinoic acid receptors and response elements present on particular genes [253]. For example, retinoic acid has been shown to stimulate the expression of 11β-HSD2 in a trophoblast-like cell line that displays a number of functional similarities to the placental syncytiotrophoblast [254]. 11β-HSD2 is a crucial enzyme that metabolizes glucocorticoids transported to the placenta from the maternal side, and a lower 11β-HSD2 activity in the placenta due to stress can lead to higher exposure of the glucocorticoid to the fetus, and consequently alter the development of the fetal HPA axis [255-257]. Gestational vitamin D status also modulates gene expression. Vitamin D acts like a steroid hormone and
interacts with both cell membrane receptors and nuclear vitamin D receptor proteins to affect
gene transcription, such as the insulin-like growth factor binding protein (IGFBP), which
affects cell differentiation, proliferation and mineral homeostasis in the offspring [252, 258].

It is also possible that the effect on the pups of feeding high amounts of fat-soluble
vitamins to the dams during pregnancy is not limited to the pregnancy period. Fat-soluble, in
contrast to water-soluble, vitamins are stored in liver and adipose tissue [259-262]. Mobilization and transfer through the milk to the pups in the early stage of lactation may have
occurred. As well the pups may have accumulated stores of the fat-soluble vitamins in the
liver and in their very limited fat stores. This may be of significance because the newborn rat
continues to undergo significant developmental changes [263, 264].

The past few decades have provided pregnant women an unparalleled opportunity to
obtain an intake of vitamins in amounts that may not have been predicted from evolution.
The effect of excess vitamin intakes by pregnant women on genetic/epigenetic changes that
adversely affect the adaptability of the offspring to their environment remains to be
determined, but there are several reasons to be concerned. First, there is a potential for
pregnant women who select a diet of foods that are rich natural sources and those that contain
added vitamins, and also consume vitamin supplements to have a chronic consumption of
vitamins at the levels above the daily upper intake levels set by the National Academy of
Sciences [233]. The high vitamin intakes at several times the requirement during pregnancy,
as found in a recent survey [111], can be easily explained. Multi-vitamin supplements
targeted to women during pregnancy contain vitamins that exceed the daily recommended
intakes [11, 17]. Second, in addition to an abundance of vitamin supplements, many foods
and beverages consumed during pregnancy contain added nutrients and the amounts are likely
underestimated, because by law, manufacturers must provide at least the labeled amounts of vitamins. Thus higher amounts are probable as manufacturers provide overage to be sure of meeting label specifications [265]. Third and of further concern are recent recommendations to increase intakes of specific vitamins during pregnancy. Motherrisk (Toronto) has proposed increasing the supplement dose of folic acid from 400 ug to 5 mg daily (12 times the RDA) for women of childbearing years and/or pregnant women. Adequate intake of Vitamin D for women 19 to 50 years is 200 IU, but the Canadian Cancer Society is now recommending 1000 IU. Much higher intakes (4000 IU) are being suggested as needed during pregnancy based on 25-hydroxy D status in plasma [266]. Finally, and relevant to the current study, consumption of a prenatal vitamin pill has been shown to produce a higher blood glucose response upon an oral sucrose load than consumption of a placebo pill in women with gestational diabetes [267]. Gestational glycemic control affects gestational outcomes in both normal and diabetic pregnant women [268, 269].

4.6 CONCLUSION

In conclusion, high multi-vitamin intake during pregnancy programmed the male offspring for the development of the components of the metabolic syndrome in adulthood, possibly by its effects on central mechanisms of food intake control.
TABLE 4.1

Blood glucose response (incremental area under the curve) during the oral glucose tolerance test in the post-weaning period\(^1\)

<table>
<thead>
<tr>
<th>Week</th>
<th>Experiment 1 (males)</th>
<th>RV</th>
<th>HV</th>
<th>PROC MIXED(^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>151 ± 12</td>
<td>169 ± 15</td>
<td></td>
<td>GD: NS</td>
</tr>
<tr>
<td>3</td>
<td>130 ± 10</td>
<td>117 ± 16</td>
<td></td>
<td>Age: NS</td>
</tr>
<tr>
<td>6</td>
<td>98 ± 16</td>
<td>97 ± 14</td>
<td></td>
<td>Interaction: P &lt; 0.01</td>
</tr>
<tr>
<td>9</td>
<td>95 ± 8</td>
<td>161 ± 16*</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Week</th>
<th>Experiment 1 (females)</th>
<th>RV</th>
<th>HV</th>
<th>PROC MIXED(^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>190 ± 16</td>
<td>164 ± 18</td>
<td></td>
<td>GD: NS</td>
</tr>
<tr>
<td>3</td>
<td>116 ± 13</td>
<td>117 ± 12</td>
<td></td>
<td>Age: P &lt; 0.01</td>
</tr>
<tr>
<td>6</td>
<td>109 ± 13</td>
<td>140 ± 15</td>
<td></td>
<td>Interaction: NS</td>
</tr>
<tr>
<td>9</td>
<td>100 ± 9</td>
<td>99 ± 16</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Week</th>
<th>Experiment 2 (males)</th>
<th>RV</th>
<th>HV</th>
<th>PROC MIXED(^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>155 ± 12</td>
<td>132 ± 11</td>
<td></td>
<td>GD: P &lt; 0.01</td>
</tr>
<tr>
<td>4</td>
<td>144 ± 11</td>
<td>149 ± 22</td>
<td></td>
<td>Age: P &lt; 0.001</td>
</tr>
<tr>
<td>9</td>
<td>126 ± 15</td>
<td>149 ± 11</td>
<td></td>
<td>Interaction: P &lt; 0.01</td>
</tr>
<tr>
<td>13</td>
<td>112 ± 14</td>
<td>108 ± 10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>108 ± 10</td>
<td>126 ± 14</td>
<td></td>
<td></td>
</tr>
<tr>
<td>23</td>
<td>95 ± 14</td>
<td>139 ± 15*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>28</td>
<td>96 ± 9</td>
<td>142 ± 18*</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^1\) Data are means ± SEM; N = 8 – 10 / group in Exp 1. N = 8 – 10 means / group in Exp 2 from Week 1 to Week 13 (each mean is the average blood glucose response of 2 pups from the same litter). At 14 wk post-weaning, one rat from each litter was sacrificed, leaving N = 10 / group from Week 18 to Week 28;

\(^2\) Data were analyzed using the PROC MIXED procedure with gestational diet and age (week) as main factors in each experiment;

* P < 0.05 by unpaired t-test at each age point (week);

RV, recommended vitamin diet; HV, high vitamin diet; iAUC, incremental area under the curve; GD, Gestational diet; NS, Not significant;
**TABLE 4.2**

*Fat pad mass at 12 weeks (Exp 1) and 14 weeks (Exp 2) post-weaning*

| Gestational Diet |  
|------------------|---
| RV | HV |

<table>
<thead>
<tr>
<th></th>
<th>g</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Experiment 1 (males)</strong></td>
<td></td>
</tr>
<tr>
<td>Body weight</td>
<td>530.6 ± 7.4</td>
</tr>
<tr>
<td>Fat pad mass(^2)</td>
<td>29.4 ± 2.0</td>
</tr>
<tr>
<td></td>
<td>559.7 ± 13.3*</td>
</tr>
<tr>
<td></td>
<td>34.7 ± 1.6*</td>
</tr>
<tr>
<td><strong>Experiment 1 (females)</strong></td>
<td></td>
</tr>
<tr>
<td>Body weight</td>
<td>330.4 ± 8.4</td>
</tr>
<tr>
<td>Fat pad mass</td>
<td>18.9 ± 2.0</td>
</tr>
<tr>
<td></td>
<td>315.2 ± 6.7</td>
</tr>
<tr>
<td></td>
<td>15.6 ± 1.0</td>
</tr>
<tr>
<td><strong>Experiment 2 (males)</strong></td>
<td></td>
</tr>
<tr>
<td>Body weight</td>
<td>564.2 ± 11.3</td>
</tr>
<tr>
<td>Fat pad mass</td>
<td>37.7 ± 3.9</td>
</tr>
<tr>
<td></td>
<td>607.0 ± 7.7*</td>
</tr>
<tr>
<td></td>
<td>48.0 ± 2.9*</td>
</tr>
</tbody>
</table>

\(^1\) Data are means ± SEM; N = 9 – 10/group. * P < 0.05 by unpaired t-test;

\(^2\) Fat pad mass, in grams, was determined by the sum of the abdominal, epididymal (only in males), and peri-renal fat pads;

RV, recommended vitamin diet; HV, high vitamin diet;
TABLE 4.3

Experiment 2: Preload effects on food intake (1 hour) after an overnight fast\(^1,4\)

<table>
<thead>
<tr>
<th>Gestational Diet</th>
<th>Water preload</th>
<th>Glucose preload(^2,5)</th>
<th>Delta(^7)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RV</td>
<td>HV</td>
<td>RV</td>
</tr>
<tr>
<td>Week</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>3.7±0.3</td>
<td>4.0±0.3</td>
<td>2.9±0.3</td>
</tr>
<tr>
<td>4</td>
<td>6.4±0.4</td>
<td>7.3±0.6</td>
<td>3.6±0.3</td>
</tr>
<tr>
<td>8</td>
<td>5.9±0.6</td>
<td>6.1±0.6</td>
<td>2.7±0.3</td>
</tr>
<tr>
<td>12</td>
<td>6.2±0.4</td>
<td>7.2±0.9</td>
<td>2.1±0.3</td>
</tr>
<tr>
<td>16</td>
<td>5.3±0.2</td>
<td>5.7±0.5</td>
<td>2.2±0.3</td>
</tr>
<tr>
<td>20</td>
<td>3.7±0.4</td>
<td>5.0±0.4*</td>
<td>1.5±0.1</td>
</tr>
</tbody>
</table>

GD \(P = 0.08\)                          P < 0.05 | NS
Age NS | P < 0.05 | NS
Interaction NS | P = 0.08 | NS

\(^1\) Data are means ± SEM; N = 8 – 10 means / group from Week 1 to Week 12 (each mean is the average food intake of 2 pups from the same litter). At 14 wk post-weaning, one rat from each litter was sacrificed, leaving N = 10 / group in Week 16 and 20;

\(^2\) Glucose was given by gavage (0.375 g glucose / ml) at 5 g / kg body weight, and water was given in the same volume as the glucose gavage;

\(^3\) Delta is calculated as: (1-h food intake after water preload) – (1-h food intake after glucose preload);

\(^4\) Food intake after water and glucose preloads and the delta were analyzed using the PROC MIXED procedure with gestational diet and age (week) as main factors. Preload effects (water and glucose) were compared using the PROC MIXED procedure with preload treatment and gestational diet as main factors. * P < 0.05 by unpaired t-test within the same week and preload;

\(^5\) The 1-h food intake after the glucose preload was lower than after the water preload in both groups (P < 0.005);

RV, recommended vitamin diet; HV, high vitamin diet; GD, Gestational diet; NS, Not significant;
TABLE 4.4

Experiment 2: Fasting plasma hormone concentrations at weaning, 14 and 28 weeks post-weaning

<table>
<thead>
<tr>
<th></th>
<th>Gestational Diet</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RV</td>
<td>HV</td>
<td></td>
</tr>
<tr>
<td>Week 0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose, mM</td>
<td>6.7 ± 0.4</td>
<td>6.9 ± 0.4</td>
<td></td>
</tr>
<tr>
<td>Insulin, ng/ml</td>
<td>0.2 ± 0.3</td>
<td>0.2 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>Insulin Resistance Index (^2)</td>
<td>1.4 ± 0.3</td>
<td>1.7 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>Ghrelin, ng/ml</td>
<td>1.5 ± 0.1</td>
<td>1.9 ± 0.1 *</td>
<td></td>
</tr>
<tr>
<td>Corticosterone, ng/ml</td>
<td>430.5 ± 28.3</td>
<td>403.3 ± 65.2</td>
<td></td>
</tr>
<tr>
<td>GLP-1, pM</td>
<td>3.4 ± 0.3</td>
<td>4.5 ± 0.3 *</td>
<td></td>
</tr>
<tr>
<td>Week 14</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose, mM</td>
<td>6.1 ± 0.2</td>
<td>6.8 ± 0.2 *</td>
<td></td>
</tr>
<tr>
<td>Insulin, ng/ml</td>
<td>2.1 ± 0.3</td>
<td>3.4 ± 0.6 †</td>
<td></td>
</tr>
<tr>
<td>Insulin Resistance Index</td>
<td>12.8 ± 1.9</td>
<td>23.0 ± 3.0 *</td>
<td></td>
</tr>
<tr>
<td>Ghrelin, ng/ml</td>
<td>2.2 ± 0.1</td>
<td>3.1 ± 0.2 ***</td>
<td></td>
</tr>
<tr>
<td>Corticosterone, ng/ml</td>
<td>213.7 ± 44.1</td>
<td>208.8 ± 49.5</td>
<td></td>
</tr>
<tr>
<td>GLP-1, pM</td>
<td>4.0 ± 0.3</td>
<td>3.3 ± 0.5</td>
<td></td>
</tr>
<tr>
<td>Week 28</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose, mM</td>
<td>6.1 ± 0.1</td>
<td>6.1 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>Insulin, ng/ml</td>
<td>2.4 ± 0.2</td>
<td>2.4 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>Insulin Resistance Index</td>
<td>14.6 ± 0.9</td>
<td>14.5 ± 1.3</td>
<td></td>
</tr>
<tr>
<td>Ghrelin, ng/ml</td>
<td>3.4 ± 0.1</td>
<td>2.3 ± 0.1 ***</td>
<td></td>
</tr>
<tr>
<td>Corticosterone, ng/ml</td>
<td>381.7 ± 79.2</td>
<td>325.2 ± 62.6</td>
<td></td>
</tr>
<tr>
<td>GLP-1, pM</td>
<td>5.8 ± 0.2</td>
<td>5.8 ± 0.1</td>
<td></td>
</tr>
</tbody>
</table>

1 Data are means ± SEM; N = 8 – 10 / group.

*** P < 0.005, ** P < 0.01, * P < 0.05, † P = 0.07 by unpaired t-test;

2 Insulin resistance index was computed as fasting glucose multiplied by fasting insulin, ng/ml · mM;

RV, recommended vitamin diet; HV, high vitamin diet;
TABLE 4.5

Experiment 2: Blood pressure at 24 weeks and 28 weeks post-weaning\textsuperscript{1,2}

<table>
<thead>
<tr>
<th></th>
<th>Gestational Diet</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RV</td>
<td>HV</td>
<td></td>
</tr>
<tr>
<td>Week 24</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Systolic BP</td>
<td>109.0 ± 6.4</td>
<td>129.9 ± 4.8*</td>
<td></td>
</tr>
<tr>
<td>Diastolic BP</td>
<td>73.5 ± 7.3</td>
<td>90.9 ± 6.4\†</td>
<td></td>
</tr>
<tr>
<td>Week 28</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Systolic BP</td>
<td>109.6 ± 4.0</td>
<td>126.4 ± 4.2&quot;</td>
<td></td>
</tr>
<tr>
<td>Diastolic BP</td>
<td>85.2 ± 7.9</td>
<td>93.5 ± 6.1</td>
<td></td>
</tr>
</tbody>
</table>

\textsuperscript{1} Data are means ± SEM; N = 9 – 10 / group.

\textsuperscript{2} Data were analyzed using the PROC MIXED procedure with gestational diet and age (week) as main factors. Gestational diet (P < 0.05), but not age, affected systolic BP.

\textsuperscript{†} P < 0.08, * P < 0.05 by unpaired t-test at each week;

RV, recommended vitamin diet; HV, high vitamin diet; BP, blood pressure;
Figure 4.1a Experiment 1: Post-weaning body weight of male offspring.

Data are means ± SEM, N = 14 / group. Gestational diet (P < 0.05) and age (P < 0.0001) affected post-weaning body weight, along with a significant gestational diet x age interaction (P < 0.01).

* P < 0.05 by unpaired t-test;

Abbreviations: RV, recommended vitamin diet; HV, high vitamin diet;
**Figure 4.1b** Experiment 1: Post-weaning body weight of female offspring.

Data are means ± SEM, N = 14 / group. Age (P < 0.0001), but not gestational diet with no interaction, led to higher post-weaning body weight.
Figure 4.1c Experiment 2: Post-weaning body weight of male offspring.

Data are means ± SEM; N = 10 means / group from Week 1 to Week 14 (each mean is the average weight of 2 pups from the same litter). At 14 wk post-weaning, one rat from each litter was sacrificed, leaving N = 10 / group from Week 15 to Week 28. Gestational diet (P < 0.01) and age (P < 0.0001) affected post-weaning body weight, along with a significant gestational diet x age interaction (P < 0.05).

* P < 0.05 by unpaired t-test;

Abbreviations: RV, recommended vitamin diet; HV, high vitamin diet;
Figure 4.2a Experiment 1: Post-weaning food intake (24-hour) of male offspring.

Data are means ± SEM; N = 10 / group. Gestational diet (P < 0.05) and age (P < 0.0005) affected post-weaning 24-h food intake, with no significant interaction.

* P < 0.05 by unpaired t-test;

Abbreviations: RV, recommended vitamin diet; HV, high vitamin diet;
Figure 4.2b Experiment 1: Post-weaning food intake (24-hour) of female offspring.

Data are means ± SEM; N = 10 / group. Age (P < 0.0001), but not gestational diet with no interaction, affected post-weaning 24-h food intake.

Abbreviations: RV, recommended vitamin diet; HV, high vitamin diet;
Figure 4.2c Experiment 2: Post-weaning food intake (24-hour) of male offspring.

N = 10 means / group in Exp 2 from Week 1 to 12 (each mean is the average food intake of 2 pups per litter). At 14 wk post-weaning, one rat per litter was sacrificed, leaving N = 10 / group from Week 15 to 28. Gestational diet (P < 0.05) and age (P < 0.0005) affected post-weaning 24-h food intake, with no significant interaction.

** P < 0.01, * P < 0.05, † P < 0.07 by unpaired t-test;

Abbreviations: RV, recommended vitamin diet; HV, high vitamin diet;
CHAPTER 5.

STUDY 2: MULTI-VITAMIN SUPPLEMENTATION OF WISTAR RATS DURING PREGNANCY ACCELERATES THE DEVELOPMENT OF OBESITY IN OFFSPRING FED AN OBESOGENIC DIET
CHAPTER 5. MULTI-VITAMIN SUPPLEMENTATION OF WISTAR RATS DURING PREGNANCY ACCELERATES THE DEVELOPMENT OF OBESITY IN OFFSPRING FED AN OBESOGENIC DIET

Preface:
To address if the obesogenic diet would accelerate the development of obesity and components of the metabolic syndrome in male and female offspring born to dams on the high multi-vitamin diet during pregnancy.

This work was published in the International Journal of Obesity (2009); Mar;33(3): 364 – 372.

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5.1 ABSTRACT

The effect of gestational multivitamin supplementation on the development of obesity in rat offspring fed an obesogenic diet was investigated. Pregnant Wistar rats (n=10/group) were fed the AIN-93G diet with the recommended vitamin content (RV) or a ten-fold increase (high vitamin, HV). At weaning, 10 males and 10 females, from separate dams, and from each gestational diet group were weaned to the liquid obesogenic diet for 48 wk post-weaning. Body weight (BW) was measured weekly, and food intake over 24-h was measured once every 3 wk for 24 wk. Every 4 wk, after an overnight fast, food intake over 1-h was measured 30 min after a gavage of water or glucose. An oral glucose tolerance test (OGTT) was performed every 3-5 wk. Post-weaning fasting glucose, insulin, ghrelin, GLP-1, and systolic blood pressure (SBP) were measured. No difference in BW at birth or litter size was observed. Males and females from HV dams gained 17% (P<0.05) and 37% (P<0.001) more BW at 48 wk post-weaning, and consumed 18% (P=0.07) and 20% (P<0.05) more food, respectively. One-hour food intake after water and glucose preload was 27% (P<0.01) and 34% (P<0.05) higher in males from HV dams. Fasting ghrelin and GLP-1 were 27% and 32% higher in males from HV dams at weaning (P<0.05). Blood glucose response to the OGGT was greater in both males and females from HV dams at 13 wk post-weaning (P<0.05), and the insulin resistance index was 76% and 43% higher in females from HV dams at 14 and 28 wk post-weaning (P<0.05). SBP was 23% and 16% higher at 44 wk post-weaning in male and females (P<0.01). High multivitamin intake during pregnancy increases the phenotypic expression of obesity and components of the metabolic syndrome in both female and male rats fed an obesogenic diet.
5.2 INTRODUCTION

The prevalence of early life onset of obesity has increased worldwide in the past three decades [189, 270]. It is generally agreed that a change in the genome alone over three decades cannot account for the rapid increase in prevalence of obesity, although genetics is a strong determinant of heritability [271]. Change in the genetic background of the population occurs slowly by assertive mating, selection, demographic changes and epigenetic effects [272]. Therefore, environmental factors are believed to be the primary catalyst, including the wide availability and variety of energy dense convenience foods, the predominance of sedentary lifestyles, and unstructured family eating habits [270, 273]. However, an interaction between the fetal genome and maternal nutrition during pregnancy also determines the response of offspring to an obesogenic environment [22, 26, 244]. Current thought is that epigenetic effects of nutrients and other dietary factors (e.g. antioxidants) during embryonic development, but not gene mutations, provide a plausible link between genetic makeup and susceptibility to developing chronic diseases [214].

Concurrent with the increased prevalence of obesity and the metabolic syndrome over the past thirty years have been several changes in dietary patterns, including increased intakes of energy, polyunsaturated and trans-fatty acids, sugars and rapidly digested carbohydrates, and bioactive components such as vitamins and minerals through supplementation and fortification of foods [11, 270]. The effect of these changes in maternal diet on the development of the offspring and risk for chronic diseases has received little investigation.

The focus of our investigations is on vitamins for several reasons. First, food fortification policies, discretionary additions of vitamins to foods and recommendations for vitamin intake during pregnancy has led to concern that for some women intakes exceed the
upper limits of intake set by the National Academy of Sciences [233]. Furthermore, this increased availability of vitamins in the maternal diet has occurred concurrently with the obesity epidemic [4, 11]. Second, vitamins involved in methylation reactions are known to alter gene expression through epigenetic modification [130, 274]. Diets containing high levels of the methyl donors, choline and betaine, and methyl metabolism cofactors, folate and vitamin B\textsubscript{12}, and fed from before pregnancy to the end of lactation to inbred mice expressing the viable yellow Agouti [42] or kinky tail genes [275] resulted in increased methylation and suppression of the gene defect. The suppression of expression of the Agouti gene (A\textsuperscript{Vy}) results in a greater number of the litters being born with the dark, rather than the yellow coat colour, and with the lean rather than the obese phenotype [42]. In contrast to the suppression of obesity in the Agouti model, we found that in an outbred rat model, which may be more relevant to human populations, feeding pregnant Wistar rats a high multivitamin diet (10 fold the control) led to male but not the female offspring with higher body weights and expressing components of the metabolic syndrome when fed the AIN-93G powder diet [150]. Third, because susceptibility to obesity is attributed to the obesogenic environment, we explored further the effect of the high multivitamin gestational diet on the expression of the obesity phenotype in the Wistar rat offspring when exposed to an obesogenic diet. We hypothesized that a liquid obesogenic diet would accentuate the effect of multivitamin supplementation of the gestational diet on the phenotypic expression of obesity in both male and female offspring of the Wistar rat.
5.3 MATERIALS AND METHODS

**Animals and Diets.** First-time pregnant Wistar rats were purchased from Charles River Inc., (Montreal, QC, Canada). Upon arrival, at 3 d of pregnancy, they were housed individually in ventilated plastic transparent cages with bedding in a 12:12 h light-dark cycle (lights on at 0600h), at a temperature of 22 ± 1 °C. The pregnant rats had free access to water by an automated water system. The University of Toronto Animal Care Committee approved the protocols and maintenance of the animals conforming to the guidelines of the Canadian Council on Animal Care.

From 3 d of pregnancy to term, dams were fed the AIN-93G diet [276] containing either the recommended vitamin (RV) level or 10 times higher vitamin (HV, high vitamin) content by addition of the AIN-93 vitamin mix. The composition (in g/kg) for the AIN-93G diet was cornstarch (529.4), high-protein casein (200), sucrose (100.1), soybean oil (70), cellulose (50), vitamin mixture (10), mineral mixture (35), choline bitartrate (2.5), and tertbutyl hydroquinone (0.014). Cornstarch, casein (87%), and cellulose were purchased from Harlan Teklad (Madison, WI). The vitamin mixture, mineral mixture, choline bitartrate, and tertbutyl hydroquinone were purchased from Dyets (Bethlehem, PA), whereas sucrose and soybean oil were purchased from local suppliers in Toronto, Canada (Allied Food Service and Loblaws, respectively). Each 10 grams of vitamin mix contains 9.75 grams of sucrose as a carrier. Hence, in the HV diet, the added sucrose was reduced to 9.5 grams to adjust for the 97.5 grams addition from the vitamin mix. The vitamin additions used in the HV diet were well below the level expected to have teratogenic effect and half the lowest intake of vitamins known to have an adverse effect in rats [116, 234]. During lactation, all dams were fed the
RV diet. At weaning (21 days after birth), pups were housed individually in ventilated transparent plastic cages with bedding, and fed the liquid obesogenic diet [249]. The obesogenic diet is a blended mixture of 330 grams of the AIN-93G diet, 330 grams of condensed milk (Loblaws, ON), 70 grams of sucrose (Allied Food Services, ON), and 270 grams of water. The macronutrient breakdown (in g/kg) for the obesogenic diet is carbohydrate (728.4), protein (147.3), and fat (78.9). The sources of fat are from soybean oil and condensed milk. The forms of fat (% of total) are saturated fatty acid (35.1), mono-unsaturated fatty acid (24.6), and poly-unsaturated fatty acid (40.4). The energy density of the obesogenic diet is 2.7 kcal/g wet weight (or 4.5 kcal/g dry weight). The diet was made fresh every 2 days and glass jars were washed every 2 days. The obesogenic diet induces over-eating in rats due to its high palatability [249, 277]. All gestational and pup diets were provided ad libitum.

**Design.** Two groups of pregnant female rats (n = 10 / group) were fed either the RV or HV diets from d 3 of pregnancy until labour. At birth, each litter was culled to 10 pups in order to minimize the difference in milk availability. Litters of less than 10 pups were excluded. During lactation, the dams were fed only the RV diet. At weaning, 10 male and 10 female rats per gestational diet group were killed, and 20 males and 20 females from each gestational group were assigned to the obesogenic diet. At 14 wk post-weaning, 10 males and 10 females from each gestational group were killed. The remaining 10 males and 10 females per gestational group were maintained to 48 wk post-weaning. Thus, 10 pups per sex (1 pup per dam) were formed for each dependent measures as recommended previously [239].
Body weight (BW) was measured at birth, and weekly from 0 to 48 wk post-weaning. Twenty-four hour food intake was measured once every 3 wk to 24 wk post-weaning. A 1-h food intake assessment was conducted every 4 wk to 20 wk post-weaning. After a 10-h overnight fast, rats were randomly assigned to be gavaged with either a glucose preload (0.375 g glucose / ml, 5 grams of glucose / kg body weight) or a water preload. 30 min after the gavage, food intake was measured for 1 h. After a washout day, rats were again fasted for 10 h overnight and gavaged with the opposite preload, 30 min after the gavage, food intake was measured for 1 h.

Oral glucose tolerance test (OGTT) was performed at 0, 4, 9, 13 and 18 wk post-weaning. Rats were fasted overnight for 10 hours before the OGTT. At the beginning of OGTT, a blood sample was withdrawn from the capillary bed of the tail tip and baseline glucose was immediately assayed using a commercial glucometer (MediSense Precision Xtra). The rats were then gavaged (0.375 g glucose / ml, 5 grams of glucose / kg body weight), and blood glucose concentrations were determined 15, 30 and 60 minutes later.

Systolic blood pressure (SBP, mmHg) was measured at 24, 28 and 44 weeks post-weaning by a non-invasive, light-based indirect blood pressure monitor (The BP-2000 Series II blood pressure monitor, Visitech Systems Inc. Apex, NC) [237]. Rats were acclimatized daily to the device, beginning 5 days prior to the measurement. On the day of measurement, between 1000h to 1300h, 5 mock measurements preceded a series of 10 measurements, of which only the latter were averaged to produce the blood pressure values.

At weaning and 14 wk post-weaning, plasma samples were collected from trunk blood obtained immediately at the neck opening upon decapitation. At 28 wk post-weaning, blood sample was withdrawn from the capillary bed of the tail tip. Plasma glucose was measured
using a plasma-compatible glucose oxidase kit (Bayers Ascensia Elite XL). Plasma insulin, ghrelin (total) and corticosterone were determined using radioimmunoassay kits (Catalogue # RI-13K and GHRT-89HK purchased from Linco Research, St. Charles, MO; and Catalogue # 07-120103 purchased from MP Biomedicals, Orangeburg, NY). Active GLP-1 concentrations in plasma was determined using an enzyme-linked immunosorbent assay (Catalogue # EGLP-35K purchased from Linco Research, St. Charles, MO).

**Statistical analysis.** For the 1-h food intake assessments, the total food intake after the water and glucose preload was analyzed, as well as the difference (delta) in food intake between the water and glucose preloads. For the OGTT, blood glucose response was calculated as the net incremental area under the curve (iAUC) of the blood glucose concentration from 0 (fasting) to 60 min after the glucose gavage. Insulin resistance index was calculated as fasting glucose multiplied by fasting insulin [238]. At weaning to 14 wk post-weaning, two male pups from the same dam were included in each group for a total of 20 pups per group. However, the BW of pups born to the same dam were averaged to obtain only one value for each litter in recognition of the reduced variance within litter compared with between litters [239].

The treatment effects on the BW, 24-h and short term food intake, glucose response and SBP in the offspring were analyzed using the PROC MIXED MODEL procedure in SAS (Version 9.1, SAS Institute Inc., Carey, NC, USA) with gestational diets and age (post-weaning period) as main factors in both experiments. In addition, the unpaired t-test was used to compare the means for the dependent measures at each of the post-weaning time points. Fasting glucose, insulin, ghrelin, corticosterone, and GLP-1 between the groups were
analyzed by unpaired t-test. Significance of difference was considered if P < 0.05. All data are expressed as means ± SE.

5.4 RESULTS

No difference was observed in litter size (13.0 ± 0.6 vs. 13.5 ± 0.5 pups per litter in RV and HV groups, respectively) or body weight at birth (8.3 ± 0.3 vs. 8.3 ± 0.2 g, n = 10 means per dam group). In male offspring, gestational diet (P < 0.01) and age (P < 0.0001) affected post-weaning body weight, and there was a significant gestational diet x age interaction (P < 0.05) because the effect of the gestational diet was greater with increasing age of the offspring (Figure 5.1a). At 12 wk post-weaning, male offspring from the HV dams were found to be 6% heavier (658.3 ± 11.2 vs. 624.2 ± 12.2 g; P < 0.05), and at 48 weeks, 17% heavier (1309.2 ± 72.0 vs. 1118.4 ± 33.9 g; P < 0.05). In female offspring, gestational diet (P < 0.005) and age (P < 0.0001) affected post-weaning body weight, and there was a significant gestational diet x age interaction (P < 0.005) (Figure 5.1b). At 7 wk post-weaning, female offspring from the HV dams were found to be 8% heavier (320.6 ± 6.0 vs. 291.1 ± 5.3 g; P < 0.05), and at 48 weeks, 37% heavier (921.6 ± 52.9 vs. 671.4 ± 34.0 g; P < 0.001).

Post-weaning 24-h food intake was affected in male offspring by gestational diet (P = 0.07) and age (P < 0.0005), but there was no gestational diet x age interaction (Figure 5.2a). At 24 wk post-weaning, male offspring from the HV dams had 22% higher 24-h food intake than those from dams on the RV diet (51.5 ± 3.1 vs. 42.1 ± 2.5 g; P < 0.05). In female offspring, gestational diet (P < 0.05) and age (P < 0.001) also affected post-weaning 24-h food intake, with no gestational diet x age interaction (Figure 5.2b). At 24 wk post-weaning,
female offspring from the HV dams had 34% higher 24-h food intake than those from dams on the RV diet (37.3 ± 3.3 vs. 27.9 ± 1.4 g; P < 0.05).

One-hour food intake after water preloads was affected by gestational diet (P < 0.01) and age (P < 0.01) in male offspring, but there was no interaction (Table 5.1). Males from the HV dams ate more after the water preloads at 1 wk (P < 0.005), 4 wk (P < 0.05), 8 wk (P < 0.05), 12 wk (P < 0.05), and 20 wk (P < 0.05) post-weaning. Gestational diet (P < 0.05), but not age, affected 1-h food intake after glucose preloads. After the glucose preloads, the males from the HV dams ate more at 4 wk (P < 0.005), and 16 wk (P < 0.05) post-weaning. Difference in food intakes after water and glucose preload, as shown by the delta value, was affected by gestational diet (P = 0.07) and age (P < 0.005). Males from the HV dams compensated less to the caloric preload (glucose) compared to those from RV dams. In female offspring, however, gestational diet did not affect 1-h food intake after water or glucose preload (Table 5.2). Females from the HV dams ate more after the glucose preloads at 4 wk (P < 0.05) post-weaning. The 1-h food intake after the glucose preload was lower than after the water preload in both male groups (P < 0.01) and female groups (P < 0.005).

Gestational diet did not affect fasting plasma glucose or insulin concentration in either of the male or female offspring at weaning (Table 5.3 and 5.4). However, fasting ghrelin and GLP-1 of the males from HV dams were 27% (P < 0.05) and 32% (P < 0.05) higher, respectively (Table 5.3). At 14 wk post-weaning, fasting ghrelin and GLP-1 were 29% (P = 0.07) and 43% (P < 0.05) higher, respectively. At 28 wk post-weaning, fasting glucose of the males from HV dams was 11% (P < 0.05) higher, but no difference was observed for fasting insulin and insulin resistance index. In female offspring from HV dams, fasting insulin and the insulin resistance index were 59% (P < 0.05) and 76% (P < 0.05) higher at 14 wk post-
weaning, and 31% (P = 0.06) and 43% (P < 0.05) at 28 wk post-weaning, respectively (Table 5.4). No difference was observed for fasting plasma corticosterone concentration in either male or female offspring at 0, 14 and 28 wk post-weaning.

In male offspring, gestational diet (P < 0.05) and post-weaning age (P < 0.01) interacted (P < 0.05) in their effect on the blood glucose response during the OGTT (Figure 5.3a). Response decreased with age in males from RV dams, but not in those from the HV dams. Blood glucose response (iAUC) at 13 and 18 wk post-weaning of the male offspring from HV dams was 40% (P < 0.05) and 45% (P < 0.05) higher, respectively. In female offspring, gestational diet (P < 0.05) and post-weaning age (P < 0.01) interacted (P < 0.01) in their effect on the blood glucose response during the OGTT (Figure 5.3b). Response decreased with age in females from RV dams, but not those from the HV dams. At 9, 13 and 18 wk post-weaning, iAUC of the female offspring from HV dams was 47% (P < 0.05), 36% (P < 0.05) and 82% (P < 0.05) higher, respectively.

Systolic blood pressure in male offspring was affected by gestational diet (P < 0.05) and age (P < 0.05) (Figure 5.4a). At 24, 28 and 44 wk post-weaning, SBP of the male offspring from HV dams was 19% (P < 0.05), 20% (P < 0.05) and 23% (P < 0.01) higher, respectively. Gestational diet (P < 0.01), but not age, affected SBP in female offspring (Figure 5.4b). At 24, 28 and 44 wk post-weaning, SBP of the female offspring from HV dams was 26% (P < 0.05), 15% (P < 0.05) and 16% (P < 0.01) higher, respectively.

5.5 DISCUSSION

The results of this study show that a high intake of multivitamins during pregnancy in Wistar rats accelerated and exacerbated the effect of an obesogenic diet on the phenotypic
expression of obesity and components of the metabolic syndrome in the offspring. Both male and female offspring born to dams fed the HV diet and weaned to an obesogenic diet exhibited increased body weight, food intake, glucose intolerance and elevated blood pressure.

The importance of the postnatal nutritional environment and sex of the offspring on the expression of the effects of the HV gestational diet is illustrated in the current study. The obesogenic diet used in the post-weaning period was modeled after a previous study that led to the development of obesity and insulin resistance in Sprague-Dawley rats when compared to a control dry diet [249, 277]. Similarly, in this study, the obesogenic diet produced the obesity phenotype in all the offspring, independent of the gestational diets. Compared to a previous study in which the male offspring were weaned to the control AIN-93G diet, a diet not known to cause obesity, the males weaned to the obesogenic diet were 31% heavier (988 g vs. 753 g) at 28 wk post-weaning [150]. Also, compared to the control AIN-93G powder diet, the obesogenic diet led to male offspring with 71% higher fasting plasma insulin (4.1 vs. 2.4 ng/ml), 83% higher insulin resistance index (26.5 vs. 14.5 ng·mM/mL) and 11% higher systolic blood pressure (130 vs. 118 mM) at 28 wk post-weaning, and 41% greater blood glucose response (165 vs. 117 min·mM) at 18 wk post-weaning [150]. In the present study, while the effect of the obesogenic diet was apparent in the rats from RV dams, the effect of the HV gestational diet was amplified. Both male and female offspring were heavier and showed an enhanced increase in components of the metabolic syndrome.

The results in our study are consistent with another study demonstrating that the effect of gestational diet on the phenotypic expression is dependent on the sex of the offspring [246]. The effect of feeding the female offspring the obesogenic diet on increased insulin
resistance, food intake and earlier weight gain is consistent with the expected. Females have been shown to develop insulin resistance later than males in life [248], and obesogenic diets accelerate their development of insulin resistance [249, 250]. In addition, after 16 wk post-weaning, the increase in food intake was associated with insulin resistance in the females but was not in the males. Their increased fasting insulin may also account for the better food intake control as shown by a greater response to the glucose preload compared with the males. Our lab has shown that hyperinsulinemic men exhibited a greater reduction food intake following a glucose load compared to normal insulinemic men [278]. Also, daily food intake was higher at all weeks except at 9 wk post-weaning in the females but only after 12 wk post-weaning in the male offspring, and increased earlier in life in the male rats compared to the females. Another difference between the sexes was shown by the lack of effect of the gestational diet on the magnitude food intake suppression by a glucose load in females, but in males, the HV diet had a less impact on food intake reduction than in those from the RV dams. Males are more susceptible than female offspring to changes in gestational nutrition [145, 247], possibly due to their faster growth rate compared to females [22]. At 13 wk post-weaning, males had higher blood glucose response during OGTT and insulin resistance index, and at 28 wk post-weaning, higher SBP than the females.

The cause of the increase in body weight and the expression of components of the metabolic syndrome may be due to the effect of the high vitamin diet on the early development of intake regulatory mechanisms in the central nervous system. Both male and female rats from the HV dams were hyperphagic in early life as shown by the higher food intake after preloads at 1 wk post-weaning. Furthermore, evidence that the HV diet affected food intake regulatory mechanisms is provided by the higher fasting ghrelin level found at
weaning and at wk 14 in the males, although this was not observed in the females. Ghrelin is an orexigenic hormone, and its higher concentration at weaning suggests that male offspring from HV dams may be programmed to hyperphagia from an early age [161]. Fasting plasma GLP-1 concentration was also increased. Although GLP-1 is recognized as a satiety hormone, it is also involved in glucoregulation [279]. Its latter role may be the most important factor here and be an early predictor of the insulin resistance in later life [161].

The effect of the high vitamin diet during pregnancy on the offspring phenotype is unlikely to have been generated from stress responses of the dams or the pups because there was no difference in litter size (13.0 ± 0.6 vs. 13.5 ± 0.5 pups per litter in control and supplemented dam groups, respectively) and birth weight of the offspring (8.3 ± 0.3 vs. 8.3 ± 0.2 g). Also, fasting corticosterone levels of the pups from both dam groups at weaning were similar (Table 5.3 and 5.4). Furthermore, in a previous study we found no effect on corticosterone levels in plasma of the dams after the lactation period [150]. Most importantly, recorded in a previous study, body weights of the RV and HV dams were similar 1 day before (369.3 ± 8.7 vs. 367.7 ± 11.1 g) or 1 day after delivery (287.5 ± 7.9 vs. 289.7 ± 8.8 g).

The fortification of 8 of the 12 required vitamins in the high vitamin diet were on average many times lower than that reported to cause teratogenic effects on the fetus. To date, the amounts that lead to toxicity for each vitamin have not been fully quantified [220, 234]. Folate addition at 20 times the recommended value in the AIN-93G diet (2 mg/kg) during pregnancy in Wistar rats has shown to lead to lower birth weight but not litter size [116]. Our high vitamin diet was much lower in vitamins involved in methyl group metabolism than those used in previous studies showing their epigenetic effects in the viable
yellow Agouti mouse. Folate, vitamin B$_{12}$, and choline fed at 9, 60, and 9 times the control diet with additions of betaine at 15 g per kg of diet during pregnancy and lactation were not reported to lead to affects on litter size or weaning weights [42, 130].

The mechanism by which the high multivitamin gestational diet modified the phenotype has not been defined, but the effect may be attributed to folate, vitamin B$_{12}$, A and D. These vitamins are known to impact on gene expression in contrast to other vitamins that function primarily in coenzyme roles. Increased CpG methylation has been found after pregnant mice were fed high dietary levels of methyl donors (betaine and choline) combined with methyl-metabolism cofactors (folate and vitamin B$_{12}$) [214]. Beside the B-complex vitamins, fat-soluble vitamins are also candidates for the observed changes. Both vitamin A and D during pregnancy affect gene expression in the offspring [251, 252]. Excess vitamin A may affect gene methylation via a stress mechanism. Its derivative, 9-cis retinoic acid, binds to retinoic X receptors [253], which may affect gene methylation through the increased transport of glucocorticoids through the placenta. Retinoic acid reduced the expression of 11β-HSD2 in a trophoblast-like cell line that displays a number of functional similarities to the placental syncytiotrophoblast [254]. 11β-HSD2 is an enzyme that metabolizes glucocorticoids transported to the placenta from the maternal side, and a reduced placental 11β-HSD2 activity due to stress can lead to higher glucocorticoid exposure to the fetus, and ultimately changes the development of the fetal HPA axis [255-257]. In contrast, non-stress related mechanisms are also relevant. Retinoic acid, in doses similar to that put in acne medication, increases the methyl transferases activity, and diminishes the availability of s-adenosyl methionine in rats [280]. Also, these doses result in altered activity in serotonergic and dopaminergic neurons through activation of retinoic acid receptors that are abundant in
the hypothalamus of adult rodent brains [281]. Thus it can be suggested that high vitamin A intake during gestation may affect the development of intake regulatory mechanisms.

Vitamin D intake during gestation also regulates gene expression. Vitamin D acts like a steroid hormone and interacts with both cell membrane receptors and nuclear vitamin D receptor proteins to modulate gene transcription, subsequently altering cell differentiation, proliferation and mineral homeostasis in the offspring [252]. 1,25-hydroxy-D3 has been reported to transcribe or repress as many as 27,091 genes [282]. In addition, neonatal rats exposed to high amounts of vitamin D can lead to long-term adverse effects on the thymic glucocorticoid receptor density [283].

The relevance of these results to vitamin intakes consumed by pregnant women to the current obesity epidemic remains uncertain. However, a recent survey estimated intakes by pregnant women in Boston, MA, USA by those in the upper third quartile to be between 2 to 7 times the RDA for 10 vitamins [111]. In addition, many multivitamin supplements recommended to pregnant women contain 2 to 5 times the requirements for B₁₂, folate and B₆ [16]. Recently, the Motherisk Program in Toronto has proposed to increase the supplement dose of folic acid from 400 µg to 5 mg daily (12 times) for women of childbearing years and/or pregnant women [284], which is much higher than the upper limit of 1000 µg set by the National Academy of Sciences, Washington, DC, USA. Adequate intake of vitamin D for women 19 to 50 years is 200 IU, but the Canadian Cancer Society is now recommending 1000 IU, and higher intakes (4000 IU) are being suggested during pregnancy and lactation based on 25-hydroxy D status in plasma [266]. Neither the presumed benefits nor the potential adverse effects of these high intakes of vitamins have been well established.
5.6 CONCLUSION

In conclusion the consumption of a high multivitamin diet during pregnancy increases the expression of postnatal obesity and metabolic syndrome in both female and male rats fed an obesogenic diet. The vitamins responsible for the observed effects remain to be identified.
## TABLE 5.1

Preload effects on food intake (1 hour) after an overnight fast in male offspring

<table>
<thead>
<tr>
<th>Gestational Diet</th>
<th>Water preload</th>
<th>Glucose preload</th>
<th>Delta</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RV</td>
<td>HV</td>
<td>RV</td>
</tr>
<tr>
<td>Week</td>
<td>g</td>
<td>g</td>
<td>g</td>
</tr>
<tr>
<td>1</td>
<td>5.3±0.3</td>
<td>7.2±0.3***</td>
<td>5.3±0.5</td>
</tr>
<tr>
<td>4</td>
<td>15.4±0.8</td>
<td>19.3±1.3*</td>
<td>11.9±0.5</td>
</tr>
<tr>
<td>8</td>
<td>13.1±0.9</td>
<td>17.8±1.7*</td>
<td>8.2±1.2</td>
</tr>
<tr>
<td>12</td>
<td>12.1±1.4</td>
<td>16.3±1.2*</td>
<td>7.1±1.2</td>
</tr>
<tr>
<td>16</td>
<td>13.5±1.0</td>
<td>15.3±1.0</td>
<td>4.4±0.9</td>
</tr>
<tr>
<td>20</td>
<td>12.6±1.1</td>
<td>15.8±0.9*</td>
<td>4.3±1.0</td>
</tr>
</tbody>
</table>

| GD              | P < 0.01     | P < 0.05       | P = 0.07 |
| Age             | P < 0.01     | NS             | P < 0.005 |
| Interaction     | NS           | NS             | NS       |

1 Data are means ± SEM; N = 8 – 10 means / group from Week 1 to Week 12 (each mean is the average food intake of 2 pups from the same litter). At 14 wk post-weaning, one rat from each litter was sacrificed, leaving N = 10 / group in Week 16 and 20;

2 Glucose was given by gavage (0.375 g glucose / ml) at 5 g / kg body weight, and water was given in the same volume as the glucose gavage;

3 Delta is calculated as: (1-h food intake after water preload) – (1-h food intake after glucose preload);

4 Food intake after water and glucose preloads and the delta were analyzed using the PROC MIXED procedure with gestational diet and age (week) as main factors. Preload effects (water and glucose) were compared using the PROC MIXED procedure with preload treatment and gestational diet as main factors. *** P < 0.005, ** P < 0.01, * P < 0.05 by unpaired t-test within the same week and preload;

5 The 1-h food intake after the glucose preload was lower than after the water preload in both groups (P < 0.01);

RV, recommended vitamin diet; HV, high vitamin diet; GD, Gestational diet; NS, Not significant;
### TABLE 5.2

Preload effects on food intake (1 hour) after an overnight fast in female offspring\(^1,4\)

<table>
<thead>
<tr>
<th>Gestational Diet</th>
<th>Water preload</th>
<th>Glucose preload(^2,3)</th>
<th>Delta(^3)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RV</td>
<td>HV</td>
<td>RV</td>
</tr>
<tr>
<td>Week</td>
<td>g</td>
<td>g</td>
<td>g</td>
</tr>
<tr>
<td>1</td>
<td>5.3±0.3</td>
<td>6.1±0.7</td>
<td>5.2±0.3</td>
</tr>
<tr>
<td>4</td>
<td>7.5±0.6</td>
<td>9.8±0.6*</td>
<td>6.2±0.9</td>
</tr>
<tr>
<td>8</td>
<td>6.8±0.8</td>
<td>7.1±0.5</td>
<td>3.9±0.4</td>
</tr>
<tr>
<td>12</td>
<td>8.6±0.7</td>
<td>8.3±0.3</td>
<td>3.6±0.7</td>
</tr>
<tr>
<td>16</td>
<td>6.4±0.5</td>
<td>7.2±0.3</td>
<td>2.2±0.5</td>
</tr>
<tr>
<td>20</td>
<td>7.0±0.7</td>
<td>7.9±0.5</td>
<td>2.7±0.6</td>
</tr>
</tbody>
</table>

| GD               | P = 0.08      | NS                       | NS          |
| Age              | NS            | P < 0.05                 | P < 0.01    |
| Interaction      | NS            | NS                       | NS          |

1 Data are means ± SEM; N = 8 – 10 means / group from Week 1 to Week 12 (each mean is the average food intake of 2 pups from the same litter). At 14 wk post-weaning, one rat from each litter was sacrificed, leaving N = 10 / group in Week 16 and 20;

2 Glucose was given by gavage (0.375 g glucose / ml) at 5 g / kg body weight, and water was given in the same volume as the glucose gavage;

3 Delta is calculated as: (1-h food intake after water preload) – (1-h food intake after glucose preload);

4 Food intake after water and glucose preloads and the delta were analyzed using the PROC MIXED procedure with gestational diet and age (week) as main factors. Preload effects (water and glucose) were compared using the PROC MIXED procedure with preload treatment and gestational diet as main factors. ** P < 0.01, * P < 0.05 by unpaired t-test within the same week and preload;

5 The 1-h food intake after the glucose preload was lower than after the water preload in both groups (P < 0.005);

RV, recommended vitamin diet; HV, high vitamin diet; GD, Gestational diet; NS, Not significant;
### TABLE 5.3

Fasting plasma hormone concentrations of male offspring at weaning, 14 and 28 weeks post-weaning\(^1\)

<table>
<thead>
<tr>
<th></th>
<th>Gestational Diet</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RV</td>
<td>HV</td>
</tr>
<tr>
<td><strong>Week 0</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose, mM</td>
<td>6.7 ± 0.4</td>
<td>6.9 ± 0.4</td>
</tr>
<tr>
<td>Insulin, ng/ml</td>
<td>0.2 ± 0.3</td>
<td>0.2 ± 0.1</td>
</tr>
<tr>
<td>Insulin Resistance Index(^2)</td>
<td>1.4 ± 0.3</td>
<td>1.7 ± 0.3</td>
</tr>
<tr>
<td>Ghrelin, ng/ml</td>
<td>1.5 ± 0.1</td>
<td>1.9 ± 0.1*</td>
</tr>
<tr>
<td>Corticosterone, ng/ml</td>
<td>430.5 ± 28.3</td>
<td>403.3 ± 65.2</td>
</tr>
<tr>
<td>GLP-1, pM</td>
<td>3.4 ± 0.3</td>
<td>4.5 ± 0.3*</td>
</tr>
<tr>
<td><strong>Week 14</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose, mM</td>
<td>6.5 ± 0.3</td>
<td>6.8 ± 0.2</td>
</tr>
<tr>
<td>Insulin, ng/ml</td>
<td>3.6 ± 0.4</td>
<td>4.3 ± 0.7</td>
</tr>
<tr>
<td>Insulin Resistance Index</td>
<td>23.4 ± 2.8</td>
<td>29.2 ± 4.2</td>
</tr>
<tr>
<td>Ghrelin, ng/ml</td>
<td>1.7 ± 0.2</td>
<td>2.2 ± 0.2</td>
</tr>
<tr>
<td>Corticosterone, ng/ml</td>
<td>161.5 ± 34.6</td>
<td>217.1 ± 41.3</td>
</tr>
<tr>
<td>GLP-1, pM</td>
<td>2.3 ± 0.2</td>
<td>3.3 ± 0.4*</td>
</tr>
<tr>
<td><strong>Week 28</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose, mM</td>
<td>6.1 ± 0.1</td>
<td>6.8 ± 0.3*</td>
</tr>
<tr>
<td>Insulin, ng/ml</td>
<td>3.8 ± 0.5</td>
<td>4.4 ± 0.7</td>
</tr>
<tr>
<td>Insulin Resistance Index</td>
<td>23.1 ± 3.2</td>
<td>30.0 ± 4.8</td>
</tr>
<tr>
<td>Ghrelin, ng/ml</td>
<td>2.4 ± 0.3</td>
<td>2.1 ± 0.2</td>
</tr>
<tr>
<td>Corticosterone, ng/ml</td>
<td>369.6 ± 43.7</td>
<td>452.2 ± 53.3</td>
</tr>
<tr>
<td>GLP-1, pM</td>
<td>5.9 ± 0.2</td>
<td>6.3 ± 0.2</td>
</tr>
</tbody>
</table>

\(^1\) Data are means ± SEM; N = 8 – 10 / group.

\(^{***} P < 0.005, ** P < 0.01, * P < 0.05\) by unpaired t-test;

\(^2\) Insulin resistance index was computed as fasting glucose multiplied by fasting insulin, ng/ml · mM;

RV, recommended vitamin diet; HV, high vitamin diet;
TABLE 5.4
Fasting plasma hormone concentrations of female offspring at weaning, 14 and 28 weeks post-weaning

<table>
<thead>
<tr>
<th>Week</th>
<th>Glucose, mM</th>
<th>Insulin, ng/ml</th>
<th>Insulin Resistance Index</th>
<th>Ghrelin, ng/ml</th>
<th>Corticosterone, ng/ml</th>
<th>GLP-1, pM</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>RV</td>
<td>7.1 ± 0.3</td>
<td>2.1 ± 0.3</td>
<td>1.5 ± 0.2</td>
<td>632.7 ± 65.8</td>
<td>2.4 ± 0.2</td>
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<td></td>
<td>HV</td>
<td>8.0 ± 0.4</td>
<td>1.7 ± 0.2</td>
<td>1.7 ± 0.2</td>
<td>603.4 ± 63.9</td>
<td>3.1 ± 0.3</td>
</tr>
<tr>
<td>14</td>
<td>RV</td>
<td>6.0 ± 0.3</td>
<td>13.3 ± 1.9</td>
<td>2.2 ± 0.3</td>
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<td>6.7 ± 0.3</td>
<td>23.4 ± 3.6</td>
<td>3.5 ± 0.5*</td>
<td>487.5 ± 60.1</td>
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<td>28</td>
<td>RV</td>
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<td>452.2 ± 53.3</td>
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</table>

1 Data are means ± SEM; N = 8 – 10 / group.

* P < 0.05 by unpaired t-test;

2 Insulin resistance index was computed as fasting glucose multiplied by fasting insulin, ng/ml · mM;

RV, recommended vitamin diet; HV, high vitamin diet;
Figure 5.1. Post-weaning body weight of (a) male offspring, and (b) female offspring.

Values are mean ± SE, n = 10 / group. ****P < 0.001, ***P < 0.005, **P < 0.01, *P < 0.05 at each time point.
Figure 5.2. Post-weaning daily food intake of (a) male offspring, and (b) female offspring.

Values are mean ± SE, n = 10 / group. *P < 0.05 at each time point.
Figure 5.3. Blood glucose response of (a) male offspring, and (b) female offspring.

Values are mean ± SE, n = 10 / group. *P < 0.05 at each time point.
Figure 5.4. Systolic blood pressure of (a) male offspring, and (b) female offspring.

Values are mean ± SE, n = 10 / group. **P < 0.01, *P < 0.05 at each time point.
CHAPTER 6.

STUDY 3: THE EFFECT OF HIGH MULTI-VITAMIN DIET DURING PREGNANCY ON FOOD INTAKE AND GLUCOSE METABOLISM IN WISTAR RAT OFFSPRING FED A LOW VITAMIN DIETS POST-WEANING
CHAPTER 6. THE EFFECT OF HIGH MULTI-VITAMIN DIET DURING PREGNANCY ON FOOD INTAKE AND GLUCOSE METABOLISM IN WISTAR RAT OFFSPRING FED LOW VITMAIN DIETS POST-WEANING

Preface:
To address the effect of high multi-vitamin intakes during pregnancy on the adaptive responses to the amplified nutrient mismatch by the post-weaning low vitamin diets of the offspring.

This work was submitted to Journal of Developmental and Origins of Health and Diseases (J DOHaD) on Sep 22, 2010, and is currently in revision as of Dec 10, 2010 (Manuscript #: 09-10-OA-0114-Anderson-R1).
6.1 ABSTRACT

Previous research has shown that offspring born to rat dams fed a high multi-vitamin diet (HV) are at increased risk of metabolic syndrome when weaned to a recommended vitamin diet (RV). Therefore we hypothesized a low vitamin post-weaning diet would enhance these characteristics in offspring born to dams fed HV diets. Wistar rats were fed during pregnancy the AIN-93G diet with or without a 10-fold increase in vitamin content. In Exp 1, at weaning, males were fed either the RV diet or a diet with 1/3 the vitamin content (1/3RV) for 12 wk. In Exp 2, at weaning, males and females were fed either the RV diet or a diet with 1/6 the vitamin content (1/6RV) for 35 wk. Body weight was measured weekly, FI daily and for 1-h after an overnight fast following water or glucose gavage at 6, 12 and 24 wk. Blood glucose and insulin responses to an oral glucose load were measured at 9 and 30 wk. Males from HV dams, compared to those from RV dams, gained more weight in Exp 1 (+7%, P<0.05) and Exp 2 (+11%, P<0.0001). The 1/6RV, but not the 1/3RV pup diet led to lower weight gain in males (-16%, P<0.0001) and females (-13%, P<0.0005), and lower FI in males (-9%, P<0.01), independent of the gestational diet. Gestational HV diet led to higher glucose response in males (+33%, P<0.05) and females (+63%, P<0.05). Females on the 1/6RV diet and from HV dams had higher 1-h FI (+36%, P<0.05) and lower insulin response (-25%, P<0.05) compared to those from RV dams. Exposure of male and female offspring to low vitamin diets did not amplify the expression of the metabolic syndrome observed in those born to dams fed a HV diet.
6.2 INTRODUCTION

Fetal programming by diets containing either inadequate or excess amount of nutrients during pregnancy compromises fetal development and affects the ability of offspring to adapt to the postnatal environment [22, 25, 26, 32]. The Predictive Adaptive Responses (PARs) hypothesis suggests that when a pre- and post-natal nutrient mismatch occurs, such that the offspring are exposed to a diet that is substantially different from that consumed by the mother, the risk of chronic disease is increased [32]. Although the PARs hypothesis is primarily based on the consequences of limiting energy and macronutrient of the pregnant mothers on the offspring, diets fed during pregnancy that are obesogenic [285, 286], high in protein [287, 288], high in saturated fatty acids [121, 289], or high in multi-vitamins [150, 290] result in offspring that are more likely to express characteristics of the metabolic syndrome, including obesity, hypertension and insulin resistance.

Therefore a logical extension of the PARs hypothesis is that offspring exposed to excess micronutrients in utero would be less able to adapt to low micronutrient environment. Partial support of this hypothesis is provided by observations of rat offspring born to dams fed a high vitamin (HV) diet and weaned to diets that contain the recommended vitamin (RV) level [150] or obesogenic [290]. Compared with pups born to dams fed the RV diet, those born to dams fed the HV diet eat more, gain more weight and adiposity, and exhibit characteristics of the metabolic syndrome [150, 290]. The mechanisms of the fetal programming by vitamin content of the maternal diet have been related to their role in the in utero development of gene expression [130, 131]. Fetal DNA expression can be modified epigenetically by methylation or acylation. In this process, dietary methyl donors
(methionine and choline) and metabolism cofactors (folate and vitamin B₁₂) are major contributors [130, 214, 275, 291, 292].

The hypothesis of this study was that increasing the mismatch in the gestational and pup diets created by feeding rat offspring a low or deficient vitamin diet at weaning would amplify the effect of high multi-vitamin intakes during pregnancy on the development of characteristics of the metabolic syndrome in the offspring.

6.3 MATERIALS AND METHODS

Animals and Diets. First-time pregnant Wistar rats were purchased from Charles River Inc., (Montreal, QC, Canada). Upon arrival, at 3 d of pregnancy, they were housed individually in ventilated plastic transparent cages with bedding in a 12-h light-dark cycle (lights on at 0600h), at a temperature of 22 ± 1 °C. The pregnant rats had free access to water by an automated water system. The University of Toronto Animal Care Committee approved the protocols and maintenance of the animals conforming to the guidelines of the Canadian Council on Animal Care.

From 3 d of pregnancy to term, dams were fed the AIN-93G diet [220] containing either the recommended (RV, recommended vitamins) or 10 times higher vitamin (HV, high vitamin) content by addition of the AIN-93 vitamin mix (Table 6.1). The vitamin additions used in the HV diet were well below the level expected to have teratogenic effect and half the lowest intake of vitamins known to have an adverse effect in rats [116, 234]. To account for the sucrose added as the carrier for the multi-vitamin mix in the HV diets, an equal amount (88.7 g / kg) of free sucrose was removed from the diet. In Exp 1, offspring were weaned to
either the RV or the 1/3RV diet with one-third the vitamin content of the RV diet. In Exp 2, male and female offspring were weaned to either the RV diet or the 1/6RV diet with one-sixth the vitamin content of the RV diet. The diets were provided in glass cups secured onto a stainless steel tray to reduce spillage. All gestational and pup diets were provided *ad libitum* in both Exp 1 and 2.

**Design.** This study consisted of two experiments. In both experiments, two groups of pregnant female rats (n = 10 per group) were fed either the RV or HV diets from d 3 of pregnancy until labour. At birth, each litter was culled to 10 pups to minimize the difference of lactation. Litters of less than 10 pups were excluded. During lactation, the dams were fed only the RV diet.

**Experiment 1: The effect of high multi-vitamin intake during pregnancy on male offspring fed a weaning diet with 1/3 RV content.** At weaning, at least one male offspring from each dam fed the RV or HV diets was randomly assigned to either the RV diet or 1/3RV diet (n = 14 / pup diet / gender). Offspring were individually caged and body weight (BW) was measured weekly from weaning to 12 wk post-weaning. Food intake for 24-h was measured once every 3 wk from 1 to 12 wk post-weaning.

At 12 wk post-weaning, after a 10-hour overnight fast, rats were randomly gavaged with either a glucose preload (0.375 g glucose/ml, 5 g glucose/kg BW) or a water preload with the same volume. Thirty minutes after the gavage, food intake was measured for one hour. The rats were then provided 24 h access to food, then fasted overnight and again gavaged, but with the opposite preload, and food intake for 1-h was measured.
An oral glucose tolerance test was performed at 9 wk post-weaning. After a 10-hour overnight fast, a blood sample was withdrawn from the capillary bed of the rat tail tip, and baseline glucose concentration was assayed using a commercial glucometer (MediSense Precision Xtra, Abbott Laboratories, Abbott Park, IL, USA). The rats were then gavaged (0.375 g glucose/ml, 5 g glucose/kg BW), and blood glucose concentrations were measured 15, 30, and 60 minutes later.

**Experiment 2: The effect of high multi-vitamin intake during pregnancy on male and female offspring fed a weaning diet with 1/6 RV content.** Because of the lack of effect of the 1/3RV diet on any measures in Exp 1, the vitamin content of the weaning diet was reduced to one-sixth of RV diet to exaggerate the mismatch between prenatal and post-weaning vitamin intake, and female offspring were added to determine the role of sex as a factor. The duration of study was extended to 35 wk post-weaning, an age where the rats reach full maturity and beginning their middle age.

At birth (after culling), the average BW of pups born to the same dam was recorded [239]. At weaning, 10 male and 10 female pups, one from each dam, were randomly allocated to either the RV or 1/6RV diet. All offspring were individually caged from weaning to 35 wk post-weaning. BW was measured at birth, and weekly from weaning to 35 wk post-weaning. Food intake was measured continuously from weaning to 25 wk post-weaning.

At 1, 2, and 3 wk post-weaning, after a 10-hour overnight fast, food intake was measured for one hour. At 6, 12 and 24 wk post-weaning, after a 10-hour overnight fast, rats were gavaged with a glucose preload (0.375 g glucose/ml, 5 g glucose/kg BW), and 30 minutes after the gavage, food intake was measured for one hour. An oral glucose tolerance
test was performed at 9 wk and 30 wk post-weaning (0.375 g glucose/ml, 5 g glucose/kg BW). At 30 wk post-weaning, a 100 µl blood sample was collected (fasting, 15, 30 and 60 minutes after gavage) and plasma insulin concentration was measured by enzyme immunoassay (catalog no. 80-INSRT-E10, Alpeo Diagnostics, Salem, NH). Blood glucose concentration was measured by a handheld commercial glucometer at each time. Fat pad mass of males (epididymal, perirenal and abdominal) and females (perirenal and abdominal) were weighed at sacrifice at 35 wk post-weaning.

**Statistical analysis.** For the oral glucose tolerance test, blood glucose response and plasma insulin response were calculated as the net incremental area under the curve (iAUC) of their respective concentrations from 0 to 60 minutes after the glucose gavage. Insulin resistance index was calculated as fasting glucose multiplied by fasting insulin [238]. In Experiment 1, cumulative food intake was calculated as the sum of the five 24-h food intake measurements.

Treatment effects on weight gain, cumulative food intake (Exp 1) and short-term food intake (Exp 1), total food intake (Exp 2), insulin resistance index and fat pad mass were analyzed using the PROC MIXED procedure in SAS (version 9.1, SAS Institute, Cary, NC) with gestational and pup diets as main factors. Treatment effects on short-term food intake (Exp 2), glucose and insulin response during the oral glucose tolerance test in the offspring were analyzed with the PROC MIXED procedure with gestational diets, pup diets and time as main factors. If an interaction was found between the effects of gestational diets, pup diets and/or time, a one-way ANOVA with post-hoc Tukey’s test was performed to identify the
effect of individual diets. Significance of difference was declared if \( P < 0.05 \). All data are expressed as means ± SEM.

6.4 RESULTS

Experiment 1: The effect of high multi-vitamin intake during pregnancy on male offspring fed a weaning diet with 1/3 RV content. Gestational diet had no effect on litter size (HV = 13.0 ± 0.5 vs. RV = 13.1 ± 0.6 pups per litter). However, at weaning, male offspring from the HV dams had 5% lower BW (HV = 69.2 ± 0.9 vs. RV = 72.5 ± 0.8 g, \( n = 28 \) / group) than those from the RV dams (\( P < 0.01 \)).

Post-weaning weight gain (\( P < 0.05 \)) and food intake (\( P < 0.01 \)) were affected by gestational diet, but not pup diet, and there was no interaction (Table 6.3). Male pups from HV dams gained 6% more weight and ate 10% more food over the 12 wk post-weaning period compared to those from RV dams.

Short-term food intake over 1-h following an overnight fast and gavaged with the water preload at 12 wk post-weaning was affected by gestational diet (\( P < 0.05 \)) and pup diet (\( P < 0.05 \)), with no interaction (Table 6.3). Pups from the HV dams ate 10% more than those from RV dams, but pups on the 1/3RV diet ate 5% less than those on the RV diet. Food intake after glucose preloads was also affected by gestational diet (\( P < 0.01 \)), but not pup diet, with no interaction. Pups from the HV dams ate 30% more than those from RV dams, suggesting that they were less sensitive to the food intake suppressing effect of glucose gavage.
Blood glucose iAUC following the glucose preload was 40% higher in rats from the HV dams (P < 0.005), but there was no effect of the pup diet nor interaction at 9 wk post-weaning (Fig 1A).

Experiment 2: The effect of high multi-vitamin intake during pregnancy on male and female offspring fed a weaning diet with 1/6 RV content. As in Exp 1, gestational diet had no effect on litter size (HV = 13.9 ± 1.1 vs. RV = 13.4 ± 0.9 pups per litter), or on birth weights (HV = 6.6 ± 0.1 vs. RV = 6.8 ± 0.1 g, n = 10 / group), or on weaning weights of males (HV = 64.5 ± 0.8 vs. RV = 65.9 ± 0.8 g, n = 20 / group) and females (HV = 61.1 ± 0.8 vs. RV = 62.9 ± 0.7, n = 20 / group).

Weight gain of the male pups did not show a main effect of either gestational diet or pup diet, but a significant interaction between gestational diet and pup diet was found (P < 0.05) (Table 6.4). The interaction arose because males on the RV diet and born to the HV dams gained 11% more weight from 0 to 35 wk post-weaning than those from RV dams (P < 0.0001), whereas no difference in weight gain due to gestational diets was found in males fed the 1/6RV diet. The 1/6RV pup diet led to a 16% less weight gain in males (P < 0.0001).

Similarly, in female pups, weight gain did not show a main effect of either gestational or pup diet, but an interaction between gestational and pup diets was found (P < 0.05) (Table 6.4). Female pups fed the 1/6RV pup diet and born to RV dams gained 21% less weight compared to those fed the RV pup diet (P < 0.05), whereas no difference in weight gain due to pup diets was found in females born to HV dams.

Total food intake of male offspring was affected by both gestational diet (P < 0.01) and pup diet (P < 0.0001) (Table 6.4), with no interaction. Males from HV dams ate 5%
more food compared to those from RV dams, even though the male pups fed the 1/6RV diet ate on average 9% less food than those fed the RV diet. In the female offspring, there was no main effect of gestational diet or pup diet, but a significant interaction was found ($P < 0.05$). Females on the RV diet, but not on the 1/6RV diet, and born to the HV dams had 7% less total food intake than those from RV dams ($P < 0.05$).

One-hour food intake after an overnight fast in males was affected by gestational diet ($P < 0.0001$) and age ($P < 0.0001$), but not pup diet, from 1 to 3 wk post-weaning (Figure 6.2A). Males from HV dams ate 33%, 23% and 28% more at 1, 2 and 3 wk post-weaning, respectively, compared to those from RV dams. In addition, an interaction between gestational and pup diets was observed ($P < 0.005$). Males on the 1/6RV diet, but not on the RV diet, and born to HV dams ate 52% and 55% more in 1-h at 2 wk ($P < 0.05$) and 3 wk ($P < 0.05$) post-weaning, respectively, than those from RV dams (Figure 6.2A). In the female offspring, gestational diet ($P < 0.05$) and pup diet ($P < 0.0005$) also affected one-hour food intake (Figure 6.2B), and an interaction between gestational diet and age was observed ($P < 0.001$). One-hour food intake was lower in females born to HV dams than those from RV dams, with the effect of the gestational diet increasing with age. At 3 wk post-weaning, females born to the HV dams ate 27% less food in 1-h than those from RV dams ($P < 0.001$). Females fed the 1/6RV diet ate 21%, 17% and 20% less at 1, 2 and 3 wk post-weaning, respectively, compared to those fed the RV diet (Figure 6.2B).

One-hour food intake of the males after glucose preloads were given at 6, 12 and 24 wk post-weaning was affected by gestational diet ($P < 0.005$), but there was no pup diet effect, age effect or interaction (Figure 6.3A). After the glucose preloads, food intake of males born to HV dams was on average 24% higher than those from RV dams. Similarly,
one-hour food intake in females born to HV dams was 15% higher (P = 0.06) than those born to RV dams, but there was no pup diet effect or interaction (Figure 6.3B).

Blood glucose response (iAUC) after an oral glucose load at 9 wk post-weaning was 26% higher in males born to the HV dams than those from the RV dams (P = 0.055), even though the glucose response was on average 24% lower in males on the 1/6RV diet compared with those on the RV diet (P < 0.05) (Figure 6.1B). A significant interaction (P < 0.05) between gestational diet and pup diet occurred, whereas males born to the HV dams and fed the 1/6RV diet, but not the RV diet, had a 73% higher glucose response than those from RV dams (P < 0.01). In female offspring, neither gestational diet (P = 0.08) nor pup diet affected the glucose response (Figure 6.1C).

Similarly in males at 30 wk post-weaning, blood glucose response was affected by gestational diet (P < 0.05) and pup diet (P < 0.05), but without an interaction (Figure 6.4A). Males born to the HV dams had 33% higher iAUC than those from the RV dams, although iAUC was again on average 24% lower in males on the 1/6RV diet compared with those on the RV diet. In female offspring, and in contrast to the absence of effect at 9 wk post-weaning, blood glucose response at 30 wk post-weaning was affected by gestational diet (P < 0.05), pup diet (P < 0.001), and a significant interaction between them (P < 0.01) (Figure 6.4B). Females on the 1/6RV diet from the HV dams had 117% higher blood glucose response (iAUC) than those from the RV dams (P < 0.05), where females on the RV diet were unaffected by gestational diet.

Plasma insulin response to the glucose load in male offspring was affected by pup diet (P < 0.01), but not gestational diet or interaction at 30 wk post-weaning (Figure 6.4C). Males on the 1/6RV diet had a 23% higher iAUC than those on the RV diet. In females,
those born to the HV dams had 20% lower insulin response than those from the RV dams (P < 0.05), and females on the 1/6RV diet had 15% lower insulin response than those on the RV diet (P < 0.05) (Figure 6.4D), with no interaction between these factors.

Insulin resistance index in male offspring at 30 wk post-weaning (RV-RV: 7.8 ± 1.1, RV-1/6RV: 8.4 ± 1.8, HV-RV: 13.7 ± 2.5, HV-1/6RV: 7.8 ± 1.0 mM·ng/ml) was not affected by gestational or pup diets, although there was a trend toward an interaction between them (P = 0.06). Males born to the HV dams and fed the RV diet had a 76% higher insulin resistance index compared to those from RV dams (P < 0.05 by unpaired t-test). Insulin resistance index in female offspring (RV-RV: 6.9 ± 1.1, RV-1/6RV: 5.7 ± 1.1, HV-RV: 5.5 ± 0.7, HV-1/6RV: 3.5 ± 0.4 mM·ng/ml) was not affected by gestational diet (P = 0.07), nor pup diet, with no interaction between them. Fat pad mass in male offspring at 35 wk post-weaning (RV-RV: 60.2 ± 6.2, RV-1/6RV: 43.5 ± 3.1, HV-RV: 69.7 ± 9.8, HV-1/6RV: 39.8 ± 2.6 g) was affected by pup diet (P < 0.05), but not gestational diet, and no interaction was found. Fat pad mass in female offspring (RV-RV: 30.3 ± 3.5, RV-1/6RV: 19.9 ± 3.2, HV-RV: 28.1 ± 3.5, HV-1/6RV: 26.8 ± 2.2 g) was not affected by gestational or pup diets, with no interaction.

6.5 DISCUSSION

The results of these studies do not support the hypothesis that Wistar rat offspring born to dams fed a high multi-vitamin diet during pregnancy and weaned to low vitamin diets exhibit an exaggerated expression of obesity and characteristics of the metabolic syndrome. Consistent with previous studies [150, 290], male rats born to dams fed the gestational HV diet exhibited higher weight gain and long-term food intake, ate more in 1 h after an
overnight fast or after a glucose preload, and had higher blood glucose response following glucose gavage. Surprisingly, these characteristics of metabolic syndrome in male rats born to the HV dams were modified little despite an extreme reduction in vitamin content of the post-weaning diet, suggesting that the programming effect of the gestational HV diet is very robust. In contrast and as shown previously [150], female rats born to HV dams did not show increased weight gain or long-term food intake, but had increased short-term appetite and hyperglycemia later in life.

The modest effect of the low vitamin pup diets on the weight gain and food intake, independent of the gestational diets, is surprising and a confounding factor in interpreting the outcomes. The vitamin content of the recommended AIN-93G growth diet [220] exceeds that required for growth by several fold [234], as shown by the absence of effect of the 1/3RV diet and the limited consequences of the 1/6RV diet on growth. Notably, in the AIN-93G diet, vitamins A, B₁₂, D and E are 11, 500, 100 and 8 fold higher than that required by the growing rat, respectively [234] (Table 6.2). The rationale for the initial investigation of the effect of the 1/3RV diet was based on two observations. First, it has been suggested that low vitamin intake leads to overeating and obesity in humans [293] and animals [294]. Second, when an obesogenic diet was offered to pups [290], both male and female offspring from HV dams became obese much more quickly and more extremely than if they were weaned to the RV diet [150]. The obesogenic diet, due to its high energy content, had a lower ratio of micronutrients to energy intake by approximately 50%, suggesting that reduced micronutrient intake may have been a factor in increasing food intake. Clearly, from the present study, it was not the case as shown by the minimal effects from the 1/3RV and 1/6RV pup diets.
A strong evidence that gestational HV diet led to \textit{in utero} programming of regulation of food intake is the altered short-term food intake during the early post-weaning period. In males, gestational HV diet led to a higher 1-h food intake after an overnight fast, which was further exaggerated by the 1/6RV diet (Fig 2). In our previous study, we observed that the fasting plasma concentration of ghrelin, an orexigenic hormone [161], was 26% higher in males born to HV dams compared to those from RV dams at weaning [290], suggesting a programmed hyperphagia phenotype in male offspring that persisted into adulthood. In females, however, the opposite was observed where females born to HV dams had a lower 1-h food intake during the first 3 weeks post-weaning, and the 1/6RV diet further lowered it. But beginning at 6 wk post-weaning, the feeding pattern changed where the females born to HV dams ate more in 1-h after a glucose load, suggesting a more complicated \textit{in utero} programming of short-term food intake regulation that involved with caloric compensation over time.

The current study, for the third time, showed that male pups born to HV dams had lower glucose tolerance and higher insulin resistance index in adulthood [150, 290]. However, their glucose response, but not insulin response, after an oral glucose load was higher compared to those born to RV dams, suggesting males born to HV dams had a lower peripheral glucose disposal capability and a sign of insulin resistance development [295]. In contrast, the low vitamin pup diet (1/6RV) led to lower glucose and higher insulin response after the glucose load regardless of the gestational diet, possibly due to its effect on lowering weight gain by 16% (Table 3) and abdominal fat pad mass by 35% (1/6RV: 41.6 ± 2.0g, RV: 64.9 ± 5.9g; P < 0.05), thus higher lean mass and better glucose disposal. In the female offspring, the gestational HV diet had the opposite effect of a higher glucose and lower
insulin response to the oral glucose load, indicating that the stimulation of insulin secretion by glucose was suppressed, a possible sign of impaired beta cell glucose sensitivity [296]. This effect of the HV diet of the dams was further exaggerated by the 1/6RV pup diet in the female offspring, as shown by the two-fold higher glucose response and 25% lower insulin response compared to all the other groups.

The sex of the offspring played a significant role in the phenotypic programming by the gestational and pup diets, where the effects in males arose primarily in long-term body weight and food intake, while females exhibited more changes in short-term food intake and glucose regulations. These results are consistent with studies showing that male rats are more susceptible to manipulation in gestational diets [145, 247, 248], possibly because of their faster growth rate and higher nutrient demands than females [22, 55]. In addition, males, but not females, born to HV dams had higher concentration of saturates and monounsaturates, higher total fat in muscle, and higher n-6 to n-3 fatty acids ratio in muscle in early adulthood [297], suggesting the males would develop insulin resistance earlier in life than females. In our previous study, female rats born to HV dams developed characteristics of metabolic syndrome only when they were weaned to an obesogenic diet [290], but not to a regular AIN-93G powdered diet [150].

Although the present study leads to the suggestion that the consequences of feeding the dams a HV diet were little affected by the vitamins content of the pup diets, a more direct test of the PARs hypothesis would include weaning the pups from dams fed the HV diet to a HV diet [25, 32], and such a study is required before it can be concluded that vitamin content of the pup diets is not a factor. Furthermore, the magnitude of the characteristics of the metabolic syndrome may be different if the gestational diet was extended to the
preconception and lactation periods [22, 26, 287, 298]. In rodents, the development of food intake regulatory systems in the hypothalamus and gastro-intestinal tracts takes place in late gestation and early post-natal period [163], and providing multi-vitamin supplementation at this critical period may induce stronger programming effects in the offspring, and a greater dependence in the vitamin content of the pup diet. Such enquiry is relevant to human application for two reasons. First, it has been shown that low micronutrient intake associates with higher risk of obesity and overeating [293]. Second, in developing countries, more mothers are receiving abundant micronutrient supplements during pregnancy and lactation. Many studies have been examining the effects of maternal supplementation on birth outcomes [299-302]. However, little attention has been placed on the infants after 1 year of age, whom are unlikely to receive micronutrient sufficient diets. This is a scenario that is similar to the present study, which combines gestational vitamin supplementation with post-weaning low vitamin environment.

6.6 CONCLUSION

In conclusion, the exposure of rat offspring to low vitamin post-weaning diets does not amplify the expression of characteristics of the metabolic syndrome observed in rats born to dams fed high multi-vitamin diets.
### TABLE 6.1
Composition of the experimental diets

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<td>Fat*†</td>
<td>70.0</td>
<td>70.0</td>
<td>70.0</td>
<td>70.0</td>
</tr>
<tr>
<td>Fiber (Cellulose) 2</td>
<td>50.0</td>
<td>50.0</td>
<td>50.0</td>
<td>50.0</td>
</tr>
<tr>
<td>Mineral mix 1</td>
<td>13.6</td>
<td>13.6</td>
<td>13.6</td>
<td>13.6</td>
</tr>
<tr>
<td>Vitamin mix 1</td>
<td>0.25</td>
<td>2.50</td>
<td>0.083</td>
<td>0.042</td>
</tr>
<tr>
<td>L-Cystine 2</td>
<td>3.0</td>
<td>3.0</td>
<td>3.0</td>
<td>3.0</td>
</tr>
<tr>
<td>Choline bitartrate 1</td>
<td>2.5</td>
<td>2.5</td>
<td>2.5</td>
<td>2.5</td>
</tr>
<tr>
<td>tert-Butylhydroquinone 2 (mg/kg)</td>
<td>14.0</td>
<td>14.0</td>
<td>14.0</td>
<td>14.0</td>
</tr>
</tbody>
</table>

1 Dyets, Inc. (Bethlehem, PA);
2 Harlan Teklad (Madison, WI);
3 Allied Food Service (Toronto, ON);
4 Loblaws (Toronto, ON);
† Fat in the RV, HV, 1/3RV and 1/6RV diets was derived from soybean oil.

* Vitamin and mineral mix contain sucrose as carrier. Values represent the absolute amount of minerals in the mineral mix, and vitamins in the vitamin mix, without the sucrose carrier.

Abbreviations: RV, recommended vitamin diet; HV, high vitamin diet; 1/3RV, diet with one-third the vitamin content of the RV diet; 1/6RV, diet with one-sixth the vitamin content of the RV diet;
TABLE 6.2

Estimated daily vitamin requirements of rats and vitamin content of the AIN-93G diet

<table>
<thead>
<tr>
<th>Vitamin</th>
<th>Estimated Requirements$^1$</th>
<th>Vitamin Content in AIN-93G Diet$^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg/kg diet</td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>0.7</td>
<td>8.0</td>
</tr>
<tr>
<td>D</td>
<td>0.025</td>
<td>2.5</td>
</tr>
<tr>
<td>E</td>
<td>18.0</td>
<td>150.0</td>
</tr>
<tr>
<td>K</td>
<td>1.0</td>
<td>0.75</td>
</tr>
<tr>
<td>Biotin</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>Folic acid</td>
<td>1.0</td>
<td>2.0</td>
</tr>
<tr>
<td>Niacin</td>
<td>15.0</td>
<td>30.0</td>
</tr>
<tr>
<td>Pantothenate</td>
<td>10.0</td>
<td>16.0</td>
</tr>
<tr>
<td>Riboflavin</td>
<td>3.0</td>
<td>6.0</td>
</tr>
<tr>
<td>Thiamin</td>
<td>4.0</td>
<td>6.0</td>
</tr>
<tr>
<td>B$_6$</td>
<td>6.0</td>
<td>7.0</td>
</tr>
<tr>
<td>B$_{12}$</td>
<td>0.05</td>
<td>25.0</td>
</tr>
</tbody>
</table>

$^1$ Values are from Nutrient Requirements of Laboratory Animals (4th Revised Edition) by the National Research Council [234].

$^2$ Values are from the Components of the AIN-93 Diets by Reeves PG [220].
### TABLE 6.3

Experiment 1: Weight gain, cumulative and short-term food intake in male offspring at 12 weeks post-weaning\(^1\)

<table>
<thead>
<tr>
<th>Gestational Diet</th>
<th>Pup Diet</th>
<th>Weight Gain</th>
<th>Cumulative FI(^2)</th>
<th>FI after water preload</th>
<th>FI after glucose preload(^3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RV</td>
<td>RV</td>
<td>459 ± 7</td>
<td>125 ± 4</td>
<td>6.1 ± 0.6</td>
<td>4.4 ± 0.6</td>
</tr>
<tr>
<td>RV</td>
<td>1/3RV</td>
<td>461 ± 11</td>
<td>122 ± 3</td>
<td>6.0 ± 0.4</td>
<td>4.7 ± 0.5</td>
</tr>
<tr>
<td>HV</td>
<td>RV</td>
<td>505 ± 13</td>
<td>138 ± 3</td>
<td>7.0 ± 0.4</td>
<td>6.1 ± 0.3</td>
</tr>
<tr>
<td>HV</td>
<td>1/3RV</td>
<td>474 ± 12</td>
<td>134 ± 3</td>
<td>6.3 ± 0.6</td>
<td>5.7 ± 0.4</td>
</tr>
</tbody>
</table>

GD | P < 0.05 | P < 0.01 | P < 0.05 | P < 0.01 | NS | NS | NS | NS |
PD | NS       | NS       | P < 0.05 | NS       | NS | NS | NS | NS |
GD x PD | NS | NS | NS | NS | NS | NS | NS | NS |

\(^1\) Data are means ± SEM; N = 12 – 14 / group. Data were analyzed using the PROC MIXED procedure with gestational diet and pup diet as main factors.

\(^2\) Cumulative food intake = Sum of five 24-h food intake measurements.

\(^3\) Glucose was given by gavage (0.375 g glucose/ml) at 5 g/kg body weight, and the water preload was the same volume.

FI, food intake; RV, recommended vitamin diet; HV, high vitamin diet; 1/3RV, diet with one-third the vitamin content of the RV diet; GD, gestational diet; PD, pup diet; NS, not significant;
TABLE 6.4
Experiment 2: Weight gain and total food intake in offspring

<table>
<thead>
<tr>
<th>Gestational Diet</th>
<th>Pup Diet</th>
<th>Weight Gain 0 – 35 wk</th>
<th>Total Food Intake 0 – 25 wk</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>g</td>
<td></td>
</tr>
<tr>
<td>Males</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RV</td>
<td>RV</td>
<td>692 ± 15&lt;sup&gt;A&lt;/sup&gt;</td>
<td>4567 ± 57</td>
</tr>
<tr>
<td>RV</td>
<td>1/6RV</td>
<td>621 ± 9&lt;sup&gt;B&lt;/sup&gt;</td>
<td>4224 ± 29</td>
</tr>
<tr>
<td>HV</td>
<td>RV</td>
<td>765 ± 26&lt;sup&gt;C&lt;/sup&gt;</td>
<td>4850 ± 89</td>
</tr>
<tr>
<td>HV</td>
<td>1/6RV</td>
<td>610 ± 23&lt;sup&gt;B&lt;/sup&gt;</td>
<td>4341 ± 89</td>
</tr>
<tr>
<td>GD</td>
<td>NS</td>
<td>P&lt;0.01</td>
<td></td>
</tr>
<tr>
<td>PD</td>
<td>NS</td>
<td>P&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>GD x PD</td>
<td>P&lt;0.05</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>Females</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RV</td>
<td>RV</td>
<td>389 ± 19&lt;sup&gt;A&lt;/sup&gt;</td>
<td>3478 ± 68&lt;sup&gt;A&lt;/sup&gt;</td>
</tr>
<tr>
<td>RV</td>
<td>1/6RV</td>
<td>307 ± 6&lt;sup&gt;B&lt;/sup&gt;</td>
<td>3312 ± 59&lt;sup&gt;AB&lt;/sup&gt;</td>
</tr>
<tr>
<td>HV</td>
<td>RV</td>
<td>346 ± 15&lt;sup&gt;AB&lt;/sup&gt;</td>
<td>3218 ± 57&lt;sup&gt;B&lt;/sup&gt;</td>
</tr>
<tr>
<td>HV</td>
<td>1/6RV</td>
<td>331 ± 6&lt;sup&gt;B&lt;/sup&gt;</td>
<td>3340 ± 85&lt;sup&gt;AB&lt;/sup&gt;</td>
</tr>
<tr>
<td>GD</td>
<td>NS</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>PD</td>
<td>NS</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>GD x PD</td>
<td>P&lt;0.05</td>
<td>P&lt;0.05</td>
<td></td>
</tr>
</tbody>
</table>

<sup>1</sup> Data are means ± SEM; N = 9 – 10 means / group. Data were analyzed using the PROC MIXED procedure with gestational diet and pup diet as main factors, followed by post-hoc Tukey’s test if an interaction was found. Values with different superscripts are significantly different, P < 0.0001 (males), P < 0.05 (females).

RV, recommended vitamin diet; HV, high vitamin diet; 1/6RV, diet with one-sixth the vitamin content of the RV diet; GD, gestational diet; PD, pup diet; NS, not significant;
Figure 6.1: Glucose response (iAUC) after a glucose load (5g/kg BW) at 9 weeks post-weaning. Data are means ± SEM; N = 7 – 10 / group in Exp 1, N = 8 – 10 / group in Exp 2. Data were analyzed using the PROC MIXED procedure with gestational diet and pup diet as main factors, followed by post-hoc Tukey’s test if an interaction was found, P < 0.01.

RV, recommended vitamin diet; HV, high vitamin diet; 1/3RV, diet with one-third the vitamin content of the RV diet; 1/6RV, diet with one-sixth the vitamin content of the RV diet; GD, gestational diet; PD, pup diet;
Figure 6.2: Experiment 2: Food intake (one hour) after an overnight fast.

Data are means ± SEM; N = 9 – 10 / group. Data were analyzed using the PROC MIXED procedure with gestational diet, pup diet and age as main factors, with statistical significance displayed to the right of each figure. Data were further analyzed with gestational diet and pup diet as main factors in every week, with statistical significance shown on the graph. Post-hoc Tukey’s test if an interaction was found within each week, P < 0.05.

RV, recommended vitamin diet; HV, high vitamin diet; 1/6RV, diet with one-sixth the vitamin content of the RV diet; GD, gestational diet; PD, pup diet; NS, not significant;
A Male offspring

![Graph showing food intake for male offspring](image)

Weeks (post-weaning) vs. Food intake (g)

- RV-RV
- RV-1/6RV
- HV-RV
- HV-1/6RV

GD: P < 0.005
GDxPD: P < 0.05

B Females offspring

![Graph showing food intake for female offspring](image)

Weeks (post-weaning) vs. Food intake (g)

- RV-RV
- RV-1/6RV
- HV-RV
- HV-1/6RV

GD: P = 0.06
PD: NS
GDxPD: NS
Age: NS

Figure 6.3: Experiment 2: Effects of glucose preloads on food intake (1 hour) after an overnight fast

Data are means ± SEM; N = 9 – 10 / group. Data were analyzed using the PROC MIXED procedure with gestational diet, pup diet and age as main factors, with statistical significance displayed to the right of each figure. Data were further analyzed with gestational diet and pup diet as main factors in every week, with statistical significance shown on the graph. Post-hoc Tukey’s test was performed if an interaction was found within each week, P < 0.05.

Glucose was given by gavage (0.375 g glucose/ml) at 5 g/kg body weight.

RV, recommended vitamin diet; HV, high vitamin diet; 1/6RV, diet with one-sixth the vitamin content of the RV diet; GD, gestational diet; PD, pup diet; NS, not significant;
Figure 6.4: Experiment 2: Glucose and insulin response (1 hour) after a glucose load (5g/kg BW) at 30 wk post-weaning.

Data are means ± SEM; N = 9 – 10 / group. Data were analyzed using the PROC MIXED procedure with gestational diet and pup diet as main factors, followed by post-hoc Tukey’s test if an interaction was found, P < 0.05.

Glucose was given by gavage (0.375 g glucose/ml) at 5 g/kg body weight.

RV, recommended vitamin diet; HV, high vitamin diet; 1/6RV, diet with one-sixth the vitamin content of the RV diet; GD, gestational diet; PD, pup diet; NS, not significant;
CHAPTER 7.

STUDY 4: MULTI-VITAMIN SUPPLEMENTATION DURING PREGNANCY ALTERS BODY WEIGHT AND MACRONUTRIENT SELECTION IN WISTAR RAT OFFSPRING
CHAPTER 7. MULTI-VITAMIN SUPPLEMENTATION DURING PREGNANCY ALTERS BODY WEIGHT AND MACRONUTRIENT SELECTION IN WISTAR RAT OFFSPRING

Preface:

To address the effect of feeding a high vitamin diet during pregnancy on food intake, the selection of protein and carbohydrate, and the expression of hypothalamic serotonergic receptors and POMC of the melanocortin system in the offspring.


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7.1 ABSTRACT

The hypothesis that vitamin content of the diet during gestation alters macronutrient choice, food intake and the expression of the serotonin receptors and pro-opiomelanocortin (POMC) in the hypothalamus of the offspring was investigated. Pregnant Wistar rats (n=10/group) were fed the AIN-93G diet containing a multi-vitamin mix at the recommended (RV) content or ten-fold higher (high vitamin, HV) content. Male offspring were weaned to a choice of 10% and 60% casein diets. Intake regulation by the serotonergic system was determined by measuring food choice daily for 7 wk, and following tryptophan (TRP) or mCPP (a serotonin receptor agonist) injections at 4 and 6 wk post-weaning. mRNA expressions of hypothalamic serotonin receptor and proopiomelanocortin (POMC) were measured at birth, weaning and sacrifice (7 wk). No differences were found in body weight at birth or weaning. HV offspring had lower food intake for the duration of the study (\(P < 0.001\)), and 11% lower body weight (\(P < 0.05\)) and 23% lower fat pad mass (\(P < 0.05\)) at 7 wk post-weaning. They selected less protein following 12 h of food deprivation (\(P < 0.05\)) and were less responsive to TRP (\(P = 0.05\)) and mCPP (\(P < 0.05\)) injections at 6 wk post-weaning. Expressions of mRNA for serotonin receptors 5-HT\(_{1A/2A/2C}\) at weaning (\(P < 0.01\)) and of POMC at weaning and 7 wk post-weaning (\(P < 0.05\)) were lower. In conclusion, intake of multi-vitamins above requirements during pregnancy affected macronutrient choice, food intake and the expression of serotonin receptors and POMC in the hypothalamus.
7.2 INTRODUCTION

Offspring of Wistar rats fed a high multi-vitamins (HV) diet during gestation have increased food intake in early life and develop characteristics of the metabolic syndrome in later life when fed either the AIN-93G powdered diet [150] or an obesogenic diet [290]. It is unclear, however, if the increase in food intake is solely driven by altered expression of feeding systems regulating energy intake or those regulating macronutrient preference. In addition to energy intake, protein and carbohydrate intake are an aspect of feeding behaviour that is regulated in animals allowed to make food choices [303-305]. The serotonin system is known to be involved in this regulation [294, 303, 306], but the role of serotonin receptors in regulating food selection has not been described. However it is known that serotonin receptors 5-HT$_{1A}$ act presynaptically, and when activated, increase food intake [307]. In contrast, increased activity of the postsynaptic receptors, 5-HT$_{2A/2C}$ has been associated with hypophagia and disruption of feeding cascade in animals [308]. 5-HT$_{2C}$ is expressed by POMC neurons, and both 5-HT$_{2C}$ and POMC co-regulate energy balance and food intake due to the direct neural circuitry between the serotonergic and melanocortin system [309-311]. Thus we hypothesized that, when compared to offspring from dams fed the RV diet, those from dams fed the HV diet during gestation have altered diet choice as well as energy intake, and that their food intake behavior could be associated with altered expressions of serotonin receptors and POMC in the hypothalamus.

Therefore, the objective of this research was to examine the effect of feeding a high multi-vitamin diet during pregnancy on food intake, the selection of protein and carbohydrate, and the expression of hypothalamic serotonergic receptors and POMC of the melanocortin system in the offspring.
7.3 MATERIALS AND METHODS

**Rats and Diets.** First-time pregnant Wistar rats (Charles River Inc., Quebec, Canada) were received on d 3 of pregnancy and housed individually in ventilated plastic transparent cages with bedding and room temperature of 22 ± 1°C in a 12-h light/12-h dark cycle (lights on at 0700 h). The pregnant rats had free access to water by an automated water system. The University of Toronto Animal Care Committee approved the protocol and maintenance of the animals conformed to the guidelines of the Canadian Council on Animal Care.

From d 3 of pregnancy to term, dams were fed the AIN-93G diet [312] containing either the recommended (RV, recommended vitamin) or 10 times higher vitamin (HV, high vitamin) content by addition of the AIN-93 vitamin mix (N = 10 dams / gestational diet). The composition (in g/kg) of the AIN-93G diet was: casein (200), cornstarch (529.4), sucrose (100), soybean oil (70), cellulose (50), vitamin mixture (10), mineral mixture (35), choline bitartrate (2.5), and tert-butylhydroquinone (0.014). Cornstarch, high-protein casein (87%) and cellulose were purchased from Harlan Teklad (Madison, WI). The vitamin mixture, mineral mixture, choline bitartrate and tert-butylhydroquinone were purchased from Dyets (Bethlehem, PA), whereas sucrose and soybean oil were purchased from local suppliers in Toronto, Canada (Allied Food Service and Loblaws, respectively). The composition of the vitamin mixture (in mg/kg RV diet) was: nicotinic acid (30), calcium pantothenate (16), pyridoxine-HCl (7), thiamin-HCl (6), riboflavin (6), folic acid (2), D-biotin (0.2), vitamin B12 (25), vitamin E as all-rac-α-tocopheryl acetate (15), vitamin A as all-trans-retinyl palmitate (8), vitamin D3 (2.5), vitamin K-1 (0.75), and powdered sucrose (9750). Because sucrose is the carrier in the vitamin mix, the 88.7 g/kg of sucrose added to the HV diet was compensated
for by removing the same amount of sucrose normally added to the RV diet. The vitamin additions used in the HV diet are below that reported to have adverse effects in rats [234], and none have been observed in previous studies [150, 290].

At birth, each litter was culled to 12 pups, and the pups were weaned at d 17 rather than d 21 because exposure to the gestational diet after that time is a determinant of protein selection by the offspring [313]. During lactation, dams were fed the RV diet. At weaning, pups were housed individually in ventilated plastic transparent cages with bedding. Male offspring, one from each dam, for each diet group (N = 10 / group), were weaned to a choice of 10% (LP, low protein) and 60% (HP, high protein) casein diets, as used in a previous study [314]. Food was available for 12 h each day (1900 to 0700h). This feeding schedule yields similar growth rates compared to 24 h ad libitum fed animals as previously reported [315]. The position of the food cups were alternated daily to control for possible position preference. The composition (in g/kg) of the LP pup diet was: casein (100), cornstarch (614), sucrose (116), soybean oil (70), cellulose (50), vitamin mixture (10), mineral mixture (35), choline bitartrate (2.5) and tert-butylhydroquinone (0.014). The composition (in g/kg) of the HP pup diet was identical except for casein (600), cornstarch (193) and sucrose (37). Pup diets had equal caloric densities of 3.95 kcal/g, were fed in jars equipped with a mesh insert to minimize spillage and were secured to a stainless steel base to add stability and catch spillage. A stainless steel partition separated the two diets in order to distinguish spillage and ensure accurate food intake measurements.

Food and macronutrient intake were measured daily, and body weight of the offspring was measured at birth, weaning and twice weekly from weaning to 7 wk post-weaning. At 4 and 6 wk post-weaning, the effect of TRP, a serotonin precursor [314], and mCPP, a 5HT2c
receptor agonist [316, 317] were used to examine serotonergic function. Macronutrient choice was measured after an overnight fast in response to saline (control), TRP (L-form, as methylester) and mCPP (1-(3-chlorophenyl)piperazine) injections. On separate days (d 1 or 3), male pups received i.p. injections of either TRP (1 mL/200 g, 75 mg/kg) or saline (1 mL/200 g) 45 min before food was provided, with a 1-d washout between the injections. Food intake of LP and HP diets was measured for 1 h after the TRP injections, and 1 and 12 h after saline injections. On d 5 or 7, male pups received i.p. injections of either mCPP (0.5 mL, 10 mg/kg) or saline (0.5 mL) 10 min before food was provided, and intake of LP and HP diets was measured over 12 h after the presentation of food cups in pups receiving mCPP, and 1 and 12 h after the presentation of food cups in pups receiving saline. Food intake data were expressed as total food intake (g), protein intake (g), carbohydrate intake (g) and protein energy intake (% of total energy intake).

At birth, weaning and 7 wk post-weaning, rats from both gestational diet groups were sacrificed by decapitation. Fat pad mass (FPM; sum of abdominal, peri-renal and epididymal fat pads) was collected upon sacrifice at 7 wk post-weaning. Brains were rapidly removed and immediately frozen by placing them on plastic cassettes on top of powdered dry ice and then stored at -80°C.

For RNA extraction, brains were removed from storage and thawed to -5°C for dissection. Each hypothalamus was dissected on a plastic cassette placed on top of chipped ice, using the posterior part of the optic chiasm as the anterior limit, the anterior part of the mammillary bodies as the posterior limit, and the lateral hypothalamic sulci as the lateral limits [318]. RNA extraction was conducted as previously reported [319]. Each dissected hypothalamic block was homogenized in 0.6 mL of TRIzol® Reagent (Invitrogen Corp.,
Grand Island, NY) using a Qiagen TissueRuptor and disposable probes (Qiagen Tech., Mississauga, ON). The homogenized samples were incubated at 15 to 30°C for 5 min and 0.12 mL of chloroform (GMD Chemicals Inc., Gibbstown, NJ) was added to the homogenized tissues. Sample tubes were capped securely and shook vigorously by hand for 15 sec. Samples were then incubated for 2 to 3 min at 15 to 30°C. Samples were centrifuged at 10,000 x g for 15 min at 4°C. Following removal from the centrifuge, the upper aqueous phase was transferred to a fresh tube. RNA was precipitated from the aqueous phase using isopropyl alcohol (Caledon Laboratories Ltd., Georgetown, ON). 0.3 mL of isopropyl alcohol was added to the aqueous phase, samples were incubated for 10 min at 15 to 30°C, and were then centrifuged at 10,000 x g for 10 min at 4°C. After centrifugation, the supernatant was removed, leaving a gel-like pellet remaining in the sample tube. The RNA pellet was then washed with 1 mL of 75% ethanol (Commercial Alcohols, Brampton, ON). The sample was mixed by vortexing and centrifuged at 6,500 x g for 5 min at 4°C. Following removal from the centrifuge, the RNA pellet was washed with 1 mL of 75% ethanol for a second time and centrifuged at 6,500 x g for 3 min at 4°C. Following centrifugation, the ethanol was removed and the remaining pellet was air-dried for 5 min. The RNA pellet was then dissolved in RNase-free water (Invitrogen Corp., Grand Island, NY) by passing the solution through a pipette tip several times and incubating the samples at 55 to 60°C for 10 min. Samples were then equally divided into two aliquots and placed in storage at -80°C until future use.

Reverse transcription was performed as previously described [319]. One hundred ng of total RNA in a 20 µL reaction was reverse transcribed using a high-capacity cDNA reverse transcription kit (Applied Biosystems Inc., Foster city, CA) according to the protocol of the
manufacturer. Samples were loaded into respective wells on a 96-well reaction card, and following a brief centrifugation (300 rpm for 3 min), the card was sealed and reverse transcription was performed using the GeneAmp® PCR System 2700 (Applied Biosystems Inc., Foster City, CA). Reactions were incubated initially at 25°C for 10 min, then at 37°C for 120 min, and finally at 85°C for 5 sec. mRNA levels of serotonin receptor 1A (5-HT₁A), 2A (5-HT₂A) and 2C (5-HT₂C), and POMC in the hypothalamus were determined at birth, weaning and 7 wk post-weaning. All of the oligonucleotide primer and fluorogenic probe sets for Taqman real-time PCR were purchased from Applied Biosystems Inc. (Foster City, CA; Table 7.1).

Real-time PCR was carried out as previously reported [319]. Nine µL of each cDNA sample, along with 11 µL of the 2X PCR master mix, were loaded into respective wells on a 96-well reaction card, followed by a brief centrifugation (3000 rpm for 3 min). The card was then sealed and real-time PCR and relative quantification was performed using the ABI PRISM 7700 Sequence Detection System (Applied Biosystems Inc., Foster City, CA). The cycle conditions were: 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec, and finally 60°C for 1 min. Negative controls lacking both mRNA and cDNA input were used in the reactions. Relative quantification values from the SDS file for all genes were normalized to the glyceraldehyde-3-phosphate dehydrogenase (GADPH) values. The ΔCₜ values were first calculated by using Cₜ for the specific gene mRNA minus Cₜ for GADPH mRNA in the sample. The mean mRNA expression (for a specific gene) from pups born to HV dams was compared to that of pup born to control dams using the formula: Relative Quantification = 2⁻ΔΔCₜ (ΔΔCₜ is the mean RVΔCₜ value minus the mean HVΔCₜ value, where a ΔΔCₜ of 1 equates a two-fold difference in cDNA added to the PCR reaction).
**Statistical analysis.** Gestational diet effect on body weight at birth, weaning and 7 wk post-weaning were analyzed by Student’s unpaired t-test. Post-weaning body weight, food and macronutrient intake data were analyzed by a two-factor repeated-measures ANOVA using the mixed model (PROC MIXED procedure in SAS) with gestational diet and day as main factors. Long-term food and macronutrient intake data were analyzed for d 1-27, 36-41 post-weaning. Weeks 4 (d 28-35) and 6 (d 42-48) were excluded from long-term food intake and selection patterns because of the experimental manipulations occurring at those times. When an interaction was declared, post-hoc comparisons were analyzed by Student’s unpaired t-tests.

1 h and 12 h food intake and diet selection at 4 and 6 wk was analyzed by two-factor repeated-measures ANOVA with gestational diet and injection as main factors. When an interaction was declared, mean comparisons were made using a one-factor ANOVA with a general linear model (GLM) procedure followed by Tukey’s post-hoc test. Treatment effects on fat pad mass at 7 wk post-weaning, and on hypothalamic mRNA gene expression at birth, weaning and 7 wk were determined by Student’s unpaired t-tests.

Significance was declared if $P < 0.05$. All data are expressed as mean ± SEM. The experimental data (e.g. body weight) of pups born to the same dam were averaged to obtain one value for the litter [239]. All statistical analyses were performed using the SAS System for Windows (Version 9.1, SAS Institute, Cary, NC).
7.4 RESULTS

There was no difference in litter size between RV and HV fed dams (13.4 ± 0.9 vs. 13.9 ± 0.6, \( P = \text{NS} \)), as previously observed. Following culling at birth, pups from RV and HV fed dams were 41.2% and 43.0% male, respectively.

**Body weight**

At birth and weaning, there was no difference in body weight between pups from HV and RV fed dams (Table 7.2). However, at 7 wk post-weaning, pups from HV fed dams weighed 14% less than RV pups \((P < 0.01)\). From weaning to sacrifice (7 wk post-weaning), there was a trend toward an effect of gestational diet \((F_{1,18} = 3.11, P = 0.09)\) and an effect of day \((F_{13,288} = 1408.83, P < 0.0001)\) (Figure 7.1). A gestational diet by day interaction \((F_{13,288} = 5.84, P < 0.01)\) on body weight gain occurred because pups from HV fed dams gained less weight than pups from RV fed dams, with the magnitude of difference between groups increasing with time. The difference was statistically different by Student’s t-test on d 8 and 12, and from d 33 to 50. At sacrifice, pups from HV fed dams had 19% less epididymal fat \((P < 0.05)\), 26% less peri-renal plus abdominal fat \((P < 0.05)\) and a 23% lower total fat pad mass (sum of epididymal, peri-renal and abdominal fat pads) as a percentage of total body weight \((P < 0.05)\) (Table 7.3).

**Long-term food and macronutrient intake**

*Food intake (g).* Gestational diet \((F_{1,22} = 6.47, P < 0.05)\) and day \((F_{32,682} = 29.85, P < 0.0001)\), with no interaction, affected total food intake (Figure 7.2A). Pups from HV fed dams ate a lower amount of food compared to pups from RV fed dams.
Protein and carbohydrate intake (g). Gestational diet did not affect protein or carbohydrate intake, however there was an effect of day (protein: $F_{32,682} = 39.56, P < 0.0001$; carbohydrate: $F_{32,682} = 17.43, P < 0.0001$) (Figure 7.2A). As pups aged, protein and carbohydrate intake increased. There was no interaction between gestational diet and day.

Protein energy intake (%). Gestational diet did not affect protein energy intake, but there was an effect of day ($F_{32,682} = 15.80, P < 0.0001$) (Figure 7.2B). As pups aged, protein energy intake increased. There was no interaction between gestational diet and day.

Short-term food and macronutrient intake

A. Saline Injection

Food, protein and carbohydrate intake (g). There was no effect of gestational diet on food intake over 1 h, following overnight food deprivation and the control saline injection prior to food access, at 4 and 6 wk post-weaning (Figure 7.3A). However, food intake over 12 h was lower in pups from the HV fed dams at wk 4 ($P < 0.05$). Gestational diet did not affect 1 h protein intake following saline injection at 4 wk, but at 6 wk, pups from HV fed dams had lower intakes ($P < 0.01$) (Figure 7.3B). But over 12 h, lower protein intake was observed at both 4 ($P < 0.01$) and 6 ($P < 0.0001$) wk. There was no effect of gestational diet on 1 or 12 h carbohydrate intake at either 4 or 6 wk (Figure 7.3C).

Protein energy intake (%). There was no effect of gestational diet on 1 h protein energy intake following overnight food deprivation at 4 wk (Figure 7.3D). However, pups from HV fed dams had a lower protein energy intake over 1 h at 6 wk ($P < 0.01$), and over 12 h at both 4 ($P < 0.05$) and 6 ($P < 0.0001$) wk, compared to pups from RV fed dams.
B. Tryptophan Injection

Food, protein and carbohydrate intake (g). There was no gestational diet effect, nor an interaction between gestational diet and injection on 1 h food (Figure 7.4A), protein (Figure 7.4B) or carbohydrate (Figure 7.4C) intake at 4 or 6 wk post-weaning. However, TRP injection decreased 1 h food and protein intake compared to the saline control at both 4 (food: $F_{1,20} = 13.38, P < 0.01$; protein: $F_{1,20} = 12.80, P < 0.01$) and 6 (food: $F_{1,20} = 7.95, P < 0.05$; protein: $F_{1,20} = 9.43, P < 0.05$) wk, but carbohydrate intake at 4 wk only ($P < 0.01$).

Protein energy intake (%). Pups from HV fed dams had a lower 1 h protein energy intake at 4 ($F_{1,21} = 4.37, P < 0.05$), but not 6 wk (Figure 7.4D). TRP injection decreased protein energy intake in both groups, but only at 6 wk ($P = 0.05$).

C. mCPP Injection

Food, protein and carbohydrate intake (g). There was no effect of gestational diet on 12 h food (Figure 7.5A), protein (Figure 7.5B) or carbohydrate (Figure 7.5C) intake at 4 or 6 wk. mCPP injection decreased food, protein and carbohydrate intake at both 4 (food: $F_{1,20} = 10.15, P < 0.05$; protein: $F_{1,20} = 7.14, P < 0.05$; carbohydrate: $F_{1,20} = 6.97, P < 0.05$) and 6 (food: $F_{1,20} = 16.48, P < 0.01$; protein: $F_{1,20} = 5.45, P < 0.01$; carbohydrate: $F_{1,20} = 13.06, P < 0.01$) wk. At 6 wk only, there was an interaction between gestational diet and injection on 12 h food ($F_{1,20} = 3.92, P = 0.06$) and protein ($F_{1,20} = 9.55, P < 0.01$), but not carbohydrate intake. In pups from RV but not HV fed dams, mCPP decreased food and protein intake ($P < 0.01$).

Protein energy intake (%). There was no effect of gestational diet or injection on 12 h protein energy intake at 4 or 6 wk (Figure 7.5D). There was an interaction between
gestational diet and injection \((F_{1,20} = 4.67, P < 0.05)\) at 6 wk only. mCPP decreased protein energy intake in pups from RV fed dams, but increased it in pups from HV fed dams.

**Hypothalamic 5-HT\(_{1A/2A/2C}\) and POMC mRNA levels**

At weaning, male pups from HV fed dams had significantly lower hypothalamic 5-HT\(_{1A}\) \((P < 0.01)\), 5-HT\(_{2A}\) \((P < 0.01)\) and 5-HT\(_{2C}\) \((P < 0.01)\) expression (Table 7.4). However, at birth and 7 wk post-weaning, expression hypothalamic 5-HT\(_{1A}\), 5-HT\(_{2A}\) and 5-HT\(_{2C}\) of the male pups was not different between the groups.

At birth, pups from RV and HV fed dams also had similar hypothalamic POMC expression levels (Table 7.5). However, at weaning and 7 wk post-weaning, male pups from HV fed dams had lower hypothalamic POMC \((P < 0.05\) at weaning and 7 wk post-weaning).

Although food selection of the female offspring was not studied, the expression of 5-HT\(_{1A}\) \((P < 0.01)\), 5-HT\(_{2A}\) \((P < 0.05)\) and 5-HT\(_{2C}\) \((P < 0.01)\) at weaning was lower in females born to the HV fed dams (Table 7.6).

**7.5 DISCUSSION**

These results support the hypothesis that increased vitamin content of the gestational diet affects both energy intake and macronutrient intake regulation and alters the expression of serotonin receptors and POMC in the hypothalamus of the offspring. Compared with pups born to dams fed the RV diet, pups born to HV dams had lower weight gain and food intake for the duration of the study. Following an overnight fast, they had lower food and protein intake when expressed as a proportion of total energy intake, reduced sensitivity to tryptophan and mCPP injections, lower serotonin receptor expression at weaning and POMC
expression at weaning and 7 wks post-weaning. However, the effects of the HV gestational diet on pups given access to a food choice feeding paradigm are in contrast to that observed in rats weaned to a single diet. Rats born to dams fed the HV diet ate more and gained more weight on both the AIN-93G diet [150] and obesogenic diet [290].

Change in the regulation of food choice in offspring due to consumption of a HV diet by the dams was demonstrated. Protein intake by those rats from the RV dams averaged approximately 25% of the total dietary energy, consistent with previous reports of protein selection in rats given similar dietary choice [320-322]. Although the long-term intake of protein was not statistically different between groups, evidence for altered regulation due to the HV gestational diet was clear in the short-term studies. Absolute protein and protein intake expressed in proportion to energy intakes were lower following an overnight fast during 12 h of feeding at 4 wk post-weaning, and during both 1 and 12 h at 6 wk post-weaning (Figure 7.3), while carbohydrate intake was unchanged.

Evidence for involvement of the serotonergic system in this food preference following an overnight fast was supported by the feeding responses to the agonists, TRP and mCPP. Because response to these agonists occurred in offspring from RV, but not HV fed dams, it can be suggested that postsynaptic sensitivity was reduced in offspring born to HV fed dams. Following TRP injection, the proportion of energy as protein decreased over 1 h at 6 wk post-weaning in pups from RV dams (Figure 7.4), and was consistent with the longer lasting effects of mCPP. Over 12 h, mCPP resulted in lower protein energy intake in pups from RV, but not HV fed dams (Figure 7.5).

Evidence that the HV diet impacted on the function of the serotonergic system is suggested by lower 5-HT1A/2A/2C receptor gene expression found in both male and female
offspring at weaning (Table 7.4 and 7.6). Unfortunately differences were not seen at birth, making the role of in utero programming of gene expression uncertain. Although the HV diet was fed only during gestation, it may have also affected postnatal development through the vitamin composition of the mothers’ milk. Higher levels of fat-soluble vitamins would be predicted to be present in the breast milk due to storage in liver and adipose tissue during gestation, and then mobilization during lactation. A single subcutaneous dose of vitamin A given to newborn male rats decreased serotonergic activity in the brain of the rats as adults [323] and in the next generation [324].

The reduced expression of $5$-HT$_{1A/2A/2C}$ receptors at weaning and of POMC at weaning and 7 wk post-weaning may have contributed to the feeding behavior of the offspring born to the HV dams. However, it does not explain why offspring selecting from high and low protein diets and born to HV fed dams weighed less and had less body fat at 7 wk post-weaning. The lower food intake in pups from HV fed dams is consistent with the results obtained after brain serotonin depletion, which results in hypophagia [307]. However, decreased $5$-HT$_{2A/2C}$ mRNA has been positively correlated with obesity and adiposity in animals [307, 308], and is consistent with higher food intake, adiposity and body weight gain in offspring fed either the AIN-93G diet [150] or the obesogenic diet [290] and born to dams fed the HV diet.

Because there is direct neuronal circuitry between the serotonergic and melanocortin systems [310, 311], the reduction of hypothalamic POMC gene expression in HV offspring at weaning and 7 wk post-weaning suggests a long-term disruption of the hypothalamic feeding regulatory system may have resulted from the gestational high vitamin diet. Downstream effectors of POMC, such as agouti-related protein (AgRP) and melanocortin-4 receptor
(MC4R), are thus prime candidates to be altered in this condition and warrant future investigation [311]. In rodents, the generation of serotonergic neurons begins as early as embryonic d 10 [325] and continues during the first 3 post-natal weeks of life [326, 327]. Although serotonin receptor expression did not differ at 7 wk post-weaning, this may be expected. Serotonin receptor gene expression is altered by frequent administration of serotonin receptor agonists [328], and because 2 doses of mCPP were given at 4 and 6 wk post-weaning, the mRNA levels measured were likely confounded. In addition to the mRNA levels of serotonin receptors and POMC, the measurement of their protein expressions would complement the findings, especially in the offspring at 7 wk post-weaning.

Why access to two diets resulted in lower food intake, body weight and body fat in offspring from the HV diets (Table 7.2 & 7.3, Figure 7.1 & 7.2A), whereas previous studies in rats fed only one diet result in increased food intake, body weight and adiposity [150, 290], is unclear. The food choice paradigm provided the rat offspring in this study was the only overall difference in study design compared to the previous publications. This choice paradigm has been widely used previously to elucidate mechanisms regulating food intake, and has illustrated that many neural pathways, including the serotonergic system, participate in both energy intake and food choice [294, 304, 305, 329]. In addition, the composition of the single diet paradigm markedly affects the regulation of food intake as illustrated by the increased body fat in rat offspring fed high fat and highly palatable diets [290]. There are many examples illustrating the effects of the pup diet and sex on the expression of intake regulatory mechanism [25, 76, 145, 150, 247]. In previous studies of the HV gestational diet, increased food intake, body weight and obesity was found in male, but not female offspring fed the AIN-93G diet [150]. However when they were fed an obesogenic diet, the female rats
ate more and developed obesity more quickly than the male rats [290]. Thus it can be concluded that the lower body weight, adiposity and food intake of pups from the HV dams arose from an interaction between the effect of the HV diet of the dams on expression of intake regulatory systems in the offspring and the food choices provided.

The relevance to humans of these effects of high multi-vitamin intakes during pregnancy on food intake, selection and body weight in the rat is uncertain at present, but it may be an indication of unexpected consequences of excess intakes on food intake regulation in humans. There is considerable evidence that many women consume high amounts of vitamins, and increased availability and consumption of fortified foods and dietary supplements have occurred in recent years. In the United States, in 2000, 28% of all adults consumed a multivitamin daily, up from 17% in 1987 [17]. Furthermore, pregnant women and women of childbearing age are advised to take a daily multi-vitamin supplement to ensure adequacy for both mother and fetus, and to protect against neural tube defects [330], and many gestational multi-vitamin supplements contain 100% or more of the recommended daily intake of one or more of the nutrients [17]. A recent survey conducted in Boston estimated micronutrient intakes by pregnant women in the upper third quartile to be 2 to 7 times the RDA for 10 vitamins [111].

7.6 CONCLUSION

In conclusion, intake of multi-vitamins above requirements during pregnancy affects body weight, food intake, macronutrient choice and expression of the serotonergic and the melanocortin systems in the hypothalamus of Wistar rats. Additional work is needed to
further elucidate which vitamins are involved and the mechanisms by which in utero programming of systems regulating food intake and macronutrient choice occurs.
**TABLE 7.1**

Probes used in real-time PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Context Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)</td>
<td>CGGGAAACCCATCACCATCTTCCAG</td>
</tr>
<tr>
<td>Serotonin Receptor 1A (5-HT1A)</td>
<td>CTAATGGGGCAGTGAGGCGAGGGTGA</td>
</tr>
<tr>
<td>Serotonin Receptor 2A (5-HT2A)</td>
<td>AACCATCTGTATGGGTACCCGTTG</td>
</tr>
<tr>
<td>Serotonin Receptor 2C (5-HT2C)</td>
<td>ATCTTTATGATTATGTCTGGCCCTT</td>
</tr>
<tr>
<td>Proopiomelanocortin (POMC)</td>
<td>GCAACCTGCTGCTTGCATCCGGGC</td>
</tr>
</tbody>
</table>
### TABLE 7.2

Body weight of the offspring at birth, weaning and 7 wk post-weaning$^{1,2}$

<table>
<thead>
<tr>
<th>GD</th>
<th>Birth</th>
<th>Weaning</th>
<th>7 wk Post-weaning</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>g</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RV</td>
<td>6.8 ± 0.4</td>
<td>54.1 ± 1.1</td>
<td>375.6 ± 7.7</td>
</tr>
<tr>
<td>HV</td>
<td>6.6 ± 0.4</td>
<td>54.1 ± 1.4</td>
<td>323.9 ± 10.6*</td>
</tr>
</tbody>
</table>

$^{1}$ Mean ± SEM, n = 10 litters/group.

$^{2}$ The body weights of pups born to the same mother were averaged to obtain one value for the litter, * $P < 0.01$ by Student’s unpaired t-test within the same column.

GD, gestational diet; RV, recommended vitamin; HV, high vitamin.
TABLE 7.3

Fat pad mass at 7 wk post-weaning\(^1\)

<table>
<thead>
<tr>
<th>GD</th>
<th>Epididymal</th>
<th>Peri-renal + abdominal</th>
<th>Total fat pad mass(^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% Body Weight</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RV</td>
<td>1.6 ± 0.1</td>
<td>1.9 ± 0.2</td>
<td>3.5 ± 0.3</td>
</tr>
<tr>
<td>HV</td>
<td>1.3 ± 0.1*</td>
<td>1.4 ± 0.1*</td>
<td>2.7 ± 0.1*</td>
</tr>
</tbody>
</table>

\(^1\) Mean ± SEM; n = 10 rats/group. Percentage of adipose tissue, (adipose tissue/body weight x 100), * \(P < 0.05\) by Student’s unpaired t-test within the same column.

\(^2\) Sum of the epididymal, peri-renal and abdominal fat pads.

GD, gestational diet; RV, recommended vitamin; HV, high vitamin.
**TABLE 7.4**

Hypothalamic 5-HT_{1A/2A/2C} mRNA expression levels at birth, weaning and 7 wk post-weaning^{1,2}

<table>
<thead>
<tr>
<th>GD</th>
<th>Birth</th>
<th>Weaning</th>
<th>7 wk Post-weaning</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5-HT_{1A}</td>
<td>5-HT_{2A}</td>
<td>5-HT_{2C}</td>
</tr>
<tr>
<td>RV</td>
<td>0.95±0.27</td>
<td>1.03±0.13</td>
<td>0.98±0.21</td>
</tr>
<tr>
<td>HV</td>
<td>1.09±0.13</td>
<td>1.03±0.07</td>
<td>0.85±0.22</td>
</tr>
</tbody>
</table>

1 Mean ± SEM; n = 5 – 6 rats/group. * P < 0.01, by Student’s unpaired t-test within the same column.

2 Data were normalized to the GAPDH values and then expressed as relative quantification.
GD, gestational diet; RV, recommended vitamin; HV, high vitamin; 5-HT_{1A}, serotonin receptor 1A; 5-HT_{2A}, serotonin receptor 2A; 5-HT_{2C}, serotonin receptor 2C; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
TABLE 7.5

Hypothalamic POMC mRNA expression levels at birth, weaning and 7 wk post-weaning\textsuperscript{1,2}

<table>
<thead>
<tr>
<th>GD</th>
<th>Birth</th>
<th>Weaning</th>
<th>7 wks post weaning</th>
</tr>
</thead>
<tbody>
<tr>
<td>RV</td>
<td>0.75 ± 0.36</td>
<td>1.13 ± 0.20</td>
<td>0.99 ± 0.18</td>
</tr>
<tr>
<td>HV</td>
<td>0.83 ± 0.30</td>
<td>0.52 ± 0.07*</td>
<td>0.57 ± 0.05*</td>
</tr>
</tbody>
</table>

\textsuperscript{1} Mean ± SEM; n = 5 – 6 rats/group. * \( P < 0.05 \) by Student’s unpaired t-test within the same column.

\textsuperscript{2} Data were normalized to the GAPDH values and then expressed as relative quantification.

GD, gestational diet; RV, recommended vitamin; HV, high vitamin; POMC, proopiomelanocortin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
TABLE 7.6
Hypothalamic 5-HT$_{1A}$/2A/2C mRNA expression levels of female offspring at weaning$^{1,2}$

<table>
<thead>
<tr>
<th>GD</th>
<th>5-HT$_{1A}$</th>
<th>5-HT$_{2A}$</th>
<th>5-HT$_{2C}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>RV</td>
<td>1.05 ± 0.09</td>
<td>0.91 ± 0.10</td>
<td>1.05 ± 0.11</td>
</tr>
<tr>
<td>HV</td>
<td>0.67 ± 0.04*</td>
<td>0.64 ± 0.05*</td>
<td>0.62 ± 0.06**</td>
</tr>
</tbody>
</table>

$^1$ Mean ± SEM; n = 5 – 6 rats/group. $^*$P < 0.05, $^{**}$P < 0.01 by Student’s unpaired t-test within the same column.
$^2$ Data were normalized to the GAPDH values and then expressed as relative quantification. GD, gestational diet; RV, recommended vitamin; HV, high vitamin; 5-HT$_{1A}$, serotonin receptor 1A; 5-HT$_{2A}$, serotonin receptor 2A; 5-HT$_{2C}$, serotonin receptor 2C; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
**Figure 7.1.** Body weight gain to 7 wk post-weaning\(^1,2\)

\(^1\) Mean ± SEM; n = 8-10 rats/group.

\(^2\) GD (\(P = 0.09\)), Day (\(P < 0.0001\)), GD x Day (\(P < 0.01\)). * Mean differences by Student’s unpaired t-test, \(P < 0.05\).

GD, gestational diet; RV, recommended vitamin; HV, high vitamin.
Figure 7.2. **A:** Long-term food⁴, protein³, carbohydrate⁴ and **B:** protein energy⁵,a intake (d 1-27 and 36-41 post-weaning)¹.

¹ Mean ± SEM; n = 8-10 rats/group for each time point.

² **Food intake:** GD (P < 0.05), Day (P < 0.0001), GD x Day (P = NS).

³ **Protein intake:** GD (P = NS), Day (P < 0.0001), GD x Day (P = NS).

⁴ **Carbohydrate intake:** GD (P = NS), Day (P < 0.0001), GD x Day (P = NS).

⁵ **Protein energy intake:** GD (P = NS), Day (P < 0.0001), GD x Day (P = NS).

ᵃ Protein Energy Intake (%) = [protein intake (g) x 4.0 (kcal/g)] ÷ [food intake (g) x 3.95 (kcal/g)] x 100%.

GD, gestational diet; RV, recommended vitamin; HV, high vitamin.
Figure 7.3.: A: Short-term food, B: protein, C: carbohydrate and D: protein energy intake following saline injection\(^1\).\(^2\).

\(^1\) Mean ± SEM, n = 6 – 9 means/group (each mean is the average intake following 2 saline injections received by each animal at 4 and 6 wk post-weaning). * Student’s unpaired t-test, \(P < 0.05\); ** \(P < 0.01\).

\(^2\) Saline intraperitoneally (1 mL/200g) 45 min prior to lights-out, or 0.5 mL 10 min prior to lights-out and access to 10% and 60% casein diets in rats adapted to a 12 h nocturnal feeding period.

\(a\) Protein Energy Intake (%) = [protein intake (g) x 4.0 (kcal/g)] ÷ [food intake (g) x 3.95 (kcal/g)] x 100%.

RV, recommended vitamin; HV, high vitamin.
Figure 7.4. Effect of tryptophan\textsuperscript{a} injection on A: food\textsuperscript{3}, B: protein\textsuperscript{4}, C: carbohydrate\textsuperscript{5} and D: protein energy\textsuperscript{6,\textit{b}} intake during a 1 h feeding period\textsuperscript{1,2}.

1 Mean ± SEM, n = 8 – 10 rats/group.
2 Macronutrient selection and food intake following saline and tryptophan injections were compared by two-way ANOVA with gestational diet and injection as main factors. * P = 0.05 by Tukey’s test.
3 Tryptophan (as methylester, 1 mL/200g, 75 mg/kg) or saline (1 mL/200g) was given intraperitoneally 45 min prior to lights-out and access to 10% and 60% casein diets in rats adapted to a 12 h nocturnal feeding period.
4 Food Intake: Week 4: GD (P = NS), Injection (P < 0.01), GD x Injection (P = NS). Week 6: GD (P = NS), Injection (P < 0.05), GD x Injection (P = NS).
5 Protein Intake: Week 4: GD (P = NS), Injection (P < 0.01), GD x Injection (P = NS). Week 6: GD (P = NS), Injection (P < 0.01), GD x Injection (P = NS).
6 Carbohydrate Intake: Week 4: GD (P = 0.09), Injection (P < 0.01), GD x Injection (P = NS). Week 6: GD (P = NS), Injection (P = NS), GD x Injection (P = NS).
7 Protein Energy Intake: Week 4: GD (P < 0.05), Injection (P = NS), GD x Injection (P = NS). Week 6: GD (P = NS), Injection (P = 0.05), GD x Injection (P = 0.09).
\textit{b} Protein Energy Intake (%) = [protein intake (g) x 4.0 (kcal/g)] ÷ [food intake (g) x 3.95 (kcal/g)] x 100%.
GD, gestational diet; RV, recommended vitamin; HV, high vitamin.
Figure 7.5. Effect of mCPP<sup>a</sup> injection on <strong>A</strong>: food<sup>3</sup>, <strong>B</strong>: protein<sup>4</sup>, <strong>C</strong>: carbohydrate<sup>5</sup> and <strong>D</strong>: protein energy<sup>6,b</sup> intake during a 12 h feeding period<sup>1,2</sup>.

1 Mean ± SEM, n = 8 – 10 rats/group.
2 Macronutrient selection and food intake following saline and mCPP injections were compared by two-way ANOVA with gestational diet and injection as main factors. * P < 0.01 by Tukey’s test.
a mCPP (0.5 mL, 10 mg/kg) or saline (0.5 mL) was given intraperitoneally 10 min prior to lights-out and access to 10% and 60% casein diets in rats adapted to a 12 h nocturnal feeding period.

3 **Food Intake:** Week 4: GD (P = NS), Injection (P < 0.01), GD x Injection (P = NS). Week 6: GD (P = NS), Injection (P < 0.0001), GD x Injection (P = 0.06).
4 **Protein Intake:** Week 4: GD (P = NS), Injection (P < 0.01), GD x Injection (P = NS). Week 6: GD (P = NS), Injection (P < 0.05), GD x Injection (P < 0.01).
5 **Carbohydrate Intake:** Week 4: GD (P = NS), Injection (P < 0.05), GD x Injection (P = NS). Week 6: GD (P = NS), Injection (P < 0.01), GD x Injection (P = NS).
6 **Protein Energy Intake:** Week 4: GD (P = NS), Injection (P = NS), GD x Injection (P = NS). Week 6: GD (P = NS), Injection (P = NS), GD x Injection (P < 0.05).
b Protein Energy Intake (g) = [protein intake (g) x 4.0 (kcal/g)] ÷ [food intake (g) x 3.95 (kcal/g)] x 100%.
GD, gestational diet; RV, recommended vitamin; HV, high vitamin.
CHAPTER 8.

GENERAL DISCUSSION


CHAPTER 8. GENERAL DISCUSSION

8.1 OVERALL FINDINGS

The results of this research support the hypothesis that high multi-vitamin intake during pregnancy (10 times as recommended in the AIN-93G diet) in rats causes compromised regulations of body weight and food intake, and results in a higher risk of developing characteristics of the metabolic syndrome in the offspring. However, sex, weaning diet composition, and the presence of diet choice altered the outcomes. Male rats born to dams fed the HV diet during pregnancy and weaned to a regular diet (RV, Study 1), an obesogenic liquid diet (Ob diet, Study 2), or a diet with 1/3 the vitamin content as the RV diet (1/3RV, Study 3) exhibited higher post-weaning weight gain, food intake and compromised glucose-insulin metabolism in adulthood compared to those from control dams. Unlike males, female offspring exhibited exaggerated weight gain and metabolic dysfunction due to the HV diet only when they were weaned to the Ob diet in Study 2. In contrast, in Study 4, males born to HV dams and weaned to the nutrient selection paradigm (a choice of high and low protein diets) exhibited a healthier phenotype, with a lower weight gain, food intake and improved glucose tolerance compared to those born to control dams. Presently, the mechanisms by which the multi-vitamin supplementation during pregnancy induced the in utero programming of adult diseases in the rat offspring are unclear.
8.2 EVIDENCE OF MULTI-VITAMIN SUPPLEMENTATION DURING PREGNANCY INDUCES PROGRAMMING OF CHRONIC DISEASES IN THE RAT OFFSPRING

The strongest indication of in utero programming of chronic diseases by the gestational vitamin supplementation was the distinctive development of appetite and metabolic dysfunctions in the offspring over time. The programming effect did not impose an immediate disease state, but instead promoted the components of metabolic syndrome and excess weight gain slowly over the post-weaning study period [150, 290]. However, two lines of evidence of early impact of the HV diet on regulatory systems were observed. One line of evidence of in utero programming of obesity and hyperphagia was the early signs of altered regulation of food intake in the offspring. In Study 1 to 3, within one week of weaning, males born to HV dams consumed more food in 1 hour after an overnight fast, which suggests that they were programmed to consume more food at an early age. Also, the fasting concentration of ghrelin, an orexigenic hormone [161], was 27% higher in male pups born to HV dams at weaning, which is consistent with early hyperphagia in the offspring. Fasting GLP-1 concentration at weaning was also increased in the male pups born to HV dams. Although GLP-1 is recognized as an anorexigenic hormone, it also plays a crucial role in glucose metabolism. Elevated levels of GLP-1 at fasting have been recognized as an early indicator of hyperglycemia and insulin resistance in adulthood [161], which is in parallel with the compromised glucose-insulin metabolism exhibited in the offspring born to HV dams [150, 290]. Overall, the early signs of hyperphagia led to higher weight gain and the onset of the compromised metabolic phenotypes at the early adulthood period (12 to 15 wk post-weaning), and persisted into adulthood (28 to 48 wk post-weaning).
A second line of evidence of *in utero* programming was the lower mRNA expressions of hypothalamic serotonin receptor (5-HT$_{1A/2A/2C}$) and POMC at weaning in male pups born to HV dams in Study 4, which may explain the observed obesity and hyperphagia in the preceding 3 studies. 5-HT$_{2C}$ is expressed by POMC neurons, and both 5-HT$_{2C}$ and POMC regulate food intake and energy homeostasis through the direct neural circuitry between the serotonergic and melanocortin systems [309-311]. The lower POMC mRNA expression in pups born to HV dams at weaning persisted to 7 wk post-weaning, which suggests a lifelong disruption of the hypothalamic feeding regulatory system and compromised eating behaviour. Also, it has been shown that lower 5-HT$_{2A/2C}$ mRNA expressions were associated with hyperphagia and higher adiposity in male rats [307, 308, 331], which is consistent with the findings in this research.

Overall, there are some inconsistencies of the *in utero* programming across the four studies. The evidence gathered in the first three studies where HV diet during pregnancy leads to higher weight gain and components of the metabolic syndrome in males were not observed in Study 4, where the postnatal diet was switched from a single diet to a nutrient selection paradigm with two diets (10% and 60% casein diets). Thus the nutrient selection paradigm highlights the importance of the post-weaning diet on the programming direction of the gestational diet, which will be discussed in Section 8.3 below. Also, the sex of the offspring was a significant factor on the final outcome of the gestational vitamin intervention, which will be discussed in Section 8.4.
8.3 THE ROLE OF POSTNATAL DIET ON FETAL PROGRAMMING

As shown in the current research, postnatal diets can exacerbate or change the effects of *in utero* programming in the rat offspring. Specifically, the Ob diet (Study 2), the low vitamin 1/6RV diet (Study 3), and the nutrient selection paradigm (NSP, Study 4) highlighted the importance of employing the appropriate diet to test the hypothesis of each study.

8.3.1 Obesogenic Liquid Diet

One of the most important findings of this research was the exaggerated development of obesity and metabolic syndrome in female offspring weaned to the Ob diet but not the AIN-93G diet after their prenatal exposure to a high-vitamin environment [290]. When weaned to the Ob diet, females born to HV dams exhibited significantly higher weight gain, food intake, insulin resistance and systolic blood pressure compared to those born to control dam. At the end of the 48 weeks post-weaning period, the Ob diet produced the obesity phenotype in all the male and female offspring, independent of the gestational diets. Nevertheless, the gestational HV diet further amplified the development of obesity and metabolic syndrome in these Ob diet-fed rat offspring. Also, among all the gestational and post-weaning diet combinations in the four studies, the HV-Ob offspring had the heaviest body weight at all post-weaning times, averaging approximately 1.3 kg at one year of age [290].

8.3.2 Low Vitamin Diets

The rationale for feeding the offspring with low vitamin diets was to test out the Predictive Adaptive Responses hypothesis, which states that when there is a mismatch
between prenatal (e.g. high vitamin) and postnatal (e.g. low vitamin) nutrient conditions, the offspring will have a higher chance of developing chronic diseases in adulthood [32]. In Study 3, two post-weaning low vitamin diets, 1/3RV and 1/6RV diets, were used to test the validity of the PARs hypothesis.

However, the 1/3RV diet did not amplify or change the effect of the gestational HV diet on the male offspring. Males born to HV dams and fed either the RV or 1/3RV diet gained more weight, consumed more food and had higher glucose intolerance compared to those born to RV dams. Notably, the 1/3RV diet did not affect weight gain nor food consumption in the male offspring. This observation was particularly important because it suggests that either male rats can maintain their normal growth at 33% of their normal vitamin requirement, or that the AIN-93G diet has more vitamins than needed for growth (Table 6.2) [220]. Obviously, the latter must be the explanation because the estimated requirements of rats for growth are not quantitative [220].

Because of the lack of response to the 1/3RV diet, the 1/6RV diet was used to test the PARs hypothesis. In males, the post-weaning 1/6RV diet negated the weight gain caused by the gestational HV diet, and led to lower daily food intake and glucose response (after an oral glucose load) compared to males weaned to the RV diet. Thus, these results in males reject the PARs hypothesis, and suggest that the 1/6RV diet was actually beneficial to male offspring exposed to in utero high vitamin environment.

In contrast, in females, the 1/6RV diet did not affect weight gain nor daily food intake, but affected metabolic regulation adversely. At 30 wk post-weaning, females weaned to the 1/6RV diet and born to HV dams had two-fold the glucose response compared to those born to control dams. Also, these females exhibited the lowest insulin response during the glucose
tolerance test, which is an early sign of diabetes development along with the observed hyperglycemia [295, 296]. Therefore, the results in females partially agreed with the PARs hypothesis, where the compromise in glucoregulation due to the gestational HV diet was exaggerated by the 1/6RV diet, but no difference was observed in overall weight gain and food intake.

At present, there is little explanation for the effects of the low vitamin diets on the expression of the metabolic programming effects of the gestational HV diet. However, it is clear that the low vitamin (1/6RV) diet is a factor and that the PARs hypothesis was of little merit, at least for these rats in the present studies and that the interaction between gestational and pup diets is much more complex than that proposed in the PARs hypothesis. In contrast, according to the PARs hypothesis, the effect of the gestational HV diet is minimized in pups by matching the vitamin content of the pup diet with the gestational HV diet. Preliminary data support this assumption in which pups born to HV dams and weaned to a HV diet did not differ in weight gain at 17 weeks post-weaning compared to the pups from RV dams (660 ± 14 vs. 664 ± 20 g, P = 0.9) (Ph.D. thesis, Clara Cho). Further investigation is necessary to determine the conditions required to amplify or reverse the effects of the gestational HV diet by varying the vitamin content of the pup diet, and the mechanisms behind the yielded phenotypes of the offspring.

8.3.3 Nutrient Selection Paradigm

Among the different postnatal diets used, the effect of the post-weaning NSP in Study 4 was the most unexpected and contradicted the previous three studies. Male pups placed on the NSP after weaning and born to HV dams had lower weight gain, adiposity, food intake,
and better glucose metabolism at 7 weeks post-weaning compared to those born to control dams. The sole presence of the choice of 10% and 60% protein casein diets (with equal caloric densities) interacted with the in utero programming effect of the gestational HV diet on macronutrient selections, especially for protein. The gestational HV diet programmed the offspring to have lower mRNA expression levels of hypothalamic serotonin receptors (5-HT$_{1A/2A/2C}$) and POMC at weaning, which are correlated with the development of obesity and adiposity in animal studies [307, 331]. However, after placing the pups on the NSP, the opposite and healthier phenotype emerged. Therefore, similar to the low vitamin diet (1/6RV) in Study 3, the NSP can be a post-weaning dietary strategy to ameliorate the compromised metabolic effects programmed by the high vitamin intake during pregnancy.

8.4 THE ROLE OF OFFSPRING SEX ON FETAL PROGRAMMING

Another confounder in interpreting the effect of the gestational diets on the offspring is illustrated by the effect of sex of the offspring in their responses to HV diet fed to the dams. In the first three studies, both male and female offspring were used. The male offspring exhibited more signs of appetite and metabolic dysregulation after exposure to the high vitamin in utero environment compared to females. This sex-specific difference is consistent with other fetal programming studies [123, 247, 332, 333], and it is crucial that examination of mechanisms of the effects of the gestational HV diet need to take the sex of the offspring into consideration.

To date, no studies, with the exception of hypertension, have focused on the sex-specific discrepancies on the mechanisms of fetal programming. Female, but not male offspring, born to lard-fed dams during pregnancy exhibited elevated systolic and diastolic
blood pressure compared to those from control dams [123]. Similarly, it has been suggested that maternal low-protein diet in rat pregnancy programs blood pressure through sex-specific mechanisms, because renal angiotensin-2 receptor mRNA expression was down-regulated in female offspring only [334].

Additional mechanisms to explain these sex-specific mechanisms maybe through the examinations of growth rate, adiposity and sex hormone interactions with those regulatory mechanisms for food intake and metabolism. Males are more susceptible to disturbance in maternal nutrition possibly because of their faster postnatal growth rate and higher nutrient demand [22, 247]. Male rat offspring had a higher insulin secretory response to glucose and higher susceptibility to develop insulin resistance if they were born to dams fed a low-protein diet during pregnancy and lactation [247, 335]. In contrast, female rats have been shown to develop insulin resistance later in life than males [248]. However, the mechanism behind this sex-specific discrepancy on obesity and metabolic dysfunction has not been fully elucidated.

8.5 STUDY DESIGN: STRENGTHS AND WEAKNESSES

The main goal of this thesis was to establish the effects of feeding a HV diet during pregnancy on the metabolic phenotype of the offspring. For that purpose the study design was adequate, although it had some disadvantages. The following discusses the advantages and disadvantages of the study design in detail.
8.5.1 Design Strengths

The strongest strength of the study design was the simplicity of the dietary intervention, which was the 10X vitamin mix supplementation during pregnancy in Wistar rats. This simple dietary manipulation led to a number of distinctive metabolic dysfunctions in the offspring, notably obesity and hyperglycemia in male offspring. A series of studies with this backbone design were subsequently performed (refer to Future Directions in Chapter 10 for ongoing studies originating from this thesis).

The repeated measures of the dependent variables in the offspring after weaning were another strength of the design. By measuring metabolic indices, such as glucose response during OGTT and 1-hour food intake, every 3 to 4 weeks after weaning, the time of the onset of components of the metabolic syndrome were identified.

Another advantage was the robustness of the experimental unit [239]. Many studies in this field of research failed to consider that the unit of measure is the dam and not the offspring, and only a very few number of dams (n = 2 – 3 per maternal dietary group) were used to carry out the experiment. Although the outcomes are measured in the offspring, the treatment is applied through random assignment of the pregnant dam to different gestational diets. It is crucial to consider that animals within a litter tend to develop more similarly to each other than those from another litter, and thus there is a potential for correlation among observations within a litter [239]. Also, the maternal variable, such as the health of the dams, may affect the long-term outcome in the offspring, which can generate statistical bias if not accounted properly. All the experiments in the current thesis utilized at least 10 pregnant dams per gestational diet group to ensure the observed effects in the offspring were not due to a few individual dams. One male and one female from each litter were randomly selected to
make up a group of 10 experimental units of pups. Thus, each pup was a representation of the litter, and the variance within each litter was further lowered.

### 8.5.2 Study Limitations

A number of limitations were embedded in the overall study design, such as the multi-vitamin supplementation itself, the dose at 10-fold the recommendation, the duration of diet intervention during pregnancy, and the lack of metabolic measurements of the dams during pregnancy.

The supplementation level at 10 times the recommended intake of AIN-93G diet has been raised as a weakness of the research. Reviewers of the first two published studies have raised the concern that 10 times the recommended vitamin intake level may be too high and caused stress in the dams or pups. However, we have shown that elevated stress was highly unlikely because neither litter size, birth weight nor corticosterone level at weaning were different compared to those on a regular gestational diet [150, 290]. Nevertheless, a dose response study where vitamin intakes at 1, 2, 5 and 10 times the recommended intake during pregnancy will be useful to determine the expression and magnitude of the components of the metabolic syndrome and obesity in the offspring.

Another confounding factor for interpreting the results and timing of in utero effect was the duration of the vitamin supplementation. The dams were only exposed to the HV diet from day 3 of fertilization to birth. Most studies that looked at the effects of maternal diets on the offspring fed the diets before fertilization and through gestation and lactation. Neither approach, however, allows one to identify the critical period of exposure. Furthermore, the multi-vitamin mix was supplemented to the dams during pregnancy only,
the pups were likely to consume milk with a higher than normal vitamin content, especially
the fat-soluble vitamins. Fat-soluble vitamins (vitamin A, D, E and K) can be stored in
maternal tissue and thus potentially mobilized and transferred through the milk to the pups.
As well the pups may have accumulated stores of the fat-soluble vitamins in the liver and in
the very limited fat stores.

A final missed opportunity of the research was the lack of food intake and metabolic
measurements of the dams during pregnancy. Such observations are required to determine
whether the effect of the gestational HV diet in the offspring was due to a change in the
metabolism of the dams, or a direct effect on offspring without affecting the dams [207, 336].
Unpublished data from our laboratory have shown that dams fed the HV diet during
pregnancy gained 8% more weight 15 weeks after delivery compared to pregnant dams fed a
control diet (Sandra Reza-Lopéz, Ph.D. thesis). Also, pups born to these dams fed control
diets during the second pregnancy also gained more weight in the post-weaning period. Thus,
it warrants further investigation to identify the underlying mechanism of in utero
programming induced by gestational HV diet.

8.6 IMPLICATION OF THE RESEARCH

In humans, with the liberalized fortification policies and an increasing abundance of
vitamin supplements in the market, it is not uncommon for pregnant women to have a
multiple-fold intake of vitamins through balanced diets and daily use of multi-vitamin
supplementation [11, 17, 337]. It was reported that the intake by pregnant women in Boston,
USA, particularly by those in the upper third quartile, ranged from 2 to 7 times the
recommended dietary allowance (RDA) for 10 vitamins [111]. However, neither the
presumed benefits nor the potential adverse effects of the high intakes of vitamins during pregnancy have been established in the offspring later in life. In fact, the 2010 Dietary Guidelines for Americans Committee (DGAC) recently published a report dismissing the use of dietary supplements among the healthy population [338]. The report stated that a daily multi-vitamin/mineral supplement does not offer health benefits to healthy Americans, and in some settings, these supplements have been associated with harmful effects.
CHAPTER 9.

SUMMARY AND CONCLUSIONS
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9.1 SPECIFIC CONCLUSIONS

Study 1: High multi-vitamin intake during pregnancy in Wistar rats programmed the male offspring for the development of the components of the metabolic syndrome in adulthood, possibly by its effects on central mechanisms of food intake control.

Study 2: The post-weaning obesogenic liquid diet amplifies the phenotypic expression of obesity and components of the metabolic syndrome in both female and male rats born to dams fed a diet supplemented with multi-vitamins.

Study 3: The exposure of rat offspring to post-weaning low vitamin diets does not amplify the expression of characteristics of the metabolic syndrome observed in male offspring born to dams fed high multi-vitamin diets. In female offspring born to HV dams, however, the low vitamin diet led to a compromised glucose-insulin metabolism in adulthood.

Study 4: High multi-vitamin intake during pregnancy affects body weight, food intake, macronutrient choice and expression of the serotonergic and the melanocortin systems in the hypothalamus of Wistar rats.

9.2 GENERAL CONCLUSION

The results of this research support the hypothesis that multi-vitamin supplementation during pregnancy in Wistar rats leads to compromised regulation of body weight, food intake
and metabolic functions in the offspring. In addition, post-weaning diets and sex of the
offspring interact with the effect of gestational HV diet, resulting in compromised
physiologic and metabolic outcomes in offspring later in life.
CHAPTER 10.

FUTURE DIRECTIONS
This research provides novel and interesting observations, but has not identified the mechanisms by which multi-vitamin supplementation during pregnancy affected the long-term health of the Wistar rat offspring. The observational data generated in this research raise a few important questions with respect to the mechanisms of metabolic programming by vitamins during pregnancy that warrant further investigation. Some of the issues that need to be addressed in the future include:

1. Which vitamin or combinations of vitamins are responsible for the observed programming effect in the offspring?

The primary candidates responsible for the observations in this thesis are assumed to be the methyl-metabolism cofactors (folate, vitamin B₆ and B₁₂) that involve in the one-carbon metabolism and the epigenetic regulation of DNA methylation [130, 131]. These specific vitamins are involved in the methyl transfer cycle, and facilitate binding of methyl groups to gene promoters. Ultimately, a number of genes that regulate body weight, food intake and metabolism may be over- or under-expressed due to the methyl binding, and affect phenotype of the offspring.

Fat soluble vitamins, especially vitamin A and D, play a role in gene expressions and hormone-like actions in the fetal development. Thus these fat-soluble vitamins should be further examined individually for their role in in utero programming of adult diseases. It is necessary to determine which vitamin or combination of vitamins are responsible for the
observed compromised metabolic phenotype in this research, and warrants further investigation to explore the mechanism behind the findings.

2. **Can the effects in the offspring due to the gestational HV diet at 10 times the recommended vitamin intake be observed under a lower multi-vitamin dose?**

   Lower doses of the multi-vitamin supplementation, such as 2 and 5 times the recommended vitamin intake, should be used to determine if any of the metabolic dysfunctions observed in this thesis will appear, and to what magnitude the dysfunction manifests. A pilot study was performed in CD-1 mice offspring born to dams fed the recommended, 2.5-fold or 5-fold the vitamin content during gestation (Daniel Cho, B.Sc. 4th year project, and Nobuhiko Okubo, M.Sc. thesis). The gestational diets led to no difference in birth weight, litter size, post-weaning weight gain and adiposity at 13 weeks post-weaning. However, it resulted in lower expression of the leptin receptor (LepR), agouti related-peptide (AgRP) and neuropeptide Y (NPY) at weaning in both male and female offspring. Also, at 13 wk post-weaning, LepR and NPY in males, AgRP in females, and pro-opiomelanocortin (POMC) in both sexes were lowered. This study suggested that *in utero* development of regulatory processes can be affected by modest increases in the vitamin content of the mouse diet during gestation without causing a change in weight gain and adiposity. Further investigation is needed to determine if prolonging the study would eventually lead to altered food intake and weight gain in these offspring.

3. **If the pup diet contains identical amount of vitamins as the gestational diet, would the compromised phenotype be cancelled out?**
This is another way to test out the Predictive Adaptive Responses hypothesis, in which the mismatch between prenatal and postnatal vitamin environment is eliminated by supplementing the pup diet with the same amount of vitamin as the gestational diet (as mentioned in Section 8.5.3). Preliminary data indicate that the weight gain caused by the HV diet during pregnancy is being cancelled out by the HV diet after weaning (Clara Cho, Ph.D. thesis). Further examinations will be necessary to describe the components of the metabolic syndromes, such as glucose-insulin metabolism, blood pressures, and adiposity.

4. Food intake data in this research have shown a possible change in the hypothalamic appetite regulation. How can future studies confirm this assumption?

In Study 4 of the current research, the mRNA expressions of hypothalamic serotonin receptors (5-HT1A/2A/2C) and POMC of the offspring born to HV dams were shown to be lowered. This is a solid indication that some alteration in the hypothalamic systems that govern energy homeostasis took place, which explains the observations of hyperphagia and weight gain in the studies.

Future studies conducted by Clara Cho and graduate students will look at the epigenetic alterations from DNA methylation in genes regulating metabolic phenotype. Also, future research will be needed to identify obesity and appetite regulating genes that have altered mRNA expression and protein level in the offspring born to HV dams. Candidate genes, such as NPY, AgRP, POMC, LepR and insulin receptor, will need to be measured in the hypothalamus of the offspring (real-time PCR). Furthermore, brain-derived neurotrophic factor (BDNF) and receptor mRNA for dopamine and serotonin from the hypothalamus will
also be measured to complement the findings and to confirm the questions that hypothalamic appetite regulation is altered by the multi-vitamin supplementation during pregnancy in Wistar rats.

5. **What is the approach to conduct similar studies in human subjects?**

There are two general approaches to determine if vitamin intake during pregnancy in humans affects the health of the offspring later in life. One approach is to conduct a retrospective study by surveying mothers of obese and non-obese adults aged 25 to 40 years old. Through food recall questionnaires, the mothers provide their supplementation habits during pregnancy, and associations between maternal vitamin intake and offspring health parameters can be drawn. However, this approach is inaccurate due to the recalling of supplementation habits from 25 to 40 years ago.

A second approach is to conduct a prospective cohort study by providing pregnant mothers a daily multi-vitamin supplementation of 50%, 100% and 200% the recommended daily allowance of all vitamins during pregnancy. Offspring will be followed to early adulthood to determine if they have a higher risk of developing obesity and components of the metabolic syndrome. This approach is both resource-intensive and time-consuming. Also, at a maternal supplementation level of 200% of the RDA of vitamins, metabolic or physiological changes in the offspring maybe difficult to detect, and higher doses may not be feasible due to ethical or possible toxicity issues.
CHAPTER 11.
REFERENCES
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