NUTRITIONALLY ADEQUATE PROTEIN SOURCES IN DIETS DURING GESTATION, LACTATION AND WEANING INFLUENCE FOOD INTAKE AND THE RISK OF CHARACTERISTICS OF METABOLIC SYNDROME IN OFFSPRING OF WISTAR RATS

by

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A thesis submitted in conformity with the requirements For the degree of Doctor of Philosophy

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ABSTRACT

The hypothesis that source of protein in a nutritionally adequate diet during gestation, lactation and weaning alters food intake and characteristics of metabolic syndrome in the offspring was investigated. Pregnant Wistar rats were randomized to either the AIN 93-G casein (C) or soy protein (S) diets (n=12/group) during gestation only or during gestation and lactation. Male offspring in each dams’ diet group were weaned to either C or S diets (n=12/group). Food intake, body weight (BW), fat pad mass, systolic (SBP) and diastolic (DBP) blood pressure, and plasma homocysteine (p<0.05) were higher in offspring born to dams fed the S diet. Fasting blood glucose (BG), BG in response to a glucose gavage and Homeostatic Model of Assessment of Insulin Resistance (HOMA-IR) index were higher only in male offspring born to dams fed the S diet. Moreover, gene expression of Agouti Related Protein (AgRP) was higher in offspring born to dams fed the S diet at weaning. Extending the dams’ diet during gestation and lactation magnified the effect of the gestational S diet on BW and composition and glucose metabolism in male offspring. Although composition of the weaning diets interacted with that of the dams’ diets, the latter was the dominant factor in determining metabolic outcomes in the offspring. In conclusion, the soy protein diet, compared to the casein diet when consumed during gestation or throughout gestation and lactation increased food intake and the presence of characteristics of metabolic syndrome in the offspring.
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LIST OF ABBREVIATIONS

AgRP: Agouti Related Protein
Alpha-MSH: α- Melanocyte-stimulating hormone
ANOVA: Analysis of Variance
ARC: Arcuate Nucleus
AUC: Area Under the Curve
BAP: Biologically Active (or Bioactive) Peptides
BBB: Blood Brain Barrier
BCAA: Branched Chain Amino Acids
BG: Blood Glucose
BP: Blood Pressure
BW: Body weight
C: Casein
CART: Cocaine-Amphetamine Related Transcript
CCK: Cholecystokinin
cDNA: Complimentary Deoxyribonucleic Acid
CHO: Carbohydrate
CRH: Corticotropin Releasing Hormone
CNS: Central nervous system
d: Day
DBP: Diastolic Blood Pressure
Delta: Change in food intake
DMN: Dorsomedial nucleus
DPP-IV: Dipeptidyl Peptidase-IV
EDTA: Ethylene Diamine Tetraacetic Acid
EIA: Enzyme Immunoassay
ELISA: Enzyme-Linked Immunosorbent Assay
Exp: Experiment
FI: Food intake
Fig: Figure
FPM: Fat Pad Mass
g: Grams
GABA: Gama amino Butyric Acid
GAPDH: Glyceraldehyde-3-Phosphate Dehydrogenase
GI: Gastro-intestinal tract
GLP-1: Glucagon-Like Peptide-1
GMP: Glycomacropeptide
GTT: Glucose Tolerance Test
h: Hour
Hcy: Homocysteine
HOMA-IR: Homeostatic Model of Assessment of Insulin Resistance
IGF-1: Insulin growth factor-1
ip: Intraperitoneal
IPP: Isolucine-proline-proline
ITT: Insulin Tolerance Test
kg: Kilograms
M: Moles
mg: Miligrams
min: Minutes
ml: Mililiters
mM: Milimoles
mRNA: Messenger Ribonucleic Acid
ng: Nanograms
NPY: Neuropeptide-Y
NS: Not significant
P: Preload
PAR: Predictive Adaptive Response
PCR: Polymerase Chain Reaction
pM: Picomoles
POMC: Pro-opiomelanocortin
PPARγ: Peroxisome proliferator-activated receptor γ
PVN: Paraventricular Nucleus
PYY: Peptide YY
RIA: Radioimmunoassay
S: Soy protein
SBP: Systolic Blood Pressure
SEM: Standard Error of the Mean
T: Time
$C_T$: Threshold Cycle

VMH: Ventromedial Nucleus of the Hypothalamus

VMN: Ventromedial Nucleus

VPP: Valine-proline-proline

Wk: Week (s)
LIST OF PUBLICATIONS AND PRESENTATIONS

ARISING FROM THESIS

**Peer Reviewed Publications:**

Jahan-mihan A, Szeto IMY, Luhovyy BL, Huot PSP, Anderson GH. Soy Protein and Casein Based Nutritionally Complete Diets Fed during Gestation and Lactation Differ in Effects on Characteristics of Metabolic Syndrome in Male Offspring of Wistar Rats (In second revision; British Journal of Nutrition)

Jahan-mihan A, Smith C, Anderson GH. Soy Protein and Casein Based Weaning Diets Differ in Effects on Food Intake and Blood Glucose Regulation in Male Wistar Rats (In press: Nutrition Research)

Jahan-mihan A, Smith C, Anderson GH. The Effect of Nutritionally Adequate Protein Sources in Maternal Diets on Development of Intake Regulatory System in Male Offspring of Wistar Rats. (In press: Am J Physiol Regul Integr Comp Physiol)

Jahan-mihan A, Smith C, Anderson GH. The Effect of Nutritionally Adequate Protein Sources in Maternal Diets on Characteristics of Metabolic Syndrome and Food Intake Regulation in Female Offspring of Wistar Rats (Submitted: Nutrients)

**Abstracts:**


**Oral Presentations:**

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CHAPTER 1

INTRODUCTION
1. INTRODUCTION

Metabolic syndrome is a cluster of metabolic disorders which may progress to type two diabetes mellitus and cardiovascular disease. It is classically defined as a combination of central obesity and insulin resistance plus any two of the following four factors: raised triglycerides, reduced HDL, raised blood pressure and raised fasting plasma glucose [1]. The prevalence of metabolic syndrome and obesity has been increasing since the mid-20th century [2]. Moreover, metabolic syndrome affects between 24% and 34% of the US population and up to 36% of Europeans aged 40-55 [3, 4]. However, the pathophysiology of metabolic syndrome is not fully understood. The overarching role of impaired insulin resistance and central obesity has been suggested [5]. More recently, an association between the pre-natal environment and the risk of metabolic syndrome in adulthood has been reported [6, 7].

Numerous epidemiological and clinical studies plus investigations in animal models provide substantial evidence that fetal and early post-natal nutrition alters development of somatic structures, endocrine systems and homeostatic mechanisms in the fetus and infant. These effects influence in later life the risk of obesity, hypertension, diabetes and other components of metabolic syndrome [8-10]. The role of early ontogeny to later life health has been named fetal programming, which is defined as the process whereby a stimulus during a critical period of early development results in long-term physiological consequences [11, 12]

The role of inadequate and excessive protein content of the maternal diet in health outcome of the offspring has been examined [12-16]. Both excessive and inadequate
amounts of protein during pregnancy influence body weight, blood pressure and metabolic and intake regulatory systems in the offspring. Low protein maternal diets increase blood pressure [14, 17], body weight [13] and adiposity [17] whilst high protein maternal diets increase body weight [18], blood pressure and food efficiency [19] and decrease energy expenditure [20, 21] in offspring of rats. However, the role of the source of protein in a nutritionally adequate diet during pregnancy, lactation and weaning has not been reported.

Casein and soy protein are two common sources of protein in the diet. In addition, milk-based and soy protein-based formulas are consumed by many infants. Although both proteins have been classified as high quality [22], they differ in their characteristics including amino acid composition, bioactive peptides, digestion kinetics and also their non-protein bioactive components [23, 24]. All of these are potential factors affecting the epigenome leading to modification of gene expression and ultimately the phenotype of the offspring.

Therefore, the objective of this study was to compare the effect of casein and soy protein as sole sources of protein in a nutritionally adequate maternal diet fed either during pregnancy alone and/or during pregnancy, lactation and after weaning on body weight, body composition, food intake, glucose homeostasis and blood pressure in the dams and in their offspring.
CHAPTER 2

LITERATURE REVIEW
2. LITERATURE REVIEW

2.1 INTRODUCTION

This review examines the current literature related to the nutritional consequences of maternal diets on characteristics of the metabolic syndrome in the offspring. In the first section, the concept of the fetal programming of chronic diseases and proposed hypotheses are reviewed. In the second section, the role of nutritional content of maternal diets on the offspring in later life is summarized. The third section focuses on the role of protein content of the diet on fetal and early post-natal programming. The fourth section addresses the physiological and metabolic characteristics of proteins and their potential mechanisms of action which may impact on fetal development and phenotype of the offspring.

2.2 FETAL PROGRAMMING

The notion that the intrauterine environment may influence the development of the fetus is not novel. However, the concept that fetal development impacts on adult diseases has arisen relatively recently and led to a revival of interest in the influence of the in utero environment on the fetus and neonate [25]. Developmental plasticity allows the fetus to adapt tissue structure in response to environmental changes. The long-term post-natal consequences of developmental plasticity have been described by a number of terms including programming [26] and metabolic imprinting [27]. Programming is described as any situation where a stimulus or an insult during development induces a
permanent physiological response [26] whilst, metabolic imprinting covers adaptive responses to specific nutritional conditions in early life that are characterized by a susceptibility limited to a critical developmental window in early life, a persistent effect lasting through adulthood, and a specific and measurable outcome [28].

2.2.1 Fetal Programming: Background

In 1934, Kermack et al. reported a fall in death rates from all causes between 1751 and 1930 in the United Kingdom and Sweden [29]. Better childhood living conditions during this period was suggested as the reason. Thereafter, Wadsworth et al. [30] found an inverse relationship between adult blood pressure and birth weight in men and women born in 1946 in the United Kingdom. In 1986, Barker and colleagues reported that the geographical distribution of mortality rates from stroke and cardiovascular diseases in England and Wales in 1968–1978 was closely related to neonatal mortality in 1921–1925. It was concluded that environmental factors, particularly nutrition during pregnancy and early life influence the risk of chronic diseases in later life.

2.2.2 Fetal Programming: Proposed Hypotheses

A plethora of hypotheses have been proposed to explain how environmental factors influence the developmental pattern in pre- and early post-natal periods that persists in later life. These are summarized in the following. First, the “early” or briefly “fetal” origins of adult disease hypothesis proposes that adulthood hypertension, insulin resistance and dyslipidemia occur due to adverse intra-uterine conditions and low birth weight [31, 32]. Hence, it has been suggested that the metabolic syndrome be renamed “the small baby syndrome” [33]. Second, the ‘thrifty phenotype hypothesis’ proposes that in response to a suboptimal fetal environment, metabolic adaptations occur to maximize
chances of surviving in conditions of post-natal food deprivation [34]. These adaptations can be beneficial if the poor conditions are continued, however, if the postnatal environment consists of a plentiful food supply, the risk of developing characteristics of the metabolic syndrome will be increased [35, 36]. This interaction between in-utero and post-natal environment has been captured in the Predictive Adaptive Response (PAR) hypothesis. According to PAR hypothesis, offspring weaned to similar diets to their mothers will adapt more appropriately to their environment than those that receive an unmatched diet [36]. Third, the catch-up growth hypothesis recognizes that there are interactions between early development and later nutritional exposure. Those that are growth restricted in the pre-natal period show a rapid post-natal increased adiposity in childhood and later adult life, associated with insulin resistance [37-39]. Finally, the fetal insulin hypothesis suggests that both low birth weight and insulin resistance may be mediated by the same inheritable genes [40] because insulin plays an important role in fetal development [41]. Similarly, the “fetal salvage” hypothesis proposes an adverse intrauterine environment influence programming of endocrine pathways, leading to permanent metabolic changes.

2.3 MATERNAL NUTRITION AND FETAL PROGRAMMING

Numerous epidemiological and clinical studies plus investigations in animal models provide substantial evidence that fetal and early post-natal nutrition alters development of somatic structure, and many regulatory systems in the fetus and infant. Many nutritional factors play a role in fetal programming including individual nutrients, energy restriction, inadequate diets and timing of insult during pregnancy or post-natal.
The role of under- and over-nutrition during pregnancy on fetal programming has been investigated in both human and animal experiments.

In the following the effects of under- and over-nutrition during pregnancy of humans and animal are reviewed. The effects of under- and over-nutrition of diets, energy and micronutrients are briefly reviewed first. However, because the focus of this thesis is on protein a later section is dedicated to a more detailed examination of the effects of over- and under-nutrition of protein, the possible mechanism by which it may affect the offspring and the rationale for the research contained in this thesis.

2.3.1 Human Studies

2.3.1.1 Under-nutrition during Pregnancy and Fetal Programming

A majority of studies have investigated the effect of under-nutrition at different stages of pregnancy. In humans, the effect of energy restriction during pregnancy on chronic diseases of the offspring depends on when it occurs during pregnancy and the post-natal environment. For example, during the Dutch famine which occurred at the end of the World War II, daily caloric intake was restricted to 400–800 kcal. Exposure of pregnant women during late gestation was associated with increased adult obesity, glucose intolerance and increased fasting pro-insulin by 50 years of age in both male and female offspring [42]. Exposure early in gestation resulted in hypertension, an exaggerated atherogenic lipid profile, higher fibrinogen concentrations and higher BMI [42, 43]. In contrast, data from individuals conceived during the siege of Leningrad did not show any association of birth weight with adult glucose homeostasis [44]. This observation has been suggested to support the predictive adaptive hypothesis. That is,
nutritional status both before and after the famine periods in Leningrad was poor and may therefore have been appropriate for the post-natal environment experienced. In contrast, under-nutrition during pregnancy followed by adequate post-natal nutrition and subsequent catch-up growth during early childhood observed in Dutch families may explain the chronic diseases found in later life.

The role of micronutrient deprivation on fetal programming of chronic diseases in humans has received little study. However, it is of importance as shown by studies of the effect of supplements in deficient populations. Offspring born to calcium supplemented mothers had lower blood pressure at 7 years of age compared with offspring born to mothers given placebo [45]. Similarly, in a prospective cohort study, an inverse relationship was found between blood pressure in offspring at 6 months of age and the intake of calcium from supplements by their mothers during pregnancy. Offspring born between January and March (lower sun exposure) had lower bone mineral content than offspring born between July and September (higher sun exposure), suggesting that vitamin D status of the mothers 6 months prior to parturition can also affect characteristics of bone in adulthood [46]. Folate deficiency during pregnancy leads to low birth weight and neural tube defects. An increased risk of hyper-homocysteinemia due to lower level of folate as caused by polymorphisms in the methylene tetrahydrofolate reductase (MTHFR) gene has been observed [47].

In summary, it is clear that both macro- and micro-nutreint deprivation during pregnancy has detrimental effects on human offspring. However, the effect of over-nutrition during pregnancy on risk of chronic disease in the offspring has received much less attention.
2.3.1.2 Over-nutrition during Pregnancy and Fetal Programming

Epidemiological evidence indicates an association between increased nutrient supply before birth and later obesity. However, few clinical studies have examined the role of gestational over-nutrition on fetal programming, limited perhaps by ethical considerations.

Intrauterine exposure to maternal obesity is associated with an increased risk of metabolic syndrome [48] and obesity [49] in later life. Obesity and morbid obesity in mothers have been associated with gestational hypertension, preeclampsia, gestational diabetes (GDM), and high fetal birth weights greater than 4000 g. GDM results in hyperglycemia and hyperinsulinemia in the fetus during late development and higher risk of obesity in later life compared to infants of nondiabetic mothers [50, 51]. Obesity during pregnancy may also influence fetal growth and post-natal outcomes independent of GDM [48, 52]. It has been suggested that in obese mothers without clinical signs of GDM, fetal hyperinsulinemia may occur due to maternal mild hyperglycemia which is below the threshold as defined for GDM.

Few studies have examined the effect of high mineral or vitamin intake during pregnancy on the metabolic phenotype of the offspring. In observational studies, maternal calcium intake from foods [53] and supplements [54], or both [55] was not found to be associated with BP. In one experimental study, no effects of calcium supplementation during pregnancy were observed on offspring BP [56]. However, in another RCT, maternal calcium supplementation was associated with a decrease in SBP at 7 y [45]. This study also reported an interaction between calcium supplementation and child body mass index (BMI) on both systolic and diastolic BP. Maternal calcium supplement
decreased the risk of high systolic and diastolic BP in children with BMI<17.5 compared with those who were born to mothers who had not taken calcium supplements, but these results were not replicated in observational studies [53, 54]. Maternal calcium supplementation has been reported to lower the risk of preeclampsia in pregnancy [57].

In summary, obesity during pregnancy has adverse effects on the offspring but studies of the consequences of high mineral or vitamin intakes during pregnancy by women are few and not conclusive.

2.3.2 Animal Studies:

Animal models have been used to examine fetal origins of characteristics of the metabolic syndrome because of their shorter life span and also because of controlled genetic and environmental influences. The effects of diet manipulation during pregnancy in animals with short gestation periods (e.g. rats, mice and guinea pigs) and in animals with longer gestation period (e.g. sheep) have been examined.

2.3.2.1 Under-nutrition during Pregnancy and Fetal Programming

Many studies have used rodents to examine gestational dietary restriction ranging from mild (30%) [58] through moderate (50%) [59] to severe (70%) [60]. Moderate to severe restriction in rats generally results in low birth weight offspring [25]. Mild restriction during the first 18 days of pregnancy resulted in higher BP in offspring at 60 days after birth [58] and lower birth weight and impaired glucose tolerance [61]. Similar findings have been observed in guinea pigs [62-64].

However, it has been reported that a balanced reduction (a 50-70% decrease) in maternal nutrient intake produces a less consistent effect on later blood pressure than does the specific restriction of maternal protein intake [58-61, 65, 66]. In addition, the
effect of energy-restricted diets during pregnancy is sex-dependent. A 50% reduction in ad libitum food intake during pregnancy in Sprague Dawley rat dams resulted a higher food intake and body weight only in female offspring at 5 weeks of age [67].

The timing of the diet restriction is also a factor influencing programming during pregnancy. For example, although offspring born to ewes under-nourished only in early and mid pregnancy were not different in birth weight, they had a slower fetal growth trajectory and reduced activity of hypothalamic-pituitary adrenal (HPA) axis and increased insulin response to glucose later in life [68, 69]. In contrast, under-nutrition in late pregnancy resulted in lower birth weight with no changes on hypothalamic-pituitary axis activity in adulthood in sheep [70]. Rats fed an energy restricted diet (50% decrease) during the first 2 wk of pregnancy but re-fed during the third week, produced male offspring that developed significant hyperphagia and obesity when fed a high-fat diet [67, 71, 72]. Offspring born to dams fed 30% of control intake throughout the whole of gestation, were smaller throughout postnatal life, but had an increase in the relative mass of the retroperitoneal fat pad at 100 days of age [73]. Food intake in the offspring of the under-nourished rats was increased early in postnatal life and was amplified by postnatal hypercaloric nutrition [73].

It is clear therefore, that under-nutrition during pregnancy in animal models, as with humans has detrimental effects on health of the offspring. However in contrast to the few studies conducted in humans, sufficient studies have been conducted in animal models to show that excess intakes of energy and of nutrients have adverse effects on the offspring.
2.3.2.2 Over-nutrition during Pregnancy and Fetal Programming

Studies of the consequence of excess energy intake in animal models are challenging to achieve due to a robust homeostatic regulation of energy intake and expenditure in most animals. However, several approaches have been successful. Injection of gold thioglucose (GTG) to damage the intake regulatory system in the ventromedial hypothalamus has been used to develop hyperphagia in animals. Using this approach in ewes resulted in intake at 155% above maintenance energy requirements in late pregnancy and increased fetal glucose and insulin concentration and subcutaneous fat in lambs in early postnatal life [74]. In another study, pregnant ewes that ate 115% more that the control fed ewes gained more weight and produced low birth weight offspring [75].

Maternal obesity during pregnancy or gestational diabetes increases risk of obesity and or glucose intolerance in offspring [50, 76]. Mimicking the intrauterine environment experienced by infants of diabetic mothers by a 30-day intrafetal glucose infusion in fetal sheep in late gestation resulted in significant fetal hyperglycaemia, hyperinsulinaemia and fat deposition [77]. The expression of mRNA for the Neuropeptide Y (NPY) or Agouti-related protein (AGRP) was not affected but was significantly up-regulated for the appetite-inhibiting precursor molecule, Proopiomelanocortin (POMC), in the hypothalamic arcuate nucleus (ARC), suggesting that during development, the central nervous system is responsive to signals of increased nutrient supply before birth and makes adaptive responses in preparation for the predicted nutritional environment [78].
Although studies are few, high fat maternal diets also affect the offspring. A high fat diet (40% of total calories) during pregnancy and lactation increased birth weight in rats. It also caused higher body fat, liver weight, fat content of the liver and blood glucose and triglyceride levels in the offspring at weaning [79]. Although it did not alter insulin sensitivity at 16 weeks of age [80] an increased visceral adiposity was observed in the adult offspring born to mothers fed a high fat diet [81]. Similarly, offspring born to rat dams fed a cafeteria diet (high in fat and simple sugars) during pregnancy and lactation had greater adiposity and elevated insulin growth factor-1 (IGF-1) and IGF-1 receptors and peroxisome proliferator-activated receptor γ (PPARγ) mRNA levels at weaning [82]. Because they did not have a higher birthweight, it can be suggested that high birthweight is not a mandatory prerequisite of adverse effects of high fat diets in later life.

Vitamins added alone or in combination above requirements to maternal diets also impact on the offspring. Agouti mouse dams fed diets supplemented with vitamin B12 at 20-60×, and folate, betaine and choline at 3-9× recommended levels had litters with a higher proportion of pseudoagouti phenotypes and fewer with the agouti phenotype. [83-85]. Pseudoagouti offspring are brown and leaner and have lower food intake than the agouti siblings that are characterized by a yellow coat colour and obesity [86, 87].

More recent studies have shown that increased intakes of vitamins during pregnancy also impact on rodents with no known single gene defects. Diets with increased folic acid content fed to pregnant rats affect the offspring. Pregnant rats allocated to 4 groups (6 in each) and were fed casein diets either with 18 g protein/100 g diet (control diet) or with 12 g protein/100 g diet supplemented with 8 mg folic acid (FAS/MP), 12 g protein/100 g diet without folic acid (FAD/MP), or 12 g protein/100 g
diet (MP) with 2 mg folic acid. Male adult offspring born to dams fed the high folate (8 mg/100g diet), low protein diet had lower docosahexaenoic acid and n-6/n-3 ratio of fatty acids in brain and higher plasma corticosterone compared with control adult offspring and also compared with offspring born to dams fed a diet with 12 g protein/100 g diet supplemented with 2 mg folic acid, suggesting that maternal folic acid supplementation decreased brain docosahexaenoic acid levels probably arising from an increase in the stress hormone corticosterone [88].

High multivitamin content in diets fed during gestation also results in the development of characteristics of the metabolic syndrome in offspring of Wistar rats [7, 89]. High multivitamin intake (10× requirements) during gestation resulted in higher body weight, food intake, and fat pad mass at 31 weeks, fasting glucose, insulin and ghrelin at 17 weeks, and systolic blood pressure at 31 weeks and impaired glucose tolerance at 26 weeks in male offspring [7].

In rats, a perinatal high salt diet resulted in hypertension in Sprague-Dawley rats in one [90], but not in another study [91]. However, in female offspring of dams fed a HS diet the pressor and tachycardic responses to 1-h of restraint were significantly enhanced, and recovery after restraint was delayed [92]. Pressor and tachycardic responses are an increased blood pressure and/or heart rate in response to treatment respectively.

In summary, excess intake of energy and micro-nutrients during pregnancy has detrimental effects on health outcomes in the offspring of animals.
2.4 MATERNAL PROTEIN INTAKE AND FETAL PROGRAMMING

The focus of this thesis is on the role of protein in the maternal diet on the phenotype of the offspring. Therefore, the following discusses the effect of maternal diets both low and high in protein content, on development and later life characteristics of the offspring. This is followed by a discussion of the mechanisms proposed to be involved in fetal programming during conditions of under- and over-nutrition. Then, the characteristics of proteins, specifically composition and their metabolic consequences that may influence fetal programming are discussed.

Proteins are known to carry a wide range of nutritional and biological functions. Nutritionally they are sources of amino acids and energy and biologically, they contribute to various regulatory systems affecting food intake and appetite, glucose and lipid metabolism, bone metabolism, blood pressure and immune function. Physico-chemical properties, amino acid composition and bioactive peptides encrypted in amino acid sequences of proteins contribute to physiological functions of proteins.

The role of protein content of maternal diets during pregnancy and post-natally on the risk of the development of characteristics of the metabolic syndrome in offspring has been investigated. Both excessive and insufficient amounts of protein in maternal diets have adverse effects on offspring [12-16], and is discussed in the following.

2.4.1 Body Weight and Composition

Both low and high protein maternal diets have detrimental effects on body weight and body composition of offspring. In humans, an association between low protein intake during pregnancy and lower placental weight and birth weight was observed in offspring
of Caucasian women in Adelaide, Australia [93] and in Southampton, UK [55]. Similarly, offspring born to women on low protein diets due to restriction of milk during pregnancy (<250 ml/d) had significantly lower birth weight compared with women who consumed more than 250 ml/d milk [94]. Surprisingly, a high protein liquid supplement containing 40 g protein added to the diets of black women during pregnancy also resulted in lower birth weight in offspring compared to those born to mothers fed a low protein supplement (6 g as 7.5% energy) beverage [95]. The underlying mechanism for the latter effect is unclear at present but might be due to changes in placenta by increased number of villous capillaries relative to control and it might reflect increased metabolic activity and cellular proliferation secondary to the high density protein supplement.

Although both maternal low and high protein diets have been reported to increase body weight of rat offspring, their effect on birth weight is not consistent. Where low protein diets during pregnancy have led to low birth weight in most studies, high protein diets have been reported to result in lower, no effect or higher birth weight. When rat dams were fed a low-protein gestational diet (90 g/kg: 9% protein diet), fetal growth was enhanced until day 20 of gestation (term = 21 day gestation), followed by a period of growth restriction over the last 2 days of gestation such that the pups tended to be of low or low-normal birth weight [96]. No effect of maternal diet on birth weight, energy expenditure, glucose tolerance, and plasma lipid levels of the offspring was observed when dams were fed a high protein (40% of total calorie) compared to normal protein (20% of total calorie) diet during pregnancy and lactation. However, the high protein diet resulted in higher blood pressure and glomerulosclerosis in male offspring, whereas increased food efficiency, higher body weight, and increased fat pads characterized in the
female offspring [19]. In another study, offspring born to rat dams fed a high protein diet (40% of total calorie) during pregnancy resulted in lower body weight on day 2 of life than controls (20% of total calorie) and greater fat mass and decreased energy expenditure in offspring at wk 9 of age. Postnatal high protein diet alone had no effect on body composition or metabolic rate [21]. In mice, a low-CHO (16.5% of total calories), high protein (%26.9 of total calories), high-unsaturated-fat diet (HFP diet) fed before and during gestation and lactation resulted in higher birth weight. However, no difference was observed in body weight in later life in offspring between experimental groups [18]. In another study, high protein (40% of total calories) diet received during pregnancy and lactation resulted in higher body weight at the beginning of puberty, persisting until the end of the experiment (wk 22) but only in female offspring of rats [19].

Protein content of the diet also influences body composition in rats. In one study, a low protein diet during pregnancy increased body weight and absolute weight of brown adipose tissue [97]. In another study, when protein restriction was prolonged throughout gestation or immediately after birth, a reduction in skeletal muscle mass resulted [98]. Furthermore, fat pad mass was also higher at wk 22 in female offspring born to mothers fed a high protein maternal diet [19] whereas the postnatal high protein diet alone had no effect on body composition or metabolic rate [21].

As would be expected from the effects of feeding dams low protein diets on body composition of the offspring, metabolic consequences also result.

**2.4.2 Glucose Regulation**

The effect of low protein diets during pregnancy and early life on the development of glucose intolerance and diabetes in offspring has been studied
extensively. However, no effect of low protein diet on glucose metabolism in dams during gestation has been reported. Offspring born to low protein (8-10%) fed dams have enhanced glucose tolerance and increased insulin sensitivity during early life [16, 99] in muscle [100] and adipose [101] tissues. However, impaired glucose tolerance occurs by 15 months of age [102] and diabetes at 17 months of age [103]. Although another study reported that fasting plasma glucose and insulin levels were normal in young offspring born to the dams fed a low-protein diet during pregnancy, impaired glucose tolerance was found in adult female offspring because insulin response to an oral glucose preload was low [104].

Gestational diets that are low in protein, but not sufficiently so that the body weight of the dams is affected, increase the risk of insulin resistance and cardiovascular disease (CVD) in the offspring in a sex-dependent manner. Male but not female Wistar rats born to low protein fed dams (8% of total calorie) were relatively hyperinsulinemic and insulin resistant at 20 weeks of age [105]. The females exhibited insulin resistance much later at 21 months of age [106]. In addition to the effect of low protein maternal diets on metabolic regulation, other physiologic systems are compromised in the offspring as illustrated by studies of blood pressure.

2.4.3. Blood Pressure Regulation

In humans, a low protein/carbohydrate ratio in the diets available during the Dutch famine had a stronger association than birthweight with high blood pressure in later life. However, the results from animal studies are contradictory. Langley and Jackson (1994) reported that feeding low-protein diets (6-12% of total calories) to rats during pregnancy resulted in an increase in blood pressure [107] whilst, Lucas (1996)
reported that low protein diets (8% of total calories) during pregnancy had no effect on blood pressure in the offspring [108]. Similarly, two widely used low protein, casein diet formulations (9% of total calories), the University of Southampton diet and that produced by the Hope Farm Company in the Netherlands have been applied to examine the effect of low protein diet on programming of blood pressure but gave contradictory results. When the Southampton diet was given to the dams, higher systolic blood pressure was found in the offspring at 4 wk of age [109, 110]. However, the Hope Farm diet, resulted in normotensive, insulin-resistant offspring [100, 108, 111]. Because these diets differ in sources of carbohydrate (starch, glucose and sucrose), fat source and content, and choline and methionine content, it is clear that the effect of the low protein diet can be modulated by other characteristics of the diet. Unfortunately they have not been identified.

Extending maternal low protein diets to the postnatal period results in a more robust effect in increasing blood pressure [112]. A low-protein diet (8% of total calories) was fed throughout gestation and lactation and to the offspring until 70 days of age when they were then fed a highly palatable cafeteria-style diet. Low protein diet during lactation significantly increased blood pressures, as it did in the cafeteria-fed rats. Authors suggested that early protein restriction and later obesity are independent risk factors for the development of hypertension. [112].

The mechanisms by which a low protein maternal diet alters blood pressure in the offspring are unknown. Increased peripheral resistance has been suggested due to lower pulse rate in the absence of cardiac hypertrophy, indicating that cardiac output is not elevated [113]. However, a low protein gestational diet has also been reported to lower heart size with no difference in pulse rate in rat offspring [114].
Although the role of low protein diets during pregnancy on blood pressure of the offspring has been investigated widely, the role of high protein diets has received less study. In one, Wistar rats were fed either a normal (20% of total calories) or high protein (40% of total calories) diet throughout pregnancy and lactation. Blood pressure was higher at wk 4 of age and persisted throughout the study in male offspring born to high protein fed dams [19]. In humans, young adults had an increased systolic blood pressure at ages 27-30 y when born to women consuming a high-meat low carbohydrate diet during pregnancy (0.45 kg meat/day) [115]

In summary, there is good evidence that consumption by rats and humans during pregnancy of both low and high protein diets affect blood pressure in the offspring. However the mechanisms behind these responses have received little examination. Thus, in the following a background is given first on proposed mechanisms of fetal programming and then followed by an exploration of how protein content and composition in diets may affect fetal programming.

2.5 MECAHNIEMS OF FETAL PROGRAMMING

The mechanisms underlying fetal programming by nutrition are not understood. However, both clinical and experimental studies demonstrated that hormones are environment-dependent organizers of the neuroendocrine system, which ultimately regulate all fundamental processes of life. Therefore, non-physiological concentrations of hormones due to altered intra-uterine and/or early post-natal environment can act as “endogenous functional teratogens” by malprogramming of the neuroendocrine-immune system leading to developmental disorders and chronic diseases in later life [116].
2.5.1 Hormonal Mechanisms of Fetal Programming

Animal studies support a role for cortisol [117], leptin [118], insulin [119-121] and ghrelin [122] in intra-uterine and early post-natal development during pregnancy plus lactation. While these hormones have been the focus, there is a high likelihood that many others are affected by the maternal diet and impact the fetus.

2.5.1.1 Corticosteroids:

Administration of glucocorticoids to pregnant animals and humans leads to intrauterine growth restriction (IUGR) [117] which has long-term clinical consequences [61]. During intrauterine under-nutrition, the fetus is exposed to higher levels of glucocorticoids thus leading to the suggestion that it is a significant factor influencing the offspring [123, 124]. Supporting this view are studies in rats. Rats exposed to excess glucocorticoids during the third week of pregnancy become hyperglycemic, glucose intolerant, and hyperinsulinemic in adult life [125]. In addition, elevated blood pressure in adults born of low birth weights have been correlated with greater corticosterone concentrations, especially in obese individuals at ages 68-78 [123].

2.5.1.2 Insulin

Insulin plays an important role in fetal growth [41] and elevated insulin is associated with development of obesity and diabetes [126]. A positive correlation between the level of amniotic insulin or perinatal hyperinsulinemia and the increase in body weight and the risk of impaired glucose tolerance in later life in offspring of diabetic mothers has been reported [119-121]. In maternal diabetes mellitus, gestational diabetes or even mildly impaired glucose tolerance, put the offspring at risk of developing obesity and glucose intolerance [127, 128].
Insulin may malorganize neuroendocrine systems by effects on hypothalamic controllers [129]. The hypothalamus is particularly sensitive to levels of circulatory hormones during the prenatal period [130]. Increased insulin concentrations within the immature hypothalamus leads to permanent dysplasia of central nervous nuclei regulating metabolism and body weight in the ventromedial hypothalamic nucleus (VMN) [128, 129, 131]. Moreover, hypothalamic resistance to the satiety signals, insulin and leptin, is associated with a life-long increase in activity and number of orexigenic peptides galanine and neuropeptide Y in rats [132, 133]. Neonatal insulin injections induce morphological alterations in hypothalamic structures that lead to the development of obesity and adult hyperinsulinaemia in rats [134].

2.5.1.3 Leptin

Leptin is an adipose-derived, anorexigenic hormone secreted in proportion to fat mass [135]. Leptin limits the expression of both NPY and agouti-related protein within the hypothalamic arcuate nucleus [136] to control weight gain, feeding behavior, and metabolism [91]. Data from human studies indicated that low maternal concentrations of plasma leptin increases risk of obesity in offspring [118]. Individuals with low birth weight tend to have higher leptin concentrations in adulthood [137].

Leptin plays a role in early post-natal development of hypothalamic circuitry [138]. Thus, it has been suggested that an abnormal profile of post-natal leptin concentrations are causative in the later development of an obesity-prone phenotype [139]. In support of this hypothesis, offspring of rats injected subcutaneously with leptin during the first ten days of life have increased food intake, body weight and reduced
responsiveness to leptin in later life associated with lower levels of ObRb expression in the hypothalamus [140].

2.5.1.4 Ghrelin

Ghrelin is an orexigenic peptide synthesized and secreted primarily from the fundic region of the stomach but also in other tissues and other parts of the gastrointestinal tract [141]. It is also expressed in human placenta. The highest levels of ghrelin are detected at mid gestation [142] suggesting that ghrelin plays an important role in pregnancy in regulating pregnancy-related maternal weight gain [143]. Plasma ghrelin concentration is also influenced by diabetes mellitus (DM) during pregnancy. In a population of pregnant women, ghrelin levels were lower in type 1 DM pregnancies at 20 and 30 weeks [122].

2.5.2 Proposed Mechanisms of Fetal Programming by Proteins

Studies of the role of protein content of maternal diets have provided evidence that it modifies the development of somatic structure and phenotype of the offspring. However physiological responses to dietary proteins are determined by not only the concentration but also by the physiologic characteristics of proteins arising from their AA composition, bioactive peptides (BAPs) and digestion kinetics. Thus, it can be predicted that nutritional adequacy of amino acids may not be the only characteristics of the maternal diet to impact the offspring.

2.5.2.1 Amino Acid Composition

Amino acids, independent of the protein content of the maternal diet may influence the risk of development of chronic diseases in offspring by affecting gene
expression presumably through one carbon pathways of epigenetic mechanisms. For example, low protein diets with similar protein content (8-9% of total calories) affect programming of blood pressure differently. The low protein Southampton diet results in higher systolic blood pressure in offspring [109, 110], whereas the Hope Farm diet had no effect [100, 108, 111]. One explanation for the differences in outcome may be based on the methionine content of the diets. In rats fed the Southampton diet which contains more added methionine than the Hope diet, increased maternal serum levels of homocysteine occur after only 4 days of feeding [144] and hyperhomocysteinemia is associated with alterations in gene methylation status in the liver of fetuses [14]. Moreover, increased Hcy concentration has been related to hypomethylation of DNA [145] and disturbed key events in organogenesis and in embryonic vasculogenesis [146].

Further support for the importance of amino acid composition of protein in fetal programming is suggested by observations that supplementing the Southampton diet with glycine as well as folate which reduces plasma homocysteine results in a normalization of blood pressure, suggesting that the methionine load contributes to the ‘Southampton’ phenotype [113]. Supplementation of the low-protein diet with threonine during the initial phases of pregnancy has been reported to both decrease [144] and increase maternal concentrations of homocysteine [14]. Supplementation of a low-protein gestational diet with taurine (2.5%) restored normal insulin secretion [147]. Taurine is also involved in homocysteine metabolism and reduces the demand for cysteine.

### 2.5.2.2 Bioactive Peptides (BAPs)

Although many physiological functions of proteins are attributed to BAPs, their role in the development of regulatory systems is unknown. BAPs have been detected in
the plasma of pregnant and lactating women [148] but whether BAPs cross the placenta and influence fetal development directly or indirectly by influencing maternal metabolism has not been shown.

BAPs have many physiological functions [149, 150]. For example, BAPs with Anigiotensin Converting Enzyme (ACE) inhibitory activity lower BP in experimental animals. Casokinins originate from all major subunits of casein, $\alpha_{s1}$-, $\beta$-, and $\kappa$-caseins [151] and their activities are much higher than those from soy protein. Vasoactive peptide [152] originates from $\alpha_{s1}$-casein fragment 25-27 and has $IC_{50}$ of 2 $\mu$M [150]. Moreover, $\beta$-casomorphins, one of the major groups of BAPs abundant in casein [153, 154], affect food intake regulation [155], gastro-intestinal motility [156], and plasma insulin concentration [157]. $\beta$-casomorphins interact with gastric opioid receptors slowing gastrointestinal motility [156, 158]. Moreover, casomorphins decrease insulin secretion in the absence of elevated glucose levels but have no effect on glucose stimulation of insulin secretion [159]. In addition, it has been proposed that BAP released from ingested proteins and delivered via portal vein may reduce first pass hepatic insulin extraction and lead to improved insulin sensitivity without resulting in increased insulin secretion [160].

The importance of absorbed peptides to physiologic regulation has been challenged because studies in pigs show that the absorption of intact peptides is below 0.1% of that ingested [161] and have a short elimination half life, estimated to be between 5 to 20 min [162]. On the other hand, oral administration of casein hydrolysate lowered both systolic and diastolic blood pressure in normotensive and hypertensive subjects [163-165]. Moreover, a meta-analysis of clinical trials revealed significant reduction of blood pressure by treatment with the tripeptides valine-proline-proline (VPP)
and isoleucine-proline-proline (IPP) [166]. These results suggest that the effects of BAPs on regulatory systems can be induced either by very low concentrations or by build-up of an active concentration in certain tissues (e.g., aorta). The aorta is a target organ of peptides VPP and IPP because both peptides were isolated intact from the aorta of spontaneously hypertensive rats that had been fed L. helveticus-fermented milk [167]. Moreover, ACE activity in spontaneously hypertensive rats (SHRs) fed L. helveticus-fermented milk containing VPP and IPP was lower in the aorta and slightly lower in the lung as compared with SHRs fed non-fermented milk [167, 168]. From these findings, orally administered VPP and IPP have been suggested to inhibit ACE activity in the aorta. Alternatively, or in addition, the effect of BAPs may be the result of their interaction with vagal receptors in the gut wall or gut lumen. The favorable effect of CCK-A receptors, mainly located in the gut, on blood pressure has been reported [169]. Devazepide, a selective CCK-A receptor antagonist, blocked the lowering effects of CCK on arterial blood pressure and heart rate in rats [169]. Moreover, hypotensive effect of BAPs with opioid activity is mediated through the vasodilatory action of binding to opiate receptors in rats [152]. Casein is a rich source of well-characterized peptides, casomorphins, with opioid activity. For each bioactive peptide, the exact mechanism needs to be elucidated.

Whether or not BAPS have any direct effect of fetal development has not been examined. However, their actions as described above could indirectly affect the fetus by impacting regulatory systems in the pregnant mother.
2.5.2.3 Digestion Kinetics

The rate of digestion of the proteins and the resulting hormonal responses in the dams and peak amino acid concentrations in the fetus may also influence the development of regulatory systems. The digestion and absorption kinetics of dietary proteins influence catabolic and anabolic activities at the whole-body level [170] and in the liver [171] and brain amino acid concentrations and neural activity [172]. Based on their rate of digestion and absorption, proteins can be classified as either “fast” or “slow” proteins [171, 173]. For example, casein is a slow protein and whey and soy proteins are fast proteins. Consistent with their composition, plasma concentrations of serine, tyrosine, valine, isoleucine, branched chain amino acids (BCAAs), lysine and total amino acids are higher, and arginine and tryptophan are lower after a casein meal compared with a soy protein meal in humans [174]. In addition, because of the more rapid absorption of soy protein, a larger portion of the AAs are degraded to urea, resulting in less protein synthesis than after consumption of casein [174]. Hormonal responses to these proteins are markedly different. For example, higher concentration of plasma insulin are found at 60 min after whey proteins compared with casein ingestion [175].

2.5.2.4 Hormones

Hormones play a key role in in-utero and post-natal development. Proteins in maternal diets may influence fetal development by their effects on hormonal responses in the mothers. The effects of low protein diet on corticosterone, insulin and leptin have been shown.

In pregnant rats fed a low protein diet, a decrease in activity of 11 β-hydroxysteroid dehydrogenase (11 β-HSD) occurs [176]. 11 β-HSD metabolizes maternal
glucocorticoid transporting to the placenta. Therefore, a decreased activity of placental 11β-HSD activity will increase fetal exposure to maternal cortisol. The detrimental effects of excess exposure of the fetus to glucocorticoids on growth, blood pressure and glucose metabolism of the offspring are well-documented [117, 123, 125]. Prenatal exposure to glucocorticoids resulted in fetal programming of chronic diseases through changes in the development of hypothalamic pituitary (HPA) axis [177, 178].

Insulin is also a factor because maternal low protein diets increase the risk of insulin resistance in male offspring at 20 weeks of age [105]. Higher concentrations of insulin within the immature hypothalamus result in permanent alterations in life-long dysplasia of central nervous nuclei regulating food intake and BW [128, 179] and may decrease sensitivity of hypothalamus to insulin and leptin [129]. Furthermore, maternal low protein diet may influence plasma concentrations of Hcy throughout altered insulin metabolism. In addition, plasma insulin in the insulin resistant rat model correlates positively with plasma Hcy [180].

Gestational protein restriction also reduces plasma leptin concentrations in mothers but not in fetus in rats [181]. This has been associated with an impact on development because exogenous administration of leptin in the last week of pregnancy and throughout lactation resulted in male offspring that were susceptible to the effect of protein deficiency. They were also more resistant to diet-induced weight gain, fat pad gain and insulin resistance when fed a high-fat diet [181].

2.5.2.5 Development of the Hypothalamus and Food Intake Regulation

The characteristics of the metabolic syndrome that appear in later life of offspring exposed to malnutrition in utero suggest that they may be simply secondary to obesity
which in turn may be a consequence of increased food intake due to altered development of intake regulation in the hypothalamus. Thus, factors affecting development of hypothalamus are reviewed here.

Components of the central neural network for regulating food intake are present before birth in rodents and higher-order mammals [182-185]. However, unlike human and sheep, the neuronal circuitry is not fully developed until 16 days after birth in rodents [182, 186, 187]. In the rat, NPY neurons first appear in the arcuate and dorsolateral hypothalamus at 14.5 days gestation [185, 188, 189] and thereafter, NPY mRNA expression rapidly increases between 2 and 15–16 days after birth and returns to adult levels at approximately 30 days of age [182]. NPY receptors are present and functional at early life as evidenced by the observation that microinjection of NPY directly into the PVN at 2 days after birth stimulates milk and water intake [190]. Moreover, it has been suggested that vagal sensory information from the gut relating to gut fullness may be important in regulating food intake in the first week after birth since during this period there is a relative dominance of NPY and α-Melanocyte-stimulating hormone (α-MSH) innervation of the PVN by efferents derived from the brain stem, rather than the arcuate nucleus [182]. However, NPY/AgRP projections from the arcuate nucleus to the Dorsomedial nucleus (DMN) are not complete until some 10–11 days after birth, and projections to the PVN do not fully develop until 15–16 days [182]. Peripheral leptin treatment at day 10 after birth reduces NPY mRNA expression in the rostral arcuate nucleus. However, it has little impact on food intake, which is in compliance with the lack of NPY projections within the hypothalamus during the early postnatal period [191].
POMC, AgRP, and Melanocyte-stimulating hormone 4 receptor (MC4R) mRNA are also all present in the hypothalamus in the early postnatal period.

In rats, the perinatal period is a critical window for the programming of postnatal appetite in the rat [192]. Plagemann and colleagues reported that increased nutritional intake due to small litters induced hyperphagia and obesity combined with hyperleptinemia, hyperglycemia, hyperinsulinemia, and insulin resistance in rats that causes various alterations in hypothalamic structures, neuropeptide levels, neuronal activity and hormonal responsiveness [179, 193, 194]. It was associated with increases in NPY and galanin expression and decreased responsiveness to leptin, insulin and neuropeptides within neurons of the ARC and PVN [147, 195, 196]. Daily insulin treatment between 8 and 11 days after birth also results in a greater body weight gain, chronic hyperinsulinemia, impaired glucose tolerance, hypertension and morphological alterations in hypothalamic structures that persist in adult life [197-199]. It supports the notion that perinatal hyperinsulinemia confers malformation of hypothalamic structures.

Although the effect of protein source in the maternal diet on intake regulation in the offspring has not been investigated, it is clear that low protein diets affect hypothalamic development. Low protein diet fed throughout gestation and lactation provoked hypoinsulinemia, normal leptin concentrations, an increase in NPY levels in the arcuate nucleus, PVN and lateral hypothalamic area, and unchanged NPY levels in the VMN [200]. Food intake of the offspring was not measured.

2.5.2.6 Development of the Pancreas and Metabolic Control

The effect of diet on the development of the endocrine pancreas during pregnancy and early post-natal period plays a key role in the development of glucose regulation and
risk of glucose intolerance in later life. Morphogenesis of the endocrine pancreas tracks a similar direction and sequence in all mammals [201]. In the rat, late pregnancy and the early post-natal period are the crucial times in the development of the pancreas [202, 203]. Initially insulin released from β-cells in the fetus is poorly responsive to glucose, but very responsive to amino acids. Apoptosis deletes many of these β-cells and replaces new islet cells that are sensitive to glucose and responsive during the acute first phase of insulin release [204]. In humans, pancreatic development starts at ~10 wk gestation, and continues throughout pregnancy and the phase of remodeling of the islets continues from late gestation onwards to at least 4 yr [201].

A low-protein diet consumed throughout pregnancy decreases the number of β-cells, insulin content in the fetal pancreatic islets and proliferation of the islet cells and increases apoptosis [205-208]. Although both energy restricted and low protein diets alter pancreas development and consequently glucose metabolism in the offspring, the mechanisms by which a low protein or energy restriction triggers pancreatic development are different. Protein restriction influences the proliferation of existing β-cells [208] whilst, energy restriction influences cellular neogenesis [209]. A low-protein gestational diet also decreases vascularization of the pancreas and decreases insulin secretion by the fetal pancreas in response to arginine and taurine [147, 196, 210].

A low protein diet during postnatal life also has a robust effect on pancreatic development. As would be expected from the known times of pancreatic development, continuing a gestational isocaloric low-protein diet during the postnatal period resulted in a greater reduction in pancreatic β-cell mass and their insulin content with smaller islets and a decrease in β-cell proliferation and an increase in β-cell apoptosis in rat offspring at
3 wk of age [208]. Consistently, the low-protein diets consumed either throughout pregnancy and postnatally or during the postnatal period alone result in fewer but larger islets in pancreas in offspring of both groups compared with those born to dams fed a low protein diet only during pregnancy [205]. However, when the low-protein diet is continued throughout pregnancy and postnatal life, there is also a decrease in islet blood vessel density and in pancreatic and islet blood flow and pancreatic insulin content [211]. Furthermore, a decline is found in insulin secretory response to both glucose and amino acids of islets from 3-month-old offspring [212]. The insulin response to an oral glucose challenge is also decreased at 3 months of age in rats born to dams fed low protein diet throughout gestation and post-natally [104].

The persistence of pancreatic malfunction in postnatal life when low protein diets are prolonged in the post-natal period may suggest that poor maternal nutrition has an adverse effect on endocrine pancreatic function as a consequence of an irreversible developmental deficit. However, the fact that the isocaloric low protein diet is higher in carbohydrates than the control as well as lower in protein may also have an independent effect on glucose metabolism.

2.6 SUMMARY

In summary, both observational and experimental studies showed that maternal diet is a major modifier of the development of regulatory systems in the offspring in-utero and post-natally. Both excessive and inadequate amounts of energy and nutrients in the maternal diet during pregnancy and lactation have detrimental effects on the offspring. Similarly, both inadequate and excess amounts of protein in maternal diets
have an impact on development and health outcomes of the offspring. However, extremes of protein intake are less likely to be a major factor affecting diets of the majority of women in developed countries. More likely, protein source may be a significant factor because many differences in health outcomes are observed in vegetarians compared with omnivores [213, 214]. Some of these differences may be attributed to source and composition of proteins consumed during pregnancy and infancy because in addition to providing essential amino acids many other physiological effects arise from their amino acid composition, BAPs and digestion kinetics. Therefore, the objective of this study was to compare the effect of casein and soy protein as sole sources of protein in a nutritionally adequate maternal diet fed either during pregnancy alone and or during pregnancy, lactation and after weaning on body weight, body composition, food intake, glucose homeostasis and blood pressure in the dams and in their offspring.
CHAPTER 3

HYPOTHESIS AND OBJECTIVES
3. HYPOTHESIS AND OBJECTIVES

3.1 GENERAL HYPOTHESIS AND OBJECTIVE:

**Hypothesis:** Nutritionally adequate protein sources in diets during gestation, lactation and weaning influence food intake regulation and the risk of developing characteristics of metabolic syndrome in offspring of Wistar rats

**Objective:** To compare the effect of casein and soy proteins as sole sources of protein in nutritionally adequate diets fed during gestation, lactation and weaning on food intake and characteristics of metabolic syndrome in the offspring of rats.

3.2 SPECIFIC HYPOTHESES AND OBJECTIVES:

To test the hypothesis, four studies were conducted:

3.2.1. Study 1

Soy protein and casein based nutritionally complete diets fed during gestation and lactation differ in effects on characteristics of metabolic syndrome in male offspring of Wistar rat

**Hypothesis:** Protein source in nutritionally adequate diets during gestation, lactation and weaning influences the risk of development of characteristics of metabolic syndrome in male offspring and the dams of Wistar rats.

**Objective:** To compare the effect of casein and soy protein as sole sources of protein in a nutritionally adequate diet fed during gestation, lactation and weaning on the risk of
development of characteristics of metabolic syndrome in male offspring and the dams of Wistar rats.

3.2.2 Study 2

The effect of protein source in the diets during gestation, lactation and weaning on the development of intake regulatory system in male offspring of Wistar rats.

**Hypothesis:** Protein source in nutritionally adequate diet during gestation, lactation and weaning influence food intake regulatory system in male offspring of Wistar rats.

**Objective:** To compare the effect of casein and soy protein as sole sources of protein in a nutritionally adequate diet fed during gestation, lactation and weaning on the development of intake regulatory system in male offspring of Wistar rats.

3.2.3 Study 3

The effect of casein and soy protein fed during gestation and lactation on characteristics of metabolic syndrome and food intake regulation in female offspring of Wistar rats.

**Hypothesis:** Protein source in nutritionally adequate diets during gestation and lactation influences the risk of development characteristics of metabolic syndrome and food intake regulatory system in female offspring of Wistar rats.

**Objective:** To compare the effect of casein and soy protein as sole sources of protein in a nutritionally adequate diet fed during gestation and lactation on the risk of development of characteristics of metabolic syndrome and food intake regulatory systems in female offspring of Wistar rats.
3.2.4. Study 4

Protein composition of the weaning diet alters food intake and blood glucose regulation after protein preloads in rats.

**Hypothesis:** Protein sources in the weaning diet influence food intake regulation and glucose metabolism in male Wistar rats.

**Objective:** To compare the effect of casein and soy protein as sources of protein in the weaning diet on food intake and satiety hormones in response to the casein and soy protein preloads and glucose metabolism in male Wistar rats.

The results from experiment (Exp) 1 in study 1 (chapter 4) and Exp 1a in study 2 (chapter 5) are obtained from the same male offspring. Moreover, male offspring in Exp 1a and 1b in chapter 5 were born to the same dams. Male offspring in Exp 2 in chapters 4 and 5 and female offspring in study 3 (chapter 6) were born to the same dams. In study 4 (chapter 7), rats were purchased at weaning from Charles River, QC, Canada.
CHAPTER 4

SOY PROTEIN AND CASEIN BASED NUTRITIONALLY COMPLETE DIETS FED DURING GESTATION AND LACTATION DIFFER IN EFFECTS ON CHARACTERISTICS OF METABOLIC SYNDROME IN MALE OFFSPRING OF WISTAR RAT
4. SOY PROTEIN AND CASEIN BASED NUTRITIONALLY COMPLETE DIETS FED DURING GESTATION AND LACTATION DIFFER IN EFFECTS ON CHARACTERISTICS OF METABOLIC SYNDROME IN MALE OFFSPRING OF WISTAR RAT

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4.1 ABSTRACT

The AIN-93G diets based on soy protein or casein were fed to pregnant Wistar rats from day 3 of gestation and compared for their effects on characteristics of metabolic syndrome in male offspring. Pregnant rats were randomized to either casein (C) or soy protein (S) diets (n=12/group) during gestation only (Exp 1) or during gestation and lactation (Exp 2). Male offspring were weaned to either C or S diets for 9 wk (Exp 1) or 15 wk (Exp 2). In Exp 1, pups born to S fed dams had higher fasting blood glucose (BG), systolic (SBP) and diastolic (DBP) blood pressure at wk 4, higher (BG) response to a glucose gavage (p<0.001) and higher body weight (BW) at wk 8 (p<0.05). In Exp 2, consumption of the S diet throughout gestation and lactation resulted in higher BW (p<0.05), DBP (p<0.005) and SBP (p<0.005) in the offspring. They also had higher HOMA-IR (p<0.05) and plasma homocysteine (p<0.05) at weaning, higher fasting BG and glucose response to glucose gavage (p<0.005) at wk 12 and higher HOMA-IR (p<0.01) at wk 15. Although composition of the weaning diets interacted with the diet of the dams, the latter was the dominant factor in determining metabolic outcomes in the offspring. In conclusion, the soy protein diet, compared to the casein diet, when consumed during gestation or throughout gestation and lactation increased the presence of characteristics of metabolic syndrome in the offspring.

Keywords: Protein source: fetal programming: metabolic syndrome: blood pressure
4.2 INTRODUCTION

Epidemiological, clinical and animal studies have shown that fetal and early postnatal nutrition alters development of somatic structure, endocrine systems and homeostatic mechanisms in the fetus and infant. These effects influence the risk of developing obesity and components of the metabolic syndrome in later life [8-10].

Both low and high protein diets fed during gestation and lactation have adverse effects on the offspring [12-16], but the mechanisms by which they bring about these effects are unclear. Rats born to dams fed a low protein diet have increased blood pressure [109], body weight [13], adiposity [97], and compromised glucose metabolism [215]. Similarly, high protein diets fed during gestation and lactation increase adiposity [21], BW [19], BP, food efficiency [19], and decrease energy expenditure in the offspring [21].

In addition to the requirement for adequate and balanced amounts of essential amino acids for protein synthesis, amino acids have potential to elicit significant effects on development through additional actions [14, 113, 144, 216, 217]. The addition of glycine [113, 217] to low protein diets ameliorates the effect of the diet on vascular dysfunction and blood pressure [113], while adding taurine normalizes insulin secretion in the offspring [147]. Although their mechanism of action remains unexplained, it may include their effects on DNA methylation. Amino acids have the potential to affect DNA methylation during fetal development either directly by involvement in DNA methylation pathways or indirectly through effects on hormonal and oxidative status of maternal blood reaching the fetus [107, 113, 144, 145]. Furthermore, because of the effects of individual amino acids on regulatory systems [14, 107, 144, 145, 147], malnutrition may
not be the only prerequisite for the effects of proteins and amino acids in maternal diets on development of the offspring. Thus, it is possible that protein source and amino acid composition of nutritionally complete maternal diets affect the phenotype of the offspring. Furthermore, whether development of the offspring is affected differently by dietary protein consumed during gestation only or throughout gestation and lactation has not been reported, but would be expected because the development of regulatory systems in rodents continues in late fetal and early postnatal life [107, 218].

The primary objective of this study was to test the hypothesis that nutritionally complete diets [219] differing in protein sources and fed during gestation alone or during gestation and lactation differ in their effects on characteristics of the metabolic syndrome in the offspring. Therefore, Wistar rat dams were fed either the AIN 93G soy protein- or casein-based diets. In addition, because the Predictive Adaptive Response Hypothesis suggests that the effect of the maternal diet on the offspring is reduced if the diet of the offspring is similar to that of the dams, two groups of offspring from both maternal groups were fed either soy protein-or casein based diets. Therefore, a secondary objective was to determine the effect of protein composition of the weaning diet on the consequences of the dams’ diets in the offspring.

4.3 EXPERIMENTAL METHODS

4.3.1 Animals and Diets

First-time pregnant Wistar rats were received at d 3 of gestation (Charles River, QC, Canada) and were housed individually in ventilated plastic transparent cages with bedding at 22±1º C and 12-h light-dark cycle (lights off at 2200h to 1000h). At weaning,
rat offspring were housed individually in ventilated plastic transparent cages with bedding. The powdered diets were provided ad libitum in stainless steel cups with a mesh disk insert to reduce spillage. All rats had free access to water throughout the experiments.

The composition (in g/kg) of the test diets was as follows. The casein diet (C) contained casein (200.0), cornstarch (529.4), sucrose (100.1), soybean oil (70.0), cellulose (50.0), vitamin mixture (10.0), mineral mixture (35.0), cystine (3.0), choline bitartrate (2.5), and tert-butyl hydroquinone (0.014). The composition of the soy diet (S) was identical to the C diet, except that soy protein replaced casein, and methionine (2.54) and cystine (2.54) were added to the soy protein diet and cystine (3.0) to the casein diet as recommended for the AIN-93G diets [219]. Amino acid content of the C and S diets as fed is shown in Table 4.1. Cornstarch, high-protein casein (87%), and cellulose were purchased from Harlan Teklad (Madison, WI). The vitamin and mineral mixtures, cystine, methionine, choline bitartrate, and tert-butyl hydroquinone were purchased from Dyets Inc. (Bethlehem, PA), sucrose from Allied Food Service (Toronto, ON, Canada), and soybean oil from Loblaws (Toronto, ON, Canada).

Genistein, daidzein and glycitein content (μg/g) of the soy protein diet was 36.1, 31.3 and 4.4, respectively.

The protocol was approved by University of Toronto Animal Care Committee and care and maintenance of the animals conformed to the guidelines of the Canadian Council on Animal Care.
4.3.2 Experimental Design

Two experiments were conducted. To test the primary hypothesis, the C and S diets were fed to the dams only during gestation in Exp 1 and throughout gestation and lactation in Exp 2. To test the Predictive Adaptive Hypothesis, male offspring from both experiments were fed either C or S diets and were followed for 9 and 15 wk post-weaning, respectively.

4.3.2.1 Exp 1: The effect of protein source in the diet fed during gestation and after weaning on components of metabolic syndrome in male offspring.

The objective of this study was to investigate the effect of C or S diets fed to pregnant dams only during gestation on postnatal development in male offspring fed either C or S diets. Two groups of pregnant rats (n=12/group) were fed either C or S diets beginning at 3 d of gestation. During lactation, both groups received only the C diet. BW of the offspring was measured at birth (day 1, after litters were culled to 10 pups) and on days 7, 14 and 21. At weaning (day 21 of age), one male offspring from each mother in each diet group was assigned to either the C or S diet (n=12/group). BW was measured weekly for 8 wk after weaning. Systolic (SBP) and diastolic (DBP) blood pressure, pulse rate and blood glucose response to a glucose load were measured at wks 4 and 8 respectively. Fat pad mass (FPM: abdominal + epididymal + perirenal fats) was measured at sacrifice at 9 wk.

4.3.2.2 Exp 2: The effect of protein source in diets fed during gestation and lactation and after weaning on components of metabolic syndrome in male offspring and in the dams.
The objective of this study was to investigate the effect of feeding the dams C and S diets throughout gestation and lactation, and to extend the duration of observation on the pups fed either C or S diets. Two groups of pregnant rats (n=12/group) were fed either the C or the S diet during gestation and lactation, and for another 6 weeks after weaning. At weaning, one male from each mother in each diet group was assigned to either C or S diet for 15 weeks (n=12/group). For the remaining pups, BW was measured at birth (day 1, after litters were culled to 10 pups) and on days 7, 14 and 21. At birth, weaning (day 21 of age) and at wk 16 post-weaning, rats (n=12/dam diet group) were sacrificed. BW was measured weekly from weaning to wk 15 after weaning. Body fat composition was determined at birth, at weaning and at wk 15. SBP and DBP were measured at wk 2 in the dams, and SBP, DBP and pulse rate at wks 4, 8 and 12 in the pups. Glucose and insulin tolerance tests were conducted at wks 4, 8 and 12.

Trunk blood of fetuses (n=5-6/group) obtained at d 20 of gestation in Exp 2 and pups at birth (n=5-6/group) in both experiments was pooled. Pups (n=12/group) were sacrificed by decapitation after a 12-hour overnight food deprivation at wk 9 in Exp 1, and at weaning and wk 15 in Exp 2. Plasma concentrations of insulin, homocysteine, corticosterone, albumin and glucose were measured.

Dams from Exp 2 on the S or C diets were sacrificed at d 14 and at d 20 of gestation (n=6/group) and at wk 6 after weaning (n=12/group). Glucose tolerance tests were conducted at 3 wk post-weaning. Plasma concentrations of glucose, insulin, corticosterone, homocysteine and albumin were measured.
4.3.3 Glucose tolerance test (GTT)

Rats were fasted overnight for 10 h. A blood sample was withdrawn from the tail vein prior to and at 15, 30, and 60 min after a glucose gavage (0.375 g glucose/ml, 5 g glucose/kg BW).

4.3.4 Insulin tolerance test (ITT)

Rats were fasted overnight for 10 h. Insulin (Humulin®-R, Eli Lilly and Company, Indianapolis, Indiana, USA) injections were given intraperitoneally (IP) (0.5 U/ml, 0.75 U insulin/kg BW) and blood obtained prior to and at 15, 30, and 60 min after an insulin injection.

4.3.5 Blood pressure

SBP and DBP were measured by the non-invasive tail-cuff method with optical plethysmography using a tail manometer-tachometer system (BP-2000, Visitech system, Apex, NC). Rats were restrained in holders on a platform with constant temperature of 30°C. They were adapted daily to the device for five days. On the day of measurement, five mock measurements preceded a series of ten measurements and only the latter were used in calculating the average as previously reported [7].

4.3.6 Blood glucose

Tail vein glucose concentration was assayed using a hand-held commercial glucose meter (MediSense Precision Xtra, Abbott Laboratories, Alameda, CA, USA) using test strips [7]. Glucose in plasma from trunk blood obtained upon decapitation was measured using a glucose oxidase kit (Ascensia Elite XL, Bayer AG, Leverkusen, Germany).
4.3.7 Blood collection

Trunk blood was collected in chilled Vacutainer tubes (BD, Franklin Lakes, NJ, USA) containing EDTA + Trasylol® (Bayer AG, Leverkusen, Germany) solution (10% blood volume, 500 KIU/ml). Blood samples were centrifuged for 20 min at 3000g and 4°C for 10 min. Plasma was separated and immediately stored at -80°C.

4.3.8 Hormone assays

Plasma insulin was measured using Insulin Enzyme Immunoassay (Cat# 80-INSRT-E01, Alpco Diagnostics, Salem, NH, USA) with assay sensitivity of 0.124 ng/ml. Plasma homocysteine was measured by Enzyme Immunoassay (Cat# 194-5361, Bio-Rad Laboratories, Inc, Hercules, CA, USA) with sensitivity of 1.0 μmol/L. Plasma albumin was measured using a colorimetric assay (Cat# 11970909, Roche Diagnostics, Indianapolis, IN, USA) with sensitivity of 0.2 g/dl. Plasma corticosterone was measured using Enzyme Immunoassay (Cat# DSL-10-81100, Beckman Coulter, Webster, TX, USA) with assay sensitivity of 1.6 ng/ml.

4.3.9 Body composition

Fat mass and lean mass were measured at birth by dual energy X-ray absorptiometry (DEXA) (pSabre, Orthometrix) applying a specialized software program (Host Software version 3.9.4; Scanner Software version 1.2.0) [220]. After sacrificing, carcasses were placed directly on the DEXA. All scans were performed at a speed of 10 mm/s and a resolution of 0.5 x 1.0 mm. At weaning and at the end of experiments, fat pad mass was measured by dissection of extracted abdominal, epididymal and perirenal fat.
4.3.10 Isoflavones measurement

Homogenized soy protein samples were analyzed for isoflavones (genistein, daidzein and glycitein) by gas chromatography mass spectrometry (GC-MS), as previously described [221]. The isoflavones analysis involved extraction of samples twice with 5 ml 70% methanol, passing a portion of extraction through a C18 solid-phase extraction column (SPE column; Octadecyl C18/14%, 200 mg/3 ml; Applied Separations, Allentown, PA), hydrolysis with β-glucuronidase (Helix Pomatia; Sigma Aldrich, St. Louis, MO) and passage through another C18 SPE column. An internal standard (5α-androstane-3β,17β-diol; Steraloids Inc, Wilton, NH) was added to the column eluent, and the sample was then derivatized with Tri-Sil Reagent (Pierce Co., Rockford, IL) before injection to the GC-MS (Agilent 6890 series GC system interfaced with an Agilent 5973 network mass selective detector; Agilent Technologies, Wilmington, DE).

4.3.11 Statistical analyses

All data are expressed as means ± SEM. The effect of dam and pup diets on BW, glucose response, SBP and DBP was analyzed by PROC MIXED MODEL procedure with diet and time as main factors in Exp 1 and 2. A one-way repeated measure analysis of variance (ANOVA) followed by post-hoc Tukey’s test was conducted when treatment effects or interactions were statistically significant. Student’s unpaired t-test was applied to compare the dependent measures (e.g. hormones) at individual time points. Blood glucose response was calculated as the total incremental area under the curve (tAUC) of the blood glucose concentration over one hour after receiving glucose gavaged for the GTT and after receiving insulin injection for the ITT. For the former the reported tAUC is positive above baseline whereas for the latter the tAUC is a negative representing the
area below baseline. The homeostasis model assessment of insulin resistance (HOMA-IR) index was calculated as fasting glucose multiplied by fasting insulin divided by 22.5 [222]. All analyses were conducted using SAS (version 9e, SAS Institute, Cary, NC). Statistical significance was defined at \( p<0.05 \).

4.4 RESULTS

4.4.1 Exp 1: The effect of protein source in the diet fed during gestation and after weaning on components of metabolic syndrome in male offspring.

Gestational diet did not affect birth weight (6.2 ± 0.2 vs. 6.3 ± 0.1 g, in rats born to dams fed the C and S diets, respectively). However, at wk 9, BW of offspring born to the S diet dams was 5% higher (450.3 ± 5.7 vs. 427.3 ± 5.3 g, \( p<0.05 \)) (Fig 4.1). At wk 4, SBP was 18% (\( p<0.05 \)), DBP was 7% (\( p<0.05 \)) and pulse rate was 6% higher (\( p<0.01 \)) in pups born to dams fed the S diet (Table 4.2). At wk 9, fasting BG was higher in rats born to the S diet dams (\( p<0.05 \)) (Table 4.3). However, the gestational diet did not affect glucose response to glucose gavage in the offspring at wk 8 (Table 4.4) or fat pad mass (15.6 ± 0.7 and 15.2 ± 0.7 g, C and S diets, respectively) at wk 9.

The pup diet influenced glucose metabolism. At wk 8, total area under the curve (\( tAUC \)) for glucose after the glucose gavage was higher in rats weaned to the S diet (\( p<0.001 \)) (Table 4.4). At wk 9, fasting BG (\( p<0.01 \)), insulin (\( p<0.005 \)), and HOMA-IR index (\( p<0.001 \)) were higher in rats weaned to the S diet (Table 4.3) but as indicated by an interaction (\( p<0.06 \)) between the gestational diets and weaning diets the effect of the weaning diet was stronger in those born to dams fed the S diet.
4.4.2 Exp 2: The effect of protein source in diets fed during gestation and lactation and after weaning on components of metabolic syndrome in male offspring and in the dams.

4.4.2.1 Offspring

There were no differences (C vs. S diets respectively) due to the protein source in the dams’ diets on the pups at birth in litter size (13.2 ± 0.7 and 13.2 ± 0.5), male/female ratio (0.53 ± 0.05 and 0.52 ± 0.03), body fat (0.30 ± 0.04 and 0.40 ± 0.05 g) or BW (6.2 ± 0.2 and 6.3 ± 0.1 g) and at weaning in BW (62.2 ± 2.1 and 56.3 ± 3.2 g) and fat pad mass (0.5 ± 0.1 and 0.4 ± 0.1 g). However, at wk 15, BW of offspring was higher by 9% (615.5 ± 8.2 vs. 564.8 ± 8.8 g) (p<0.05) if born to S vs. C diet (Fig 4.2). Their abdominal fat (22.0 ± 1.5 vs. 18.2 ± 1.0 g) (p<0.05) and fat pad mass was also larger in offspring (34.9 ± 2.4 vs. 30.9 ± 1.8 g) (p<0.05) born to S diet fed dams at wk 15.

There was no effect of protein source in diets fed throughout gestation and lactation on fasting plasma glucose at birth or at weaning in the offspring (Table 4.5). However, the dams’ diet affected glucose metabolism in later life. Fasting blood glucose at wk 15 was higher in offspring born to dams on the S diet (p<0.005) (Table 4.3). Blood glucose (tAUC) response to the glucose gavage was also higher at wk 4, 8 and 12 (p<0.002) in offspring from the S diet dams. The effect of the dams’ diet became stronger with time (p<0.0001) as shown by the interaction (p<0.05) of the dams’ diet with time. The effect of insulin injections (ITT) on blood glucose were not affected by dams’ or weaning diets or by time. The source of protein in the dams’ diets had no effect on plasma insulin in the fetus at d 20 (p=0.08) or in the pups at birth (p=0.08) (Table 4.5). However, at weaning, offspring born to the dams fed the S diet had higher fasting plasma
insulin (p<0.05), HOMA-IR (p<0.05) and homocysteine (p<0.05) (Table 4.5), and at wk 15, higher HOMA-IR (p<0.01) (Table 4.3). Higher SBP and DBP were found in the offspring born to the S diet dams at 4, 8, and 12 wk (p<0.05) (Table 4.2). This effect of the gestation and lactation diet of the dam interacted with the weaning diet (p<0.01), explained by much higher SBP in pup consuming the S diet and born to mothers fed the S diet. Pulse rate of the offspring was also higher at 4, 8, and 12 wk in the offspring born to dams fed the S diet (p<0.05). Overall pulse rate decreased with time (p<0.05) (Table 4.2). Dams’ diet had no effect on fasting plasma corticosterone in the offspring (Tables 4.3, 4.5). Pup diet had no statistically significant effect on any of the parameters measured.

4.4.2.2 Dams

BW of the dams was not affected by their diet during gestation (Fig 4.3). However, BW was higher after parturition and at wk 1 of lactation in dams fed the C diet (p<0.05). Fasting plasma glucose was higher in S fed dams at d 14 (p=0.06) and at d 20 (p<0.05), but not at wk 6 after weaning. No dams’ diet effects were found in dams’ fasting plasma insulin at d 14 or at d 20 of gestation, but insulin was higher at wk 6 in dams fed the S diet (p<0.05), as was the HOMA-IR index at d 20 gestation (p<0.05) and wk 6 after weaning (p<0.05) (Table 4.6). The diets of the dams did not affect their fasting plasma homocysteine, corticosterone or albumin (Table 4.6), SBP and DBP at wk 2 after weaning (Table 4.2), GTT and ITT at wk 4 after weaning or fat pad mass at d 14, d 20 or wk 6 after weaning (data not shown).
4.5 DISCUSSION

The results of these experiments support the hypothesis that nutritionally complete diets differing in protein sources and fed during gestation alone or during gestation and lactation differ in their effects on characteristics of the metabolic syndrome in the offspring. The soy protein diet, compared with the casein-based diet, increased BW, body fat, SBP, DBP, and led to impaired insulin sensitivity and glucose tolerance in the male offspring. However, protein source in the pups’ diet impacted little on the primary effects of protein source in the dams’ diets on the offspring.

Extending the duration of the test diets from gestation alone in Exp 1 to gestation and lactation in Exp 2 resulted in a more robust effect of the S diet on BW, body composition and glucose metabolism in the offspring. The S diet fed during gestation and lactation increased BW of the offspring 4 wk earlier (wk 4 vs. wk 8) than when fed only during gestation and also led to impaired glucose metabolism. Furthermore it made clear that the increase in body weight was arising from increased body fat. Thus protein composition in the diet consumed during lactation also affected development and is consistent with continued development of intake regulatory systems of the hypothalamus and gastro-intestinal tract in rodents in late pregnancy and the early post-natal period [107, 223].

The effects of protein source in diets fed to the dams throughout gestation and lactation was not modified by the composition of diets fed the pups. This was surprising because the Predictive Adaptive Response Hypothesis is based on the premise that offspring weaned to similar diets as their mothers will adapt more appropriately to their
postnatal environment than those receiving an unmatched diet [36]. Clearly, the adverse
effect of the S diet fed to the dams on BP (Table 4.2) and glucose regulation (Tables 4.3,
4.4, 4.5) in the offspring was not diminished by feeding the pups with S diets.

The more favorable effect of casein on blood pressure (Table 4.2) may have its
origins in both the amino acids and bioactive peptides (BAPs) it contains. Casein and soy
protein differ in amino acid composition [224] (Table 4.1). Soy protein contains only half
the proline content of casein and may be a factor because the high blood pressure of
offspring born to dams fed low protein gestational diets is reduced by adding glycine,
proline and/or threonine [113, 225]. Consumption of hydrolysates of proteins lowers
blood pressure in both humans and animals [226, 227], suggesting that BAPs may be a
factor. For example, in hypertensive humans, consumption of a daily dose of casein
hydrolysate (0.49 g/d) containing the peptides valine-proline-proline and isoleucine-
proline-proline, known to inhibit in vitro angiotensin converting enzyme (ACE), lowered
both systolic and diastolic blood pressure [163]. In hypertensive rats, oral administration
of derivatives of casein hydrolysate (32mg/kg BW/d) significantly decreased blood
pressure [227]. Based on in vitro measures of ACE activity, BAPs have been found to be
more abundant and inhibitory in casein [150] and than in soy protein [151, 228] but their
role in vivo is uncertain because of the low absorption of the active peptides [161].
However, casein is also rich in casomorphins capable of activating opioid receptors in the
enteric nervous system and on the vagus, resulting in vasodilation [150, 226, 229].

A weakness of the present study is that the effects observed could be due to a
combination of the effects of the proteins and the amino acid additions to the diets. Thus
it cannot be concluded that soy protein alone had adverse effects compared with casein.
To provide an adequate diet, as recommended in the AIN 93G diets, both methionine and cystine were added as free amino acids to the soy protein diet and cystine was added to the casein diet. These additions may be important because methionine content of the diet has a direct relationship with plasma homocysteine and increased homocysteine concentrations have been related to hypomethylation of DNA [145] and disturbed key events in organogenesis and in embryonic vasculogenesis [146]. In the present study, plasma homocysteine was 48% higher at weaning and 7% higher at wk 15 in the offspring born to the S diet fed dams (Tables 4.3, 4.5). The origin of this higher concentration of homocysteine is puzzling because total methionine content of the diets was similar (4.0 vs. 4.5 g/kg diet). However, 54% of the methionine in the soy protein diet was in the form of free amino acid and would be expected to result in a faster absorption compared with methionine released during digestion. Cystine was also added to both diets (Table 4.1). The effect of cysteine on homocysteine is unclear because previous studies report that cysteine both increases [230, 231] and decreases [232] homocysteine in humans.

The higher concentrations of homocysteine may also be attributed to insulin resistance as it occurred in the offspring with higher HOMA-IR indices at weaning (Table 4.5) and at wk 15 (Tables 4.3). Previous studies have found that insulin resistance provokes hyper-homocysteinemia in both humans [233] and rats [234]. Insulin regulates plasma homocysteine concentration via hepatic cystathionine β-synthase, a key enzyme involved in the trans-sulfuration activity [235].

Finally, the effect on the dams by the soy protein diet consumed during gestation and lactation may have contributed to metabolic differences in the offspring (Table 4.6).
The S diet fed dams had higher blood glucose and HOMA-IR index values, concurrently with a trend to higher plasma insulin in their fetuses at d 20 of gestation. Glucose intolerance during gestation is known to lead to overweight, impaired glucose tolerance, hyperinsulinemia and insulin resistance in juvenile and adult rat offspring, irrespective of any genetic disposition [128, 131, 236]. Although the increased body weight observed in infants from mothers that have Type 2 diabetes was not observed in the rat offspring at birth, much higher blood glucose concentrations and insulin resistance than found here are required. [50, 51]. In the present studies, while plasma glucose concentrations in the S fed dams were significantly higher (averaging 6.5 mM) at d 20 of gestation, these concentrations would not be sufficient to classify them as diabetic and unlikely to affect the body weight of their offspring at birth.

Many physiological properties of soy protein are attributed to its isoflavones content, specifically genistein [237-241] or interactions between isoflavones and other components in the diet (e.g. amino acids) [24]. However, the isoflavone content of the soy protein is an unlikely factor in the results obtained as the levels were below that associated with physiological effects. The genistein content of the S diet was 36 μg/g of the diets, well below that reported (250 μg/g diet) in the maternal diet to affect epigenetic and phenotypic changes in mice [242]. Similarly, the results obtained from the present study cannot be explained by stress responses of the dams or pups, for two reasons. First, there were no differences due to diet in litter size or birth weight of the offspring, or in BW of dams at arrival, d 14 and d 20 of gestation (Fig 4.3). Secondly, no differences were found in fasting corticosterone levels in either dams at d 14, d 20 of gestation and wk 6 post-weaning or pups at weaning and wk 15 (Tables 4.3, 4.5, 4.6).
In summary, this is the first study to show that nutritionally complete diets based on soy protein or casein and fed to rat dams affect the metabolic phenotype of the offspring. This observation is of particular significance because the majority of animal studies aimed at understanding the effects of maternal diets on development are based on malnutrition models. In contrast, this study shows that metabolic outcomes in the offspring may differ among “normal” diets used for maintenance of the dams during pregnancy and lactation. For example, the source of protein in lab chow diets is highly variable and often includes herring fish, whey and plant proteins. In addition, there are several recommended AIN-93 diets with various sources of proteins (e.g. casein, soy and whey protein). Clearly, even though judged to be nutritionally complete diets, protein source and many other aspects of test diets fed during pregnancy and lactation in animal models of development could be a factor explaining variance in outcomes among studies. The relevance of these results to humans is uncertain because human diets during gestation contain many mixed protein sources. On the other hand, infants are weaned to formula that are dependent on single sources of proteins or their hydrolysates during early life and may be a factor affecting development of regulatory systems and later life outcomes.

4.6 CONCLUSION

Soy protein, when compared to casein based AIN 93G diets and fed during gestation or during gestation and lactation, increased the risk of developing characteristics of the metabolic syndrome in the offspring.
Acknowledgments

The authors thank the management and technicians at the Department of Comparative Medicine at the University of Toronto. All the authors contributed to the preparation of the paper and read and approved the final manuscript. G.H. Anderson and A. Jahan-mihan conceptualized and designed the research. A. Jahan-mihan, P.S. Huot, C. Smith, A. Hamedani and B.L. Luhovyy conducted the research. A. Jahan-mihan analyzed the data. A. Jahan-mihan, G.H. Anderson and I.M. Szeto wrote the paper. G.H. Anderson had primary responsibility for final content.

Authors declare no conflict of interest. This study was supported by the Natural Sciences and Engineering Research Council of Canada.
The amino acid content of the diets is calculated based on purity of the protein sources (87% and 90% for casein and soy protein respectively) and the totals include the addition of the free amino acids.

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Micellar Casein</th>
<th>Isolated Soy Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine</td>
<td>4.7</td>
<td>6.7</td>
</tr>
<tr>
<td>Arginine</td>
<td>5.7</td>
<td>11.9</td>
</tr>
<tr>
<td>Aspartic acid + Asn</td>
<td>10.6</td>
<td>18.0</td>
</tr>
<tr>
<td>Cystine</td>
<td>3.5</td>
<td>4.5</td>
</tr>
<tr>
<td>Glutamic acid + Gln</td>
<td>34.8</td>
<td>29.9</td>
</tr>
<tr>
<td>Glycine</td>
<td>2.8</td>
<td>6.7</td>
</tr>
<tr>
<td>Histidine</td>
<td>4.4</td>
<td>4.1</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>8.7</td>
<td>7.7</td>
</tr>
<tr>
<td>Leucine</td>
<td>13.9</td>
<td>12.8</td>
</tr>
<tr>
<td>Lysine</td>
<td>12.2</td>
<td>9.9</td>
</tr>
<tr>
<td>Methionine</td>
<td>4.0</td>
<td>4.5</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>7.7</td>
<td>8.1</td>
</tr>
<tr>
<td>Proline</td>
<td>16.0</td>
<td>7.9</td>
</tr>
<tr>
<td>Serine</td>
<td>8.4</td>
<td>8.1</td>
</tr>
<tr>
<td>Threonine</td>
<td>6.6</td>
<td>5.9</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>1.7</td>
<td>2.2</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>8.0</td>
<td>5.9</td>
</tr>
<tr>
<td>Valine</td>
<td>10.4</td>
<td>7.9</td>
</tr>
</tbody>
</table>

1 The amino acid content of the diets is calculated based on purity of the protein sources (87% and 90% for casein and soy protein respectively) and the totals include the addition of the free amino acids.

2 Isolated soy protein: Dyets Inc; Micellar casein: Harlan Teklad

3 Cystine was added to both casein (3g/kg diet) and soy protein (2.54g/kg diet) diets.

4 Methionine was added to only the soy protein diet (2.54 g/kg diet)
Fig. 4.1. Expt 1: Effect of protein source during gestation on post-weaning BW of male offspring. Data are means ± SEM; n= 12/group; BW was analyzed by MIXED model followed by Tukey’s post-hoc test with gestational diet, weaning diet and time as main factors: Gestational diet (NS); Weaning diet (NS); Time (p<0.0001); interaction of gestational diet and time (p<0.005); * p<0.05; Data are pooled for pup diet to present the effect of gestational diet alone on BW. C: Casein; S: Soy protein; NS: Not significant.
Table 4.2. Effect of source of protein in diets of dams and offspring on systolic and diastolic blood pressure and pulse rate1 (Mean values with their standard errors)

<table>
<thead>
<tr>
<th>Dams’ Diet</th>
<th>C</th>
<th>S</th>
<th>Weaning Diet</th>
<th>C</th>
<th>S</th>
<th>p values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Systolic BP (mmHg)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wk</td>
<td>Experiment 1 (Gestational Diet)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>128 ± 2.2</td>
<td>130 ± 3.5</td>
<td>137 ± 10.9</td>
<td>134 ± 3.9</td>
<td></td>
<td>D: p &lt;0.05</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>W: NS</td>
</tr>
<tr>
<td>Experiment 2 (Gestation and Lactation Diet)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>120 ± 3.8ab</td>
<td>110 ± 5.8a</td>
<td>119 ± 5.9ab</td>
<td>132 ± 7.0b</td>
<td></td>
<td>D: p &lt;0.005</td>
</tr>
<tr>
<td>8</td>
<td>130 ± 4.9ab</td>
<td>113 ± 5.5a</td>
<td>127 ± 5.0ab</td>
<td>136 ± 5.6b</td>
<td></td>
<td>D×W: p &lt;0.01</td>
</tr>
<tr>
<td>12</td>
<td>119 ± 5.9ab</td>
<td>103 ± 10.3a</td>
<td>142 ± 10.6b</td>
<td>131 ± 9.4ab</td>
<td></td>
<td>T: NS</td>
</tr>
<tr>
<td>Diastolic BP (mmHg)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Experiment 1 (Gestational Diet)</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>93 ± 4.8</td>
<td>100 ± 3.2</td>
<td>110 ± 5.4</td>
<td>104 ± 5.6</td>
<td></td>
<td>D: p &lt;0.05</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>W: NS</td>
</tr>
<tr>
<td>Experiment 2 (Gestation and Lactation Diet)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>99 ± 3.9</td>
<td>95 ± 8.1</td>
<td>100 ± 4.9</td>
<td>111 ± 7.6</td>
<td></td>
<td>D: p &lt;0.005</td>
</tr>
<tr>
<td>8</td>
<td>105 ± 6.1</td>
<td>90 ± 7.3</td>
<td>106 ± 6.6</td>
<td>118 ± 7.6</td>
<td></td>
<td>W: NS</td>
</tr>
<tr>
<td>12</td>
<td>82 ± 8.9</td>
<td>89 ± 13.6</td>
<td>107.9 ± 9.4</td>
<td>115 ± 10.0</td>
<td></td>
<td>T: NS</td>
</tr>
<tr>
<td>Pulse (BPM)2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Experiment 1 (Gestational Diet)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>417 ± 7.3</td>
<td>426 ± 11.9</td>
<td>447 ± 6.1</td>
<td>445 ± 9.3</td>
<td></td>
<td>D: p &lt;0.001</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>W: NS</td>
</tr>
<tr>
<td>Experiment 2 (Gestation and Lactation Diet)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>388 ± 4.6</td>
<td>387 ± 9.3</td>
<td>408 ± 10.3</td>
<td>399 ± 8.7</td>
<td></td>
<td>D: p &lt;0.05</td>
</tr>
<tr>
<td>8</td>
<td>382 ± 6.7</td>
<td>357 ± 12.4</td>
<td>397 ± 10.8</td>
<td>394 ± 9.6</td>
<td></td>
<td>T: p &lt;0.05</td>
</tr>
<tr>
<td>12</td>
<td>379 ± 6.4</td>
<td>378 ± 5.6</td>
<td>384 ± 7.6</td>
<td>378 ± 9.1</td>
<td></td>
<td>W: NS</td>
</tr>
</tbody>
</table>

C: Casein; S: Soy protein; D: Dams’ diet; W: Weaning diet; T: Time; NS: Not Significant

1 MIXED model with dams’ diet and weaning diet (Expt 1) and dams’ diet, weaning diet and time (Expt 2) as main factors followed by Tukey’s post-hoc test when interaction was significant; values in a row at each time point with different superscript letters are significantly different, p<0.05; n=11-12/group

2 BPM: Beats per minute
Table 4.3. Effect of source of protein in diets of dams and offspring on fasting plasma measures in the offspring\(^1\) (Mean values with their standard errors)

<table>
<thead>
<tr>
<th>Dams’ Diet</th>
<th>C</th>
<th>S</th>
<th>Weaning Diet</th>
<th>C</th>
<th>S</th>
<th>p&lt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wk 9 post-weaning</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose, mM</td>
<td>5.6 ± 0.3</td>
<td>6.1 ± 0.3</td>
<td>5.8 ± 0.4</td>
<td>6.9 ± 0.2</td>
<td>0.05</td>
<td>0.01</td>
</tr>
<tr>
<td>Insulin, ng/ml</td>
<td>0.67 ± 0.20</td>
<td>1.03 ± 0.08</td>
<td>0.49 ± 0.10</td>
<td>1.40 ± 0.18</td>
<td>NS</td>
<td>0.005</td>
</tr>
<tr>
<td>HOMA-IR (^2)</td>
<td>0.16 ± 0.50</td>
<td>0.28 ± 0.02</td>
<td>0.13 ± 0.04</td>
<td>0.43 ± 0.06</td>
<td>NS</td>
<td>0.001</td>
</tr>
<tr>
<td>Corticosterone, ng/ml</td>
<td>249.1 ± 11.4</td>
<td>255.8 ± 11.6</td>
<td>229.3 ± 9.8</td>
<td>236.8 ± 8.0</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Homocysteine, μmol/L</td>
<td>3.1 ± 0.1</td>
<td>3.5 ± 0.7</td>
<td>3.7 ± 0.7</td>
<td>3.9 ± 0.4</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Wk 15 post-weaning</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose, mM</td>
<td>5.0 ± 0.2</td>
<td>5.2 ± 0.2</td>
<td>5.5 ± 0.2</td>
<td>5.8 ± 0.2</td>
<td>0.005</td>
<td>NS</td>
</tr>
<tr>
<td>Insulin, ng/ml</td>
<td>1.92 ± 0.30</td>
<td>2.25 ± 0.07</td>
<td>2.33 ± 0.22</td>
<td>2.20 ± 0.15</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>0.43 ± 0.10</td>
<td>0.50 ± 0.03</td>
<td>0.57 ± 0.05</td>
<td>0.58 ± 0.03</td>
<td>0.01</td>
<td>NS</td>
</tr>
<tr>
<td>Corticosterone, ng/ml</td>
<td>330.0 ± 19.6</td>
<td>315.6 ± 14.3</td>
<td>331.2 ± 37.9</td>
<td>318.6 ± 9.0</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Homocysteine, μmol/L</td>
<td>8.8 ± 0.6</td>
<td>8.4 ± 1.1</td>
<td>9.4 ± 1.2</td>
<td>9.0 ± 0.6</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

C: Casein; S: Soy protein; D: Dams’ diet; W: Weaning diet; NS: Not significant
\(^1\) MIXED model with dams and weaning diets as main factors. Expt 1: Interaction for HOMA-IR: \(p=0.06; n=5-6/group\)
\(^2\) HOMA-IR index was calculated as fasting glucose (mM) multiplied by fasting insulin (ng/ml) divided by 22.5
Exp 2: Effect of protein source during gestation and lactation on post-weaning BW of male offspring

Data are means ± SEM; n=12/ group; BW was analyzed by MIXED model followed by Tukey’s post-hoc test with the diets fed during gestation and lactation (dams’ diets), weaning diets and time as main factors: Gestation and lactation diet (p<0.002); Weaning diet (NS), Dams’ diet × time, (p<0.01); * p<0.05
C: Casein; S: Soy protein; NS: Not significant
Data are pooled for pup diet to present the effect of the dams’ diet alone on BW
Table 4.4. Effect of source of protein in diets of dams and offspring on blood glucose response during the oral glucose and insulin tolerance tests

<table>
<thead>
<tr>
<th>Dams’ Diet</th>
<th>Weaning Diet</th>
<th>GTT 2 (min mmol/L)</th>
<th>C</th>
<th>S</th>
<th>W: p &lt;0.001</th>
<th>Experiment 2 (Gestation and Lactation Diet)</th>
<th>D: p &lt;0.005</th>
<th>T: p &lt;0.0001</th>
<th>D×T: p &lt;0.05</th>
<th>W: NS</th>
<th>Experiment 1 (Gestational Diet)</th>
<th>D: NS</th>
<th>W: p &lt;0.001</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>C</td>
<td>S</td>
<td></td>
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<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>514 ± 16.8</td>
<td>497 ± 9.3</td>
<td>517 ± 11.8</td>
<td>496 ± 12.0</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>405 ± 10.4</td>
<td>405 ± 7.0</td>
<td>445 ± 12.6</td>
<td>426 ± 9.6</td>
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<td></td>
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<td></td>
<td>389 ± 6.7</td>
<td>417 ± 3.8</td>
<td>440 ± 7.23</td>
<td>436 ± 6.7</td>
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<td>514 ± 16.8</td>
<td>497 ± 9.3</td>
<td>517 ± 11.8</td>
<td>496 ± 12.0</td>
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<td>8</td>
<td>405 ± 10.4</td>
<td>405 ± 7.0</td>
<td>445 ± 12.6</td>
<td>426 ± 9.6</td>
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<td>12</td>
<td>389 ± 6.7</td>
<td>417 ± 3.8</td>
<td>440 ± 7.23</td>
<td>436 ± 6.7</td>
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<td>C</td>
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<td>224 ± 12.8</td>
<td>257 ± 13.9</td>
<td>236 ± 11.4</td>
<td>233 ± 11.8</td>
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<td>8</td>
<td>226 ± 5.6</td>
<td>242 ± 9.0</td>
<td>248 ± 9.9</td>
<td>262 ± 14.3</td>
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<td>12</td>
<td>224 ± 8.5</td>
<td>241 ± 13.4</td>
<td>233 ± 7.2</td>
<td>233 ± 8.5</td>
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</table>
| C: Casein; S: Soy protein; D: Dams’ diet; W: Weaning diet; T: Time; NS: Not significant
1 MIXED model with dams’ and weaning diets as main factors; n= 10-12 / group
2 GTT (Glucose tolerance test): After overnight fasting rats received glucose (0.375 g glucose/ml, 5 g glucose/kg BW) by gavage and blood glucose was measured prior to and 15, 30, and 60 min later
3 ITT (Insulin tolerance test): Insulin (Humulin® R, Eli Lilly and Company, Indianapolis, Indiana, USA) Injections were given intraperitoneally (IP) (0.5 U/ml, 0.75 U insulin/kg BW) and blood glucose was measured prior to and 15, 30, and 60 min later
Table 4.5. Exp 2: Effect of source of protein fed during gestation and lactation on fasting plasma measures in offspring 1
(Mean values with their standard errors)

<table>
<thead>
<tr>
<th>Gestation and Lactation Diet</th>
<th>C</th>
<th>S</th>
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<tbody>
<tr>
<td>Fetus (Day 20 Pregnancy)</td>
<td></td>
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<tr>
<td>Insulin, ng/ml</td>
<td>0.28 ± 0.03</td>
<td>0.41 ± 0.06*</td>
</tr>
<tr>
<td>Birth</td>
<td></td>
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</tr>
<tr>
<td>Glucose, mM</td>
<td>5.2 ± 0.2</td>
<td>5.4 ± 0.3</td>
</tr>
<tr>
<td>Insulin, ng/ml</td>
<td>0.27 ± 0.07</td>
<td>0.49 ± 0.08*</td>
</tr>
<tr>
<td>HOMA-IR^2</td>
<td>0.06 ± 0.01</td>
<td>0.11 ± 0.02</td>
</tr>
<tr>
<td>Weaning</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose, mM</td>
<td>5.2 ± 0.2</td>
<td>5.3 ± 0.1</td>
</tr>
<tr>
<td>Insulin, ng/ml</td>
<td>0.12 ± 0.01^a</td>
<td>0.18 ± 0.02^b</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>0.03 ± 0.002^a</td>
<td>0.04 ± 0.001^b</td>
</tr>
<tr>
<td>Corticosterone, ng/ml</td>
<td>299.2 ± 29.2</td>
<td>351.7 ± 59.7</td>
</tr>
<tr>
<td>Homocysteine, μmol/L</td>
<td>5.7 ± 0.2^a</td>
<td>8.5 ± 0.9^b</td>
</tr>
</tbody>
</table>

C: Casein; S: Soy protein

1 Unpaired t-test; values in a row with different superscript letters are significantly different, p<0.05; * p=0.08; n=5-6/group;
2 HOMA-IR index was calculated as fasting glucose (mM) multiplied by fasting insulin (ng/ml) divided by 22.5
Fig. 4.3. Exp 2: Effect of source of protein during gestation, lactation and 6 wks post-weaning on BW of dams.
Data are means ± SEM; n= 12/ group; BW was analyzed by MIXED model followed by Tukey’s post-hoc test with diet and time as main factors: Diet (NS); Time (p<0.0001);
Diet × time (p=0.08); * p<0.05
C: Casein; S: Soy protein
Table 4.6. Exp 2: Effect of source of protein fed during gestation and lactation on fasting plasma measures in the dams \(^1\) (Mean values with their standard errors)

<table>
<thead>
<tr>
<th>Diet</th>
<th>C</th>
<th>S</th>
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<tbody>
<tr>
<td>Day 14 pregnancy</td>
<td></td>
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<tr>
<td>Glucose, mM</td>
<td>6.5 ± 0.2 (^a)μ</td>
<td>7.1 ± 0.2 (^b)</td>
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<tr>
<td>Insulin, ng/ml</td>
<td>1.9 ± 0.2</td>
<td>1.9 ± 0.1</td>
</tr>
<tr>
<td>HOMA-IR (^2)</td>
<td>0.54 ± 0.07</td>
<td>0.61 ± 0.03</td>
</tr>
<tr>
<td>Corticosterone, ng/ml</td>
<td>192 ± 28</td>
<td>228 ± 47</td>
</tr>
<tr>
<td>Homocysteine, μmol/L</td>
<td>5.5 ± 1.0</td>
<td>4.0 ± 0.8</td>
</tr>
<tr>
<td>Albumin g/L</td>
<td>40.8 ± 0.8</td>
<td>41.2 ± 0.9</td>
</tr>
<tr>
<td>Day 20 pregnancy</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose, mM</td>
<td>5.6 ± 0.2 (^a)</td>
<td>6.5 ± 0.3 (^b)</td>
</tr>
<tr>
<td>Insulin, ng/ml</td>
<td>1.3 ± 0.2</td>
<td>1.9 ± 0.3</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>0.32 ± 0.04 (^a)</td>
<td>0.55 ± 0.09 (^b)</td>
</tr>
<tr>
<td>Corticosterone, ng/ml</td>
<td>340.1 ± 30.4</td>
<td>387.9 ± 45.1</td>
</tr>
<tr>
<td>Homocysteine, μmol/L</td>
<td>5.7 ± 1.4</td>
<td>5.6 ± 1.3</td>
</tr>
<tr>
<td>Albumin g/L</td>
<td>35.8 ± 0.1</td>
<td>36.7 ± 1.8</td>
</tr>
<tr>
<td>Wk 6 post-weaning</td>
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<tr>
<td>Glucose, mM</td>
<td>5.8 ± 0.3</td>
<td>5.9 ± 0.2</td>
</tr>
<tr>
<td>Insulin, ng/ml</td>
<td>1.9 ± 0.1 (^a)</td>
<td>2.3 ± 0.1 (^b)</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>0.46 ± 0.05 (^a)</td>
<td>0.60 ± 0.03 (^b)</td>
</tr>
<tr>
<td>Corticosterone, ng/ml</td>
<td>212.7 ± 16.2</td>
<td>238.8 ± 33.0</td>
</tr>
<tr>
<td>Homocysteine, μmol/L</td>
<td>4.7 ± 0.8</td>
<td>5.8 ± 1.7</td>
</tr>
<tr>
<td>Albumin g/L</td>
<td>39.7 ± 2.0</td>
<td>36.3 ± 1.5</td>
</tr>
</tbody>
</table>

C: Casein; S: Soy protein
\(^1\) Unpaired \(t\)-test, values in a row at each time point with different superscript letters are significantly different, \(p<0.05; \(^{\mu} p=0.06;\)
\(n=5-6/group;\)
\(^2\) HOMA-IR index was calculated as fasting glucose (mM) multiplied by fasting insulin (ng/ml) divided by 22.5
CHAPTER 5

THE EFFECT OF PROTEIN SOURCE IN DIETS FED DURING GESTATION AND LACTATION ON FOOD INTAKE REGULATION IN MALE OFFSPRING OF WISTAR RATS
5. THE EFFECT OF PROTEIN SOURCE IN DIETS FED DURING GESTATION AND LACTATION ON FOOD INTAKE REGULATION IN MALE OFFSPRING OF WISTAR RATS\textsuperscript{1,3}

Alireza Jahan-mihan, Chris Smith; G. Harvey Anderson\textsuperscript{2}

Department of Nutritional Sciences, Faculty of Medicine, University of Toronto, Toronto, ON, Canada, M5S 3E2

FOOTNOTES

Running Head: Maternal proteins and food intake

Submitted to: Am J Physiol Regul Integr Comp Physiol

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The final version of this chapter is available in Am J Physiol Regul Integr Comp Physiol.
5.1 ABSTRACT

We hypothesized that protein source in the nutritionally adequate AIN 93G diets fed during gestation, lactation and weaning, influences food intake (FI) regulation in male offspring of Wistar rats. Pregnant rats were fed either the recommended casein (C) or soy protein (S) diet during gestation (Exp 1) or during gestation and lactation (Exp 2). Pups (n=12/group) weaned to either C or S diets were followed for 9 wks (Exp 1) or for 14 wks (Exp 2). At termination, body weight was higher by 5.4% and 9.4%, respectively in offspring born to S diet fed dams. Altered FI regulation was shown by failure of devazepide (CCK-A receptor blocker) to block FI reduction after protein preloads in rats born to the S diet whereas it had a strong effect on those born to C fed dams (p<0.005). Similarly, naloxone (opioid receptor blocker) blocked FI reduction more after casein than after soy protein preloads (p<0.01). In Exp 2, offspring born to dams fed the S diet had higher hypothalamic gene expression of agouti related protein (AgRP) at weaning (p<0.05) and higher FI was found throughout post-weaning (p<0.0001). FI reduction after protein preloads at wk 7 and after glucose preloads at wk 13 was greater in offspring born to the C diet fed dams (p<0.05). Plasma insulin at weaning and insulin, ghrelin and GLP-1 at wk 15 were higher in those born to the S diet fed dams (all p<0.05). In conclusion, nutritionally complete diets based on casein and soy protein consumed during gestation and lactation differ in their effects on BW and FI regulation in the offspring. Extending the diet from gestation alone to throughout gestation and lactation exaggerated the adverse effects of the S diet. However, the diet consumed post-weaning had little effect on the outcome.

**Key words:** protein, food intake, body weight, programming, offspring
5.2 INTRODUCTION

Numerous epidemiological and experimental studies indicate that programming of obesity and related disorders is associated with alterations in food intake regulation [19, 200]. In animal models it is clear that both under- [61] and over-nutrition [74] during gestation result in higher food intake in their offspring. Moreover, it has been suggested that obesity in the offspring is due to in utero programming of food intake regulatory systems and that hyperphagia is an underlying cause of obesity [61, 243]. These effects of malnutrition may have an origin in their effect on programming of postnatal appetite [192]. Development of intake regulatory systems in the GI tract and hypothalamus in rodents occurs primarily during late gestation and early postnatal life [107, 223].

Several studies have associated alterations in hypothalamic regulation of food intake with increased weight gain due to maternal diets. Malnutrition (50% diet restriction) during gestation results in higher food intake and body weight (BW) associated with higher hypothalamic mRNA expression of Ob-Rb in offspring of mice [244]. Rats born to dams fed high vitamin diets become obese and have higher food intake associated with higher mRNA expression of hypothalamic orexigenic peptide Agouti-related protein (AgRP) and plasma insulin [245]. Similarly, rats born to dams fed a high fat diet during gestation had higher BW and energy intake and concomitantly had higher hypothalamic mRNA expression of leptin receptor, Pro-opiomelanocortin (POMC) and neuropeptide-Y (NPY) [246]. In addition, the protein content of maternal diets has also been shown to affect food intake regulation in the offspring. A high protein diet during gestation resulted in higher BW in the offspring [18] and this was associated with increased food efficiency [19] and lower energy expenditure [20, 21]. Low protein
(9% protein) diets fed during gestation and lactation to rats also have been reported to lead to higher food intake [243] in adulthood and also provoke hypoinsulinemia and increased NPY levels in the arcuate nucleus, PVN and lateral hypothalamic area [200]. Moreover, after 7 days of receiving a low protein diet (10% protein), male rats had higher food intake and concomitantly increased hypothalamic AgRP mRNA levels compared with controls fed a 20% protein diet [247].

Although both low and high protein diets fed during gestation and lactation affect food intake and BW in the offspring, the role of source of protein and its composition in nutritionally complete diets consumed during gestation has not been reported. In addition to providing for the requirement for adequate and balanced amounts of essential amino acids for protein synthesis, amino acids have potential to elicit significant effects on development thorough additional actions [14, 113, 144, 216, 217]. Because of the effects of individual amino acids on regulatory systems [14, 107, 144, 145, 147], malnutrition is not the only prerequisite for the observed effects of proteins and amino acids in maternal diets on development in the offspring. Therefore, it is possible that protein source and amino acid composition of nutritionally complete maternal diets affect development of food intake regulatory systems of the offspring.

Nutritionally complete soy (S) protein compared with casein (C) based diets fed to rats during gestation and lactation resulted in higher body weight, body fat, insulin resistance and elevated blood pressure in the offspring [248]. Because many of the characteristics of the metabolic syndrome are associated with increased food intake, it can be suggested that the S diet adversely affected development of food intake regulatory systems.
Therefore, the primary objective of this study was to test the hypothesis that nutritionally complete diets [219] differing in protein sources and fed during gestation alone or during gestation and lactation differ in their effects on the regulation of food intake in the offspring. Therefore, Wistar rat dams were fed either the AIN 93G soy protein- or casein-based diets. However, because the Predictive Adaptive Response (PAR) Hypothesis suggests that the effect of the maternal diet on the offspring is reduced if the diet of the offspring is similar to that of the dams, two groups of offspring from both maternal groups were fed either soy protein or casein based diets. Therefore, a secondary objective was to determine the effect of protein composition of the weaning diet on the consequences of the dams’ diets on food intake regulation in the offspring.

5.3 MATERIALS AND METHODS

5.3.1 Animals and diets

First-time pregnant Wistar rats were received at d 3 of gestation (Charles River, QC, Canada) and were housed individually in ventilated plastic transparent cages with bedding at 22±1° C and 12-h light-dark cycle (lights off at 2200h to 1000h). At weaning, rat offspring were housed individually in ventilated plastic transparent cages with bedding. The powdered diets were provided ad libitum in stainless steel cups with a mesh disk insert to reduce spillage. All rats had free access to water throughout the experiments.

The composition (in g/kg) of the test diets was as follows. The casein diet (C) contained casein (200.0), cornstarch (529.4), sucrose (100.1), soybean oil (70.0), cellulose (50.0), vitamin mixture (10.0), mineral mixture (35.0), cystine (3.0), choline...
bitartrate (2.5), and tert-butyl hydroquinone (0.014). In addition, methionine (2.54) and cystine (2.54) were added to the AIN-93G soy protein diet and cystine (3.0) was added to the casein diet, as recommended [219].

Amino acid content of the C and S diets as fed is shown in Table 5.1. Cornstarch, high-protein casein (87%), and cellulose were purchased from Harlan Teklad (Madison, WI). The vitamin and mineral mixtures, cystine, methionine, choline bitartrate, and tert-butyl hydroquinone were purchased from Dyets Inc. (Bethlehem, PA), sucrose from Allied Food Service (Toronto, ON, Canada), and soybean oil from Loblaws (Toronto, ON, Canada).

Genistein, daidzein and glycitein content (μg/g) of the soy protein diet was 36.1, 31.3 and 4.4, respectively.

The protocol was approved by University of Toronto Animal Care Committee and care and maintenance of the animals conformed to the guidelines of the Canadian Council on Animal Care.

5.3.2 Design

Two experiments were conducted. The C and S diets were fed to the dams during gestation alone (Exp 1) or during gestation and lactation (Exp 2). To test the PAR hypothesis the male pups from both dam diet groups were randomized to either the C or S diets. In Exp 1, the effect of the C and S diets fed to the dams during gestation alone and in the pup diets on food intake, intake regulatory hormones and satiety signaling via CCK-A and peripheral opioid receptors were studied to 9 wk post-weaning. In Exp 2, the effect of the C and S diets fed to the dams during both gestation and lactation and also in the pup diets to 15 wk post weaning on intake regulation was studied. The rationale for
feeding the dams throughout gestation and lactation was twofold. First, most studies of
the effect of maternal nutrition on the offspring feed test diets throughout gestation and
lactation. Second, the development of intake regulatory systems in the GI tract and
hypothalamus in rodents continues into early postnatal life [107, 223]. Extending the
duration of the study in Exp 2 was done to examine the effects of the diets until 15 wks
post-weaning when the offspring were in adulthood.

5.3.2.1 Exp 1: The effect of protein source in the diet of dams during gestation
and in the pup diets on food intake regulation in male offspring of Wistar rats.

This study includes two parts. In part a, the effect of gestational diet on
cumulative food intake to wk 4 and 1 h food intake at wk 6 in response to protein
preloads in the offspring was examined. In part b, the effect of gestational diet on 1 h
food intake at wk 6 and 7 in response to protein preloads with or without CCK-A and
peripheral opioid receptor blockers was examined. Food and water were provided ad
libitum to wk 5. At wk 5, to adapt rats to the procedure of 1 h food intake measurement
after an overnight in response to the preloads, food was provided only during dark
periods (12 h/d) and cumulative food intake measurement was ceased.

Pregnant rats (n=15/group) received either the C or S diets during gestation and
received only the C diet during lactation. Litters were culled to 10 at birth. At weaning
(day 21 of age), one male offspring from each mother in each diet group was assigned to
either the C or S diet (n=12/group) (part a) or to the C diet (n=15/group) (part b) for 9
weeks. BW was measured at birth, at weaning and at the end of study.

5.3.2.2. Exp 2: The effect of protein source in the dam (gestation and lactation)
and pup diets on food intake regulation in offspring and in the dams of Wistar rats.
Pregnant rats (n=12/group) were fed either the C or the S diets during gestation and lactation. At birth, litters were culled to 10 pups for each mother. At weaning, at d 21, one male from each mother in each maternal diet group was assigned to either C or S weaning diets (n=12/group). Additional pups were sacrificed and intake regulatory hormones were measured (n=6/group) at weaning. Offspring were followed for 14 weeks. BW was measured at birth, at weaning and at the end of study. Rats had access to food during dark periods (12 h/d) and food intake in the offspring was measured weekly throughout the study. Food intake (1 h) after an overnight fast was measured in response to water and protein preloads at wk 7 and in response to water and glucose preloads at wk 13.

Trunk blood of fetuses (n=5-6) at d 20 of gestation in Exp 2 and pups (n=5-6) at birth in Exp 1 and 2 from each dams’ diet group was obtained by pooling (n=3-4/sample) to attain sufficient blood volume required for hormonal assays. Pups (n=12/group) were sacrificed by decapitation after an overnight fast at wk 9 in Exp 1, and at weaning and at wk 15 after weaning in Exp 2. Blood concentrations of insulin, GLP-1 (Glucagon-Like Peptide-1), ghrelin and PYY (Peptide YY) were measured. Hypothalamus at birth and at weaning was extracted and relative mRNA expression of POMC, leptin receptor, NPY and AgRP was measured.

5.3.3 Preloads

Micellar casein was obtained from American Casein Co (Burlington, N.J.) and isolated soy protein from General Nutrition Products (Greenville, S.C.). Each rat received 3 g/kg BW protein/6 ml distilled water by gavage. Glucose was supplied by EMD™
(Gibbstown, NJ, USA) and each rat received 3 g/kg BW glucose/6 ml distilled water by gavage. The control preload was 6 ml distilled water.

5.3.4 Receptor blockers

Devazepide (donated by ML laboratories PLC, London, UK) was suspended in methyl cellulose (BDH Toronto, Toronto, Canada) [155]. The methocel solution was prepared by adding 0.25 of methyl cellulose powder to 100g of hot (80°C) deionized water, stirred for 1 minute and allowed to chill to 5° C for 2-3 h. The solution was stirred every 0.5 h until it was clear with no visible particles. To mix in devazepide (0.5 g/ml), a glass homogenizer (Tissue Grinder, Pyrex Brand, No. 7725; Thomas Scientific, Swedesboro, NJ) was used. Each rat received 0.25 mg/kg BW, a dose which given alone, does not affect food intake [155].

Peripheral opioid receptor blocker, naloxone methiodide (Sigma Chemical Co. St Louis, MO), was diluted in saline and used within one hour of preparation at a dose of 1.0 mg/kg body weight. Injections were given intraperitoneally (i.p.) in a volume of 1.0 ml.

5.3.5 Short-term food intake

Short-term food intake (1h) was measured in overnight fasted rats and 30 min after control (water) and protein preloads at wk 6 in Exp 1 (part a), at weeks 6 and 7 (part b) and at wk 7 in Exp 2 and 30 min after water and glucose preload at wk 13 after weaning in Exp 2. Before testing, rats were adapted to the experimental procedures. They were gavaged with water over 7 days before the adaptation test, performed as follows. Food was deprived for 12 h before measurement. On d 1, one half of the rats were gavaged with water preload, whereas the rest were untreated. On the next day, this testing
order was reversed. In Exp 1, part b, rats were also injected with saline (i.p.) over 7 days before the adaptation test. The experiment began when it was determined that the processes of gavaging and injection did not affect food intake.

A randomized design was applied to examine the effect of preloads (casein, soy protein or glucose) on short-term food intake. For testing the effect of protein preloads, on the first day of experiment and after 12 h fasting, at 2130 h rats received in random order casein, soy protein or water control preloads by gavage (3g/kg BW/6ml distilled water). Food was introduced at 2200 h and was measured for 1 h. After a wash-out day, the testing order was reversed. The same protocol was applied to test the effect of glucose preload. Food intake was measured to the nearest 0.1 g under red light during the dark cycle.

In Exp 1 (part b), at wk 6, rats (n=15/group) in each group were allocated to 5 groups and randomly assigned to receive, prior to access to food, one of five treatments with one day washout in between: 1) Water control (by gavage, 6 ml) plus methocel (vehicle for devazepide; i.p.1 ml); 2) Casein; 3) Casein plus devazepide; 4) Soy protein and 5) Soy protein plus devazepide. The design was identical when naloxone methiodide was used as the opioid receptor blocker. Devazepide (CCK-A receptor blocker) at 2125 h and naloxone methiodide (peripheral opioid receptor blocker) were applied at 0955 h. Preloads were given at 0930 h and food was introduced at 1000 h and was measured for 1 h.

5.3.6 Long-term food intake

Dams and weaned rats were housed individually in ventilated plastic transparent cages with bedding. All maternal and pup diets were provided *ad libitum* in jars with a
mesh disk insert to minimize spillage. Food intake was measured throughout wk 1-4 after weaning in Exp 1 and for 14 wks after weaning in Exp 2.

5.3.7 Blood collection

Rats were sacrificed by decapitation after an overnight food deprivation at the end of the experiments. Trunk blood was collected in chilled vacutainer tubes containing EDTA + trasylol solution (10% blood volume). Blood samples were centrifuged within 20 min after decapitation at 3000×g, 4°C for 10 min. Plasma was extracted and immediately stored at -80°C. Concentrations of insulin, GLP-1, PYY, ghrelin and glucose were measured.

5.3.8 Hormone assays

Total plasma PYY concentrations were measured using radioimmunoassay (RIA) method (Cat. # RMPYY-68HK, Millipore Research Inc, St. Charles, MO) with assay sensitivity of 15.6 pg/ml. Total plasma ghrelin concentrations were measured using radioimmunoassay (RIA) (Cat. # GHRT-89HK, Linco Research Inc, St. Charles, MO) with assay sensitivity of 93 pg/ml. Plasma insulin concentrations were measured using Enzyme Immunoassay (EIA) (Cat. # 80-INSRT-E01, Alpco Diagnostics, Salem, NH, USA) with assay sensitivity of 0.124 ng/ml. Plasma GLP-1 concentrations were measured using ELISA (Cat. # EGLP-35K, Linco Research Inc, St. Charles, MO) with assay sensitivity of 2 pM.

5.3.9 RNA extraction

As previously described [249], the brains were removed after decapitation and frozen and stored at -80°C. Thereafter, brains were removed from the storage and thawed
at -5°C. Each hypothalamus was dissected on a plastic cassette placed on top of chipped ice. To dissect the hypothalamus, 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 were used [250]. Each dissected hypothalamic block was homogenized in TRIzol® Reagent (Invitrogen Corp, Long Island, NY) and then incubated at 15-30° C for 5 minutes and 0.12 mL of chloroform (GMD chemicals Inc., Gibbstown, NJ) was added. Tissue homogenization and RNA extraction was conducted according to the protocol from Invitrogen Inc. (Carlsbad, CA). Samples were incubated at 15-30° C for 3 minutes prior to centrifuge (at 10,000×g for 15 minutes at 4 °C). The aqueous phase was transferred to a fresh tube and mixed with 0.3 mL of isopropyl alcohol (Caledon Laboratories Ltd., Georgetown, ON) and incubated at 15-30° C for 10 minutes. After centrifuge (at 10,000×g for 10 minutes at 4 °C) the pellet was washed twice with 75% ethanol, dissolved in RNase free water and incubated in a water-bath at 55-60° C for 10 minutes. Samples were placed in storage at -80° C. Then the integrity of the RNA was quantified using a UV-Agilent 8453.

5.3.10 Reverse Transcription and Real-time PCR.

RNA was reverse transcribed into cDNA using the High Capacity cDNA archive kit (Aplied Biosystems Inc, Foster City, CA) following the manufacturer’s protocol. This was followed by real-time RT-PCR (ABI PRISM 7700 Sequence Detection System, Applied Biosystems Inc., Foster City, CA) to determine mRNA levels of 5 hypothalamic appetite-regulatory genes: POMC, leptin receptor, NPY and AgRP. All of the oligonucleotide primer and fluorogenic probe sets for 51 TaqmanTM real-time PCR were purchased from Applied Biosystems Inc. (Foster City, CA) (Table 5.2). Glyceraldehyde-
3-phosphate dehydrogenase (GAPDH) was used as an endogenous control. The quantification values for each were normalized to respective GAPDH values to calculate the relative mRNA levels of each gene.

5.3.11 Statistical analyses

All data are expressed as means ± SEM. The effect of maternal diet on body weight and food intake was analyzed by MIXED MODEL with dam and pup diets and time as main factors in Exp 1 & 2. A one-way repeated measure analysis of variance (ANOVA) with post-hoc Tukey’s test was conducted when treatment effects or interactions were statistically significant. Student’s unpaired t-test was applied to compare the dependent measures at each time point. Food intake in response to protein preloads was analyzed by using PROC MIXED MODEL procedure with dam and pup diets, preload and time as main factors. The effect of the diets and preloads on plasma hormone concentrations was also analyzed by using PROC MIXED MODEL procedure with dam and pup diets and preload as main factors. All analyses were conducted using SAS (version 9e, SAS Institute, Cary, NC). Statistical significance was defined at p<0.05.

5.4 RESULTS

5.4.1 Exp 1 The effect of protein source in the diet of dams during gestation and in the pup diets on food intake regulation in male offspring of Wistar rats.

5.4.1.1 Part a. The diet fed during gestation did not affect BW at birth (C: 6.2 ± 0.2; S: 6.3 ± 0.1) or at weaning (C: 52.2 ± 1.3; S: 50.2 ± 0.9). However, offspring born to dams on the S diet were heavier at wk 9 (450.3 ± 5.7 vs. 427.3 ± 5.3 g) (p<0.05).
Food intake at wks 1-4 post-weaning was influenced by an interaction between gestational and weaning diets \((p<0.05)\) (Table 5.3). It was lower in pups that received the same diet as their mothers. At wk 6, food intake \((1\ \text{h})\) was lower after protein preloads than after water preloads \((p<0.0001)\) (Table 5.4) but was not affected by gestational diet. Decrease in food intake after protein preloads compared with after water was not affected by either gestational or weaning diet or their interaction.

Plasma insulin at wk 9 was influenced by both protein composition of the weaning diet and preload \((p<0.0001)\) but not by the dams’ diets (Table 5.5). It was higher in rats weaned to the S diet and after soy protein preloads compared to casein and water preloads. It was also higher after casein than the water preloads. Plasma GLP-1 was not influenced by either weaning or dams’ diet or their interaction but was affected by the preload \((p<0.0001)\) and by an interaction between preload and weaning diet \((p<0.05)\) (Table 5.5). Plasma GLP-1 was higher after soy protein than casein and was higher after casein than water preload (Table 5.5). Plasma concentrations of PYY were not influenced by either diets or preloads (Table 5.5). Plasma ghrelin was reduced by protein preloads compared with water \((p<0.0001)\) but was not affected by either dams’ or weaning diets (Table 5.5).

5.4.1.2 Part b.

a) CCK-A receptor blocker. Food intake \((1\ \text{h})\) in response to protein preloads was influenced by both dams’ diet \((p<0.05)\) and preload \((p<0.0001)\) at wk 6. It was reduced more in rats born to the S diet fed dams and after protein preloads than after water preloads (Table 5.6). Neither the dams’ diet nor preloads influenced the magnitude of food intake reduction after protein preloads (food intake after water minus after protein preloads).
The effect of devazepide (CCK-A receptor blocker) on food intake in response to protein preloads was influenced by the dams’ diet \( (p<0.005) \). Devazepide blocked food intake reduction after protein preloads more in rats born to the dams fed the C diet than in those born to dams fed the S diet.

b) Peripheral opioid receptor blocker. Food intake \( (1 \text{ h}) \) was influenced by preload composition \( (p<0.0001) \) (Table 5.6). It was lower after soy protein and casein preloads compared with water. The magnitude of food intake reduction after protein preloads was not influenced by either maternal diet or preload. However, the effect of naloxone methiodide (peripheral opioid receptor blocker) on food intake in response to protein preloads was influenced by preload \( (p<0.01) \). Naloxone blocked food intake reduction after casein preloads to a greater extent than after soy protein preloads in offspring born to dams fed both C and S diets (Table 5.6).

5.4.2 Exp 2 The effect of protein source in the dam (gestation and lactation) and pup diets on food intake regulation in offspring and in the dams of Wistar rats.

Protein source in the maternal diets had no effect on BW at birth \( (6.3 \pm 0.1 \text{ and } 6.4 \pm 0.1 \text{ g}) \) or at weaning \( (C: 62.2 \pm 2.1 \text{ and } S: 56.3 \pm 3.2 \text{ g}) \). However, offspring born to dams fed the S diet were 11% heavier at wk 15 \( (C: 576.2 \pm 10.0 \text{ and } S: 615.3 \pm 8.8 \text{ g}) \) \( (p<0.005) \).

Maternal diet had no effect on plasma insulin in the fetus at d 20 or in pups at birth \( (p=0.08) \), but at weaning it was higher in offspring born to dams fed the S diet (Table 5.7). Maternal diet had no effect on hypothalamic mRNA expression of leptin receptor, POMC, NPY or AgRP at birth but mRNA expression of AgRP at weaning was higher in offspring born to dams fed the S diet \( (p<0.05) \) (Fig 5.1). The mean
amplification CT for GAPDH was not influenced by the diet at any measured time (data not shown).

Food intake was affected by maternal diet, time and by their interaction (all p<0.0001) (Fig 5.2). It was higher in offspring born to the S diet fed dams throughout wk 4-11. At wk 7, food intake (1 h) was influenced by maternal diet (p<0.005) and preload (p<0.0005) and also by an interaction of maternal and weaning diets (p<0.001) (Table 5.8). It was lower in offspring born to the C diet fed dams and also was lower in offspring that received the same diet as their mothers. Food intake (1 h) was also lower after protein preloads than after the water control (p<0.0005). The magnitude of food intake reduction after protein preloads (decrease in food intake after protein preloads compared with after water) was influenced by the dams’ diet (p<0.05). The effect of protein preloads on food intake was stronger in rats born to the dams fed the C diet.

At wk 13, food intake (1 h) was influenced by the dams’ (p<0.0005) and weaning diets (p<0.001) and by preloads (p<0.0001) (Table 5.9). It was higher in offspring born to the S diet fed dams and in offspring weaned to the S diet. Food intake reduction after glucose preload (decrease in food intake after glucose compared with after water) was influenced by the dams’ diet (p<0.05) (Table 5.9) and was lower in offspring born to dams fed the S diet.

At wk 15, plasma insulin was higher in rats born to dams fed the S diet (p<0.05) (Table 5.10). The effect of preload on plasma insulin approached statistical significance and was higher after protein preloads than after water preload (p=0.06). Plasma concentrations of GLP-1 were increased by the protein preloads (p<0.05) and more so if the rats were born to dams fed the S diet (p<0.05) (Table 5.10). Neither maternal nor
weaning diet affected plasma concentrations of PYY in response to preloads (Table 5.10). Plasma ghrelin in response to preloads was higher in rats born to dams fed the S diet (p<0.01) and also was higher after water preloads than after protein preloads (p<0.05) (Table 5.10).

5.5 DISCUSSION

The results of these experiments support the hypothesis that the composition of protein in nutritionally adequate diets consumed during gestation and lactation influences food intake regulation in the offspring. Offspring born to dams fed the S diet during gestation and lactation had increased food intake and BW compared with those born to the C diet fed dams. In addition, decreased response to glucose preloads, higher insulin at weaning and 15 wks post-weaning and higher mRNA expression of AgRP at weaning and higher insulin, GLP-1 and ghrelin, 30 min after protein preloads characterized the offspring born to dams fed soy protein. In addition, the PAR hypothesis was not supported. Rats born to dams fed soy protein but fed the C diet at weaning were less adversely affected than those maintained on the S diet.

Higher food intake and body weight in offspring born to dams fed the S diet may be attributed to its effect on the development of the intake regulatory system for several reasons: First, the development of insulin resistance as shown by higher plasma insulin concentrations at d 20 of gestation and at birth in offspring born to dams fed the S diet would be expected to influence development of the intake regulatory system. Higher concentrations of insulin within the immature hypothalamus have been associated with greater food intake and BW in rats [128, 179].
Second, mRNA expressions of AgRP in the hypothalamus and plasma concentrations of ghrelin were higher in offspring born to dams fed the S diet. Both ghrelin and AgRP are orexigenic [251]. Ghrelin stimulates feeding through activation of NPY/AgRP in hypothalamus in rats [251]. Furthermore, it may be that differences in amino acid composition of soy protein and casein accounted for the increase in AgRP mRNA expression in the offspring from the dams fed the S diet. Rats placed on a low protein diet (10% of calories) exhibited increased food intake and hypothalamic AgRP mRNA expression. In vitro, hypothalamic cells reduce AgRP mRNA expression in response to increased amino acid concentration [247].

Third, the stronger blocking effect of devazepide (CCK-A receptor blocker) on food intake reduction after protein preloads in rats born to the dams fed the C diet indicates that both central and peripheral intake regulatory system were altered by source of protein in the dams’ diet. CCK-A receptors are mainly located in the gastro-intestinal tract and mediate the short-term satiety effect of proteins [252] and the stronger blocking effect of devazepide observed in offspring born to dams fed the C diet demonstrates more of the satiety effect of protein preloads was mediated through CCK-A receptors.

Fourth, both long-term food intake and 1 h food intake in response to preloads were influenced by the dams’ diet. Food intake over the study period was higher and short-term food intake was less responsive to preloads in offspring born to dams fed the S diet indicating that both short- and long-term intake regulatory systems were affected by the dams’ diet.

The current study also showed that postnatal environment prior to weaning was a factor affecting the expression of the effect of source of protein in the dams’ diet.
Extending the test diet to gestation and lactation (Exp 2) exaggerated the effect of the S diet on the increase in food intake, BW and plasma concentrations of insulin and GLP-1 in the offspring compared to when the diet was fed only during gestation. In addition, while mRNA expression of intake regulatory genes was not altered at birth, higher mRNA expression of AgRP was found at weaning in offspring born to dams fed the S diet (Fig 5.1) consistent with continuing development of neuronal circuitry until 16 days after birth in rodents [182, 186, 187]. Fasting concentrations of insulin were affected by the weaning diet in Exp 1 when the diets were fed to the dams only during gestation and were higher in offspring weaned to the S diets. Similarly, weaning diet influenced fasting concentrations of PYY in Exp 1 and they were higher in offspring weaned to the C diet. Conversely, extending the dams’ diet throughout lactation (Exp 2) abolished the effect of the weaning diets found in Exp 1 on higher fasting insulin and PYY and the interaction between the dams’ and weaning diets in influencing the food intake reduction after protein preloads. Possibly the latter is explained by exposure of the pups to the diet, as rats start to eat a solid diet by 15 days of postnatal age [253]. Previous studies have shown that the protein composition of diets fed to rats at d 17 of age affects food selection behavior [172].

Rats at weaning were fed either C or S diet in Experiments 1a and 2 as a test of the PAR hypothesis which states that offspring weaned to similar diets as their mothers will adapt more appropriately to their postnatal environment than those that receive an unmatched diet [36]. However, our observations in the current study do not support the PAR hypothesis and show that the weaning diet had little influence on the effects of the dam’s diets. In Exp 1a the weaning diet had no effect on cumulative 4 wk FI (Table 5.3)
or 1h FI in response to protein preloads (Table 5.4) or on response of insulin, ghrelin, GLP-1 or PYY to protein preloads (Table 5.5). In Exp 2, neither cumulative 14 wk FI (Fig 5.2) or 1 h FI in response to protein preloads at wk 7 (Table 5.8) or glucose preloads at wk 13 (Table 5.9). Similarly, at wk 15 (Table 5.10) higher insulin, GlP-1 and ghrelin, following protein preloads characterized the offspring from the S diet fed dams and this response was not affected by the composition of the weaning diet.

The results obtained from the present study cannot be explained by stress responses of the dams or pups. First, there were no group differences due to diet in litter size or birth weight of the offspring or in BW of dams at arrival, d 14 or d 20 of gestation. Secondly, as reported previously, no difference was found in fasting corticosterone level (ng/ml) in dams at d 14 (192 ± 28 and 228 ± 47) or d 20 (340 ± 34 and 388 ± 45) of gestation or at wk 6 after weaning (213 ± 16 and 239 ± 33) or pups at weaning (299 ± 29 and 352 ± 60) [248].

However, there are limiting factors in this study that make it difficult to compare the effect of gestational diet in Exp 1 and extended dams’ diet in Exp 2: First, hormone measurements were conducted at different stages of life (during maturation at wk 9 in Exp 1 vs. after maturation at wk 15 in Exp 2). Second, food intake was measured only for 4 wks in Exp 1 while it was measured for 14 wks after weaning in Exp 2.

The mechanisms by which protein source in the diet fed during gestation and lactation influences food intake regulation in offspring remain to be investigated. However, the difference in characteristics of casein and soy protein including amino acid composition, bioactive peptides (BAP) and digestion kinetics may play a role. Arginine, one of the most potent insulinotropic amino acids [254-256], which is almost twofold
higher in soy protein than casein, may have contributed to higher in utero insulin exposure of offspring born to dams fed the S diet. Proline which is twofold higher in casein than soy protein can influence glucose metabolism by stimulating glucose uptake in tissues [257]. Moreover, proline-rich bioactive peptides are more resistant to digestion which is essential for their further physiological functions [258]. Furthermore, many BAPs have been identified in casein and soy protein which are known to affect release of peptide hormones in the gastro-intestinal tract [226, 259-261] and to be absorbed and present in blood [262]. The digestion kinetics of proteins also influence their metabolic activities [170] and brain amino acid concentrations and neural activity [172]. Furthermore, increased homocysteine concentrations at weaning in offspring born to S diet fed dams have been observed [248]. Homocysteine concentrations in plasma have been related to hypomethylation of DNA [145] and disturbed key events in organogenesis and in embryonic vasculogenesis [146].

5.6 CONCLUSION

Nutritionally complete diets based on casein and soy protein consumed during gestation and lactation differ in their effects on BW and FI regulation in the offspring. The increased BW of pups born to dams fed the S diets is associated with alteration in the food intake regulatory system that favor increased food intake. Extending the diet from gestation alone to throughout gestation and lactation exaggerated these effects of the S diet. However, the diet consumed post-weaning had little effect on the outcome of the dams’ diets.
5.7 PERSPECTIVES AND SIGNIFICANCE

This observation that diets routinely used in animal studies and believed to be nutritionally equal led to different physiological outcomes is of particular significance because the majority of animal studies aimed at understanding the effects of maternal diets on development are based on diets that induce under-nutrition. In contrast, this study shows that food intake regulation in the offspring differs among “normal” diets often used for maintenance of rodent dams during gestation and lactation. Clearly, even though judged to be nutritionally complete diets, protein source and many other aspects of test diets fed during gestation and lactation in animal models of development could be a factor explaining variance in outcomes among studies. The diets fed were chosen because these proteins are prevalent in the human diet and are the foundation for two recommended diets for rodent studies. Although the relevance of these results to humans is uncertain because human diets during gestation contain many mixed protein sources, infants may be a vulnerable group. Infants are weaned to formula that contain a constant source of protein (e.g. whey/casein mixtures, soy protein) or their hydrolysates, with or without amino acid additions, during early life. However, the effects of the protein and amino acid composition of infant formula on FI regulatory systems and later life outcomes remain to be determined.
The amino acid content of the diets is calculated based on purity of the protein sources (87% and 90% for casein and soy protein respectively; Cystine was added to both casein (3g/kg diet) and soy protein (2.54g/kg diet) diets; Methionine was added to soy protein diet (2.54 g/kg diet); Isolated soy protein: Dyets Inc; Micellar casein: Harlan Teklad

**Table 5.1. Amino acid composition of casein and soy protein AIN-93G diets (/kg diet)**

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Micellar Casein</th>
<th>Isolated Soy Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine</td>
<td>4.7</td>
<td>6.7</td>
</tr>
<tr>
<td>Arginine</td>
<td>5.7</td>
<td>11.9</td>
</tr>
<tr>
<td>Aspartic acid + Asn</td>
<td>10.6</td>
<td>18.0</td>
</tr>
<tr>
<td>Cystine</td>
<td>3.5</td>
<td>4.5</td>
</tr>
<tr>
<td>Glutamic acid + Gln</td>
<td>34.8</td>
<td>29.9</td>
</tr>
<tr>
<td>Glycine</td>
<td>2.8</td>
<td>6.7</td>
</tr>
<tr>
<td>Histidine</td>
<td>4.4</td>
<td>4.1</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>8.7</td>
<td>7.7</td>
</tr>
<tr>
<td>Leucine</td>
<td>13.9</td>
<td>12.8</td>
</tr>
<tr>
<td>Lysine</td>
<td>12.2</td>
<td>9.9</td>
</tr>
<tr>
<td>Methionine</td>
<td>4.0</td>
<td>4.5</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>7.7</td>
<td>8.1</td>
</tr>
<tr>
<td>Proline</td>
<td>16.0</td>
<td>7.9</td>
</tr>
<tr>
<td>Serine</td>
<td>8.4</td>
<td>8.1</td>
</tr>
<tr>
<td>Threonine</td>
<td>6.6</td>
<td>5.9</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>1.7</td>
<td>2.2</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>8.0</td>
<td>5.9</td>
</tr>
<tr>
<td>Valine</td>
<td>10.4</td>
<td>7.9</td>
</tr>
<tr>
<td>Gene</td>
<td>Context Sequence</td>
<td></td>
</tr>
<tr>
<td>-------------------------------------------</td>
<td>-----------------------------------------</td>
<td></td>
</tr>
<tr>
<td>Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)</td>
<td>CGGAAAACCACCCATCACCCTTCCAG</td>
<td></td>
</tr>
<tr>
<td>Neuropeptide Y (NPY)</td>
<td>CCCGCCGCCCATGATGCTAGGTAAC</td>
<td></td>
</tr>
<tr>
<td>Agouti Related Protein (AgRP)</td>
<td>GCTTTGGCAGAGTGCTAGATCCAC</td>
<td></td>
</tr>
<tr>
<td>Pro-opiomelanocortin (POMC)</td>
<td>GCAACCTGCTGGCTTGCATCCGGGC</td>
<td></td>
</tr>
<tr>
<td>Leptin Receptor</td>
<td>TGTTACACTGGGAATTCTGTATGT</td>
<td></td>
</tr>
</tbody>
</table>

Probes were supplied by Applied Biosystems Inc.
Table 5.3. Exp 1 (part a): Effect of protein source in gestational and in weaning diets on food intake of male offspring at wk 1-4 post-weaning

<table>
<thead>
<tr>
<th>Dams’ Diet</th>
<th>Weaning Diet</th>
<th>C</th>
<th>S</th>
<th>C</th>
<th>S</th>
<th>p Values</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(g)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Week 1</td>
<td>87 ± 3.3</td>
<td>91 ± 3.0</td>
<td>100 ± 2.9</td>
<td>88 ± 2.2</td>
<td>D: NS</td>
</tr>
<tr>
<td></td>
<td>Week 2</td>
<td>129 ± 5.5</td>
<td>144 ± 3.9</td>
<td>137 ± 3.2</td>
<td>140 ± 3.5</td>
<td>W: NS</td>
</tr>
<tr>
<td></td>
<td>Week 3</td>
<td>145 ± 3.9</td>
<td>152 ± 3.6</td>
<td>150 ± 3.5</td>
<td>147 ± 3.7</td>
<td>T: &lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>Week 4</td>
<td>153 ± 5.8</td>
<td>165 ± 4.2</td>
<td>169 ± 3.6</td>
<td>162 ± 2.6</td>
<td>D × W: &lt;0.05</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>W × T: &lt;0.005</td>
</tr>
</tbody>
</table>

Data are means ± SE; n= 12/ group. C: Casein diet; S: Soy protein; D: Dams’ diet; W: Weaning diet; T: Time; NS: Not significant; Food intake was analyzed by MIXED model with gestational and weaning diets and time as main factors.
Table 5.4. Exp 1 (part a): Effect of protein source in the gestational and in the pup diets on food intake (1 h) in response to protein preloads in male offspring at wk 6

| Preload      | Casein  | Soy Protein | Casein  | Soy Protein | Casein  | Soy Protein | Casein  | Soy Protein | Casein  | Soy Protein | Casein  | Soy Protein | Casein  | Soy Protein | Casein  | Soy Protein | Casein  | Soy Protein | Casein  | Soy Protein | Casein  | Soy Protein | Casein  | Soy Protein | Casein  | Soy Protein |
|--------------|---------|-------------|---------|-------------|---------|-------------|---------|-------------|---------|-------------|---------|-------------|---------|-------------|---------|-------------|---------|-------------|---------|-------------|---------|-------------|---------|-------------|---------|-------------|---------|-------------|---------|-------------|---------|-------------|
| Water        | 4.6 ± 0.2| 4.7 ± 0.5   | 4.5 ± 0.3| 4.1 ± 0.5   | 4.5 ± 0.3| 4.0 ± 0.3   | 5.2 ± 0.6| 5.3 ± 0.4   |         |             |         |             |         |             |         |             |         |             |         |             |         |             |         |             |         |             |
| Protein      | 2.8 ± 0.3| 2.1 ± 0.6   | 2.7 ± 0.3| 3.6 ± 0.3   | 3.3 ± 0.3| 2.5 ± 0.4   | 3.2 ± 0.3| 3.1 ± 0.3   |         |             |         |             |         |             |         |             |         |             |         |             |         |             |         |             |         |             |
| Water - Protein | 1.7 ± 0.3 *| 1.5 ± 0.4 *| 1.8 ± 0.3 *| 0.5 ± 0.5   | 1.2 ± 0.5 *| 1.5 ± 0.4 *| 2.0 ± 0.5 *| 2.1 ± 0.5 *|         |             |         |             |         |             |         |             |         |             |         |             |         |             |         |             |         |             |

Values are means ± SE; n=11-12/group; C: Casein diet; S: Soy protein diet; D: Dams’ diet; W: Weaning diet; P: Preload; NS: Not significant; Casein: American Casein Co (Burlington, N.J.) was given at 3g/kg BW by gavage 30 min before introducing the food; Isolated soy protein: General nutrition products (Greenville, S.C.) was given at 3g/kg BW by gavage 30 min before introducing the food; Distilled water (control): 6 ml; Water-Protein: Calculated as food intake after water preload - food intake after protein preload; Data were analyzed by MIXED model with maternal diet, weaning diet, preload and time as main factors; * Significant food intake suppression: p<0.05; † p=0.08
Table 5.5. Exp 1 (part a): Effect of protein source in the gestational and in the pup diets on plasma measures in response to preloads at wk 9 post-weaning

<table>
<thead>
<tr>
<th>Dams’ Diet Weaning Diet</th>
<th>C</th>
<th>S</th>
<th>S</th>
<th>p Values</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Preload</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Insulin, ng/ml</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Water</td>
<td>0.68 ± 0.12</td>
<td>1.03 ± 0.08</td>
<td>0.49 ± 0.14</td>
<td>1.39 ± 0.18</td>
</tr>
<tr>
<td>Casein</td>
<td>1.65 ± 0.20</td>
<td>2.13 ± 0.25</td>
<td>1.59 ± 0.39</td>
<td>2.15 ± 0.10</td>
</tr>
<tr>
<td>Soy Protein</td>
<td>1.99 ± 0.41</td>
<td>2.84 ± 0.12</td>
<td>1.72 ± 0.31</td>
<td>2.96 ± 0.30</td>
</tr>
<tr>
<td>GLP-1, pM</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Water</td>
<td>2.8 ± 0.21b</td>
<td>1.9 ± 0.23c</td>
<td>2.1 ± 0.33b</td>
<td>2.2 ± 0.35b</td>
</tr>
<tr>
<td>Casein</td>
<td>4.0 ± 0.41a</td>
<td>3.6 ± 0.26b</td>
<td>3.7 ± 0.26a</td>
<td>2.6 ± 0.30b</td>
</tr>
<tr>
<td>Soy Protein</td>
<td>4.4 ± 0.33a</td>
<td>5.0 ± 0.50a</td>
<td>4.8 ± 0.58a</td>
<td>5.6 ± 0.47a</td>
</tr>
<tr>
<td>PYY, pM</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Water</td>
<td>49.8 ± 4.47</td>
<td>43.1 ± 4.48</td>
<td>50.5 ± 2.13</td>
<td>37.9 ± 3.80</td>
</tr>
<tr>
<td>Casein</td>
<td>52.3 ± 5.10</td>
<td>59.3 ± 2.58</td>
<td>47.6 ± 7.83</td>
<td>43.9 ± 4.25</td>
</tr>
<tr>
<td>Soy Protein</td>
<td>48.7 ± 5.14</td>
<td>52.3 ± 5.89</td>
<td>40.7 ± 8.12</td>
<td>50.7 ± 5.99</td>
</tr>
<tr>
<td>Ghrelin, ng/ml</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Water</td>
<td>3.6 ± 0.36</td>
<td>2.4 ± 0.21</td>
<td>2.9 ± 0.34</td>
<td>3.8 ± 0.55</td>
</tr>
<tr>
<td>Casein</td>
<td>1.7 ± 0.23</td>
<td>1.7 ± 0.26</td>
<td>1.7 ± 0.43</td>
<td>1.5 ± 0.13</td>
</tr>
<tr>
<td>Soy Protein</td>
<td>1.3 ± 0.75</td>
<td>1.8 ± 0.36</td>
<td>1.7 ± 0.34</td>
<td>1.6 ± 0.28</td>
</tr>
</tbody>
</table>

Data are means ± SE, n=6/group; C: Casein diet; S: Soy protein diet; D: Dams’ diet; W: Weaning diet; P: Preload; NS: Not significant; Casein: American Casein Co (Burlington, N.J.) was given at 3g/kg BW by gavage 30 min before blood withdrawal; Isolated soy protein: General nutrition products (Greenville, S.C.) was given at 3g/kg BW by gavage 30 min before blood withdrawal; MIXED model followed by Tukey’s post-hoc test with dams’ and weaning diets and preloads as main factors: Values in a column in each diet group with different superscript letters are significantly different, p<0.05
Table 5.6. Exp 1 (part b): Effect of source of protein during gestation on food intake (1 h) after protein preloads with and without CCK-A and opioid receptor blockers at wk 6 post-weaning

<table>
<thead>
<tr>
<th>Dams' Diet</th>
<th>C</th>
<th>S</th>
<th>p Values</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Casein</td>
<td>Casein</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Soy Protein</td>
<td>Soy Protein</td>
<td></td>
</tr>
<tr>
<td>Preload</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Naloxone (g)</td>
<td>Water</td>
<td>4.9 ± 0.3</td>
<td>5.3 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>Protein</td>
<td>2.5 ± 0.3</td>
<td>2.5 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>Protein + Naloxone</td>
<td>4.3 ± 0.5</td>
<td>4.9 ± 0.5</td>
</tr>
<tr>
<td>Water- Protein</td>
<td>2.4 ± 0.5 *</td>
<td>2.1 ± 0.5 *</td>
<td>2.8 ± 0.4</td>
</tr>
<tr>
<td>(Protein + Naloxone) - Protein</td>
<td>1.9 ± 0.5 *</td>
<td>0.8 ± 0.4</td>
<td>2.5 ± 0.5 *</td>
</tr>
<tr>
<td>Devazepide</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Water</td>
<td>5.4 ± 0.6</td>
<td>4.4 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>Protein</td>
<td>2.7 ± 0.4</td>
<td>2.9 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>Protein + Devazepide</td>
<td>5.6 ± 0.5</td>
<td>3.9 ± 0.4</td>
</tr>
<tr>
<td>Water - Protein</td>
<td>2.8 ± 0.6 *</td>
<td>3.0 ± 0.5 *</td>
<td>1.5 ± 0.6</td>
</tr>
<tr>
<td>(Protein + Devazepide) - Protein</td>
<td>3.0 ± 0.6 *</td>
<td>2.8 ± 0.6 *</td>
<td>1.0 ± 0.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>D×P: NS</td>
</tr>
</tbody>
</table>

Data are means ± SE; n=10-12/group; C: Casein diet; S: Soy protein diet; D: Diet; P: Preload; NS: Not significant; Casein: American Casein Co (Burlington, NJ.) was given at 3g/kg BW by gavage 30 min before introducing the food; Isolated soy protein: General nutrition products (Greenville, S.C.) was given at 3g/kg BW by gavage 30 min before introducing the food; Distilled water (control): 6 ml; Devazepide: CCK-A receptor blocker: ML laboratories, PLC, London, UK was given at 2.5 mg/kg BW intraperitoneal (IP) injection at 35 min before introducing the food; Naloxone: opioid receptor blocker: Sigma Chemical Co. St Louis, MO, was given at a dose of 1.0 mg/kg body weight by intraperitoneal (IP) injection at 5 min before introducing the food; Water-Protein: Calculated as food intake after water preload - food intake after protein preload; (Protein + receptor blocker)-Protein: Calculated as food intake after co-administration of preload and receptor blocker - food intake after preload; MIXED model with diet, preload and time as main factors. * Significant food intake suppression: p<0.05
Table 5.7. Exp 2: Effect of source of protein during gestation and lactation on fasting plasma measures to weaning in offspring

<table>
<thead>
<tr>
<th></th>
<th>Gestation and Lactation Diet</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C</td>
<td>S</td>
</tr>
<tr>
<td><strong>Fetus (Day 20 Gestation)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Insulin, ng/ml</td>
<td>0.28 ± 0.03</td>
<td>0.41 ± 0.06*</td>
</tr>
<tr>
<td><strong>Birth</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Insulin, ng/ml</td>
<td>0.27 ± 0.07</td>
<td>0.49 ± 0.08*</td>
</tr>
<tr>
<td><strong>Weaning</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Insulin, ng/ml</td>
<td>0.12 ± 0.01a</td>
<td>0.18 ± 0.02b</td>
</tr>
<tr>
<td>Ghrelin, ng/ml</td>
<td>2.3 ± 0.19</td>
<td>1.9 ± 0.15</td>
</tr>
<tr>
<td>GLP-1, pM</td>
<td>2.7 ± 0.11</td>
<td>3.6 ± 0.5</td>
</tr>
<tr>
<td>PYY, pM</td>
<td>53.0 ± 4.76</td>
<td>46.0 ± 5.40</td>
</tr>
</tbody>
</table>

Data are means ± SE; n=5-6/group; C: Casein; S: Soy protein; Values in a row with different superscript letters are significantly different, p<0.05; * p=0.08; Unpaired t-test
Fig. 5.1. Exp 2: Effect of source of protein during gestation and lactation on relative mRNA expression of hypothalamic NPY, AgRP, POMC and leptin receptor in offspring at birth and at weaning.

Data are means ± SE; n= 9-10/ group; Unpaired t-test: * p<0.05
Fig. 2.2. Exp 2: The effect of source of protein during gestation and lactation on food intake of male offspring.

Data are means ± SE; n= 12/group. C: Casein; S: Soy protein; Food intake was analyzed by MIXED model with maternal diet and time as main factors. Time: \( p<0.0001 \), Diet: \( p<0.0001 \); Interaction: \( p<0.0001 \); * \( p<0.05 \); Data is pooled for pup diet to present the effect of dams’ diet alone on food intake.
### Table 5.8. Exp 2: Effect of source of protein during gestation and lactation and weaning on food intake (1 h) in response to protein preloads at wk 7 post-weaning

Data are means ± SE; n=11-12/group; C: Casein; S: Soy protein; D: Dams’ diet; W: Weaning diet; P: Preload; NS: Not significant; Casein: American Casein Co (Burlington, N.J.); Isolated soy protein: General nutrition products (Greenville, S.C.); Protein preloads were given at 3g/kg BW by gavage 30 min before introducing the food; Distilled water (control): 6 ml; Water-Protein: food intake after water preload – food intake after protein preload; Food intake was analyzed by MIXED model followed by Tukey’s post-hoc test with maternal diet, weaning diet and preload as main factors. Values in a row with different superscript letters are significantly different, p<0.05; * Significant food intake suppression: p<0.05; † p=0.07

<table>
<thead>
<tr>
<th>Preload</th>
<th>C</th>
<th>S</th>
<th>C</th>
<th>S</th>
<th>p Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>8.6 ± 0.5b</td>
<td>9.4 ± 0.6ab</td>
<td>11.3 ± 1.4a</td>
<td>8.8 ± 0.9b</td>
<td>D: &lt;0.005</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>W: NS</td>
</tr>
<tr>
<td>Casein</td>
<td>5.4 ± 0.7b</td>
<td>7.5 ± 0.6ab</td>
<td>9.6 ± 1.4a</td>
<td>7.2 ± 0.8ab</td>
<td>P: &lt;0.0005</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>D × W: &lt;0.001</td>
</tr>
<tr>
<td>Soy Protein</td>
<td>5.3 ± 0.7</td>
<td>6.9 ± 0.7</td>
<td>8.8 ± 1.4</td>
<td>7.3 ± 0.9</td>
<td></td>
</tr>
</tbody>
</table>

### Water - Protein

<table>
<thead>
<tr>
<th>Preload</th>
<th>C</th>
<th>S</th>
<th>C</th>
<th>S</th>
<th>p Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein</td>
<td>3.3 ± 0.6*</td>
<td>1.9 ± 0.7*</td>
<td>1.6 ± 0.7</td>
<td>1.6 ± 0.6</td>
<td>D: &lt;0.05</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>W: NS‰</td>
</tr>
<tr>
<td>Soy Protein</td>
<td>3.4 ± 0.5*</td>
<td>2.4 ± 0.6*</td>
<td>2.5 ± 0.6*</td>
<td>2.6 ± 1.6*</td>
<td>P: NS</td>
</tr>
</tbody>
</table>
Table 5.9. Exp 2: Effect of source of protein during gestation and lactation and weaning on food intake (1 h) in response to glucose and water preloads at wk 13 post-weaning

<table>
<thead>
<tr>
<th>Dams’ Diet</th>
<th>Weaning Diet</th>
<th>Water</th>
<th>Glucose</th>
<th>Water - Glucose</th>
<th>p Values</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C</td>
<td>S</td>
<td>C</td>
<td>S</td>
<td></td>
</tr>
<tr>
<td>Preload</td>
<td>C</td>
<td>S</td>
<td>C</td>
<td>S</td>
<td></td>
</tr>
</tbody>
</table>
| Water      | 5.6 ± 0.4    | 6.4 ± 0.4 | 6.2 ± 0.4 | 7.0 ± 0.3 | D: <0.0005  
|            |              |       | W: <0.001 |               |         |
| Glucose    | 3.2 ± 0.2\(^b\) | 4.3 ± 0.3\(^a\) | 4.7 ± 0.2\(^a\) | 5.3 ± 0.3\(^a\) | P: <0.0001 |
| Water - Glucose | 2.4 ± 0.4\(^*\) | 2.1 ± 0.3\(^*\) | 1.5 ± 0.3 | 1.8 ± 0.3 | D: <0.05  
|            |              |       | W: NS   |               |         |
|            |              |       | P: NS   |               |         |

Data are means ± SE; n=11-12/group; C: Casein; S: Soy protein; D: Dams’ diet; W: Weaning diet; P: Preload; NS: Not significant; Glucose preload was given by gavage (0.375 g/ml) at 5g/kg BW; water preload was given in the same volume as glucose preload; Water – Glucose: food intake after water preload – food intake after glucose preload; Food intake was analyzed by MIXED model with maternal diet, weaning diet and preload as main factors; Values in a row with different superscript letters are significantly different, p<0.05; * Significant food intake suppression: p<0.05
Table 5.10. Exp 2: Effect of source of protein during gestation and lactation and weaning on plasma measures in response to water and protein preloads in male offspring at wk 15

<table>
<thead>
<tr>
<th>Dams’ Diet</th>
<th>C</th>
<th>S</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Weaning Diet</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Preload</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Insulin, ng/ml</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Water</td>
<td>1.47 ± 0.19</td>
<td>1.95 ± 0.14</td>
<td>2.06 ± 0.27</td>
<td>2.14 ± 0.09</td>
<td>D: &lt;0.05</td>
<td></td>
</tr>
<tr>
<td>Casein</td>
<td>2.01 ± 0.06</td>
<td>1.97 ± 0.14</td>
<td>2.02 ± 0.11</td>
<td>1.93 ± 0.11</td>
<td>W: NS</td>
<td></td>
</tr>
<tr>
<td>Soy Protein</td>
<td>2.06 ± 0.07</td>
<td>2.05 ± 0.15</td>
<td>2.10 ± 0.04</td>
<td>2.15 ± 0.09</td>
<td>P: =0.06</td>
<td></td>
</tr>
<tr>
<td><strong>GLP-1, pM</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Water</td>
<td>2.00 ± 0.26</td>
<td>2.50 ± 0.26</td>
<td>2.57 ± 0.20</td>
<td>1.99 ± 0.13</td>
<td>D: &lt;0.05</td>
<td></td>
</tr>
<tr>
<td>Casein</td>
<td>3.63 ± 0.84</td>
<td>6.58 ± 0.88</td>
<td>7.97 ± 1.16</td>
<td>5.13 ± 1.06</td>
<td>W: NS</td>
<td></td>
</tr>
<tr>
<td>Soy Protein</td>
<td>8.06 ± 1.87</td>
<td>6.83 ± 0.79</td>
<td>8.94 ± 0.76</td>
<td>9.40 ± 1.65</td>
<td>P: &lt; 0.05</td>
<td></td>
</tr>
<tr>
<td><strong>PYY, pM</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Water</td>
<td>47.00 ± 6.32</td>
<td>43.10 ± 2.13</td>
<td>41.12 ± 4.74</td>
<td>43.16 ± 5.71</td>
<td>D: NS</td>
<td></td>
</tr>
<tr>
<td>Casein</td>
<td>50.23 ± 5.40</td>
<td>48.28 ± 2.17</td>
<td>42.20 ± 1.42</td>
<td>48.48 ± 1.43</td>
<td>W: NS</td>
<td></td>
</tr>
<tr>
<td>Soy Protein</td>
<td>50.13 ± 4.04</td>
<td>47.98 ± 4.19</td>
<td>44.35 ± 3.70</td>
<td>47.80 ± 8.57</td>
<td>P: NS</td>
<td></td>
</tr>
<tr>
<td><strong>Ghrelin, ng/ml</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Water</td>
<td>1.98 ± 0.21</td>
<td>1.75 ± 0.20</td>
<td>3.77 ± 0.10</td>
<td>2.67 ± 0.35</td>
<td>D: &lt;0.01</td>
<td></td>
</tr>
<tr>
<td>Casein</td>
<td>1.53 ± 0.21</td>
<td>1.53 ± 0.16</td>
<td>1.55 ± 0.20</td>
<td>1.78 ± 0.98</td>
<td>W: NS</td>
<td></td>
</tr>
<tr>
<td>Soy Protein</td>
<td>1.70 ± 0.29</td>
<td>1.42 ± 0.11</td>
<td>2.89 ± 0.48</td>
<td>1.83 ± 0.19</td>
<td>P: &lt; 0.05</td>
<td></td>
</tr>
</tbody>
</table>

Data are means ± SE; n=6/group; C: Casein diet; S: Soy protein diet; D: Diet; P: Preload; W: Weaning Diet; NS: Not significant; Casein: American Casein Co (Burlington, N.J.) was given at 3g/kg BW by gavage 30 min before blood withdrawal; Isolated soy protein: General nutrition products (Greenville, S.C.) was given at 3g/kg BW by gavage 30 min before blood withdrawal MIXED model followed by Tukey’s post-hoc test with dams’ and weaning diets and preload as main factors.
CHAPTER 6

CASEIN AND SOY PROTEIN IN MATERNAL DIETS AFFECT
CHARACTERISTICS OF METABOLIC SYNDROME AND FOOD
INTAKE IN FEMALE OFFSPRING OF RATS
6. CASEIN AND SOY PROTEIN IN MATERNAL DIET ALTER
CHARACTERISTICS OF METABOLIC SYNDROME AND FOOD INTAKE IN
FEMALE OFFSPRING OF RATS

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Running Head: Maternal proteins and metabolic syndrome
Submitted to: Nutrients

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H. Anderson, no conflicts of interest.

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6.1 ABSTRACT

The AIN-93G diets based on soy protein (S) or casein (C) were fed to two groups (n=12/group) of pregnant Wistar rats from day 3 of gestation and throughout lactation. Their effects on characteristics of metabolic syndrome and food intake regulation in female pups maintained for 15 wk on the C diet were compared. Body weight (BW) and food intake (FI) were measured weekly. Fat pad mass was measured at birth, at weaning and at wk 15. Glucose and insulin tolerance tests were conducted at wks 8 and 12 and systolic (SBP) and diastolic (DBP) blood pressure were measured at wks 4, 8 and 12. Plasma was collected at weaning and the end of the studies for glucose, insulin, GLP-1, PYY and ghrelin. FI in response to protein preloads was measured at wk 7. Feeding the S diet throughout gestation and lactation resulted in higher SBP (p<0.005), FI (p<0.05) and GLP-1 and lower PYY at weaning and higher BW during weeks 11-15 and fat pad mass at wk 15 (all p<0.05). However, no sign of insulin resistance was found, nor was short-term FI in response to protein preloads affected. In conclusion, the AIN-93 G diet based on soy protein compared to casein when consumed throughout gestation and lactation increased the presence of characteristics of metabolic syndrome in female offspring.

**Keywords:** protein, programming, metabolic syndrome, food intake, female offspring
6.2 INTRODUCTION

Numerous epidemiological and clinical studies plus investigations in animal models provide substantial evidence that fetal and early post-natal nutrition alters development of somatic structures, endocrine systems and homeostatic mechanisms in the fetus and infant. These effects influence the risk of obesity, hypertension, diabetes and other components of metabolic syndrome in later life [8-10].

Both low and high protein diets fed during gestation and lactation have detrimental effects on the physiologic and metabolic phenotype of offspring in animals [12, 16, 263]. In addition, source of protein in nutritionally complete diets fed to pregnant rats influences phenotype of the male offspring [248]. Casein and soy protein are two prevalent sources of proteins in human diets [264] and are the proteins most often used in rodent test diets for metabolic studies [265, 266]. We previously reported that soy protein-based diets compared with casein-based diets fed during gestation alone or gestation and lactation increased the presence of characteristics of the metabolic syndrome in male offspring of Wistar rats [248]. However, there is substantial evidence indicating that the dams’ diet affects phenotypes of the offspring in a sex-dependent manner [19, 67, 92, 104-106]. For example, when rat dams were fed a high protein (40% of total calories) diet during gestation and lactation, blood pressure and glomerulosclerosis were elevated in male offspring, whereas increased food efficiency, body weight and fat pads were observed in female offspring [19]. In another study, impaired glucose tolerance was found in adult females and their insulin response to an oral glucose preload was low if they were born to rat dams fed a low protein (8% protein) diet during gestation [210]. In contrast, male but not female Wistar rats born to dams fed
low protein (8% of total calories) diets were relatively hyperinsulinemic and insulin resistant at 20 weeks of age [105].

Therefore, the present report is based on the two observations that the effects of maternal diets on rat offspring are sex dependent [19] and that AIN 93G diets based on soy protein and casein differ in their effects on metabolic syndrome and food intake in male rats (Chapters 4 and 5). The specific objective of this study was to report the effect of the AIN-93 G diets based on soy protein or casein and fed during gestation and lactation on characteristics of metabolic syndrome and food intake regulation in female offspring of Wistar rats.

6.3 METHODS AND PROCEDURES

6.3.1 Design

Two groups of pregnant rats (n=12/group) were fed either the casein (C) or the soy protein (S) diets during gestation and lactation. At weaning, one female from each mother in each diet group was assigned to the C diet for 15 weeks (n=12/group). For the remaining pups, BW was measured at birth (day 1, after litters were culled to 10 pups) and on days 7, 14 and 21. At birth, weaning (day 21 of age) and wk 16 post-weaning, rats (n=12/dam diet group) were sacrificed. BW was measured weekly from weaning to wk 15 after weaning. Body fat composition was determined at birth, at weaning and at wk 15. SBP, DBP and pulse rate were measured at wks 4, 8 and 12 in the pups. Glucose and insulin tolerance tests were conducted at wks 8 and 12.

Trunk blood of fetuses (n=5-6) at d 20 of gestation in Exp 2 and pups (n=5-6) at birth in Exp 1 and 2 from each dams’ diet group was obtained by pooling (n=3-4/sample)
to attain sufficient blood volume required for hormonal assays. At wk 15, pups (n=12/group) were sacrificed by decapitation after a 12-hour overnight food deprivation at weaning and at the end of the experiment (wk 15) for blood collection. At wk 15, pups in each diet group were allocated to two groups of six and received either glucose or water preload and were sacrificed 30 min later. Plasma concentrations of glucose, insulin, GLP-1, ghrelin, PYY, homocysteine, corticosterone and albumin were measured at weaning and at wk 15.

The protocol was approved by the University of Toronto Animal Care Committee and care and maintenance of the animals conformed to the guidelines of the Canadian Council on Animal Care.

6.3.2 Animals and diets

First-time pregnant Wistar rats were received at d 3 of gestation (Charles River, QC, Canada) and were housed individually in ventilated plastic transparent cages with bedding at 22±1°C and 12-h light-dark cycle (lights off at 2200h to 1000h). At weaning, female offspring were housed individually in ventilated plastic transparent cages with bedding. The powdered diets were provided *ad libitum* in stainless steel cups with a mesh disk insert to reduce spillage. All rats had free access to water throughout the experiments.

The composition (in g/kg) of the test diets was as follows. The casein diet (C) contained casein (200.0), cornstarch (529.4), sucrose (100.1), soybean oil (70.0), cellulose (50.0), vitamin mixture (10.0), mineral mixture (35.0), cystine (3.0), choline bitartrate (2.5), and tert-butyl hydroquinone (0.014). The composition of the soy diet (S) was identical to the C diet, except that soy protein replaced casein, and methionine (2.54)
and cystine (2.54) were added as recommended for the AIN-93G soy protein diet [219].

Amino acid content of the C and S diets as fed is shown in Table 6.1. Cornstarch, high-protein casein (87%), and cellulose were purchased from Harlan Teklad (Madison, WI). The vitamin and mineral mixtures, cystine, methionine, choline bitartrate, and tert-butyl hydroquinone were purchased from Dyets Inc. (Bethlehem, PA), sucrose from Allied Food Service (Toronto, ON, Canada), and soybean oil from Loblaws (Toronto, ON, Canada).

Genistein, daidzein and glycitein content (μg/g) of the soy protein diet was 36.1, 31.3 and 4.4, respectively.

6.3.3 Glucose tolerance test (GTT)

Rats were fasted overnight for 10 h. A blood sample was withdrawn from the tail vein prior to and at 15, 30, and 60 min after a glucose gavage (0.375 g glucose/ml, 5 g glucose/kg BW).

6.3.4 Insulin tolerance test (ITT)

Rats were fasted overnight for 10 h. Insulin (Humulin®-R, Eli Lilly and Company, Indianapolis, IN) injections were given intraperitoneally (IP) (0.5 U/ml, 0.75 U insulin/kg BW) and blood obtained prior to and at 15, 30, and 60 min after an insulin injection.

6.3.5 Blood pressure

Systolic (SBP) and diastolic blood pressure (DBP) were measured by the non-invasive tail-cuff method with optical plethysmography using a tail manometer-tachometer system (BP-2000, Visitech system, Apex, NC). Rats were restrained in
holders on a platform with constant temperature of 30°C. They were adapted daily to the
device for five days. On the day of measurement, five mock measurements preceded a
series of ten measurements and only the latter were used in calculating the average as
previously reported [7].

6.3.6 Blood glucose
Tail vein glucose concentration was assayed using a hand-held commercial
glucose meter (MediSense Precision Xtra, Abbott Laboratories, Alameda, CA) using test
strips [7]. Glucose in plasma from trunk blood obtained upon decapitation was measured
using a glucose oxidase kit (Ascensia Elite XL, Bayer AG, Leverkusen, Germany).

6.3.7 Short-term food intake
Short-term food intake (1h) was measured 30 min after water and protein preloads
at wk 7. Before testing, rats were adapted to the experimental procedures. They were
gavaged with water over 7 days before the adaptation test, performed as follows. Food
was deprived for 12 h before measurement. On d 1, one half of the rats were fed the
protein preload, whereas the rest were untreated. On the next day, this testing order was
reversed. The experiment began when it was determined that the process of gavaging did
not affect food intake.

At wk 7 after weaning, a randomized design was applied to examine the effect of
preloads (casein, soy protein or glucose) on short-term food intake. For testing the effect
of protein preloads, on the first day of experiment and after 12 h fasting, at 2130 h rats
received either casein, soy protein (3g / kg BW/6 ml distilled water) or water control (6
ml distilled water) as preload by gavage in random order. Food was introduced at 2200 h
and was measured for 1 h in both experiments. After a wash-out day, the testing order
was reversed. Ultimately, all rats received all treatments in random order. Food intake was measured to the nearest 0.1 g, under red light.

**6.3.8 Long-term food intake**

Weaned rats were housed individually in ventilated plastic transparent cages with bedding. Diets were provided ad libitum. The powdered diet was provided in jars with a mesh disk insert to minimize spillage. Food intake was measured weekly for 14 wks after weaning. Food intake over 24 h was not measured during wk 7-8 when preload experiments were conducted.

**6.3.9 Blood collection**

Trunk blood was collected in chilled Vacutainer tubes (BD, Franklin Lakes, NJ, USA) containing EDTA + Trasylol® (Bayer AG, Leverkusen, Germany) solution (10% blood volume, 500 KIU/ml). Blood samples were centrifuged for 20 min at 3000g and 4°C for 10 min. Plasma was separated and immediately stored at -80°C.

**6.3.10 Hormone assays**

Plasma insulin was measured using Insulin Enzyme Immunoassay (Cat# 80-INSRT-E01, Alpco Diagnostics, Salem, NH) with assay sensitivity of 0.124 ng/ml. Plasma homocysteine was measured by Enzyme Immunoassay (Cat# 194-5361, Bio-Rad Laboratories, Inc, Hercules, CA) with assay sensitivity of 1.0 μmol/L. Plasma albumin was measured using a colorimetric assay (Cat# 11970909, Roche Diagnostics, Indianapolis, IN) with assay sensitivity of 0.2 g/dl. Plasma corticosterone was measured using Enzyme Immunoassay (Cat# DSL-10-81100, Beckman Coulter, Webster, TX, USA) with assay sensitivity of 1.6 ng/ml. Total plasma PYY concentrations were
measured using radioimmunoassay (RIA) method (Cat. # RMPYY-68HK, Millipore Research Inc, St. Charles, MO) with assay sensitivity of 15.6 pg/ml. Total plasma ghrelin concentrations were measured using radioimmunoassay (RIA) method (Cat. # GHRT-89HK, Linco Research Inc, St. Charles, MO) with assay sensitivity of 93 pg/ml. Plasma GLP-1 concentrations were measured using ELISA method (Cat. # EGLP-35K, Linco Research Inc, St. Charles, MO) with assay sensitivity of 2 pM.

6.3.11 Body composition

Fat mass and lean mass were measured at birth by dual energy X-ray absorptiometry (DEXA) (pSabre, Orthometrix Inc., White Plains, NY) applying a specialized software program (Host Software version 3.9.4; Scanner Software version 1.2.0) [220]. After sacrificing, carcasses were placed directly on the DEXA. All scans were performed at a speed of 10 mm/s and a resolution of 0.5 x 1.0 mm. At weaning and at the end of experiments, fat pad mass was measured by dissection of extracted abdominal and perirenal fat.

6.3.12 Isoflavones measurement

Homogenized soy protein samples were analyzed for isoflavones (genistein, daidzein and glycine) by gas chromatography mass spectrometry (GC-MS), as previously described [221]. The isoflavones analysis involved extraction of samples twice with 5 ml 70% methanol, passing a portion of extraction through a C18 solid-phase extraction column (SPE column; Octadecyl C18/14%, 200 mg/3 ml; Applied Separations, Allentown, PA), hydrolysis with β-glucuronidase (Helix Pomatia; Sigma Aldrich, St. Louis, MO) and passage through another C18 SPE column. An internal standard (5α-androstane-3β,17β-diol; Steraloids Inc, Wilton, NH) was added to the column eluent, and
the sample was then derivatized with Tri-Sil Reagent (Pierce Co., Rockford, IL) before injection to the GC-MS (Agilent 6890 series GC system interfaced with an Agilent 5973 network mass selective detector; Agilent Technologies, Wilmington, DE).

6.3.13 Statistical analyses

All data are expressed as means ± SEM. The effect of dams’ diet on BW, food intake, glucose response, SBP and DBP was analyzed by PROC MIXED MODEL procedure with diet and time as main factors. A one-way repeated measures analysis of variance (ANOVA) followed by post-hoc Tukey’s test was conducted when treatment effects or interactions were statistically significant. Student’s unpaired t-test was applied to compare the dependent measures (e.g. hormones) at individual time points. Blood glucose response was calculated as the total incremental area under the curve (tAUC) of the blood glucose concentration over one hour after receiving glucose by gavage for the GTT and after receiving insulin injection for the ITT. For the former the reported tAUC is positive above baseline whereas for the latter the tAUC is a negative representing the area below baseline. The homeostasis model assessment of insulin resistance (HOMA-IR) index was calculated as fasting glucose multiplied by fasting insulin divided by 22.5 [222]. Food intake in response to protein preloads was analyzed by using PROC MIXED MODEL procedure with diet, and time as main factors. To examine the suppressant effect of protein preload on food intake, the delta of food intake (food intake after water control– food intake after nutrient preload) was calculated. The effect of the diets and preloads on plasma hormone concentrations was also analyzed by using PROC MIXED MODEL procedure with diet and preload as main factors. All analyses were conducted
using SAS (version 9e, SAS Institute, Cary, NC). Statistical significance was defined at $p<0.05$.

6.4 RESULTS

There were no differences due to the dams’ diets (C vs. S diets, respectively) on litter size ($13.2 \pm 0.7$ and $13.2 \pm 0.5$) or male/female ratio ($0.53 \pm 0.05$ and $0.52 \pm 0.03$), or on the pups’ body fat or body weight at birth (Table 6.2). There were no differences due to protein source in the dams’ diet in plasma insulin concentration in the fetus at d 20 of gestation ($0.28 \pm 0.03$ vs. $0.41 \pm 0.06$) or in the pups at birth ($0.27 \pm 0.07$ vs. $0.49 \pm 0.08$) (all $p=0.08$).

BW was not influenced by the dams’ diet ($p=0.07$) but was affected by time ($p<0.0001$) with no interaction between diet and time. However, BW was higher by 12% in female offspring born to S diet fed dams at wk 14 ($p<0.05$) (Fig 6.1), with the difference becoming statistically significant at wk 11 ($p<0.05$). Dams’ diet had no effect on BW and fat pad mass at weaning (Table 6.2) but by 15 wk post-weaning, fat pad mass was higher in offspring born to dams on the S diet ($p<0.05$) (Table 6.2).

Food intake was influenced by time ($p<0.0001$) and dams’ diet ($p<0.05$). It was higher in offspring born to the S diet fed dams throughout weeks 13-14 (Fig 6.2). At wk 7, food intake suppression of protein preloads (delta of food intake after water preload and after protein preload) was not influenced by the dams’ diet at 1h (Table 6.3). At weaning, fasting plasma GLP-1 higher and PYY lower in rats born to dams fed the S diet compared to those born to C diet fed dams. ($p<0.05$) (Table 6.4). Dams’ diet had no effect on fasting plasma concentrations of ghrelin, homocysteine and corticosterone at
weaning or at wk 15 (Table 6.4). However, plasma GLP-1 was affected by preload at wk 15 and was higher in pups from both dam groups after glucose preload than after water control (p<0.0005) (Table 6.5).

Dams’ diet influenced SBP which was higher in rats born to dams fed the S diet (p<0.05). Pulse rate was affected by an interaction between diet and time (p<0.005) (Table 6.6). At wk 12, the maternal S diet resulted in higher pulse rate (p<0.05) in the offspring (Table 6.6).

Dams’ diet had no effect on HOMA-IR index and fasting plasma concentrations of either glucose or insulin at weaning and wk 15 (Table 6.4). No effect of dams’ diet on glucose response (tAUC) to the glucose preload and to the insulin injection at wk 8 and wk 12 was observed (Table 6.7).

6.5 DISCUSSION

The results of the present study indicate that the soy protein diet fed during gestation and lactation increased the presence of characteristics of metabolic syndrome in the female offspring compared with those born to dams fed the casein diet. However, compared with previous reports of the effects of feeding these two diets during gestation and lactation to dams on male pups, the effects on the females were less severe.

These results are consistent with our previous observation in male pups indicating that source of protein in a nutritionally adequate diet consumed during pregnancy and lactation alters metabolic and physiologic phenotype of the offspring. In female offspring, the soy protein-based diet, when compared with the casein-based diet, resulted in higher food intake, body weight and systolic blood pressure, similar to effects in male
offspring. However, unlike our observation in males, the dams’ diet had no effect on glucose response to glucose preloads or on the HOMA-IR index, as indicators of insulin resistance, and also had no effect on plasma concentrations of glucose, insulin or GLP-1 at wk 15 (Table 6.4). Because female rats develop insulin resistance later in life (e.g. 21 months) than males [106] it may be suggested that the present study of 14 wk duration post-weaning was too short to show an effect in females. At 20 wk of age, male but not female Wistar rats born to low protein fed dams (8% of total calories) were relatively hyperinsulinemic and insulin resistance [105].

However, female offspring were more resistant to the effect of protein source in the dams’ diet on several measures reported to be affected in the male offspring. In the previous study, the increase in BW of male offspring born to dams fed the S diet started at wk 4 [267], but this effect was delayed to wk 11 in the females (Fig 6.1). Similarly an increase in food intake was found beginning at wk 4 in males born to dams fed the S diet [267] but not until wk 13 in females (Fig 6.2). Moreover, the S diet resulted in higher systolic and diastolic blood pressure and pulse rate in male offspring [248] but only systolic blood pressure was affected in female offspring. Furthermore, food intake (1 h) in response to protein preloads was not influenced by the dams’ diet in females but was in males [267]. At weaning, in female offspring born to dams fed the C diet, plasma PYY, an anorexigenic compound [268], was higher, and consistent with their lower food intake. However, a causative relationship is in doubt because there was no effect of the dams’ diet on PYY at wk 15. Similarly, plasma PYY concentrations was not influenced by the dams’ diet in male offspring while food intake was higher in those born to dams fed the S diet [267].
The mechanisms by which source of protein can influence phenotype of offspring are uncertain at present. However, there are many differences in characteristics of casein and soy protein. These include amino acid composition, bioactive peptides (BAP) and digestion kinetics. For example, arginine, which is almost twofold higher in soy protein than casein, is one of the most potent insulinotropic amino acids [254-256] and may have contributed to higher in utero insulin exposure of offspring born to dams fed the S diet [248]. Furthermore, many BAPs have been identified in both casein and soy protein. For example, casein is rich in casomorphins capable of activating opioid receptors in the enteric nervous system and on the vagus, resulting in lower blood pressure through vasodilation [150, 226, 229]. Moreover, casein and soy protein differ in digestion kinetics. Casein is a slow protein while soy protein is classified as fast protein [173]. The digestion kinetics of proteins influence their metabolic activities [170], brain amino acid concentrations and neural activity [172, 269]. For example, lower protein synthesis after ingestion of soy protein compared with animal proteins [270] could be explained by the fact that the pattern of amino acids reaching the liver is not only more rapid but also more unbalanced in composition than milk protein [271, 272].

The results obtained from the present study cannot be attributed to isoflavone content of soy protein. The genistein content of the S diet was only 36 μg/g of the diets, far below that reported (250 μg/g diet) in the maternal diet to affect epigenetic and phenotypic changes in mice [242]. Moreover, they cannot be explained by stress responses of the dams or pups because: there were no differences due to diet in litter size or birth weight of the offspring, or in BW of dams at arrival, d 14 and d 20 of gestation and no difference was found in fasting corticosterone level in pups at weaning and wk 15.
6.6 CONCLUSION

Protein source in nutritionally adequate diets fed to rat dams during gestation and lactation influence the physiologic and metabolic phenotype of the female offspring. Soy protein, when compared to casein based diets, increased food intake and the risk of developing characteristics of the metabolic syndrome.

Acknowledgements

The authors thank the management and technicians at the Department of Comparative Medicine at the University of Toronto. All the authors contributed to the preparation of the paper and read and approved the final manuscript. G.H. Anderson and A. Jahan-mihan conceptualized and designed the research. A. Jahan-mihan, C. Smith and A. Hamedani conducted the research. A. Jahan-mihan analyzed the data. A. Jahan-mihan, G.H. Anderson and C. Smith wrote the paper. G.H. Anderson had primary responsibility for final content. This study was supported by the Natural Sciences and Engineering Research Council of Canada.
The amino acid content of the diets is calculated based on purity of the protein sources (87% and 90% for casein and soy protein respectively).

Cystine was added to both casein (3g/kg diet) and soy protein (2.54g/kg diet) diets.

Isolated soy protein: Dyets Inc; Micellar casein: Harlan Teklad

### Table 6.1 Amino acid composition of casein and soy protein AIN-93G diets (/kg diet)  

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Micellar Casein</th>
<th>Isolated Soy Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine</td>
<td>4.7</td>
<td>6.7</td>
</tr>
<tr>
<td>Arginine</td>
<td>5.7</td>
<td>11.9</td>
</tr>
<tr>
<td>Aspartic acid + Asn</td>
<td>10.6</td>
<td>18.0</td>
</tr>
<tr>
<td>Cystine (^2)</td>
<td>3.5</td>
<td>4.5</td>
</tr>
<tr>
<td>Glutamic acid + Gln</td>
<td>34.8</td>
<td>29.9</td>
</tr>
<tr>
<td>Glycine</td>
<td>2.8</td>
<td>6.7</td>
</tr>
<tr>
<td>Histidine</td>
<td>4.4</td>
<td>4.1</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>8.7</td>
<td>7.7</td>
</tr>
<tr>
<td>Leucine</td>
<td>13.9</td>
<td>12.8</td>
</tr>
<tr>
<td>Lysine</td>
<td>12.2</td>
<td>9.9</td>
</tr>
<tr>
<td>Methionine (^3)</td>
<td>4.0</td>
<td>4.5</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>7.7</td>
<td>8.1</td>
</tr>
<tr>
<td>Proline</td>
<td>16.0</td>
<td>7.9</td>
</tr>
<tr>
<td>Serine</td>
<td>8.4</td>
<td>8.1</td>
</tr>
<tr>
<td>Threonine</td>
<td>6.6</td>
<td>5.9</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>1.7</td>
<td>2.2</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>8.0</td>
<td>5.9</td>
</tr>
<tr>
<td>Valine</td>
<td>10.4</td>
<td>7.9</td>
</tr>
</tbody>
</table>

\(^1\) The amino acid content of the diets is calculated based on purity of the protein sources (87% and 90% for casein and soy protein respectively).

\(^2\) Cystine was added to both casein (3g/kg diet) and soy protein (2.54g/kg diet) diets.

\(^3\) Methionine was added to soy protein diet (2.54 g/kg diet)

\(^4\) Isolated soy protein: Dyets Inc; Micellar casein: Harlan Teklad
Data are means ± SEM; n=11-12/group; Unpaired t-test; values in a row with different superscript letters are significantly different, p<0.05
C: Casein; S: Soy protein
TF: Total fat (was measured by DEXA at birth)
FPM: Fat pad mass (Abdominal + perirenal)

<table>
<thead>
<tr>
<th></th>
<th>Dams' Diet</th>
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</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>C</td>
<td>S</td>
</tr>
<tr>
<td><strong>Birth</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BW (g)</td>
<td>6.2 ± 0.2</td>
<td>6.3 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>TF(g)</td>
<td>0.3 ± 0.0</td>
<td>0.4 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>TF / BW ratio (%BW)</td>
<td>6.2 ± 0.8</td>
<td>7.2 ± 0.8</td>
<td></td>
</tr>
<tr>
<td><strong>Weaning</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BW (g)</td>
<td>59.7 ± 2.5</td>
<td>55.2 ± 2.0</td>
<td></td>
</tr>
<tr>
<td>FPM (g)</td>
<td>0.5 ± 0.0</td>
<td>0.4 ± 0.0</td>
<td></td>
</tr>
<tr>
<td>FPM / BW ratio (%BW)</td>
<td>0.8 ± 0.0</td>
<td>0.7 ± 0.0</td>
<td></td>
</tr>
<tr>
<td><strong>Wk 15 post-weaning</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BW (g)</td>
<td>322.0 ± 8.9a</td>
<td>360.0 ± 12.0b</td>
<td></td>
</tr>
<tr>
<td>FPM (g)</td>
<td>20.2 ± 1.2a</td>
<td>26.3 ± 2.5b</td>
<td></td>
</tr>
<tr>
<td>FPM / BW ratio (%BW)</td>
<td>6.2 ± 0.3</td>
<td>7.2 ± 0.5</td>
<td></td>
</tr>
</tbody>
</table>
**Fig 6.1** Effect of source of protein in dams’ diet on post-weaning BW of female offspring

Data are means ± SEM; n=12/group; BW was analyzed by MIXED model with maternal and time as main factors: Time (p<0.0001); Maternal Diet: (p=0.07); * p<0.05

C: Casein; S: Soy protein
The effect of source of protein in dams’ diet on food intake of female offspring at post-weaning.

Data are means ± SEM; n=12/group. Food intake was analyzed by MIXED model with maternal diet and time as main factors. Time: \( p<0.0001 \). Diet: \( p<0.05 \); * \( p<0.05 \)

C: Casein; S: Soy protein

Food intake was not measured during wk 7-8 when preload measures were conducted.
Table 6.3  Effect of source of protein in dams’ diet on food intake (1 h) in response to protein preloads in female offspring at wk 7 post-weaning

<table>
<thead>
<tr>
<th>Dams' Diet</th>
<th>C</th>
<th>S</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water Preload</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(g)</td>
<td>6.5 ± 0.4</td>
<td>6.4 ± 0.5</td>
</tr>
<tr>
<td>Protein Preload</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Casein Preload</td>
<td>4.4 ± 0.4</td>
<td>5.2 ± 0.7</td>
</tr>
<tr>
<td>Soy Protein Preload</td>
<td>3.8 ± 0.5</td>
<td>4.3 ± 0.5</td>
</tr>
<tr>
<td>Water - Protein</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Casein Preload</td>
<td>2.1 ± 0.6*</td>
<td>1.2 ± 0.7</td>
</tr>
<tr>
<td>Soy Protein Preload</td>
<td>2.7 ± 0.8*</td>
<td>2.2 ± 0.8*</td>
</tr>
</tbody>
</table>

Data are means ± SEM; n=11-12/group; Food intake was analyzed by MIXED model with maternal diet and preload as main factors: Total food intake: Diet: NS, Preload: p<0.0001; Water – Protein: Diet: NS, Preload: NS ;Significant food intake suppression: p<0.05 Protein preload was given by gavage at 3g/kg BW/6 ml; water preload was given in the same volume as protein preload Water - Protein: food intake after water preload – food intake after protein preload C: Casein; S: Soy protein Total food intake: Food intake after either preloads D: Dams’ diet; P: Preload; T: Time; NS: Not significant
Table 6.4  Effect of source of protein in the diet during gestation and lactation on fasting plasma measures in female offspring

<table>
<thead>
<tr>
<th>Dams’ Diet</th>
<th>C</th>
<th>S</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Weaning</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose, mM</td>
<td>5.7 ± 0.24</td>
<td>5.5 ± 0.20</td>
</tr>
<tr>
<td>Insulin, ng/ml</td>
<td>0.2 ± 0.01</td>
<td>0.2 ± 0.03</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>0.04 ± 0.01</td>
<td>0.05 ± 0.01</td>
</tr>
<tr>
<td>Corticosterone, ng/ml</td>
<td>250.8 ± 58.9</td>
<td>288.2 ± 48.7</td>
</tr>
<tr>
<td>Homocysteine, μmol/L</td>
<td>6.0 ± 0.95</td>
<td>7.6 ± 1.73</td>
</tr>
<tr>
<td>Ghrelin, ng/ml</td>
<td>2.2 ± 0.13</td>
<td>2.4 ± 0.31</td>
</tr>
<tr>
<td>GLP-1, pM</td>
<td>2.5 ± 0.23&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.4 ± 0.32&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>PYY, pM</td>
<td>59.1 ± 3.14&lt;sup&gt;a&lt;/sup&gt;</td>
<td>43.4 ± 2.28&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Wk 15 post-weaning</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose, mM</td>
<td>5.2 ± 0.11</td>
<td>4.9 ± 0.24</td>
</tr>
<tr>
<td>Insulin, ng/ml</td>
<td>3.9 ± 0.48</td>
<td>3.8 ± 0.54</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>0.91 ± 0.10</td>
<td>0.86 ± 0.13</td>
</tr>
<tr>
<td>Corticosterone, ng/ml</td>
<td>295.2 ± 17.9</td>
<td>314.2 ± 28.7</td>
</tr>
<tr>
<td>Homocysteine, μmol/L</td>
<td>7.1 ± 0.87</td>
<td>8.3 ± 1.27</td>
</tr>
<tr>
<td>Ghrelin, ng/ml</td>
<td>3.6 ± 0.34</td>
<td>3.9 ± 1.27</td>
</tr>
<tr>
<td>GLP-1, pM</td>
<td>2.5 ± 0.23</td>
<td>2.7 ± 0.44</td>
</tr>
<tr>
<td>PYY, pM</td>
<td>53.1 ± 5.80</td>
<td>42.9 ± 2.39</td>
</tr>
</tbody>
</table>

C: Casein; S: Soy protein Student’s unpaired t-test; values in a row at each time point with different superscript letters are significantly different, p<0.05; n=5-6/group
HOMA-IR index was calculated as fasting glucose (mM) multiplied by fasting insulin (ng/ml) divided by 22.5
Table 6.5  Exp 2: Effect of source of protein in dams’ diet on plasma measures in response to water and glucose preloads in female offspring at wk 15

<table>
<thead>
<tr>
<th>Dams' Diet</th>
<th>Water Preload</th>
<th>Glucose Preload</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C</td>
<td>S</td>
</tr>
<tr>
<td>Insulin, ng/ml</td>
<td>3.9 ± 0.48</td>
<td>3.8 ± 0.54</td>
</tr>
<tr>
<td>GLP-1, pM</td>
<td>2.5 ± 0.23</td>
<td>2.7 ± 0.44</td>
</tr>
<tr>
<td>Glucose, mM</td>
<td>5.2 ± 0.11</td>
<td>4.9 ± 0.24</td>
</tr>
</tbody>
</table>

Data are means ± SEM, n=6/group; MIXED model with main effect of the diet and preload. Insulin: Diet: NS; Preload: NS; GLP-1: Diet: NS; Preload: p<0.0005; Glucose: Diet: NS; Preload: p<0.01 C: Casein; S: Soy protein; D: Diet; P: Preload; NS: Not significant; Glucose: Glucose was given at 3 g/kg BW by gavage 30 min before blood withdrawal.
Table 6.6  Effect of source of protein in dams’ diet on systolic and diastolic blood pressure and pulse rate in female offspring

<table>
<thead>
<tr>
<th>Dams’ Diet</th>
<th>C</th>
<th>S</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Systolic BP (mmHg)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Wk</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>118 ± 3.9</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>122 ± 4.5</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>119 ± 5.7</td>
</tr>
<tr>
<td></td>
<td>Diastolic BP (mmHg)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>93 ± 8.9</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>93 ± 9.1</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>91 ± 8.8</td>
</tr>
<tr>
<td></td>
<td>Pulse (BPM)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>396 ± 14.8</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>393 ± 7.3</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>375 ± 7.9(^a)</td>
</tr>
</tbody>
</table>

Data are means ± SEM. (n=11-12 rat/group); MIXED model with dams diet and time as main factors followed by Tukey’s post-hoc test when interaction was significant: Systolic BP: Diet: p<0.05; Diastolic BP: Diet: NS; Pulse: Interaction of Diet and Time: p<0.005
Values in a row at each time point with different superscript letters are significantly different, p<0.05
C: Casein; S: Soy protein; D: Dams diet; T: Time; NS: Not significant
BPM: Beat per minute
CHAPTER 7

PROTEIN COMPOSITION OF THE WEANING DIET ALTERS FOOD INTAKE AND BLOOD GLUCOSE REGULATION AFTER PROTEIN PRELOADS IN RATS
7. PROTEIN COMPOSITION OF THE WEANING DIET ALTERS FOOD INTAKE AND BLOOD GLUCOSE REGULATION AFTER PROTEIN PRELOADS IN RATS 1,2,3

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Nutrition Research

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7.1 ABSTRACT

We hypothesized that protein source in the weaning diet alters blood glucose and food intake regulation after protein preloads in rats. In Exp 1, male Wistar rats (n=21/group) received either casein (C) or soy protein (S) AIN-93 G diets for seven weeks. In Exp 2, three groups of rats were formed (n=21/group). Group CS was weaned to the C diet for six weeks followed by the S diet for another seven weeks. The diet sequence was reversed for group SC. Group CC received the C diet throughout 13 wks. Body weight and cumulative food intake (FI) were not affected by the diets in either experiment. In Exp 1, in fasted rats, soy protein preloads reduced FI over 1h more in the C diet group (p<0.05). However, the effect of casein preloads was not affected by the diet. A CCK-A receptor blocker prevented this effect of soy protein in rats fed C but not S diet (p<0.05). At wk 7, plasma insulin (p<0.005), glucose (p<0.05) and HOMA-IR index (p<0.005) were higher in rats fed the S diet. In Exp 2, FI was again suppressed most strongly by soy protein preloads in rats fed the C diet (p<0.05). At wk 13, soy protein preloads increased insulin and the insulin/glucose ratio more than either casein or water (p<0.05) in the CS group. In conclusion, protein composition of the recent diet was a factor in determining FI and blood glucose regulation following soy protein, but not casein preloads and protein source of the first diet exposure had no lasting effects.

KEY WORDS: food intake, glucose, weaning, rat, casein, soy protein
7.2 INTRODUCTION

Adaptation to the source and amount of dietary protein continues throughout life. The weaning period is characterized by a rapid adaptation to ingestion and digestion of food sources that differ in the quantity and quality of proteins compared with maternal milk [273]. For example, in pigs after weaning, activity of pancreatic proteases is affected by both protein source and the amount in the feed [274, 275] and their digestion of vegetable protein increases through 35 d of age. In contrast, digestion of cow’s milk protein is high immediately after weaning and does not increase further with age [276-278].

Adaptation to the amount of protein in the diet also occurs. Food intake (FI) initially is suppressed by high protein diets compared with low protein diets [158]. However, the satiating response to a high protein diet appears to be transient in both humans and animals. A standard protein meal was found to be less satiating in human subjects previously adapted to a high- compared with a low-protein diet [279]. Similarly, rats fed high protein diets initially decrease FI, but within ten days they increase their energy intake to that of the controls [280], regardless of the type of protein in the diet [281]. The normalization of FI following high protein diets has been associated with a rapid increase in catabolism of amino acids and a reduction of plasma and brain amino acid concentrations [280, 282, 283].

Although the capacity of the body to rapidly adjust to protein intake by modifying metabolism suggests that the source and amount of protein consumed postnatally is unlikely to affect obesity and chronic diseases in later life, it has been proposed that infant formula, due to its high protein content compared with human milk, increases the
risk of infants becoming obese and developing chronic diseases [284]. Therefore, understanding the effect of source and quantity of protein fed in early life is important because, unlike the varied sources of protein in the adult diet, the majority of the protein obtained in early life tends to be from a single source, usually mother’s milk, cow’s milk or soy protein based formula [285]. Furthermore, milk and soy beverages are often a constant and primary source of protein well after breast feeding is terminated [264].

Therefore, the hypothesis of this study was that protein source in the weaning diet determines the regulation of blood glucose and FI in rats. The objective of these two studies was to compare the effect of casein (C) and soy protein (S) in nutritionally adequate weaning diets on regulation of blood glucose and FI in rats. The primary dependent measures were body weight (BW), cumulative FI and FI (1h), glucose, insulin, CCK, and GLP-1 following soy protein and casein preloads.

### 7.3 MATERIALS AND METHODS

#### 7.3.1 Experimental Design

Two experiments were conducted:

**7.3.1.1 Exp 1**: Effect of protein sources in the weaning diet to 7 wk post-weaning on food intake and glucose metabolism

The objective was to compare the effect of weaning diets containing either C or S on BW, FI, glucose and satiety hormones in response to casein and soy protein preloads.
Weaned male Wistar rats (n=21/group) received either C or S AIN 93-G diets for 7 weeks. BW and FI were measured weekly. FI (1 h) at 6 wk was measured after an overnight fast in response to either casein or soy protein preloads prior to and following a CCK-A receptor blocker (devazepide). Devazapide was injected intra-peritoneally (i.p.) 5 minutes before gavage with the protein or water treatments at 30 min before the rats had access to food. Rats in each diet group were allocated to 3 groups of seven and randomly assigned to receive one of the following three treatments prior to food access: water control (6 ml), protein (3 g/kg BW/6 ml distilled water) and protein plus devazepide (0.25 mg/kg BW/ 1 ml methocel). Each group then received the remaining two treatments with one day washout in between. Upon completion of the treatment with the first protein (e.g. casein or soy protein), rats were again randomized to treatments with the second protein as the preload. In each diet group, the familiar protein that was the same as that in the diet was fed in the first preload study and the second preload treatment were with the unfamiliar protein (i.e., soy protein preload given to those on the C diet and vice versa). Plasma concentrations of glucose, insulin, CCK and GLP-1 following protein preloads were measured at wk 7.

7.3.1.2 Exp 2: Effect of crossing over C and S in diets to 13 wk post-weaning on food intake and glucose metabolism.

The objective was to determine if protein source in the weaning diet has long lasting effects that persist throughout changes in protein source later in life. At weaning, rats were allocated to three groups (n=21/group): During the first 6 wk, 2 groups were fed the C diet while the third group received the S diet. After measurement of FI (1 h) in response to the preloads of casein, soy protein and water at wk 6, the group on the S diet
was fed the C diet (group SC), one of the groups on the C diet was fed the S diet (group CS) and another group remained on the C diet (group CC) for a further six weeks (growth diets). FI (1 h) was measured again during wk 12. Thirty minutes before access to the diet, half of the rats in each group received the familiar protein preload while the other half received water. After a wash-out day, the preload treatments were reversed. After another wash out day, the treatments were repeated but the protein preload contained the unfamiliar protein.

BW was measured weekly and FI over 24 h was measured at weeks 1-4 and 7-10. Because of the FI measurement in response to protein preloads, 24 h FI was not measured at weeks 5-6 and 10-12.

At the end of each experiment (at wk 7 in Exp 1 and at wk 13 in Exp 2), rats in each diet group (n=21/group) were allocated to three groups of seven. After a 12 h fasting period, groups received a gavage of casein, soy protein or water and the rats were sacrificed by decapitation 30 min later. Plasma concentrations of CCK, GLP-1, insulin and glucose were measured.

7.3.2 Animals and diets

Male weanling Wistar rats (Charles River, QC, Canada) were received at 21 days of age and BW of 50 to 55g. Rats were housed individually in ventilated plastic cages at 22±1°C and 12-h light-dark cycle (lights off at 1000 h), and had free access to water and a pellet diet (Rodent Laboratory Chow 5001; Lab Chows, Strathory, Canada). On day 2, the pellet diet was replaced with either casein (C) or soy protein (S) based AIN-93 G diets prepared on the premises. The composition (per kg diet) of the AIN-93G casein diet was casein (200g), cornstarch (529.4g), sucrose (100.1g), soybean oil (70g), cellulose
(50g), vitamin mixture (10g), mineral mixture (35g), cystine (3g), choline bitartrate (2.5g), and tert-butyl hydroquinone (0.014g). Soy protein diet had the following modifications: casein was replaced by soy protein, and the additions of methionine (2.54g) and cystine (2.54g) were made according to recommendations for the AIN-93G diet to provide similar total sulphur amino acid content [219]. Cornstarch, high-protein casein (87%), and cellulose were purchased from Harlan Teklad (Madison, WI). Vitamin mixture, mineral mixture, cystine, methionine, choline bitartrate, and tert-butyl hydroquinone were purchased from Dyets (Bethlehem, PA), sucrose from Allied Food Service (Toronto, ON, Canada), and soybean oil from Loblaws (Toronto, ON, Canada). Water was ad libitum and food was provided from 1000 h to 2200 h. The protocol was approved by University of Toronto Animal Care Committee and care and maintenance of the animals were conformed to the guidelines of the Canadian Council on Animal Care.

7.3.3 Protein preloads

Micellar casein was obtained from American Casein Co. (Burlington, NJ) and isolated soy protein was from General Nutrition Products (Greenville, SC). Each rat received 3 g/kg BW protein/6 ml distilled water by gavage.

7.3.4. CCK-A receptor blocker

Devazepide (donated by ML laboratories PLC, London, UK) was suspended in methyl cellulose (BDH Toronto, Toronto, ON, Canada) [155]. The methocel solution was prepared by adding 0.25 of methyl cellulose powder to 100g of hot (80°C) deionized water, stirred for 1 minute and allowed to chill to 5° C for 2-3 h. The solution was stirred every 0.5 h until it was clear with no visible particles. To mix in devazepide (0.5 g/ml), a glass homogenizer (Tissue Grinder, Pyrex Brand, No. 7725; Thomas Scientific,
Swedesboro, NJ) was used. Each rat received 0.25 mg/kg BW, a dose which given alone does not affect FI [155]. Injections were given intraperitoneally (i.p.) in a volume of 1.0 ml.

7.3.5 Food intake (1 h)

FI over 1 h following an overnight fast was measured after protein preloads at wk 6 in Exp 1 and at wk 6 and wk 12 in Exp 2. Before testing, rats were adapted over 7 days to the injections and gavages. They were gavaged and/or injected with water and saline (0.9%) over 4 days. Then food was removed for 12 h and on day 5, half of the rats received the gavage and injection treatments while the other half were untreated. On day 6, the testing order was reversed. The experiment began only when it was determined that there was no effect of gavage or injection on FI.

7.3.6 Food intake (24 h)

The powdered diet was provided in jars with a mesh disk insert to minimize spillage. FI was measured over a 24 h period once per week.

7.3.7 Blood glucose measurement

Tail vein glucose concentration was assayed using a hand-held commercial glucometer (MediSense Precision Xtra, Abbot laboratories, Alameda, CA) using test strips [7]. The accuracy and variance of the glucometer and test strips were examined by comparing with a commercial human serum standard (6.3 mmol/L, Assayed Human Multi-Sera, Randox Laboratories Canada Ltd, Mississauga, ON, Canada). Glucose in plasma from trunk blood obtained upon decapitation was measured using a glucose oxidase kit (Ascensia Elite XL, Bayer AG, Leverkusen, Germany).
7.3.8 **Blood collection**

Trunk blood was collected in chilled vacutainer tubes (BD, Franklin Lakes, NJ, USA) containing an EDTA + trasylol® (Bayer AG, Leverkusen, Germany) solution (10% blood volume). A dipeptidyl peptidase IV inhibitor (Cat. # DPP4, Linco Research Inc, St. Charles, MO) was added at 1% ratio for the determination of active GLP-1 in plasma. Blood samples were centrifuged for 20 min at 3000×g and 4°C for 10 min. Plasma was separated and immediately stored at -80°C.

7.3.9 **Hormone assays**

Plasma CCK concentrations were determined by radioimmunoassay (RIA) (Cat# RB 302, Euria CCK, Euro-Diagnostica AB, Malmo, Sweden) with assay sensitivity of 0.3 pmol/L. Plasma GLP-1 concentrations were determined by the ELISA method (Cat. # EGLP-35K, Linco Research Inc, St. Charles, MO) with assay sensitivity of 2 pM. Plasma insulin concentrations were determined by RIA (Cat. # RI-13K, Linco Research Inc, St. Charles, MO) with assay sensitivity of 0.124 ng/ml.

7.3.10 **Statistical analyses**

Data are expressed as means ± SEM. BW and FI (24h) were analyzed by using the PROC MIXED MODEL procedure with weaning diet and time (Exp 1) and diet, time and diet sequence (Exp 2) as main factors. FI (1 h) in response to protein preloads was analyzed by PROC MIXED MODEL procedure with weaning diet and preload (Exp 1) and diet, preload and diet sequence (Exp 2) as main factors. The effect of the diets and preloads on plasma glucose, insulin/glucose ratio and hormones were also analyzed by PROC MIXED MODEL procedure with weaning diet and preload (Exp 1) and diet sequence and preload (Exp 2) as main factors. The effect of the diets on HOMA-IR index
was analyzed by unpaired t-test in Exp 1 and by one-way analysis of variance (ANOVA) in Exp 2. When treatment interactions were found to be statistically significant, a one-way repeated measure ANOVA with post-hoc Tukey’s was conducted. All analyses were conducted using SAS (version 9e, SAS Institute, Cary, NC). Statistical significance was defined at \( p < 0.05 \).

### 7.4 RESULTS

#### 7.4.1 Exp 1. Effect of protein sources in the weaning diet to 7 wk post-weaning on food intake and glucose metabolism

Neither BW (Table 7.1) nor 24 h FI (Table 7.2) was affected by the composition of the weaning diet. However, BW over 4 wk was affected by time (\( p < 0.0001 \)) (Table 7.1). The interaction indicated that BW was slightly higher over the duration of the study in those fed the S diet. FI (1 h) after an overnight fast was influenced by diet (\( p < 0.005 \)), preload (\( p < 0.0001 \)) but not their interaction (\( p = 0.08 \)) (Table 7.3). It was lower in rats fed the S diet than in those fed the C diet. FI was lower after soy protein preloads than after casein preloads in rats on both weaning diets. FI suppression (the delta of FI after water control and FI after the protein preloads) was influenced by preload (\( p < 0.05 \)) and the interaction between diet and preload (\( p < 0.01 \)). Preloads of soy protein suppressed FI more than casein, but an interaction occurred because of greater suppression by soy protein preloads in rats on the C diet compared with the S diet, whereas the converse occurred after the casein preload. When devazapide was given with the protein gavage, it blocked the effect of soy protein preloads but only in rats fed the C diet and blocked the effect of casein only in rats fed the S diet (\( p < 0.05 \)).
Plasma glucose (p<0.05) and insulin (p<0.005) concentrations and HOMA-IR index (0.4 ± 0.06 vs. 0.7 ± 0.06) (p<0.005) were higher in rats fed the S diet. Moreover, plasma insulin concentrations in response to preloads were higher after the soy protein preload than after the casein preload at 6 wk (p<0.001) (Table 7.4). Plasma CCK concentrations in response to preloads were higher after soy compared with casein (p<0.05). Plasma GLP-1 concentrations were not affected by either diets or preloads (Table 7.4).

7.4.2 Exp 2. Effect of crossing over C and S in diets to 13 wk post-weaning on food intake and glucose metabolism.

BW was not affected by diet or diet sequence but was influenced by time and by the interaction between diet and diet sequence (p<0.0001) (Table 7.5). The interaction is explained by a smaller weight gain of 160 g from 6 wk to 12 wk in the rats switched from C to S diet compared to a gain of 176 g in the SC and CC groups. FI (24 h) was not affected by either diet or diet sequence but was affected by time and the interaction between diet sequence and time (all p<0.0001) (Table 7.6). Rats maintained on the same diet, the CC diet group, had higher FI, primarily in wk 7-10, compared with the other groups. At wk 7, it was higher in group CC than in groups CS and SC and at wk 9 higher than group CS (p<0.05) (Table 7.6).

FI over 1 h following the water preload at weeks 6 and 12 was affected by diet sequence (p<0.0001) and by preload (p<0.0001) (Table 7.7). FI was higher after 6 wk of the C diet in group CS than in group SC fed the S diet and at wk 12 was higher in group CC than both other groups. Protein preloads compared with water reduced FI. FI was also affected by interactions between preload and diet (p<0.001), between diet and diet
sequence (p<0.0001) and between preload and diet (p<0.001) (Table 7.7). It was lower after soy protein preloads compared with casein preloads in rats that received the C diet. Conversely, FI was lower after casein preloads in rats consuming the S diet than in those consuming the C diet. The interaction of diet with diet sequence is primary explained by the fact that rats ate more at wk 12 than at wk 6.

FI suppression by protein preloads was influenced by diet (p<0.01), diet sequence (p<0.05) and their interaction (p<0.0005). Overall FI suppression after preloads was greater in rats fed the C diet than the S diet. In addition, soy protein preloads suppressed FI more in rats fed the C diet than the S diet at wk 6. Similarly, at wk 12, FI suppression after the soy protein preload was greater after the C diet than after the S diet. However, diet had no effect on the magnitude of suppression after the casein preload (Table 7.6).

Plasma concentrations of glucose, insulin and GLP-1 and insulin to glucose ratio (I/G) following the water gavage at wk 13 were not affected by diet sequence, preload or by the interaction between diet sequence and preload (Table 7.8). However, insulin (p<0.01) and I/G ratio (p<0.05) were affected by preload. Casein preloads had no effect on plasma glucose but increased insulin in SC and I/G ratio most in the CS diet sequence groups compared to water. In contrast, the soy protein gavage increased insulin and I/G ratio most in the CS group, with less effect in the diet sequence groups SC and CC. HOMA-IR index (0.35 ± 0.06, 0.30 ± 0.03, 0.37 ± 0.08 in CS, SC and CC groups respectively) was also not affected by diet. No difference was found in plasma glucose and GLP-1 concentrations in response to casein, soy protein or water preload (Table 7.8).
7.5 DISCUSSION

The hypothesis that protein source in the early postnatal diet may contribute to obesity and metabolic diseases in later life was not supported. The early weaning diet had no long lasting effect. However, protein composition of the most recent diet was a greater determinant of FI and glucose regulation. The effects of soy protein but not casein preloads were different in rats fed the C and S diets. Its effect was greater on FI in rats fed C diets, and insulin and I/G ratio were greater in those fed S diets. Both preloads had greater effects on insulin and I/G ratio at seven, but not at 13 wk.

Several lines of evidence show that protein composition of the recent diet is a primary determinant of FI and metabolic responses following protein preloads and that the first diet exposure has no long lasting effects. First, the effect of the S diet during weaning to 7 wk (Exp 1) on blood glucose regulation and insulin resistance was not seen when the S diet was followed by 6 wk of the C diet (Exp 2). At 7 wk, the S weaning diet resulted in higher blood glucose and insulin concentrations and HOMA-IR index compared with the C diet (Table 7.4). However, switching these rats to the C diet at 6 wk eliminated this effect by 13 wk. Furthermore, switching the rats weaned to C to the S diet had no negative effects, suggesting that the effect of the S diet at 7 wk would not have persisted to 13 wk. However, the failure to wean a group fed the S diet throughout (SS group) in the study, makes uncertain the long-term effect of a soy protein- compared with a casein-based diet.

Second, soy protein preloads consistently resulted in higher plasma insulin concentrations compared to casein, especially if the diet also contained soy protein
(Tables 7.4, 7.8). This observation is consistent with its more rapid digestion compared with casein [173] (Table 7.4) and the higher plasma concentrations of arginine and tryptophan, the most potent insulinotropic amino acids [254, 255], after soy protein compared with casein consumption [174]. Conversely, β-casomorphins, bioactive peptides encrypted in casein, reduce the plasma insulin concentration arising from casein consumption in rats [159].

Third, a novel observation of these studies is that adaptation to the protein source of the chronic diet results in decreased sensitivity to its effect on regulatory mechanisms affecting FI. In general, the protein preload that reflected the composition of the diet, the familiar protein, resulted in less suppression of FI and stimulation of satiety hormones than the unfamiliar proteins. Soy protein suppressed short-term FI (1 h) more than casein, with the strongest effect on rats that were recently consuming casein (Tables 7.3, 7.7). Devazapide blocked suppression of FI by soy protein, but not casein preloads in rats fed the C diet. However, plasma CCK concentrations were not different after casein and soy protein preloads suggesting that the action of CCK was reduced in rats fed the C diet, perhaps due to desensitization of the CCK receptors. Adaptive responses of CCK activity occur in response to the chronic diet [286, 287]. Exogenous CCK given to rats fed a high fat or high protein diet suppressed FI markedly less than in rats fed an isoenergetic low fat or low protein diet, which simulate CCK less [288, 289]. Moreover, continuous CCK infusion leads to down-regulation of the CCK-receptor gene expression in the central branch of the rat hypothalamo-pituitary-adrenal axis [290] accounting for desensitization of CCK receptors.
Previous assumptions on the role of the weaning diet on obesity and chronic diseases in animal models have been based on comparison of diets that are inadequate or excessive in protein and fed during gestation and weaning [13, 14, 21] and also on comparison of breast fed vs. formula fed infants in clinical trials [284]. However, we have also shown that protein source is a factor. BW in offspring born to dams fed S diets compared with C diets during pregnancy and lactation and risk of metabolic syndrome was increased but the weaning diet had no effect [248]. The weaning period is less likely to be a factor because most of the development in FI and other regulatory systems in the rat are completed by the time the rat is weaned.

The present study was designed to assess the impact of protein source in the weaning diet when fed in the recommended amounts in nutritionally complete diets. These results suggested that rats at weaning are capable of adapting to the source of protein with no permanent alteration of phenotype. However, the present study did not address directly the effect of source of protein during the normal lactation period in rats when many regulatory systems are undergoing final stages of development. Moreover, the effect of a diet containing protein above requirements in early life on glucose and FI regulation in adulthood has been expressed as a concern for human infants [284]. Human infants fed a milk based formula similar in protein concentration to mothers’ milk have similar length and BW at two years of age, whereas the high protein cow’s milk formula resulted in higher BW for length [284]. Similarly, rats fed a high protein weaning diet (40% of total calories) had higher BW, fat mass and plasma glucose in females exposed to high fat diet at 4 months of age compared with regular protein weaning diet (20% of
total calories) [291]. Therefore, quantity may be more important than source in the
weaning diet.

7.6 CONCLUSION

Protein composition of the diet most recently consumed affects blood glucose and
food intake in response to soy protein but not casein preloads.
Table 7.1  Exp 1: Effect of protein source in weaning diet on BW of male offspring (n=21/group)

<table>
<thead>
<tr>
<th>Weaning Diet</th>
<th>C</th>
<th>S</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arrival</td>
<td>52.5 ± 0.9</td>
<td>52.4 ± 0.7</td>
<td>D: NS</td>
</tr>
<tr>
<td>Wk 1</td>
<td>87.8 ± 1.1</td>
<td>91.9 ± 1.3</td>
<td>T: &lt;0.0001</td>
</tr>
<tr>
<td>Wk 2</td>
<td>150.6 ± 1.5</td>
<td>152.5 ± 1.3</td>
<td>D × T: &lt;0.05</td>
</tr>
<tr>
<td>Wk 3</td>
<td>206.5 ± 2.0</td>
<td>211.8 ± 1.9</td>
<td></td>
</tr>
<tr>
<td>Wk 4</td>
<td>263.9 ± 2.4</td>
<td>267.5 ± 2.8</td>
<td></td>
</tr>
</tbody>
</table>

Data are means (g) ± SEM; BW was analyzed by MIXED model with weaning diet and time as main factors; C: Casein; S: Soy protein; D: Diet; T: Time; NS: Not significant
**Table 7.2** Effect of protein source in weaning diet on food intake (24 h) (n=21/group)

<table>
<thead>
<tr>
<th>Weaning Diet</th>
<th>C</th>
<th>S</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wk 1</td>
<td>14.3 ± 0.3</td>
<td>14.0 ± 0.3</td>
<td>D: NS</td>
</tr>
<tr>
<td>Wk 2</td>
<td>17.9 ± 0.3</td>
<td>17.0 ± 0.5</td>
<td>T: &lt;0.0001</td>
</tr>
<tr>
<td>Wk 3</td>
<td>21.2 ± 0.5</td>
<td>20.3 ± 0.3</td>
<td>D × T: NS*</td>
</tr>
<tr>
<td>Wk 4</td>
<td>24.1 ± 0.5</td>
<td>23.5 ± 0.3</td>
<td></td>
</tr>
</tbody>
</table>

Data are means (g) ± SEM; food intake was analyzed by MIXED model with weaning diet and time as main factors; C: Casein; S: Soy protein; D: Diet; T: Time; NS: Not significant; *p=0.08
Table 7.3  Exp 1: Effect of C and S diets on food intake (1 h) in response to protein preloads and devazepide at wk 6 (n=20-21/group)

<table>
<thead>
<tr>
<th>Diet Preload</th>
<th>C</th>
<th>Soy Protein</th>
<th>S</th>
<th>Soy Protein</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water (control)</td>
<td>8.4 ± 0.38a</td>
<td>7.6 ± 0.35a</td>
<td>7.6 ± 0.41a</td>
<td>5.7 ± 0.54b</td>
<td>D: &lt;0.005</td>
</tr>
<tr>
<td>Protein</td>
<td>7.8 ± 0.56a</td>
<td>4.0 ± 0.44b</td>
<td>5.7 ± 0.50b</td>
<td>4.3 ± 0.45b</td>
<td>P: &lt;0.0001</td>
</tr>
<tr>
<td>Protein + Devazepide</td>
<td>7.7 ± 0.47</td>
<td>6.0 ± 0.44</td>
<td>6.8 ± 0.45</td>
<td>4.5 ± 0.45</td>
<td>D×P: =0.08</td>
</tr>
<tr>
<td>Control - Protein</td>
<td>0.5 ± 0.60b</td>
<td>3.5 ± 0.66a</td>
<td>1.8 ± 0.34ab</td>
<td>1.4 ± 0.56b</td>
<td>D: NS</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>D: NS</td>
<td></td>
<td>P: &lt;0.05</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>D×P: &lt;0.01</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Protein + Devazepide)- Protein</td>
<td>-0.1 ± 0.59</td>
<td>2.1 ± 0.92</td>
<td>1.1 ± 0.47</td>
<td>0.2 ± 0.39</td>
<td>D: NS</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>D: NS</td>
<td></td>
<td>P: NS</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>D×P: &lt;0.05</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Data are means (g) ± SEM; MIXED model followed by Tukey’s post-hoc test with weaning diet and preload as main factors: Values in a row with different superscript letters are significantly different, *p*<0.05; C: Casein diet; S: Soy protein diet; D: Diet; P: Preload; NS: Not significant; Water: 6 ml distilled water, Protein preloads were given at 3 g/kg BW by gavage 30 min before introducing the food; Devazepide: CCK-A receptor blocker: ML laboratories, PLC, London, UK was given at 2.5 mg/kg BW intraperitoneal (IP) injection at 35 min before introducing the food.
### Table 7.4 Exp 1: Plasma measures in response to preloads at wk 7 (n=6/group)

<table>
<thead>
<tr>
<th>Weaning Diet</th>
<th>C</th>
<th>S</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Preload</td>
<td>Water</td>
<td>Casein</td>
<td>Soy protein</td>
</tr>
<tr>
<td>Glucose, mM</td>
<td>5.7 ± 0.31</td>
<td>5.8 ± 0.17</td>
<td>5.5 ± 0.31</td>
</tr>
<tr>
<td>Insulin, ng/ml</td>
<td>0.4 ± 0.06</td>
<td>0.6 ± 0.05</td>
<td>0.8 ± 0.11</td>
</tr>
<tr>
<td>Insulin/Glucose ratio</td>
<td>0.06 ± 0.01</td>
<td>0.09 ± 0.01</td>
<td>0.14 ± 0.02</td>
</tr>
<tr>
<td>GLP-1, pM</td>
<td>3.9 ± 0.76</td>
<td>4.6 ± 1.19</td>
<td>4.7 ± 1.16</td>
</tr>
<tr>
<td>CCK, pM</td>
<td>&lt;0.03</td>
<td>2.2 ± 0.34</td>
<td>2.5 ± 0.53</td>
</tr>
</tbody>
</table>

Data are means ± SEM; MIXED model followed by Tukey’s post-hoc test with main effect of the diet and preload. Different letters are different in each row for each group: *p<0.05; * p=0.08; C: Casein diet; S: Soy protein diet; D: Diet; P: Preload; NS: Not significant; Water: 6 ml distilled water; Protein preloads were given at 3 g/kg BW by gavage 30 min before blood withdrawal.
Table 7.5  Exp 2: Effect of protein source in post-weaning diet on BW in male offspring (n=20-21/group)

<table>
<thead>
<tr>
<th>Group (Diet sequence)</th>
<th>CS</th>
<th>SC</th>
<th>CC</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Weaning Diet</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arrival</td>
<td>51 ± 0.7</td>
<td>51 ± 0.9</td>
<td>51 ± 0.8</td>
<td>D: NS</td>
</tr>
<tr>
<td>Wk 1</td>
<td>86 ± 1.7</td>
<td>86 ± 1.5</td>
<td>85 ± 1.6</td>
<td>DS: NS</td>
</tr>
<tr>
<td>Wk 2</td>
<td>147 ± 2.1</td>
<td>146 ± 2.3</td>
<td>145 ± 2.0</td>
<td>T: &lt;0.0001</td>
</tr>
<tr>
<td>Wk 3</td>
<td>203 ± 3.8</td>
<td>207 ± 2.9</td>
<td>199 ± 1.9</td>
<td>DS×T: NS</td>
</tr>
<tr>
<td>Wk 4</td>
<td>252 ± 2.9</td>
<td>257 ± 3.7</td>
<td>252 ± 3.2</td>
<td>D×T: NS</td>
</tr>
<tr>
<td>Wk 5</td>
<td>292 ± 6.5</td>
<td>288 ± 4.9</td>
<td>285 ± 4.2</td>
<td>DS×D: &lt;0.0001</td>
</tr>
<tr>
<td>Wk 6</td>
<td>331 ± 5.4</td>
<td>326 ± 6.1</td>
<td>335 ± 5.1</td>
<td></td>
</tr>
<tr>
<td>Growth Diet</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wk 7</td>
<td>S</td>
<td>C</td>
<td>C</td>
<td></td>
</tr>
<tr>
<td>Wk 8</td>
<td>377 ± 5.0</td>
<td>381 ± 4.9</td>
<td>383 ± 4.5</td>
<td></td>
</tr>
<tr>
<td>Wk 9</td>
<td>423 ± 6.3</td>
<td>429 ± 6.4</td>
<td>443 ± 5.6</td>
<td></td>
</tr>
<tr>
<td>Wk 10</td>
<td>459 ± 6.5</td>
<td>463 ± 6.7</td>
<td>473 ± 6.8</td>
<td></td>
</tr>
<tr>
<td>Wk 11</td>
<td>481 ± 7.2</td>
<td>488 ± 8.7</td>
<td>492 ± 10.1</td>
<td></td>
</tr>
<tr>
<td>Wk 12</td>
<td>495 ± 6.8</td>
<td>502 ± 9.1</td>
<td>509 ± 9.7</td>
<td></td>
</tr>
</tbody>
</table>

Data are means (g) ± SEM; BW was analyzed by MIXED Model with group (diet sequence), diet (weaning vs. growth) and time (wk) as main factors. D: Diet, DS: Diet sequence, T: Time; NS: Not significant; C: Casein diet; S: Soy protein diet; CS: C followed by S diet; SC: S followed by C diet; CC: C Diet throughout.
Table 7.6  Exp 2: Effect of protein source in weaning and growth diets on food intake (24 h) (n=20-21/group)

<table>
<thead>
<tr>
<th>Group (Diet sequence)</th>
<th>Weaning Diet</th>
<th>Growth Diet</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CS</td>
<td>SC</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>S</td>
</tr>
<tr>
<td>Wk 1</td>
<td>13.8 ± 0.4</td>
<td>14.2 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>D: NS</td>
<td></td>
</tr>
<tr>
<td>Wk 2</td>
<td>19.3 ± 0.6</td>
<td>20.0 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>DS: NS</td>
<td></td>
</tr>
<tr>
<td>Wk 3</td>
<td>22.2 ± 0.6</td>
<td>22.5 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>T: &lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>Wk 4</td>
<td>23.5 ± 0.5</td>
<td>23.8 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>D×DS: &lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>Wk 7</td>
<td>29.7 ± 1.1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>34.5 ± 1.1&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Wk 8</td>
<td>27.6 ± 0.6</td>
<td>29.3 ± 0.8</td>
</tr>
<tr>
<td>Wk 9</td>
<td>26.9 ± 0.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>29.2 ± 0.9&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>Wk 10</td>
<td>25.2 ± 0.6</td>
<td>24.5 ± 0.8</td>
</tr>
</tbody>
</table>

Data are means (g) ± SEM; MIXED model followed by Tukey’s post-hoc test with group (diet sequence), and time (wk) as main factors: Values in each row with different superscript letters are significantly different, p<0.05: C: Casein diet; S: Soy protein diet; D: Diet; DS: Diet sequence; NS: Not significant; CS: C followed by S diet; SC: S followed by C diet; CC: C Diet throughout
Table 7.7 Exp 2: Effect of weaning and growth diets on food intake (1 h) in response to protein preloads given by gavage to fasting rats at weeks 6 and 12 (n=20-21/group)

<table>
<thead>
<tr>
<th>Group (Diet Sequence)</th>
<th>Wk 6</th>
<th>Wk 12</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CS C</td>
<td>SC S</td>
<td>CS C</td>
</tr>
<tr>
<td>Diet</td>
<td>Preload</td>
<td>Total Intake:</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Water (control)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>8.9 ± 0.46&lt;sup&gt;a&lt;/sup&gt;</td>
<td>11.5 ± 0.64</td>
<td>12.3 ± 0.83</td>
</tr>
<tr>
<td></td>
<td>7.4 ± 0.29&lt;sup&gt;b&lt;/sup&gt;</td>
<td>11.4 ± 0.56</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>12.3 ± 0.83</td>
<td></td>
</tr>
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<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Casein</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>7.7 ± 0.53&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8.7 ± 0.73&lt;sup&gt;b&lt;/sup&gt;</td>
<td>11.1 ± 0.69&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>6.3 ± 0.27&lt;sup&gt;b&lt;/sup&gt;</td>
<td>8.6 ± 0.49&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Water-Casein</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.1 ± 0.48</td>
<td>2.7 ± 0.73</td>
<td>1.2 ± 0.87</td>
</tr>
<tr>
<td></td>
<td>1.0 ± 0.39</td>
<td>2.8 ± 0.59</td>
<td></td>
</tr>
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<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Water (control)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>8.8 ± 0.37&lt;sup&gt;a&lt;/sup&gt;</td>
<td>9.5 ± 0.60&lt;sup&gt;b&lt;/sup&gt;</td>
<td>12.7 ± 1.01&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>7.6 ± 0.32&lt;sup&gt;b&lt;/sup&gt;</td>
<td>11.6 ± 0.66&lt;sup&gt;ab&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>12.7 ± 1.01&lt;sup&gt;a&lt;/sup&gt;</td>
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<td></td>
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<td></td>
</tr>
<tr>
<td></td>
<td>Soy Protein</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>5.6 ± 0.42</td>
<td>8.1 ± 0.61</td>
<td>8.8 ± 1.05</td>
</tr>
<tr>
<td></td>
<td>6.7 ± 0.39</td>
<td>6.0 ± 0.86</td>
<td></td>
</tr>
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</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Water-Soy protein</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>3.2 ± 0.41&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.4 ± 0.91&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.9 ± 1.30&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>0.9 ± 0.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.6 ± 0.87&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Data are means (g) ± SEM; MIXED model followed by Tukey’s post-hoc test with group, diet and preload as main factors; Values in a row at each wk with different superscript letters are significantly different, p<0.05; C: Casein diet; S: Soy protein diet; D: Diet; DS: Diet sequence; P: Preload; T: Time; NS: Not significant; CS: C followed by S diet; SC: S followed by C diet; CC: C diet throughout; Water: 6 ml distilled water; Protein preloads were given at 3 g/kg BW by gavage 30 min before introducing the food.
Table 7.8 Exp 2: Plasma measures in response to water, casein and soy protein preloads at wk 13 (n=6/group)

<table>
<thead>
<tr>
<th>Group (Diet Sequence)</th>
<th>Preload</th>
<th>CS</th>
<th>SC</th>
<th>CC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Water</td>
<td>Casein</td>
<td>Soy protein</td>
<td>Water</td>
</tr>
<tr>
<td>Glucose, mM</td>
<td>7.6 ± 0.25</td>
<td>7.4 ± 0.20</td>
<td>7.2 ± 0.11</td>
<td>7.1 ± 0.22</td>
</tr>
<tr>
<td>Insulin, ng/ml</td>
<td>1.1 ± 0.15</td>
<td>1.5 ± 0.22</td>
<td>1.8 ± 0.15</td>
<td>1.0 ± 0.09</td>
</tr>
<tr>
<td>Insulin/Glucose ratio</td>
<td>0.14 ± 0.02</td>
<td>0.20 ± 0.03</td>
<td>0.26 ± 0.08</td>
<td>0.13 ± 0.01</td>
</tr>
<tr>
<td>GLP-1, pM</td>
<td>3.8 ± 0.61</td>
<td>5.0 ± 0.79</td>
<td>5.4 ± 0.82</td>
<td>3.9 ± 0.62</td>
</tr>
</tbody>
</table>

Data are means ± SEM; MIXED model with main effect of the diet sequence (group) and preload. No interactions were detected; DS: Diet sequence (group); P: Preload; NS: Not significant; CS: C followed by S diet; SC: S followed by C diet; CC: C Diet throughout; Protein preloads were given at 3 g/kg BW by gavage 30 min before blood withdrawal.
CHAPTER 8

GENERAL DISCUSSION
8. GENERAL DISCUSSION

This research has yielded an observation that is both novel and significant, namely, that the source of protein in nutritionally complete diets fed during pre- and post-natal development affects the development of characteristics of the metabolic syndrome and the intake regulatory system in offspring of Wistar rats. Thus, the results support the hypothesis of the thesis. Compared with the casein diet, the soy protein diet ingested during gestation and lactation increased food intake, BW, body fat, SBP, and DBP in both male and female offspring, and led to impaired insulin sensitivity and glucose tolerance in dams and male offspring. Protein source in the weaning diet, though, failed to modulate the primary effects of the protein source in the diets of the dams and had no permanent effect on food intake, body weight, or glucose metabolism. The soy protein diet fed post-weaning, however, also contributed to increased body weight and insulin resistance, compared to the casein diet.

8.1 FACTORS AFFECTING OUTCOMES

Factors affecting the outcome included the timing of exposure in both the dams and the weaning diets and the sex of the offspring. However, the effects of protein source in the maternal diet on the offspring are clearly related to characteristics of the proteins fed (e.g. amino acid composition, BAPs, and digestion kinetics), and their impact on development of regulatory systems in utero and early postnatal life and on glucoregulation in the dams. These factors are discussed in the following pages.
8.1.1 Timing of Exposure

The timing of exposure influenced the effect of the dams’ diet on phenotype of the offspring (Chapters 4 and 5). In the present research, extending the duration of the dams’ diet from gestation only to gestation and lactation showed that the primary effects of protein source in the dams diet was reproducible. In addition, extending the diets through lactation identified the lactation period as being important in development as it resulted in a more robust effect on BW, body composition, and glucose metabolism in the offspring. Higher BW in male offspring born to dams fed the S diet, compared with those born to C-diet-fed dams, was observed 4 weeks earlier, and impaired glucose tolerance was found when the diet was extended to the lactation period. Therefore, the composition of the protein consumed during lactation alone may have had a bearing on the health of the offspring. This notion is consistent with sustained development of intake regulatory systems in the hypothalamus and in the gastro-intestinal tract, as well as pancreas of rodents in late pregnancy and the early post-natal period [107, 223]. Our observation that the hypothalamic gene expression of AgRP was not altered at birth but rather at weaning suggests a role for protein source in the diet during lactation.

The effects of the diets fed to the dams throughout gestation and lactation were not averted by feeding the pups the same diet as the mothers. This observation runs counter to the PAR hypothesis, which holds that offspring weaned on diets similar to their mothers’ will adapt more appropriately than those on an unmatched diet [36]. However, in our studies (Chapter 4), the adverse effects of the S diet fed to the dams on BP (Table 7.2) and glucose regulation (Tables 7.3, 7.4, 7.5) were actually more apparent.
if the offspring had been provided with the S diet rather than the C diet. Thus, it is evident that the PAR hypothesis had little application.

Protein source in the weaning diet had no persistent effect on body weight, intake regulation, or blood pressure (Chapter 7). The effect of the protein composition of the first diet exposure was transient, and its effect on food intake and glucose metabolism was altered by the growth diet. Therefore, the mechanism by which protein source influences the phenotype of offspring clearly depends on the timing of the exposure. It may be that protein source during gestation and lactation triggers metabolic and intake regulatory systems by programming the regulatory system of the offspring during development, while the effect during weaning is more likely a modulation of an adaptation to the protein source. It is, therefore, transient, as signaled in Chapter 5 by a decreased sensitivity to the effect of protein source on regulatory mechanisms affecting food intake.

Adaptation to the protein source is shown from the fact that the familiar protein caused less suppression of food intake and stimulation of regulatory hormones than did the unfamiliar proteins. That is, soy protein preload suppressed food intake more when it was given after the C diet than after the S diet, whereas the effect of casein was relatively greater after the S diet than after the C diet.

Further evidence that adaptation to the familiar protein occurs to reduce food-intake inhibitory signals was produced by assessing CCK concentrations in plasma and the food intake responses to the protein preloads when given with the CCK-A receptor blocker. In the present study, desensitization of CCK receptors due to chronic intake of soy protein may be the mechanism. Further evidence of this resistance to CCK has been
provided by previous studies [286, 292, 293]. In rats fed high-fat or high-protein diets, exogenous CCK suppressed food intake to a markedly smaller degree than in rats fed an isoenergetic, low-fat, or low-protein diet [288, 289]. Moreover, continuous CCK infusion led to down-regulation of receptor gene expression in the central branch of the hypothalamus-pituitary-adrenal axis [290], accounting for the desensitization of CCK receptors.

8.1.2 Sex of the Offspring

Consistent with previous studies reported by us [89] and others [106], male offspring (Chapters 4 and 5) were more affected by the maternal diet than were female offspring (Chapter 6). However, the influence of the maternal diet on phenotype in female offspring may need more time to be expressed. For example, in the present study, the effect of the maternal diet on BW began at wk 4 in males but at wk 11 in females. It is also reported that females develop insulin resistance later in life than males [106], which may suggest that the time frame of the present study (14 wk after weaning) was not long enough to permit the maternal diet’s effect on glucose metabolism in females to be detected.

8.1.3 The Role of Characteristics of the Protein Sources

The outcomes of these studies cannot be attributed to nutritional inadequacy in the diets. More likely, the effects of the protein source are due to the specific characteristics of casein and soy protein including amino acid composition, bioactive peptides (BAPs) encrypted in their structure, as well as digestion kinetics that influence their physiologic and metabolic functions. Casein and soy protein differ in amino acid composition [174]:
Soy protein has nearly twice as much alanine, aspartate, and glycine but only one-half the proline content of casein. This may be significant because the detrimental effects of low-protein gestational diets on blood pressure were reduced by adding glycine, proline, and/or threonine [113, 225]. Furthermore, the concentration of the amino acid arginine was twice as high in the S diet. Higher plasma concentrations of arginine and tryptophan, the most potent insulinotropic amino acids [254, 255] after soy protein, compared to casein meals [174], may contribute to higher concentrations of plasma insulin after soy protein preloads.

8.2 PROTEIN SOURCE AND REGULATORY MECHANISMS

Although this research is primarily descriptive in nature, various approaches were used to begin exploring underlying mechanisms. The risk of characteristics of the metabolic syndrome was examined by testing body weight and composition, plasma glucose in response to administration of glucose and insulin, HOMA-IR index, and systolic and diastolic blood pressure. Furthermore, the influence of the maternal diet on the development of the intake regulatory system was tested by measuring cumulative food intake and food intake in response to proteins and glucose preloads as end-point markers. To elucidate underlying mechanisms in both periphery and hypothalamus, various methods were applied. First, measurements were taken of plasma concentrations of intake regulatory hormones (e.g. GLP-1, PYY, ghrelin, and CCK) at fasting and also in response to preloads. Second, receptor blockers were applied in order to examine the effect of protein source in the dams’ diet on the mediating role of CCK-A and opioid receptors in the satiety effect of protein preloads. Third, gene expression of intake
regulatory receptors including POMC, NPY, AgRP, ghrelin, and the leptin receptor in the hypothalamus in response to protein source of the dams’ diet was measured. In addition, plasma homocysteine concentrations, an indicator of methylation in DNA, as one of the mechanisms of programming, were measured.

The obesogenic effect and insulin resistance in weaned rats born to dams fed the S diet are apparent, but the mechanisms through which soy protein exhibits these effects during development needs to be explored in future studies. The influence on the weaned rat is more likely due to some property of the protein that has arisen because of its constant ingestion and metabolic adaptations, rather than to an alteration in gene expression. However this hypothesis remains to be explored. Similarly the reason for the increased insulin resistance in rats fed the soy protein diet after weaning needs to be explored. Nevertheless, the current studies provide some indications of possible mechanisms for the effects of the protein sources in the maternal diets on the offspring and these are discussed in the following pages.

Alterations in food intake regulation in the offspring are clearly shown in Chapters 5 to 7. In these chapters evidence is provided to support the hypothesis that it is the altered development of the food intake regulatory systems in the hypothalamus that is a primary factor leading to increased food intake, body fat content and insulin resistance in the offspring. For example, gene methylation in the hypothalamus of the offspring in utero due to amino acid composition of the diets and insulin resistance may be the cause of the increased food intake and metabolic consequences.

To provide an adequate diet, both methionine and cystine were added to the soy protein diet as free amino acids. These additions may be important because the
methionine content of the diet has a direct relationship with plasma Hcy levels and because increased Hcy concentrations have been associated with the hypomethylation of DNA [145]. The reactions caused by the combination of these elements have been shown to disturb key events in organogenesis and in embryonic vasculogenesis [146].

In the present study, plasma Hcy was 48% higher at weaning and 7% higher at wk 15 in male offspring born to the S-diet-fed dams (Tables 7.3, 7.5). What caused this higher concentration of Hcy is puzzling because the methionine content of the diets was similar (4.0 vs. 4.5 g/kg diet). However, 54% of the methionine in the soy protein diet was in the form of the free amino acid and would be expected to result in a faster absorption, compared with methionine released during digestion. Cystine was also added to both diets (Table 7.1), but its effect on Hcy is unclear because previous studies report that cystine both increases [230, 231] and decreases [232] Hcy in humans.

The increased expression of AgRP in the hypothalamus of the rats at weaning, and the concomitantly higher plasma concentrations of homocysteine in rats born to dams fed the S diet, suggest that altered methylation of genes during development is a factor. AgRP is orexigenic and increases appetite. [251]. The differences in amino acid composition of soy and casein may account for increased AgRP gene expression in the offspring of the dams fed the S diet. Rats placed on a low-protein diet (10% of calories) exhibited increased food intake and hypothalamic AgRP gene expression. In vitro, hypothalamic cells reduce AgRP expression in response to increased amino acid concentration [247].

In addition, the higher concentrations of Hcy found in Study 1 may be attributed to insulin resistance independently of the sulfur amino acid content of the diets. The
elevated concentrations of Hcy occurred in the offspring with higher HOMA-IR indices at weaning and at wk 15. Insulin resistance provokes hyper-homocysteinemia in both humans [233] and rats [234]. Insulin regulates plasma Hcy concentration via hepatic cystathionine β-synthase, a key enzyme involved in trans-sulfuration activity [235].

Higher concentrations of insulin within the immature hypothalamus cause permanent alterations in life-long dysplasia of the central nervous nuclei regulating food intake and BW [128, 179]. Furthermore, higher concentrations of insulin lead to elevated plasma homocysteine levels [234] that may in turn result in hypomethylation in DNA [145], causing further adverse effects on phenotype of offspring [107, 113].

Some of the effects of the maternal diets may also be due to their influence on the dams observed very early in the life of the offspring, suggesting that in utero programming occurred but that little direct effect was detected. For example, a higher HOMA-IR index observed in the dams, concurrently with a trend towards higher plasma insulin in their fetuses at d 20 of gestation, may have contributed to altered fetal development.

The results obtained from these studies cannot be attributed to genistein content of the soy protein because it made up only 36 μg/g of the diets, well below the level (250 μg/g diet) reported in the maternal diet to affect epigenetic and phenotypic changes in mice [242]. Nor can these results be explained by stress responses of the dams or pups, because corticosterone concentrations measured at weaning and at the end of the studies were within normal range.
8.3 STUDY IMPLICATIONS

As noted previously, the results of these studies have yielded an observation that is both novel and significant. The overall implication of these studies is that development of regulatory systems in the fetus and early postnatal life of the rats is more sensitive to the protein component of the maternal diet than has been previously thought. Whether or not, this hypothesis applies to other nutrients remains to be seen. However it is clear that this needs to be explored and dependence on malnutrition models to inform on the relationships between maternal diet and development may be misleading to the importance of nutrient content of maternal diets understood to be nutritionally adequate.

Most animal studies aimed at understanding the effects of maternal diets on development are based on malnutrition models. In contrast, this study shows that metabolic outcomes in the offspring of animal models may differ among “normal” diets used for maintenance of the dams during pregnancy and lactation. For example, the source of protein in lab-chow diets is highly variable and often includes herring fish, whey, and plant proteins. In addition, there are several recommended AIN 93 diets with varied sources of proteins (e.g. casein, soy, and whey protein). Clearly, protein source could be one of the many aspects of the diets that account for the variance in outcomes among studies of the effect of maternal diets on the offspring.

Although the implications of these results for humans may be challenged because human diets during gestation feature a mixture of many protein sources. However, human infants after birth are exposed to a long duration of feeding of a constant protein source. If not breast fed, infants ingest a formula that depends on single sources of proteins or
their hydrolysates, and this may be a factor affecting the development of regulatory systems as considerable neural development continues after birth and this may affect and later-life outcomes. Furthermore, premature babies have long exposure to formulas with a fixed protein quantity and source and often this occurs at very early stages of neural development when gene methylation is occurring.
CHAPTER 9

CONCLUSION
9. CONCLUSION

9.1 SPECIFIC CONCLUSIONS

9.1.1 Study 1. Protein source in diets fed to rat dams during gestation and lactation affected the metabolic phenotype of the offspring. Soy protein, when compared to casein-based diets, increased the risk of developing characteristics of the metabolic syndrome.

9.1.2 Study 2. Protein source during gestation and lactation in a nutritionally adequate diet influenced BW, food intake, and insulin metabolism in the offspring of rats. Maternal diet enhanced the effect of gestational diet on BW, food intake, and insulin metabolism.

9.1.3 Study 3. Protein source during gestation and lactation in a nutritionally adequate diet influenced BW, food intake, and systolic blood pressure in the female offspring of rats.

9.1.4 Study 4. The effects of protein source in the weaning diet on the regulation of food intake and glucose in rats were transient, and the protein source of the most recent diet determined the response to familiar and unfamiliar protein preloads.

9.2 GENERAL CONCLUSION

Protein source in nutritionally complete diets fed to rat dams during pregnancy and lactation affects the development of food intake regulatory system and characteristics of the metabolic syndrome in the offspring. Soy protein, when compared to casein-based diets, increases food intake and the presence of characteristics of the metabolic syndrome.
The effects of protein source in the weaning diet on the regulation of glucose and food intake are transient, and the protein source of the most recent diet determines the response to familiar and unfamiliar protein preloads. Soy protein, when compared with casein, generally exhibits a detrimental effect on glucose metabolism.
CHAPTER 10

FUTURE DIRECTIONS
10. FUTURE DIRECTIONS

The present study showed that the protein source in adequate diets fed during gestation, lactation, and weaning influences the development of food intake regulation and the risk of characteristics of the metabolic syndrome in offspring of Wistar rats. However, these results also give rise to several questions that need to be addressed by further studies. The following are suggestions for future research.

10.1 CHARACTERISTICS OF PROTEINS

The outcomes of this research were more likely due to the respective characteristics of casein and soy proteins. Amino acid composition, digestion kinetics, and BAPs encrypted in these proteins are major factors influencing physiological responses to the proteins. The following two approaches could help determine which characteristics of the proteins are involved.

First, examine the effect of a hydrolysate form vs. an amino-acid-based form of a certain protein in the diets fed during gestation and lactation. Such a study would help clarify whether the effect of the proteins was due to their amino acid composition, or to BAPs, or to both. Casein could be a logical choice because, in the present studies, casein, as compared to soy protein, generally demonstrated a more favorable effect on the health of the offspring.

Second, compare two proteins with similar digestion kinetics (e.g., soy protein vs. whey protein), which would help clarify whether the difference in digestion kinetics
(casein as a slow protein vs. soy protein as a fast protein) was a factor in the present study.

10.2 REGULATORY SYSTEMS

In the present study, protein source of the dams’ diet influenced food intake, blood pressure, and metabolic regulatory systems. However, the mechanisms by which protein source exhibits a systemic effect on the development of various regulatory systems are still elusive. This systemic effect most likely arises from the influence of the development of various key organs including brain, GI tract, liver, and adipose tissue. These effects, though, at least partly, could be secondary to or strengthened by increased body weight and fat mass observed in offspring born to dams fed the soy protein diet. Therefore, to understand the underlying mechanisms, the following approaches could be helpful:

10.2.1 Food-intake regulatory system

Altered hypothalamic development at weaning observed in the present study. However, receptors in other areas of the CNS (e.g., central opioids or serotonin receptors) could be targets for protein-derived bioactive components, including amino acids. For example, amino acid composition and concentration in the CNS affect the intake regulatory system through serotonin receptors [283]. Therefore, testing in the dams’ diet the effect of protein source on the gene expression of opioid and serotonin receptors in the CNS of offspring, and testing food selection by exposing rats to diets with various protein contents as end-point biomarkers, could be instructive.
Moreover, it is not clear whether the effect of protein source on the intake regulatory system is permanent. Therefore, it could be helpful to follow the offspring for a longer period (e.g., 12 months), measuring food intake and gene expression of intake regulatory receptors in the CNS as well as the intake regulatory receptors in the GI tract at different life stages (birth, weaning, maturation, and at month 12).

In the present study, a lowered response to familiar protein versus unfamiliar protein occurred at weaning, which suggested an adaptation to the protein source of the chronic diet. The mechanisms, however, are elusive. Therefore, it could be helpful to examine the effect of the chronic intake of protein source on the activity of the gut peptides mediating the satiety effect of proteins including CCK-A, GLP-1, and PYY by measuring their gene expression in both the hypothalamus and the GI tract.

### 10.2.2 Glucose metabolism

Glucose metabolism of offspring was altered by the source of protein in diets fed to dams during gestation and lactation, as evidenced by impaired HOMA-IR index and plasma glucose response to glucose and insulin administered to offspring born to soy-protein-fed dams compared to casein-diet-fed dams. However, underlying mechanisms still need to be explored. The hypothalamus, liver, and GI tract are major players in the regulation of glucose metabolism. Therefore, it could be informative to examine key parameters in each organ as well as their interactions through hypothalamus-liver-GI tract axes during development. Hypothalamic glucose sensing, activation or suppression of liver glucose production (which represents as net contribution of gluconeogenesis and glycogenolysis), activity of gut receptors involved in glucose sensing (e.g., GLP-1 and amylin receptors), and rate of glucose uptake in myocytes are all main players in glucose
homeostasis. Therefore, glucose production in the liver, glucose uptake in the periphery, and activity of related gut receptors are potential means of measurement. Furthermore, the role of the hypothalamus could be examined by measuring glucose production in the liver in response to the intrahypothalamic infusion of glucose. The role of gut receptors could be examined by applying specific gut receptor agonists and antagonists.

Moreover, in the present study, protein source in the dams’ diet did not influence glucose metabolism in female offspring. However, insulin resistance occurs later in female than in male offspring [106]. This suggested that the period of the present study (14 wk after weaning) may not have been long enough to allow the effect of maternal diet on glucose metabolism in females to be detected. Therefore, following the female offspring for a longer period (e.g. 12 months) could lead to a better understanding of the mechanisms.

**10.2.3 Blood pressure regulatory system**

Protein source in the maternal diet also altered blood pressure in the offspring. However, the mechanisms are elusive at present. Proteins influence blood pressure, and this effect is source dependent [294]. BAPs, with the inhibitory effect of an angiotensin-converting enzyme (ACE), have been identified in various protein sources. Therefore, measurement of ACE activity is a potential way of discovering the underlying mechanism.
CHAPTER 11

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11. REFERENCES

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CHAPTER 12

APPENDICES
12. APPENDICES

12.1 APPENDIX 1. EFFECT OF SOURCE PROTEIN IN THE DAMS’ DIET ON BLOOD GLUCOSE RESPONSE DURING THE ORAL GLUCOSE AND INSULIN TOLERANCE TESTS IN FEMALE OFFSPRING

Data are means ± SEM. (n=11-12 rat/group); MIXED model with dams’ diet and time as main factors; n= 10-12 / group

GTT (Glucose tolerance test): After overnight fasting rats received glucose (0.375 g glucose/ml, 5 g glucose/kg BW) by gavage and blood glucose was measured prior to and 15, 30, and 60 min later

ITT (Insulin tolerance test): Insulin (Humulin® R, Eli Lilly and Company, Indianapolis, Indiana, USA) Injections were given intraperitoneally (IP) (0.5 U/ml, 0.75 U insulin/kg BW) and blood glucose was measured prior to and 15, 30, and 60 min later

C: Casein; S: Soy protein

<table>
<thead>
<tr>
<th>Dams’ Diet</th>
<th>C</th>
<th>S</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>GTT (min mmol/L)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wk</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>438 ± 11.2</td>
<td>450 ± 10.2</td>
</tr>
<tr>
<td>12</td>
<td>382 ± 9.4</td>
<td>402 ± 10.9</td>
</tr>
<tr>
<td><strong>ITT (min mmol/L)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>214 ± 8.4</td>
<td>203 ± 8.0</td>
</tr>
<tr>
<td>12</td>
<td>204 ± 27.0</td>
<td>228 ± 9.2</td>
</tr>
</tbody>
</table>
### APPENDIX 2. EFFECT OF SOURCE OF PROTEIN IN THE DAMS’ DIETS

ON FASTING CONCENTRATIONS OF INTAKE

REGULATORY HORMONES IN THE DAMS

<table>
<thead>
<tr>
<th>Diet</th>
<th>C</th>
<th>S</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><strong>Gestation</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Day 14</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Insulin, ng/ml</td>
<td>1.9 ± 0.20</td>
<td>1.9 ± 0.10</td>
</tr>
<tr>
<td>Ghrelin, ng/ml</td>
<td>2.0 ± 0.22</td>
<td>1.9 ± 0.44</td>
</tr>
<tr>
<td>GLP-1, pM</td>
<td>2.62 ± 0.16</td>
<td>3.03 ± 0.50</td>
</tr>
<tr>
<td>PYY, pM</td>
<td>38.5 ± 4.79</td>
<td>37.7 ± 3.58</td>
</tr>
<tr>
<td><strong>Day 20</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Insulin, ng/ml</td>
<td>1.3 ± 0.20</td>
<td>1.9 ± 0.30</td>
</tr>
<tr>
<td>Ghrelin, ng/ml</td>
<td>1.4 ± 0.06</td>
<td>1.8 ± 0.13</td>
</tr>
<tr>
<td>GLP-1, pM</td>
<td>3.8 ± 0.47</td>
<td>4.6 ± 0.67</td>
</tr>
<tr>
<td>PYY, pM</td>
<td>37.6 ± 2.37</td>
<td>42.0 ± 2.14</td>
</tr>
<tr>
<td><strong>Post-weaning</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Insulin, ng/ml</td>
<td>1.9 ± 0.10&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.3 ± 0.10&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Ghrelin, ng/ml</td>
<td>4.8 ± 1.18</td>
<td>6.3 ± 0.97</td>
</tr>
<tr>
<td>GLP-1, pM</td>
<td>2.9 ± 0.14</td>
<td>2.9 ± 0.17</td>
</tr>
<tr>
<td>PYY, pM</td>
<td>48.1 ± 4.10</td>
<td>37.7 ± 3.58</td>
</tr>
</tbody>
</table>

Data are means ± SEM, n=6/group; C: Casein diet; S: Soy protein diet; Unpaired t-test; Values in a row with different superscript letters are significantly different, p<0.05
12.3 APPENDIX 3. EFFECT OF SOURCE OF PROTEIN IN THE DAMS’ DIETS ON BODY WEIGHT AND BODY COMPOSITION OF THE DAMS

<table>
<thead>
<tr>
<th>Diet</th>
<th>Day 14</th>
<th>Day 20</th>
<th>Post-weaning</th>
<th>Week 6</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Gestation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>302.2 ± 13.5</td>
<td>303.2 ± 8.2</td>
<td>415.2 ± 14.8</td>
<td>342.5 ± 7.3</td>
</tr>
<tr>
<td>S</td>
<td>303.2 ± 8.2</td>
<td>384.0 ± 9.8</td>
<td>384.0 ± 9.8</td>
<td>347.0 ± 7.8</td>
</tr>
<tr>
<td></td>
<td>11.3 ± 1.0</td>
<td>11.7 ± 1.3</td>
<td>17.3 ± 1.7</td>
<td>19.9 ± 1.2</td>
</tr>
<tr>
<td></td>
<td>3.8 ± 0.2</td>
<td>3.9 ± 0.4</td>
<td>4.2 ± 0.1</td>
<td>5.8 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>3.9 ± 0.4</td>
<td>3.8 ± 0.4</td>
<td>3.8 ± 0.4</td>
<td>5.6 ± 0.3</td>
</tr>
<tr>
<td>C</td>
<td>303.2 ± 8.2</td>
<td>384.0 ± 9.8</td>
<td>384.0 ± 9.8</td>
<td>347.0 ± 7.8</td>
</tr>
<tr>
<td>S</td>
<td>314.7 ± 12.2</td>
<td>385.1 ± 10.0</td>
<td>415.2 ± 14.8</td>
<td>390.5 ± 11.2</td>
</tr>
<tr>
<td></td>
<td>11.7 ± 1.3</td>
<td>14.4 ± 1.0</td>
<td>17.3 ± 1.7</td>
<td>19.9 ± 1.2</td>
</tr>
<tr>
<td></td>
<td>3.9 ± 0.4</td>
<td>3.8 ± 0.4</td>
<td>4.2 ± 0.1</td>
<td>5.8 ± 0.3</td>
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<tr>
<td></td>
<td>3.8 ± 0.4</td>
<td>3.8 ± 0.4</td>
<td>3.8 ± 0.4</td>
<td>5.6 ± 0.3</td>
</tr>
</tbody>
</table>

Data are means ± SEM; n=11-12/group; Unpaired t-test; values in a row with different superscript letters are significantly different, p<0.05
C: Casein; S: Soy protein
TF: Total fat (was measured by DEXA at birth)
FPM: Fat pad mass (Abdominal + perirenal)