Post-weaning metabolic programming by dietary monosaccharides

Lianne M. S. Bouwman

Thesis committee

Promotor
Prof. Dr Jaap Keijer
Professor of Human and Animal Physiology
Wageningen University & Research

Co-promotors
Dr Evert M. van Schothorst
Associate professor, Human and Animal Physiology
Wageningen University & Research

Dr Annemarie Oosting
Sr Team Leader Developmental Biology & Nutritional Programming, Early Life Nutrition
Danone Nutricia Research, Nutricia Advanced Medical Nutrition, Utrecht

Other members
Prof. Dr Tiny van Boekel, Wageningen University & Research
Prof. Dr Catalina Picó Segura, University of the Balearic Islands, Spain
Prof. Dr Edwin Mariman, Maastricht University, The Netherlands
Dr Nils Billecke, Cargill R&D centre Europe, Vilvoorde, Belgium

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Chapter 1

General Introduction

Background

In the 1980s and 1990s, low-fat high-carbohydrate diets (with ~30% energy from fat) were promoted as they were considered to be healthy, good for the heart, and to contribute to weight control [1]. The advice on low fat diet could not prevent the rise in obesity [2], and the focus on fat as adverse component of the diet started to shift to carbohydrates. Nowadays, the view that carbohydrates are "unhealthy" components of the diet is more common, at least for the general public, and also increasingly for health policy makers [3]. Scientific evidence is, however, inconclusive if dietary carbohydrate intake is a causal factor in the rise of obesity [3]. There is still scientific debate whether only the energy balance is relevant to the development of obesity regardless of the macronutrient or type of product ("a calorie is a calorie") [4], or that certain diets (high in high-glycaemic load carbohydrates) contribute disproportionally to the development of obesity [5]. Overall health outcomes for carbohydrates, which are rather diverse in form, are more likely to depend on the quality of the carbohydrate than on the (relative) quantity [2]. Nutritional guidelines have recommendations on the type of carbohydrates that are best to consume; low intake of simple sugars and refined carbohydrates and high intakes of dietary fibres is recommended (in the USA and The Netherlands) [6, 7]. Guidelines on the relative energy intake are not very strict. In the USA the contribution of carbohydrates to total daily energy consumptions is recommended to be between 45-65% [6]; in The Netherlands an even wider guideline is given, with 40-70% of daily energy from carbohydrates [7].

Structure dietary carbohydrates

Carbohydrates ($C_n(H_2O)_n$) are polyhydroxy aldehydes or ketones [8, 9]. When a carbohydrate consists of one aldehyde or ketone unit, it is called a monosaccharide [9]. Three types of monosaccharides are most relevant in our diet: the hexoses glucose, fructose, and galactose (Fig. 1A). These monosaccharides share the same molecular formula ($C_6H_{12}O_6$), but their structural formula differs (Fig. 1A). Monosaccharides are the building blocks for all other carbohydrates. Monosaccharides can be linked by O-glycosidic bonds [8]; when a carbohydrate consists of two monosaccharide units linked together, it is called a disaccharide. Maltose (a dimer of two glucose moieties), sucrose (a dimer of one glucose and one fructose moiety), and lactose (a dimer of one glucose and one galactose moiety) (Fig. 1B) are the disaccharides most present in our diet. In addition, our diet contains oligosaccharides: short chains of 3-10 monosaccharides. Longer chains of monosaccharides are referred to as polysaccharides, and can contain up to thousands of monosaccharide units (usually glucose); starch is an example of a dietary polysaccharide. When the sugar-units of a polysaccharide are connected by linkages that cannot be digested by the human body, the polysaccharide is called a (dietary) fibre.
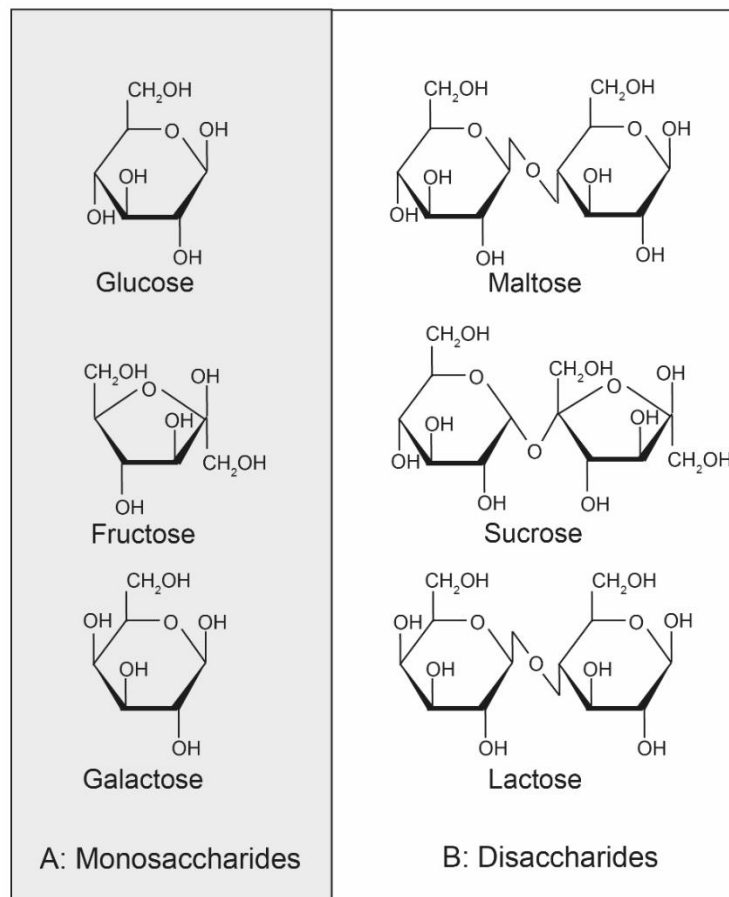
Figure 1. Molecular structure of common monosaccharides (A) and dissaccharides (B) in our diet.

Basic glucose metabolism

Upon intake, carbohydrates are broken down into monosaccharides, that can be absorbed by the enterocytes in the small intestine. After absorption, glucose is secreted into the portal vein. Glucose enters β-cells in the pancreas via SLC2A2 (also known as GLUT2) transporters, and stimulates insulin release. Insulin is a key hormone that stimulates the switch from fat oxidation in fasted conditions, to glucose oxidation in a number of tissues. Glucose enters the liver via SLC2A2 transporters, and is subsequently phosphorylated by insulin-activated hexokinase; this phosphorylation "traps" glucose inside the hepatocyte. Glucose is broken down into pyruvate via glycolysis: a sequence of enzymatic reactions, that provide cellular energy (ATP). Pyruvate can be converted to lactate (rapid energy, low ATP yield) or can go into the mitochondria, and be completely oxidized in the TCA cycle (slower process, high ATP yield). Insulin also stimulates glycogenesis (the formation of glycogen out of glucose) in hepatic tissue and the conversion of glucose into fat molecules: *de novo lipogenesis.* In muscle tissue and adipose tissue, insulin stimulation causes translocation of SLC2A4 (also known as GLUT4) transporters to the cell membrane, leading to an influx of glucose. This glucose is stored as glycogen in muscle, or can be converted to fatty acids via *de novo lipogenesis* in adipocytes. In addition, insulin inhibits a number of processes, such as gluconeogenesis in the liver, and lipolysis in the adipocytes.

In fasting conditions, hepatic glycogen stores are broken down to maintain blood glucose levels. In addition, gluconeogenesis, the process of glucose synthesis out of amino acids and glycerol, becomes more important. Hepatic gluconeogenesis is most well-known, however intestinal gluconeogenesis and in particular gluconeogenesis in the kidney are more important in fasting conditions and after protein rich meals [10].

Galactose metabolism

Galactose is absorbed from the intestinal lumen into the enterocyte in the same way as glucose, via the SLC1A5 (also known as SGLT1) transporter, and exits the basolateral side via SLC2A2. The main site of galactose metabolism is the liver [11-13], where a set of enzymes transforms galactose into glucose-1-phosphate (See Fig. 2); a pathway known as the Leloir pathway [14]. First, galactose (β-D-Galactose) is transformed by galactose mutarotase (to α-D-galactose), and then phosphorylated by galactokinase. Next, phosphorylated galactose is converted to UDP-galactose, by galactose uridylyl transferase, whereby one UDP-glucose molecule is converted to glucose-1-phosphate. The UDP-galactose is converted to UDP-glucose by galactose epimerase. The glucose-1-phospate formed in this pathway can go into glycolysis, or into glycogenesis (depending on cellular conditions). De novo synthesis of galactose takes place as well [15]. Galactose hardly stimulates pancreatic insulin release (summarized in [16]).

Fructose metabolism

Fructose is taken up via the SLC2A5 (also known as GLUT5) transporter into the enterocytes, and exits via basolateral SLC2A2 transporters, to go into the bloodstream. The liver is a major site of fructose metabolism (See Fig 2.), where fructose is phosphorylated by ketohexokinase, and the phosphorylated product (fructose -1 phosphate) is broken down into dihydroxyacetone-phosphate and glyceraldehyde. These products can be further metabolized in glycolysis. Fructose metabolism bypasses phosphofructokinase, the rate-limiting and highly regulated enzyme in glycolysis. In other tissues, fructose is converted to fructose 6-phosphate by hexokinase, and thereby enters glycolysis upstream of phosphofructokinase. Recently it was shown that fructose, when given in low quantities, is mainly metabolized in the intestine; yet in high, supraphysiologic conditions, more hepatic fructose metabolism as well as microbial fructose fermentation in the colon take place [17].

Carbohydrate intake during lactation

The main carbohydrate in human breastmilk is lactose. Human milk has the highest lactose concentrations of all lactating species [18]. For the determination of lactose, enzymatic colorimetric methods are used most often; these methods are based on hydrolysis of lactose followed by a colorimetric reaction of one of the constituting monosaccharides [23]. Lactose
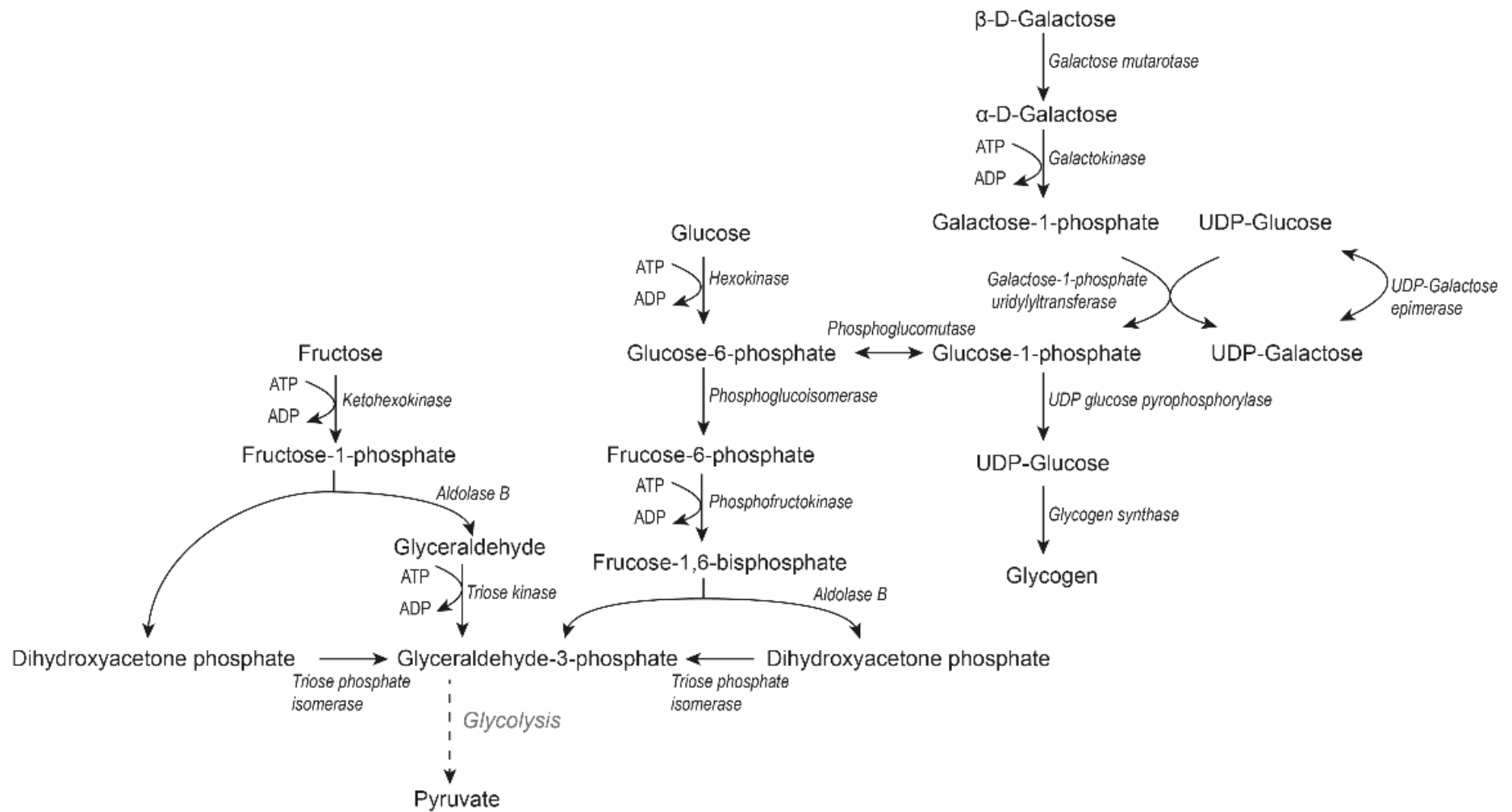
Figure 2. Hepatic metabolism of glucose, galactose, and fructose. Figure based on [14, 19-22].

content of breastmilk is about 64-76 g/L [23, 24], although 56-57 g/L (by liquid chromatography with tandem mass spectrometry) [25], and 82-88 g/L (by [1]H 1D NOESY spectroscopy) have also been reported [26]. The concentration of lactose in breastmilk is stable over the stage of lactation (1-12 months) [25, 27], and over the time of day [28]. Higher lactose concentrations are found in women that produce a higher total volume of breastmilk [29], and high lactose concentrations are associated with a higher number of feedings per day [28].

Besides lactose, human breastmilk contains 10-15 g / L of complex sugars and carbohydrates, referred to as human milk oligosaccharides (HMOs) [30]. HMOs can be between 3-22 sugar units long, and approximately 200 different types of HMOs have been described, all with lactose at the reducing end [31]. These HMOs cannot be digested and absorbed by infants, and are therefore considered non-nutritive [24]. HMOs do have an immunomodulating function, as they help prevent bacterial adhesion, and they are prebiotics, that stimulate the abundance of beneficial bacterial strains in the intestines [32]. Some glucose is also present (255 mg/L) in breastmilk, but this is very little compared with lactose content (76 g/L in same samples) [33]. Glucose concentrations are increased in breastmilk from mothers with type 1 and type 2 diabetes [34]. Recently, some fructose has been found in breastmilk in trace amounts (6.7 mg/L), likely as a consequence of maternal fructose intake [33].

Relative energy contribution of carbohydrates during lactation
Only lactose is relevant for the relative energy contribution of carbohydrates during lactation, because HMOs do not directly contribute to energy intake of the infant [24], and because glucose and fructose are only present in very small amounts. Although absolute lactose concentrations in breastmilk are quite stable [24], the relative energy contribution of lactose is variable, as this depends on protein and fat content of the breastmilk as well. Absolute protein concentrations decrease (about 25% decrease) in the first half year of lactation [27], but remain relatively stable afterwards. Most variable is the absolute fat content in human milk [28]. Fat content varies over the time of day [28, 35]. In addition, fat content in hindmilk (milk produced at the end of a feeding session) is about two- to three-fold higher than the amount of fat in foremilk (milk produced at the beginning of a feeding session) [28, 36]. Finally, experts state that it is difficult to determine fat content of breastmilk accurately, because of the non-linearity of fat increase during a feeding session, and because babies may not drink all milk available in a breast [37]. In general, lipids provide 40 to 55 % of the energy provided to the infant [18]. Based on concentrations of protein, fat, and lactose reported by Ballard *et al.* [24], we calculated that approximately 40 to 49% of energy in breastmilk originates from lactose. Prentice *et al.* (2016), who measured lactose with [1]H 1D NOESY spectroscopy, reported higher relative contributions, with a median of 55en% coming from lactose [26]. Overall, it is clear that the contribution of lactose to total energy in human breastmilk is substantial.

Weaning period

The lactation period, where (breast) milk is the only source of nutrition, is followed by the weaning period. Weaning is described as " the entire process during which the infant changes from full dependence on breastmilk to complete independence from it" [38]. The weaning period is also referred to as the complementary feeding period, whereby the WHO defines complementary feeding as: "the process starting when breastmilk alone is no longer sufficient to meet the nutritional requirements of infants so that other foods and liquids are needed" [39, 40]. At the end of the weaning period, an infant or child depends fully on "solid" foods. There is some discrepancy in the advice on when to start with complementary feeding. The WHO recommends 6 months of exclusive breastfeeding, and then complementary foods can be added into the diet [40]. The European Society for Peadiatric Gastroenterology, Hepatology, and Nutrition (ESPGHAN) recommends first complementary foods to be given between 4 to 6 months, not later [39]. Additionally, full breastfeeding in the first 17 weeks of life, and predominant breastfeeding for 6 months is advised [39]. Introduction of complementary foods before 4 months of age is discouraged, as there is some evidence that it may increase the risk of overweight during childhood [41].

Average age at which weaning is complete, is when a child is 29 months old in traditional, non-industrial, societies [42]. However, the range of weaning ages reported is very broad: between 12 months and 66 months have been documented [42]. Age at weaning is a bit more difficult to establish nowadays, because infants that discontinue breastfeeding can be transferred to a breastmilk replacer or get mixed feeding with breastmilk and breastmilk replacer. Follow-on formula's, and young child formulas (YCFs, also known as growing up milks, or toddler's milks) are such replacers [43]. In addition, there are infants that are never breastfed and depend on formula feeding, as breastfeeding is not always possible, or not the wish of the parents. When infants and children no longer depend on breastmilk or replacers, and complementary foods, but have switched to full consumption of regular "family" foods, it can be considered that they are completely weaned.

Weaning foods

Weaning is characterized by a diversification of the diet, and a change in macronutrient profiles. During weaning, the relative contribution of fat to energy intake decreases [44, 45], to ~ 30 % energy from fat when an infant is nine months old [44]. The contribution of carbohydrates to total energy intake increases to 47-55 en% in an one-year-old, and remains at this level for the second year of life [46]. The contribution of lactose to total energy intake decreases during weaning, as a result of the lower milk intake [46].

There are various (non-scientific) sources with recommendations and information on complementary feeding, but there are few scientific guidelines on complementary feeding [47]. ESGHAN prescribes that complementary foods should be rich in iron, and without added salt or sugar [39]. Because high intake of protein has been associated with higher body weight and fat mass in childhood [48], it is recommended that energy from protein during the weaning period should not exceed 15%, as higher protein intakes are associated with

overweight and obesity [39, 49]. High adherence to recommended weaning diet is associated with lower childhood adiposity [50, 51].

Breastfeeding and its impact on health
The WHO recommends exclusive breastfeeding for 6 months, because it reduces the risk of mortality and infection related morbidities in infants [52].Duration of breastfeeding varies widely, and differs over varies regions worldwide. Average breastfeeding duration is much shorter in high and upper-middle income countries [52]. At 6 months of age, over 90% of the children in low-income countries are breastfed, while this is approximately 40% in high income countries. Similarly, at 12 months, over 80% of children in low income countries receive breastmilk, while this is about 20% in high income countries (Europe, North America) [52]. In addition to the short-term benefits of breastfeeding compared with formula feeding, effects have been reported lasting beyond the breastfeeding period [52]. Breastfeeding has been shown to reduce the risk of obesity and of type 2 diabetes in offspring [53]. Several underlying mechanisms have been proposed, including a lower concentration of protein in breastmilk compared with formulae [54], the presence of bioactive compounds in breastmilk including leptin, adiponectin, ghrelin and insulin [55], and the beneficial effect of breastmilk on the intestinal microbiome composition [56]. Apart from beneficial effects on offspring, prolonged lactation appears beneficial for mothers as well, as longer lactation duration has been associated with reduced risk of breast-cancer [57, 58], ovarian cancer [57], type 2 diabetes [59], and non-alcoholic fatty liver disease [60].

Developmental origins of health and disease

The effects of breastfeeding on later life metabolic health supports the notion that conditions in early life can have long lasting effects on health and disease risk in adulthood, a concept known as the "Developmental Origins of Health and Disease" paradigm, or "DOHaD" [61, 62]. Especially the risk of chronic, noncommunicable diseases is influenced by conditions during critical periods of development. Early life nutrition is one of the determinants of later life (metabolic) health [63]. With the high prevalence of obesity and its concurrent metabolic dysfunctions, and limited effectiveness of treatment, early prevention has increasingly become of interest. Interventions in early life are thought to have great potential for improving public health (Fig. 3), especially for metabolic disorders [64]. An extra advantage is that parents are particularly willing to make lifestyle changes and implement healthy diet choices in this period [65].

Many epidemiological studies have linked risk of cardiovascular and metabolic diseases to early life nutrition, also known as nutritional programming. One of the most illustrious studies dates back to 1976, when Ravelli *et al.* studied the obesity rates of 19-year old men in the Dutch military records [66]. They reported an increase of obesity rates in those exposed to the Dutch Famine of 1944-'45 (also known as the Hunger Winter) during the first two trimesters

of the pregnancy; and a decrease of obesity rates in those exposed to famine during the last trimester and the first months of life [66]. In other investigations, exposure to the Dutch Famine *in utero* has been linked to increased risk of coronary disease, mental disorders such as schizophrenia and depression, altered lipid metabolism, airway disease, and deteriorated glucose tolerance [67, 68].
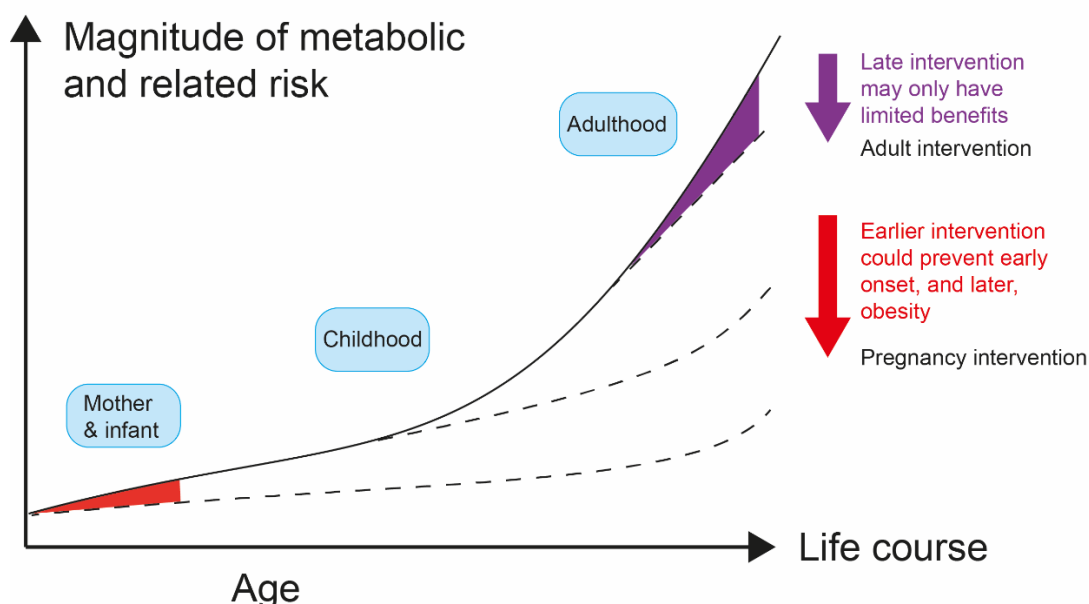
Figure 3. Relation between early life interventions and the potential to reduce later life disease risk. Figure adapted from Symonds *et al.* [64], who adapted it from Godfrey *et al.* [69].

Cohort studies, that track individuals from pregnancy, throughout infancy, childhood, and puberty into (early) adulthood, are extremely valuable for determining effect size of early life conditions on later life health and disease [70]. However, while these studies clearly link early life conditions with later life health outcomes, these studies do not allow for evidence beyond association, i.e they do not provide causation or mechanisms. Animal models provide an experimental alternative, and offer the opportunity to control the early life intervention, allowing to study effects of specific nutrients and the mechanisms underlying nutritional programming. Effects of caloric and protein restriction during pregnancy on later life susceptibility to non-communicable diseases, originally found in human epidemiological data, have been substantiated by numerous animal studies (reviewed in [71, 72]). From these studies, it has become clear that the severity of the intervention and the timing of the intervention affect the outcome, and that results can be sex-specific [71].

Mechanisms

Mechanisms underlying nutritional programming are not completely understood, although several processes have been found to contribute. For example, structural changes to organs under development may affect later life risk. Undernutrition during nephrogenesis leads to a

lower nephron number, which increases the risk of kidney disease in later life [73, 74]. Also the endocrine pancreas is sensitive to low protein conditions and caloric restriction. Both can result in low β-cell mass and reduced vascularisation [72, 75, 76]. Undernutrition in the perinatal period can have effects on brain structures, such as the hypothalamus [73, 77]. Secondly, epigenetic mechanisms are considered to play a role, as such mechanisms enable different phenotypes to develop from one genotype [78]. Although there are different epigenetic mechanisms (including histone modification and expression of noncoding RNA), the most studied mechanism is DNA methylation [79]. DNA methylation in metabolic organs (adipose tissue, liver, pancreas, skeletal muscle) can influence metabolic processes, such as insulin secretion and signalling, adipose tissue differentiation, and glucose and lipid processing [79]. Another process that may underlie lasting effects of early life nutrition on risk for non-communicable diseases is cellular aging, in particular telomere length and oxidative stress [72].

Mouse model for nutritional programming

The C57BL/6J mouse strain is frequently used for metabolic research. It is a favourite model for diet-induced obesity, because this strain displays rapid weight gain and develops hyperglycaemia and hyperinsulinemia on a high-fat diet [80]. Because of the short pregnancy and suckling periods and the short lifespan (approximately 2 years) of these mice, they are also suitable for studying nutritional programming, particularly in investigating the relation between early life interventions and risk of metabolic disorders in later life. Pregnancy lasts 19.5 days for C57BL/6J mice, and pups depend fully on suckling until 16 days postnatal (PN16). After PN16, transition to solid food starts. In laboratory conditions, pups are weaned and taken from their mother and housed separately on PN21. This moment is close to the moment when weaning is complete in pups of the house mouse, which are fully weaned by PN23 [81]. When groups of mice receive a different treatment in early life, but all are placed on the same diet afterwards, it is possible to study programming effects. When C57BL/6 mice are placed on a high-fat (or western style) diet after the intervention, the nutritional programming study is combined with a model for diet-induced obesity in adulthood.

Critical periods of development

Foetal life and infancy are unequivocally recognized as critical periods of development during which nutritional programming can occur [82, 83]. The recognition of puberty as critical period of development is increasing. Severe protein restriction (4% w/w vs 23%) during puberty causes higher adiposity and pancreatic islet dysfunction in adulthood in male rats [84, 85]. Data from the Dutch Famine show that exposure to severe undernutrition during adolescence (ages 11-14) increases the risk of type 2 diabetes and peripheral arterial diseases in women, but exposure during childhood or pre-adolescence (ages 2-5 and 6-10) does not [86].

Childhood, with or without the adolescent period, can be a critical period of development as well. Distress in early childhood is associated with a range of health problems in adulthood, both of physical and mental nature [87]. Self-reported exposure to the Dutch Famine in early life (ages 0-21) was associated with increased risk for type 2 diabetes [88] and COPD and asthma [89]. Enriched environmental conditions in the post-weaning period in rats (PN21-PN94) beneficially affect depressive- and anxiety-like behaviours [90].

With a murine model as described above, beneficial health effects have been found with nutritional interventions around weaning. N-3 long chain poly unsaturated fatty acids fed from PN2 to PN42, reduce fat mass development in adulthood, with smaller adipocytes and lower HOMA-IR [91]. Feeding of synbiotics (PN2-PN42) reduces body weight and fat mass in adulthood, and lowers plasma insulin levels and liver triglycerides [92]. Nutritional programming effects induced in the (post-)weaning period (PN16-PN42) have been found with a formula (Nuturis), with large lipid droplets coated with milk fat globule membrane fragments that mimic the lipid droplets found in breastmilk more than regular formula lipid droplets [93]. This formula reduces FM gain in adulthood in male mice [93-95], with reductions in insulin resistance [93], plasma lipids [93] and cholesterol [93, 95], inguinal (subcutaneous) adipocyte size [95], and increases markers of mitochondrial capacity in white adipose tissue (retroperitoneal) and muscle (*m. tibialis*) [96]. In addition, feeding of Nuturis formula from in the weaning and post-weaning period reduces visceral fat mass, fasted blood glucose concentrations and triglyceride concentrations in a rat model of intra-uterine growth restriction – a well-known model of adverse metabolic programming [97]. In addition, medium chain fatty acids (PN16-PN42) program for reduced fat accumulation and improved insulin sensitivity in WSD challenged male mice [98], and program a moderate reduction in adipocyte size in WSD challenged male rats [98]. Wielinga *et al*. showed lasting programming effects of supplementation with arachidonic acid and docosahexaenoic acid. The intervention (in four-weeks-old mice) lasted eight weeks, and was followed by a high-fat high-carbohydrate diet, and resulted in reduced fat mass, lower leptin concentrations, and smaller adipocyte sizes [99].

Aims and outline of this thesis

Although evidence is limited, previous studies have shown that nutritional programming can occur in the weaning and post-weaning period. These studies have focussed on undernutrition, rearing conditions, or lipids; however, evidence of nutritional programming by carbohydrates is lacking so far. Given the lack of scientific guidelines for the weaning period, and the potential impact of nutrition in this period, we studied the effects of post-weaning monosaccharides on long-term metabolic health in this thesis.

We used the C57BL/6JRcc mouse model for nutritional programming and compared the effects of fructose and galactose to glucose, the "control" monosaccharide. The diversity in carbohydrates in the diet of infants and children increases drastically in the weaning period:

lactose (glucose + galactose) intake decreases, while the intake of other (fruit) sugars (fructose and sucrose: glucose + fructose) increases. While we focused on effects of these mono-saccharides, we also included starch in the diet of our mouse studies, to have a more realistic diet and dietary profile that models carbohydrate intake during weaning in infants and young children.

The first aim of this thesis was to establish whether the post-weaning period can be considered a critical period of development, susceptible to nutritional programming by the monosaccharides fructose and galactose, with glucose as control. This was studied in a pre-clinical mouse model, and the research focussed on long-term effects on body composition and metabolic health. Secondly, this thesis aimed to study if post-weaning programming by monosaccharides is different for males and females. Thirdly, this thesis aimed to substantiate mechanisms underlying the potential programming effects.

Chapter 2 describes a mouse study where the effects of fructose in the post-weaning diet on later life health are compared with the effects of glucose. Both male and female mice, fed a high-fat diet in adulthood, were studied in this chapter. The effects of the post-weaning intervention on body weight, body composition, glucose tolerance, and whole body metabolism in an obesogenic environment were analysed.

For additional insight in metabolic effects of fructose, in **Chapter 3,** the direct effects of fructose, fructose and glucose in a 1:1 ratio, and glucose, are studied in adult male and female mice on a moderate high-fat diet background.

The programming effects of galactose and glucose in a 1:1 ratio in the post-weaning period are compared with the effects of glucose alone in **Chapter 4.** Both males and females are studied in this chapter, but because fat mass appeared to be metabolically programmed in females alone, most analyses focussed on females.

Chapter 5 continues on the findings described in **Chapter 4**, yet zooming in on the direct effects of galactose *vs* glucose in the post-weaning diet in females at PN42. Transcriptomics analyses were used to study hepatic responses at the molecular level.

In **Chapter 6** the effects of post-weaning galactose *vs* glucose on later life metabolic functions is investigated in females, with in depth analysis of whole body metabolism and insulin resistance development.

Finally, the main findings of this thesis are discussed in **Chapter 7**, the general discussion, along with methodological considerations and the implications of the results.

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Chapter 2

No adverse programming by post-weaning dietary fructose of body weight, adiposity, glucose tolerance or metabolic flexibility

Lianne M. S. Bouwman*,

José M. S. Fernández-Calleja*,

Hans J. M. Swarts,

Inge van der Stelt,

Annemarie Oosting,

Jaap Keijer,

Evert M. van Schothorst.

Abstract

Scope: Metabolic programming can occur not only in the perinatal period, but also post weaning. This study aims to assess whether fructose, in comparison to glucose, in the post-weaning diet programs body weight, adiposity, glucose tolerance, metabolic flexibility and health at adult age.

Methods & Results: Three-week-old male and female C57BL/6JRccHsd mice are given an intervention diet with 32 energy percent (en%) glucose or fructose for only 3 weeks. Next, all animals are switched to the same 40 en% high fat diet for 9 weeks. Neither body weight nor adiposity differs significantly between the animals fed glucose or fructose diets at any point during the study in both sexes. Glucose tolerance in adulthood is not affected by the post-weaning diet, nor are activity, energy expenditure and metabolic flexibility, as measured by indirect calorimetry. At the end of the study, only in females fasting serum insulin levels and HOMA-IR index are lower in post-weaning fructose versus glucose diet (P = 0.02), without differences in pancreatic β-cell mass.

Conclusions: Our present findings indicate no adverse programming of body weight, adiposity, glucose tolerance, and metabolic flexibility by dietary (solid) fructose in comparison to glucose in the post-weaning diet in mice.

Introduction

Obesity is considered a major public health problem. In 2014, worldwide over 600 million adults and 41 million children younger than five years of age were obese (whttp://www.who.int/mediacentre/factsheets/fs311/en/). Excessive intake of high energy foods and low physical activity are considered the most important factors contributing to the high global obesity prevalence. Furthermore, it has been shown that early life nutrition influences the susceptibility of an individual to obesity in later life, and also type 2 diabetes and cardiovascular diseases [1-3]. This is "nutritional programming", defined as the process whereby "a stimulus or insult (a nutrient) operating at a critical or sensitive period of development results in a long-standing or life-long effect on the structure or function of the organism"[4]. It is of imminent importance for the development of preventive strategies to understand how early life diet affects later life health.

Focus in nutritional programming research has mainly been on caloric or protein restriction of mothers (in the perinatal period). However, evidence suggests that also carbohydrates can have programming effects. Supplementing maternal diet during gestation and lactation with sucrose reduced insulin sensitivity in the adult offspring in rats [5, 6]. In mice, providing dams before and throughout gestation and lactation with sucrose led to hyperinsulinemia and decreased glucose tolerance in female offspring when adult, while food intake was increased in both sexes [7]. Moreover, insulin levels were higher in weanlings of rat dams whose drinking water was supplemented with fructose, compared to weanlings of dams who received only tap water; glucose in drinking water of the dams did not affect insulin levels in offspring at weaning [8]. Programming effects of carbohydrates have also been observed when exposure was only during lactation [9, 10]. Formula feeding with a high-carbohydrate formula (56 energy percent (en%) carbohydrates) increased β-cell mass, hyperinsulinemia and body weight (BW) in male rats, compared to rats reared by a dam on natural rat milk (~8 en% carbohydrates) [10]. Similarly, in female pups, artificial rearing with high carbohydrate formula led to higher food intake, BW, and insulin levels in adulthood [9]. These studies show that carbohydrates in the perinatal period can program metabolic health.

Moreover, also the period directly following lactation, the post-weaning period, can be considered as a critical window of development, impacting lifelong metabolic health, although this is much less investigated. For example, post-weaning dietary lipids arachidonic acid/docosahexaenoic acid supplementation prevented excessive BW gain in later life, and affected lipid metabolism beneficially [11]. Similarly, the structure of lipid droplets (size and phospholipid coating) in the post-weaning diet also affected body composition (BC) and metabolic profile [12]. Little is known on post-weaning programming by sugars, although sucrose (in comparison to starch) in weaning diet of rats was shown to increase circulating insulin and cholesterol levels at later life, when rats were switched to the starch diet [13].

Sugars are a major component of the diet, in particular in infants and children. Overall, sugar energy intake in children represents 25% of total energy intake, being relatively higher than in adults with about 20 en% intake [14]. High sugar diets are causally related to obesity, and consumption of sugar-sweetened beverages is causally related to type 2 diabetes [15]. Because of this high intake of simple carbohydrates and their role in the development of obesity, it is important to study their potential for nutritional programming in the post-lactation period. While the amount of sugar in diets have been causally related to metabolic health, the effects of type of sugar are less clear [15]. It has been postulated that especially high fructose consumption leads to higher adiposity and increased risk of metabolic syndrome [16, 17]. However, this theory is under debate, as it is considered that fructose may not contribute more to metabolic syndrome than glucose [18-20].

Underlying the debate on the different risks of glucose and fructose is the knowledge that the metabolism and intestinal uptake of the two monosaccharides is different. Circulating glucose is selectively taken up in various peripheral organs, while the majority of fructose is metabolized in the liver. There, fructose enters glycolysis, bypassing the rate-limiting and tightly controlled step by phosphofructokinase. Part of glucose is also metabolized in the liver, yet at high concentrations of intracellular ATP inhibiting phosphofructokinase, glucose goes into glycogenesis to be stored [21]. Moreover, glucose causes an insulin response in the blood, while fructose does not [22].

In summary, young children are exposed to high dosages of simple sugars. As early childhood is considered a critical period of development, and glucose and fructose are metabolized differently, their respective effects on lifelong metabolic function and health might be different. Therefore, the aim of this study was to investigate whether fructose in comparison to glucose in the post-weaning diet programs (negatively) for bodyweight, adiposity, glucose tolerance, and metabolic flexibility at adult age.

Materials and Methods

Animals and experimental setup

All experimental procedures were approved by the Animal Experimental Committee (DEC 2014085, Wageningen) and complied with the principles of good laboratory animal care following the EU-directive for the protection of animals used for scientific purposes (2010/63/EU). All experiments were carried out at controlled laboratory conditions (23°C ± 1°C; 12:12 light dark cycle) with *ad libitum* access to food, unless stated otherwise.

Male and female C57BL/6JRccHsd mice (Harlan Laboratories BV, Horst, The Netherlands) were time mated and kept on a semi-synthetic purified low fat breeding diet (Research Diet Services BV, Wijk bij Duurstede, The Netherlands) (see Supporting Information S1). One or two days

after birth, litters were standardized to six pups per nest with at least two pups of each sex. Pups were used for the intervention study; see Fig. 1A for an overview of the setup. Pups (males: n = 12 per group, females n = 14 per group) were weaned three weeks after birth, stratified by body weight (BW), housed individually and placed on the post-weaning intervention diet for three weeks. The post-weaning diets (Research Diet Services) contained 20 en% protein, 16 en% fat and 64 en% carbohydrates of which 32 en% starch and 32 en% monosaccharides, being either glucose or fructose. At 6 weeks of age, all animals were switched to a high fat (HF) diet with 40 en% fat (for details on the dietary composition see Supporting Information S1). GLU and FRU are used to refer to animals on the glucose and fructose diets before diet switch, HF is added for the period after the diet switch (i.e. GLU-HF and FRU-HF). Food was refreshed every week. Food intake was determined by subtracting the weight of remaining pellets from the weight of the pellets provided. BW was measured weekly throughout the study. Body composition (BC) - lean mass (LM) and fat mass (FM) - was measured using the EchoMRI 100V (EchoMedical Systems, Houston, TX, USA). BC measurements were carried out weekly during the post-weaning intervention period, and bi-weekly once animals were on HF.

Diets

Diets were an adaptation from the BIOCLAIMS diet [23], adjusted for the fat content as recommended by AIN-93 for growing and lactating animals [24]. Moreover, all fructose was omitted from the breeding diet, to prevent contact with fructose in dams and offspring before feeding the post-weaning diet.

Indirect Calorimetry (InCa)

A subset of the mice was placed into the Phenomaster LabMaster Metabolism Research Platform (TSE systems GmbH, Bad Homburg, Germany) for measurements of activity, energy expenditure (EE), respiratory exchange ratio (RER) and food intake (FI) as described previously [25]. The animals were placed in the InCa at the end of the monosaccharide diet intervention, and again at the end of the HF period (Fig. 1A). Measurements consisted of 24 h adaptation, 24 h recording of basal conditions, and a fasting-refeeding challenge. The fasting-refeeding challenge assesses metabolic flexibility *in vivo,* by determining an individual's ability to switch from fat oxidation to carbohydrate oxidation after a period of fasting, as determined by the RER. The challenge consisted of restricted feeding of own diet (1.5 g for low fat diets, 1.1 g for HF) 2 h before dark phase started. The next morning, fasting state was verified by low RER. The animals were refed *ad libitum* with the low fat glucose diet (Supporting Information S1), 1 h before dark phase on that day (25 h after the restricted feeding). The animals were taken out of the InCa 15 h after the refeeding, during the next light phase. BW and BC were determined before and after the InCa measurements. Animals that did not go into the InCa system, were fasted and refed according to the same procedure to ensure identical treatment.

Oral glucose tolerance test (OGTT)

In week 11, OGTT analyses were performed as previously published [26], with minor modifications. Briefly, food was removed 1 h after start of the light phase. Mice remained without food for the following 5 h, after which blood glucose was measured via a tail cut with the Freestyle blood glucose system (Abbott Diabetes Care, Hoofddorp, The Netherlands) and 2 g glucose/kg BW was given by oral gavage. Fifteen, 30, 45, 60, 90, and 120 min after glucose administration, glucose concentration was determined (Freestyle). Glucose tolerance was analysed with time course data and incremental area under the curve (iAUC).

Sacrifice

In week 15, animals were fasted for 2-5 h at the start of the light period, and sacrificed by decapitation to prevent effects of anesthesia on metabolic parameters including glucose levels [27]. Animals were sacrificed in random order (males, females, and treatments randomized). A drop of blood was used to measure blood glucose levels (Freestyle), the rest of the blood was collected in MiniCollect® serum tubes (Greiner Bio one B.V., Alphen aan de Rijn, The Netherlands), and centrifuged for 10 min at 3000 x g and 4°C to obtain serum. Serum samples were aliquoted and stored at -80°C. Liver and gonadal fat pads were collected, weighed, and snap frozen in liquid nitrogen. Mesenteric fat and pancreas were excised at once, separated based on density by placing it in PBS, cut and weighed. Pancreata were fixated in 4% PAF overnight, and embedded in paraffin.

Serum measurements

Serum leptin and adiponectin were measured using Bio-Plex Pro mouse diabetes assays (Bio-Rad laboratories, Veenendaal, the Netherlands) in accordance with the manufacturer's instructions. Samples were tested in duplicate; leptin samples were diluted ten times, adiponectin samples were diluted 1600 times. Insulin levels were measured using an Ultra Sensitive Mouse Insulin ELISA Kit (ChrystalChem, Downers Grove, Illinois, United States) according to the manufacturer's instructions. Samples were tested in duplicate and averaged. HOMA-IR was calculated with the formula HOMA-IR = (glucose)* (insulin)/14.1, according to van Dijk *et al.* [28], where 14.1 is a C57BL/6J mice adjusted factor (instead of 22.5 used for humans).

Liver triglycerides

Liver triglyceride (TG) levels were determined using the Liquicolor kit (Human, Wiesbaden, Germany). Briefly, liver tissue (20 mg/mL) was placed in 10mM Tris, 2mM EDTA, 250 mM sucrose buffer, pH 7.5, and homogenized by sonication. Protein content of the liver homogenates was measured using a DC protein Assay (Bio-Rad) according to the manufacturer's instructions. Samples were tested in triplo, 100 μL of reagent was added to 5 μL sample.

Immunohistochemistry of the pancreas

Paraffin-embedded pancreata were cut into sections of 5 μm. Five sections per sample, spaced at least 200 μm apart, were mounted on Superfrost plus slides (Menzel, Braunschweig, Germany) for immunohistochemistry, as published [29]. In detail, sections were deparaffinised, rehydrated, and incubated in 1% H_2O_2 in methanol for 20 min. After microwave antigen retrieval in sodium citrate buffer, sections were blocked with 5% goat serum in PBS for 30 min. Sections were incubated at 4°C overnight with primary rabbit anti-insulin antibody (Cell signaling Technology, Leiden, The Netherlands; 1:500). Negative controls were incubated with rabbit IgG solution (Vector Laboratories, Burlingame, California, United states). Sections were incubated for 60 min at room temperature with goat anti-rabbit biotinylated antibody (Vector Laboratories; 1:200) followed by 60 min incubation with Vectastain Elite ABC kit (Vector Laboratories; 1:2000). For visualization, a 3-3'diaminobenzidine kit (Immpact DAB, Vector Laboratories; 1:200) was used. Sections were counterstained with Hematoxylin QS (Vector laboratories). Specific staining was absent in controls. Pictures were taken with a Leica DM6 microscope (Leica Microsystems, Wetzlar, Germany) and merged to overview pictures of whole sections with LasX pc software (Leica). Pancreatic β-cell area was determined with the ROI manager in ImageJ-software, version 1.51f (http://rsbweb.nih.gov/ij/). DAB-positive areas were manually encircled, and expressed as percentage of automatically calculated total area. β-cell mass was estimated by multiplying the percentage of insulin positive surface area by the pancreas weight.

Hardness

Hardness of dietary pellets was measured using a Kahl device, as described [23].

Statistics

Adiposity was calculated by dividing EchoMRI-determined FM by BW. Data were analyzed in GraphPad Prism, version 5.04 (GraphPad Software Inc., San Diego, CA, USA). Data were checked for normality by D'Agostino and Pearson omnibus normality test. Normally distributed data were compared with an independent Students' t-test, with Welch's correction when applicable. Not-normally distributed data were log (or square root) transformed and rechecked for normality. BW, FM, LM, and adiposity were checked by two-way ANOVA Findings were compared within sex. All data are shown as mean ± SD. Significance was defined at $P < 0.05$.

Results

Body weight (BW) did not significantly differ between the animals fed with fructose or glucose enriched diets in the 3 week post-weaning period, nor during the following HF period (Fig. 1B for males, Fig. 1C for females). These animals are referred to as GLU or FRU when on the intervention diets, or as GLU-HF and FRU-HF during the high fat feeding period. Fat mass (FM) was not significantly different at any point in males (Fig. 1D) nor females (Fig. 1E), although
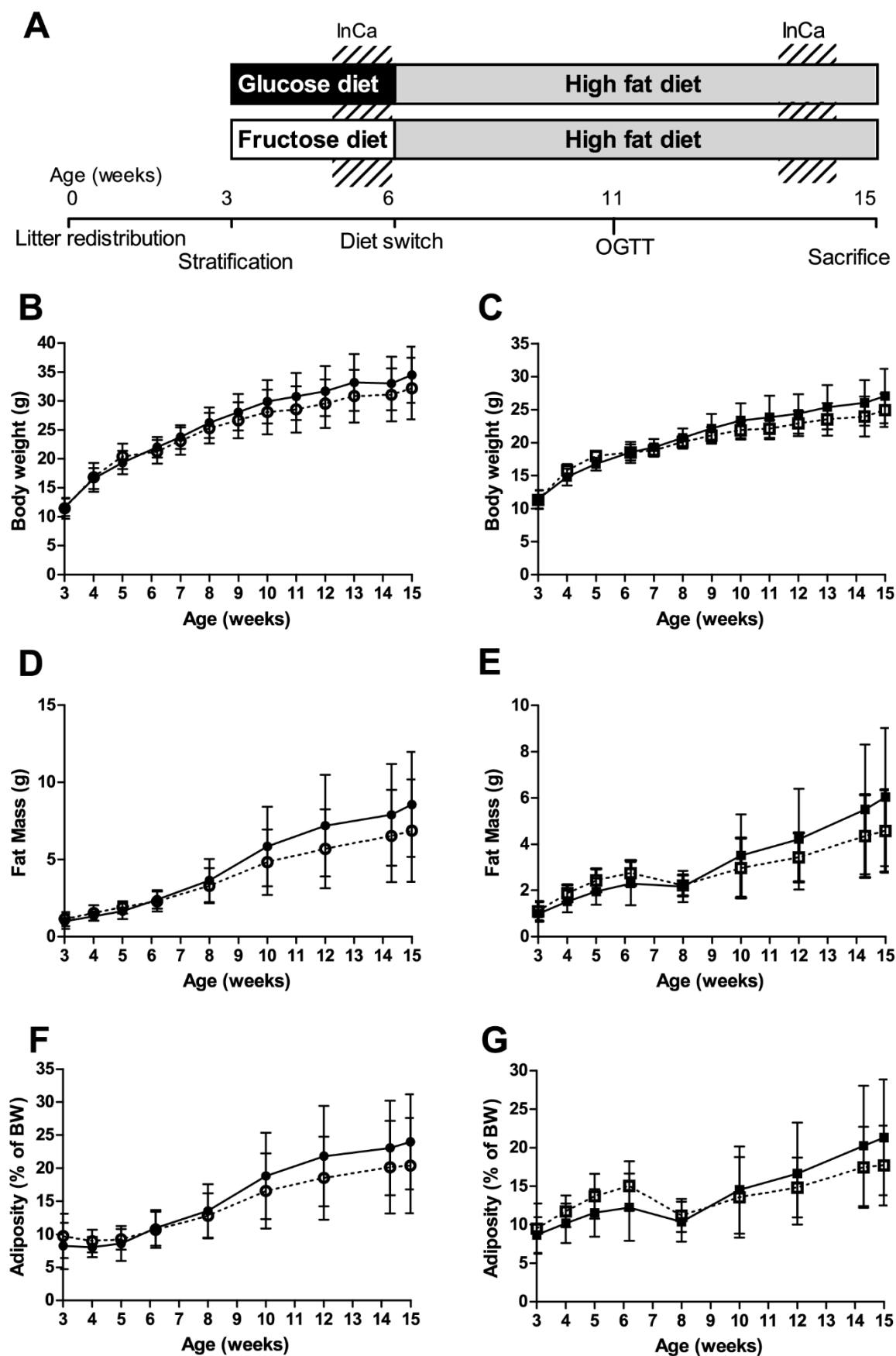
(Legend on the next page)

Figure 1. Study setup, body weight (BW) and body composition development over time. (A) Schematic overview of the study setup. Briefly, litters were standardized to six pups per nest. In week 3, pups were stratified by BW and placed on a glucose or fructose diet (animals in these groups are GLU or FRU). In week 6, all animals were switched to the same high fat diet (HF; animals are referred to as GLU-HF or FRU-HF). Animals were tested in the indirect calorimetry (InCa) system in week 5 and week 14, an oral glucose tolerance test (OGTT) was performed in week 11. Animals were sacrificed in week 15. (B) BW over the intervention period (week 3-6) and HF period (week 6-15) for males and (C) females, (D) fat mass (FM) for males and (E) females, (F) adiposity for males and (G) females. Black symbols and lines represent GLU until week 6 and GLU-HF from week 7 till week 15; open symbols and dotted lines represent FRU groups until week 6 and FRU-HF groups from week 7 till week 15. Values represent mean ± SD, n = 12 for males, n = 14 for females.

there was a trend for higher FM in FRU females in week 6 (P = 0.058, Table 1). However, the interaction post-weaning diet x time was significant by two-way ANOVA for BW in males and females, and for FM in females. Similarly, adiposity was not significantly affected by the post-weaning diet (Fig. 1F for males and 1G for females), nor during the HF period. In females, the interaction post-weaning diet x time was significant. Lean mass was not different between groups at any point in the study (data not shown). Thus, body composition parameters did not differ at any specific time point, but the two-way ANOVA analysis indicated a lower FM for the FRU-HF females.

Table 1. Body weight (BW), fat mass (FM) and cumulative food intake after the 3 weeks intervention and subsequent 9 weeks high fat (HF) diet.

	Cumulative food intake intervention (g)	BW after intervention (g)	FM after intervention (g)		Cumulative food intake HF(g)	BW after HF period (g)	FM after HF period (g)
GLU Males	63 ± 3	22.0 ± 1.7	2.4 ± 0.6	GLU-HF Males	172 ± 13	34.5 ± 4.9	8.6 ± 3.4
FRU Males	71 ± 8**	21.2 ± 2.0	2.3 ± 0.7	FRU-HF Males	171 ± 20	32.2 ± 5.3	6.9 ± 3.3
GLU Females	59 ± 5	18.5 ± 1.6	2.3 ± 0.9	GLU-HF Females	162 ± 15	27.1 ± 4.1	6.0 ±3.0
FRU Females	69 ± 7***	18.5 ± 1.2	2.8 ± 0.5	FRU-HF Females	154 ± 18	24.9 ± 2.5	4.6 ± 1.8

Values represent mean ± SD, n = 12 for males, n = 14 for females. ** Differs significantly between GLU males and FRU males (P < 0.01). *** Differs significantly between GLU females and FRU females (P < 0.001).

Food intake measurements during the intervention seem to suggest a higher intake of the fructose intervention diet in both sexes (Table 1). However, the fructose intervention diet was more brittle and had a wetter appearance than the glucose intervention diet. This was reflected in the hardness of the diets, being 24.5 ± 3.0 kgf for glucose intervention diet and 4.1 ± 0.5 kgf for the fructose diet. Moreover, spillage of diet seemed to occur more often in

the fructose group, thus likely overestimating food intake in the FRU animals. Interestingly, during the measurement in the InCa in week 5, no differences in 24 h FI were found between GLU and FRU (Table 2). Food intake of the HF was not affected by the post-weaning intervention in regular measurements (Table 1) or during the 24 h basal measurement in the InCa (Table 2).

Energy expenditure (EE) and activity were not significantly different between GLU or FRU males, nor between GLU-HF and FRU-HF males (Table 2). For the females, also no difference in EE or activity between GLU and FRU was found, nor for GLU-HF versus FRU-HF, although in week 14 EE was higher and activity was lower compared to week 5 (see Table 2), likely due to increased BW and FM. RER values during 24 h basal measurements were not different between GLU males and FRU males (Fig. 2A). During the fasting-refeeding measurements, RER values in fasted state were similar (Fig. 2B and C), while RER was higher in the 2 h following the refeeding challenge in GLU compared to FRU males (Fig. 2B and C). GLU males ate more in the 2 h following the refeeding than the FRU males (Fig. 2D). The GLU-HF and FRU-HF males did not differ in RER during the 24 h basal measurements (Fig. 2E) or during the fasting-refeeding challenge (Fig. 2F and G). Food intake in the 2 h after the refeeding was not different for GLU-HF and FRU-HF males (Fig. 2H). As expected, RER showed a circadian rhythm over 24 h (Fig. 2A), which was dampened when animals were fed the HF diet (Fig. 2E). For females, no difference in RER during the basal measurements nor during the fasting-refeeding challenges were found between GLU and FRU animals in week 5 (Fig. 3A-D), nor for GLU-HF and FRU-HF animals in week 14 (Fig. 3E-G). Thus, energy metabolism parameters were not significantly different between the diets.

Table 2. Energy expenditure, activity, and food intake during the 24 h basal indirect calorimetry (InCa) measurements.

	Average EE (KJ/h)	Total activity (counts)	Cumulative FI (g)
Week 5:			
GLU Males	1.84 ± 0.13	$4.48 \times 10^4 \pm 7.28 \times 10^3$	4.14 ± 0.57
FRU Males	1.81 ± 0.08	$4.23 \times 10^4 \pm 2.92 \times 10^3$	3.30 ± 1.4
Week 14:			
GLU-HF Males	2.19 ± 0.06	$2.97 \times 10^4 \pm 7.28 \times 10^3$	3.44 ± 0.57
FRU-HF Males	2.16 ± 0.19	$3.28 \times 10^4 \pm 7.00 \times 10^3$	3.29 ± 0.44
Week 5:			
GLU Females	1.73 ± 0.13	$5.98 \times 10^4 \pm 8.05 \times 10^3$	3.57 ± 0.79
FRU Females	1.70 ± 0.15	$5.61 \times 10^4 \pm 1.13 \times 10^4$	3.36 ± 0.69
Week 14:			
GLU-HF Females	1.95 ± 0.19	$3.92 \times 10^4 \pm 1.08 \times 10^4$	3.09 ± 0.70
FRU-HF Females	1.98 ± 0.20	$4.39 \times 10^4 \pm 1.17 \times 10^4$	3.65 ± 0.74

Results are given for 24 h basal measurements. Energy expenditure (EE) is given as the average per hour of the 24 h measurements. Activity and food intake (FI) are cumulative over the 24 h. No significant differences were observed between dietary groups.
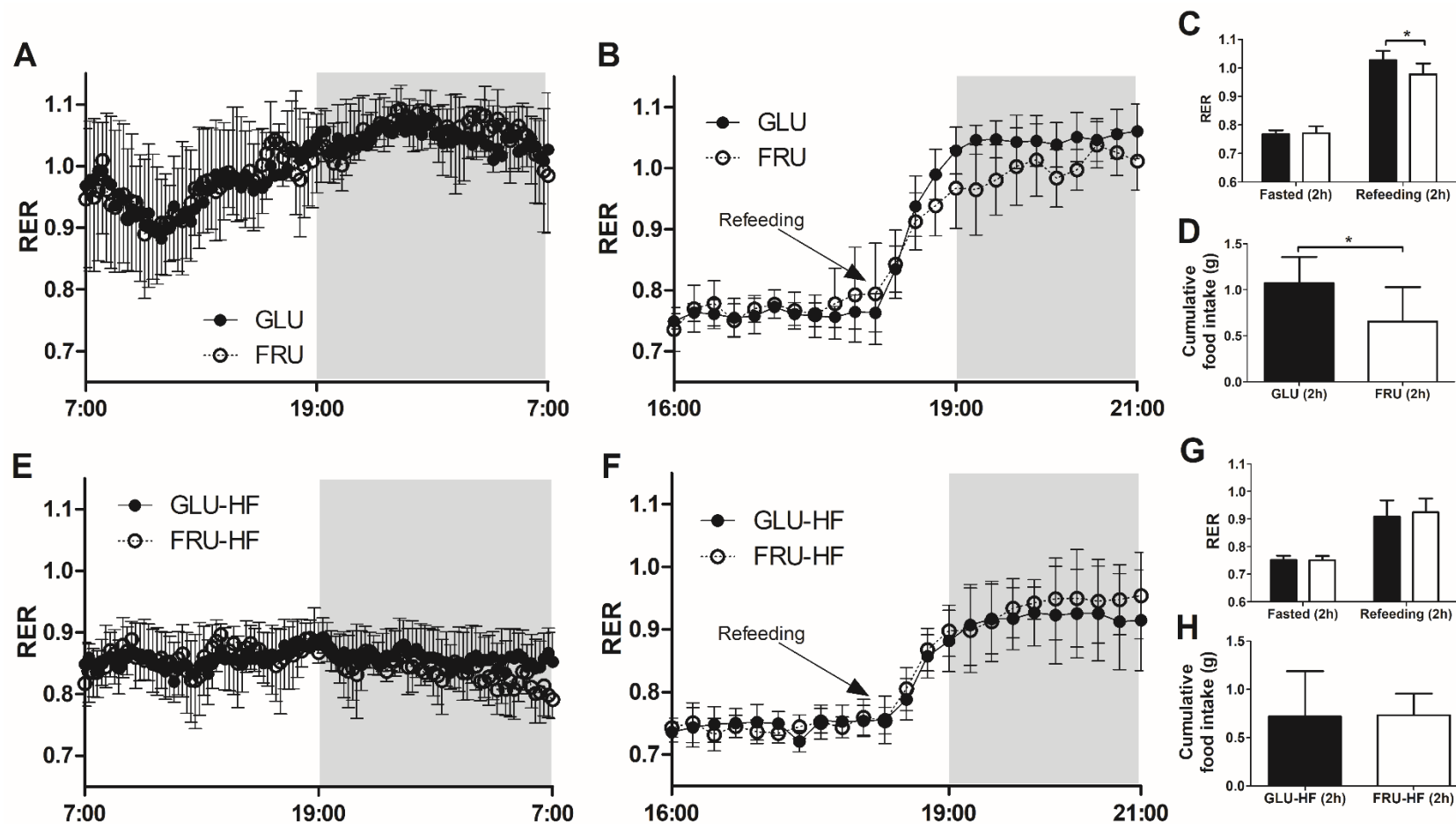
Figure 2. Respiratory exchange ratio (RER) during basal conditions and fasting-refeeding challenge in males during the intervention (week 5) and during the HF diet (week 14). (A) RER for males on glucose or fructose post-weaning diet (week 5) during 24 h basal measurement and (B) during fasting-refeeding challenge. (C) Average RER for fasting-refeeding challenge, values represent the average of 2 h before and 2 h after refeeding for males on GLU or FRU diet (week 5). (D) Cumulative food intake for the first 2 h after refeeding in the fasting-refeeding challenge for GLU and FRU males (week 5). (E) RER for GLU-HF and FRU-HF males (week 14) during 24 h basal measurement and (F) during fasting-refeeding challenge. (G) Average RER for fasting-refeeding challenge, values represent the average of 2 h before and 2 h after refeeding for GLU-HF and FRU-HF males (week 14). (H) Cumulative food intake for the first 2 h after refeeding in the fasting-refeeding challenge for GLU-HF and FRU-HF males (week 14). Values represent mean ± SD, n = 6-9. *: $P < 0.05$.
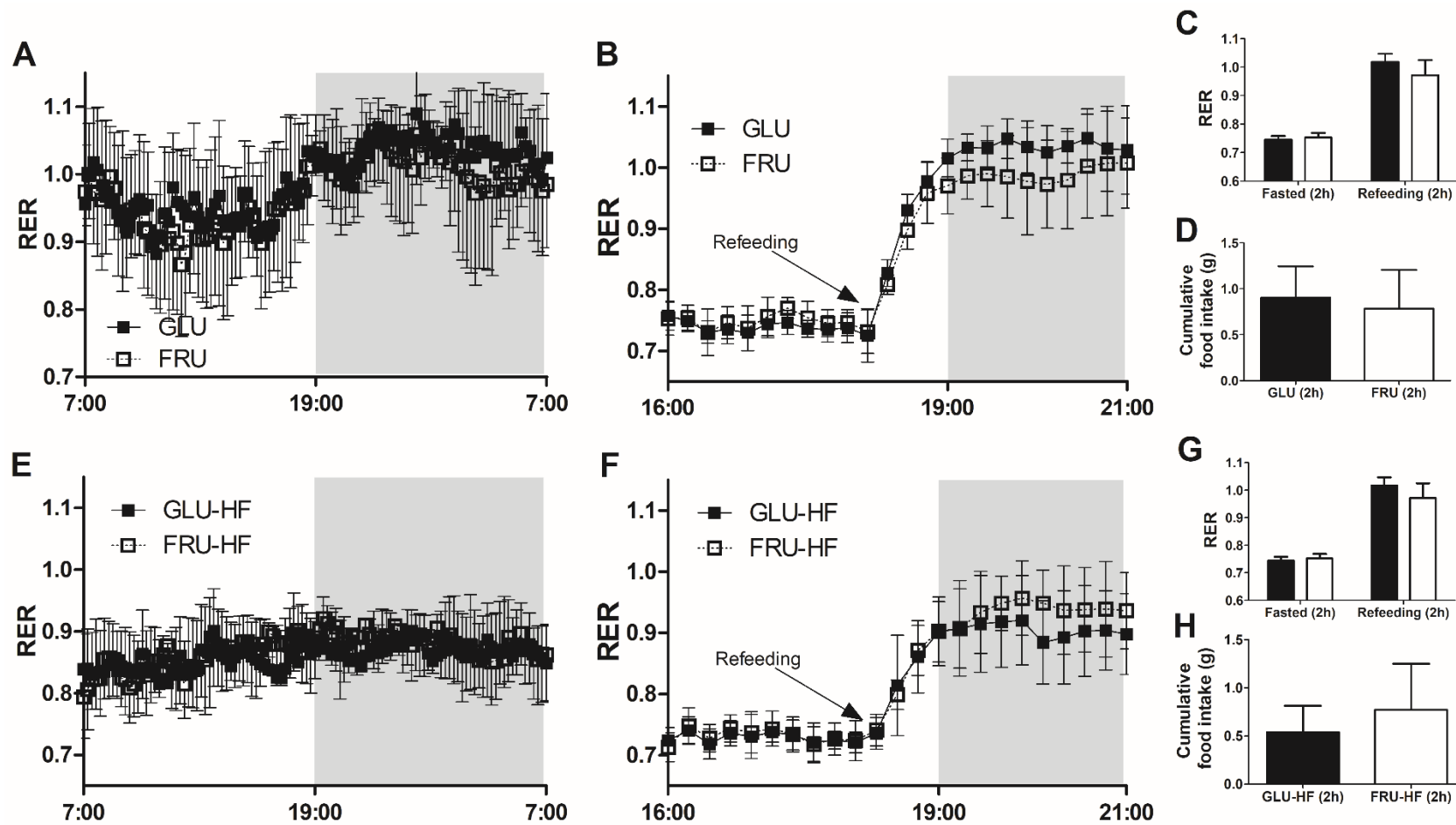
Figure 3. Respiratory exchange ratio (RER) during normal conditions and fasting-refeeding challenge in females during the intervention (week 5) and during the HF diet (week 14). (A) RER for females on glucose or fructose post-weaning diet (week 5) during 24 h basal measurements and (B) during fasting-refeeding challenge. (C) Average RER for fasting-refeeding challenge, values represent the average of 2 h before and 2 h after refeeding for females on GLU or FRU diet (week 5). (D) Cumulative food intake for the first 2 h after the refeeding in the fasting-refeeding challenge for GLU and FRU females (week 5). (E) RER for GLU-HF and FRU-HF females (week 14) during 24 h basal measurement and (F) during fasting-refeeding challenge. (G) Average RER for fasting-refeeding challenge, values represent the average of 2 h before and 2 h after refeeding for GLU-HF and FRU-HF (week 14). (H) Cumulative food intake for the first 2 h after the refeeding in the fasting-refeeding challenge for GLU-HF and FRU-HF females (week 14). Values represent mean ± SD, n = 8-9.
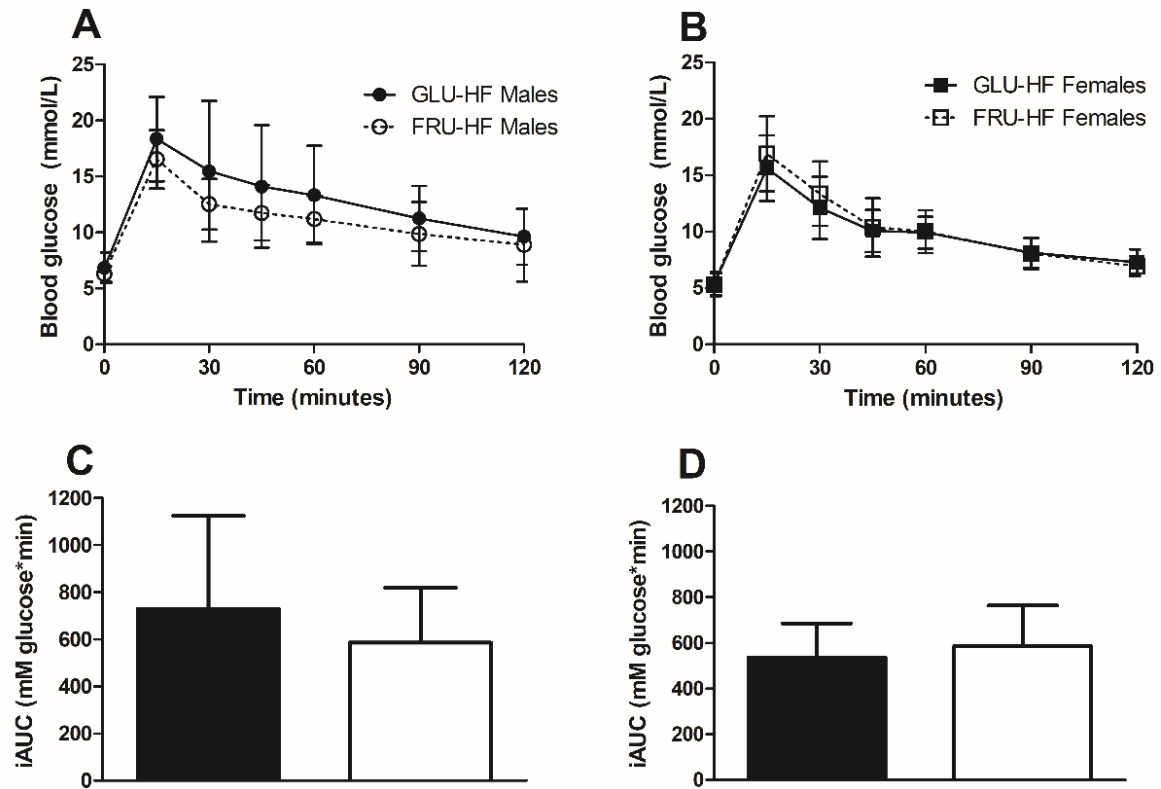
Figure 4. Oral glucose tolerance test (OGTT) in week 11. (A) Blood glucose levels for males and (B) females. (C) Incremental area under the curve (iAUC) data for males and (D) females. Black bars represent GLU-HF groups, white bars FRU-HF groups. Values represent mean ± SD, n = 12 for males, n = 14 for females.

Table 3. Organ weights, serum parameters and hepatic TG levels at section (week 15).

	Organ weights				Serum Parameters		
	Liver weight (g)	Pancreas weight (g)	Mesenteric WAT weight (g)	Gonadal WAT weight (g)	Leptin (ng/ml)	Adiponectin (μg/mL)	Liver TG (μg/mg protein)
GLU-HF Males	1.19 ± 0.35	0.50 ± 0.15	0.63 ± 0.27	0.66 ± 0.29	29.55 ± 16.99	14.23 ± 2.96	341 ± 226
FRU-HF Males	1.10 ± 0.38	0.44 ± 0.12	0.51 ± 0.25	0.59 ± 0.34	20.63 ± 12.70	14.92 ± 3.88	254 ± 136
GLU-HF Females	0.90 ± 0.20	0.35 ± 0.09	0.43 ± 0.26	0.41 ± 0.25	14.46 ± 9.70	17.55 ± 4.13	194 ± 94
FRU-HF Females	0.83 ± 0.17	0.33 ± 0.07	0.30 ± 0.10	0.30 ± 0.13	8.66 ± 4.57	17.74 ± 4.47	178 ± 64

Data represent mean ± SD; n = 11-12 for males, n = 14 for females. No significant differences were observed between dietary groups.

Basal blood glucose levels were similar, and the response to the OGTT in week 11 was not significantly different between GLU-HF and FRU-HF in both males and females (Fig. 4).

At sacrifice, liver, pancreas, mesenteric white adipose tissue (WAT) and gonadal WAT weights were not different between GLU-HF and FRU-HF (Table 3), reflecting whole body FM obser-
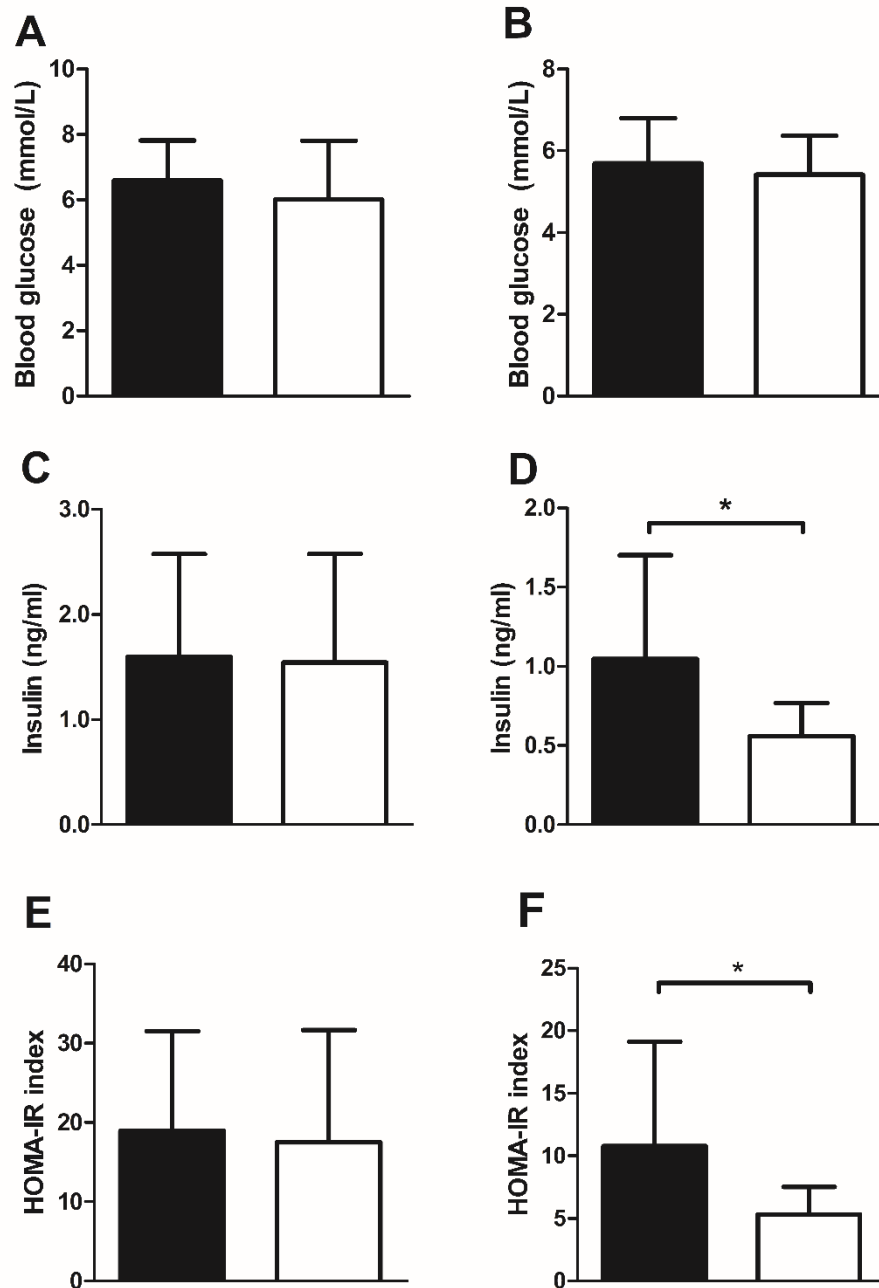
Figure 5. Blood glucose, insulin levels, and HOMA-IR index week 15. (A) Blood glucose levels (2 h fasted) for males and (B) females. (C) Serum insulin levels (ng/mL) males and (D) females. (E) HOMA-IR index for males and (F) females. Black bars represent GLU-HF groups, white bars for the FRU-HF groups. Values represent mean ± SD, n = 12 for males, n = 13-14 for females. *: $P < 0.05$.

vations. There was a trend for higher circulating leptin levels in GLU-HF females compared to FRU-HF females (P = 0.058), while adiponectin levels were not different (Table 3). In males, no difference in leptin or adiponectin levels was found (Table 3). Liver TG content, in week 15 was not significantly altered by post-weaning diet for males or females (Table 3). Insulin levels in week 15 were significantly higher for GLU-HF females than FRU-HF females while blood glucose levels were not significantly affected (Fig. 5B and D), even though these mice received the same HF diet for nine weeks. Consequently, HOMA-IR was higher for GLU-

HF compared to FRU-HF females (Fig. 5F), suggesting higher insulin resistance in GLU-HF females than in FRU-HF females. In males, blood glucose, insulin or HOMA-IR indexes were not significantly different in week 15 (Fig. 5A, C and E).

Pancreata of females were analysed histologically (Supporting Information Fig. S2) to see whether the effect on serum insulin was reflected in β-cell density or mass. The percentage of β-cell area (0.67% ± 0.17% and 0.75% ± 0.23% of total pancreas area) was not different between the GLU-HF and FRU-HF females, nor was β-cell mass (Fig. 6).
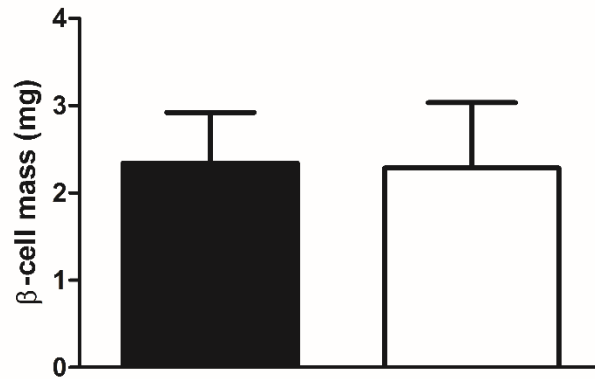

Figure 6. Pancreatic β-cell mass in females in week 15. Black bars represent GLU-HF females, white bars represent FRU-HF females. Values represent mean ± SD, *n* = 6.

Discussion

Overall, the post-weaning 3 week intervention with fructose versus glucose as part of pelletized diets neither significantly affected BW or BC (adiposity) directly, nor did it differently affect BW or adiposity in later life when the animals were switched to a fructose-free HF diet for nine weeks. Remarkably, serum insulin levels at the end of the study appeared to be lower in females fed fructose post-weaning compared with females fed glucose post-weaning.

In this study, two challenge tests were included to assess metabolic health: the fasting-refeeding challenge in InCa, and the classical OGTT. It is nowadays recognized that health is not a static state, but includes adaptation to changing environmental conditions and the flexibility to cope with these: the better the ability to adapt the healthier [30]. The ability to adapt, or phenotypic flexibility, may be especially useful in nutrition research, where effects are usually small [30]. Metabolic flexibility, or "the ability of a system to adjust fuel oxidation to fuel availability" [31], was tested with the fasting-refeeding challenge both during the post-weaning intervention and during the HF period. The fasting-refeeding challenge did not show differences in the males on HF or in the females at both time points, indicating the metabolic flexibility is not affected nor programmed. Unfortunately, male GLU mice had a higher feed intake upon refeeding than FRU male mice, thereby hampering the ability to draw conclusions regarding the metabolic flexibility of these mice, as higher food intake can cause a higher RER.

The OGTT is a classically used challenge test. In this study, the OGTT in week 11 indicated that glucose tolerance was not affected by the type of monosaccharide in the post-weaning diet. However, basal insulin levels in the females in week 15 (thus after nine weeks HF diet) suggest that insulin sensitivity was lower in the GLU-HF females than in the FRU-HF females. Possibly, insulin sensitivity was altered, yet this was not reflected by the OGTT in week 11, likely because impaired insulin sensitivity precedes altered glucose tolerance [32]. Thus, insulin levels may have risen higher in GLU-HF females than in FRU-HF females during the OGTT in week 11, yet were still adequate for normal glucose homeostasis. Further studies are needed to elucidate whether extending the 40 en% HF diet period will ultimately lead to altered glucose tolerance by metabolic programming in early life.

The difference in fasting serum insulin levels between GLU-HF and FRU-HF females could not be explained by a difference in pancreatic β-cell mass (Fig. 6). Our intervention started at the post-weaning state, thus excluding the transition phase from lactation to solid foods during weaning, which was shown to trigger a discrete maturation step of β-cells, elevating the mitogenic and secretory responses to glucose in mice [33]. Nonetheless, it might be that effects of the post-weaning intervention on β-cell mass were overruled by effects of the HF diet given in later life, as a HF diet can also lead to increased β-cell mass and enlarged islets [34]. Alternatively, peripheral insulin resistance in skeletal muscle, liver, or adipose tissue might underlie increased insulin production without altered β-cell mass. Measurements of circulating insulin levels during an OGTT challenge, an euglycemic insulin clamp study, or an insulin inhibition test will be best for future in-depth studies focussed on insulin sensitivity in females. Overall, the evidence is not strong enough to conclude fructose versus glucose post-weaning on later life insulin sensitivity is beneficial, but with confidence we can conclude that fructose compared with glucose does not show adverse effects on later life health for all parameters analysed.

To investigate the metabolic programming of the post-weaning diet on later life health, the fructose and glucose diets were replaced by a high-fat diet after 3 weeks. It was recently shown that continuing on a fructose-rich diet (compared with a glucose rich diet) affects BW gain adversely: young male mice on a fructose diet (18 en%) gained more BW than on a glucose diet (18 en%), although this difference only appeared after 4 weeks [35]. Here, fat mass in females had a tendency to be higher in the FRU group at the end of the intervention, suggesting fat mass could be negatively affected if the diet had been continued. Interestingly however, the trend in fat mass did not persevere when the females were fed high fat diet, and serum leptin levels, a marker for adiposity, in week 15 even showed a reverse trend. In addition, the two-way ANOVA analysis showed a significant interaction between post-weaning diet and time. This may suggest that the females fed the (more adverse) fructose diet are slightly better able to cope with the adverse later life HF diet environment. These results in the females could be in line with the mismatch hypothesis in the developmental origins of

health and disease [36], which postulates that a mismatch between the early life and the mature environment increases the risk of metabolic disease.

A limitation in this study is the quantification of FI during the three week intervention period with glucose and fructose diets, which was far less reliable for the fructose diet due to its crumbliness. The lower hardness and the wet appearance of the fructose intervention diet can be explained by the fact that fructose is more hygroscopic (water attracting) than glucose [37]. That FI was not higher when measured in the 24 h basal measurement in the InCa (Table 1) seemed to suggest that intake of fructose and glucose diets were not different. Also the HF diet was crumbly due to its increased lipid content, but both groups received the same diet likely affecting FI measurements similarly.

Glucose and fructose are considered to have differential effects on brain appetite and reward pathways, as it is thought that fructose stimulates food intake via hypothalamic signalling, while glucose inhibits food intake (reviewed in [38]). The difference in appetite effects is likely hormone-mediated, as insulin, leptin and glucagon-like peptide-1 release are stimulated by glucose, yet not by fructose, while the reduction in ghrelin is more pronounced with glucose intake. Yet, even if the animals on the fructose intervention diet had a higher intake, this did not result in negative effects on the parameters studied here. This suggests that fructose indeed does not program adversely compared with glucose. In fact, insulin levels and insulin resistance as indicated by HOMA-IR index appeared more adverse in GLU-HF females compared with FRU-HF females, even after five weeks HF feeding.

In conclusion, this study showed that, at least when incorporated in solid food, fructose and glucose are comparable given their direct physiological effect. Moreover, no adverse programming effects of dietary fructose in the post-weaning diet in comparison with glucose on body weight, adiposity, glucose tolerance and metabolic flexibility were observed. If anything, for females fructose rather than glucose in post-weaning diet possibly enhanced insulin sensitivity in adulthood. Our results underscore and warrant future nutritional studies that are carefully designed to exclude effects of caloric load and focus on understanding mechanisms of effect of individual monosaccharides.

Author contributions

LB and JFC contributed equally to this work. AO, EvS, JFC, JK, HS and LB designed animal studies; JFC, HS and LB executed the animal studies, JFC and HS executed InCa experiments; EvS, JFC and HS analysed InCa data, IvdS and LB performed laboratory analysis, which was analysed by EvS, IvdS, JK and LB; AO, EvS, IvdS, JFC, JK, HS and LB wrote or critically revised the manuscript.

Acknowledgements

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Supporting Information

Table S1. Composition of the intervention diets.

| | Breeding diet | Post-weaning diets | | High fat (HF) diet |
		Glucose (GLU)	Fructose (FRU)	
Casein	200.0	200.0	200.0	235.0
L-cysteine	3.0	3.0	3.0	3.0
Wheat starch	380.0	153.1	153.1	285.0
Maltodextrin	100.0	153.1	153.1	100.0
Maltose	100.0	0.0	0.0	0.0
Glucose	50.0	323.7	0.0	70.0
Fructose	0.0	0.0	323.7	0.0
Coconut oil	12.6	12.6	12.6	37.8
Sunflower oil	49.0	49.0	49.0	147.0
Flaxseed oil	8.4	8.4	8.4	25.2
Cholesterol	0.03	0.03	0.03	0.097
Cellulose	50.0	50.0	50.0	50.0
Mineral mix	35.0	35.0	35.0	35.0
Vitamin mix	10.0	10.0	10.0	10.0
Choline bitartrate	2.5	2.5	2.5	2.5
Total energy (kcal/kg)	4032	4032	4032	4732
Protein en%	20	20	20	20
CHO en%	64	64	64	40
Fat en%	16	16	16	40

Ingredients are given in gram / kilogram of diet, unless otherwise stated.

Figure S2. Representative overview pictures of pancreatic DAB-insulin staining.
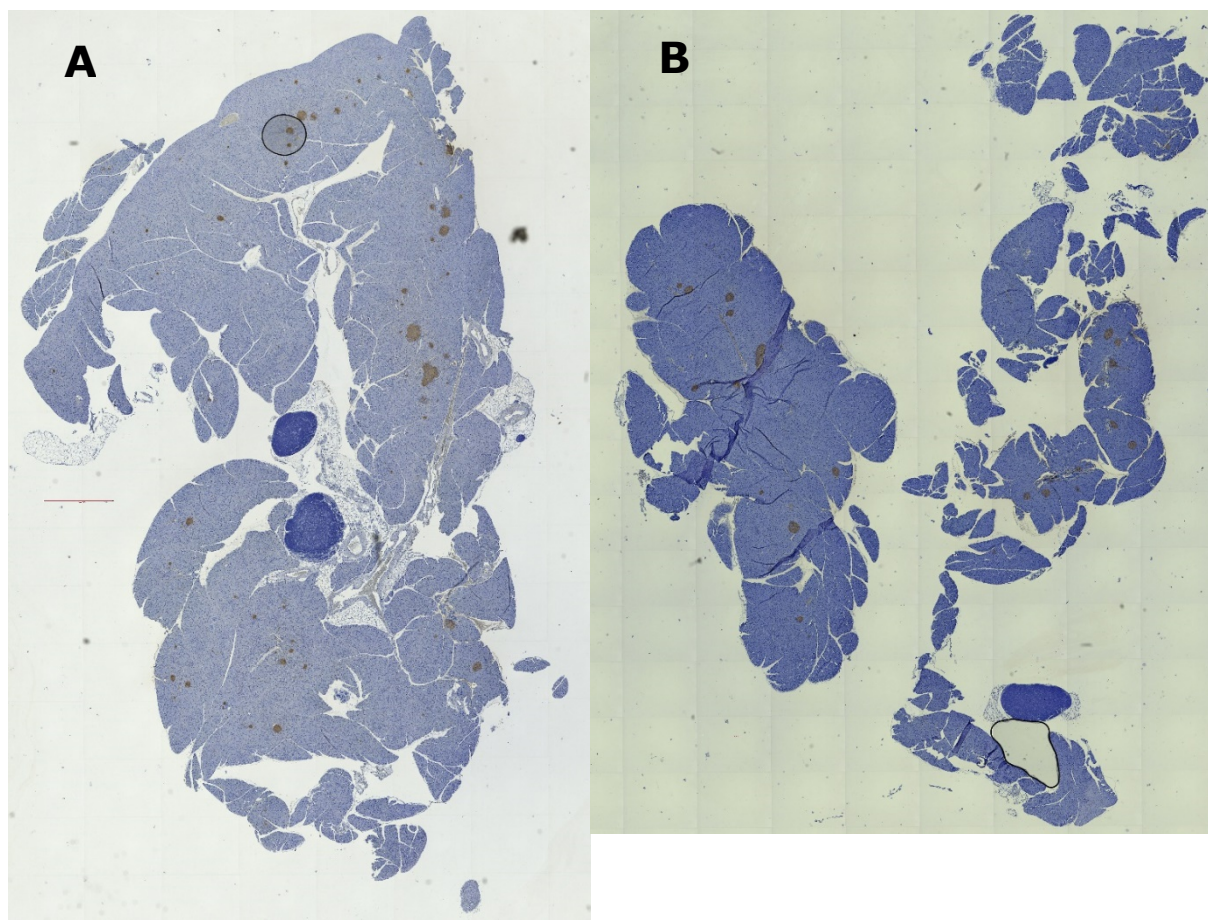
 Figure S2. Representative overview picture of DAB staining for insulin counterstained with hematoxylin in pancreas of (A) a GLU-HFD female and (B) a FRU-HFD female, used to estimate beta cell islet area. Brown staining indicates DAB colouring. Photographs were taken at the same magnification, and merged into overview pictures with LasX pc software (Leica Microsystems, Wetzlar, Germany).

Chapter 3

Metabolic effects of the dietary monosaccharides fructose, fructose-glucose or glucose in mice fed a starch-containing moderate high-fat diet

Lianne M. S. Bouwman,

Arie G. Nieuwenhuizen,

Hans J.M. Swarts,

Rosaria Piga,

Evert M. van Schothorst,

Jaap Keijer.

Abstract

Fructose consumption has been linked to obesity and to increased hepatic *de novo* lipogenesis (DNL). Excessive caloric intake often confounds the results of fructose studies, and experimental diets are generally low-fat diets, not representative for westernized diets. Here, we compared the effects of dietary fructose with those of dietary glucose, in adult male and female mice on a starch-containing moderate high-fat (HF) diet. After five weeks fattening on a HF high-glucose (HF-G) diet, mice were stratified per sex and assigned to one of the three intervention diets for six weeks: HF high-fructose (HF-F), HF with equimolar glucose and fructose (HF-GF), or HF-G. Body weight (BW) and food intake were measured weekly. Indirect calorimetry was performed in week five; animals were sacrificed in food-deprived state in week six. Data were analysed within sex. BW gain was similar among animals on the HF-G, HF-GF, and HF-F diets. Cumulative food intake was slightly lower in HF-F animals (both sexes). However, energy expenditure was not affected, nor were circulating insulin and glucose concentrations, and hepatic triglyceride levels at endpoint. Hepatic gene expression analysis showed only minor alterations in hexokinase expression in males, and no alterations in sugar transporters, glycolysis, or DNL related enzymes. In females, no consistent alterations in hepatic or small intestine gene expression were seen. Concluding, partial or complete replacement of dietary glucose with fructose does not increase caloric intake, and does not affect BW, hepatic triglyceride levels, or insulin concentrations in male and female mice on a moderate high-fat diet.

Keywords

Indirect Calorimetry, isocaloric, liver metabolism, energy intake, adiposity, small intestine

Introduction

The worldwide increase in prevalence of obesity is a major health concern [1]. The cause of this increase is considered to be multifactorial, with dietary habits and a sedentary life style as two main contributors. In addition, it has been hypothesized in the early 2000s that the increase in obesity and its related health problems are caused by a rise in fructose consumption, in particular by fructose coming from beverages [2]. A decade later, Bray *et al.* (still) state that the consumption of sugar sweetened beverages is contributing to the high incidence of obesity, and that fructose "has critical adverse effects" [3]. Others also suggested that fructose exerts detrimental health effects, much worse than caloric equivalents, and state that its effects resemble those of alcohol [4]. However, this theory is controversial, as several research groups suggest that it is not the increased sugar or fructose consumption per se that is contributing to the pandemic of obesity, but the overall high caloric intake [5-7].

Glucose and fructose are both monosaccharides and they have the same molecular formula ($C_6H_{12}O_6$), yet glucose has an aldehyde group at the carbon 1 atom, while fructose has a keto-group on its carbon 2 atom [8]. This structural difference causes the monosaccharides to be absorbed and metabolized differently. Intestinal uptake of glucose is mainly active and occurs with sodium cotransport by the sodium-dependent glucose transporter 1 (SLC5A1, also known as SGLT1), while intestinal uptake of fructose occurs passively via the SLC2A5 (also known as GLUT5) transporter [9]. Both glucose and fructose exit the intestinal cells via the SLC2A2 (also known as GLUT2) transporter by passive transport, reach the liver via the portal vein, and enter the hepatocytes via the SLC2A2 transporter [10]. Importantly, glucose is taken up into multiple tissues under the influence of insulin, while fructose is mainly taken up by the liver in an insulin independent manner [11]. Once inside the hepatocyte, glycolysis takes place, converting glucose into pyruvate. In detail, glucose is metabolised by hexokinase (HK) or glucokinase (GK) to glucose-6-phosphate, isomerized into fructose-6-phosphate and subsequently phosphorylated into fructose 1,6-biphosphate by phosphofructokinase (PFK). Fructose 1,6-biphosphate is then cleaved by aldolase B (ALDOB) into dihydroxyacetone phosphate and glyceraldehyde-3-phosphate [12]. Triose phosphate isomerase (TPI) converts dihydroxyacetone-phosphate into glyceraldehyde-3-phosphate, which in turn is converted by glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and after several other enzymatic steps, pyruvate is formed [13]. Fructose, however, is metabolised by ketohexokinase (KHK) into fructose-1-phosphate. Fructose-1-phosphate is also broken down by ALDOB. The products formed are dihydroxyacetone phosphate, and glyceraldehyde [12]. Both are subsequently converted to glyceraldehyde-3-phosphate, by TPI and Triokinase and FMN cyclase (TKFC) and a small fraction of glyceraldehyde is converted to glycerol [13].

Activity of PFK depends on the energy level of the cell. When the latter is high, ATP and citrate levels are high, inhibiting PFK activity [12]. KHK activity, however, is not regulated the by energy status of the cell [12]. Therefore, it is believed that in fed conditions fructose is directed towards *de novo* lipogenesis (DNL), while glucose is directed to glycogenesis [14]. This is used as explanation for hepatic lipid accumulation in studies with fructose supplementation in the drinking water. Others however, showed that fructose also contributes to glycogenesis [15]. Scientific evidence underlying the potential adverse health effects of fructose is often derived from animal studies, yet the results of these studies are often difficult to translate to the human situation. Fructose in animal studies is generally given in very high doses (up to 60 energy percent (en%) from fructose) and the animals are on a low-fat dietary background. In contrast, the contribution of fructose to total energy in the human diet is about 9% (median intake in The Netherlands [16] and the US [17]), and 17.8 en% for the 95th percentile fructose consumption [17]. Moreover, the low-fat dietary background in most animal studies is also not representative for the human situation; for example, the median intake from fats in adults is approximately 34 en% in The Netherlands [18].

There is accumulating evidence that sex differences exist in the response to fructose. Several human intervention studies have shown that the effects of fructose are attenuated in females. Acute fructose consumption leads to higher uric acid and lactate responses in males than in females [19] and enhanced hepatic DNL stimulation [20]. In addition, the effects of fructose seem blunted in females with six days overfeeding [21] and after a six-week fructose drink intervention [22]. Several studies in animal models focus mainly on the effects of long-term fructose intake on liver health. Female mice were are more susceptible to liver damage than males when their drinking water is supplemented with 30% fructose for 16 weeks, even though males have higher weight gain [23]. Liver weight and visceral adiposity increase more in female rats compared with their male counterparts with a fructose intervention of nine weeks, yet in males insulin sensitivity and blood pressure are affected [24]. Underlying alterations in hepatic metabolism by two weeks fructose supplementation are more severe in females, and mediated by hepatocyte nuclear factor 4 (HNF4), while in males the reduction of peroxisome proliferator-activated receptor α (PPARα) causes the alterations in liver [25]. However, in all these studies fructose was provided in the drinking water, causing an increased liquid intake and a decreased food intake, which cumulatively resulted in an alteration in the ratio of carbohydrate intake to fat intake, as well as in a net increase in caloric intake in the fructose groups. Therefore, the effects cannot simply be regarded as the effects of fructose as such, as they may result from a higher caloric intake.

The aim of this study was, therefore, to elucidate the effects of dietary fructose, glucose, and glucose-fructose in a 1:1 ratio (all in isocaloric amounts) on body weight development and hepatic gene expression, in mice on a moderate high-fat diet. Fructose and glucose were administered as

part of the pelletized diet, to circumvent the confounding effects of overconsumption of fructose in the drinking water. The diet had a moderate high-fat content and contained starch, to provide a relevant dietary context to the monosaccharides. The diet with glucose and fructose in a 1:1 ratio, representing the common dietary sugar sucrose, was used, because of dietary relevance, and because an interaction of glucose and fructose on gene expression in the liver has been reported [26]. Moreover, the diets were administered to both males and females to study whether the effects of this intervention is affected by sex.

Materials & Methods

The experiment was performed according to the Dutch Animal Experimentation Act (1996). The experimental protocol was approved by the Animal Welfare Committee of Wageningen University, Wageningen, The Netherlands (DEC 2010115.d).

Animals & experimental procedure

Thirty male and thirty female C57BL/6JOlaHsd mice of nine weeks of age were purchased from Harlan Laboratories (Horst, The Netherlands). Mice were individually housed in Makrolon II cages with standard bedding of wood chips and shavings, with *ad libitum* access to food and water and were maintained under controlled environmental conditions (temperature 21±1°C; relative humidity 52.5 ± 2.5%; 12h-12h light-dark cycle, lights on at 5.00h). Animals were fed a semi-synthetic moderate high-fat (35.9 en% fat) high-glucose diet (HF-G; Table 1) obtained from Research Diet Services B.V. (Wijk bij Duurstede, The Netherlands). This diet is an adaptation of the published BIOCLAIMS high-fat diet [27], which is known for the induction of insulin resistance [28]. After five weeks of fattening, the animals were stratified by body weight and assigned to one of three dietary groups (*n* = 10 per group, per sex): a moderate high-fat high-glucose (HF-G) diet, a moderate high-fat glucose and fructose (HF-GF) diet, or a moderate high-fat high-fructose (HF-F) diet; these diets only differed in their monosaccharides composition. The detailed dietary compositions can be found in Table 1. Given the monosaccharide content, the diets contained: 28.6 en% glucose and 0 en% fructose (HF-G), 14.3 en% glucose and 14.3 en% fructose (HF-GF), and 0 en% glucose and 28.6 en% fructose (HF-F). All diets contained starch (15.3 en%). The calculation of the required group size was based on past experience with the detection of statistically significant diet-induced alterations in molecular markers. The dietary intervention lasted for a total of six weeks, and body weight and food intake were determined weekly in the morning.

Indirect calorimetry

In week five of the intervention, animals were placed into a PhenoMaster Indirect Calorimetry System (TSE Systems, Bad Homburg, Germany). Rates of oxygen consumption and carbon dioxide

production were measured for each animal once every twelve minutes. O_2 consumption, CO_2 production and activity were measured as previously described [29]. Energy expenditure was calculated every 12 minutes (previously described [29]) and expressed as kcal/h. The average of all the energy expenditure values was taken and multiplied by 24 to obtain the energy expenditure in kcal/day.

Table 1. Composition of the intervention diets. Ingredients are given in gram / kilogram of diet. All diets are moderate high-fat and isocaloric.

	Moderate high-fat - glucose (HF-G)	Moderate high-fat - glucose-fructose (HF-GF)	Moderate high-fat- fructose (HF-F)
Casein	225	225	225
Wheat starch	172	172	172
Dextrose	322.5	161.25	0
Fructose	0	161.25	322.5
Lipids*	180	180	180
Cholesterol	0.097	0.097	0.097
Cellulose	50	50	50
Mineral mixture	35	35	35
Vitamin mixture	10	10	10
Choline bitartrate	2.5	2.5	2.5
L-Cysteine	3	3	3
Total	1000	1000	1000
Total energy (kcal)	4510	4510	4510
Protein en%	20.2	20.2	20.2
CHO en%	43.9	43.9	43.9
Fat en%	35.9	35.9	35.9

*The lipid fraction contained 18% cocos oil, 70% sunflower oil, and 12% flaxseed oil (wt/wt) to ensure a health fatty acid profile, as in the BIOCLAIMS diet [27].

Sacrifice

At the end of the sixth week of the intervention, animals were food-deprived for at least two hours during the light phase. A tail incision was made and blood glucose concentrations were measured with a Freestyle blood glucose system (Abbott Diabetes Care, Hoofddorp, The Netherlands); no anaesthesia was used, to prevent effects on blood glucose concentrations [30]. Blood was collected in Microvette CB 300 EDTA tubes (Sarstedt, Nürnberg, Germany) to obtain plasma samples by centrifugation at 2000 *g*. Immediately afterwards, the animals were anesthetized with a mixture of 5% isoflurane in 1:1 gas mixture of nitrous oxide and oxygen for

one minute, and decapitated. Liver and gonadal fat pads (epididymal fat pad in males, fat pads around uterus in females) were collected, weighed, snap frozen in liquid nitrogen and stored at -80° C. The small intestine was collected, cut open longitudinally, rinsed, and scraped. Cell scrapings were snap frozen in liquid nitrogen, and stored at -80° C.

Plasma measurements

Plasma insulin concentrations were analysed in plasma using a Mouse insulin (TMB) ELISA kit (Biovendor GmbH, Kassel, Germany) according to the manufacturer's instructions. Plasma triglyceride (TG) concentrations were measured using a Triglyceride liquicolor Kit (Human, Wiesbaden, Germany). HOMA-IR was calculated from insulin concentrations and blood glucose levels, using a C57BL/6J mice-adjusted factor of 14.1, as previously described [31].

Liver measurements

Liver tissue was defrosted and dissolved in Tris-EDTA buffer (20mg/ml). The TG level was determined using the Instruchemie Triglycerides Liquicolor kit (Human GmbH) according to the manufacturer's instructions. Input was corrected for wet tissue weight. Liver glycogen levels were determined according to a method previously described [32]. Briefly, liver tissue (caudate lobe) was grinded and dissolved in chloric acid solution (7%). Samples were centrifuged at 830 g for 15 minutes (4° C) and the supernatant was stored at -80 °C for 1.5 hours. Petroleum ether was added to the frozen sample and after shaking, the aqueous layer was collected and stored on ice. A total amount of 260 μL reagent solution (0.90 M calcium chloride; 1.31 mM iodine; 11.4 mM potassium iodine) was added to 10 μL sample. After 10 minutes incubation, the absorbance was measured at 460 nm.

Gene expression analysis

Liver or intestinal tissue was grinded and homogenized in liquid nitrogen. Subsequently, RNA was isolated using a RNeasy Mini Kit (Qiagen, Venlo, The Netherlands) according to the manufacturers' instructions. RNA yield and purity were evaluated using a Nanodrop Spectrophotometer (Isogen Life Science, Maarssen, The Netherlands) and RNA integrity was measured on the Experion automated electrophoresis system (Bio-Rad, Veenendaal, The Netherlands). cDNA was synthesized using the iScript cDNA synthesis kit (Bio-Rad). Reverse-transcription quantitative real-time polymerase chain reaction (RT-qPCR) experiments were performed using iQ SYBR Green Supermix (Bio-Rad). As regard the standard curves, serial dilutions of pooled cDNA from all samples were made. Samples were tested in duplicate; male and female samples were tested in separate runs. An overview of the target genes, primer sequences and annealing temperatures can be found in Table 2. The expression of target genes was normalized against the geometrical mean of the reference gene(s), and normalized against the mean of the glucose group set at 1.0. For intestinal gene expression, Ribosomal protein S15 (*Rps15*) and

Calnexin (*Canx*) were used as reference genes. As regard the hepatic gene expression, *Rps15* and Hypoxanthine phosphoribosyltransferase 1 (*Hprt1*) were used as reference genes for the expression of *Slc2a2, Slc2a5*, *Hk1*, *Hk2*, *Hk4*, *Khk*, *Aldob*, *Tkfc*, *Gapdh* and *Tpi1*; *Canx* was used as reference gene for DNL genes acetyl-CoA carboxylase beta (*Acacb*), fatty acid synthase (*Fasn*), elongation of long chain fatty acids family member 6 (*Elovl6*) and stearoyl-coenzyme A desaturase 1 (*Scd1*) as well as microsomal triglyceride transfer protein (*Mttp1*), a protein involved in hepatic lipid export.

Table 2. Target genes, primer sequences and annealing temperatures in RT-qPCR.

Gene	Forward Primer 5'-'3'	Reverse Primer 5'-3'	Annealing Temperature
Acacb	TCTTCACGTTCAGAGCGAGGGAT	ATCTTGTGGTTGGCACAGGGCA	55.6°C
Aldob	TACCAGATACAACCCCAATAGTCAGG	TCAACTCATTCTCTACACTCCATAGC	61.5°C
*Canx**	GCAGCGACCTATGATTGACAACC	GCTCCAAACCAATAGCACTGAAAGG	60.0°C
Elovl6	GGCACTAAGACCGCAAGGCA	GCTACGTGTTCTCTGCGCCT	60.0°C
Fasn	AGTTAGAGCAGGACAAGCCCAAG	GTGCAGAGCTGTGCTCCTGA	60.0°C.
Gapdh	AAGGCTGTGGGCAAGGTCATC	CGAAGGTGGAAGAGTGGGAGTTG	61.5°C
Hk1	ACGCTCGGTGCCATCTTGAAC	CCTTGCCACTGCCACTCTCC	61.5°C
Hk2	ATCAAAGAGAACAAGGGCGAGGAG	GCGGAGGAAGCGGACATCAC	61.5°C
Hk4	CCTGGGCTTCACCTTCTCCTTC	CCTCACATTGGCGGTCTTCATAG	61.5°C
*Hprt1**	TGACACTGGTAAAACAATGCAAACTTTG	GAGGTCCTTTTCACCAGCAAGCT	61.5°C
Khk	GCAGCGGATAGAGGAGCACAATG	CCAGGCACAGACAAGCGTAGC	61.5°C
Mttp1	GTGGAGGAATCCTGATGGTGA	TGATCTTAGGTGTACTTTTGCCC	61°C
*Rps15**	CGGAGATGGTGGGTAGCATGG	ACGGGTTTGTAGGTGATGGAGAAC	60°C
Scd1	TCATGGTCCTGCTGCACTTGG	CTGTGGCTCCAGAGGCGATG	60°C
Slc2a2	CACACCAGCATACACAACACCAG	GGACACAGACAGAGACCAGAGC	61.5°C
Slc2a5	TCCTCCTCCTCCCGTTCTTTCC	CTTCTCAGCCTCATCCTCCTTCC	61.5°C
Slc2a7	TCGGTGGTGAGGACAGAGATTG	AGCAGCAGTGAGGATACAGACG	61.5°C
Slc5a1	TCGTCATCTACTTCGTGGTGGTG	CCTGCGGCTGCTCCTGTG	61.5°C
Tkfc	GGGCAGCAGCACAGGAGTTC	AGGATGGCACGGAAGATGGC	61.5°C
Tpi1	GGACTGGCAAGACGGCAAC	GCAGGCAGGTAGAGGGATGG	61.5°C

* Transcripts used for normalization.

Statistical analysis

Statistical analysis was performed using GraphPad Prism version 5.04 (Graphpad Software, San Diego, USA). Comparisons were made between the different diets within each sex. Data were checked for normality by a D'Agostino & Pearson omnibus normality test. If data were not normally distributed, they were log converted and rechecked for normality. A one-way ANOVA

with post-hoc Tukey comparison was used for normally distributed datasets. In case of not-normally distributed data, a Kruskal-Wallis test was performed, followed by a Dunn's Multiple comparison test. Body weight and RER curves were analysed by 2-way ANOVA. *P*-values < 0.05 were considered statistically significant.

Results

Body weight was not significantly different among animals on a moderate high-fat diet rich in glucose (HF-G), fructose (HF-F), or glucose-fructose in a 1:1 ratio (HF-GF), neither for males (Fig. 1A) nor for females (Fig. 1B). Only in male mice, the interaction time x diet was significant: HF-GF males gained more body weight than HF-G males or HF-F males. Cumulative caloric intake was significantly higher in HF-G animals compared with HF-F animals in both sexes (Table 3). As diets were isocaloric, differences in caloric intake were due to a difference in food intake. Animals on the HF-GF diet had an intermediate food intake.

Indirect calorimetry measurements in week five showed that mean RER values were higher in the dark phase than in the light phase in both males (Fig. 2A, Table 3) and females (Fig. 2B, Table3), indicating relatively more carbohydrate metabolism during the dark phase and more lipid metabolism in the light phase. However, no effect of diet on RER was found in either sex (Fig. 2, Table 3). Twenty-four hour energy expenditure was not affected by the diet, in males as well as females (Table 3).
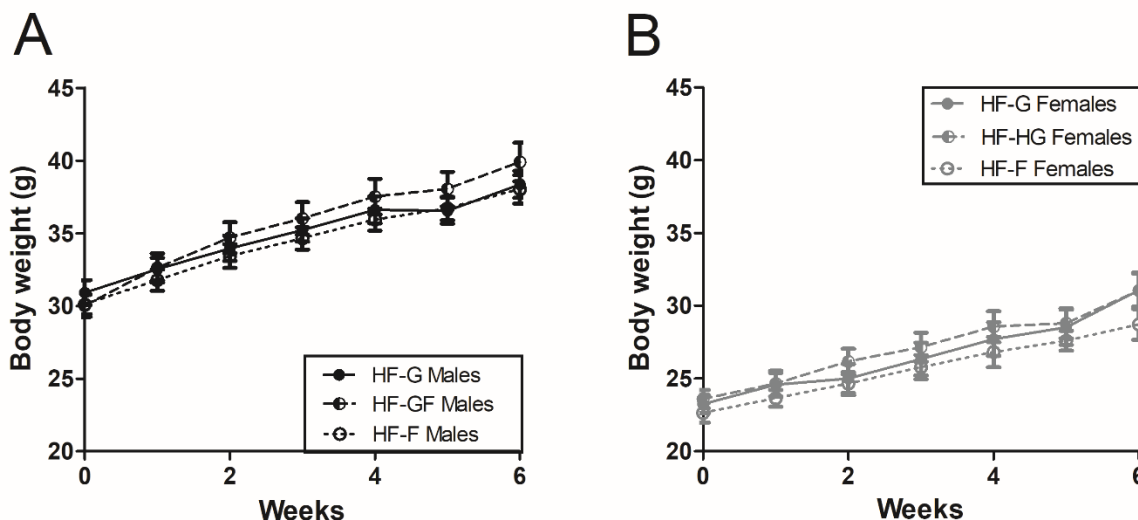

Figure 1. Body weight development. Body weight development during the intervention period for males (A) and females (B) when giving a 35.9 en% high fat diet containing 28.6 en% of glucose (HF-G, solid dots and lines), 14.3 en%-14.3 en% glucose and fructose (HF-GF, half-open dots and striped lines), or 28.6en% fructose (HF-F, open dots and dotted lines). Values are expressed as mean ± SEM; *n* = 10.
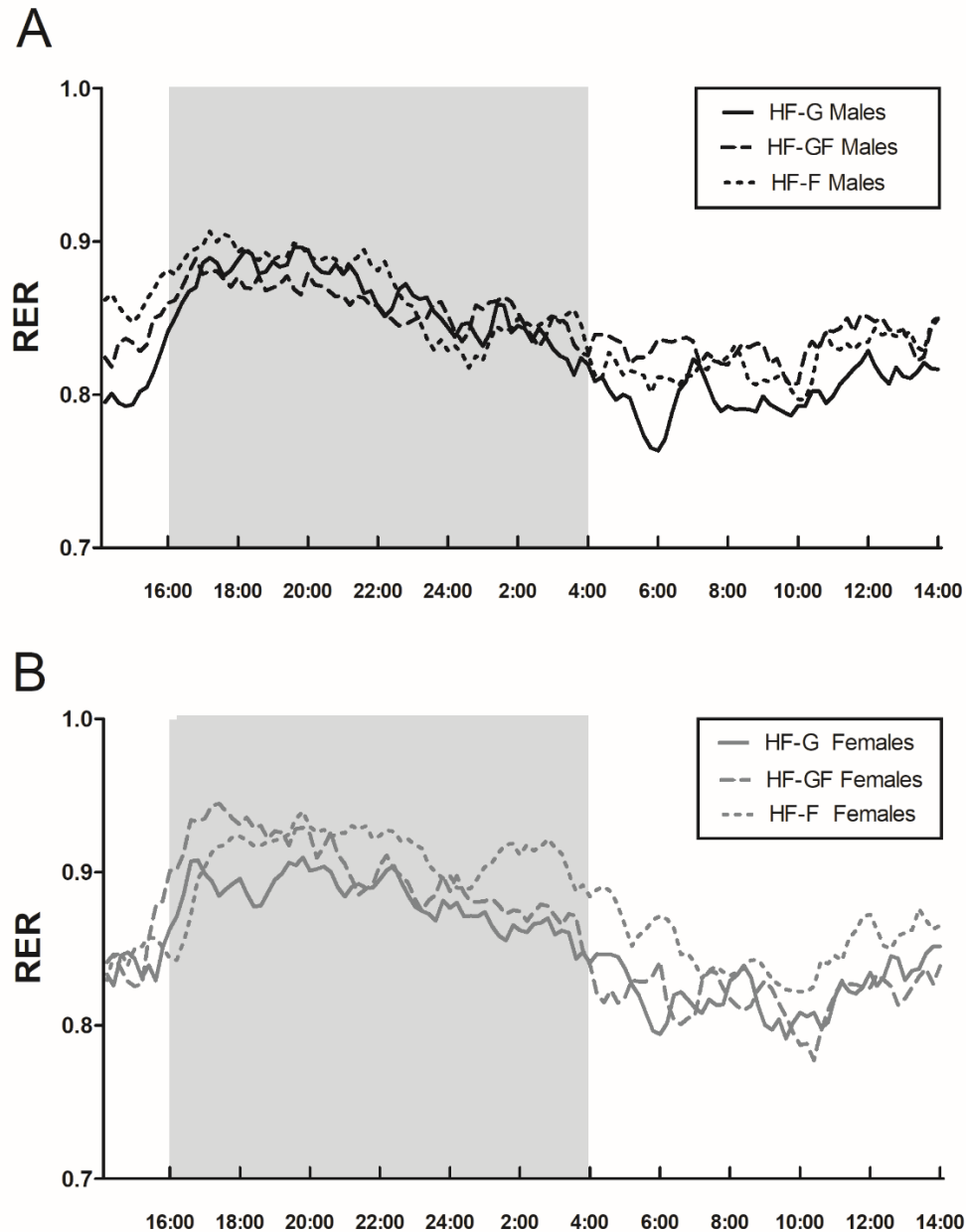
Figure 2. Mean Respiratory Exchange Ratio (RER) over a 24 hour period. RER was measured by indirect calorimetry, in male (A) and in female (B) mice. The grey shaded area represents the dark phase (when animals are active), the white shaded areas represent the light phase (when animals are inactive). Solid lines represent HF-G, striped lines represent HF-GF, and dotted lines represent HF-F. Lines show mean of the group (n = 10).

Gonadal fat pad weight was similar among HF-G, HF-GF and HF-F males, suggesting no difference in adiposity at the end of the intervention (Table 3). Gonadal fat pad weight was also similar among HF-G, HF-GF and HF-F females. Liver weight was not affected by diet in either sex. In males, plasma insulin concentrations and blood glucose concentrations did not differ significantly among

groups (Fig. 3A and 3B). As a consequence, HOMA-IR was not significantly different (Fig. 3C). For the females, plasma insulin concentrations were not different among the diets (Fig. 3D), and although a trend was observed for the HF-F females to have a lower blood glucose concentrations than the HF-G females (Fig. 3E), this effect was not significant ($P = 0.054$). HOMA-IR index was not significantly affected by the diet in females (Fig. 3F). Overall, this suggests no effect on glucose homeostasis by the diet in both sexes.

Table 3. Food intake, energy expenditure, RER, and organ weights.

		Cumulative feed intake (g)	Energy expenditure (kcal/day)	Average RER (dark phase)	Average RER (light phase)	Gonadal fat pad weight (g)	Liver weight (g)
Males	HF-G	149 ± 10[a]	12.55 ± 0.53	0.86 ± 0.02	0.80 ± 0.03	1.95 ±0.29	1.43 ± 0.15
	HF-GF	146 ± 8[a,b]	13.10 ± 0.61	0.86 ± 0.02	0.83 ± 0.03	2.12 ± 0.33	1.52 ± 0.21
	HF-F	140 ± 8[b]	12.82 ± 0.41	0.87 ± 0.02	0.82 ± 0.03	2.07 ± 0.40	1.50 ± 0.12
Females	HF-G	156 ± 10[x]	11.93 ± 0.66	0.88 ± 0.06	0.82 ± 0.06	1.35 ± 0.46	1.10 ± 0.15
	HF-GF	150 ± 13[x,y]	11.93 ± 0.52	0.90 ± 0.04	0.82 ± 0.04	1.33 ± 0.50	1.13 ± 0.12
	HF-F	138 ± 19[y]	11.69 ± 0.59	0.91 ± 0.04	0.85 ± 0.05	1.13 ± 0.34	1.14 ± 0.16

The cumulative caloric intake is calculated over the six weeks experimental period. The mean energy expenditure is calculated over a 24- hour period in the indirect calorimetry system. The mean respiratory exchange ratio (RER) is calculated over a 10-hour (light phase) or 12-hour (dark phase) period. Gonadal fat pad weight and liver weight were measured at sacrifice. Different superscript letters indicate significant differences ($P < 0.05$), data are analysed within sex. Values are expressed as mean ± SD, $n = 10$.

Plasma TG concentrations were significantly higher in HF-F males compared with HF-G males, with intermediate TG concentrations in HF-GF male mice (Fig. 4A). HF-GF females had significantly higher plasma TG concentrations than HF-G and HF-F females (Fig. 4C). In contrast to plasma TG concentrations, total hepatic TG content was not affected by diet (Fig. 4B for males, 4D for females). Hepatic glycogen levels did not differ among the diets in both sexes (data not shown).

The mRNA expression of the fructose-related enzyme ketohexokinase (*Khk*) was increased in both fructose fed male groups (HF-GF and HF-F) (Fig. 5A). Hexokinase 4 (*Hk4*) was the only hexokinase for which the expression was significantly increased in the HF-F males compared with HF-G males, while the expression of other hexokinases was not affected. The fructose content of the diet did not alter the hepatic expression of monosaccharide transporters (*Slc2a2* and *Slc2a5*), glycolysis related enzymes (*AldoB, Gapdh, Tkfc*, and *Tpi*) and DNL-related enzymes (*Acacb, Elovl6, Fasn* and *Scd1*) and *Mttp1* in males.
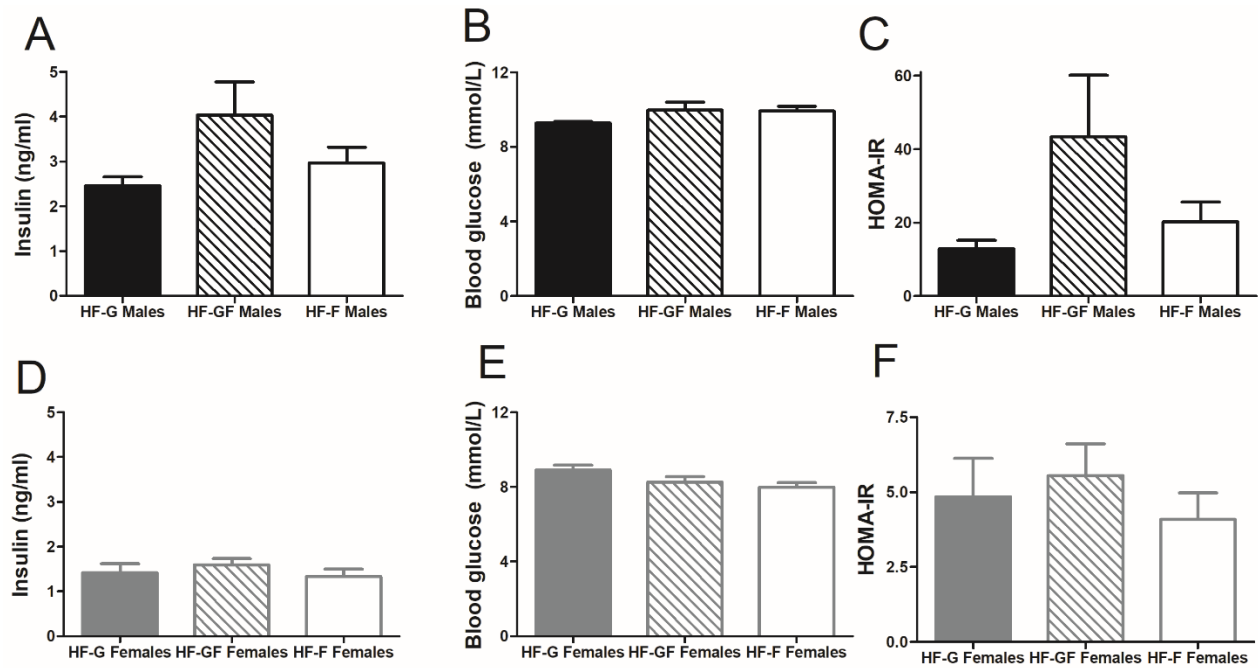
Figure 3. Plasma insulin, blood glucose and HOMA -IR. Plasma insulin concentrations in male (A) and in female (D) mice, blood glucose concentrations in males (B) and in females (E) and HOMA-IR in males (C) and in females (F). Solid bars represent HF-G diet, striped bars represent HF-GF, and white bars represent HF-F. Values are expressed as mean ± SEM, n = 10 or n = 8 (HF-G males insulin and HF-G males HOMA-IR).
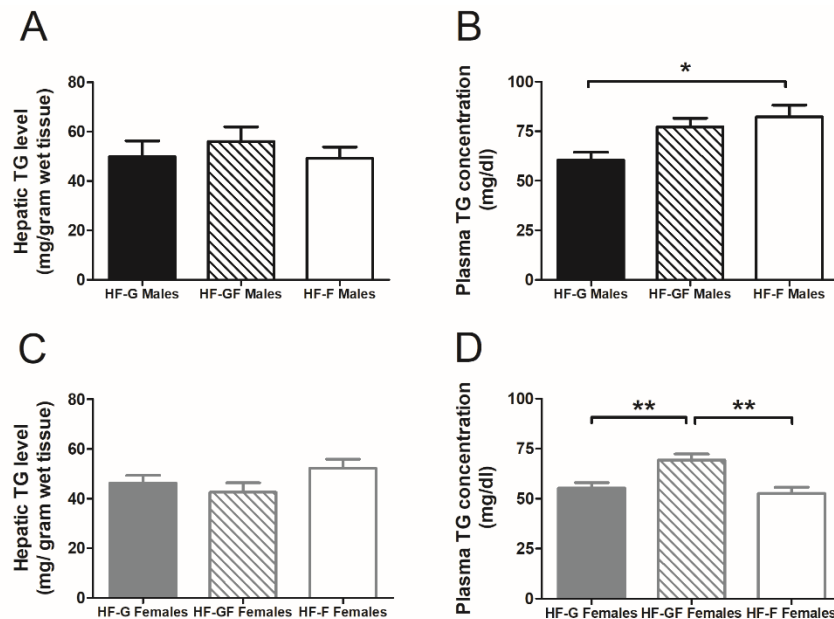

Figure 4. Plasma TG concentrations and hepatic TG levels. Plasma TG concentrations in male (A) and female (C) mice. Liver TG levels in males (B) and females (D). Solid bars represent HF-G diet, striped bars represent HF-GF, and white bars represent HF-F. Values are expressed as mean ± SEM, n = 8 for the plasma measurement in the HF-G male group, n = 10 for all other groups. *: $P < 0.05$; **: $P < 0.01$.
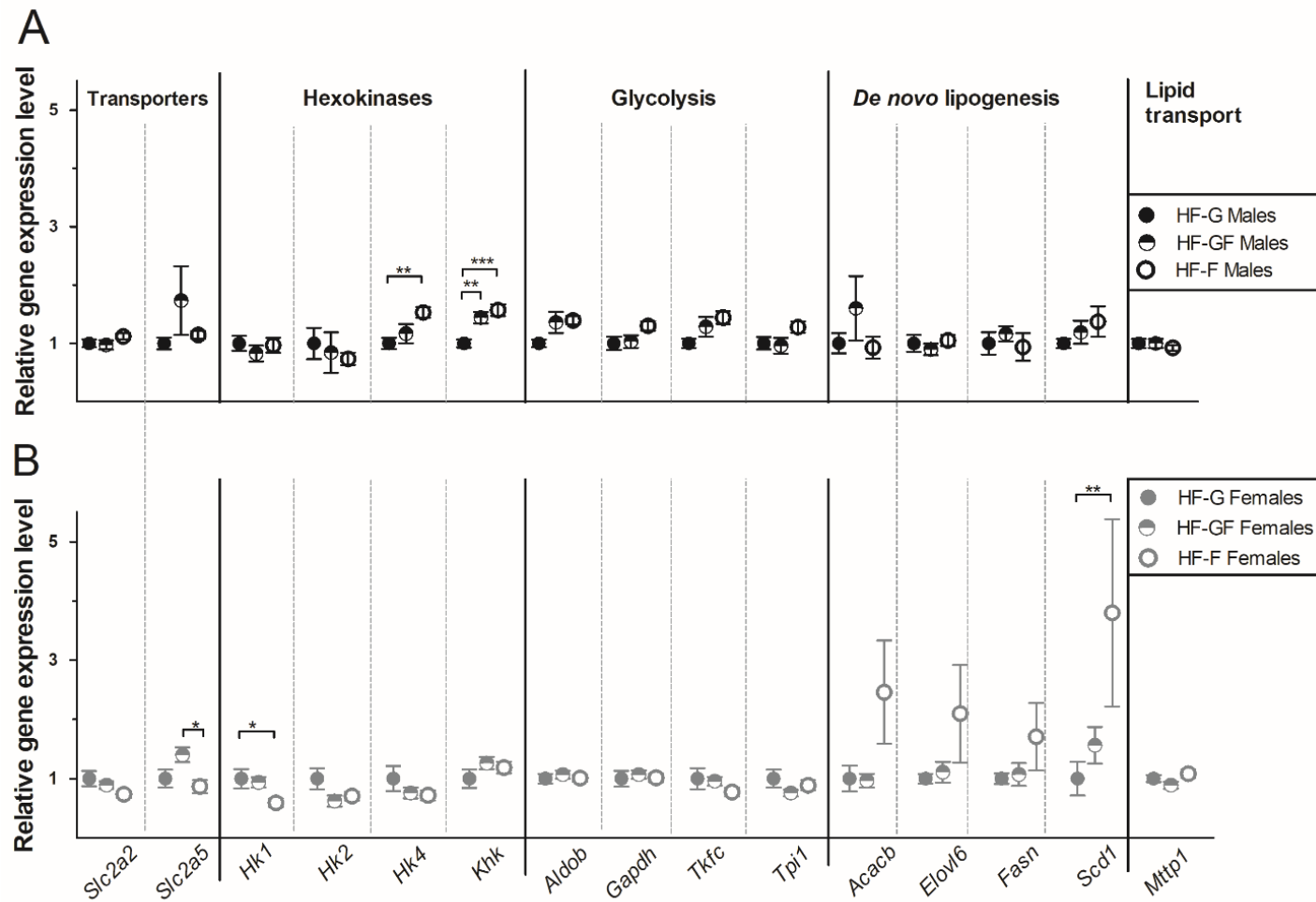
Figure 5. Hepatic gene expression. Expression of enzymes involved in monosaccharide transport, initial glucose conversion (hexokinases), glycolysis, *de* novo lipogenesis and lipid transport in liver tissue for (A) male and (B) female mice. *Acacb*: Acetyl-CoA carboxylase beta; *Aldob*: Aldolase B; *Elovl6*: Elongation of long chain fatty acids family member 6; *Fasn*: Fatty acid synthase; *Gapdh*: Glyceraldehyde-3-phosphate dehydrogenase; *Hk*: Hexokinase; *Khk*: Ketohexokinase; *Mttp*: Microsomal triglyceride transfer protein; *Scd1*: Stearoyl-coenzyme A desaturase 1; *Slc2a*: Solute Carrier Family 2; *Tkfc*: Triokinase, FMN cyclase; *Tpi*: Triose phosphate isomerase. Solid dots represent HF-G diet, half-open dots represent HF-GF, and open dots represent HF-F. Values are expressed as mean ± SEM, *n* = 9-10. *: $P < 0.05$; **: $P < 0.01$; *** : $P < 0.001$.

A significant upregulation of *Slc2a5* was found in the HF-GF females compared with HF-F females (Fig. 5B). *Hk1* was downregulated in the HF-F females compared with the HF-G females. The hepatic expression of other kinases (*Hk2*, *Hk4* and *Khk*) and *Slc2a2* was not affected by the fructose content of the diet in females, nor were glycolysis-related enzymes or *Mttp1*. DNL-related enzyme expression was not significantly altered, except for a higher *Scd1* expression in HF-F females compared with HF-G females.

Finally, intestinal gene expression was studied in the females. No significant changes were observed in the expression of sugar transporters (*Slc2a2, Slc2a5, Slc2a7,* and *Slc5a1*), hexokinases, or glycolysis-related enzymes (Fig. 6).
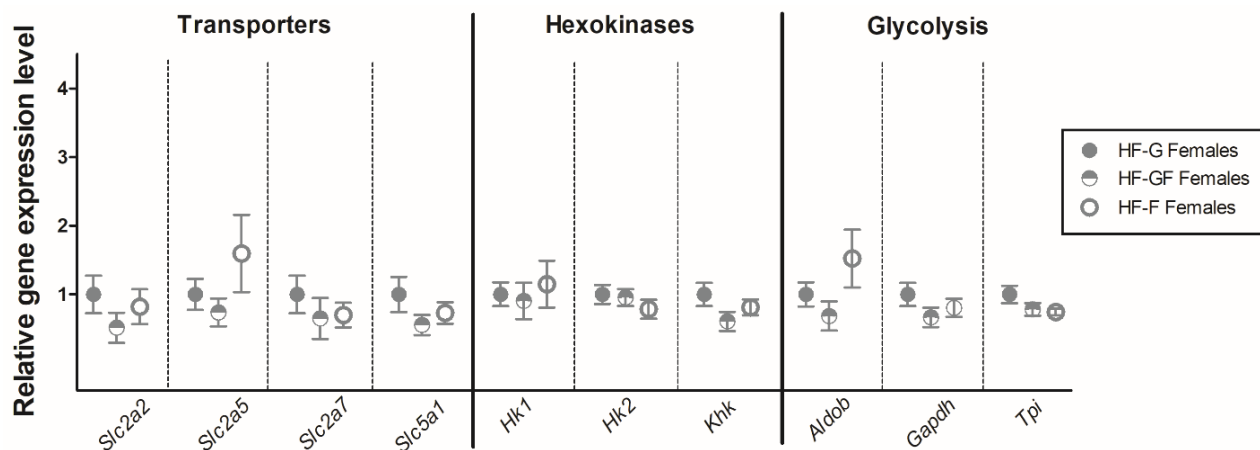

Figure 6. Intestinal gene expression in female mice**.** Expression of enzymes involved in monosaccharide transport, initial glucose conversion (hexokinases) and glycolysis, in the intestinal tissue of female mice. *Aldob*: Aldolase B; *Gapdh*: Glyceraldehyde-3-phosphate dehydrogenase; *Hk*: Hexokinase; *Khk*: Ketohexokinase; *Slc2a*: Solute Carrier Family 2; *Slc5a*: Solute Carrier Family 5; *Tpi*: Triose phosphate isomerase. Solid dots represent HF-G diet, half-open dots represent HF-GF, and open dots represent HF-F. Values are expressed as mean ± SEM, *n* = 10.

Discussion

Animals fed a Westernized moderate high-fat diet in which half or all of the dietary monosaccharide glucose was replaced with fructose did not show altered HF-induced body weight or adiposity, neither in males nor in females. Nevertheless, plasma TG concentrations were affected by the fructose content, as HF-F males had higher TG concentrations than HF-G males, and HF-GF females had higher plasma TG concentrations than HF-G and HF-F females. Liver TG and glycogen levels were not altered by the dietary fructose. From these data we conclude that isocaloric dietary fructose does not affect body composition nor liver energy stores. These data are in line with a meta-analysis in humans, isocaloric studies concluding that fructose has no

extra effect on body weight than other carbohydrates [33]. The same group reported that fructose intake, compared with glucose intake, can increase circulating uric acid and TG concentrations, but does not worsen insulin concentrations, markers of fatty liver or atherogenic aspects of the lipid profile [5]. Fructose may even have positive effects on body weight and blood pressure in human intervention studies [5]. This fully agrees with our observation that plasma TG concentrations were increased by the fructose content in the diet, especially in the combined fructose-glucose exposure.

Our data are in striking contrast to other studies that show increased adiposity, increased liver weight and increased hepatic TG levels resulting from fructose intake. In the large majority of cases, these effects can be explained by excess caloric intake, in particular due to supplementation with fructose via the drinking water or the lack of an adequate control group [23-25]. Effects of fructose feeding mimic those of high-fat feeding [34, 35]. In addition, fructose can exaggerate the obesogenic effects of high-fat diet and promote insulin resistance [36, 37]. But because these studies often do not have an isocaloric glucose group (eg. [34-37]), it is not clear whether these effects are fructose specific, or that similar effects would be seen with glucose. Indeed, also maltodextrin exaggerate the effects of a high-fat diet [38]. With similar caloric intake, fructose administered via the drinking water gives worse metabolic outcomes than glucose administered via the drinking water in animals on a high-fat diet [39]. The overall macronutrient intake was also shifted to some extend in that study [39]. In our study, fructose and glucose were administered as part of the pelletized diet in an isocaloric manner, to circumvent the confounding effects of overconsumption of fructose in the drinking water, and to prevent shifts in macronutrient intake. Although overconsumption and shifts in macronutrient intake were prevented, the fructose diet displayed a significantly lower energy intake in both sexes: HF-F males consumed 6% less than HF-G males, while HF-F females consumed 12 % less than HF-G females. Our data contrast with a study where 18 en% of fructose and 18 en% of glucose were given as part of a normal-fat diet in young male mice, resulting in increased body weight, liver weight, and fat mass by fructose compared with glucose [40]. Of importance, also the absolute caloric intake was higher in the fructose group, despite isocaloric amounts of the sugars in the experimental diets. The overall lower caloric intake may have had some protective influence in our study. Taken together, the way of administration of fructose (as part of the diet of in a liquid), and effects on overall energy intake seems to affect the outcomes of studies.

To substantiate potential functional differences between fructose, glucose-fructose and glucose, hepatic gene expression was analysed, which showed minor changes. The upregulation of KHK in HF-GF and HF-F compared with the HF-G was significant in males, and may be expected as KHK is the first enzyme metabolizing cellular fructose. The limited effect size in other genes may be due to the timing of the analysis: lipogenesis related gene expression in liver was upregulated after 5

days of fructose feeding compared with a starch control, but the difference did not persist after 45 days of fructose feeding [41]. Alternatively, because the animals were killed in the daytime, two hours after removal of the diets, they were possibly in the post-prandial state, which may have limited the gene expression effects of fructose. However, this argument is counteracted by the observed upregulation of *Slc5a2* in HF-GF fed males and females. The absence of pronounced gene expression effects is in agreement with the absence of differential effects of the monosaccharides on body and liver composition.

Our results did show increased plasma TG concentrations with fructose in the diet (Fig 4). Because hepatic gene expression did not indicate an increase in DNL, these increased concentrations may result from decreased clearance. In fact, decreased lipid clearance can be caused by sucrose [42] and fructose [43]. Increased circulating TG with fructose in the diet was also seen in human males [22, 44], but not in females [22]. Moreover, as in our study, no differential effects on hepatic TG levels were seen in humans with 25 en% from glucose or from fructose, neither with isocaloric feeding nor with hypocaloric feeding [45]. The absence of a difference between glucose and fructose, but higher hepatic TG levels in the hypercaloric condition compared with the isocaloric condition confirm the notion that it is particularly the energy intake, rather than specific effects of fructose compared with glucose, that results in the adverse metabolic effects.

In summary, our physiological data indicate that, within an isocaloric pelletized diet, fructose does not result in worse physiological effects than glucose or glucose-fructose.

Author contributions
JK, AN, and EvS designed the experiment; HS and AN carried out the animal experiment; RP and AN performed the lab experiments; LB, EvS, and JK interpreted the results; LB drafted the manuscript LB; EvS and JK revised the manuscript.

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Chapter 4

Replacing part of glucose with galactose in the post-weaning diet protects female but not male mice from high-fat diet-induced adiposity in later life

Lianne M. S. Bouwman,

José M. S. Fernández-Calleja,

Inge van der Stelt,

Annemarie Oosting,

Jaap Keijer,

Evert M. van Schothorst.

Abstract

Background: Duration of breastfeeding is positively associated with decreased adiposity and increased metabolic health in later life, which might be related to galactose.

Objective: The objective was to investigate if partial replacement of glucose with galactose in the post-weaning diet had a metabolic programming effect.

Methods: Male and female mice (C57BL/6JRccHsd) received an isocaloric diet (16 energy% fat; 64 energy% carbohydrates; 20 energy% protein) with either glucose (32 energy%) (GLU) or glucose + galactose (GLU+GAL, 16 energy% each) for three weeks post weaning. Afterwards, all mice were switched to the same 40 energy% high-fat diet (HFD) for nine weeks to evaluate potential programming effects in an obesogenic environment. Data were analysed within sex.

Results: Female body weight (-14%) and fat mass (-47%) were significantly less at the end of the HFD period (both $P < 0.001$) among those fed GLU+GAL vs GLU, effects in males were in line with these findings, but non-significant. Food intake was affected in GLU+GAL-fed females (+8% on post-weaning diet, - 9% on HFD) vs GLU-fed females, but not for hypothalamic transcript levels at endpoint. Also, in GLU+GAL-fed females, serum insulin concentrations (-48%, $P < 0.05$) and associated HOMA-IR were significantly lower ($P < 0.05$) at endpoint, but there were no changes in pancreas morphology. In GLU+GAL-fed females, expression of *Irs2* ($P < 0.001$, Fold change (FC) = -1.4; $P < 0.0001$, FC = -1.3), and adipocyte size markers *Lep* ($P < 0.05$, FC = -1.7; $P < 0.05$, FC = -2.7) and *Mest* ($P < 0.05$, FC = -5.0; $P < 0.05$, FC = -3.5) was lower in gonadal and subcutaneous white adipose tissue (WAT). Expression of *Irs1* ($P < 0.05$, FC = -1.3) was only lower in subcutaneous WAT in GLU+GAL-fed females.

Conclusions: Partial replacement of glucose with galactose, resulting in a 1:1 ratio mimicking lactose, in a three-week post-weaning diet lowered body weight, adiposity, HOMA-IR, and expression of WAT insulin signalling in HFD-challenged female mice in later life. This suggests that prolonged galactose intake may improve metabolic and overall health in later life.

Keywords

Introduction

Quality of early life diet can have lasting effects, ameliorating glucose and lipid metabolism as well as attenuating adiposity and obesity, provided this nutritional exposure occurs during a critical window of development. This phenomenon was reported first by Barker, who hypothesized that foetal undernutrition affects morbidity and mortality in adulthood [1]. Later, data from, in particular, the Dutch Famine Cohort showed that early life undernutrition increased later life susceptibility to diseases like type 2 diabetes [2]. Since then, a substantial body of literature has supported the impact of maternal environment on the phenotype of the offspring [3]. While effects of under- and over-nutrition during pregnancy as well as nursing on later life health are well established, the impact of early life nutrition (weaning and early post-weaning) has been studied less, although animal studies have shown lasting effects on later life metabolic health [4-7].

Weaning is characterized by a change in nutritional profile [8] and diversification from lactose as the primary carbohydrate source to a variety of sugars and polysaccharides [9]. In humans, timing of first weaning foods, types of weaning foods - which may include fruits and vegetables, dairy, and cereals -, and duration of weaning all vary according to cultural and individual preferences [10]. As a result, there is a great variability among individuals in types and amounts of carbohydrates to which they are exposed in late infancy and early childhood (during and shortly after weaning).

Lactose, a disaccharide of glucose and galactose, is the main carbohydrate in breastmilk, providing ~44% of its energy [11]; *i.e.* ~22% of total energy from glucose and ~22% from galactose. The relative contribution of carbohydrates to total energy intake in young children (2-3 years) in the Netherlands increases during the post-weaning phase to approximately 58% [12], but decreases again to ~48% in adulthood (19-30 years, [13]). In the post-weaning diet, energy from carbohydrates comes mainly from glucose, in form of simple sugars and a variety of (complex) polysaccharides. Fructose also has a sizable contribution to energy intake in post-weaning diets (9% of the energy; 7-69 years [14]), while galactose barely contributes. Current nutritional guidelines are not specific about the type of carbohydrate to give during weaning and (early) post-weaning periods, although products containing added sugar, fruit juices, and sugar sweetened beverages are discouraged [15]. However, because the early post-weaning period covers a critical period of developmental period that is amenable to nutritional programming, it is important to elucidate the differential effects of carbohydrates, and clarify to what extent carbohydrates can affect long-term (adult) health.

Extended breastfeeding (> 12 months of age) is associated with protection from obesity, and data suggest the effect is due to milk constituents rather than other environmental factors [16-18]. Since lactose is the main energy source during breastfeeding, it is tempting to speculate that high intakes of lactose might explain, at least in part, these health benefits.

Further, half of the monosaccharides derived from lactose are galactose, a monosaccharide that, otherwise, is hardly present in the diet of older infants and young children.

Currently, there is some evidence for nutritional programming by carbohydrates, which focusses on pregnancy and/or nursing periods (on various parameters). A high carbohydrate formula given to rats during suckling caused lasting alterations in hypothalamic gene expression, which led to hyperphagia, greater body weight gain, and higher circulating leptin concentrations in adulthood, a marker of higher fat [19]. Similarly, sucrose feeding during pregnancy and lactation led to increased hepatic triglycerides content and lower insulin and glucose tolerance in rat offspring [20]. Overall, fructose intake during pregnancy and lactation increased the chance of metabolic disturbances in hepatic and adipose tissue of offspring (reviewed in [21]). To our knowledge, nutritional programming studies with galactose are lacking, thus far.

In the present study, we aimed to establish whether galactose can impact long-term metabolic health. More specifically, whether partial replacement of glucose by galactose in the post-weaning diet, mimicking lactose, affects body composition and/or metabolic profiles including ectopic lipid storage, using a mouse model of nutritional programming. To assess this, C57BL/6JRccHsd mice were given a starch-based diet containing additional monosaccharides at 32 energy percent (en%) from glucose or 16 en% glucose + 16 en% galactose, to mimic lactose intake, for three weeks post weaning, before being switched to a galactose-free high-fat diet (HFD) for nine weeks. We hypothesized that including galactose in the post-weaning diet would protect the mice from adiposity in later life. We, therefore, focussed molecular analyses on mainly adipose tissue, but also on liver triglycerides, pancreas morphology, and hypothalamic gene expression. White adipose tissue (WAT) metabolic pathways were examined in more detail.

Materials and methods

Ethical approval

All experimental procedures were approved by the Animal Experimental Committee (DEC 2014085, Wageningen, The Netherlands) and were carried out in accordance with the principles of good laboratory animal care, following the European Union Directive for the protection of animals used for scientific purposes (86/609/EEC). This experiment was included as part of a larger study investigating the nutritional programming effects of different monosaccharides during the post-weaning period, reducing the number of experimental animals used, in line with the 3R principles. More specifically, the glucose control group was also used as controls for an experiment investigating post-weaning programming effects of fructose. Data from this control glucose group [*i.e.* body weight (BW), food intake (FI), fat mass (FM), lean mass (LM), organ weights, glucose tolerance, circulating leptin concentrations, serum insulin concentrations, and hepatic TG content] have been reported previously [22].

Diets

Diets were ordered from Research Diet Services BV (Wijk bij Duurstede, The Netherlands). Compositions of breeding and post-weaning diets were in accordance with AIN-93 guidelines for growing rodents [23]; see Supplemental Table 1 for a complete overview of the compositions. The post-weaning diets differed only in monosaccharide composition: we compared a glucose only diet (GLU) with one containing a 1:1 mixture of glucose and galactose (GLU+GAL) mimicking lactose. A 1:1 mixture was used rather than lactose, to prevent problems associated with lactose intolerance, due to lactase deficiency, in mice after weaning. Both post-weaning diets had 32 en% from monosaccharides (*i.e.* 32 en% glucose in GLU or 16 en% galactose and 16 en% glucose in GLU+GAL) and contained 16 en% fat, 20 en% protein, and 32 en% starch, as well as mineral and vitamin mixes compliant with AIN-93 guidelines [23].

After the three-week post-weaning period, all mice were switched to the same high-fat diet (HFD) with 40 en% fat, 20 en% protein, and 40 en% carbohydrates (starch and sucrose) as well as mineral and vitamin mixes (Supplemental Table 1). The HFD corresponded to the human macronutrient intake profile and induced adiposity [24, 25].

Animals, study design, and measurements

Male and female C57BL/6JRccHsd mice were obtained from Harlan (Harlan Laboratories BV, Horst, The Netherlands). C57BL/6JRccHsd mice are an established model for diet-induced obesity and, as humans, have an intact nicotinamide nucleotide transhydrogenase gene, which is absent in many BL/6j strains. The mice were given a semi-synthetic breeding diet (Research Diet Services, 16 en% fat, 20en% protein, 64en% carbohydrates, Supplemental Table1) and time-mated. Nests were standardized to 6 pups per nest 0-2 days after birth, with 2 to 4 female pups per nest. Female and male pups were included in the experiments. After weaning at three weeks of age, the pups were stratified by sex and body weight, maximising the number of nests contributing to the dietary groups, and given either the GLU post-weaning diet or the GLU+GAL post-weaning diet. After three weeks post-weaning intervention, all the animals were switched to the same HFD for nine weeks. Ultimately, males were $n = 12$ per group, while GLU-fed females were $n = 14$ and GLU+GAL-fed females were $n = 13$. BW and FI were measured weekly. Energy intake was calculated by multiplying food intake (in grams) by diet energy content (Supplemental Table 1). Body composition (BC) was determined (without anaesthesia) using an EchoMRI 100V (EchoMedical Systems, Houston, TX, USA) [26], and measured weekly during the post-weaning period and bi-weekly during the HFD period. Food and water were available *ad libitum*. During all procedures, the mice were kept in a controlled environment (23°C ± 1°C; 12h:12h light dark cycle).

Oral glucose tolerance test

During week 11, an oral glucose tolerance test (OGTT) was performed, as described previously [22].

Organ collection and processing

At the end of the HFD feeding period, the animals were fasted for two to 5.5 hours from the start of the light phase, before being sacrificed; decapitation was in a random order. Blood glucose concentrations were measured using a standardized method (Freestyle). MiniCollect® serum tubes (Greiner Bio-One B.V., Alphen aan de Rijn, The Netherlands) were used to collect serum. Livers were collected, weighed, and snap-frozen in liquid nitrogen. Gonadal white adipose tissue (gWAT) was excised; left fat pads were snap-frozen in liquid nitrogen, while right fat pads were weighed and fixed in paraformaldehyde. Subcutaneous WAT (sWAT) was collected from the inguinal region and snap-frozen in liquid nitrogen. Pancreatic tissue was separated from mesenteric WAT, based on density in PBS; both organs were weighed, and pancreatic tissue was fixated in paraformaldehyde. Hypothalami were collected and snap-frozen in liquid nitrogen. All samples were stored at -80°C until analysis.

Serum measurements

Serum leptin was measured using Bio-Plex Pro mouse diabetes assays (Bio-Rad laboratories, Veenendaal, The Netherlands) in accordance with the manufacturer's instructions. Serum insulin was measured using the Ultra-Sensitive Mouse Insulin ELISA Kit (ChrystalChem, Downers Grove, Illinois, United States) following the manufacturer's instructions. Serum measurements were done in duplicates. HOMA-IR was calculated from serum insulin (in milliunits per liter) and whole blood glucose (in millimoles per liter) using a factor 14.1, which is applicable for C57BL/6J mice [27].

TG extraction and measurement

Hepatic triglyceride (TG) content was measured using the Liquicolor kit (Human, Wiesbaden, Germany) and expressed per protein content, as published previously [22].

Pancreatic β-cell mass and β-cell area

Analysis of pancreatic β-cell mass and area was performed as published elsewhere [22], with the modification that β-cell area was determined using Adobe Photoshop.

Analysis of adipocyte size gonadal white adipose tissue

Adipocyte size was determined using images of Mayer's haematoxylin-stained gWAT; details can be found in the Supplemental Methods.

RNA extraction

Total RNA was extracted from hypothalami, gWAT, and sWAT. Tissues were ground with mortar and pestle in liquid nitrogen before being dissolved in TRIzol reagent (Invitrogen, Breda, The Netherlands). After TRIzol extraction, additional chloroform, phenol/chloroform/isoamyl-alcohol, and chloroform extraction steps were performed. Total RNA was precipitated with isopropanol, washed with ethanol, and dissolved in DNase/RNase-free water. RNA

concentrations were measured using a Nanodrop spectrophotometer (IsoGen Life Science, Maarsen, The Netherlands). RNA quality was checked using an Experion automated electrophoresis system (Bio-Rad). Additionally, sWAT RNA quality was checked on an Agilent 2200 Tapestation (Agilent Technologies Inc, Santa Clara, CA, USA) with RNA ScreenTape (Agilent). Subsequently, RNA was converted to cDNA using an iScript kit (Bio-Rad) according to the manufacturer's instructions (input: 1 μg RNA for gWAT and sWAT, 22.5 ng RNA for hypothalamus).

RT-qPCR

Transcript expression was measured using iQ SYBR Green Supermix (Bio-Rad) on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad), with 3 minutes at 95°C, 40 cycles of 15 seconds at 95°C, and 45 seconds at the annealing and elongation temperature, followed by melt-curve analysis. Primers were designed using NCBI Primer-Blast (NCBI Website); an overview of primer sequences and annealing temperatures is given in Supplemental Table 2. cDNA from all samples was pooled and serial dilutions were used for standard curves; for each transcript, two negative controls were also included (water and a sample without reverse transcriptase). Samples were measured in a 100-fold dilution; samples and standards were measured in duplicate. Low-level expressed genes were pre-amplified with SsoAdvanced PreAmp Supermix (Bio-Rad) to ensure good technical results. For gWAT, pre-amplification consisted of 3 minutes at 95°C, followed by 10 cycles of 15 seconds at 95°C, and 45 seconds at 58°C. For sWAT, pre-amplification was performed in 100-fold diluted cDNA for 15 cycles (genes that were pre-amplified are listed in Supplemental Table 2). Pre-amplification of hypothalamic samples, carried out for all target and reference genes, was for 16 cycles. A cDNA pool of the pre-amplified samples was made for the standard curves and RT-qPCR was carried out as described above. Negative controls were included in the pre-amplification. Stable gene expression levels were determined using CFX Manager software, (version 3.1, Bio-Rad), and data normalized against stable reference genes, namely *Rplp0,Canx, B2m,* and *Rps15* for gWAT, *Rplp0, B2m,* and *Rps15* for sWAT, and *B2m* and *Rps15* for hypothalami. Data were normalized to expression in the GLU-fed group, which was set to 1 for each gene.

Statistics

Data were analysed within sex, for each sex separately, because many physiological parameters differ between the sexes, and programming effects are often sex-dependent. Statistical analyses were performed in GraphPad Prism, version 5.04 (GraphPad Software Inc., San Diego, CA, USA). Two-way repeated measures ANOVA was used for analysis of BW, FM, and LM, with post-weaning diet as the between-subject factor, time as the within-subject factor, and group x time interaction. Post-weaning and HFD periods were studied separately. When the effects of post-weaning diet were significant, *post-hoc* Bonferroni analysis was performed on all time points. OGTT was analysed with two-way repeated measures ANOVA. Other parameters were analysed using the Students' *t*-test (normally distributed data) or *t*-test with Welch correction (normally distributed with unequal variances). D'Agostino &

Pearson omnibus normality tests were used to test for normality; data that were not normally distributed were log-transformed and retested for normality. A Mann-Whitney U test was applied on original data when transformed data were also not normally distributed. The gene expression analyses were targeted and not corrected for multiple comparisons [28, 29] and raw individual P values are used. Results are given as mean ± SEM, and P values < 0.05 were considered significant.

Results

Male body composition development

During the three-week post-weaning period, BW, LM, and FM increased significantly in both GLU- and GLU+GAL-fed males (Fig. 1A, B, and C; Supplemental Table 3 for an overview of statistical parameters from Two-way ANOVAs). Post-weaning diet x time tended to affect BW (P = 0.088) and FM (P = 0.096), but post-weaning diets had no significant effect overall (P = 0.28 for BW, P = 0.28 for FM). Similarly, LM was not affected significantly (Fig. 1C; Supplemental Table 3).

During the HFD period, BW, FM, and LM increased significantly in both GLU- and GLU+GAL-fed males (Fig. 1A, B, and C; Supplemental Table 3). Post-weaning diet x time tended to affect BW (P = 0.094), *i.e.* BW increased less in GLU+GAL-fed males than in those receiving GLU. There were, however, no significant post-weaning diet x time interactions for FM or LM (Supplemental Table 3). Post-weaning diets tended to affect BW during HFD feeding (P = 0.061), but post-weaning diets had no effect on FM (P = 0.19). Post-weaning diet affected LM significantly during the HFD period, (P = 0.007); post-hoc analysis indicated this was significant in weeks 8-12, but not subsequently (Fig. 1C). Energy intake was higher among GLU+GAL-fed males than among GLU-fed males post-weaning (Figure 1D), but the same during HFD-feeding (Fig. 1E). Consequently, cumulative energy intakes were similar over the entire 12-week study period (data not shown).

Female body composition development

During the post-weaning period, BW, FM, and LM of GLU- and GLU+GAL-fed females increased significantly (Fig. 2A, B, and C; Supplemental Table 3). Post-weaning diet x time was different for GLU- and GLU-GAL fed females (P = 0.031). A trend for post-weaning diet x time was seen in LM (P = 0.058), but not FM (P = 0.62). Overall, the diets had no significant effects on BW, LM and FM. GLU+GAL-fed females consumed significantly more of the post-weaning diet than GLU-fed females (Fig. 2D).

During the HFD period, BW, FM, and LM also increased significantly in both GLU- and GLU+GAL-fed females (Fig. 2A, B, and C; Supplemental Table 3). Post-weaning x time was
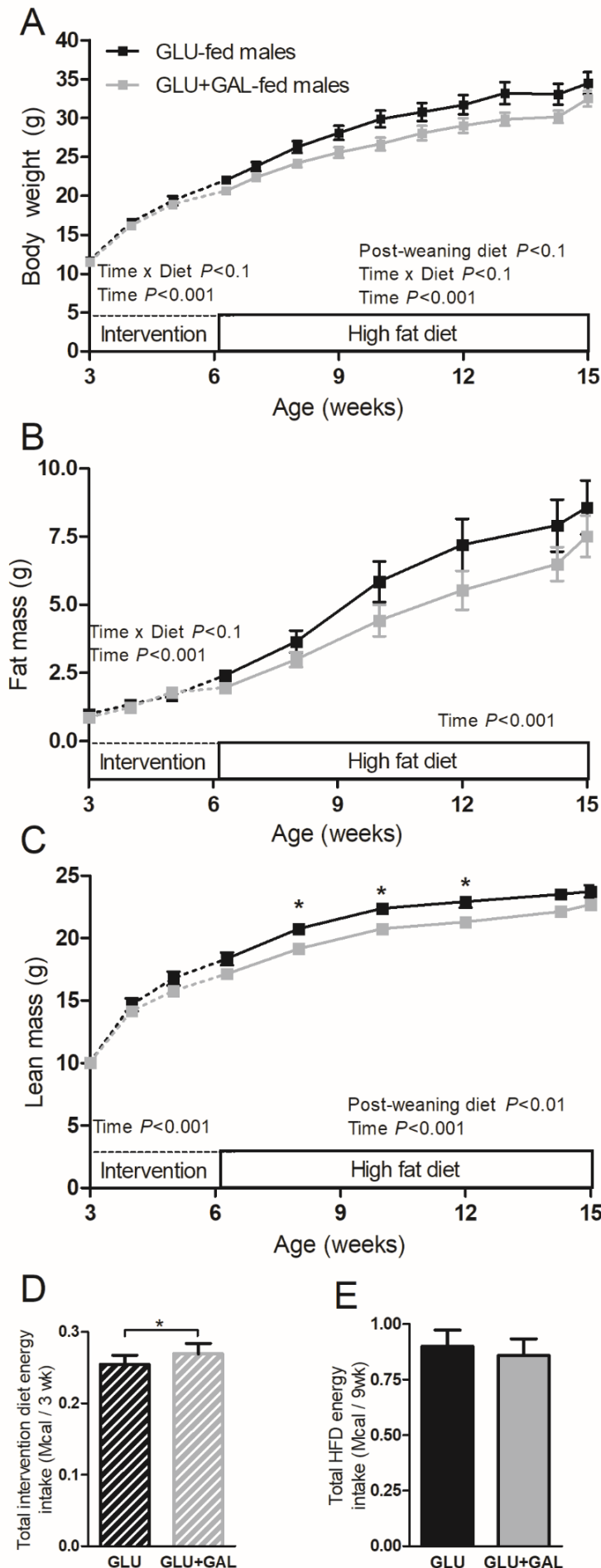
Figure 1. Longitudinal body weight (A), fat mass (B), and lean mass (C) development of male mice; total energy intake during post-weaning (D) and HFD period (E), for male mice fed GLU or GLU+GAL diets post weaning for 3 weeks and thereafter a HFD for 9 weeks. Group (post-weaning GLU *vs* GLU+GAL diet), time, and group by time effects were determined by repeated-measures ANOVA for BW, FM, and LM; post-weaning and HFD periods were analysed separately. Values are given as mean ± SEM, *n* = 11-12. * Groups differ, P < 0.05. Data on BW, FM, and energy intake from the control GLU-fed group have been published [22].
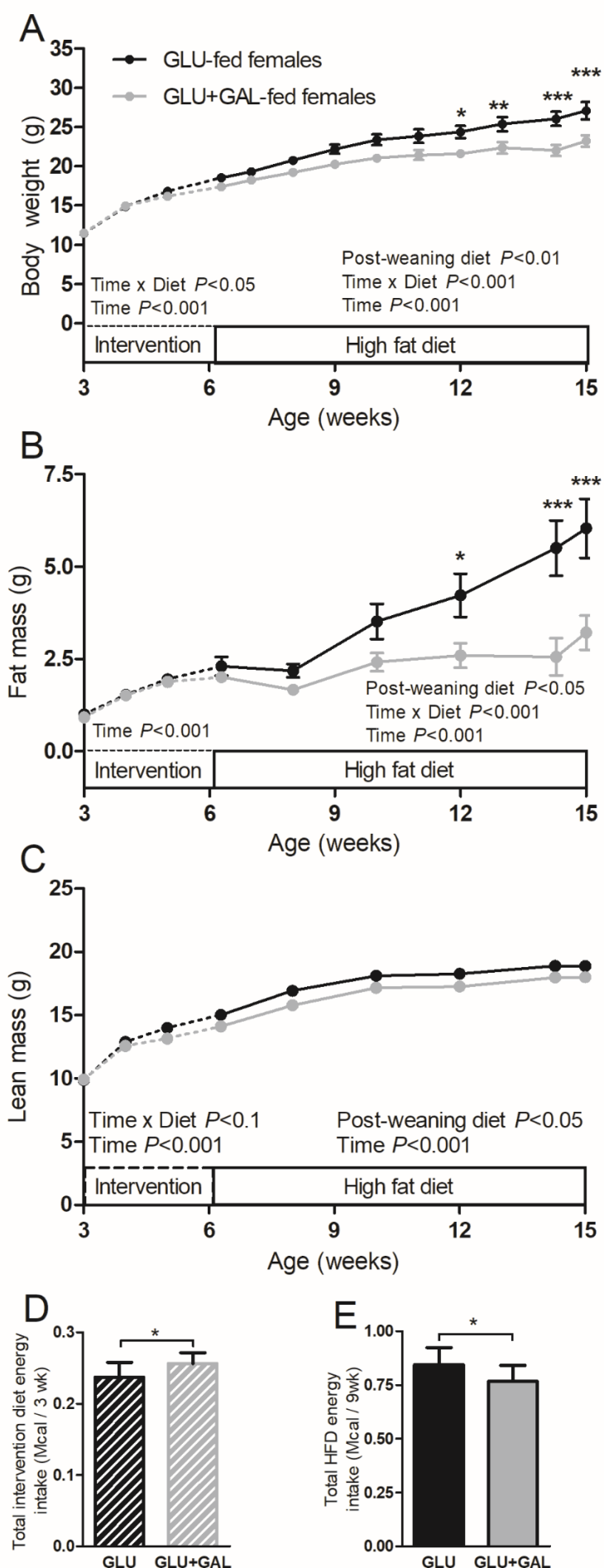
Figure 2. Longitudinal body weight (A), fat mass (B), and lean mass (C) development of female mice; total energy intake during post-weaning (D) and HFD period (E), for female mice fed GLU or GLU+GAL diets post weaning for 3 weeks and thereafter a HFD for 9 weeks. Group (post-weaning GLU *vs* GLU+GAL diet), time, and group by time effects were determined by repeated-measures ANOVA for BW, FM, and LM; post-weaning and HFD periods were analysed separately. Values are given as mean ± SEM, *n* = 12-14. * Groups differ, $P < 0.05$, ** groups differ, $P < 0.01$, *** groups differ, $P < 0.001$. Data on BW, FM, and energy intake from the control GLU-fed group have been published [22].

significantly different during the HFD period for BW ($P < 0.0001$) and FM ($P < 0.0001$): BW and FM increased less in the GLU+GAL-fed females than in GLU-fed females. Post-weaning diets also had a significant effect on BW ($P = 0.008$) and FM ($P = 0.01$) during the HFD period: BW and FM were significantly lower in GLU+GAL-fed females from week 12 onwards. No post-weaning x time effect was observed for LM ($P = 0.97$), LM gain was the same in both groups. However, while the post-weaning diets had a significant impact on overall LM ($P = 0.021$), *post-hoc* analysis showed this was not significant at individual time points. Cumulatively, GLU+GAL-fed female mice consumed less HFD than GLU-fed female mice (Fig. 2E), and tended to have lower energy intakes over the entire 12 weeks study (1.05 ± 0.02 Mcal/12 wk vs 1.10 ± 0.02 Mcal /12 wk; $P = 0.09$).
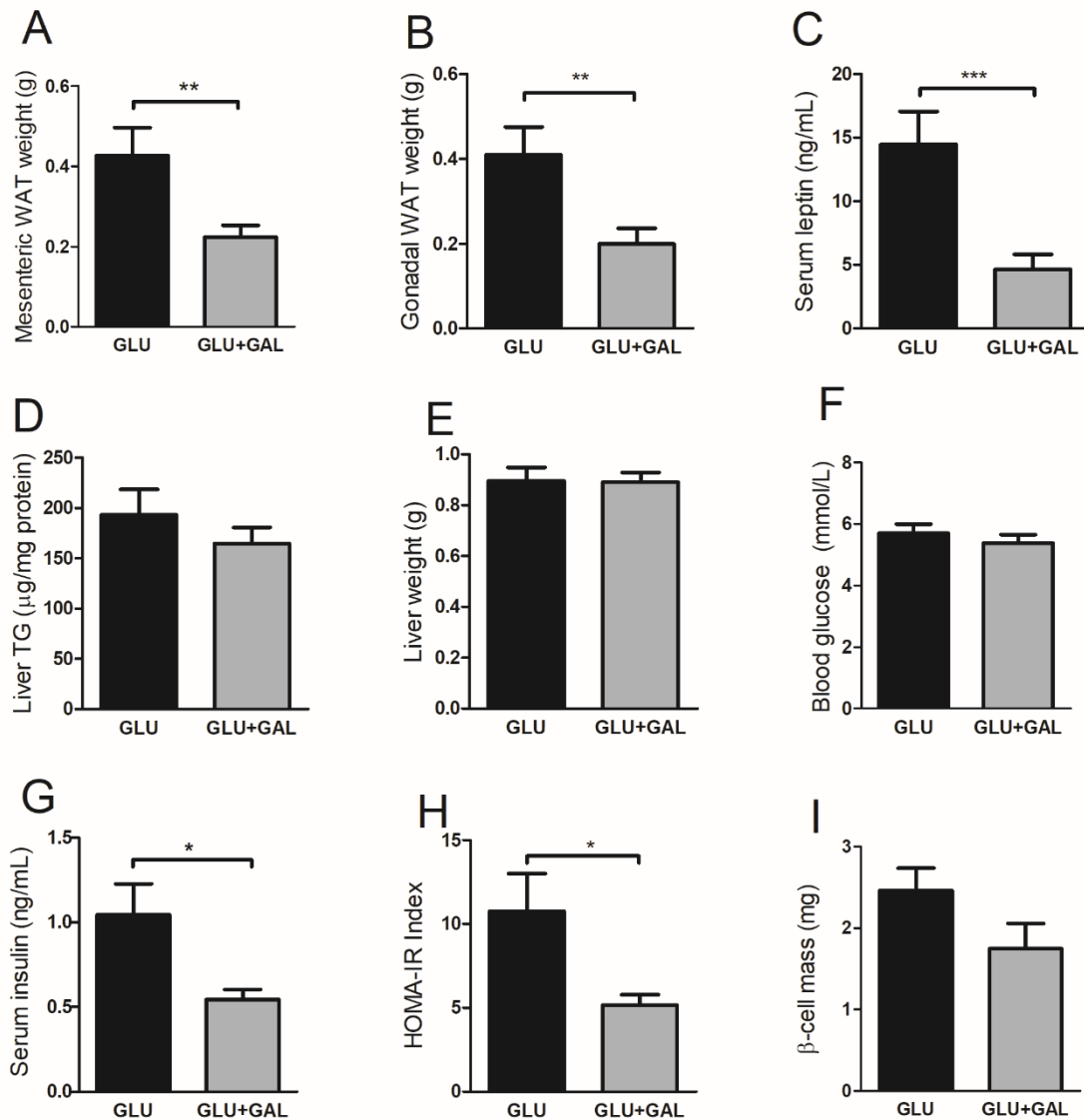
Figure 3. Mesenteric WAT (A), gonadal WAT (B), serum leptin concentrations (C), hepatic TGs (D), liver weight (E), whole blood glucose concentrations (F), serum insulin concentrations (G), HOMA-IR (H) and pancreatic β-cell mass (I) in female mice fed GLU or GLU+GAL diets post weaning for 3 weeks and thereafter a HFD for 9 weeks. Values are given as mean ± SEM, $n = 13$-14, $n = 6$ for β-cell mass. * Bracketed groups differ, $P < 0.05$, ** bracketed groups differ, $P < 0.01$, *** bracketed groups differ, $P < 0.001$. Data from the GLU-fed group have been published [22].

Although patterns of development in BW, FM and LM during the post-weaning and HFD periods were similar for both sexes, at the end of the study, diet affected BW and BC significantly only in females. Therefore, subsequent analyses focussed only on females and, in particular, FM-related parameters.

Metabolic characterization of programming in females

GLU+GAL-fed females had less mesenteric (Fig. 3A) and gonadal WAT (gWAT; Fig. 3B) than GLU-fed females. Average serum leptin concentrations in GLU+GAL-fed females were approximately one third of the average concentration in GLU-fed females (Fig. 3C). Hepatic triglyceride (TG) content was similar in both groups (Fig. 3D), as was liver weight (Fig. 3E). Blood glucose concentrations were also similar (Fig. 3F), but serum insulin concentrations were lower in GLU+GAL-fed females (Fig. 3G), indicating greater insulin sensitivity in GLU+GAL-fed females after the HFD period. Indeed, HOMA-IR, a surrogate marker for insulin resistance that is widely used but has its limitations [27], was also lower for GLU+GAL-fed females (P = 0.03, Fig. 3H). Analysis of the incremental area-under-the-curve from oral glucose tolerance tests performed four weeks earlier (week 11) showed a trend for better glucose tolerance in GLU+GAL-fed females than in GLU-fed females (P = 0.09, Fig. 4A and B). Immunohistological analysis of pancreata from GLU- and GLU+GAL-fed females (Supplemental Fig. 1A and 1B) showed that both β-cell areas (Supplemental Fig. 1C, P = 0.37) and masses (Fig. 3I, P = 0.12) were, however, similar.
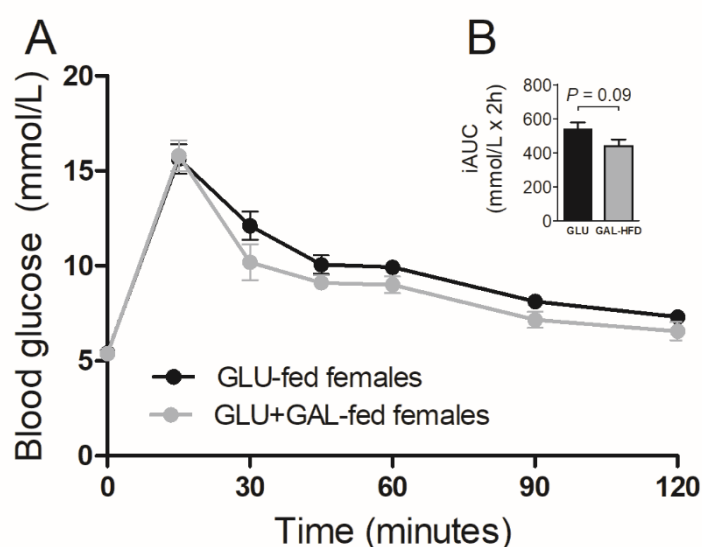
Figure 4. Blood glucose curves (A) and incremental Area Under the Curve (iAUC) (B) from the oral glucose tolerance test performed in week 11 in female mice (fed GLU or GLU+GAL diets post weaning for 3 weeks and thereafter a HFD for 5 weeks.). Values are given as mean ± SEM, n = 13-14. Data from the GLU-fed group have been published [22].

Because both energy intakes and serum leptin concentrations were different between GLU- and GLU+GAL-fed females (Fig. 2D and 2E; Fig. 3C), hypothalamic gene expression of

orexigenic and anorexigenic transcripts and leptin signalling were examined. However, no significant differences were found between GLU- and GLU+GAL-fed females (Fig. 5).

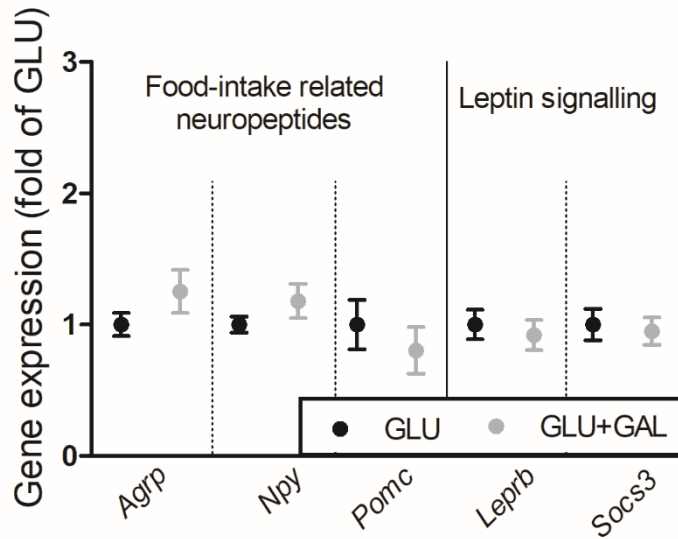**Figure 5.** Gene expression in hypothalamus of female mice fed GLU- or GLU+GAL diets post weaning for 3 weeks and thereafter a HFD for 9 weeks. *Agrp*: agouti-related peptide; *Npy*: neuropeptide Y; *Pomc*: pro-opiomelanocortin; *Leprb:* leptin receptor long isoform; *Socs3:* suppressor of cytokine signalling 3. Values are given as mean ± SEM, *n* = 11-14.

Histological and detailed molecular analyses of gWAT depots showed a trend for smaller unilocular adipocytes (diameter < 50 μm) in GLU+GAL-fed females than in GLU-fed females (*P* = 0.07) (Supplemental Fig. 2). Leptin gene expression was lower in gWAT as well as sWAT of GLU+GAL-fed females (Fig. 6 and 7). Expression of *Mest,* a marker for adipose expandability, was also lower among GLU+GAL-fed females in both WAT depots. However, no differences in gWAT expression of *Fabp4* and *Plin1,* adipocyte differentiations markers, were observed (Fig. 6).

To explore differences associated with the lower HOMA-IR index, insulin signalling was also analysed. Strikingly, *Irs2* expression was significantly lower in gWAT of GLU+GAL-fed females than in gWAT of GLU-fed females. Expression of *Insr* and *Irs1* in gWAT were the same (Fig. 6). Insulin signalling linked gene expression in sWAT was significantly affected:*Irs2* expression was significantly lower in GLU+GAL- *versus* GLU-fed females (Fig. 7), and although *Insr* expression was similar in both groups, *Irs1* was lower in GLU+GAL-fed females (Fig. 7). In gWAT, expression of genes associated with fatty acid synthesis and elongation, lipolysis and fatty acid oxidation, tricarboxylic acid (TCA) cycle and carbohydrate metabolism were not affected by post-weaning diet composition (Fig. 6). Likewise, in sWAT, fatty acid oxidation and *Pck1* were not regulated differentially. Although *Lipe* expression was significantly lower in GLU+GAL-fed females, other lipid metabolism related genes (*Acacb, Acadl,* and *Pnpla2*) were unaffected by the post-weaning diet (Fig. 7).

Figure 6. Gene expression in gWAT of female mice fed GLU or GLU+GAL diets post weaning for 3 weeks and thereafter a HFD for 9 weeks. *Adipoq*: adiponectin; *Lep*: leptin; *Mest*: mesoderm-specific transcript homolog protein; *Fabp4:* fatty acid binding protein 4; *Plin1*: perilipin 1; *Irs1*: insulin receptor substrate 1; *Irs2*: insulin receptor substrate 2; *Insr*: Insulin receptor; *Acly*: ATP-citrate lyase; *Elovl6*: elongation of long-chain fatty acids family member 6; *Fasn*: fatty acid synthase; *Cpt1a*: carnitine palmitoyltransferase I; *Hadha*: hydroxyacyl-CoA dehydrogenase/3-ketoacyl-CoA thiolase / enoyl-coA hydratase; *Pnpla2:* Patatin like phospholipase domain containing 2; *Cs*: citrate synthase, *Sucgl1*: Succinate CoA ligase subunit alpha; *Pck1*: phosphoenolpyruvate carboxykinase 1; *Pdk4*:Pyruvate dehydrogenase kinase 4. * Bracketed gene differs between groups, $P < 0.05$, ** bracketed gene differs between groups, $P < 0.01$. Values are given as mean ± SEM, n = 9-13.
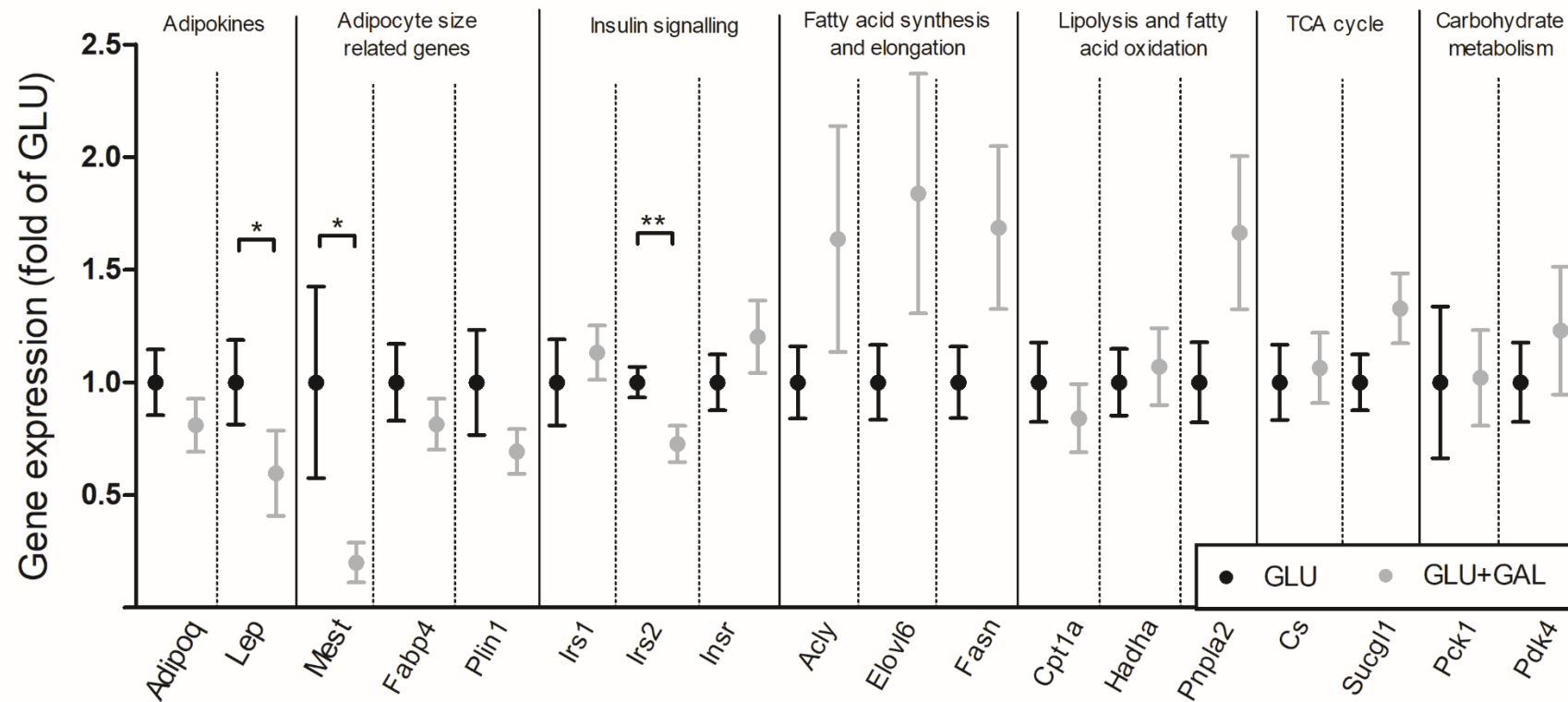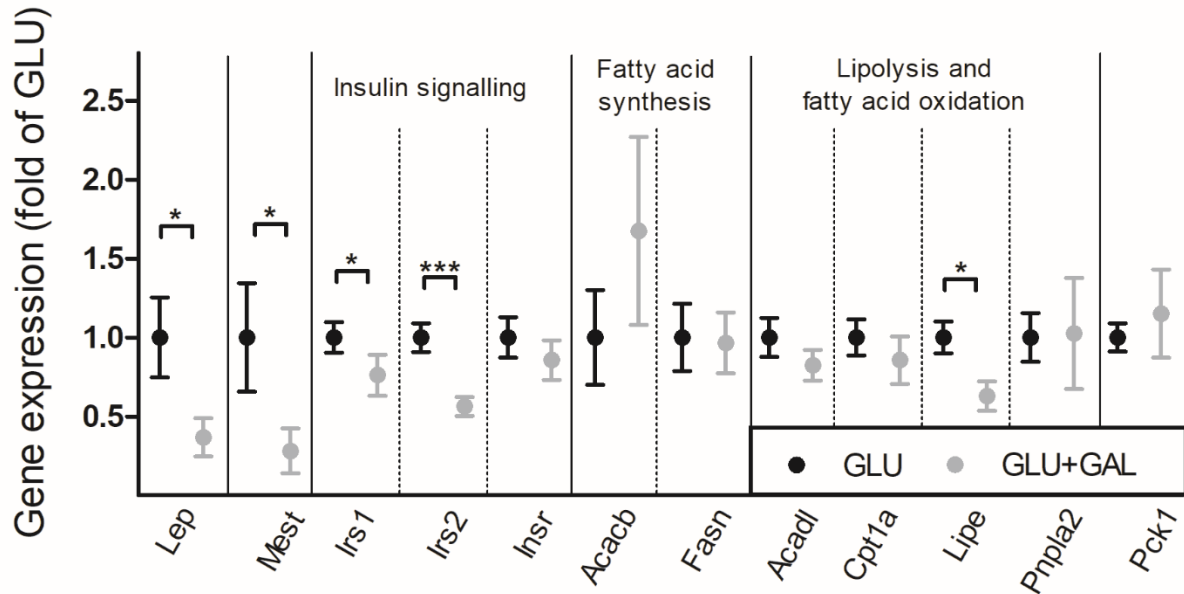
Figure 7. Gene expression in sWAT of female mice fed GLU or GLU+GAL diets post weaning for 3 weeks and thereafter a HFD for 9 weeks. *Lep*: leptin; *Mest*: mesoderm-specific transcript homolog protein*; Irs1*: insulin receptor substrate 1; *Irs2*: insulin receptor substrate 2; *Insr*: Insulin receptor; *Acacb*: Acetyl-CoA carboxylase 2; *Fasn*: fatty acid synthase; *Acadl:* Acyl-CoA dehydrogenase; *Cpt1a*: carnitine palmitoyltransferase I; *Lipe*: hormone-sensitive lipase; *Pnpla2:* Patatin like phospholipase domain containing 2; *Pck1*: phosphoenolpyruvate carboxykinase 1. * Bracketed gene differs between groups, $P < 0.05$, *** bracketed gene differ between groups, $P < 0.001$. Values are given as mean ± SEM, n = 10-14.

Discussion

In this study, we showed that replacing half of the glucose with galactose, compared with glucose alone, in an otherwise isocaloric three-week post-weaning diet, attenuated later life HFD-induced adiposity in females. In males, only subtle or non-significant effects were seen; body weight tended to be lower in male mice fed GLU+GAL post weaning than in male mice fed GLU (P = 0.06). Insulin resistance was lower in GLU+GAL-fed females in later life, with a likely role for insulin signalling via *Irs2* in WAT depots, based on reduced *Irs2* transcription levels. Such differences might be considered a form of nutritional programming, as defined by Lucas: "a stimulus or insult (a nutrient) operating at a critical or sensitive period of development results in a long-standing or life-long effect on the structure or function of the organism"[30], even though the effects emerged partially during the post-weaning period.

We explored brain and adipose tissues to find what might mediate galactose-related nutritional programming during the post-weaning period in GLU+GAL-fed females. The fact that food intake was higher during post-weaning period but was lower during the HFD period for GLU+GAL fed females versus GLU-fed females, suggested the hypothalamus (Fig. 5) could have a role, as it is the regulatory centre for energy intake in the brain. Hypothalamic gene expression, related to satiety and to leptin signalling, has been shown to be susceptible to

nutritional programming [31, 32]. However, no changes in hypothalamic expression of orexi- or anorexigenic (satiety related) transcripts *Agrp*, *Npy*, and *Pomc* were found. Also, there were no effects on expression of the leptin receptor *Leprb*, or the leptin receptor-activation marker *Socs3*, despite differences in circulating leptin concentrations between GLU- and GLU+GAL-fed females (Fig. 3C). This contrasts with programming by dietary carbohydrates in nursing rats, where a high-carbohydrate formula during nursing was associated with upregulation of *Socs3* and *Leprb* in adult males [33]. Also, exposure to a cafeteria diet during weaning affected directly energy balance-related gene expression in the hypothalami of male offspring [34]. In female rats, hypothalamic programming has been reported during gestation [31, 32], but not post-natal period [33, 34]. Although highly speculative, our results might be explained if the post-weaning period is not critical for hypothalamic programming in females.

Reduced adult adiposity observed in GLU+GAL-fed females might be due to differential effects on insulin signalling. The insulin response provoked by galactose (considering the direct effect) was small compared with the response to glucose (summarized in [35]). Moreover, galactose has been reported to improve insulin sensitivity in rats [36]. Thus, if also true in this nutritional model, insulin peaks in GLU+GAL-fed mice might be lower than in their GLU-fed counterparts. Differences in insulin release during a critical period of rapid growth and development might affect pancreatic development or the insulin response of peripheral tissues, ultimately impacting susceptibility to increased adiposity. While we obtained no evidential support for the first notion, the second notion is supported by our findings in peripheral WAT, where *Irs2* was down-regulated, suggesting less insulin was needed for adequate signal transduction.

Molecular analysis focussed on females because proposed programming effects of the GLU+GAL diet on adiposity were clear in this sex. A sex-dependent difference in metabolic programming has been seen previously [37-41]. This may be due to (one of) the various differences that exist between males and females, including circulating steroid hormones and body composition [42] and growth patterns. Independent of the origins of such differences, our results highlight the fact that sex-related dimorphism should be considered when studying metabolic programming effects in any species, including humans.

Our results suggest that a mixture of glucose and galactose, mimicking lactose, given in the post-nursing period, might protect against HFD-induced insulin resistance in a mouse model for human nutrition. It remains to be seen whether this finding can be translated to humans and whether lactose, indeed, explains the beneficial health effects of extended breastfeeding during the transition to solid foods. Some support for this theory has been provided by a meta-analysis of prospective cohort studies showing that consumption of dairy products, the major source of dietary lactose, reduces the risk of being overweight or obese in both children and adolescents [43]. Another meta-analysis found an inverse relationship between dairy consumption and adiposity in adolescents, although no effect was found in pre- and school-aged children [44]. Although it is tempting to speculate that these effects are due to lactose,

other dairy components, such as calcium and high-quality protein might also have a role [43], although adding lactose to a high-fat diet was associated with reduced weight gain and adiposity in rats, again favouring the direct effect of galactose [45].

We used glucose and galactose in a 1:1 ratio rather than lactose to ensure lactase deficiency did not interfere with our results, and because galactose metabolism is influenced by co-ingestion with glucose. Plasma galactose concentrations are lower when consumed with equimolar amounts of glucose[46], because of increased splanchnic clearance [47]. This is important in potential future applications, especially where galactose and glucose are used in preference to lactose, since the majority of adult humans (about 70%) are lactase deficient [48].

In conclusion, this study showed that replacing glucose with galactose in a post-weaning diet, in a 1:1 ratio (mimicking lactose), had beneficial metabolic programming effects in female mice, over glucose alone, characterized by lower adiposity, body weight, HOMA-IR index, and expression of insulin signalling components in WAT. Future research is needed to elucidate the mechanism(s) underlying these benefits and impact of such dietary modification on adiposity, insulin sensitivity, and energy balance in adulthood, as well as its translatability to humans. Nevertheless, this knowledge could be useful for determining (ga)lactose concentrations in infant and toddler formulas. Similarly, consumption of lactose – in those who are lactose tolerant as adults – might be beneficial over glucose alone, due to the persistence of galactose in the diet.

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Supplemental Material

Supplemental methods

Analysis of adipocyte size gWAT

After fixating, gWAT tissue samples were dehydrated and paraffin-embedded. Six samples per treatment were selected with the average and standard deviation of the FM (GLU: 6.19 ± 2.43 g; GLU+GAL: 3.24 ± 1.08 g; mean ± SD) and gWAT weight (GLU: 0.41 ± 0.16; GLU+GAL: 0.19 ± 0.08) being an accurate representation of the whole group (total FM: GLU: 6.04 ± 2.99 g; GLU+GAL: 3.21 ± 1.68 g; gWAT weight: GLU: 0.41 ± 0.25; GLU+GAL: 0.20 ± 0.13). Samples were cut in 5 μm sections using a HM350 microtome (Microm, Heidelberg, Germany) and mounted on Superfrost glass slides (Menzel-Gläser, Braunschweig, Germany). Four sections per sample were used, with a distance of 150 μm between sections to prevent double counting of the same adipocyte. Slides were deparaffinized, stained with Mayer's haematoxylin, and counterstained with eosin Y. Slides were dehydrated and dried overnight at 37°C. Pictures were taken with the Fluorescent rhodamine filter on the Leica DM6B microscope (Leica Microsystems, Wetzlar, Germany). Cell size was determined with the adipocyte Pipeline (Rodeheffer Lab) in CellProfiler software (Broad Institute Inc, Cambridge, MA, USA) for at least 1,000 intact adipocytes per sample. Area per adipocyte in pixels was converted to diameter in μm and used for further analysis. Broken adipocytes and areas with multilocular adipocytes were excluded.

Supplemental Table 1. Compositions of diets used in the study.

	Breeding diet	Glucose (GLU) (Post-weaning diet)	Glucose+ Galactose (GLU+GAL) (Post-weaning diet)	High fat diet (HFD)
Casein	200	200	200	235
L-cysteine	3	3	3	3
Wheat starch	380	153.1	153.1	285
Maltodextrin	100	153.1	153.1	100
Maltose	100	0	0	0
Glucose	50	323.7	161.9	70
Galactose	0	0	161.9	0
Coconut oil	12.6	12.6	12.6	37.8
Sunflower oil	49	49	49	147
Flaxseed oil	8.4	8.4	8.4	25.2
Cholesterol	0.03	0.03	0.03	0.097
Cellulose	50	50	50	50
Mineral mix*	35	35	35	35
Vitamin mix*	10	10	10	10
Choline bitartrate	2.5	2.5	2.5	2.5
Total energy (kcal/kg)	4032	4032	4032	4732
Protein en%	20	20	20	20
Carbohydrates en%	64	64	64	40
Fat en%	16	16	16	40

Ingredients are given in gram / kilogram, unless stated otherwise. En% = energy percentage. *Vitamin and mineral mixes as published previously [23]. Wheat starch was used as carrier for these mixes and this is taken along for energy density estimations of total diet and carbohydrates contributions.

Supplemental Table 2. Primer sequences, annealing temperatures, and pre-amplification.

| Gene Symbol	Target/ reference	Forward Primer	Reverse Primer	Annealing temperature	Pre-amplification
		gWAT			
Adipoq	Target	GGCAGGCATCCCAGGACATC	GCGGCTTCTCCAGGCTCTC	58.0	10 cycles
Acly	Target	TGGGCTTCATTGGGCACTACC	AGGGCTCCTGGCTCAGTTACA	61.0	10 cycles
Cpt1b	Target	ACCCCTAAGGATGCCATTCTTG	GCGGAAGCACACCAGGCAGTA	62.5	10 cycles
Cs	Target	ACAGTGAAAGCAACTTCGCC	GTCAATGGCTCCGATACTGC	57.5	10 cycles
Elovl6	Target	GGCACTAAGACCGCAAGGCA	GCTACGTGTTCTCTGCGCCT	60.5	10 cycles
Fabp4	Target	AATCACCGCAGACGACAGGAAG	TGCCCTTTCATAAACTCTTGTGGAAG	60.0	-
Fasn	Target	AGTTAGAGCAGGACAAGCCCAAG	TTCAGTGAGGCGTAGTAGACAGTG	58.0	-
Hadha	Target	GCTTTCGTCCTCTTCTGCTCA	AATGCAGCCTCTGGAGCGTA	58.0	10 cycles
Insr	Target	CATCATGTGGTCCGCCTTCT	CCGGTGCACAAACTTCTTGG	60.0	10 cycles
Irs1	Target	TTAGGCAGCAATGAGGGCAA	TCTTCATTCTGCTGTGATGTCCA	60.0	10 cycles
Irs2	Target	GCACCTATGCAAGCATCGAC	GCGCTTCACTCTTTCACGAC	60.0	10 cycles
Lep	Target	GGCTTTGGTCCTATCTGTCTTATGTTC	CCCTCTGCTTGGCGGATACC	63.5	10 cycles
Mest	Target	GATTCGCAACAATGACGGCA	ATCCAGAATCGACACTGTGG	56.0	10 cycles
Pck1	Target	GTTTGTAGGAGCAGCCATGAGATC	CCAGAGGAACTTGCCATCTTTGTC	58.0	10 cycles
Pdk4	Target	TCAGTGACTCAAAGACGGGAAACC	TGTGGTGAAGGTGTGAAGGAACG	62.5	10 cycles
Plin1	Target	CCAGGCTGTCTCCTCTACCAAAG	TCGATGTCTCGGAATTCGCTCTC	60.0	10 cycles
Pnpla2	Target	ACCACCCTTTCCAACATGCTACC	GCTACCCGTCTGCTCTTTCATCC	57.5	-
Suclg1	Target	TCAGCAGGCTTTGGAGTACG	TGTCAGTCTGTGCTTGACCC	58.0	-
B2m	Reference	CCCCACTGAGACTGATACATACGC	AGAAACTGGATTTGTAATTAAGCAGGTTC	60.0	-
Canx	Reference	GCAGCGACCTATGATTGACAACC	GCTCCAAACCAATAGCACTGAAAGG	57.5	10 cycles
Rplp0	Reference	CAATAAGGTGCCAGCTGCTGCTCG	GAAGAAGGAGGTCTTCTCGGGTCCT	63.5	-
Rps15	Reference	CGGAGATGGTGGGTAGCATGG	ACGGGTTTGTAGGTGATGGAGAAC	60.0	-
		sWAT			
Acacb	Target	TCTTCACGTTCAGAGCGAGGGAT	ATCTTGTGGTTGGCACAGGGCA	55.6	15 cycles
Acadl	Target	TGCACACATACAGACGGTGCAGC	GCAGAACCGGAGTCCAGACGT	60.0	-
Cpt1a	Target	See above		57.0	15 cycles
Fasn	Target	See above		58.0	15 cycles
Insr	Target	See above		60.0	15 cycles
Irs1	Target	See above		60.0	15 cycles
Irs2	Target	See above		60.0	15 cycles
Lep	Target	See above		60.0	15 cycles
Lipe	Target	CTCCAAGCAGGGCAAAGAAG	ACTGTGTCATCGTGCGTAAATCC	57.0	15 cycles
Mest	Target	See above		56.0	15 cycles
Pck1	Target	ACTGTTGGCTGGCTCTCACTG	TCTCAAAGTCCTCTTCCGACATCC	58.0	-
Pnpla2	Target	See above		60.0	-
B2m	Reference	See above		60.0	-
Rplp0	Reference	See above		60.0	-
Rps15	Reference	See above		60.0	-
		Hypothalamus			
Agrp	Target	CTACGTGCTACTGCCGCTTC	CTACAGAGGTTCGTGGCCG	58.0	16 cycles
Leprb	Target	GGTTTCACCAAAGATGCTATCGAC	CAGGCTCCAGAAGAAGAGGAC	58.0	16 cycles
Npy	Target	CCCGCCACGATGCTAGGTA	TCAGCCAGAATGCCCAAACA	58.0	16 cycles
Pomc	Target	CTTTCCGCGACAGGGGTC	CGTACTTCCGGGGGTTTTCA	62.0	16 cycles
Socs3	Target	CTCGCCTCGGGGACCATAGG	CGGGAAACTTGCTGTGGGTGAC	58.0	16 cycles
B2m	Reference	See above		60.0	16 cycles
Rps15	Reference	See above		60.0	16 cycles

Supplemental Table 3. Outcomes two-way repeated ANOVA'S for body weight, fat mass, and lean mass for male and female mice, fed GLU or GLU+GAL diet for 3 weeks post weaning, and thereafter a HFD for 9 weeks. Post-weaning and HFD periods were studied separately.

Sex	Para-meter	Period	Post-weaning diet*		Interaction Time x post-weaning diet		Time		Subjects	
			F value	*P* value	F value	*P* value	F value	*P* value	F value	*P* value
M	BW	Post-weaning	1.204	0.2843	2.268	0.0888	542.9	< 0.0001	9.266	< 0.0001
M	FM	Post-weaning	1.205	0.2842	2.205	0.0957	46.33	< 0.0001	2.437	0.0029
M	LM	Post-weaning	1.856	0.1869	2.165	0.1004	350.1	< 0.0001	7.902	< 0.0001
M	BW	HFD	3.906	0.0608	1.689	0.0936	224	< 0.0001	54.05	< 0.0001
M	FM	HFD	1.828	0.1901	0.819	0.5388	73.53	< 0.0001	14.35	< 0.0001
M	LM	HFD	8.885	0.0069	0.704	0.6218	192	< 0.0001	15.22	< 0.0001
F	BW	Post-weaning	0.797	0.3805	3.109	0.0314	275.3	< 0.0001	7.363	< 0.0001
F	FM	Post-weaning	0.687	0.4151	0.589	0.6242	41.47	< 0.0001	3.006	< 0.0001
F	LM	Post-weaning	1.662	0.2091	2.601	0.0583	208.4	< 0.0001	7.936	< 0.0001
F	BW	HFD	8.254	0.0082	5.147	< 0.0001	108.7	< 0.0001	34.41	< 0.0001
F	FM	HFD	7.746	0.0101	8.366	< 0.0001	28.89	< 0.0001	12.15	< 0.0001
F	LM	HFD	6.029	0.0214	0.170	0.9732	152.9	< 0.0001	16.03	< 0.0001

* Effect of the diet the mice received post-weaning: this means the direct effect is tested in the analysis on the post-weaning period, and the sustained (metabolic programmed) effect is tested in the HFD period.
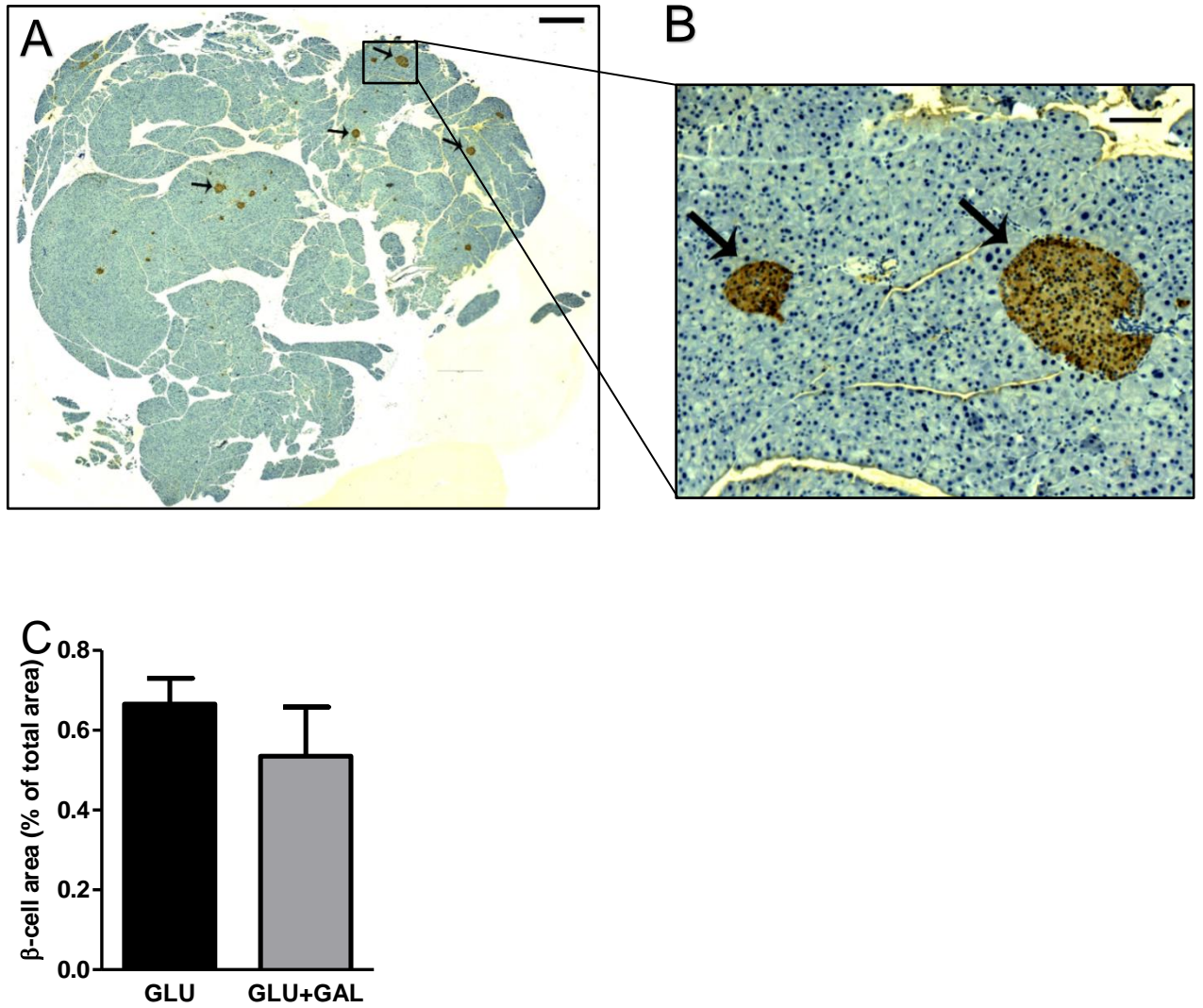
Supplemental Figure 1. Immunohistochemical analysis of the pancreas of female mice fed GLU or GLU+GAL diets post weaning for 3 weeks and thereafter a HFD for 9 weeks. (A) Mosaic picture of a whole pancreatic slice with DAB-staining for insulin, scale bar = 1 mm, 5x magnification, and (B) magnification of Islets of Langerhans stained for insulin, scale bar = 100 μm, 20x magnification. (C) Pancreatic β-cell area as percentage of total area. Values are given as mean ± SEM, n = 6.
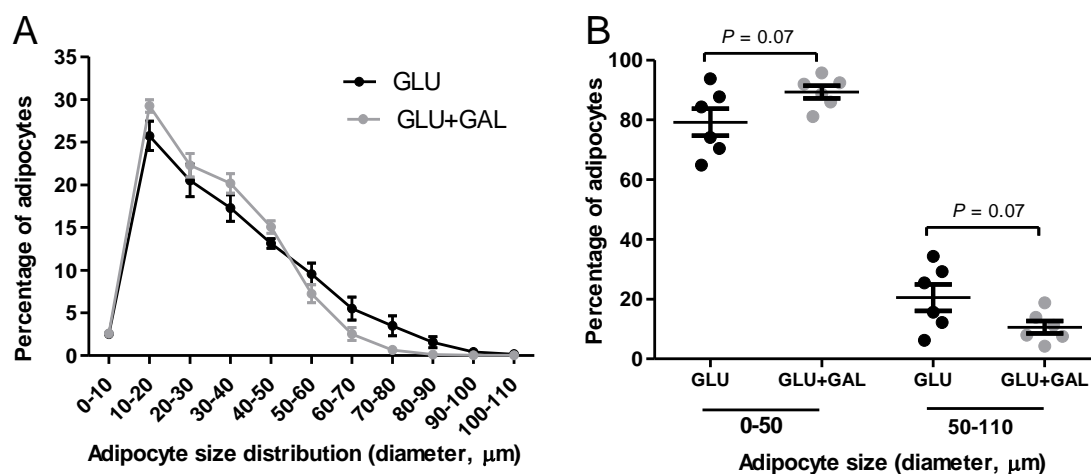
Supplemental Figure 2. Unilocular adipocytes size of the gonadal WAT depot of female mice fed GLU or GLU+GAL diets post weaning for 3 weeks and thereafter a HFD for 9 weeks. In figure (A) the adipocytes are clustered per bin of 10 μm. Two-way ANOVA showed a significant effect of early life diet ($P < 0.05$), of bin ($P < 0.001$), and of the interaction ($P < 0.05$); however, *post-hoc* tests showed no differences in bins. Figure (B) was created from the same data, although here only two bins were made: adipocytes < 50 μm in diameter, and of adipocytes ≥50 μm in diameter. Here a trend ($P = 0.07$) was observed for more smaller adipocytes and less larger adipocytes in the GLU+GAL-fed females than in the GLU-fed females. Values are given as mean ± SEM, *n* = 6, with individual dots representing individual mice (B).

Chapter 5

Partial replacement of glucose by galactose in the post-weaning diet improves parameters of hepatic health

Lianne M. S. Bouwman,

Hans J. M. Swarts,

José M. S. Fernández-Calleja,

Henk Schols,

Inge van der Stelt,

Annemarie Oosting,

Jaap Keijer,

Evert M. van Schothorst.

Abstract

Replacing part of glucose with galactose in the post-weaning diet beneficially affects later life metabolic health in female mice. The liver is the main site of galactose metabolism, but the direct effects of this dietary intervention on the liver in the post-weaning period are not known. The aim of this study was to elucidate this.

Weanling female mice (C57BL/6JRccHsd) were fed a starch containing diet with glucose (32 en%) monosaccharide (GLU), or a diet with glucose and galactose (1:1 both 16 en%) (GLU+GAL). Body weight, body composition, and food intake were determined weekly. After three weeks, mice were sacrificed, and serum and liver tissues were collected. Global hepatic mRNA expression was analysed and hepatic triglyceride (TG) and glycogen contents were determined by enzymatic assays.

Body weight and body composition were similar in both groups, despite higher food intake in mice on GLU+GAL diet. Hepatic TG content was lower in GLU+GAL-fed than GLU-fed females, while glycogen levels were unaffected. Analysis of global expression patterns of hepatic mRNA showed that mainly inflammation-related pathways were affected by the diet, which were predominantly downregulated in GLU+GAL-fed females compared with GLU-fed females. This reduction in inflammation in GLU+GAL-fed females was also reflected by decreased serum concentrations of acute phase protein Serum amyloid A 3.

In conclusion, replacing part of glucose with galactose in the post-weaning diet reduces hepatic TG content and hepatic inflammation.

Introduction

Breastmilk is the sole source of nutrition for offspring in the lactation period. The lactation period is followed by the weaning period: a period when solid foods are gradually introduced in the diet [1]. The weaning period is characterized by a diversification of the diet [2], in particular in the carbohydrate fraction. The main source of carbohydrate in breastmilk and infant formula is lactose, a disaccharide of the monosaccharides glucose and galactose [3], next to lower levels of the mammal milk oligosaccharides sialyllactose and fucosyllactose. In contrast, weaning foods contain a wide range of carbohydrates that are glucose and fructosebased [3]. Due to the gradual reduction in milk intake, the relative intake from lactose decreases gradually during weaning [3], and, consequently, so does the intake of galactose.

In contrast to glucose, which is extensively metabolised in the periphery [4], galactose is mainly metabolized in the liver [5-7]. Galactose elimination from the blood is used as a (clinical) measure for hepatic function, although outcome is to some extent dependent on clearance by kidney cells and the magnitude of hepatic blood flow [8]. Liver glycogen content was found to increase more rapidly after a glucose bolus than after a galactose bolus in adult rats [4]. However, an interaction exists between galactose and glucose metabolism. In humans, depleted liver glycogen replenishes more quickly with a drink containing maltodextrin and galactose, than with a drink with maltodextrin and glucose [9]. In rats, the relative contribution of galactose to hepatic glycogen synthesis is larger than that of glucose, when 10% of a glucose load is replaced with galactose [10]. Furthermore, splanchnic clearance of galactose is higher when consumed in combination with glucose [11].

Metabolism of galactose is via the Leloir pathway, that starts with the epimerization of α-D-galactose to β-D-galactose by galactose mutarotase (GALM), followed by the phosphorylation of β-D-galactose by galactokinase (GALK). Subsequently, galactose-1 phosphate is converted to UDP-galactose by galactose-1-phosphate uridylyl transferase (GALT) utilising UDP-glucose and releasing glucose-1 phosphate. The UDP-galactose is converted to UDP-Glucose by UDP-galactose-4-epimerase (GALE) [12]. Rate limiting in the elimination of galactose from the circulation is the activity of GALK [4, 13-15].

In a previous study, we investigated the programming effects of replacing part of the glucose with galactose in the post-weaning period in a mouse model of nutritional programming [16]. This replacement of glucose with galactose protected female mice against later-life body weight gain and adiposity. Given these long term effects and the important role of the liver in galactose metabolism, we are here interested in the direct effects on the liver, which may provide additional insight into the mechanisms underlying the beneficial programming effect.

The aim of this study was, therefore, to determine the effect of replacing half of glucose with galactose (16en%) in a post-weaning, starch containing (32 en%) diet (given for three weeks) on energy balance, and analysis focussed specifically on liver function and health by gene

expression. A diet with galactose in a 1:1 ratio with glucose (both 16 en%) was compared with a diet with glucose (32 en%) as monosaccharide. The study was performed in female mice, as we found metabolic programming only in this sex [16].

Materials and Methods

Animals and experimental setup

All animal experimental procedures were approved by the national and local Animal Experimental Committees (AVD1040020171668) and executed in compliance with EU directive 2010/63/EU. Animals were housed at 23°C ± 1°C, with a 12:12 light dark cycle, in Makrolon type II cages with bedding and nesting material, with ad *libitum* access to water and food. Breeding pairs (C57BL/6JRccHsd mice) were purchased from Envigo (Horst, The Netherlands) and time-mated while on a standard rodent chow diet (AM-II, AB Diets, Woerden, The Netherlands). Litters were randomized, and standardized to six pups per nest, with two to four female pups per litter, at postnatal day (PN) one or two. At PN21 (±1), female pups were weighed and their body composition (fat mass (FM) and lean mass (LM)) were determined with an EchoMRI 100V (Echo Medical Systems, Houston, TX, USA). Next, female pups were stratified by body weight (BW) and FM, housed individually, and placed on either a diet with 32 energy % (en%) glucose (*n* = 12; GLU) or a diet with 16 en% glucose and 16 en% galactose (*n* = 12; GLU+GAL) for three weeks. For better comparison, the monosaccharides glucose and galactose were used rather than the disaccharide lactose, because the (post-) weaning decline in lactase activity in mice [17], does not allow the use of lactose in this animal model. Diets were purchased from Research Diet Services BV (Wijk bij Duurstede, The Netherlands); details on the dietary composition are in Supplemental Table S1. BW, FM, LM, and food intake (FI) were determined weekly. Water intake (72 h) was recorded in a subset of the animals by subtracting the weight of the water bottle on PN39 from the weight of the water bottle on PN36. At PN42, food was removed at the start of the light phase. BW, FM, and LM were determined, and animals were sacrificed two to five hours after the start of the light phase by decapitation. Whole blood was collected in MiniCollect serum tubes (Greiner-Bio one B.V., Alphen aan de Rijn, The Netherlands), centrifuged at 3000g for 10 minutes (4°C), aliquoted, and stored at -80°C. Livers were collected and weighed, lobes were separated, snap frozen in liquid nitrogen, and stored at -80°C.

Indirect calorimetry

In a parallel cohort of female mice, treated identically as described above (*n* = 12 per group), indirect calorimetry measurements were performed from PN40 till PN42, using a Phenomaster LabMaster Metabolism Research Platform (TSE systems GmbH, Bad Homburg, Germany). Continuous measurements of oxygen consumption, carbon dioxide production, hydrogen production, activity, water intake, and FI were executed as described [18, 19], with the subsequent calculations of respiratory exchange ratio (RER), energy expenditure (EE).

Measurement intervals were 1.32 min per cage, which resulted in 3 measurements per cage per hour. The first day of the measurements was considered as adaptation and the next 24 h (one 12h light phase and one 12h dark phase) were analysed. For this cohort, only indirect calorimetry data are shown.

Serum measurements

Commercial assays were used to measure serum concentrations of the following hormones and metabolites: insulin (Ultra-Sensitive Mouse Insulin ELISA Kit, Crystal Chem, Elk Grove Village, IL, USA), glucose (glucose GOD-PAP kit, Roche, Mannheim, Germany), leptin (Bio-Plex Pro Mouse diabetes assays, Bio-Rad laboratories, Veenendaal, The Netherlands), triglycerides (TG) (liquicolour kit, Human), free fatty acids (FFA) (NEFA-HR(s), Fujifilm Wako Chemicals Europe GMBH, Neuss, Germany), ALT (ALT Activity Assay, Sigma-Aldrich, Zwijndrecht, The Netherlands) and Serum amyloid A 3 (SAA3) (Mouse SAA-3 ELISA, Merck chemicals B.V., Amsterdam, The Netherlands). All assays were performed according to the manufacturers' instructions; samples were tested in duplicate. HOMA-IR index was calculated as published [20] based on serum insulin and serum glucose concentrations, using a mouse-adjusted correction factor.

Hepatic measurements

Hepatic TG levels were determined in the right lobe as described previously [21]. Hepatic glycogen content in the right lobe was determined with the Abcam Glycogen Assay kit ab169558 (Abcam, Cambridge, UK). Briefly, liver tissue (50 mg/ml) was homogenized with a plastic pestle in demineralised water on ice, boiled for 10 minutes, and centrifuged at 13140 g for 10 minutes. Supernatant was diluted 80-fold in demineralised water, and 10 μl was used. The assay was carried out according to the manufacturer's instructions. Samples were tested in duplicate.

RNA isolation

Total RNA was isolated from the right liver lobe with RNeasy columns (Qiagen, Venlo, The Netherlands). RNA integrity was checked on an Agilent 2200 Tapestation according to the manufacturer's instructions (Agilent Technologies Inc, Santa Clara, CA, USA).

Genome wide gene expression

Two hundred ng RNA of each sample (n = 12 per group) was assayed using mouse whole genome 8×60K v.2 microarrays (Agilent Technologies Inc.), as published [22, 23]. Signals were extracted (Feature extraction version 10.7.3.1., Agilent), quality controlled and normalized as described [22]. All microarray data are Minimum Information About a Microarray Experiment (MIAME) compliant and stored in Gene Expression Omnibus (GEO; GSE124929). Analysis of pathway maps using all expressed genes with P-value < 0.05 was performed with MetaCore (version 6.37; Thomson Reuters, New York, NY, USA). Genes enriched in the top 10 pathway maps were gathered and presented as a heatmap using GeneMaths XT 2.12 (Applied Maths,

Sint-Martens-Latem, Belgium), based on transcript mean centred data. In addition, a pre-defined set of genes (i.e. genes involved in galactose metabolism, insulin signalling, pyruvate dehydrogenation, gluconeogenesis, fatty acid synthesis, lipolysis and fatty acid beta-oxidation) was studied separately, we show the most significant probe per gene. Statistics were performed on log 2 transformed data.

RT-qPCR

To confirm micro-array results, quantitative Real-Time Reverse-Transcription Polymerase Chain Reaction (RT-qPCR) was carried out as described [24]. Data were normalized against β-2 microglobulin (*B2m*), Calnexin (*Canx*), and ribosomal protein S15 (*Rps15*). Average gene expression per gene of GLU-fed female mice was set to 1. For an overview of primer sequences and annealing temperatures, see Supplemental Table S2.

Western Blot

Frozen liver tissue was grinded on liquid nitrogen, placed in ice-cold Tris-NaCL lysis buffer (50mM, 150mM) pH 7.4, with glycerol (10%), triton (1%) EDTA (1 mM), trichostatin A (2 μM), nicotinamide (10 mM), complete protease inhibitor cocktail (Roche), and phosphatase inhibitor-Mix I (Roche) and stored at -80°C. Next, samples were thawed on ice, homogenized by sonication, centrifuged for 10 minutes (13140g) at 4°C. Protein concentrations were determined in the supernatant with the DC protein assay (Bio-Rad). Subsequently, samples were diluted to the same concentration, mixed with LDS loading (Fisher scientific, Landsmeer, The Netherlands) buffer and DTT, and heated to 95°C for 10 minutes. Samples were loaded (25 μg protein for signal transducer and activator of transcription 1 (STAT1); 10 μg protein for SAA3) on 4-12% Nupage Bis-Tris gels (Fisher scientific) and ran for 40 minutes at 110 V and 60 minutes at 150 V. Next, protein was transferred (1 hour for STAT1, 45 minutes for SAA3, 300 mA) to a methanol-activated Immobilon-FL PVDF membrane (0.45 μm pore size, Merck). Protein transfer was verified with a Ponceau staining, and blots were rinsed with TBS+ 0.1% tween (TBSt) until no more Ponceau staining was visible. Membranes were blocked with TBSt+ 5% BSA for 1 hour at room temperature. Blots were cut, and incubated overnight at 4°C with anti-STAT1 antibody (1:200; Abcam Cat. # ab2415; Abcam); Anti-SAA3 antibody (1:2000; Abclonal Cat. # A11948; Abclonal, Woburn, MA, US) or β-ACTIN antibody (1:5000; Abcam Cat. # ab2415; Abcam). Blots were washed with TBSt (6x 5min) and incubated with secondary antibody at room temperature for 1 hour; using donkey-anti rabbit IRDye800 (1:15000; LI-COR # 926-32213; LI-COR , Lincoln, NE, USA) for STAT1 and SAA3; and donkey-anti mouse IRDye680 (1:10000; LI-COR # 926-32222; LI-COR) for β-ACTIN. After washing (4x5 min TBSt, 1x 5min TBS), blots were scanned at 800 nm and at 700 nm (β-ACTIN) on an Odyssey scanner (LI-COR). Intensities were quantified using Odyssey software V3.0 (LI-COR).

Statistics

Graphpad Prism 5.04 (Graphpad Software, San Diego, CA, USA) was used for statistical analysis. BW, FM, LM, and FI were analysed using a two-way ANOVA (diet and time as

variables), with *post-hoc* Bonferroni testing. For all other data, independent Students' *t*-tests (with Welch's correction when applicable) were used to compare normally distributed data; Mann-Whitney U tests were used when data were not normally distributed. Normality was checked using a Saphiro-Wilk normality test; when data were not normally distributed, data were log transformed and rechecked for normality. *P*-values < 0.05 were considered significant, and indicated as follows: * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$.

Results

Body weight and food intake

Mice fed the glucose+galactose diet (GLU+GAL) for three weeks had similar body weights as mice fed the glucose diet (GLU) (Fig. 1A). In line with this finding, body composition analysis showed that lean mass (Fig. 1B) and fat mass (Fig. 1C) were not affected by the post-weaning diet. In addition, gonadal and mesenteric fat pad weights were similar among both groups (data not shown). Food intake was, however, consistently higher on GLU+GAL diet in the three weeks of the intervention, and cumulatively the mice on the GLU+GAL diet consumed approximately 8.5% more than the mice on the GLU diet (Fig. 1D).
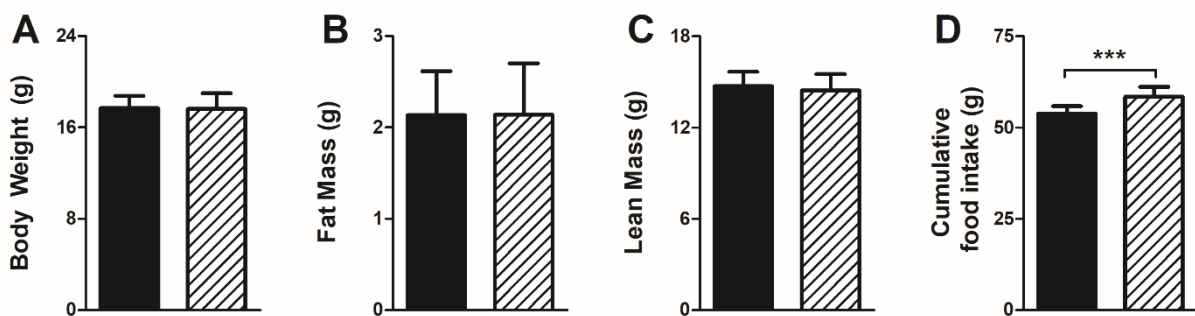

Figure 1. Body weight, body composition, and cumulative food intake in females fed control GLU diet (black bars) or GLU+GAL diet (striped bars). (A) Body weight, (B) lean mass, and (C) fat mass at the end of the study. (D) Cumulative food intake over the three-week study period. Values are expressed as mean ± SD (*n* = 12).

Indirect calorimetry

To investigate metabolism and substrate utilisation of animals on the GLU and GLU+GAL diets in more detail, animals were studied in an indirect calorimetry system. RER patterns showed no clear difference between GLU- and GLU+GAL fed females (Fig. 2A). No differences were found between GLU- and GLU+GAL-fed females in average 24 h RER (Fig. 2B) , nor in average RER in the light or the dark phase separately (Table 1). In contrast, the maximum RER reached during the 24 h measurement was significantly higher in GLU-fed females than in GLU+GAL-fed females (Fig. 2C), while the minimal RER was not affected (Fig. 2D). Energy expenditure (EE) was not different during the 24 h period (Fig. 2E), nor during the light phase or the dark phase only (Table 1), nor was activity different (Fig. 2F and Table 1). During the 24 h indirect
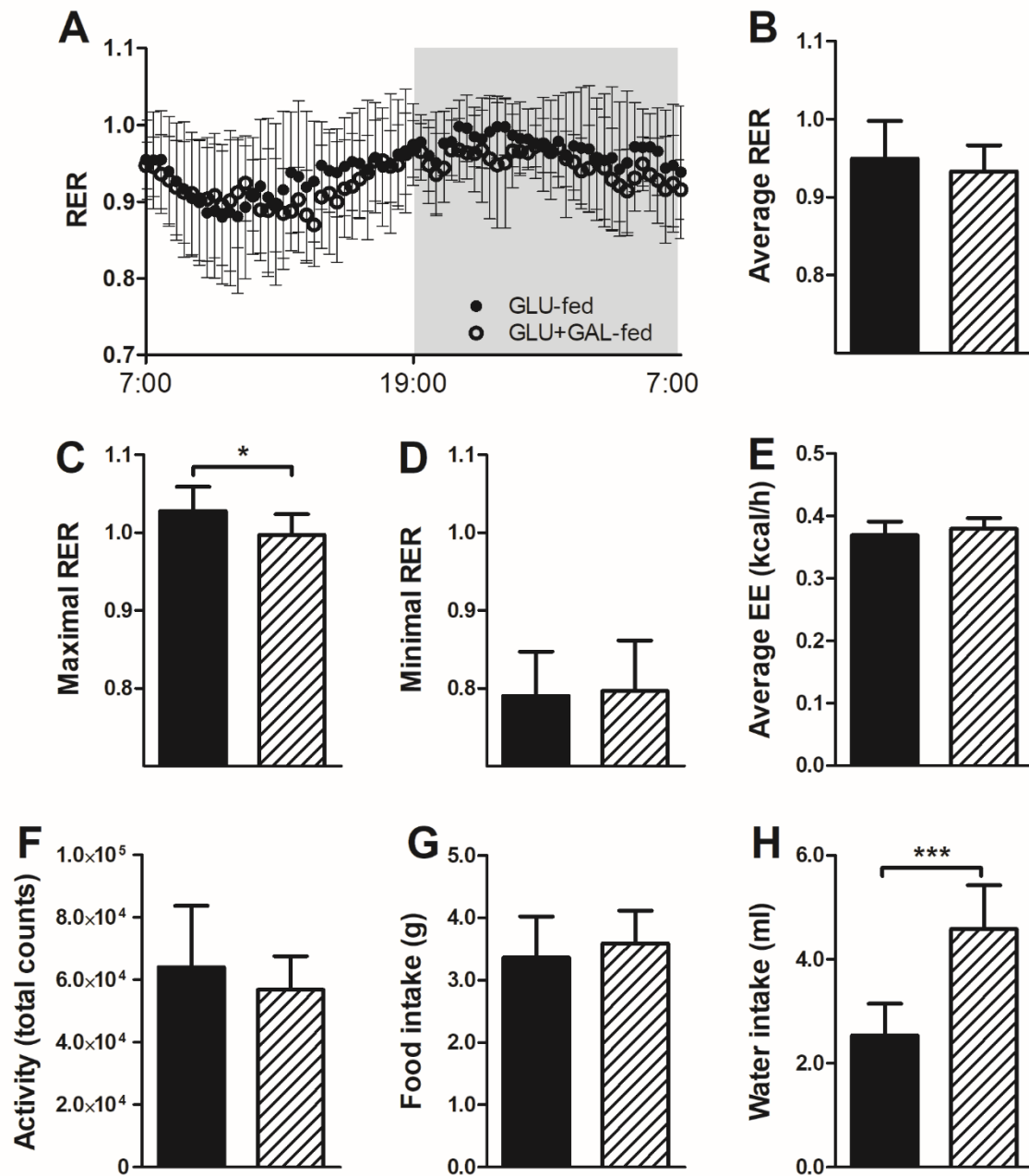
Figure 2. Twenty-four hour indirect calorimetry recordings of females fed control GLU diet (black dots and black bars) or GLU+GAL diet (white dots and striped bars). (A) 24 h Respiratory exchange ratio (RER) pattern, (B) average 24 h RER, (C) average of the three highest consecutive RER values), (D) average the three lowest consecutive RER values, (E) 24 h energy expenditure (EE), (F) total activity, (G) 24 h food intake, and (H) 24 h Water intake. Values are expressed as mean ± SD (n = 11-12).

calorimetry recording, there was no significant difference in food intake between the groups (Fig. 2G). Water intake was, however, significantly higher in the animals on the galactose diet (Fig. 2H), both during the light phase and the dark phase (Table 1). Hydrogen production was not affected by the diet (data not shown).

Water intake was also significantly increased in GLU+GAL-fed females outside the indirect calorimetry system, as 72 hours water intake in normal home cages was increased by ~70% (8.6 ± 1.3 mL in GLU *vs* 14.7 ± 2.2 mL in GLU+GAL; P < 0.0001; *n* = 11 - 12). The analysis of urinary galactose content in a subset of animals, showed that urine of GLU+GAL-fed females contained detectable levels of galactose, while galactose was absent from the urine of GLU-fed females (Supplemental Fig. S1).

Table 1. Indirect calorimetry results for respiratory exchange ratio (RER), energy expenditure (EE), activity, food intake, and water intake, split for light phase and dark phase. Values are expressed as mean ± SD (*n* = 11-12).

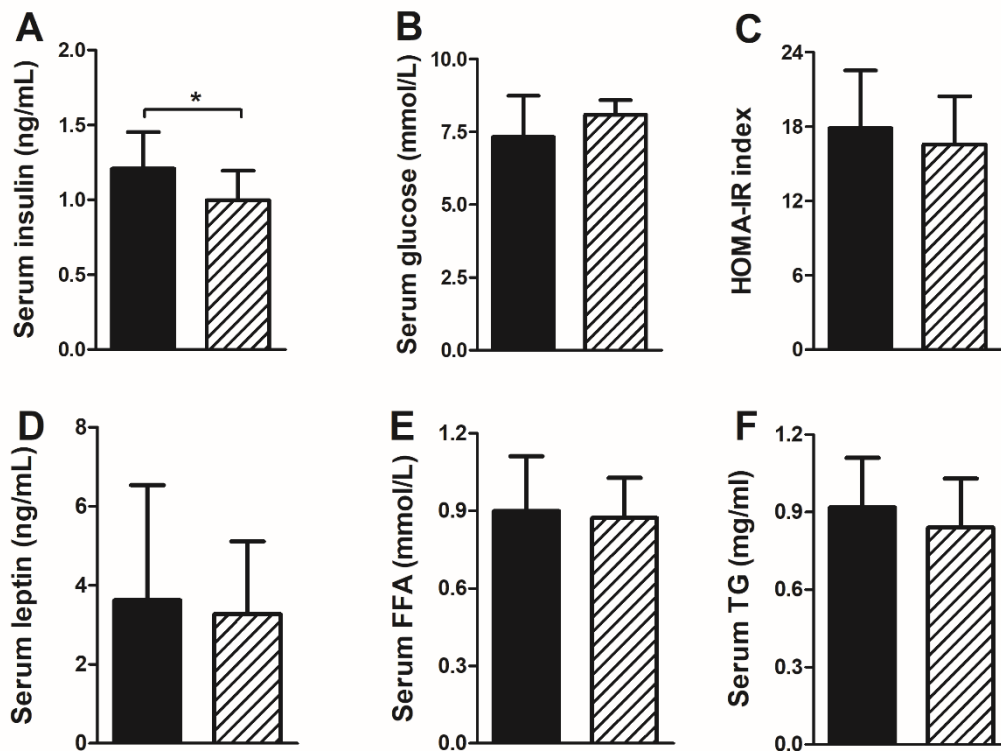|	Average RER	Average EE (kcal/h)	Activity(total counts*1000)	Food intake (g)	Water intake (mL)
			Light phase		
GLU	0.927 ± 0.056	0.328 ± 0.027	15.0 ± 5.7	1.04 ± 0.41	0.67 ± 0.29
GLU+GAL	0.917 ± 0.038	0.347 ± 0.019	16.0 ± 3.9	1.11 ± 0.30	1.35 ± 0.35***
			Dark phase		
GLU	0.970 ± 0.049	0.411 ± 0.023	49.0 ± 15.8	2.32 ± 0.58	1.82 ± 0.49
GLU+GAL	0.950 ± 0.036	0.412 ± 0.018	40.8 ± 9.98	2.48 ± 0.48	3.23 ± 0.64***

Figure 3. Serum concentrations and HOMA-IR in females fed control GLU diet (black bars) or GLU+GAL diet (striped bars). (A) Serum insulin concentrations, (B) serum glucose concentrations, (C) HOMA-IR index, (D) serum leptin concentrations, (E) serum FFA concentration, and (F) serum TG concentrations. Values are expressed as mean ± SD (*n* = 10-12).

Serum and hepatic parameters

Despite higher overall food intake, serum insulin concentrations were significantly lower in the GLU+GAL animals (Fig. 3A). Serum glucose concentrations were not significantly different (Fig. 3B), neither was the HOMA-IR (Fig. 3C), a surrogate marker for insulin resistance. Neither serum leptin (Fig. 3D), nor serum FFA (Fig. 3E) nor serum TG (Fig. 3F) were significantly affected by the different monosaccharide composition of the diets.

Overall liver mass was not affected (Fig. 4A), yet analysis of hepatic triglyceride (TG) levels indicated that these were lower in the GLU+GAL-fed females (Fig. 4B). Hepatic glycogen levels was not affected by the diet (Fig. 4C). Serum ALT, a marker for hepatocellular damage [25], was not different between GLU- and GLU+GAL-fed mice (Fig. 4D).
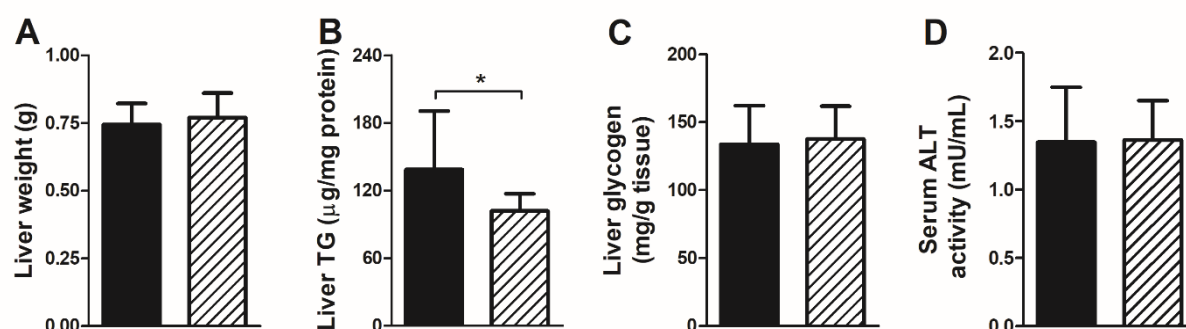

Figure 4. Liver-related parameters in females fed control GLU diet (black bars) or GLU+GAL diet (striped bars). (A) Whole liver weight, (B) liver TG content, (C) liver glycogen content, (D) and serum ALT concentrations. Values are expressed as mean ± SD (n = 11-12).

Transcriptomics

Next, we analysed the hepatic transcriptome. Pathway analysis showed that the ten most affected pathways were inflammation related (Fig. 5A). With genes occurring in two or more pathways within these ten most regulated pathways, a heatmap was created (Fig. 5B). This shows that the majority of the (immune-related) regulated genes were lower expressed in the livers of GLU+GAL-fed *vs* GLU-fed females (Fig. 5). Next, carbohydrate and lipid metabolism genes, gene involved in galactose metabolism, insulin signalling, pyruvate dehydrogenation, gluconeogenesis, fatty acid synthesis, lipolysis and fatty acid beta-oxidation, were studied separately. *Galk2* expression was slightly higher in GLU+GAL-fed females, and *Elovl5* expression slightly lower (Supplemental Fig. 2) In addition, expression of *Akt1* and *Pdpk1* were significantly different between groups, although in opposite directions (downregulated and upregulated in GLU+GAL-fed females, respectively). All other genes were expressed similarly in both dietary groups (Supplemental Fig. 2), in agreement with the lack of appearance of these processes in the pathway analysis.
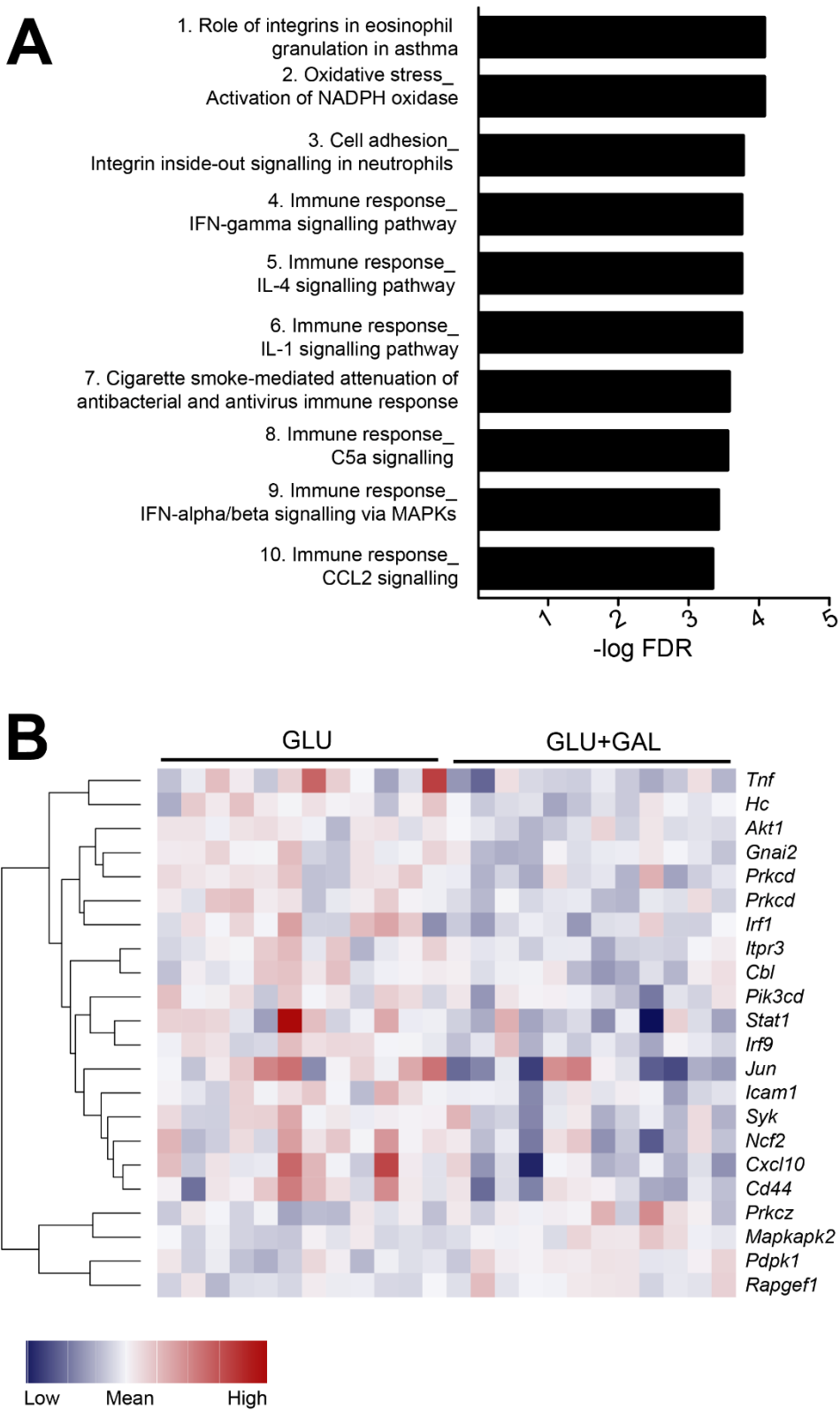
Figure 5. Most affected pathways and concurrent genes in whole genome gene expression analysis of liver tissue. (A) The most affected pathways in hepatic gene expression in GLU+GAL- *vs* GLU-fed female mice, with their relative false discovery rate (FDR)-adjusted *P*-value. (B) Heat map with cluster analysis was performed on probes occurring in at least 2 of the 10 most regulated pathways with a significant difference. Heat map columns (Legend continues on next page)

represent individual samples, colours indicate mean transcript centered data. *Akt1:* Thymoma viral proto-oncogene 1; *Cbl:* Casitas B-lineage lymphoma; *Cd44:* CD44 antigen; *Cxcl10:* Chemokine (C-X-C motif) ligand 10; *Gnai2:* Guanine nucleotide binding protein (G protein), alpha inhibiting 2; *Hc:* Hemolytic complement; *Icam1:* Intercellular adhesion molecule 1; *Irf1:* Interferon regulatory factor 1; *Irf9:* Interferon regulatory factor 9; *Itpr3*: Iinositol 1,4,5-triphosphate receptor 3; *Jun:* jun proto-oncogene; *Mapkapk2:* MAP kinase-activated protein kinase 2; *Ncf2:* Neutrophil cytosolic factor 2; *Pdpk1:* 3-phosphoinositide dependent protein kinase 1; *Pik3cd:* Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit delta; *Prkcd:* Protein kinase C, delta; *Prkcz:* Protein kinase C, zeta; *Rapgef1:*Rap guanine nucleotide exchange factor (GEF) 1; *Stat1:*Signal transducer and activator of transcription 1; *Syk:* Spleen tyrosine kinase; *Tnf:* Tumor necrosis factor.

Figure 6. Hepatic *Saa3* gene and SAA3 protein expression in females fed control GLU diet (black bars) or GLU+GAL diet (striped bars). (A) Hepatic *Saa3* by microarray analysis, (B) hepatic *Saa3* expression by RT-qPCR, (C) representative picture of SAA3 western blot in liver samples using β-actin as loading control, (D) semi-quantification of hepatic SAA3 protein by western blot, and (E) serum SAA3 concentrations. Values are expressed as mean ± SD (n = 11-12).

Protein expression

To verify the effect on inflammation, acute phase protein SAA3, was studied in detail. The microarray data showed a significant downregulation of hepatic *Saa3* mRNA expression in the

livers of GLU+GAL-fed females (Fig. 6A), and this was confirmed by RT-qPCR (Fig. 6B). Although SAA3 protein concentrations in liver homogenates were unaffected (Fig. 6C and 6D), serum SAA3 concentrations were significantly lower in GLU+GAL-fed females than in GLU-fed females (Fig. 6E). The microarray data also showed a significant lower expression of *Stat1* mRNA in the GLU+GAL fed females (fold change -1.3, data not shown). This was also measured by RT-qPCR, but the difference did not reach statistical significance. Similarly, a non-significant downregulation was found in both the α- and the β-isoform of STAT1 protein in GLU+GAL-fed females (Fig. 7).
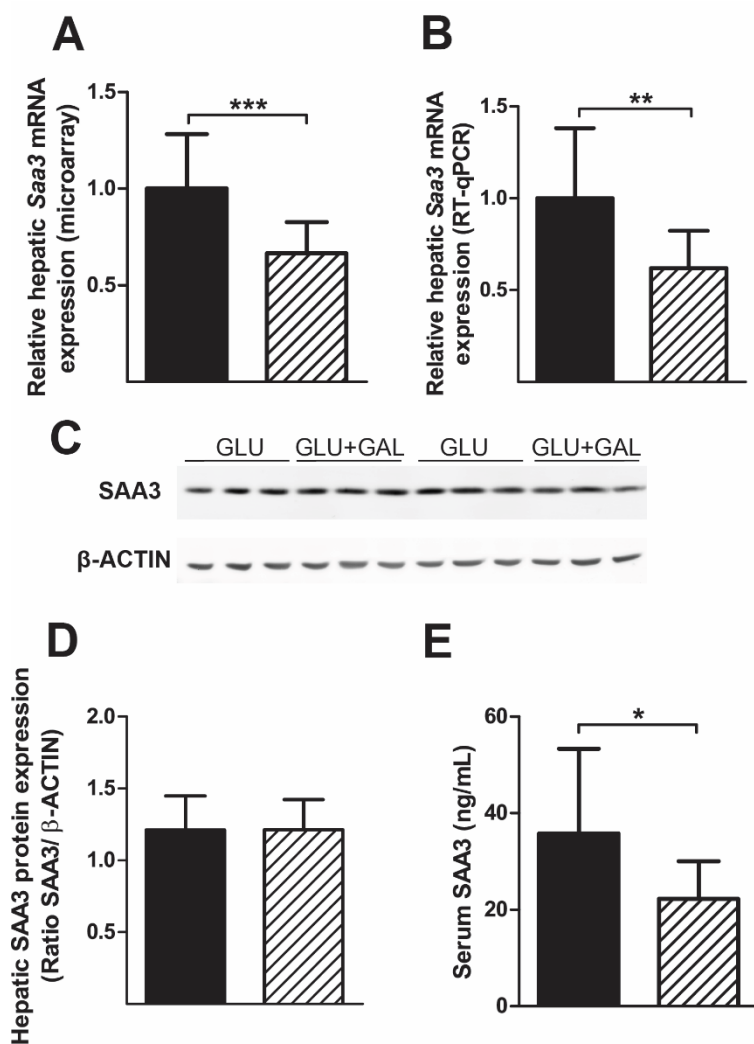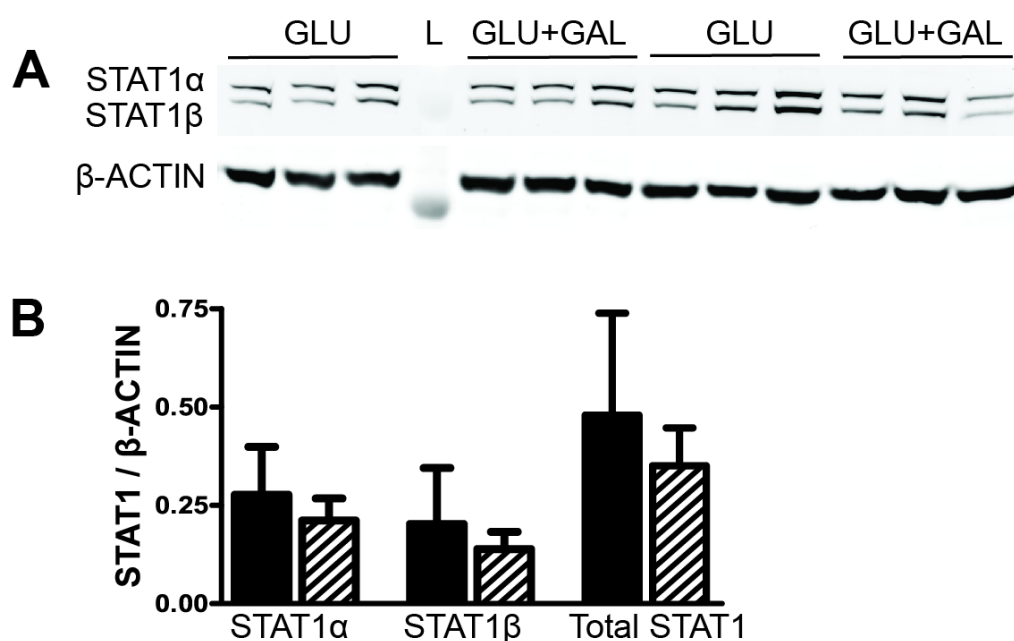

Figure 7. Hepatic STAT1 protein expression in females fed control GLU diet (black bars) or GLU+GAL diet (striped bars). (A) Representative picture of STAT1 western blot on liver samples using β-actin as loading control. (B) Semi-quantification of STAT1α, STAT1β, and the combination of both isoforms. L = ladder; values are expressed as mean ± SD (n = 10-12).

Discussion

In this study, we investigated the effect of replacing part of dietary glucose with galactose on energy balance and hepatic gene expression in the post-weaning period in female mice. This replacement resulted in slightly higher food intake (~ 8.5%) and substantially higher water intake (~2 fold), without differences in body weight, body composition, or energy expenditure. In addition, it led to lower fasted serum insulin concentrations and lower hepatic triglyceride levels. In depth analysis of hepatic gene expression indicated a downregulation of inflammation-related pathways, and this downregulation was confirmed by lower serum SAA3 concentrations.

In general, hepatic lipid storage is considered an adverse condition. Accumulation of fats in the liver that is not caused by (excessive) alcohol intake is called non-alcoholic fatty liver

disease (NAFLD). In 45-55% of human patients with NAFLD, the disease develops into non-alcoholic steatohepatitis (NASH) over a three-year period, a process that is characterized by increased liver inflammation [26]. NASH may subsequently lead to hepatic cirrhosis and hepatocellular carcinomas [26]. NAFLD is linked to excessive dietary fat intake, but hepatic TG accumulation can also be caused by high glucose intake. Four weeks of feeding with a high glucose diet (60% w/w) was shown to increase hepatic TG levels in adult male mice [27]. In humans, overfeeding with glucose (3.0 g /kg BW /day, corresponding to a 37% increase in energy intake) caused an increase in hepatic lipids of about 60% [28]. Hepatic lipid accumulation predisposes to activation of Kupffer cells, macrophages that reside in the liver, and the recruitment of peripheral macrophages, which plays central role in disease progression [29]. Here, the GLU+GAL-fed females had lower hepatic TG levels and a lower inflammation-related gene expression, thus likely healthier livers than GLU-fed females. Serum SAA3 concentrations and hepatic SAA3 expression were lower in GLU+GAL-fed females, and since SAA3 is mainly expressed by macrophages [30], this lower hepatic SAA3 expression is likely the result of lower macrophage activation and infiltration in the liver. The transcriptome results indicate that immune cell related pathways were affected, rather than general or hepatocyte specific pathways (Fig. 5B). This difference in liver health occurred without differences in serum ALT concentrations (Fig. 4D), but this is plausible given the fact that normal serum ALT concentrations can be present in patients with NAFLD, even those with hepatic steatosis [31, 32].

Similar to the results obtained here, food intake on the GLU+GAL diet was increased in female and male mice in our previous study [16]. In both studies the increased food intake did not result in increased body weight or fat mass. Our current indirect calorimetry measurements indicate that the higher food intake did not result from a higher energy expenditure. The indirect calorimetry measurements strikingly showed that GLU+GAL-fed females had a much higher water intake, which was confirmed in a subset of animals in regular housing. Food and water intake generally have a linear relationship of about 1:1 in rodents [33, 34]. Although water intake was not exactly proportional to the increase in food intake, the increase in water and food intake led us to hypothesize that the increase in food and water intake were to compensate higher urinary losses, possibly related to loss of galactose. Indeed, we found galactose to be present in the urine of GLU+GAL-fed females, with widely varying concentrations (Supplemental Fig. S1). Galactose has been found in urine before, and the amount is related to intake. High concentrations of urinary lactose correlate with high dairy intake, the major source of dietary galactose [35]. The relative excretion of galactose depends on the dose given; for example, about one percent of galactose was lost in 24 h urine in healthy human subjects with a 7 mg/kg BW dose of galactose [36], as well as in wild type mice after a 10 μmol single gavage dose (GALT knockouts excreted ~35%) [37]. However, 35% of a 1g/kg BW galactose dose was lost in urine in the first two hours after administration in adult rats [4], and 51% was lost after a 4 g/kg BW dose of galactose [38]. A healthy kidney reabsorbs most glucose after filtration, but SGLT2, the transporter responsible for about 90% of the glucose transport, transports galactose with only 16% efficiency (for the human variant) [39].

We speculate that because of the lower galactose reabsorption, more water is retained in the urine, leading to greater urinary water loss as well, and thus causing the animals on the GLU+GAL diet to drink more to compensate. Unfortunately, we were unable to do a 24 h collection of urine and the loss of galactose could not be determined quantitatively. Hence, we could not calculate whether the loss of galactose could fully explain the need for the increased food intake to maintain energy balance. However in case it would fully explain the increased food intake, about 40% of the ingested galactose would be lost (under the assumptions that the energy need is the same for both dietary groups and that all other nutrients are used completely). We did not observe any difference between the groups in hydrogen production by the gut microbiota, suggesting that a similar amount of nutrients reached the colon and that galactose, like glucose, is indeed fully absorbed in the small intestine.

Results of the whole genome gene expression analysis showed no clear molecular adaption to the galactose in the post-weaning, because only the *Galk2* transcript was modestly upregulated, and not the other transcripts in the Leloir pathway (*Galm, Galk1, Galt, Gale*). Because GALK activity is considered rate-limiting in the elimination of galactose from the circulation [4, 7, 13-15], a minor adaptation may be present. This may be because mice may have a limited ability to adjust hepatic metabolism to dietary galactose. In line, four weeks of galactose diet (40 en%) did not increase oxidation rate of 1-^{14}C-labelled galactose in wild type mice [40]. Alternatively, regular transcript levels are high enough to allow for an efficient metabolism of galactose.

The urinary loss of galactose and concomitant increase in food intake resulted in an altered macronutrient usage. This is supported by maximal RER, which was lower in the GLU+GAL fed females compared with the GLU fed females (Fig. 2C). This may have led to a lower hepatic lipid load or a lower hepatic *de novo lipogenesis* in GLU+GAL fed females, resulting in lower hepatic TG levels (Fig. 4B). It is likely that, overall, carbohydrates were sufficiently available for the GLU+GAL-fed females as the diet contained 32 en% starch and 16 en% glucose. This is supported by similar hepatic glycogen levels (Fig. 3C).

Concluding, replacing part of glucose with galactose in the post-weaning diet, affects energy intake but not body weight nor body composition. Hepatic health is positively affected by the replacement, with a lower inflammatory gene expression profile, and a lower hepatic triglyceride level, suggesting a reduced risk to develop fatty liver disease. Since hepatic liver inflammation and lipid accumulation are associated not only with NALFD, but also with increased insulin resistance [41], the changes observed in the liver of these 6 week old female mice, may be related to the increased later-life insulin sensitivity that was seen for mice on the GLU-GAL diet [16].

Authors' disclosure

AO is employee of Danone Nutricia Research B.V., Utrecht, The Netherlands. The other authors declare no conflict of interest.

Author contributions

LB, JFC, HSc, AO, JK, and EvS designed the research; LB, HSw, JFC, and IvdS performed the experiments; LB, IvdS, HSc, and EvS analyzed data; LB drafted the manuscript. LB, EvS, AO, and JK interpreted the data and finalized the manuscript. All authors read and approved the final manuscript.

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Supplemental material

Supplemental Table 1. Dietary composition and energy content.

	Glucose diet		Glucose+Galactose diet	
	Mass (g)	Energy (kcal)	Mass (g)	Energy (kcal)
Casein	200.0	800	200.0	800
L-cysteine	3.0	12	3.0	12
Wheat starch	278.0	1112	278.0	1112
Sucrose	0.0	0	0.0	0
Glucose	322.0	1288	161.0	644
Fructose	29.5	118	29.5	118
Galactose	0.0	0	161.0	644
Coconut oil	12.6	113	12.6	113
Sunflower oil	49.0	441	49.0	441
Flaxseed oil	8.4	76	8.4	76
Palm oil	0.0	0	0.0	0
Cholesterol	0.030	0	0.030	0
Cellulose	50.0	0	50.0	0
Mineral mix*	35.0	31	35.0	31
Vitamin mix*	10.0	39	10.0	39
Choline bitartrate	2.5	0	2.5	0
Total energy (kcal/kg)	1000	4030	1000	4030
Protein en%		20		20
CHO en%		64		64
Fat en%		16		16

* Wheat starch as carrier for the mineral and vitamin mix.

Supplemental Table 2. Overview Primer sequences and annealing temperatures.

Gene	Type	Forward Primer (5'-3')	Reverse Primer (5'-3')	Annealing temperature
S100a8	Target	ACTTCGAGGAGTTCCTTGCG	TGCTACTCCTTGTGGCTGTC	58°C
Saa3	Target	AAAGAAGCTGGTCAAGGGTC	TGTCCCGTGAACTTCTGAAC	58°C
Stat1	Target	CCTGCGTGCAGTGAGTGAGT	CCACTGTGACATCCTTGAGATTCC	60°C
B2m	Reference	CCCCACTGAGACTGATACATACGC	AGAAACTGGATTTGTAATTAAGCAGGTTC	60°C
Canx	Reference	GCAGCGACCTATGATTGACAACC	GCTCCAAACCAATAGCACTGAAAGG	60°C
Rps15	Reference	CGGAGATGGTGGGTAGCATGG	ACGGGTTTGTAGGTGATGGAGAAC	60°C

Supplemental Method

High-Performance Anion-Exchange Chromatography (HPAEC)

Urine was collected from a subset of mice, once during the light-phase without controlling for the conditions, and once food deprived at sacrifice. Mice were placed in a clean, empty cage; their urogenital area was gently touched to stimulate urination if needed. Urine was pipetted and stored at -20°C. Unfortunately, urine samples could not be collected for all mice. HPAEC was performed on a ICS5000 High Performance Anion Exchange Chromatography system with Pulsed Amperometric detection (ICS5000 ED) (Dionex Corporation, Sunnyvale, CA, USA) equipped with a CarboPac PA-1 column (250 mm × 2 mm i.d.) and a CarboPac PA guard column (25 mm × 2 mm i.d.). The two mobile phases were (A) 0.1 M NaOH and (B) 1 M NaOAc in 0.1 M NaOH and the column temperature was 20 °C. We used the following gradient method at a flow rate of 0.4 mL/min: 0-27 min 100% water; followed by 5 min 100% B and 10 min 100% A with an equilibration period with 100% water for 15 min. Post column NaOH, 0.5 M was added to the column effluent to keep the solvent flow to the detector at pH 12.5. Data handling was done using Chromeleon version 7 (Thermo Fisher Scientific, Waltham, MA, USA). Galactose was used as standard. For samples without a detectable galactose peak, urinary galactose levels were set at half the level of the lowest standard (n = 7 for GLU-fed females in not controlled conditions, and n = 3 for food deprived animals).

Supplemental Figures
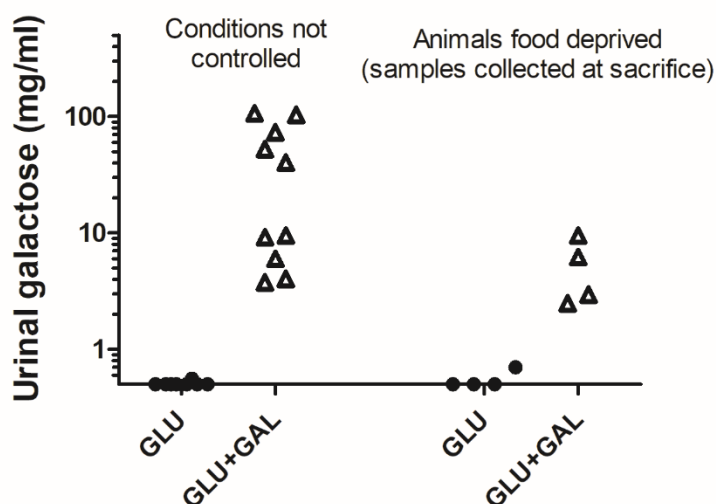
Supplemental Figure 1. Urinary galactose levels as determined by HPAEC. For samples without a detectable galactose peak, urinary galactose levels were set at half the level of the lowest standard (and multiplied by the dilution factor 400; resulting in 0.50 mg/mL). Dots represent individual samples of GLU-fed females, triangles represent individual samples of GLU+GAL-fed females (n = 4 - 10).
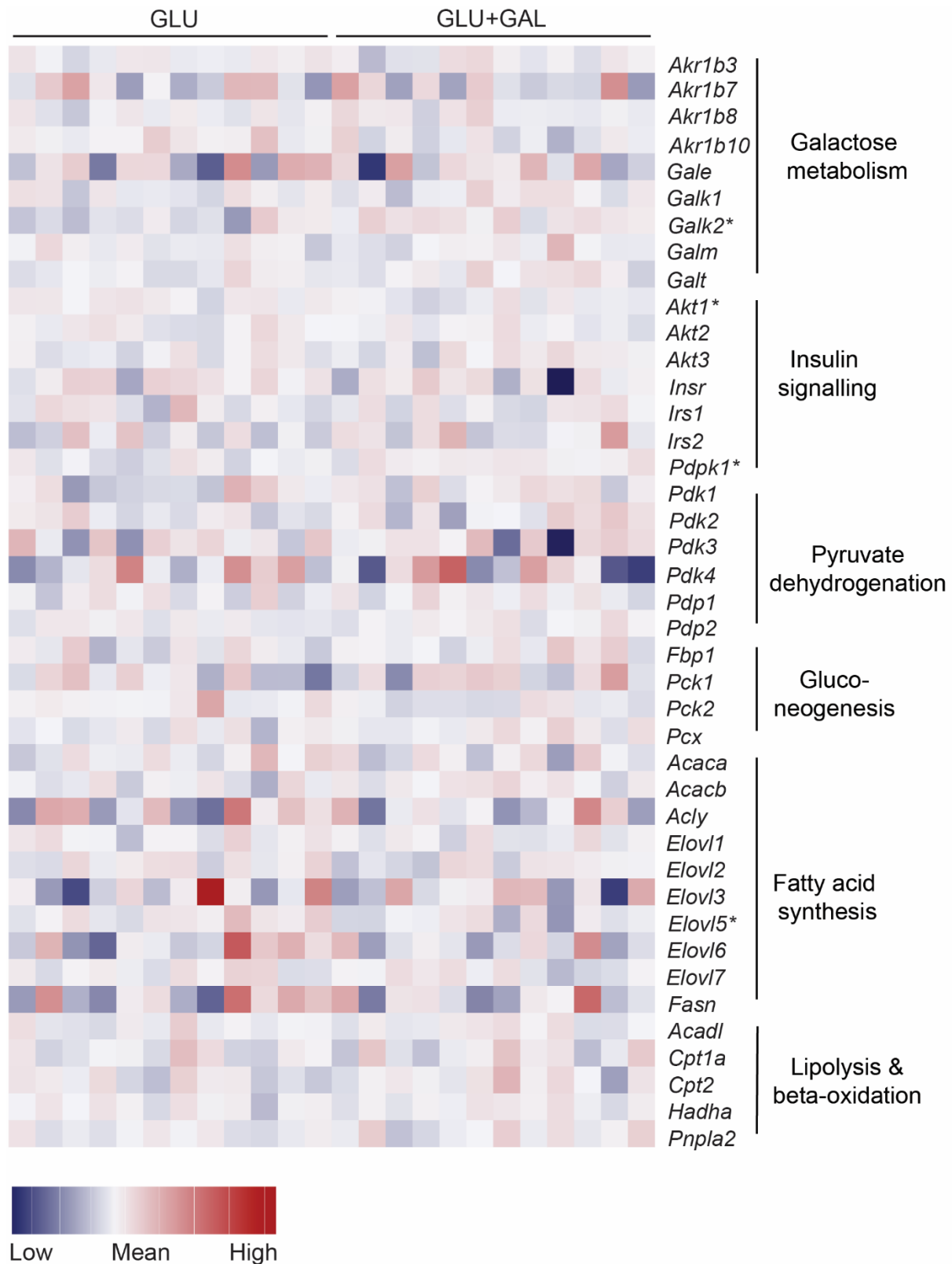
Supplemental Figure 2. Heatmap of hepatic gene expression. Heat mapping was performed on transcripts related to galactose metabolism, insulin signalling, pyruvate dehydrogenation, gluconeogenesis, fatty acid synthesis, and lipolysis and beta-oxidation. Expression values were mean centred per gene, heat map squares (Legend continues on next page)

represent individual samples. For genes with multiple spots, the spots with the lowest P-values were selected for visualization and analysis. * Significantly different in Student's t-test (no correction for multiple testing applied). *Acaca*: Acetyl-Coenzyme A carboxylase alpha; *Acacb*: Acetyl-Coenzyme A carboxylase beta; *Acadl*: Acyl-Coenzyme A dehydrogenase, long-chain; *Acly*: ATP citrate lyase; *Akr1b10*: Aldo-keto reductase family 1, member B10 (aldose reductase); *Akr1b3*: Aldo-keto reductase family 1, member B3 (aldose reductase); *Akr1b7*: Aldo-keto reductase family 1, member B7; *Akr1b8*: Aldo-keto reductase family 1, member B8; *Akt1*: Thymoma viral proto-oncogene 1; *Akt2*: Thymoma viral proto-oncogene 2; *Akt3*: Thymoma viral proto-oncogene 3; *Cpt1a*: Carnitine palmitoyltransferase 1a; *Cpt2*: Carnitine palmitoyltransferase 2; *Elovl1*: Elongation of very long chain fatty acids – family member 1; *Elovl2*: Elongation of very long chain fatty acids – family member 2; *Elovl3*: Elongation of very long chain fatty acids – family member 3; *Elovl5*: Elongation of very long chain fatty acids – family member 5; *Elovl6*: Elongation of very long chain fatty acids – family member 6; *Elovl7*: Elongation of very long chain fatty acids – family member 7; *Fasn*: Fatty acid synthase; *Fbp1*: Fructose bisphosphatase 1; *Gale*: Galactose-4-epimerase, UDP; *Galk1*: Galactokinase 1; *Galk2*: Galactokinase 2; *Galm*: Galactose mutarotase; *Galt*: Galactose-1-phosphate uridyl transferase; *Hadha*: Hydroxyacyl-Coenzyme A dehydrogenase/3-ketoacyl-Coenzyme A thiolase/enoyl-Coenzyme A hydratase, alpha subunit; *Insr*: Insulin receptor; *Irs1*: Insulin receptor substrate 1; *Irs2*: Insulin receptor substrate 2; *Pck1*: Phosphoenolpyruvate carboxykinase 1, cytosolic; *Pck2*: Phosphoenolpyruvate carboxykinase 2 (mitochondrial); *Pcx*: Pyruvate carboxylase; *Pdk1*: Pyruvate dehydrogenase kinase, isoenzyme 1; *Pdk2*: Pyruvate dehydrogenase kinase, isoenzyme 2; *Pdk3*: Pyruvate dehydrogenase kinase, isoenzyme 3; *Pdk4*: Pyruvate dehydrogenase kinase, isoenzyme 4; *Pdp1*: Pyruvate dehyrogenase phosphatase catalytic subunit 1; *Pdp2*: Pyruvate dehyrogenase phosphatase catalytic subunit 2; *Pdpk1*: 3-Phosphoinositide dependent protein kinase 1; *Pnpla2*: Patatin-like phospholipase domain containing protein 2.

Chapter 6

The effect of post-weaning dietary galactose on nutritional programming of insulin resistance in adult female mice

Lianne M. S. Bouwman,

Annemarie Oosting,

José M. S. Fernández-Calleja,

Jaap Keijer,

Evert M. van Schothorst.

Abstract

Replacing part of glucose with galactose in the post-weaning diet may protect from later life adiposity. The aim of this study was to evaluate if galactose in the post-weaning diet reduces the development of insulin resistance. Three-week-old female C57BL/6JRccHsd mice were fed a diet with glucose + galactose (GLU+GAL; 16 energy% (en%) each) or control diet with glucose (GLU; 32 en%) for three weeks, and switched to the same high fat diet (HFD) afterwards. After five weeks HFD, an oral glucose tolerance test was performed. After nine weeks HFD, animals were sacrificed fifteen minutes after a glucose bolus. Body weight and body composition were not affected by the post-weaning diet, neither during the post-weaning period, nor during the HFD period. The oral glucose tolerance showed no differences in glucose tolerance, only the iAUC for insulin was higher in GLU+GAL-fed females than GLU-fed females (P = 0.03) Serum insulin concentrations were not different at the end of the study, nor were AKT phosphorylation and insulin-related gene expression in gonadal white adipose tissue. Concluding, galactose in the post-weaning diet did not have a significant effect on body composition or insulin signalling in adulthood in this study.

Keywords: Nutritional programming; galactose; insulin resistance; adipose tissue.

Introduction

Nutrition in early life, encountered in a critical period of development, can have long-lasting effects on body composition and metabolism, a process referred to as nutritional programming [1]. Nutritional programming has been shown to affect glycaemic control and insulin resistance and other features of obesity and associated metabolic disease [2, 3]. With the increased prevalence of metabolic disease and the concurrent rise in costs of this condition [4], the interest in the development of preventive strategies has increased as well. To reduce susceptibility to metabolic syndrome population wide, full understanding of the early life conditions that influence disease risks is essential.

One of the hallmarks of metabolic disease is deterioration of glycaemic control and the development of insulin resistance [5]. The risk of offspring developing insulin resistance was shown to be influenced by the maternal diet [6]. Animal models clearly demonstrated that beneficial or adverse effects on later life insulin resistance depend on timing of exposure and the type and amount of nutrients, with often with sex-specific effects. For example, mild calorie restriction of rat dams during pregnancy increased the risk for development of insulin resistance in their offspring, while mild calorie restriction during lactation gave a protective effect [7, 8].

Programming effects on insulin signalling have been studied with various carbohydrate-based interventions as well. For example, carbohydrate-rich supplementation of dam's feed during the second part of gestation period caused insulin resistance in adult male rat offspring [9]. Feeding dams a diet rich in sucrose during pregnancy and lactation decreased insulin sensitivity in adult male rat offspring [10]. However, another study in mice showed that continuous sucrose feeding of dams, from preconception until end of lactation, increased body weight, fat mass, and insulin resistance in adult female offspring, with males remaining unaffected [11]. Finally, high-carbohydrate formula in the lactation period increased body weight and plasma insulin concentrations in female [12] and male [13] rats. The type of intervention and experimental setups of these studies were very different, and the observed effects variable and often sex-specific, with possibly a modulating role for the timing of exposure. Although these differences in study setup make it difficult to compare the outcomes, nutritional programming with carbohydrates is evident, and effects on insulin signalling seem to be adverse.

Nutritional programming can be the result of exposure during pregnancy and lactation, but it can also be achieved in the immediate post-weaning period. This has been demonstrated especially for exposure to dietary lipids. Multiple rodent studies have shown that the dietary lipid structure in the post-weaning period given for a brief period can beneficially program metabolic health in later life [14-18]. Likewise, medium-chain fatty acids in the early post-weaning diet program for lower adiposity with smaller adipocytes in later life in male mice, with a tendency for improved glucose tolerance [19]. Continuous feeding of both pre- and

probiotics during lactation and the early post-weaning period can program for lower fat mass accumulation in adulthood, with improved insulin sensitivity [20]. In summary, metabolically programming of body composition and metabolic health by dietary lipids given within the post-weaning period is proven, but programming by post-weaning carbohydrates is much less established.

Previously, we have investigated to what extend different carbohydrate fractions of the post-weaning diet can program metabolic health upon an adult obesogenic environment. A lowly digestible starch diet fed in the post-weaning period affected metabolic flexibility beneficially in high fat diet (HFD)-challenged female mice, compared with a highly digestible starch diet [21]. A post-weaning isocaloric diet with 32 energy % (en%) fructose instead of glucose reduced serum insulin concentrations and HOMA-IR in adult HFD-fed female mice, without significant changes in body weight or fat mass [22]. Replacing part of glucose with galactose in the post-weaning diet, to mimic an extended exposure to lactose present in milk, gave the most striking effect: it reduced body weight, fat mass, and circulating insulin concentrations in adult HFD-fed female mice [23].

Because glycaemic control and insulin signalling are often affected in models of nutritional programming, have a central role in metabolism and metabolic flexibility, and are pivotal for carbohydrate metabolism, we intended to examine the beneficial programming effect of the partial replacement of glucose with galactose towards glycaemic control, insulin signalling and metabolic flexibility. In a sense, the exposure to glucose plus galactose (compared with glucose alone) thus mimics exposure to nutritional products containing lactose, as for instance human milk, milk formulae or dairy products, compared with other types of weaning foods. To examine effects of galactose on glycemic control, insulin signalling and metabolic flexibility, an independent dietary intervention in female C57BL/6J mice was performed. We hypothesized that the increased insulin sensitivity in the female offspring mice would be reflected in an improved glycemic response and/or a lower insulin response during the oral glucose tolerance test in the HFD period, in lower circulating insulin concentrations at the endpoint in a glucose-challenged state, and, at the cellular level, in increased AKT phosphorylation levels in gonadal WAT (gWAT), since insulin signalling in target organs, including WAT, converges ultimately in the downstream signalling and phosphorylation of protein kinase B/AKT [24].

Materials and methods

Diets

Two semi-synthetic post-weaning diets and one semi-synthetic high fat diet (HFD) were ordered from Research Diet Services BV (Wijk bij Duurstede, The Netherlands). Post-weaning diets were composed in accordance with AIN-93 guidelines for growing rodents [25]. Detailed composition can be found in Supplemental Table 1. The macronutrient composition of the

post-weaning diets was 20 en% protein, 16 en% fat, and 64 en% carbohydrates. The carbohydrates in the post-weaning diets were half wheat starch (32 en %), and half monosaccharides (32 en%), i.e only glucose in the glucose diet (GLU), and a glucose and galactose combination (in 1:1 ratio; 16 en% each) in the glucose + galactose diet (GLU+GAL) (Supplemental Table 1). The HFD contained 20 en% protein, 40 en% fat (mainly palm oil based) and 40 en% carbohydrates (Supplemental Table 1). This type of HFD was shown to induce a more adverse metabolic state after long-term feeding than the BIOCLAIMS high-fat diet consisting of mainly poly-unsaturated fatty acids [26], which was used in our previous study [23] (**Chapter 4**). To all diets, vitamin and mineral mixes were added, to ensure nutrient recommendations were met [25].

Animals and ethical approval

Ethical approval for the animal experiments and procedures was granted by national and local Animal Experimental Committees (AVD1040020171668). Experiments were executed following the EU directive 2010/63/EU. All experiments were carried out at 23 ± 1 °C, with a 12:12 light dark cycle, and *ad libitum* access to food, unless stated otherwise. Breeding pairs (C57BL/6JRccHsd mice) were ordered from Envigo (Horst, The Netherlands). After an adaptation period of two to five weeks in the animal facilities, mice were time-mated. At postnatal day (PN)1 or PN2, nests were cross-fostered and standardized to 6 pups per nest, with 2-4 females per nest. During adaptation, pregnancy, and lactation, dams were fed a standard breeding chow (AM-II, AB Diets, Woerden, The Netherlands). At PN21 (±1), female pups were stratified by body weight (BW) and fat mass (FM) – as determined by EchoMRI 100V (EchoMedical Systems, Houston, TX, USA). One group was assigned to the GLU diet (*n* = 14), the other group to the GLU+GAL diet (*n* = 14). The mice were housed individually. After three weeks on these diets, all animals were switched to the HFD for nine weeks. Another subset of animals (GLU, *n* = 12) and GLU+GAL, *n* = 12) were killed at PN42 for molecular evaluation of direct effects, as reported elsewhere [27].

Experimental setup and measurements

Body weight was measured weekly. Body composition, consisting of FM and lean mass (LM), was measured weekly in the three-week post-weaning period, and biweekly in the HFD period. Food intake was determined weekly by subtracting the weight of remaining food pellets from the weight of the food pellets provided. Some animals on the HFD crumbled pellets without eating all, causing food crumbs to be dispersed within the bedding, and making food intake quantification impossible. These animals were excluded from the regular HFD food intake measurements (*n* = 5 for GLU; *n* = 2 for GLU+GAL). Drink intake (72 h) was measured in a subset of animals by subtracting the weight of the water bottle on PN39 from PN36, and PN53 from PN50. Indirect calorimetry (InCa) measurements were performed twice (*n* = 12 animals per group); from PN40 till PN42 at the end of the intervention period, and from PN93 till PN98 near the end of HFD-feeding.

Oral glucose tolerance test

On PN77, an oral glucose tolerance test (OGTT) was performed. Mice were fasted for five hours. A tail cut was made for whole blood glucose measurements and plasma collection. A glucose bolus (2.0 g/kg BW) was given by oral gavage. Blood glucose was measured with a Freestyle blood glucose system (Abbott Diabetes Care, Hoofddorp, The Netherlands) at timepoints t = 0, 15, 30, 45, 60, 90, and 120 min. Blood plasma was collected using Microvette® tubes (Starstedt, Nümbrecht, Germany) at timepoints t = 0, t = 15, and t = 30 min; samples were centrifuged for 5 min at 2000 g, and plasma was stored in -80°C until further analysis. Unfortunately, one of the GLU mice died unexpectedly during this procedure, and data from this individual mouse have been excluded from the start of the HFD onwards.

Indirect Calorimetry

Measurements of activity, energy expenditure, respiratory exchange ratio (RER), food intake, and water intake were measured with the Phenomaster LabMaster Metabolism Research Platform (TSE systems GmbH, Bad Homburg, Germany) as described previously [28]. The system was recently extended with hydrogen and methane sensors to measure gut microbiota fermentation gasses [29]. The first day in the InCa was considered adaptation period, the next 24 h (one light phase and one dark phase) were analysed. For the calorimetry session on PN40-42, basal measurements were performed, and these have been reported previously [27], while for the calorimetry session on PN93-98, basal measurements were followed by a fasting-and-refeeding (F&R) challenge to assess metabolic flexibility in vivo, as published [26, 30].

Before the F&R challenge, settings were adjusted to record only six cages (instead of twelve), with an interval of 1.25 min per cage, resulting in a resolution of six data points per hour (vs three in basal measurements). Preceding the night before the F&R challenge, mice were fed a restricted amount of HFD (1.55 g, representing ~50 % of their daily intake). The next day, fasting state was verified (RER of ~0.7 when fasting) and mice remained fasted until 2 h prior to the next dark phase. At that time, animals of both dietary groups were refed with 0.9 g of the GLU diet (high carbohydrate diet, to enhance the visibility of the switch from fat to glucose oxidation). The next morning, half an hour after the start of the light phase, animals were given *ad libitum* access to HFD again. Animals that had not finished the 0.9 g pellet provided in the F&R challenge by the start of the next light phase, were excluded from the analysis (*n* = 2 for GLU, *n* = 1 for GLU+GAL). The animals that were not included in the InCa measurements due to capacity constraints (*n* = 1 for GLU; *n* = 2 for GLU+GAL) were food restricted and refed in the same way in their home cage to keep treatments similar.

Dissection

On PN105, animals were food deprived at the start of the light phase for at least 2 hours. Fifteen min before sacrifice, animals were given a glucose bolus (2.0 g/kg BW) by oral gavage, to have them in a challenged state with peaking insulin concentrations, upon sacrifice. Animals were decapitated, and a drop of blood was used to measure whole blood glucose concentrations (Freestyle). The remainder of the blood was collected in MiniCollect® serum

tubes (Greiner Bio one B.V., Alphen aan de Rijn, The Netherlands), centrifuged for 10 min at 3000 *g* at 4°C, and obtained serum was aliquoted and stored at -80°C. Liver and gonadal white adipose tissue (WAT) depots were collected, weighed, snap-frozen in liquid nitrogen and stored at -80°C until further analysis.

Serum and plasma analyses

Serum leptin and adiponectin concentrations were determined with Bio-Plex Pro Mouse diabetes assays (Bio-Rad laboratories, Veenendaal, The Netherlands), serum glucose concentrations with a glucose GOD-PAP kit (Roche, Mannheim, Germany), and serum free fatty acids with the NEFA-HR(s) kit (Fujifilm Wako Chemicals Europe GMBH, Neuss, Germany). Serum insulin (sacrifice) and plasma insulin (OGTT) concentrations were measured with an Ultra-Sensitive Mouse Insulin ELISA Kit (ChrystalChem, Downers Grove, Illinois, United States). All assays were performed according to the manufacturers' instructions. Samples were tested in duplicates, except for the plasma insulin measurements of the OGTT due to the limited amount of sample available. A HOMA-IR was calculated with the C57BL/6J mouse specific formula [31], using whole blood glucose and plasma insulin concentrations at t = 0 from the OGTT.

Gene expression

Total RNA was extracted from gonadal WAT (gWAT) using RNeasy columns (Qiagen, Venlo, The Netherlands), according to the manufacturer's instructions. RNA integrity was verified on the Agilent 2200 Tapestation (Agilent Technologies Inc, Santa Clara, CA, USA). cDNA Synthesis and RT-qPCR were executed as described [23] without pre-amplification. Primer sequences and annealing temperatures can be found in Supplemental Table 2.

Western Blot

Western blot for pAKT-Ser473, total AKT, and β-ACTIN was performed on gWAT protein extracts as described [32].

Statistical analysis

Data were analysed with GraphPad Prism, version 5.04 (GraphPad Software Inc., San Diego, CA, USA). Two-way repeated measures ANOVA was used for analysis of BW, FM, and LM, with post-weaning diet as the between-subject factor, and time as the within-subject factor, and group x time interaction. Post-weaning and HFD periods were studied separately. OGTT data were analysed with two-way repeated measures ANOVA. Other parameters were analysed with a Students' *t*-test for normally distributed data, a *t*-test with Welch correction for normally distributed data with unequal variances, or a Mann-Whitney U for not normally distributed data. D'Agostino & Pearson omnibus normality tests were used to test for normality; data were log-transformed when original distribution was not normal, and retested for normality. Results are given as mean ± SD unless stated otherwise; *P*-values < 0.05 were considered significant.

Results

BW, FM, and LM increased significantly over time for both GLU- and GLU+GAL-fed mice during the post-weaning as well as the subsequent HFD period (Fig. 1; Supplemental Table 3 for F- and *P*-values). There was no effect of the post-weaning diet on BW, FM and LM during the post-weaning phase, only time x post-weaning diet tended to be affected for LM (*P* = 0.096). These effects resembled those found in a previous study with the same design and intervention [27]. For the HFD period, there was also no effect of post-weaning diet on BW, FM, and LM. Time x post-weaning diet tended to be affected for body weight (*P* = 0.0912). Overall, post-weaning diet did not affect BW, FM, and LM directly, nor in a programmed manner.

At the end of the HFD period, organ weights indicated that pancreata of GLU+GAL-fed females weighed significantly more than pancreata of GLU-fed female (Table 1). Liver weight was not affected by post-weaning diet, nor were gonadal and mesenteric WAT weights (Table 1). Food intake was significantly higher in the animals on the GLU+GAL diet in the post-weaning period (*P* < 0.001; Fig. 2A). Cumulatively, GLU+GAL-fed females ate approximately 11% more food than GLU-fed females in this period (60.0 ± 2.9 g vs 53.9 ± 2.9 g); comparable to previously described findings [27]. Water intake was almost two-fold higher in the animals on the GLU+GAL diet (Fig. 2B). Food intake was no longer different between the two groups during the HFD period (Fig. 2C). The drop in food intake in week 8 of the HFD period was the result of the F&R challenge given in that week. Water intake in the HFD period was not affected by the post-weaning diet (Fig. 2D), and water intake on HFD, about 3 mL per day, was similar to the water intake of animals on GLU diet (Fig. 2B).

Table 1. Organ weights GLU- and GLU+GAL-fed females after HFD (PN105).

	Liver weight (g)	gWAT weight (g)	mWAT weight (g)	Pancreas weight (g)
GLU	1.22 ± 0.43	0.62 ± 0.27	0.59 ± 0.29	0.25 ± 0.03
GLU+GAL	1.19 ± 0.24	0.70 ± 0.23	0.67 ± 0.21	0.29 ± 0.07*

* *P*-value <0.05 between dietary groups. Values are expressed as mean ± SD, *n* = 13-14.

After 5 weeks on the HFD, an OGTT was performed (Fig. 3). Glucose tolerance was not significantly different between GLU- and GLU+GAL-fed females, neither blood glucose concentrations (Fig. 3A) nor iAUCs were glucose affected (Fig. 3B). Plasma insulin concentrations were not significantly different between GLU- and GLU+GAL-fed females at any time point (Fig. 3C), but insulin iAUC suggested that GLU+GAL-fed females had a lower insulin sensitivity (Fig. 3D). The HOMA-IR, however, indicated that insulin resistance was similar among the groups (Fig. 3E).

Animals were studied in the indirect calorimetry system in week 8 of the HFD period. Food intake results of the InCa indicate that the GLU+GAL-fed (3.50 ± 0.84 g) animals ate more of
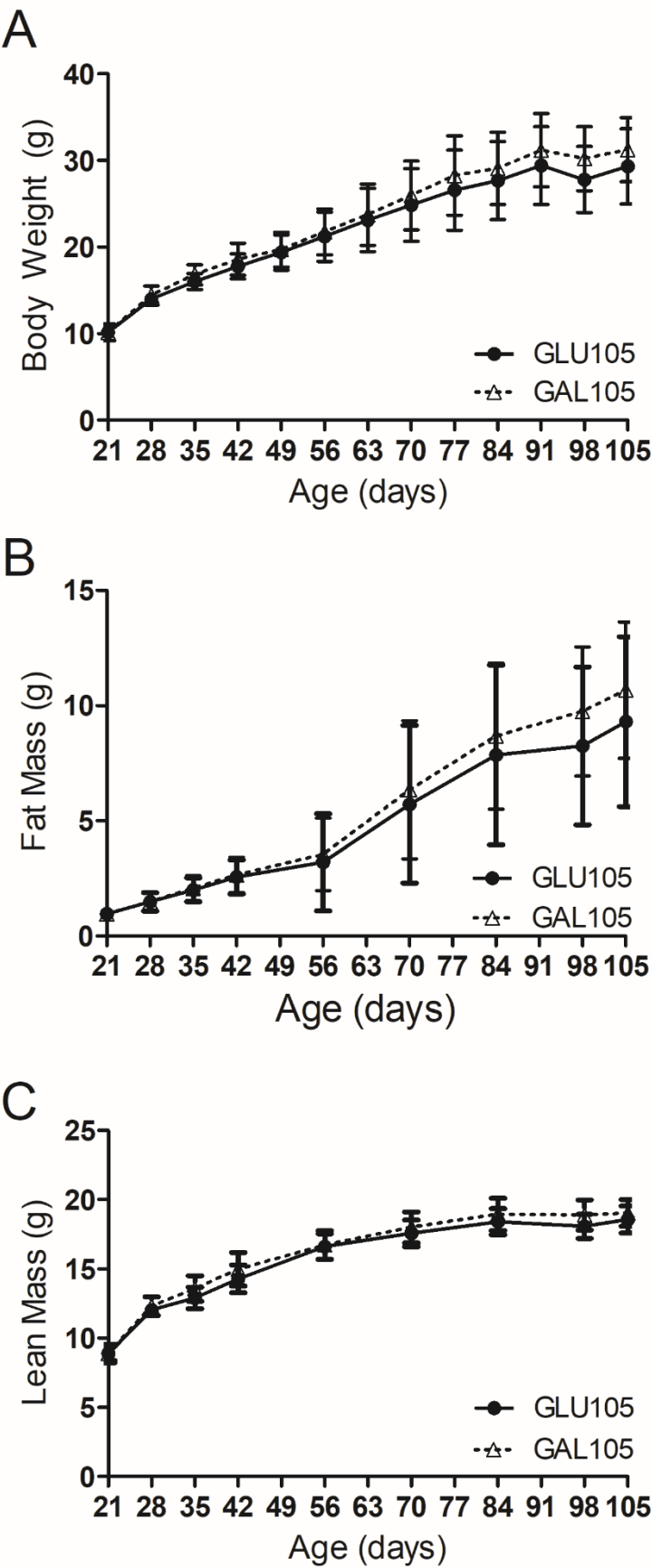
Figure 1. Body weight, lean mass, and fat mass development in GLU- and GLU+GAL-fed females. A) Body weight, B) fat mass, and C) lean mass development during intervention (PN21-PN42) and consecutive HFD (PN42-PN105) periods. Values represent mean ± SD, n = 13-14.

the HFD than the GLU-fed animals (2.22 ± 1.62 g) in the 24 h InCa measurement. This food intake deviated from the "normal" food intake on the HFD (Fig. 2C), that was 3.04 ± 0.20 g for GLU-fed and 3.10 ± 0.19 for GLU+GAL-fed females (average daily intake calculated over weeks 2 -7). This suggests that the GLU-fed females ate less than usual in the 24 h InCa measurements, while the GLU+GAL-fed females ate more. Given the effects food intake has on RER, EE, and possibly activity, the InCa measurements were considered to be not representable for the normal situation, and thus not further analysed. After the basal measurements, a F&R challenge was performed, in order to study metabolic flexibility. It was visually observed that all animals found the food provided for refeeding, and that all animals returned to sleep before finishing. This was reflected in the RER curves, that peaked more than once (Supplemental Fig. 1). This precluded analysis of metabolic flexibility, because not all food was consumed at once.
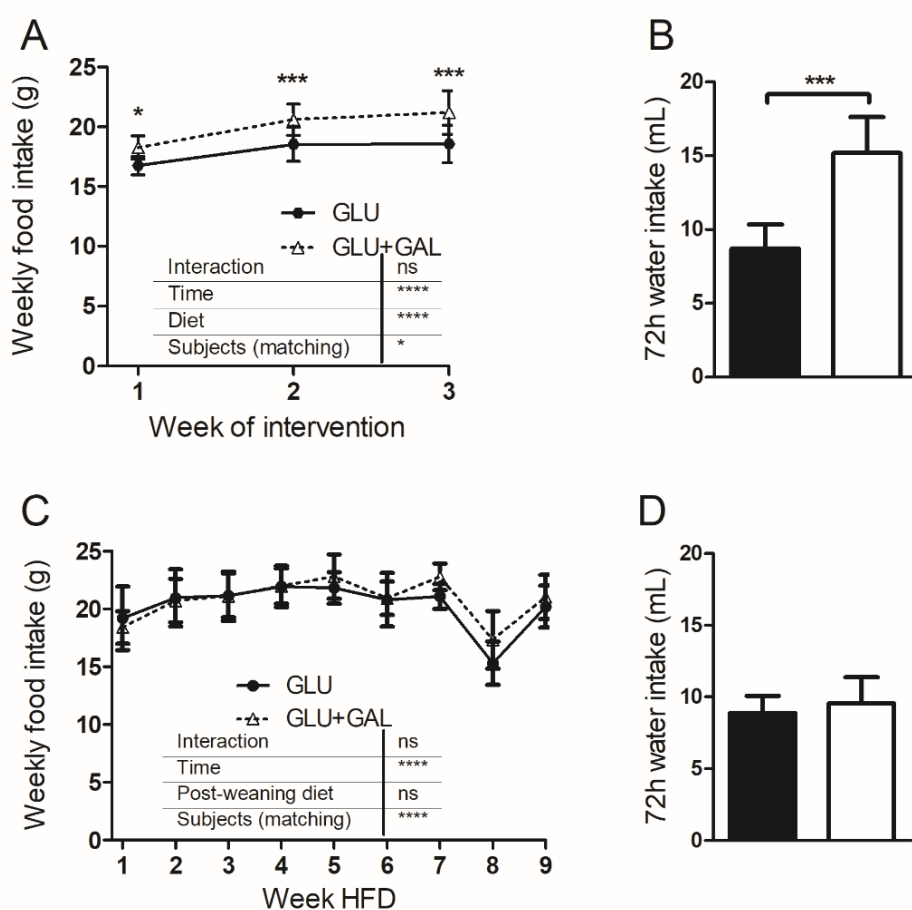
Figure 2. Food intake and water intake on intervention diet and subsequent HFD. A) Weekly food intake of GLU- and GLU+GAL-fed females (*n* = 14) in post-weaning phase, B) 72 h water intake in post-weaning phase PN36-PN39 (*n* = 7; taken from [27], C) weekly food intake on HFD (GLU-fed (*n* = 8) and GAL+GLU-fed (*n* = 12), D) 72 h water intake when on HFD (*n* = 7; PN50-PN53). Black bars represent GLU-fed females; white bars represent GLU+GAL-fed females. Values are expressed as mean ± SD.
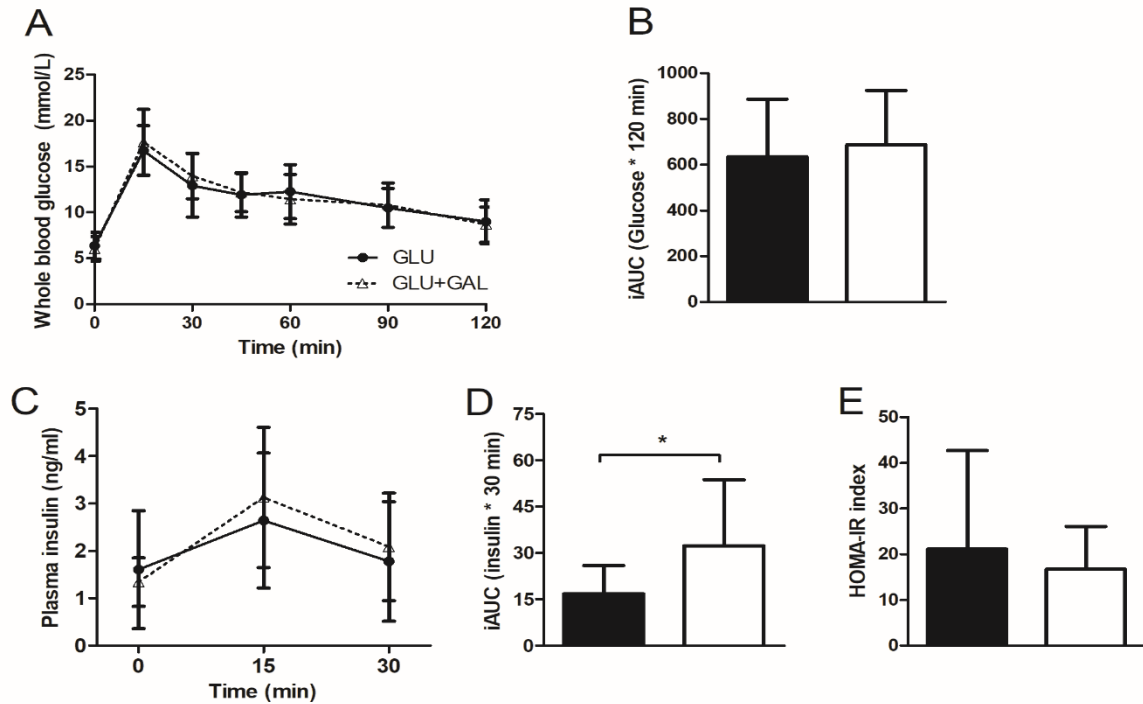
Figure 3. Glucose tolerance test (2.0 mg glucose / kg BW) in GLU- and GLU+GAL-fed females (PN77). A) Blood glucose curves over time, B) iAUC glucose curve, C) plasma insulin concentrations over time, D) iAUC insulin curve and E) HOMA-IR (calculated with blood glucose concentrations and plasma insulin concentrations of t = 0). Black bars represent GLU-fed females; white bars represent GLU+GAL-fed females. Values are expressed as mean ± SD, n = 12-14.

Figure 4. Serum parameters in GLU- and GLU+GAL-fed females at end of study (PN105) upon a glucose challenge (15 min after 2.0 mg/kg BW glucose). A) Serum insulin, B) serum glucose, C) serum adiponectin, D) serum leptin, E) serum free fatty acids. Black bars represent GLU-fed females; white bars represent GLU+GAL-fed females. Values are expressed as mean ± SD, n = 13-14.

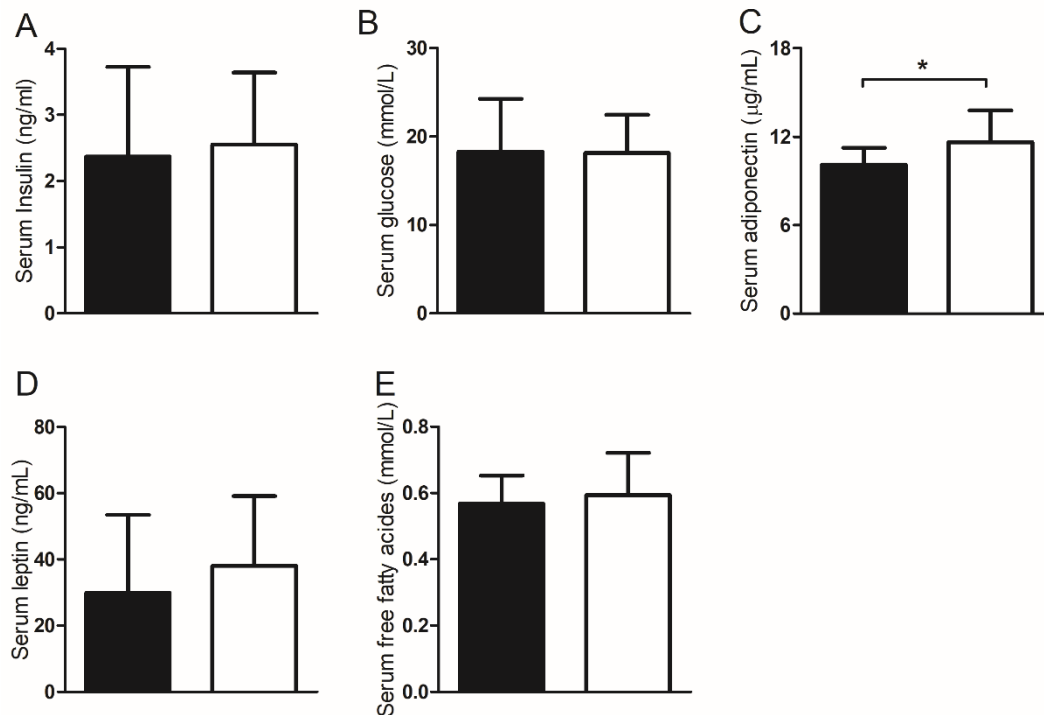At the end of the study, animals were sacrificed in a challenged state by giving food-deprived animals a glucose bolus of 2.0 g glucose / kg BW 15 min before sacrifice. Serum glucose (Fig. 4A) and insulin (Fig. 4B) concentrations were not different between the groups. Serum adiponectin concentrations were significantly higher in GLU+GAL-fed females (Fig. 4C). Serum leptin, an indicator for whole body adiposity, was not affected (Fig. 4D). Serum FFA concentrations were not affected by the post-weaning diet (Fig. 4E). Insulin signalling, as measured by AKT Ser473 phosphorylation in gWAT was not significantly different between GLU- and GLU+GAL-fed females (Fig. 5). Analysis of gene expression in gWAT indicated that expression of several genes involved in insulin signalling, lipolysis, inflammation, and WAT expendability (Fig. 6) were all not programmed by the post-weaning diet.
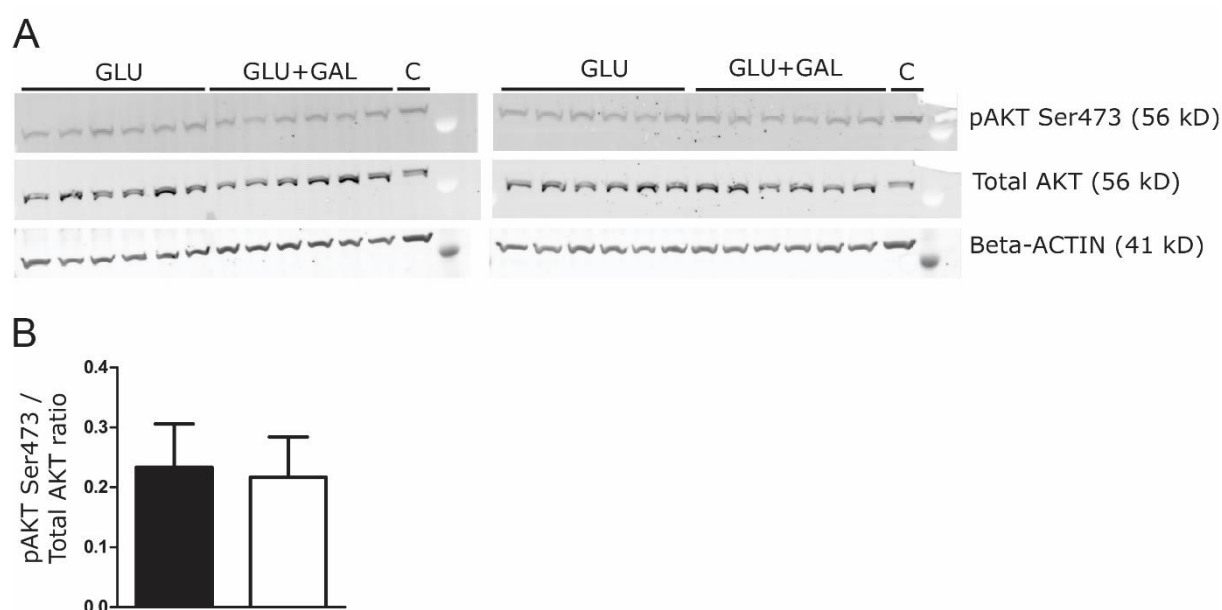
Figure 5. Western blot of AKT activation in gWAT of GLU- and GLU+GAL-fed females, PN105, 15 min after 2.0 mg/kg BW glucose. A) overview of blot results of activated pAKTser473, total AKT, and β-actin (loading control), B) quantification of pAKTser473 / total AKT ratio as marker of insulin signalling. C represents a control sample from epididymal WAT from a previous study [32]. Black bar represents GLU-fed females; white bar represents GLU+GAL-fed females. Values are expressed as mean ± SD, n = 12.

Discussion

Despite previously found programming effects on adult fat mass and metabolic profile, this study showed no long-term effects of early post-weaning galactose on adult glycemic control, insulin signalling and metabolic flexibility. The current data showed that body weight and body composition were not affected by replacing part of the glucose with galactose in the post-weaning diet, neither directly nor on the long term (programmed). Fasting insulin and glucose concentrations were not affected, although the amount of insulin needed to clear a glucose bolus was higher in GLU+GAL-fed females in later life, as indicated by iAUC in the OGTT. After

Figure 6. Gonadal WAT gene expression in GLU and GLU+GAL – fed females, PN105. *Irs1*: insulin receptor substrate 1; *Irs2*: insulin receptor substrate 2; *Tbc1d4*: Tuberculosis 1 domain family member 4; *Atgl*: Adipose triglyceride lipase; *Cpt1a*: carnitine palmitoyltransferase I; *Lpl*: Lipoprotein lipase; *Mrc1*: Mannose receptor C type 1; *Saa3*: Seru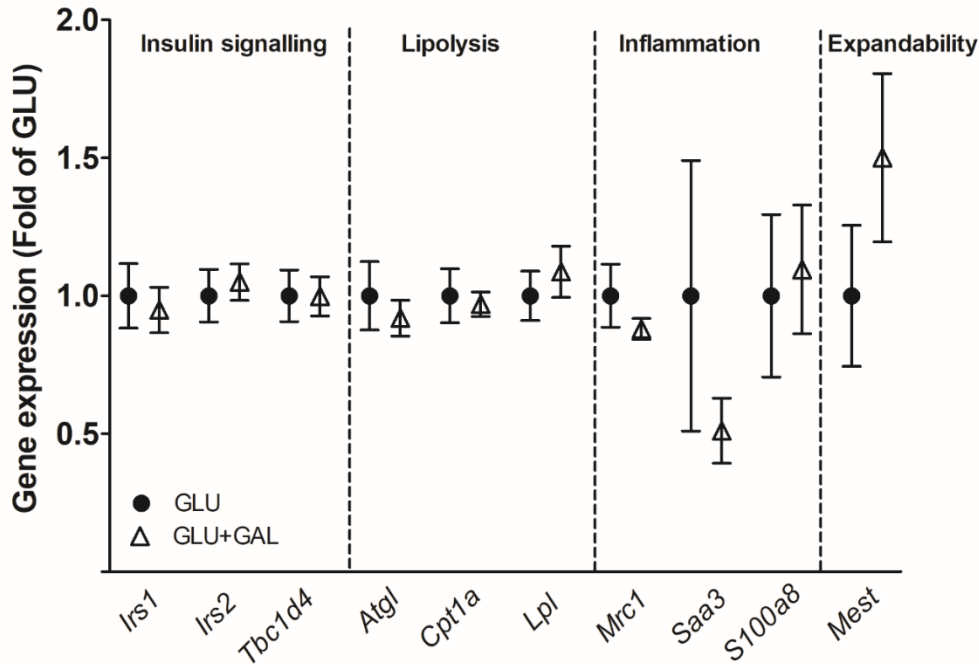m amyloid A 3; *S100a8*: S100 calcium-binding protein A8; *Mest*: mesoderm-specific transcript homolog protein; Values are expressed as mean ± SEM, *n* = 10.

nine weeks of HFD-feeding, pancreas weight was higher in GLU+GAL-fed females. Insulin signalling in gWAT was not significantly affected by the post-weaning diet.

The metabolic programming effect of replacing part of glucose with galactose in the post-weaning diet on adiposity that was observed in a previous study with similar setup [23] was not seen here. Although largely similar, certain aspects were different between the studies, for instance the composition of the HFDs and the breeding diets. The HFD in this study was mainly palm oil based and thus rich in saturated fatty acids, while in our previous study a HFD rich in poly-unsaturated fatty acids was used, based on the BIOCLAIMS HFD [33, 34]. We previously showed that these two HFDs, that differ in the degree of saturated versus unsaturated fatty acids, did not show significant differences on BW or FM in adult male mice after 27 weeks of feeding [26]. However, these diets resulted in a different metabolic flexibility, which was associated with an increased adipocyte size, and an increased muscle and liver lipid accumulation [26]. Possibly, the more adverse high-fat diet used in this study compared with the diet used in our previous study, prevented or suppressed potential beneficial programming effects of dietary galactose given in the post-weaning period, which may explain the disparity between both studies.

Another explanation for the discrepancy between the studies may be the breeding diet: a semi-synthetic breeding diet was used in the previous study [23], and a breeding chow was

used in this study. We observed much better breeding outcomes using chow-fed compared with semi-synthetic diet-fed dams (unpublished observations from our lab), but the reason for this difference in breeding success on these diets remains elusive, as there are numerous differences between semi-synthetic and chow diets [26]. In addition, chows suffer large batch to batch variations and are generally not well defined. Given the importance of the perinatal environment on later life metabolic health [6], it is plausible that the susceptibility of the animals to weight gain and the development of insulin resistance was different between the studies, due to the different breeding diets. The composition of chows and semi-synthetic diets is very different with regards to the fibre content, which is much higher in chows than in semi-synthetic diets [35, 36]. It is well known that high fibre content positively affects metabolic health [37]; thus the metabolic health of the dams may have been different between studies, which in turn could have resulted in different peri-natal programming. In addition, chow and semi-synthetic diets differently affect microbiota composition [36]. Microbiota composition is linked to linked to adiposity, and can transferred between animals [38]. Thus, the intestinal microbiota was likely different between the two studies (both for the breeding dams and for the experimental females), which might explain the different results.

In line with a general lack of effect in this study, the programming effects on insulin sensitivity were not as hypothesized; the insulin iAUC of the OGTT even indicated a lower rather than improved insulin sensitivity in the GLU+GAL-fed females, even though at start of the OGTT fasted basal concentrations of insulin and glucose were similar. As the females were sacrificed in a challenged state at the end of the study, neither basal fasted insulin concentrations nor HOMA-IR could be determined. Overall, it appears that insulin sensitivity was not positively programmed by GLU+GAL-feeding in the post-weaning period.

Multiple studies have measured the direct effects of glucose and galactose on insulin release. In humans, a galactose drink gives a lower increase in circulating insulin concentrations than a glucose drink [39-42]. In addition, the insulin response of a drink with only galactose gives a lower insulin increase than a drink with glucose and galactose (1:1) [43, 44]. Moreover, on the long term, feeding rats with a diet with 15% galactose, compared with 15% glucose, for nine weeks improves hepatic insulin sensitivity in adult males [45]. Thus, it is likely that the galactose diet had a direct effect on insulin concentrations; indeed, the fasting serum insulin concentrations at the end of the post-weaning intervention period support this [27]. However, in this study, replacing part of the glucose with galactose in the post-weaning diet had no beneficial effect on the long term, as hypothesized, even though serum adiponectin concentrations appeared to be higher in the galactose-fed mice, and this hormone is considered an insulin sensitizer [46].

Because the most striking finding of the previous study was the difference in fat mass [23], our analyses were focussed on gWAT as well. Here, no difference in *Irs2* expression in WAT was found. Whether this is due to use of alternative signalling within this tissue, e.g. via IRS1, to altered circulating insulin concentrations, or the overall insulin sensitised state, remains to be studied. Previously, it was reported that in livers of HFD- vs LFD-fed mice, *Irs1* expression

was increased while *Irs2* expression was reduced [47]. In subcutaneous adipose tissue of insulin resistant versus insulin sensitive women, IRS2 appeared lower expressed [48]. Whether this represents tissue depot-specifics, or differences between human and mice, warrants future studies.

One of the metabolic markers included in this study was the measurement of metabolic flexibility using a F&R challenge. Metabolic flexibility, or capacity to adapt substrate usage to substrate availability, depends for a large extend on the insulin release and insulin sensitivity of an individual [49]. Unfortunately, as the mice did not finish consuming the food pellet provided in the refeeding phase quickly, the change in "substrate availability" to the body was not the same for all animals; and analysing the response would thus not give reliable outcomes on the metabolic flexibility. A difference in food intake in the refeeding was also seen in young male mice in another study where we refed *ad libitum* [22]. Here, we attempted to eliminate this problem by standardizing the amount of food provided, but unfortunately this did not solve the issue. Although providing a test meal as challenge has as advantage that it most naturally and realistically tests metabolic flexibility, instantaneous intake is more difficult to control. This disadvantage can be tackled by providing a liquid meal by gavage, although this gives a more artificial setting. On the other hand, such a procedure (gavage with a liquid meal) mimics the PhenFlex test meal, a nutritional challenge test optimized for the human situation, more closely [50].

Concluding, replacing part of glucose with galactose in the post-weaning diet did not beneficially affect later life health parameters in adult female mice in an obesogenic environment. Insulin signalling may have been affected to some extent by the post-weaning replacement, although it is not clear if the effects are long-term, nor whether these are beneficial. It is likely that the impact of nutrition during gestation, a well-established critical period of development, influences the long-term impact of nutritional interventions during subsequent developmental periods including the post-weaning period.

Author contributions

LB, AO, JFC, JK and EvS designed the research, LB performed the experiments; LB, EvS, AO, and JK interpreted the data; LB drafted the manuscript; LB, AO, JK and EvS critically revised the manuscript.

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Supplemental material

Supplemental Table 1: Composition of the experimental diets.

	Glucose diet (GLU)		Galactose diet (GLU+GAL)		High fat diet (HFD)	
	Mass (g)	**Energy (kcal)**	**Mass (g)**	**Energy (kcal)**	**Mass (g)**	**Energy (kcal)**
Casein	200.0	800	200.0	800	233.0	932
L-cysteine	3.0	12	3.0	12	4.0	16
Wheat starch	278.0	1112	278.0	1112	300.0	1200
Sucrose	0.0	0	0.0	0	155.4	622
Glucose	322.0	1288	161.0	644	0.0	0
Fructose	29.5	118	29.5	118	0.0	0
Galactose	0.0	0	161.0	644	0.0	0
Cocos oil	12.6	113	12.6	113	0.0	0
Sunflower oil	49.0	441	49.0	441	0.0	0
Flaxseed oil	8.4	76	8.4	76	4.0	36
Palm oil	0.0	0	0.0	0	206.0	1854
Cholesterol	0.030	0	0.030	0	0.097	0
Cellulose	50.0	0	50.0	0	50.0	0
Mineral mix*	35.0	31	35.0	31	35.0	31
Vitamin mix*	10.0	39	10.0	39	10.0	39
Choline bitartrate	2.5	0	2.5	0	2.5	0
Total energy (kcal/kg)		4030		4030		4730
Protein en%		20		20		20
Carb en%		64		48		40
Fat en%		16		16		40

Supplemental Table 2: Primer sequences and annealing temperatures RT-qPCR in gonadal WAT.

Genes	Forward Primer (F) Reverse Primer (R)	Annealing Temperature (°C)
Irs1	F: TTAGGCAGCAATGAGGGCAA R: TCTTCATTCTGCTGTGATGTCCA	60
Irs2	F: GCACCTATGCAAGCATCGAC R: GCGCTTCACTCTTTCACGAC	60
Tbc1d4 (As160)	F: CTGGAAGCAAGAAGAGATGAGC R: ATTTCCCCTCGCCGACTTTT	62
Lpl	F: GGACTGAGAATGGCAAGCAACAC R: GCAGTTCTCCGATGTCCACCTC	60
Atgl	F: ACCACCCTTTCCAACATGCTACC R: GCTACCCGTCTGCTCTTTCATCC	58
Cpt1a	F: AAAGATCAATCGGACCCTAGACA R: CAGCGAGTAGCGCATAGTCA	57
Mest	F: GATTCGCAACAATGACGGCA R: ATCCAGAATCGACACTGTGG	57
S100a8	F: ACTTCGAGGAGTTCCTTGCG R: TGCTACTCCTTGTGGCTGTC	58
Saa3	F: AAAGAAGCTGGTCAAGGGTC R: TGTCCCGTGAACTTCTGAAC	58
Mrc1	F: GTTATGAAAGGCAAGGATGGATAC R: TCAGTGAAGGTGGATAGAGTGG	58
B2m (reference)	F: CCCCACTGAGACTGATACATACGC R: AGAAACTGGATTTGTAATTAAGCAGGTTC	60
Canx (reference)	F: GCAGCGACCTATGATTGACAACC R: GCTCCAAACCAATAGCACTGAAAGG	60
Rps15 (reference)	F: CGGAGATGGTGGGTAGCATGG R: ACGGGTTTGTAGGTGATGGAGAAC	60

Supplemental Table 3. F- and *P*-values of two-way repeated measures ANOVA's on body weight (BW), lean mass (LM), and fat mass (FM); separate for post-weaning and HFD period.

Parameter	Period	Post-weaning diet*		Interaction		Time		Subjects	
		F-value	*P*-value	F-value	*P*-value	F-value	*P*-value	F-value	*P*-value
BW	Post-weaning	1.888	0.1817	1.389	0.2526	494.8	< 0.0001	5.390	< 0.0001
FM	Post-weaning	0.07241	0.7900	0.09480	0.9627	89.96	< 0.0001	3.468	< 0.0001
LM	Post-weaning	2.849	0.1034	2.190	0.0959	497.8	< 0.0001	4.775	< 0.0001
BW	HFD	0.9269	0.3449	1.695	0.0912	283.1	< 0.0001	61.17	< 0.0001
FM	HFD	0.6429	0.4302	1.139	0.3432	136.1	< 0.0001	21.11	< 0.0001
LM	HFD	1.678	0.2070	1.731	0.1322	349.1	< 0.0001	26.97	< 0.0001

* Effect of the diet the animals received post-weaning: this means the direct effect is tested in the analysis on the post-weaning period, and the sustained (programmed) effect is tested in the HFD period.

Supplemental Table 4. F- and *P*-values two-way repeated measures ANOVA's on food intake; separate for post-weaning and HFD period.

Parameter	Period	Post-weaning diet*		Interaction		Time		Subjects	
		F-value	*P*-value	F-value	*P*-value	F-value	*P*-value	F-value	*P*-value
Food intake	Post-weaning	31.97	< 0.0001	1.575	0.2168	34.63	< 0.0001	2.072	0.0128
Food intake	HFD	0.7806	0.3886	1.946	0.0574	31.81	< 0.0001	6.779	< 0.0001

* Effect of the diet the animals received post-weaning: this means the direct effect is tested in the analysis on the post-weaning period, and the sustained (programmed) effect is tested in the HFD period.
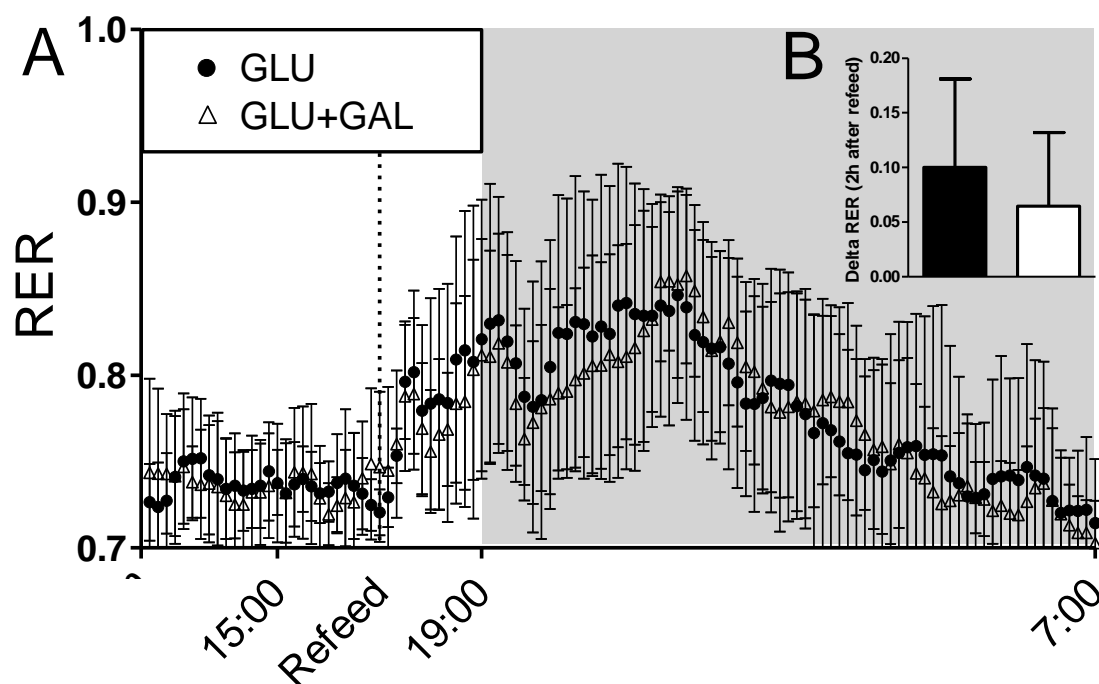
Supplemental Figure 1. RER patterns in the fasting & refeeding challenge (PN97 / PN98) for GLU- and GLU+GAL fed females. After 8 weeks HFD-feeding, mice were fasted and all refed using a high glucose-containing meal at 17:00u. A) RER pattern over time. B) Delta RER in the two hours after refeed (From 17:00 u to 19:00 u). Data are expressed as mean ± SD (n = 10 GLU-fed, n = 11 GLU+GAL-fed).

Chapter 7

General discussion

This thesis aimed to establish to what extend the post-weaning period is amendable to nutritional programming by the monosaccharides fructose and galactose, with glucose as control, using a pre-clinical mouse model. The research focused on long-term effects on body composition and metabolic health. In addition, this thesis aimed to assess if males and females were equally susceptible to programming by monosaccharides in the post-weaning period, and if any underlying mechanisms could be identified.

In this chapter, the main results of the thesis will be summarized, and the main question of the thesis will be discussed: Is there nutritional programming by post-weaning monosaccharides? In addition, certain choices in the study design will be discussed. Subsequently, the discussion will focus on galactose: the potential reason for the high amount of (ga)lactose in milk, and other findings with galactose. Next, some considerations for the translation of the findings to the human situation will be described. The last paragraph concern implication of this research and conclusions.

Main findings

In **Chapter 2** we found no adverse nutritional programming by fructose in the post-weaning diet on body weight, body composition, glucose tolerance or metabolic flexibility in adulthood. Circulating insulin concentrations and HOMA-IR were attenuated in females that had fructose post-weaning, while in males no significant differences were found.

In **Chapter 3**, direct effects of fructose in adult animals were examined, as an extension to **Chapter 2**. It was shown that body weight (BW), circulating insulin concentrations, and energy expenditure were not differentially affected by fructose, or by fructose and glucose in a 1:1 ratio, when compared to glucose, in adult male and female mice on a moderate high-fat diet (HFD). So in both chapters we did not see worse effects of fructose compared to glucose.

Galactose in a 1:1 ratio with glucose (GLU+GAL), compared to glucose only (GLU), in the post-weaning period programmed for lower adiposity in later life in female mice, reported in **Chapter 4.** This was associated with lower circulating insulin concentrations, reduced food intake on the HFD, a tendency for smaller adipocytes, and lower *Irs2* expression in white adipose tissue depots. In males, no significant effects on body weight and insulin concentrations were found and therefore no subsequent analyses were done in this sex.

In **Chapter 5**, describing direct effects of a post-weaning GLU+GAL versus GLU diet in female mice, it was confirmed that body weight development was similar, despite increased food intake on the GLU+GAL diet. Water intake was also higher with the GLU+GAL diet, and fasting serum insulin concentrations were decreased. Hepatic triglyceride levels were lowered by the GLU+GAL diet, and inflammation related gene expression was decreased in hepatic tissue.

In **Chapter 6** no significant effect of post-weaning GLU+GAL (compared with GLU) on later life adiposity was found in female mice. No evidence for better insulin signalling with GLU+GAL in the post-weaning diet was found; in fact, the OGTT suggested a higher insulin resistance in the females fed the GLU+GAL diet post weaning.

Only varying one nutritional component, in this case monosaccharides, allows for precise comparisons in a study. With the broad scope of energy metabolism-related parameters analysed, it gives a valuable overview of the consequences of the dietary exposure. Including state-of-the-art new technologies in the indirect calorimetry system [1] enabled simultaneous microbiota analysis as well, with only minor inflictions on animal welfare.

Nutritional programming

The main aim of this thesis was to see to what extend the post-weaning period is susceptible to nutritional programming by dietary monosaccharides. So, pivotal to this question are whether the post-weaning period is a critical period of development, and whether there are long-lasting or life-long consequences of the nutritional interventions. As introduced in **Chapter 1**, some studies show that dietary interventions in the post-weaning period have lasting effects. However, these interventions started at postnatal day (PN) 16, thus before or during weaning, [2-6], and not at PN21, solely post weaning, as we did in our studies. Nevertheless, growth of the animals is considerable in the period we studied: fat mass of the mice doubled and BW almost doubled in this period (**Chapter 2**, **Chapter 4**, and **Chapter 6).** In **Chapter 2**, little support was found for lasting effects of fructose (compared to glucose) in the post-weaning period. For males, no evidence for nutritional programming was found, while for females a significant reduction of serum insulin concentrations was observed. This difference between the males and females suggests that there may be some sexual dimorphism in programmed susceptibility to the development of HFD-induced insulin resistance. For galactose, however, the results presented in **Chapter 4** showed programming effects, at least in females, suggesting that the post-weaning period may be amenable to nutritional programming by carbohydrates. However, the programming effect of galactose (given in a 1:1 ratio with glucose), was not confirmed in the second study (**Chapter 6**). Thus, although there is evidence for programming in the post-weaning period, the effect is modest, and the effect likely depends on experimental conditions.

By definition, programming is "a long-standing of life-long alteration in structure or function" resulting from a nutritional cue in early life, is programming [7]. In case of life-long effects, the choice of time point in an experiment to assess effects should not affect the outcome; regardless of the time point chosen, the effect should be always visible. For example, nephron number will always be lower in subjects that suffered from undernutrition compared to those who did not [8], regardless of the age chosen. Nonetheless, a functional difference (disadvantage) may appear only later in life. The definition of programming also includes "long-standing" effects, but what can be considered "long-standing" is open for interpretation. The time point we have chosen to look for programming effects in this thesis was post-natal day 105 (PN105). The choice was loosely based on the timeframe used by others [4, 5, 9]. It resulted in a later life period (when the animals were fed HFD) that was three times the length of the intervention period. We considered these effects to be long-

standing.

Not necessarily all effects that result from a post-weaning intervention will last into adulthood, not even structural ones. In a mouse study in our lab with post-weaning a highly- or lowly-digestible starch diet, the intestines were longer in mice fed the lowly-digestible starch after three weeks on the diet, but the difference was no longer present in mice that were fed a HFD for nine weeks thereafter [10].

In **Chapter 4,** we reported a programming effect on body weight, which was clear from week 12 onwards. This indicates that the timeframe selected seems justified, although we cannot rule out that parameters that were significantly different at PN105 would have remained significant with an even longer lasting experiment. However, the effects on BW and FM from week 12 onwards till end of the study provides some suggestion that this is a biological relevant observation.

Differences induced by interventions in adulthood, for example on BW, will last some time, even though these interventions are not in a critical period of development. In a study where adult mice were either kept on or switched between a high (40 en%) and a low (10en%) fat diet, the effect on BW induced by the previous diet was lost in two to three weeks (Fig. 1) [11]. In line, the effects of previous diet disappeared within two weeks, when switching between 60 en% and 10 en% fat diets after six weeks [12]. Differences in BW induced by three to five weeks of 20% energy restricting on a medium-high-fat diet (30 en% fat), disappear even more quickly [13]. These patterns are clearly different than the changes in BW we observed in **Chapter 4**, where we observed a difference in BW that gradually increased in the HFD period.
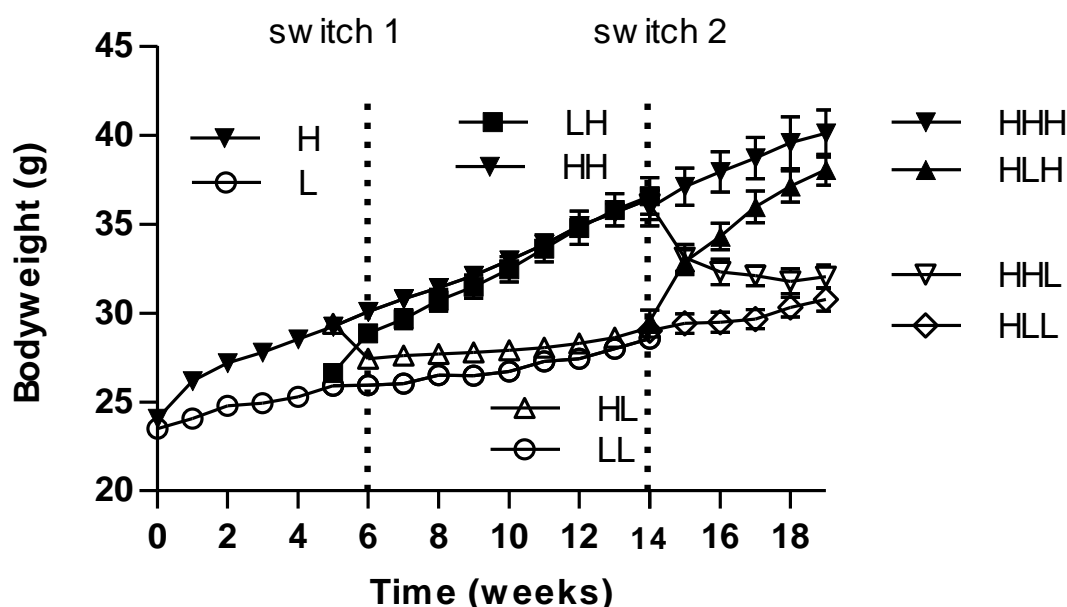
Figure 1. Body weight development for adult male mice switched between high fat (H; 40 en% fat) and low fat (L; 10 en% fat) diets. After the first switch in week 5, the BW of HL-group was similar to the LL group, and significantly lower than the LH and the HH groups from week 7 onwards. After the second switch in week 14, BW of the HHL-group was similar to the HLL group, and significantly lower than the HLH and HHH groups from week 17 onwards. Values represent mean ± SEM. Adapted from: Hoevenaars *et al*. [11].

Throughout this thesis (in particular in **Chapter 2**, **Chapter 4**, and **Chapter 6**) we have followed the definition for nutritional programming by Lucas [7]; "the concept that a stimulus or insult operating at a critical or sensitive period of development could result in a long-standing or life-long effect on the structure or function of the organism". However, some have argued that "programming" is a misleading term [14]. "Programming" may imply that the outcome of a nutritional insult in early life is fixed, while there actually is always plasticity, because the outcome of a nutritional insult in early life will always depend on the conditions an individual experiences in later life as well. Therefore, the term "conditioning" may be more appropriate. In this thesis, a similar dietary intervention did not give the same outcome in two separate nutritional programming experiments (**Chapter 4** versus **Chapter 6**). As discussed in **Chapter 6**, certain conditions were different between the two experiments, in particular the type of chow during pregnancy, lactation, and weaning, and the type of fat in the HFD. These slightly different conditions may be the reason that a significant difference in fat mass development was shown in one, but not in the other study. Thus also in our experiments, the term "conditioning" may be slightly better in covering the process. For ease and consistence, we will keep using "programming". However, to highlight that susceptibility can be influenced, we will redefine programming as: "the concept that a stimulus or insult operating at a critical or sensitive period of development results in a later life effect on the structure, function or susceptibility of the organism".

Galactose

As discussed in **Chapter 1**, lactose is present in human breast milk in large quantities. As a result, the dietary contribution of galactose is considerable in new-borns. Exposure to lactose, and thus galactose, decreases during weaning. After weaning, dairy products are the major source of lactose and galactose in adults. Lactose represents about 3% of the total energy intake per day for Dutch adults [15]. Similar intake levels (in g/day) were reported in a study in the UK [16]. Certain fruits and vegetables [17] and legumes [18] contain free galactose, but in very limited amount, thus these foods hardly contribute to overall galactose intake in the general population.

It raises the question why (ga)lactose is present in high concentrations in (human) milk. As discussed briefly in **Chapter 1,** milk has an immunomodulative role, besides its nutritional role. In fact, on an evolutionary scale, it is believed that the immunomodulative role of milk came first [19, 20] before the nutritional role of milk became important. Urashima *et al.* hypothesized that oligosaccharides were present in milk first, and because all oligosaccharides have lactose as a basis, high lactose enabled sufficient oligosaccharide formation. From there, the use of lactose as a source of nutrition by the young evolved [21]. In addition, lactose and its derivatives (such as human milk oligosaccharides) are suitable for storage in the mammary gland, in contrast to glucose [19]. Moreover, lactose has an osmoregulatory role [19].

In a study comparing the fat, protein, energy, and dry matter content in the milk of various

lactating species (not including humans), it was found that the composition of milk is likely the result of evolutionary history, maternal diet, and relative length of lactation period [22]. Sugar content of milk is probably the result of the same evolutionary drivers, although sugar content was not specifically analysed in that study, because of very low sugar concentrations in milk in some species [22]. Certain large mammalian species, such as seals whales and bears, have (very) low-sugar, high-fat milk. These animals fast for long periods of time during lactation [23]. Species that have a high sugar content in their milk, forage and feed during lactation. Primates have milk that is high in lactose, and low in protein and fat [24]. Primate milk is relatively low in energy, because frequent suckling is possible; and because infancy is relatively long [25]. The concentrations of lactose in human milk are not exceptional among primates [25], although the high galacto-oligosaccharide content and the type of galacto-oligosaccharides, are unique to human milk [21, 25].

In **Chapter 5**, we observed that adding galactose to the diet results in higher drink intake. How does the increased drink intake link with the presence of lactose (and thus galactose) in the milk? Based on our results we might speculate that (ga)lactose in milk is a way to ensure an infant keeps drinking. So far, no clear evidence for this theory was found in literature. However, the high (ga)lactose content in milk evolved in circumstances where frequent suckling was possible.

Whatever reason underlies the high concentrations of lactose in milk is, human infants encounter high levels of dietary lactose and therefore galactose. Thus there will be certain (metabolic) consequences of the exposure to high levels of galactose.

In our research, we aimed to see long-lasting effects of monosaccharides on metabolic health and body composition. From that perspective, we judged the effect of galactose as beneficial, in particular the lower fat mass in adulthood of females fed galactose post-weaning (**Chapter 4**). Below we discuss a number of studies, with very different focus (on aging and on female reproductive effects), that report other galactose-induced effects as adverse.

Chronic administration of D-galactose in rodents is used as an artificial model for aging research. D-galactose is the stereo-isomer of galactose that is naturally present in lactose and that is used in cellular metabolism in e.g. the glycosylation of proteins. With subcutaneous or intraperitoneal injections (or sometimes oral administration) of D-galactose for six or more weeks in young adult rodents, morphological and biochemical changes are induced that mimic those occurring with aging [26]. The D-galactose model is frequently used to study the aging brain and concurrent defects in memory and learning [26], but it is also applied to study aging in other tissues, such as the heart [27]. It is a quick and low-cost way of inducing accelerated aging in an animal model [28]. It is not clear what mechanisms underlie the D-galactose aging model, but several theories exists. These include the formation of reactive oxygen species (ROS), the formation of advanced glycation end products, an increased inflammation, and apoptosis [26]. A meta-analysis commented on inconsistencies and heterogeneity in the results of the D-galactose model [29]. For example, there are cases where the D-galactose

model was not able to elicit results [28]. Nevertheless, an effect of oxidative stress, with decreased superoxide dismutase and glutathione peroxidase activities, and increased malondialdehyde concentrations was observed in the meta-analysis [29]. It has been reported that the effects of D-galactose on memory function depend on sex and age, with young females being more resistant [30], but this was not confirmed [29]. Because of the use of the D-galactose model in aging research, we need to carefully consider if a similar effect could be relevant in our studies, where dietary galactose was given to young mice. We did not do any ageing-related brain analysis or behavioural testing, but we did measure the activity of female mice on the GLU+GAL diet in the calorimetry system, as part of our metabolic analysis. There was no difference in the activity of mice on either the GLU+GAL or GLU diet (**Chapter 5**). Furthermore, contrary to the D-galactose model that predicts increased inflammation [26], our data from **Chapter 5** show a slightly lower inflammation-related gene expression in the liver of mice on the GLU+GAL diet; and this was confirmed by serum SAA3 concentrations. This micro-array analysis (**Chapter 5**) also did not give an indication for increased ROS formation. We specifically checked micro-array results to see if transcripts that are generally affected by high ROS levels were regulated in our dataset, but we found no significant effect on *Sod1* (*Isoforms 1-3*), *Cat, Gtx* (isoforms 1-4, 6 and 7) and *Txn1*. For *Txn2*, 2 out of 3 distinct probes showed no significant regulation. In addition, catalase expression was not affected in intestinal cells (analysed by qPCR, data not shown). Overall, this suggests an absence of induction of ROS in our studies.

There are a number of differences that may partly explain this discrepancy. First of all, there is the length of exposure, which is typically 6 weeks of more in the D-galactose experiment. Remarkably, the exposure in our experiments was similar, as our dietary invention of three weeks was directly after the 3 weeks lactation period where the pups were exposed to (ga)lactose in milk. A big difference is that in the D-galactose model mice are exposed to galactose after a period of absence, which possibly may affect the way galactose is handled. Second, the dose of galactose has to be considered. Dosages in the D-galactose aging model range from 50 - 1250 mg galactose per kg BW per day [28], but doses of 50 - 300 mg galactose per kg BW are most frequently used [26, 29]. Because we provided the galactose in the diet, an exact dose cannot be given, but we can make an estimation (data **Chapter 5** and **Chapter 6**). The average female mouse on the GLU+GAL diet ate 2.82 gram of the diet per day with 16% w/w of galactose, which amounts to 0.45 gram of galactose per day (calculated over the entire intervention period). Average body weight at PN35 (after 2 weeks on the diet) was 16.6 g, which would equal to ~ 27 g galactose per kg BW per day. The dosage in **Chapter 4** (with very similar diet composition) will be of similar order of magnitude. Thus the daily dose of galactose in our study was about 100 - 500 times higher than the dose typically given by injections (50 - 300 mg per kg BW). Finally, there is a difference in the route of administration. Route of administration can significantly affect the outcome. For example, oral butyrate supplementation suppresses food intake, while intravenous administration does not, even though higher circulating levels of butyrate are reached with the latter method [31]. The

subcutaneous and intraperitoneal injections in the D-galactose model, will give a single, high peak level per day, and will reach systemic circulation without a "buffering" organ. In contrast, dietary galactose will be taken in over the day, and will first be taken up by enterocytes, before going into the portal vein. Hepatic clearance of galactose will be considerable [32], decreasing systemic concentrations of D-galactose. Even though concentrations that reach systemic circulation may be similar because of the higher daily dose, the dietary galactose will lead to more gradual increases and multiple peaks in circulating galactose concentrations.

Speculating, because of differences in the route of administration, effects on blood pressure will be different as well. It might be that effects of blood pressure underlie the D-galactose-induced effects in the brain in the aging model. In detail, circulating concentrations will peak rapidly following an injection with galactose. This will instantly lead to galactose excretion (or incomplete galactose reabsorption) in the kidney. The galactose in the urine will lead to higher water loss, whereby blood viscosity increases and blood volume (slightly) decreases, leading to hypotension. This hypotension will lead to increased drink intake, but might also influence the brain. In fact, chronic hypotension is associated with a diminished cognitive function, via decreased cerebral blood supply [33]. In addition, orthostatic hypotension, rapid decreases in blood pressure due to standing up, is associated with incident dementia [34, 35] and cognitive decline [36] in humans. Therefore it might be the fluctuations in blood pressure that underlie the age-inducing effects in the brain seen in the D-galactose model. In our model with dietary galactose, however, increases in circulating galactose concentrations will change less abruptly. Compensatory drink intake might be more gradual as well, leading to less fluctuations in blood pressure, thereby minimizing the induction of brain damage.

Overall, our data, in particular from the micro-array in the liver, suggest that the effects of the D-galactose model are not relevant here. But because no analysis on the brain was done in animals at PN42, we cannot fully exclude that similar, so-called age-inducing, effects that are seen in the D-galactose model are occurring here. Therefore it will be worthwhile to do some brain analyses of animals on a GLU+GAL diet in follow-up studies, for instance into ROS levels and inflammation markers, so it can be verified to see if any aging-like effects are induced by galactose in the diet. Nevertheless, that being said, the D-galactose model with its negative effects is in contrast with the natural occurring high levels of galactose in (human) milk. Dosages of (ga)lactose to which children and infants are exposed, are particularly high; while the early post-natal (and weaning) period is one of extensive neurological development [37, 38]. It is highly counterintuitive that galactose would negatively affect brain development in babies.

In addition, adverse effects on female reproductive function have been shown in animal models by galactose. In young female mice, two to six weeks on a 50% (w/w) galactose diet decreases ovulatory activity [39]. In addition, this 50% galactose diet significantly reduces the number of ovulating oocytes upon gonadotropin stimulation, but one week of normal diet after seven weeks of galactose diet, normalizes the ovulation upon gonadotropin stimulation

[39]. A maternal diet with 50% of galactose reduces oocyte number in offspring, but also significantly hinders growth of the pups (two- to three times smaller than normal) [40]. Three weeks of a 20% (w/w) galactose diet in (pre-) pubertal female rats reduces the number of growing and antral follicles [41]. A diet with 50% galactose is a very high dose so in our studies with 16 en% (which was also 16 % w/w) effects may be less pronounced or even absent. However, the difference between our GLU+GAL diets and the 20% (w/w) galactose diet in the rat study [41], was not so large. These findings prompted us to do some initial analyses to screen if ovarian function was affected in our nutritional studies.

Ovary weight at end of the study (PN105) was not affected by galactose in the post-weaning diet (Fig. 2A, using tissue from the mice described in **Chapter 4**). Serum concentrations of anti-Müllerian hormone (AMH), a marker for ovarian reserve, were also not significantly different between females fed GLU and GLU+GAL in early life (Fig. 2B), suggesting an absence of adverse effects. However, as these measurements were performed after nine weeks of HFD, and certain effects of dietary galactose may be reversible [39], it was relevant to also study the ovaries of animals immediately after three weeks of the GLU+GAL diet (study described in **Chapter 5**). Preliminary results of histological examination of the ovaries at PN42 showed no significant effect on the numbers of antral follicles, pre-antral follicles, and *corpora lutea* (n=4-5 per group, data not shown). Therefore, we consider that the potential adverse effects of galactose on the ovaries were not of relevance in our studies.
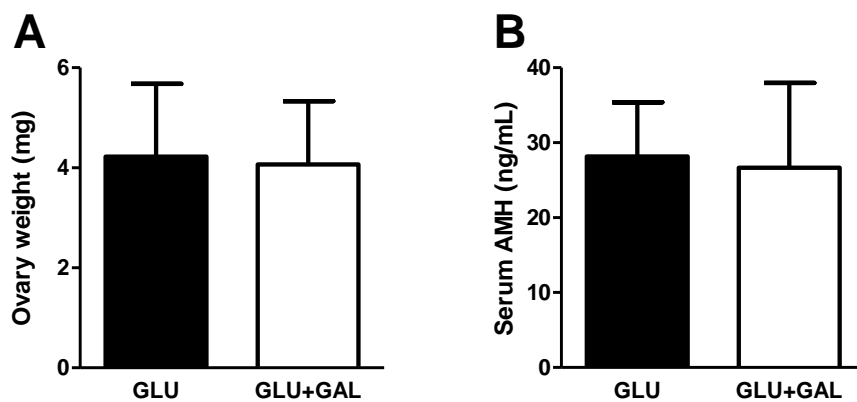

Figure 2. Ovarian function. Ovarian weight (A) and serum concentrations of Anti-Müllerian Hormone (AMH) (B) in females at PN105, who were fed GLU diet or GLU+GAL diet from PN21-42, and thereafter HFD till PN105. This mouse study is reported in **Chapter 4**. Values represent mean ± SD, n=13-14.

Overall, our results do not suggest that dietary galactose induced ageing-like effects or decreased reproductive function in our studies. Thus, we can still consider that effects of dietary galactose in the post-weaning period are beneficial directly (**Chapter 5**) or on the long-term (**Chapter 4**). However, it would be good to include some brain (and ovarian) analysis in follow-up studies into potential beneficial metabolic effects of dietary galactose, to exclude that the dietary intervention induces unwanted, negative side- effects. Analysis directly after three weeks of the diet would be most relevant for studying the direct effect. In addition, analysis in longer experiments, with HFD periods lasting for 26 weeks or longer, will give more

insight into the susceptibility in late adulthood.

Tissues and mechanisms

Besides establishing whether the post-weaning period is susceptible to nutritional programming by monosaccharides, this thesis also aimed at identifying mechanisms associated with programming effects. After observing the programming of lower fat mass development in adulthood by galactose given in the post-weaning period (compared with glucose only) (**Chapter 4**), we aimed at establishing which mechanism may be involved (**Chapter 4, 5 and 6**). Our main hypothesis was that altered insulin signalling underlies the metabolic programming effect. Indeed, **Chapter 4** showed a programmed effect on basal serum insulin concentrations, and downregulation of *Irs2* expression in gonadal and subcutaneous adipose tissue, and of *Irs1* expression in subcutaneous tissue. In **Chapter 5**, it was shown that there was a direct effect of three weeks dietary galactose on serum insulin concentrations. In **Chapter 6**, we intended to study how insulin signalling was affected by the post-weaning intervention with galactose. Therefore, the mice were challenged with a glucose bolus before sacrifice. However, no evidence for reduced insulin resistance in adult females fed galactose post weaning was found. The OGTT results in this chapter even pointed towards a higher insulin resistance in the females that had galactose in early life, but no significant effect remained until the endpoint, as determined by circulating insulin and glucose levels.

To clarify in what way insulin signalling is really affected, another repetition of the study with GLU+GAL and GLU diets is advised. Such a study could be more informative if it would include a hyperinsulinaemic-euglycaemic clamp (HIEC) for insulin sensitivity measurements. This technique is considered as golden standard for human research [42, 43]. Also in mice research, HIEC experiments are most informative on insulin sensitivity, although it is technically challenging to perform studies with this technique [44]. The frequently used glucose tolerance tests have a somewhat limited sensitivity, and the outcomes of the HOMA-IR do not always predict insulin sensitivity accurately [44, 45]. Furthermore, studying epigenetic marks related to insulin signalling would be interesting and could provide more conclusive answers.

In our studies, females seemed more susceptible to programming by galactose. Similarly, females are more susceptible to adverse *in utero* programming by glucose availability [46].

Another mechanism we investigated, was hypothalamic programming. Hypothalamus circuits involved in energy homeostasis and food intake regulation develop mainly postnatal in mice, although connectivity within these circuits is established in the four weeks after birth [47]. Leptin [48, 49] and insulin [50] are thought to affect hypothalamic circuit formation [47]. Our intervention, starting at PN21, overlapped partially with the last stretch of the hypothalamic circuit formation, and may thus have had some impact. Insulin concentrations probably were slightly affected by the diet, supported by the finding of **Chapter 5** that showed lower serum insulin concentrations in food-deprived animals that received the GLU+GAL diet for three weeks. There is no reason to believe that the leptin concentrations were different during this

phase of development, since a similar increase in fat mass was observed in the first week of the intervention period (**Chapter 4** and **Chapter 6**), and leptin levels are strongly correlated with fat mass [51]. In **Chapter 4**, the hypothalamus was studied in female mice at PN105, but no long term effects on neuropeptide gene expression or leptin signalling were found (Fig. 5 in **Chapter 4**). The method of studying mRNA expression in the whole hypothalamus, may be considered as relatively crude, and thus subtle differences may have been overlooked, such as differences in connectivity in parts of the hypothalamus. Immunohistochemistry of different brain areas may be used to enhance spatial resolution, and would provide information about the functional protein level, rather than the mRNA level. Alternatively, our intervention may not have had lasting effects, because the intervention period only partially covered the period of hypothalamic development. Studies starting at PN2 or even PN16 have at least a larger overlap with this hypothalamus developmental timeframe, which might in part explain larger differences in those studies. Nevertheless, it cannot be ruled out that lipids have a larger effect than carbohydrates, and future studies are warranted to elucidate existence of nutritional differential effects.

Food intake was increased on the GLU+GAL diet compared with intake on the GLU diet, in males and females (**Chapter 4**). This increased food intake on the GLU+GAL diet during the post-weaning intervention period was replicated both in **Chapter 5** and **Chapter 6**. However, the increased food intake on the HFD in females as observed in **Chapter 4**, was not seen in **Chapter 6**. Thus, there was no clear incentive to study this further.

In **Chapter 5** we observed a strong increase in drink intake in animals on the GLU+GAL diet, and galactose was found in the urine of these animals. The formation of nephrons starts in the foetal phase and is completed before birth [52], thus nephron number will likely not be affected in our studies. It would be interesting to see if there is any adaptation in sugar transporter expression, and to see if there is any adaptation possible.

Extrapolation to human situation

A pre-clinical mouse model was used in our studies to investigate the nutritional programming effects of monosaccharides in the post-weaning period, but eventually we intend to translate these findings into predictions for the human situation. Below certain aspects that have to be taken into account when translating the findings (on galactose) to the human situation, will be discussed. Based on promising results in animal studies, an intervention may be tested in humans. This requires safety testing first. For example, nutritional programming effects of large lipid droplets coated with milk fat globule membrane fragments in rodents [2-5, 53] (**Chapter 1**), was recently studied for safety in human infants [54], after it was successfully tested in human adult subjects for safety and metabolic parameters [55]. Whether the formula will have similar long-term effects on body composition, like those found in mice, remains to be confirmed. The formula was tested in both rodents and humans, despite the clear differences in mouse and human lipid metabolism, like for example the HDL and LDL

fractions of circulating lipids [56].

Developmental periods of mice and humans do not align, even when lifespan is taken into account. For example, certain developmental processes that take place during the final trimester of pregnancy in humans, occur (partly) after birth in rodents, such as the formation of functional connections in the hypothalamus [47], and the saccular formation in lung development [57]. Overall, development to sexual maturity is much faster in mice than in humans, also in relative terms. Maturation in early life (birth to one month of age in mice) is about 150 times faster in mice than in humans, while maturation in older mice (6+ months of age in mice) is only 25 times faster than in humans [58]. For translation of the mouse results to the human situation, developmental periods should be compared. Similar insults during similar phases of development are likely to have similar programming effects, even though life stages may not be the same in two species.

Infants are probably well adapted to metabolise galactose. Galactose utilisation is higher in infants than in adults [59]. Galactose elimination capacity (in µmol/min/kg body weight) decreases with age: the capacity in adulthood is about half of the capacity in one-year-olds [60]. Glucose levels rise much more after galactose administration in new-borns than in children and adults [61]. In elderly, galactose elimination capacity declines further, but this is considered to be the result from a decrease in liver functionality [62]. Animal models have also shown that galactose utilisation is higher in new-borns than in adult animals [59]. Therefore, it is likely that galactose metabolism is enhanced in mouse pups compared to adult mice as well. As discussed in **Chapter 1,** lactose concentrations in human milk are around 64-76 g/L [63, 64]. In mouse milk, lactose concentrations of 1.1 – 3% have been reported [23, 65, 66]. Mouse pups may be less adapted to galactose usage than human babies, simply due to lower exposure to lactose, and thus galactose, in the lactation period.

Implications

Breast milk is considered the ideal food for infants, and thus breast milk composition is the basis for infant formula formulations. As discussed in **Chapter 1**, lactose is the main carbohydrate in breast milk, and other sugars are present in trace amounts. Nonetheless, European regulations state that infant formula's may contain other sugars, such as sucrose, glucose, maltose, and maltodextrins [67]. Lactose content should be at least 1.1 g per 100 kJ, but as 2.2-3.4 g per 100 kJ carbohydrates should be present, also other carbohydrates are allowed. But, results of our studies might suggest that a higher lactose content, similar as has been measured in breast milk, may be advisable.

As higher lactose (and thus galactose) content might increase drink intake, it is important what kind of drink is given. A large part of the toddlers (in Western societies) consume fruit juices and/or sugar sweetened beverages [68, 69] that contribute significantly to additional sugar intake. High intake of sugar-sweetened beverages and free sugars is associated with higher incidence of dental caries and adiposity [70]. Adiposity in the childhood phase often persists

into adulthood [71], thus lowering the risk of adiposity in early life is important. The advice to caregivers is to limit the juice and sugar-sweetened beverage intake of toddlers and children, and to replace it with water, or with milk products [70]. As long as this advice is taken to heart, no negative consequences follow from increased drink intake, and additional (ga)lactose may present a minor beneficial effect.

During weaning, complementary foods are added to the diet. These foods are easily digestible, and rich in (fruit) sugars [72]. Cultural differences exist, with e.g. in Sri Lanka, weaning foods being mainly rice-based. Our results suggest that it might not matter if it is in particular glucose or fructose in these foods.

As early life growth trajectories determine later life susceptibility to overweight and obesity, the role of carbohydrates and in particular sugars in the weaning period and early post-weaning period is very important.

Conclusions

The results presented in this thesis indicate that the post-weaning period may be susceptible to nutritional programming by dietary monosaccharides, in particular by galactose. The effect, found in a mouse model, is modest, and highlight that conditions (perinatally and/or in adulthood) can affect outcomes. Our studies suggest that fructose in the post-weaning period has no major impact on body composition and metabolic health in later life, at least when compared to glucose. With regards to sexual dimorphisms in programming, our studies suggest that females are more susceptible to nutritional programming by monosaccharides in the post-weaning period than males. Although there are clues that insulin signalling could be involved in the programming effect with galactose, it is currently not clear if this is indeed the main mechanism involved.

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Summary

Nutrition in early life can have lasting effects on metabolism: nutritional programming. Pregnancy and lactation are well established critical periods of development impacting lifelong health, but development continues after these periods. Therefore, nutrition in later periods, such as the weaning and (early) post-weaning period, may also induce lasting metabolic effects. After lactation, when an infant relies solely on milk for nutrition, the weaning period commences. In this phase, solid foods are gradually introduced; it is a period of great diversification of the diet. The transition to solid foods introduces a major change in carbohydrate exposure. The main carbohydrate in the lactation period is lactose, a dimer of glucose and galactose. Gradually, a variety of di- and polysaccharides are introduced that are mainly glucose based, but can also contain fructose.

Whether or not exposure to different types of monosaccharides at weaning has lasting effects on metabolism and metabolic health is not known. The aim of this thesis was to establish whether the post-weaning period is a critical developmental period in which exposure to different types of monosaccharides can lead to programming of adult metabolic health. We were particularly interested in galactose and fructose. Our interest in galactose was because this sugar almost disappears from the diet after weaning. Our interest in fructose was because it appears in the diet at weaning, and is suggested to be more detrimental to health compared to glucose, which is always present and is taken as a reference. Thus we compared effects of the dietary monosaccharides fructose and galactose, to those of glucose. Analyses were focussed on body composition and metabolic health. A mouse model for nutritional programming was used. In this model, newly weaned C57BL/6JRccHsd mice were fed with diets differing in monosaccharide content for three weeks. Afterwards, all mice were fed an obesogenic high-fat diet (HFD) for nine weeks.

Chapter 2 describes a mouse study where the effects of fructose in the post-weaning diet on later life health were compared to the effects of glucose alone. Body weight, body composition, and organ weights were similar in both groups after the nine-week HFD period. Indirect calorimetry analyses indicated that respiratory exchange ratio's (RER's), energy expenditure, activity, and metabolic flexibility were not different between fructose- and glucose fed animals, neither directly, nor when on the HFD. Serum insulin concentrations were significantly lower in females fed fructose post weaning, than in females fed glucose post weaning, while serum insulin concentrations were not significantly affected in males. From these data we concluded that fructose and glucose are comparable in their direct effect, and there is no adverse programming of fructose compared to glucose in the post-weaning period.

For additional insight in metabolic effects of fructose, the direct effects of fructose, fructose and glucose in a 1:1 ratio, and glucose, were studied in adult male and female mice on a moderate HFD (**Chapter 3**). Mice on the HFD with fructose had slightly lower energy intakes overall. Body weight was not affected by the monosaccharide composition of the diet, and nor were plasma insulin concentrations. Hepatic gene expression analyses showed minor

upregulation of hexokinase expression in fructose fed compared to glucose fed males, without significant alterations in sugar transporters, or glycolysis- or *de novo* lipogenesis-related enzymes. Gene expression in the liver and intestine of female mice showed no consistent differences. Overall, our physiological data indicate that isocaloric dietary fructose does not result in more adverse physiological effects than a diet containing glucose-fructose or glucose.

Next, we examined the effects of galactose. The programming effect of post-weaning galactose and glucose in a 1:1 ratio mimicking the milk sugar lactose was compared to the effect of glucose alone (**Chapter 4**). In females, body weight and fat mass gain on the HFD were significantly lower in animals fed galactose post weaning. These females had lower circulating serum insulin concentrations, lower adipose depots weights, with a tendency towards smaller adipocytes in gonadal white adipose tissue, and altered insulin-signalling-related gene expression. Although food intake was significantly higher in the post-weaning period, and significantly lower in the HFD period, no effects in hypothalamic gene expression on food-intake related neuropeptides or leptin signalling were found. In males, fat mass development was not affected by post-weaning monosaccharides. Concluding, this study showed that replacing glucose with galactose in a post-weaning diet, in a 1:1 ratio (mimicking lactose), had beneficial metabolic programming effects in female mice, over glucose alone.

Chapter 5 shows the direct effect of post-weaning galactose and glucose in a 1:1 ratio compared to glucose alone in females. Females on the galactose diet had a higher food intake and a two-fold higher drink intake than females on the glucose diet. High-performance anion-exchange chromatography analyses indicated galactose presence in the urine of females on the galactose diet. Indirect calorimetry measurements showed no significant effect on energy expenditure or average RER, but maximal RER in the dark phase was lower in females on the galactose diet. Serum insulin concentrations and hepatic triglyceride levels were lower in females on the galactose diet. Transcriptomic analysis of the liver indicated that the gene expression profiles in metabolic pathways were not significantly affected by the diet, but inflammation-related gene expression profiles were slightly downregulated in galactose-fed females. Concluding, replacing part of glucose with galactose in the post-weaning diet reduces hepatic TG content and hepatic inflammation, implying immediate beneficial effects.

In a second mouse study examining the metabolic programming effects of post-weaning galactose in females, presented in **Chapter 6,** no differences in body weight gain and fat mass gain were seen in the HFD period. The oral glucose tolerance test showed no difference in glucose tolerance, but indicated that circulating plasma insulin concentrations were relatively more increased in females fed galactose as analysed by the insulin incremental area under the curve. At the end of the study, no significant differences were found in circulating insulin concentrations, AKT phosphorylation and insulin-related gene expression in gonadal white adipose tissue. Concluding, replacing part of glucose with galactose in the post-weaning diet did not beneficially affect body composition or insulin signalling in adult female mice in an

obesogenic environment in this study. Differences between the results of **Chapter 4** and **Chapter 6** may be due to differences in the experimental conditions.

 In **Chapter** 7 the findings of this thesis are discussed. Concluding, this thesis shows that the post-weaning period may be susceptible for nutritional programming by dietary monosaccharides, in particular by galactose. The effect is modest, and inconclusive as two studies yielded different outcomes. Although there are some clues that insulin signalling is involved, it is so far not clear what the main mechanism is. Females seem to be more susceptible to programming by monosaccharides in the post-weaning period than males. Our results suggest that there are no major differences in metabolic programming by fructose and glucose in the post-weaning period. Also no major differences were seen between fructose and glucose in adult mice. Extending the period of galactose intake after weaning may be beneficial as it seems to protect against liver inflammation.

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About the author

Curriculum Vitae

Lianne Margarethe Sophie Bouwman was born on the 11th of January 1989 in Zevenaar, The Netherlands. In 2007, she completed pre-university education at the Liemers College in Zevenaar. In the same year, she started her BSc program Life Sciences and Technology at the University of Groningen, with the Major Behaviour and Neurosciences. After completion of the Minor Education & Public Administration, she received her *cum laude* Bachelor degree in 2011.

In 2012, Lianne started her MSc Food Safety at Wageningen University. She did her MSc thesis at the department of Food Toxicology at the Wageningen University, where she worked on *in vitro* assays for the detecting of thyroid peroxidase inhibition and thyroid receptor-coregulator interactions. She conducted her internship at the department Neurotoxicology, Institute for Risk Assessment Sciences, in Utrecht, where she studied the effects of PFOS and PFOA on $GABA_A$ receptor function and spontaneous activity in neuronal networks. She completed her MSc education by the end of 2014.

In January 2015, Lianne started with her PhD research in the NWO-TTW-funded project "Optimizing early-life dietary carbohydrates to improve later-life metabolic health", at the chair group of Human and Animal Physiology at Wageningen University. During her PhD project, she participated in the educational program of the Graduate School VLAG. Lianne attended several national and international conferences and courses. In addition, she was involved in teaching activities of the chair group and supervised several MSc students during their MSc thesis.

Currently, she is working at Wageningen Food Safety Research as Researcher in the BU Toxicology Novel Foods & Agrochains, and at Wageningen University within the department of Human and Animal Physiology on education.

List of Publications

Peer-reviewed publications

- **Bouwman LMS**, Fernandez-Calleja JMS, Swarts HJM, van der Stelt I, Oosting A, Keijer J, et al. No Adverse Programming by Post-Weaning Dietary Fructose of Body Weight, Adiposity, Glucose Tolerance, or Metabolic Flexibility. *Mol Nutr Food Res* 2018; 62. DOI:10.1002/mnfr.201700315
- Fernandez-Calleja JMS, **Bouwman LMS**, Swarts HJM, Oosting A, Keijer J, van Schothorst EM. Direct and Long-Term Metabolic Consequences of Lowly vs. Highly-Digestible Starch in the Early Post-Weaning Diet of Mice. *Nutrients* 2018; 10. DOI:10.3390/nu10111788
- Fernandez-Calleja JMS, Konstanti P, Swarts HJM, **Bouwman LMS**, Garcia-Campayo V, Billecke N, et al. Non-invasive continuous real-time in vivo analysis of microbial hydrogen production shows adaptation to fermentable carbohydrates in mice. *Sci Rep* 2018; 8:15351. DOI:10.1038/s41598-018-33619-0
- **Bouwman LMS**, Fernandez-Calleja JMS, van der Stelt I, Oosting A, Keijer J, van Schothorst EM. Replacing part of glucose with galactose in the post-weaning diet protects female but not male mice from high-fat diet-induced adiposity in later life. *J Nutr* 2019; 148. DOI:10.1093/jn/nxz028
- Fernandez-Calleja JMS, **Bouwman LMS**, Swarts HJM, Oosting A, Keijer J, van Schothorst EM. Extended indirect calorimetry with isotopic CO_2 sensors for prolonged and continuous quantification of exogenous vs. total substrate oxidation in mice. *Sci Rep* 2019*, 9:11507. DOI:10.1038/s41598-019-47977-w
- **Bouwman LMS**, Swarts HJM, Fernandez-Calleja JMS, van der Stelt I, Oosting A, Keijer J, et al. Partial replacement of glucose by galactose in the post-weaning diet improves parameters of hepatic health. *J Nutr Biochem, Revised version accepted.* DOI:10.1016/j.jnutbio.2019.108223

Expected publications

- **Bouwman LMS**, Niewenhuizen AG, Swarts HJM, Piga R, van Schothorst EM, Keijer J. Metabolic effects of the dietary monosaccharides fructose, fructose-glucose or glucose in mice fed a starch-containing moderate high-fat diet. *Submitted*.
- Tukker AM, **Bouwman LMS**, Van Kleef RGDM, Hendriks HS, Legler J, Westerink RHS. Acute in vitro exposure to perfluorooctane sulfonate (PFOS) and perfluorooctanoate (PFOA) inhibits human GABAA receptor function and affects spontaneous neuronal network activity of primary rat cortical neurons and hiPSC-derived neuronal networks. *Submitted*.
- **Bouwman LMS**, Oosting A, Fernandez-Calleja JMS, Keijer J, van Schothorst EM. The effect of post-weaning dietary galactose on nutritional programming of insulin resistance in adult female mice. *In advanced stage of preparation*.

Overview of completed training activities

Discipline specific activities

Course Laboratory Animal Science, Utrecht University	(Utrecht, NL, 2014)
Course Energy Metabolism and Body Composition, VLAG	(Wageningen, NL, 2016)
Course Epigenesis and Epigenetics, VLAG, *poster presentation*	(Wageningen, NL, 2017)
Dutch Nutritional Science days, NAV	(Heeze, NL, 2015, 2016)
Dutch Nutritional Science days, NAV, *oral presentation*	(Heeze, NL, 2017, 2018)
Symposium Early life Nutrition and growth, Nutricia research	(Groningen, NL, 2017)
Symposium Guts 2 Move, HAP	(Wageningen, NL, 2017)
Conference Power of Programming, *poster presentation*	(Munich, DE, 2016)
Conference Early Life Nutrition and Growth, *poster presentation*	(Amsterdam, NL, 2017)

General courses

Course Bridging across Cultural Differences	(WGS, Wageningen, 2016)
Course Techniques for Writing and Presenting a Scientific Paper	(WGS, Wageningen, 2016)
Course Teaching and Supervision MSc Thesis Students	(WGS, Wageningen, 2016)
Course Effective behaviour in your Professional Surroundings	(WGS, Wageningen, 2017)
Course Career Perspectives	(WGS, Wageningen, 2018)
PhD Week	(VLAG, Baarlo, 2015)
PhD Workshop Carousel	(WGS, Wageningen, 2015)
PhD Workshop Carousel	(WGS, Wageningen, 2017)

Optional activities

Preparation of research proposal	(HAP, 2015)
Weekly HAP group meeting *oral presentation*	(HAP, 2015-2018)
Project progress meetings *oral presentations*	(TTW NWO, 2015-2019)
Review RMC Proposals	(WIAS, 2016)
Essentials of Nutritional Physiology	(WUR, 2017)
Journal Club HAP	(2017-2018)

HAP: Department of Human & Animal Physiology, Wageningen University