José M. S. Fernández-Calleja

EXTENDED INDIRECT CALORIMETRY AS A PHYSIOLOGICAL PHENOTYPING TOOL IN MOUSE NUTRITIONAL INTERVENTION STUDIES, WITH A FOCUS ON METABOLIC PROGRAMMING BY STARCHES

Propositions

1. Incorporating new gas sensors into mouse indirect calorimetry systems not only serves scientific progress but also contributes to the refinement principle in humane animal research.
   (this thesis)

2. Childhood and adolescence should be recognised as sensitive periods of development within the theoretical framework of nutritional programming.
   (this thesis)

3. If we want to keep using the word "race" in the human biomedical sciences we must make clear whether we refer to the biological definition or the social construct.

4. Tiny effects of the gut microbiome on obesity can still help your way into Nature if you present them as percentages of fat gain (Turnbaugh *et al.*, Nature 2006).

5. The multiverse theory is not scientific.

6. Before we can study metabolically healthy obesity (Cardenas-Sanchez *et al.*, J Adolesc Health 2019) we should have a solid definition of health.

7. Today's scientists should be more like the scientists of yore, marvelled at the sight of a new star in the firmament rather than a new star in our statistical outputs.

8. Female mice are the most unsuspected victims of male chauvinism in science.

Propositions belonging to the thesis, entitled

Extended indirect calorimetry as a physiological phenotyping tool in mouse nutritional intervention studies, with a focus on metabolic programming by starches

José M. S. Fernández-Calleja

Wageningen, 19 November 2019

Extended indirect calorimetry as a physiological phenotyping tool in mouse nutritional intervention studies, with a focus on metabolic programming by starches

José M. S. Fernández-Calleja

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 Michiel Kleerebezem, Wageningen University & Research
Prof. Dr Paula Oliver, University of the Balearic Islands, Spain
Prof. Dr Ko Willems van Dijk, Leiden University Medical Center
Dr Wilma Steegenga, Wageningen University & Research

This research was conducted under the auspices of the Graduate School VLAG (Advanced studies in Food Technology, Agrobiotechnology, Nutrition and Health Sciences).

Extended indirect calorimetry as a physiological phenotyping tool in mouse nutritional intervention studies, with a focus on metabolic programming by starches

José M. S. Fernández-Calleja

Thesis

submitted in fulfilment of the requirements for the degree of doctor
at Wageningen University
by the authority of the Rector Magnificus,
Prof. Dr A.P.J. Mol,
in the presence of the
Thesis Committee appointed by the Academic Board
to be defended in public
on Tuesday 19 November 2019
at 1:30 p.m. in the Aula.

José M. S. Fernández-Calleja
Extended indirect calorimetry as a physiological phenotyping tool in mouse nutritional intervention studies, with a focus on metabolic programming by starches,
192 pages.

PhD thesis, Wageningen University, Wageningen, the Netherlands (2019)
With references, with summary in English and Spanish

ISBN 978-94-6395-111-1
DOI 10.18174/499124

Table of Contents

Chapter 1

General Introduction

In this Chapter, I will present the fundamental concepts and recurring themes of this thesis. First, the theoretical background of indirect calorimetry (InCa) and some shortcomings of this technique are introduced. Then, the known health implications of dietary starches and their digestibility are introduced, emphasising on their apparent sex-dependent effects indicated by rodent models. We then move on to the concept of metabolic programming and how the potential of post-weaning starches to modulate metabolic health later in life is understudied. Having identified technological and scientific knowledge gaps in these areas, the aims of this thesis are delineated, along with a brief description of how these aims were addressed in each Chapter.

Indirect calorimetry

InCa is a technique used to estimate energy expenditure (EE) at the whole body level based on measurement of oxygen (O_2) consumption and carbon dioxide (CO_2) production. In the cell, acetyl-CoA derived from glucose, fatty acids, and amino acids is used to produce ATP in a series of redox reactions that consume O_2 and produce CO_2 and water. The commercial application of this technique to human physiology goes back to the beginning of the 20th century[1], and is at the centre of numerous metabolic studies in humans and other animals, including rodents[2], production animals[3], fish[4], birds[5] and insects[6,7]. InCa remains the gold standard to measure energy expenditure in clinical settings[8,9], despite the availability of other well-established techniques (*e.g.* direct calorimetry and the double labelled water method, DLW). While these and other alternatives are valuable in certain situations, their intrinsic challenges and limitations have favoured the use of InCa to estimate EE[10]. For example, measuring O_2 and CO_2 exchange by InCa can be done much more accurately than measuring heat production by direct calorimtery[10], and EE estimated by InCa is not affected by macronutrient imbalance, a common source of error in DLW studies when unaccounted for[11]. InCa is based on long-established notions, like the conservation of energy in closed systems, and assumptions, like the precise stoichiometry of substrate oxidation reactions. Thus, the volumes of O_2 and CO_2 can be used to calculate the rate at which a mixture of nutrients is utilised by the body to produce ATP.

InCa has evolved across scientific fields. The first commercial InCa systems in the 1920s were only capable of measuring O_2, and only decades later were they able to accurately measure both O_2 and CO_2 thanks to the invention of gas analysers that did not rely on material balance[12]. Further developments led the design of portable and inexpensive devices for humans[12], as well as airtight respiration chambers for both production animals and humans, ideal when free movement or grouped housing are needed[13]. In rodents, InCa systems (currently produced by three main manufacturers, Columbus Instruments International, TSE Systems, and Sable Systems International) not only offer robust measurements of gas exchange, but also simultaneous records of food intake, temperature, and physical activity[10]. For mice in particular, there is consistent progress in developing the InCa technique in theory and practice. For example, efforts are ongoing to increase the time resolution of O_2 and CO_2 measurements[2], improve the interpretation of EE in light of body mass and compositional data[2,14], and even to validate EE measurements without knowledge of CO_2 production[15]. Clearly, InCa is a current, non-invasive, and evolving

technique that offers researchers working in rodent models an excellent tool to study whole body metabolism.

For each of the different substrates, coefficients for nutrient oxidation (Table 1) represent the basis of InCa, and are used to calculate EE and substrate use under many experimental conditions. The oxidation of carbohydrate and fat mixtures is easily estimated based on the ratio of CO_2 production to O_2 consumption, called respiratory quotient (RQ, when referring to cellular metabolism) or respiratory exchange ratio (RER, for whole body level metabolism). Thus, an RER of 1 indicates net carbohydrate oxidation and 0.7 indicates net fat oxidation, based on the stoichiometry of oxidation reactions. For instance, 6 mol of CO_2 are produced and 6 mol of O_2 are consumed in the complete combustion of 1 mol of glucose (CO_2/O_2 = 1.0), and 16 mol of CO_2 are produced and 23 mol of O_2 are consumed for every mol of palmitate oxidised (CO_2/O_2 = 0.7). As protein oxidation is generally constant and not including this process overestimates EE very marginally[2], protein oxidation is often neglected in rodent studies and referred to as non-protein RER. For the remainder of this thesis, I will use the term RER to refer to non-protein RER. Many other coefficients and formulas have been developed to account for the oxidation of unusual substrates (*e.g.* alcohol) and other products of substrate oxidation reactions (*e.g.* urea, ketone bodies)[16]. Some of these substrates and products may become relevant under certain circumstances. For instance, a correction for biosynthesis of ketone bodies excreted in urine and breath (thus, exceeding their oxidation) may be in order when subjects consume ketogenic diets[17]. Therefore, O_2 and CO_2 measurements safely indicate metabolic rate and the net balance of substrates oxidised at the whole body level in most conditions. But what relevant phenotypic information are we missing when we only measure O_2 and CO_2?

Table 1. Calorimetric coefficients used for the calculations of EE and fuel utilisation

	O_2 consumed ($l\ g^{-1}$)	CO_2 produced ($l\ g^{-1}$)	Energy produced ($kJ\ g^{-1}$)	Energy equivalent of O_2 ($kJ\ l^{-1}$)	RQ
Carbohydrate	0.89	0.89	17.48	21.12	1.000
Fat	2.02	1.43	39.60	19.61	0.710
Protein	1.03	0.85	20.18	19.48	0.833

Based on Elia and Livesey[16]. These coefficients vary depending on the exact composition of model carbohydrates, fat, and proteins. A glucan residue, dioelylpalmitate, and Kleiber's standard protein, respectively, are reported in this table. Respiratory quotient (RQ), in contrast to respiratory exchange ratio (RER), is the preferred term here because RQ is independent of non-metabolic processes (hypo- or hyper-ventilation or changes in the bicarbonate pool).

This thesis is focussed on two pertinent examples where current mouse InCa systems could be improved to study the phenotype of the animal in a better integrated manner, beyond whole body metabolism. Firstly, one way in which InCa systems could be extended to obtain more complete information about substrate metabolism is to include analysis of fermentation (summarised in Figure 1). The gut microbiota obtain nutrients from the host's diet or the host itself (*e.g.* mucins) for microbial growth and energy production, a process generally called fermentation[18]. Some of the by-products generated in fermentation are waste and some can be used by both microbes and host to *e.g.* obtain more energy and

modulate cellular processes. Major gaseous products of fermentation are hydrogen (H_2), methane (CH_4), and hydrogen sulfide (H_2S). The stoichiometry of fermentation is complex, but in general H_2 is produced by hydrogenogens and subsequently used by methanogens (archaea) releasing CH_4, acetogens to produce acetate, or sulfate reducers releasing H_2S (ref. 19). If the carbohydrates ingested by the host are fermentable, some 3-5% of the energy contained in these carbohydrates will be excreted in the faeces and fermentation gases[20]. Energy lost as H_2 (−12.75 kJ l^{-1}, or 25.50 kJ l^{-1} O_2) and CH_4 (−39.71 kJ l^{-1}, or 19.86 kJ l^{-1} O_2) can be adjusted for in InCa equations for the calculation of energy expenditure and substrate oxidation[16,21], although this is mostly done for ruminants as they heavily rely on fermentation. There have been sporadic studies measuring H_2 and CH_4 production in rats and mice, but this has been done outside the context of energy metabolism[22-26] or only at discrete time points[27,28]. Furthermore, the importance of measuring fermentation gases goes beyond energy balance as they could provide a non-invasive way to study diet-host-microbiota interactions. As relevant as they might be, there are currently no commercial rodent systems capable of measuring the fermentation gases H_2 and CH_4 in real time or in conjunction with other physiological gases.

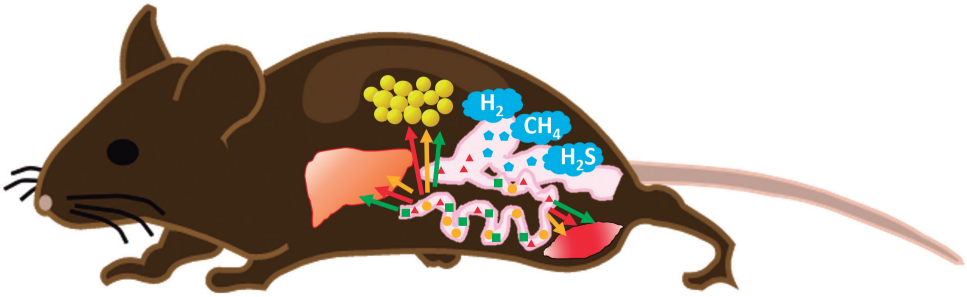


Figure 1. Where fermentation and host energy metabolism meet. Carbohydrates (red triangles), fats (orange circles), and proteins (green squares) consumed by the host go through the digestive process, starting from the mouth (not shown) and culminating in the small intestine. These nutrients are absorbed and delivered to the periphery to serve in biological processes like synthesis of structural molecules, energy storage (*e.g.* fat storage in white adipose tissue, WAT), and cellular and mitochondrial energy production (consuming O_2 and releasing CO_2 in the process). Some nutrients that resist digestion reach compartments of the gastrointestinal tract abundant in microbes, who in turn are capable to ferment these nutrients and produce metabolites like short-chain fatty acids (SCFA, blue pentagons), and gases like H_2, CH_4, and H_2S. SCFA can be excreted, utilised by other microbes, or absorbed by the host (not shown) for the production of energy. SCFA can also act as molecular signals to several tissues like WAT, liver, and muscle. Fermentation gases can be excreted directly, feed microbial metabolic pathways (especially H_2), or be absorbed in the blood stream to be ultimately exhaled via the lungs.

The second example pertains to the measurement of the oxidation of specific substrates. RER computed from VO_2 (the volumetric rate of O_2 consumption) and VCO_2 (the volumetric rate of CO_2 production) only reflects the overall oxidation of substrates in the body and thus lacks detailed information of the specific endogenous or exogenous substrates being oxidized. A way to partly overcome this limitation is to use ingested or infused isotopic tracers[29]. Isotopes are atoms of a given element varying in the amount of neutrons, and they can be radioactive (*e.g.* ^{14}C) or stable (non-radioactive, *e.g.* ^{13}C). Isotopic tracers are

compounds (*e.g.* ^{13}C-labelled glucose) that are metabolised in the same way as their tracee (*e.g.* normal glucose), but that can be differentially detected (*e.g.* based on their radioactivity or mass difference). Isotopic enrichment can be achieved artificially (*e.g.* by growing plants in $^{13}CO_2$ rich atmospheres), but it is also present in nature at lower levels. For instance, C4 plants like maize are naturally enriched in ^{13}C compared to C3 plants like wheat[30]. Thus, it is possible to distinguish between oxidation of endogenous (body) and exogenous (ingested) fuels using isotopic tracers in combination with a method to detect the resulting isotopically labelled CO_2. This distinction is important when one wishes to measure the oxidative disposal of newly ingested nutrients irrespective of their availability in body stores, *e.g.* in studying how recently ingested nutrients are used to fuel physical activity[31,32], or when the (altered) oxidation of specific nutrients may indicate a (patho) physiological condition, like *Helicobacter pylori* infection or delayed gastric emptying assessed by $^{13}CO_2$ breath tests using ^{13}C urea and ^{13}C octanoic acid, respectively[29].

Nowadays stable isotopes are preferred over radioactive isotopic tracers due to health concerns[33]. This also means that the method of detection of isotopically-labelled CO_2 became more difficult: $^{14}CO_2$ can be readily measured with a scintillation counter, but $^{13}CO_2$ is usually detected by expensive mass spectroscopy[30]. To complicate matters, breath samples can be stored for future analysis, but the decision of how many samples are to be collected at what intervals must be based on a priori knowledge of the expected outcomes (*e.g.* oxidation of glucose goes much faster than oxidation of fat) and, of course, the ability to collect those samples. A good example here is an ambitious paper on mouse metabolism using stable isotopic tracers, where 13 people were acknowledged only for collecting hourly breath samples for $^{13}CO_2$ enrichment analysis[34]. Although studies using metabolic tracers and measuring $^{13}CO_2$ enrichment are present, only two have combined this with InCa[34,35], which is a prerequisite to quantify true oxidation rates because $^{13}CO_2$ enrichment only provides qualitative data[36]. The problem is not, however, that there are no other ways of measuring $^{13}CO_2$. There are in fact other real-time, less expensive ways[30], and at least one of them has been implemented already in InCa studies on production animals[37]. But, for some unknown reason and despite the wide availability of commercial InCa systems for rodents, there are currently no systems on the market that integrate $^{13}CO_2$ enrichment analysis into InCa.

Starches

Starches are polymers of glucose produced by plants, and as such, they constitute a major component of human diets. There are two main starch polymers and both are present in almost all starch sources: amylose and amylopectin (Figure 2). Amylose is a non-branching chain linked by α-1,4' glycosidic bonds, and amylopectin is very branched and linked by both α-1,4' and α-1,6' glycosidic bonds[38]. The content of amylose and amylopectin in starch sources and the configuration they take as crystalline granules is variable and influences the ability of salivary and pancreatic amylase to digest these starches[38]. Starch digestibility can also be influenced by processing, for instance, heating starches in water disrupts their crystalline structure and renders them more digestible[39].

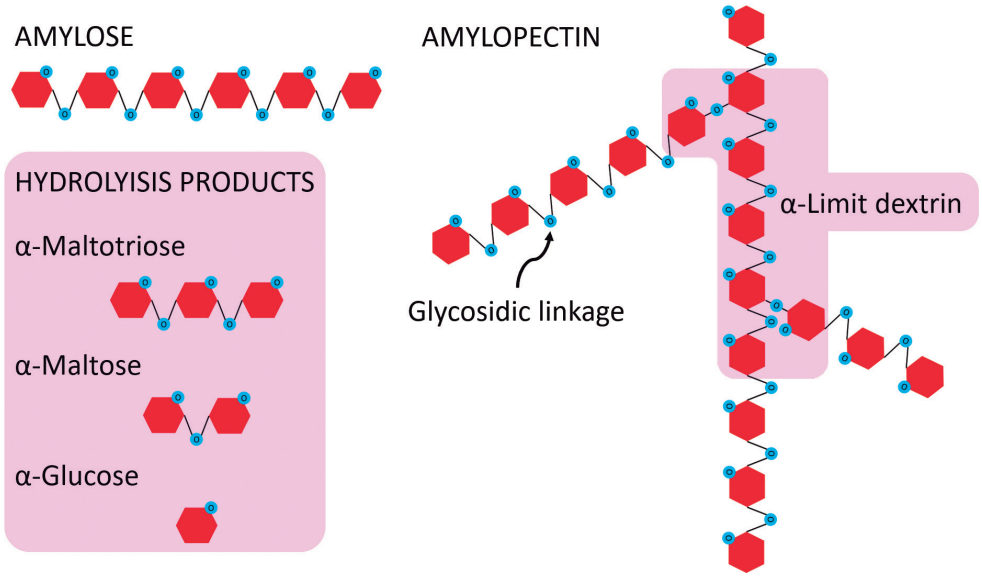


Figure 2. Simplified representation of the molecular structure of starch and starch digestion products. Amylose is a non-branching glucose polymer linked by α-1,4 glycosidic linkages, whereas amylopectin is a branched glucose polymer linked by α-1,4' and α-1,6' linkages. Some of the products formed upon enzymatic digestion of starches are shown in purple. Oxygen atoms in ether and glycosidic bonds are shown in blue. Based on Colonna *et al.*[39]

Based on their *in vitro* digestibility (using an enzyme mixture containing α-amylase) and observations in human ileostomies, starch fractions have been classified as rapidly digestible starch (RDS, digested within 20 min), slowly digestible starch (SDS, 20 – 120 min), and resistant starch (RS, not digested within 120 min and assumed to escape absorption by the small intestine)[40]. A single starch source may form different proportions of RDS, SDS, and RS, and starch sources high in amylose are more likely to form RS[18]. This classification is however not universally accepted nor consistently used throughout the scientific literature[41,42]. Therefore, I will circumvent using these definitions by referring to starches simply as lowly digestible or highly digestible starches.

The rate of starch digestion can affect postprandial glycaemia and gut-microbial activity. The glycaemic response to foods is scored by the glycaemic index (GI), a measure of how blood glucose levels change after a meal[43]. Lowly digestible starches can sometimes[44], but not always[45], be low GI foods. Undigested starch can thus potentially become available to gut microbes as a substrate for fermentation, resulting in the production of gases (*e.g.* H_2, CH_4) and other metabolites[46]. A class of these metabolites are the group of organic acids known as short-chain fatty acids (SCFAs). The main SCFAs are acetate, propionate, and butyrate, and each of them can have different physiological effects. For example, the liver uses acetate to obtain energy and synthesise lipids, and propionate to synthesise glucose[47], and colonocytes obtain energy from butyrate oxidation[48].

Human and rodent studies indicate that starch digestibility may have important health consequences. For instance, lowly digestible starches could be helpful in managing diabetes and reduce obesity and colon cancer risk[49]. It is thought that these effects could

be mediated by a reduced glycaemic response to lowly digestible starches, dilution of the energy density of foods, increased satiety, increased fat oxidation, and their impact on gut microbiota composition and activity (*e.g.* production of SCFAs)[42,49]. While the evidence for these effects in humans is very limited and still needs to be complemented by more long-term feeding studies[50], evidence that lowly digestible starches have a positive impact on metabolic health in rodent models is promising. One of the most consistent effects of lowly *vs* highly digestible starches in male rodents is a decrease in adiposity[51]. White adipose tissue (WAT) is an important organ in the regulation of energy homeostasis, through its capacity to store energy in the form of triglycerides and its endocrine functions (*i.e.* secretion of adipokines like leptin and adiponectin). Feeding lowly *vs* highly digestible starches has been shown to preserve the function of WAT by preventing adipocyte hypertrophy[52,53], maintaining adipocyte insulin sensitivity[52,53], and reducing macrophage recruitment to this tissue[54]. Insulin resistance in WAT may also impair the capacity of an organism to adjust fuel utilisation to fuel availability, a concept known as metabolic flexibility[55], by a failure of WAT to suppress lipolysis upon insulin stimulation. At the same time, increased macrophage infiltration in WAT can indicate an inflammatory status and has been associated with a reduced metabolic flexibility[56]. There is indeed evidence that lowly digestible starches preserve metabolic flexibility, as seen by a greater switch to glucose oxidation in response to a carbohydrate-rich meal in male mice that had been on a lowly *vs* highly digestible starch diet for several weeks[57].

Although the physiological effects of starches have been a subject of study for decades, it is poorly recognised that these effects may be different in females and males. Studies like the one by Robertson *et al.*[58] show that the same starchy meal is oxidised more extensively by women than by men, and that this is probably due to the higher insulin sensitivity of women. How starch digestibility and sex interact is even less studied, and the available evidence comes from rodent models of dietary GI interventions. From these studies (reviewed by Campbell *et al.*[51]) it can be concluded that body weight, body fat, and glucose tolerance are improved by low *vs* high GI diets (mainly represented by lowly *vs* highly digestible starches) only in males, although less than a quarter of the available studies included females. Moreover, other aspects of glucose metabolism besides glucose tolerance and fasting glucose and insulin levels have not been sufficiently studied in both sexes. For example, male mice have an better capacity to oxidise exogenous glucose after long-term consumption of lowly digestible starches compared to males fed a highly digestible starch diet[57]. This is, however, not known for females. Therefore, an important step to understand the metabolic implications of dietary starches would be to provide more evidence for head-to-head comparisons of the physiological effects of starches of different digestibility in females *vs* males.

Metabolic programming

It is increasingly recognised that not everyone living in an unhealthy environment is equally vulnerable to it. It is thought that disease risk late in life is in part due to different environmental influences (including nutrition) early in development, according to the developmental origins of health and disease (DOHaD) theory[59,60]. This phenomenon

(sometimes also referred to as *metabolic*, *nutritional*, or *developmental programming*) has been repeatedly demonstrated, from its first associations based on historical demographical data from 1934 (ref. 61), to the experimental work linking early life environment to cardiovascular and metabolic disease in large and small animals in the 1970s [59], to epidemiological work on foetal growth restriction and postnatal cardiovascular disease risk[62], to recent advances on the epigenetic mechanisms driving metabolic programming[63].

For these exposures or environmental cues to have a long-lasting impact, it is key that they take place during periods of developmental plasticity, like perinatal life and early childhood. This is a good reason why policy makers are now taking a life-course approach to public health, targeting sensitive periods of development like childhood[64], and why scientific journals are devoting special attention to the role of health during critical stages of life in the prevention of non-communicable diseases[65,66]. Experimental animal studies are critical to progress research in the DOHaD field as it requires early interventions, but its effects on later life require model animals with a short life cycle. For instance, adjusting the precise types and quantities of nutrients in infant formulas can only be reconsidered if there is sufficient backing from model organisms about safety and efficacy. In this regard, mice and rats are very useful models to investigate nutritional programming effects, particularly because of their relatively quick generation time and the vast amount of metabolic and molecular knowledge and tools available for these species[10,67]. We indeed owe a large deal of our understanding of metabolic programming mechanisms to rodent studies[68,69]. Promisingly, at least one dietary concept to prevent adult obesity starting in early life, *i.e.* the modification of the physical structure of milk fat globules in infant formula to resemble breast milk more closely, has been successfully taken from mouse models to the first clinical trials in humans[70,71].

Despite the fact that the sensitivity of the developing organism to nutritional stimuli is undisputed, there is still little scientific evidence to recommend macronutrient intakes for infants and young children. The case of carbohydrates is particularly understudied. The proportion of dietary carbohydrates dramatically increases in the transition from lactation to complementary feeding[72], yet all we know is that added sugars should be avoided to prevent caries and that because high glycaemic responses can be detrimental for adults we should assume high-glycaemic index foods are also negative for the young[73]. In this regard, human milk, containing lactose, produces a low glycaemic response[74]. Moreover, the introduction of solid foods during weaning is the greatest determinant of early life gut microbiota composition[75]. The development of a healthy symbiosis between the gut microbiota and the host in this period could determine the risk of obesity and other non-communicable diseases[76]. Accordingly, an altered gut microbiota composition by exposure to antibiotics during the gestation, lactation, and early post-weaning periods has been shown to promote obesity in mice[77]. In this sense, the type of starches that babies are weaned onto could have metabolic programming effects on later life health. At present, there is only one starch-based study that has directly asked this kind of question using a rat model, and the results suggest that lowly digestible starches given to pregnant dams and to their offspring during early life may protect females from metabolic impairment, as shown by the better glucose tolerance and lower visceral adiposity of female offspring at

the end of the study[78]. This study, however, was mainly focussed on the role of maternal diet and the study was terminated at the beginning of adulthood. Whether long-term effects on metabolic health can be induced solely by the digestibility of the starches first encountered by an organism and whether those effects persist well beyond adolescence remains to be tested.

Aims

The overall aims of this thesis are twofold: 1) to develop and show the added value of an extended mouse metabolic phenotyping tool based on InCa for the real-time study of microbiota activity and the oxidation of exogenous *vs* endogenous substrates; and 2) to apply this tool to study the direct and metabolic programming effects of starches consumed during the early post-weaning period.

In particular, we aim to establish whether additional gas sensors can be integrated into an InCa system, thus an extended InCa (eInCa) system, to study microbiota activity (**Chapter 2**) and the specific oxidation of exogenous substrates (**Chapter 3**). We also aim to examine whether consuming starches of low *vs* high digestibility acutely affect the oxidation of the starch molecule, and whether this is sex-dependent (**Chapter 4**). Finally, we aim to explore whether and to what extent starches of different digestibility consumed during the early post-weaning period can improve metabolic health later in life, and whether this is sex-dependent (**Chapter 5**).

Our experimental approach towards these aims is summarised as follows:

- **Chapter 2** describes a proof-of-principle study of the added value of H_2 and CH_4 sensors coupled to a commercial InCa system. The system is tested by examining mice acutely and chronically exposed to lowly and highly digestible starch diets. Other fermentation parameters and the relationship of H_2 with faecal microbial communities are explored to support the validity of the approach to study microbiota activity.

- **Chapter 3** describes the performance of $^{13}CO_2$ and $^{12}CO_2$ sensors incorporated into the same commercial InCa system. The validity of the new sensors to measure $^{13}CO_2$ enrichment is tested by examining mice fed diets with natural variation in ^{13}C content. The system is further tested using lean and obese mice fed a liquid mixed meal containing ^{13}C glucose or ^{13}C palmitate to quantify exogenous substrate oxidation rates. The results are compared to conventional measurements of RER as the standard indicator of metabolic flexibility to illustrate the added value of isotopic CO_2 sensors in tandem with conventional InCa in mice.

- **Chapter 4** tests the hypothesis that a short-term intervention with lowly *vs* highly digestible starches acutely influences the oxidative disposal of the starch molecule, in young female and male mice. Selected parameters related to the digestion and partitioning of a single starch bolus are further investigated in females.

- **Chapter 5** tests the hypothesis that lowly *vs* highly digestible starches in the early post-weaning diet program the adult metabolic phenotype, in female and male mice. Metabolic phenotyping in this study includes body weight and composition, glucose tolerance, metabolic flexibility, whole body metabolism, and adipose tissue morphology and inflammation markers. Additionally, the direct effects of starch digestibility in the early post-weaning period are examined.

Finally, **Chapter 6** offers a general discussion of the technological and scientific contributions of this thesis, and future perspectives on eInCa and nutritional programming studies in mouse models.

References

1 Atwater, W. O. & Benedict, F. G. *A Respiration Calorimeter with Appliances for the Direct Determination of Oxygen*. (Carnegie Institution of Washington, Washington, D.C., 1905).

2 Even, P. C. & Nadkarni, N. A. Indirect calorimetry in laboratory mice and rats: principles, practical considerations, interpretation and perspectives. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **303**, R459-476 (2012).

3 Gerrits, W. J. J., Borne, J. J. G. C. v. d. & Labussière, E. in *Indirect Calorimetry* Ch. 1, 19-34 (Wageningen Academic Publishers, Wageningen, 2015).

4 Nelson, J. A. Oxygen consumption rate v. rate of energy utilization of fishes: a comparison and brief history of the two measurements. *J. Fish. Biol.* **88**, 10-25 (2016).

5 Walsberg, G. E. & Wolf, B. O. Variation in the respiratory quotient of birds and implications for indirect calorimetry using measurements of carbon-dioxide production. *J. Exp. Biol.* **198**, 213-219 (1995).

6 Yatsenko, A. S., Marrone, A. K., Kucherenko, M. M. & Shcherbata, H. R. Measurement of metabolic rate in Drosophila using respirometry. *J. Vis. Exp.*, e51681 (2014).

7 Lighton, J. R., Brownell, P. H., Joos, B. & Turner, R. J. Low metabolic rate in scorpions: implications for population biomass and cannibalism. *J. Exp. Biol.* **204**, 607-613 (2001).

8 Haugen, H. A., Chan, L. N. & Li, F. Indirect calorimetry: a practical guide for clinicians. *Nutr. Clin. Pract.* **22**, 377-388 (2007).

9 da Rocha, E. E., Alves, V. G. & da Fonseca, R. B. Indirect calorimetry: methodology, instruments and clinical application. *Curr. Opin. Clin. Nutr. Metab. Care* **9**, 247-256 (2006).

10 Speakman, J. R. Measuring energy metabolism in the mouse - theoretical, practical, and analytical considerations. *Front. Physiol.* **4**, 34 (2013).

11 Hall, K. D. *et al.* Methodologic considerations for measuring energy expenditure differences between diets varying in carbohydrate using the doubly labeled water method. *Am. J. Clin. Nutr.* **109**, 1328-1334 (2019).

12 Frankenfield, D. C. On heat, respiration, and calorimetry. *Nutrition* **26**, 939-950 (2010).

13 Heetkamp, M. J. W. *et al.* in *Indirect calorimetry* Ch. 2, 35-56 (Wageningen Academic Publishers, Wageningen, 2015).

14 Tschop, M. H. *et al.* A guide to analysis of mouse energy metabolism. *Nat. Methods* **9**, 57-63 (2012).

15 Kaiyala, K. J., Wisse, B. E. & Lighton, J. R. B. Validation of an equation for energy expenditure that does not require the respiratory quotient. *PLoS One* **14**, e0211585 (2019).

16 Elia, M. & Livesey, G. Energy expenditure and fuel selection in biological systems: the theory and practice of calculations based on indirect calorimetry and tracer methods. *World Rev. Nutr. Diet.* **70**, 68-131 (1992).

17 Schutz, Y. & Ravussin, E. Respiratory quotients lower than 0.70 in ketogenic diets. *Am. J. Clin. Nutr.* **33**, 1317-1319 (1980).

18 Englyst, K. N., Liu, S. & Englyst, H. N. Nutritional characterization and measurement of dietary carbohydrates. *Eur. J. Clin. Nutr.* **61 Suppl 1**, S19-39 (2007).

19 Wolf, P. G., Biswas, A., Morales, S. E., Greening, C. & Gaskins, H. R. H_2 metabolism is widespread and diverse among human colonic microbes. *Gut Microbes* **7**, 235-245 (2016).

20 Elia, M. & Cummings, J. H. Physiological aspects of energy metabolism and gastrointestinal effects of carbohydrates. *Eur. J. Clin. Nutr.* **61 Suppl 1**, S40-74 (2007).

21 Poppitt, S. D. *et al.* Circadian patterns of total 24-h hydrogen and methane excretion in humans ingesting nonstarch polysaccharide (NSP) diets and the implications for indirect calorimetric and $D_2{}^{18}O$ methodologies. *Eur. J. Clin. Nutr.* **50**, 524-534 (1996).

22 Hartmann, L., Taras, D., Ramlage, B. & Blaut, M. A new technique to determine hydrogen excreted by gnotobiotic rats. *Lab. Anim.* **34**, 162-170 (2000).

23 Ostrander, C. R., Stevenson, D. R., Neu, J., Kerner, J. A. & Moses, S. W. A sensitive analytical apparatus for measuring hydrogen production rates. I. Application to studies in small animals. Evidence of the effects of an α-glucosidehydrolase inhibitor in the rat. *Anal. Biochem.* **119**, 378-386 (1982).

24 Dufourlescoat, C., Lecoz, Y., Andrieux, C. & Szylit, O. Effects of nature, size and level of incorporation of dietary fibers on colonic functions in germ-free rats and in heteroxenic rats inoculated with a human flora. *Food. Hydrocoll.* **9**, 9-15 (1995).

25 Rodkey, F. L., Collison, H. A. & O'Neal, J. D. Carbon monoxide and methane production in rats, guinea pigs, and germ-free rats. *J. Appl. Physiol.* **33**, 256-260 (1972).

26 Tuboly, E. *et al.* Determination of endogenous methane formation by photoacoustic spectroscopy. *J. Breath Res.* **7**, 046004 (2013).

27 Isken, F., Klaus, S., Osterhoff, M., Pfeiffer, A. F. & Weickert, M. O. Effects of long-term soluble vs. insoluble dietary fiber intake on high-fat diet-induced obesity in C57BL/6J mice. *J. Nutr. Biochem.* **21**, 278-284 (2010).

28 Walter, D. J., Eastwood, M. A., Brydon, W. G. & Elton, R. A. An experimental design to study colonic fibre fermentation in the rat: the duration of feeding. *Br. J. Nutr.* **55**, 465-479 (1986).

29 Van den Borne, J., Heetkamp, M. J. W., Alferink, s. J. J. & Gerrits, W. in *Indirect Calorimetry* Ch. 5, 87-114 (Wageningen Academic Publishers, Wageningen, 2015).

30 McCue, M. D. & Welch, K. C. ^{13}C-Breath testing in animals: theory, applications, and future directions. *J. Comp. Physiol. B* **186**, 265-285 (2015).

31 Jeukendrup, A. E., Mensink, M., Saris, W. H. & Wagenmakers, A. J. Exogenous glucose oxidation during exercise in endurance-trained and untrained subjects. *J. Appl. Physiol. (1985)* **82**, 835-840 (1997).

32 Sonko, B. J. *et al.* Ingested fat oxidation contributes 8% of 24-h total energy expenditure in moderately obese subjects. *J. Nutr.* **135**, 2159-2165 (2005).

33 McCue, M. D. Tracking the oxidative and nonoxidative fates of isotopically labeled nutrients in animals. *Bioscience* **61**, 217-230 (2011).

34 McCue, M. D. & Pollock, E. D. Measurements of substrate oxidation using $^{13}CO_2$-breath testing reveals shifts in fuel mix during starvation. *J. Comp. Physiol. B.* **183**, 1039-1052 (2013).

35 Ishihara, K. *et al.* A soybean peptide isolate diet promotes postprandial carbohydrate oxidation and energy expenditure in type II diabetic mice. *J. Nutr.* **133**, 752-757 (2003).

36 Welch, K. C., Jr., Peronnet, F., Hatch, K. A., Voigt, C. C. & McCue, M. D. Carbon stable-isotope tracking in breath for comparative studies of fuel use. *Ann. N. Y. Acad. Sci.* **1365**, 15-32 (2016).

37 Alferink, S. J. J. *et al.* in *Progress in Research on Energy and Protein Metabolism*. (Wageningen Academic Publishers, Wageningen, 2003).

38 Cummings, J. H. & Stephen, A. M. Carbohydrate terminology and classification. *Eur. J. Clin. Nutr.* **61 Suppl 1**, S5-18 (2007).

39 Colonna, P., Leloup, V. & Buleon, A. Limiting factors of starch hydrolysis. *Eur. J. Clin. Nutr.* **46 Suppl 2**, S17-32 (1992).

40 Englyst, H. N., Kingman, S. M. & Cummings, J. H. Classification and measurement of nutritionally important starch fractions. *Eur. J. Clin. Nutr.* **46 Suppl 2**, S33-50 (1992).

41 Garcia-Campayo, V., Han, S., Vercauteren, R. & Franck, A. Digestion of food ingredients and food using an in vitro model integrating intestinal mucosal enzymes. *Food Nutr. Sci.* **09**, 711-734 (2018).

42 Keenan, M. J. *et al.* Role of resistant starch in improving gut health, adiposity, and insulin resistance. *Adv. Nutr.* **6**, 198-205 (2015).

43 Jenkins, D. J. *et al.* Glycemic index of foods: a physiological basis for carbohydrate exchange. *Am. J. Clin. Nutr.* **34**, 362-366 (1981).

44 Zhang, G. & Hamaker, B. R. Slowly digestible starch: concept, mechanism, and proposed extended glycemic index. *Crit. Rev. Food Sci. Nutr.* **49**, 852-867 (2009).

45 Eelderink, C. *et al.* Slowly and rapidly digestible starchy foods can elicit a similar glycemic response because of differential tissue glucose uptake in healthy men. *Am. J. Clin. Nutr.* **96**, 1017-1024 (2012).

46 Englyst, K. N. & Englyst, H. N. Carbohydrate bioavailability. *Brit. J. Nutr.* **94**, 1 (2007).

47 den Besten, G. *et al.* The role of short-chain fatty acids in the interplay between diet, gut microbiota, and host energy metabolism. *J. Lipid. Res.* **54**, 2325-2340 (2013).

48 Bird, A. R., Brown, I. L. & Topping, D. L. Starches, resistant starches, the gut microflora and human health. *Curr. Issues Intest. Microbiol.* **1**, 25-37 (2000).

49 Birt, D. F. *et al.* Resistant starch: promise for improving human health. *Adv. Nutr.* **4**, 587-601 (2013).

50 Robertson, M. D. Dietary-resistant starch and glucose metabolism. *Curr. Opin. Clin. Nutr. Metab. Care* **15**, 362-367 (2012).

51 Campbell, G. J., Senior, A. M. & Bell-Anderson, K. S. Metabolic effects of high glycaemic index diets: a systematic review and meta-analysis of feeding studies in mice and rats. *Nutrients* **9**, 646 (2017).

52 So, P. W. *et al.* Impact of resistant starch on body fat patterning and central appetite regulation. *PLoS One* **2**, e1309 (2007).

53 Kabir, M. *et al.* Dietary amylose-amylopectin starch content affects glucose and lipid metabolism in adipocytes of normal and diabetic rats. *J. Nutr.* **128**, 35-43 (1998).

54 Bindels, L. B. *et al.* Resistant starch can improve insulin sensitivity independently of the gut microbiota. *Microbiome* **5**, 12 (2017).

55 Galgani, J. E., Moro, C. & Ravussin, E. Metabolic flexibility and insulin resistance. *Am. J. Physiol. Endocrinol. Metab.* **295**, E1009-1017 (2008).

56 Sparks, L. M. *et al.* Relation of adipose tissue to metabolic flexibility. *Diabetes Res. Clin. Pract.* **83**, 32-43 (2009).

57 Isken, F. *et al.* Impairment of fat oxidation under high- vs. low-glycemic index diet occurs before the development of an obese phenotype. *Am. J. Physiol. Endocrinol. Metab.* **298**, E287-295 (2010).

58 Robertson, M. D., Livesey, G. & Mathers, J. C. Quantitative kinetics of glucose appearance and disposal following a ^{13}C-labelled starch-rich meal: comparison of male and female subjects. *Brit. J. Nutr.* **87**, 569 (2007).

59 Gluckman, P. D., Hanson, M. A. & Buklijas, T. A conceptual framework for the developmental origins of health and disease. *J. Dev. Orig. Health Dis.* **1**, 6-18 (2010).

60 Gluckman, P. D., Hanson, M. A. & Beedle, A. S. Early life events and their consequences for later disease: a life history and evolutionary perspective. *Am. J. Hum. Biol.* **19**, 1-19 (2007).

61 Kermack, W. O., McKendrick, A. G. & McKinlay, P. L. Death-rates in Great Britain and Sweden. Some general regularities and their significance. *Int. J. Epidemiol.* **30**, 678-683 (2001).

62 Barker, D. J. The fetal and infant origins of adult disease. *BMJ* **301**, 1111 (1990).

63 Heard, E. & Martienssen, R. A. Transgenerational epigenetic inheritance: myths and mechanisms. *Cell* **157**, 95-109 (2014).

64 Jacob, C. M., Cooper, C., Baird, J. & Hanson, M. *What quantitative and qualitative methods have been developed to measure the implementation of a life-course approach in public health policies at the national level?*, https://www.ncbi.nlm.nih.gov/pubmed/30869851 (2019).

65 The Lancet. Campaigning for preconception health. *Lancet* **391** (2018).

66 Gluckman, P. D., Hanson, M. A. & Low, F. M. Evolutionary and developmental mismatches are consequences of adaptive developmental plasticity in humans and have implications for later disease risk. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* **374**, 20180109 (2019).

67 McMullen, S. & Mostyn, A. Animal models for the study of the developmental origins of health and disease. *Proc. Nutr. Soc.* **68**, 306-320 (2009).

68 Seki, Y., Williams, L., Vuguin, P. M. & Charron, M. J. Minireview: epigenetic programming of diabetes and obesity: animal models. *Endocrinology* **153**, 1031-1038 (2012).

69 Langley-Evans, S. C. & McMullen, S. Developmental origins of adult disease. *Med. Princ. Pract.* **19**, 87-98 (2010).

70 Breij, L. M. *et al.* An infant formula with large, milk phospholipid-coated lipid droplets containing a mixture of dairy and vegetable lipids supports adequate growth and is well tolerated in healthy, term infants. *Am. J. Clin. Nutr.* **109**, 586-596 (2019).

71 Oosting, A. *et al.* Size and phospholipid coating of lipid droplets in the diet of young mice modify body fat accumulation in adulthood. *Pediatr. Res.* **72**, 362-369 (2012).

72 Shaoul, R., Tiosano, D. & Hochberg, Z. Evo-devo of child growth: the role of weaning in the transition from infancy to childhood. *Crit. Rev. Food Sci. Nutr.* **56**, 887-895 (2016).

73 Stephen, A. *et al.* The role and requirements of digestible dietary carbohydrates in infants and toddlers. *Eur. J. Clin. Nutr.* **66**, 765-779 (2012).

74 Wright, C. J., Atkinson, F. S., Ramalingam, N., Buyken, A. E. & Brand-Miller, J. C. Effects of human milk and formula on postprandial glycaemia and insulinaemia. *Eur. J. Clin. Nutr.* **69**, 939-943 (2015).

75 Tilg, H. & Moschen, A. R. Food, immunity, and the microbiome. *Gastroenterology* **148**, 1107-1119 (2015).

76 Wopereis, H., Oozeer, R., Knipping, K., Belzer, C. & Knol, J. The first thousand days - intestinal microbiology of early life: establishing a symbiosis. *Pediatr. Allergy Immunol.* **25**, 428-438 (2014).

77 Cox, L. M. *et al.* Altering the intestinal microbiota during a critical developmental window has lasting metabolic consequences. *Cell* **158**, 705-721 (2014).

78 Gugusheff, J. *et al.* The effect of maternal and post-weaning low and high glycaemic index diets on glucose tolerance, fat deposition and hepatic function in rat offspring. *J. Dev. Orig. Health Dis.*, 1-10 (2015).

Chapter 2

Non-Invasive Continuous Real-Time *In Vivo* Analysis of Microbial Hydrogen Production Shows Adaptation to Fermentable Carbohydrates in Mice

José M. S. Fernández-Calleja

Prokopis Konstanti

Hans J. M. Swarts

Lianne M. S. Bouwman

Vicenta Garcia-Campayo

Nils Billecke

Annemarie Oosting

Hauke Smidt

Jaap Keijer

Evert M. van Schothorst

Scientific Reports (2018) 8:15351 DOI:10.1038/s41598-018-33619-0

Abstract

Real time *in vivo* methods are needed to better understand the interplay between diet and the gastrointestinal microbiota. Therefore, a rodent indirect calorimetry system was equipped with hydrogen (H_2) and methane (CH_4) sensors. H_2 production was readily detected in C57BL/6J mice and followed a circadian rhythm. H_2 production was increased within 12 hours after first exposure to a lowly-digestible starch diet (LDD) compared to a highly-digestible starch diet (HDD). Marked differences were observed in the faecal microbiota of animals fed the LDD and HDD diets. H_2 was identified as a key variable explaining the variation in microbial communities, with specific taxa (including *Bacteroides* and *Parasutterella*) correlating with H_2 production upon LDD-feeding. CH_4 production was undetectable which was in line with absence of CH_4 producers in the gut. We conclude that real-time *in vivo* monitoring of gases provides a non-invasive time-resolved system to explore the interplay between nutrition and gut microbes in a mouse model, and demonstrates potential for translation to other animal models and human studies.

Keywords: Indirect calorimetry, microbiota, Bacteroides, hydrogen, starch, amylose, amylopectin, dietary carbohydrates, C57BL mice, nutritional physiology.

Introduction

Carbohydrates are a major dietary constituent of humans and rodents. Not all carbohydrates are metabolically equal. Most dietary carbohydrates, including several sugars and starches high in amylopectin content, are readily digested and thus absorbed early in the gastro-intestinal tract, making them quickly available to the organism[1]. Other carbohydrates, such as amylose-rich starches, are only available to the organism after fermentation by the intestinal microbiota[2], which results in a more gradual release to the organism. Microbial fermentation results in a variety of metabolic products, including short chain fatty acids (SCFA), which are thought to mediate the beneficial health effects of the intestinal microbial community[3]. Glucose and other monosaccharides, present as such in the diet or becoming available from highly-digestible carbohydrates, are readily taken up via transporters from the small intestinal lumen into the body. This occurs primarily in the jejunum, the proximal part of the small intestine[4]. Carbohydrates that are less readily digestible reach the caecum and colon, where most of the intestinal microbiota reside[5]. Specific microbial communities utilize these substrates, in the process generating metabolites that are absorbed by the body, or are excreted as gases or in the faeces. Major digestion products are SCFA, which are known to influence host physiology, acting as energy substrates and as signalling molecules[3]. Other digestion products are the microbial fermentation gases hydrogen (H_2), methane (CH_4), and hydrogen sulphide (H_2S)[6].

Since the studies of Gordon *et al.*[7], it is increasingly realized that the small and large intestinal microbiota not only plays a major role in gastrointestinal health but also in the host's metabolic health[8,9]. However, how the microbial community affects metabolic health and how this can be beneficially modulated by nutrition and specific nutrients is far less well established. While a variety of cross-sectional methods can be applied to analyse changes in intestinal microbiota in rodents at specific time points, longitudinal measurements in rodent and human studies mainly rely on sampling of the faeces, long after food-microbiota interactions have already taken place throughout the gastrointestinal tract. Continuous measurements of fermentation gas emissions are already in place for ruminants like cattle and sheep[10-12], as they are known to fully rely on microbiota fermentation in rumen and hindgut to digest cellulose, being distinct from monogastric organisms including rodents and humans. Furthermore, recent studies showed strong correlations between dynamics of metabolite production and microbiota composition and activity in dairy cows[13,14]. Measurements of H_2 and CH_4 as indicators of human gut microbial activity *in vivo* have been used before[15-19], but these are in fact single-time-point gas measurements that lack the information that continuous analysis can provide.

Therefore, our study objective was to apply a simple non-invasive method to monitor the effect of diet on intestinal microbiota in real time using a human-relevant model, which we envisioned as a powerful tool to better understand the direct impact of nutrition on the microbiota and by extension of diet-microbiota interactions on human health.

C57BL/6J mice are the most widely used model in medical and nutritional health research and have shown their validity in dissecting microbe-host interactions and causality testing.

However, analysis of fermentation gases in mice and other rodent models is a largely unexplored area. As is the case in humans, single-time-point measurements of H_2 (ref. 20-23) and CH_4 (ref. 24-26) have been reported for mice and rats. This is critical, because only continuous measurements allow to faithfully study the time-resolved kinetics of digestion and metabolism of nutrients reaching the gut microbiota.

Indirect calorimetry makes use of the measurement of oxygen (O_2) and carbon dioxide (CO_2), as well as food and water intake and locomotor activity, to analyse energy metabolism. We have equipped a commercially available indirect calorimetry system with sensors for H_2 and CH_4, allowing continuous measurements of release of these gases non-invasively in real time. We applied this extended system to explore the adaptation of gut microbiota to highly- and lowly-digestible carbohydrates. To the best of our knowledge, this is the first time that food-microbiota interactions have been studied continuously, non-invasively and in real time in a murine model.

Materials and Methods

Coupling of hydrogen (H_2) and methane (CH_4) sensors into indirect calorimetry system

A PhenoMaster indirect calorimetry system (TSE Systems, Bad Homburg, Germany) was extended by coupling a Sensepoint XCD H_2 gas analyser (Honeywell Analytics, Hegnau, Switzerland) and a CH_4 gas analyser (ABB Automation GmbH, Frankfurt am Main, Germany) in a closed circuit in series in front of a Siemens High-Speed Sensor Unit containing the O_2 and CO_2 sensors. This order was chosen to prevent dilution of the sample with reference air, which is required by the Siemens unit. The H_2 sensor has a stability of < ± 2% full scale deflection (fsd)/yr representing < 2 ppm/yr as it was adjusted to a measuring range from 0 to 100 ppm. The CH_4 sensor has a zero drift of ≤ 1% of span per week and a measuring range from 0 to 500 ppm. A two point calibration of both H_2 and CH_4 analysers was performed within 24 h before each animal experiment. The calibration procedure was carried out using three gas mixtures (Linde Gas Benelux BV, Dieren, The Netherlands): zero (20.947% O_2 and N_2), span H_2 (98.8 ppm H_2 and synthetic air), and span CH_4 (0.521% CO_2, 450 ppm CH_4, and N_2). The zero calibration mixture was flushed through the system for 10 min and ADC signals were assigned H_2 and CH_4 values of 0 ppm. Thereafter, each of the span gas mixtures was run for 10 min and ADC signals assigned 98.8 ppm H_2 and 450 ppm CH_4, accordingly. For O_2 and CO_2 calibration, the routine indicated in the TSE manufacturer's manual was followed, using an additional gas mixture (0.999% CO_2 and N_2) for the span calibration point. Animals were measured as previously described[27] with minor adjustments for the newly coupled sensors. These include the adjustment of airflow to 0.43 l min^{-1} and the measuring time per cage set to 1.5 min. Data was recorded using an updated, customized, version of the TSE PhenoMaster software (V5.8.0) specially developed for the incorporation of H_2 and CH_4 measurements.

Animal experiments and sample harvest

All animal experiments were approved by the Animal Ethical Committee (DEC 2014085.h) and performed in accordance to EU directive 2010/63/EU. Female and male

C57BL/6JRccHsd mice (Harlan Laboratories BV, Horst, The Netherlands) were housed in Makrolon II cages enriched with wood chips and wood shavings, with free access to drinking water, at 23°C ± 1 °C and a 12:12 h light:dark cycle. Standard rodent chow (RMH-B, AB Diets, Woerden, The Netherlands) was provided exclusively and continuously since weaning, unless specified. Three different studies were conducted to investigate diet-host-microbiota interactions upon provision of diets containing starches with differences in digestibility (the experimenter was not blinded to the diets that the animals were given).

Study 1 (long-term exposure, post-weaning): Mice were mated and the offspring reassigned to a foster dam 1 or 2 days after birth to obtain standardized litters. Males and females were stratified by body weight at post-natal day (PN) 21, housed individually and randomly assigned to be fed a highly- or a lowly-digestible starch diet (HDD and LDD, respectively; see below). The randomisation was achieved by generating a column of random numbers in a spreadsheet and sorting each diet and animal number according to the column of random numbers from smallest to largest. From PN36-42, a subgroup of mice was measured in the indirect calorimetry system with *ad libitum* access to the experimental diets (males: *n*=12 per diet, females HDD *n*=12, LDD *n*=11). Fresh faecal pellets were sampled on PN39 (*n*=6 per diet and sex) and stored at -80 °C for intestinal microbiota analysis. Another subgroup of female mice was culled on PN42 for collection of caecum (*n*=6) and colon contents (*n*=5 HDD, *n*=7 LDD), and the faeces produced during the last week before sacrifice were collected for gross energy measurements (see *In vivo* diet digestibility). Before sacrifice, food was removed 1 h after the start of the light phase and animals decapitated 2-6 h after removal of food. Caecum and colon contents were immediately frozen in liquid nitrogen, and stored at -80 °C until analysis.

Study 2 (short-term exposure with fasting, adult): Eight-month-old female mice were individually housed in indirect calorimetry cages. After a 2-day adaptation period, mice were allowed a restricted amount (1.1 g) of chow 1 h before the onset of the dark phase to induce a fasting state in early morning, as published[28]. At the end of the light phase at 18.00 h, mice were re-fed with a restricted amount (1.1 g) of chow, or first-time HDD or LDD (the refeeding diet was assigned randomly; *n*=4 per dietary group). Shortly before the following dark phase mice received access to the same diet they were allocated the day before, but now *ad libitum*. Indirect calorimetry measurements continued for an additional 5.5 d.

Study 3 (short-term exposure without fasting, adult): Ten-month-old female mice were individually housed in indirect calorimetry cages. After a 2-day adaptation period, mice were provided clean bedding and given *ad libitum* first-time access to either HDD or LDD (random assignment, *n*=6) shortly before the dark phase and for the remaining experimental period. Measurements continued for an additional 4.5 d. Faecal pellets produced after the introduction of the new diets were collected from the bedding at the end of the experiment and stored at -80 °C.

Experimental diets

Both the HDD and the LDD satisfy the nutrient requirements for rodent growth and lactation (AIN-93G)[29], with appropriate levels of mono- and poly-unsaturated fatty acids. The macronutrient composition was 20.1 energy percentage (en%) protein, 54.9 en% carbohydrates, and 25 en% fat (Table 1), with starch being the sole source of carbohydrates. The starch fraction (Cargill BV, Sas van Gent, The Netherlands) of the HDD was composed of 100% amylopectin (which is highly digestible), while that of the LDD was a mixture of 60% amylose (which resists complete digestion) and 40% amylopectin. The diets were pelleted by Research Diet Services BV, Wijk bij Duurstede, The Netherlands.

Table 1. Diet composition

Component	Diet	
	HDD	LDD
Casein	212.2	212.2
L-Cysteine	3.0	3.0
Amylose mix (AmyloGel 03003)	0.0	568.6
Amylopectin (C*Gel 04201)	568.6	0.0
Coconut oil	21.4	21.4
Sunflower oil	83.1	83.1
Flaxseed oil	14.2	14.2
Cholesterol	0.03	0.03
Cellulose	50.0	50.0
Mineral mix (AIN-93G-MX)	35.0	35.0
Vitamin mix (AIN-93-VX)	10.0	10.0
Choline bitartrate	2.5	2.5
Total (g)	1000.0	1000.0
Gross energy density (kJ g^{-1})[a]	18.9	19.5
Calculated energy density (kJ g^{-1})[b]	17.9	17.9
Protein (en%)[b]	20	20
Carbohydrate (en%)[b]	55	55
Fat (en%)[b]	25	25

Values are g kg^{-1} of diet unless otherwise specified. [a]Measured by bomb calorimetry, [b]calculated based on Atwater's nutritional values. HDD, highly-digestible starch diet; LDD, lowly-digestible starch diet.

In vivo *diet digestibility*

Total faeces produced from PN 35-42 (Study 1) were recovered from the bedding of a subgroup of randomly selected animals (*n*=4 per sex and diet). Food intake was recorded over the same period. Gross energy in faeces and food was determined in blinded samples

using a C7000 bomb calorimeter (IKA, Staufen, Germany) and diet digestibility was calculated as published[30].

In vitro *carbohydrate digestibility*

The *in vitro* digestibility of starches in the experimental diets was determined in blinded samples in triplicate[31]. Briefly, 5 intact pellets of each diet were cryoground to homogeneous particle size and weighed separately into 3 tubes (70 mg). Each sample was digested in a 15-ml tube by adding cocktail solutions (modified from Versantvoort *et al.*[32]) and digestive enzymes at 37 °C in three sequential steps to represent the oral (5 min), gastric (2 h), and duodenal (6 h) phases. Two blanks containing only enzymes and solutions were included. Samples were taken at several time points during the gastric and duodenal phases and centrifuged. Clean supernatants were recovered and free glucose content was determined by the glucose oxidase peroxidase method[33]. Starch digestion was expressed as the percentage of total glucose released based on the amount of starches in the diets.

Quantification of SCFA in intestinal digesta by gas chromatography (GC)

Short-chain fatty acids in caecum- and colon-contents were determined as previously reported[34], with some modifications. Samples (about 25 mg) were weighed, thawed, homogenized in 100 µl of ultrapure water, and centrifuged for 3 min at 21,382 *g*. To 50 µl of supernatant, 100 µl of 2-ethylbutyric acid solution (0.45 mg ml^{-1}) were added as internal standard. An external standard curve was prepared containing 50 µl of a mixture of acetic, propionic, butyric, valeric, isobutyric, and isovaleric acid at concentrations ranging from 0.002 mg ml^{-1} to 0.8 mg ml^{-1}, to which 100 µl of internal standard were added. Blanks containing only water or water and internal standard were included for quality control. HCl and oxalic acid were added to all samples, blanks, and standards in order to protonate the SCFA. Gas chromatography was performed on a FOCUS GC apparatus coupled to a flame ionization detector (Interscience, Breda, The Netherlands). Samples were injected (1 µl) into an CP-FFAP CB column (25 m × 0.53 mm × 1.00 µm; Agilent Technologies, Santa Clara, CA, USA). Helium served as carrier gas at a pressure of 40 kPa. The initial oven temperature was 100 °C with 0.5 min hold, ramped to 180 °C at 8 °C min^{-1} with 1 min hold, and finally ramped to 200 °C at 20 °C min^{-1} with 5 min hold. Peak identities and areas were analysed with Xcalibur software (version 2.2; Thermo Scientific, Waltham, MA, USA). Concentrations of SCFA were normalised to the internal standard and expressed relative to original sample weight.

Microbiota analysis

Microbial DNA was isolated from faecal pellets using the Maxwell® 16 Instrument (Promega, Leiden, The Netherlands). Faecal pellets were added to a bead-beating tube with 350 µl Stool Transport and Recovery (STAR) buffer, 0.25 g of sterilized zirconia beads (0.1 mm), and three glass beads (2.5 mm). Faecal pellets were homogenized by bead-beating three times (60 s × 5.5 ms) and incubation for 15 min at 95 °C at 100 rpm. Samples were then centrifuged for 5 min at 4 °C and 14,000 *g* and supernatants transferred to sterile tubes. Pellets were re-processed using 200 µl STAR buffer and both supernatants were pooled.

DNA purification was performed with a customized kit (AS1220; Promega) using 250 µl of the final supernatant pool. DNA was eluted in 50 µl of DNAse- RNAse-free water and its concentration measured using a DS-11 FX+ Spectrophotometer/Fluorometer (DeNovix Inc., Wilmington, USA). The V4 region of 16S ribosomal RNA (rRNA) gene was amplified in duplicate PCR reactions for each sample in a total reaction volume of 50 µl. The master mix contained 1 µl of a unique barcoded primer, 515F-n and 806R-n (10 µM each per reaction), 1 µl dNTPs mixture, 0.5 µl Phusion Green Hot Start II High-Fidelity DNA Polymerase (2 U/µl; Thermo Scientific, Landsmeer, The Netherlands), 10 µl 5× Phusion Green HF Buffer, and 36.5 µl DNAse- RNAse-free water. The amplification program included 30 s of initial denaturation step at 98°C, followed by 25 cycles of denaturation at 98 °C for 10 s, annealing at 50 °C for 10 s, elongation at 72 °C for 10 s, and a final extension step at 72 °C for 7 min. The PCR product was visualised in 1% agarose gel (~290 bp) and purified with CleanPCR kit (CleanNA, Alphen aan den Rijn, The Netherlands). The concentration of the purified PCR product was measured with Qubit dsDNA BR Assay Kit (Invitrogen, Caliornia, USA) and 200 ng of microbial DNA from each sample were pooled for the creation of the final amplicon library which was sequenced (150bp, paired-end) on the Illumina HiSeq2000 platform (GATC Biotech, Constance, Germany).

Microbiota data processing and analysis

Data filtering and taxonomy assignment were performed using the NG-Tax pipeline[35]. Briefly, an OTU table was created for each sample with the most abundant sequences. Low abundance OTU's were discarded, using a minimum relative abundance threshold of 0.1%. Two distinct in-house assembled mock communities were included in the library and were compared with their theoretical composition for quality control (Fig. S1). Calculations for α- and β-diversity analyses were performed using the publicly available Microbiome R package (version 1.2.1)[36]. Adonis permutational multivariate analysis of variance (PERMANOVA) using either the weighted or unweighted Unifrac distances were performed with the Vegan package (version 2.5-2) and were used to determine the amount of variation explained by the grouping variables. Linear Discriminant Analysis (LDA) Effect Size (LEfSe) was applied to determine the differences between the microbial communities of HDD- and LDD-fed mice using a publicly available pipeline (http://huttenhower.sph.harvard.edu/galaxy/)[37]; the threshold for the logarithmic LDA score was set to 2.0. *P* values for Kruskal-Wallis and Wilcoxon tests for the LEfSe analysis were set to 0.05. For non-parametric Student's *t*-tests, reads were transformed to their relative abundances and tests were carried out with 999 permutations using QIIME (version 1; http://qiime.org/index.html)[38]. Statistical significance was determined using the Benjamini-Hochberg false discovery rate (FDR) adjustment.

Data analysis

Statistical analysis was performed using GraphPad Prism 5.04 (GraphPad, San Diego, CA, USA), unless stated otherwise. All data was tested for normality using the D'Agostino and Pearson omnibus test and its distribution was normalized by log transformation when applicable. Comparisons between two groups were made using unpaired two-tailed Student's *t*-tests (for data with normal distribution) or two-tailed Mann-Whitney *U* tests

(VH_2 during light phase between HDD and LDD). Comparisons between more than two groups were made by one-way analysis of variance (ANOVA) with *post hoc* Bonferroni's test for multiple comparisons. Time course data (H_2 evolution) was analysed by repeated measures two-way ANOVA with Bonferroni's *post hoc* test. When sample sizes being compared were similar and relatively large (n > 5), similarity of variances was not taken into account. All data is reported as mean ± s.d. Statistical significance was set at 5%, with levels indicated as $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$, and $^{****}P < 0.0001$. Sample size was not determined statistically as the effect size was unknown, but it was based on our previous results on the use of indirect calorimetry to assess metabolic flexibility[28,39].

Results

In vitro *reflects* in vivo *diet digestibility*

To confirm the difference in digestibility of the two starches incorporated into our experimental diets (Table 1), an *in vitro* model that mimics food digestion for the oral, gastric and small intestinal phases was used. The lowly-digestible starch diet (LDD) showed a slower and 14% less complete carbohydrate digestion than the highly-digestible starch diet (HDD; Figure 1). In addition, we quantified food intake and faecal energy content in female and male mice habituated to the experimental diets (Table 2). Daily faecal mass was increased in all mice fed LDD, whereas faecal energy density was increased in LDD females only. LDD mice lost on average twice as much energy in faeces compared to HDD mice. With similar food and energy intake, the diet digestibility was 6% lower in LDD *vs* HDD fed mice (Table 2). Taken together, both *in vitro* and *in vivo* analyses showed a reduced digestibility of the LDD *vs* HDD.

Table 2. Dietary *in vivo* digestibility of the experimental diets.

	Females			Males		
	HDD	LDD	*P* value	HDD	LDD	*P* value
Food intake (g)	2.53 ± 0.05	2.71 ± 0.24	0.1942	2.82 ± 0.21	2.86 ± 0.37	0.8489
Gross energy intake (kJ)	48.01 ± 0.92	53.08 ± 4.76	0.0816	53.36 ± 4.01	55.88 ± 7.18	0.5634
Faeces weight (g)	0.20 ± 0.01	0.41 ± 0.06	**0.0006**	0.24 ± 0.02	0.45 ± 0.05	**0.0002**
Faeces gross energy (kJ g^{-1})	15.48 ± 0.26	16.18 ± 0.24	**0.0072**	15.94 ± 0.27	16.01 ± 0.23	0.7227
Faeces energy loss (kJ)	3.10 ± 0.12	6.68 ± 1.08	**0.0006**	3.74 ± 0.22	7.26 ± 0.78	**0.0001**
Digestible energy intake (kJ)	44.91 ± 0.91	46.40 ± 3.70	0.4645	49.63 ± 3.80	48.62 ± 6.44	0.7967
Diet digestibility (%)	93.6 ± 0.2	87.5 ± 0.9	**< 0.0001**	93.0 ± 0.2	87.0 ± 0.6	**< 0.0001**

Energy balance calculated over the third week of exposure to the diets in females and males (*n*=4 per sex and diet) and expressed per day. Statistical comparisons by Student's *t*-test within sex. Data reported as mean ± s.d.

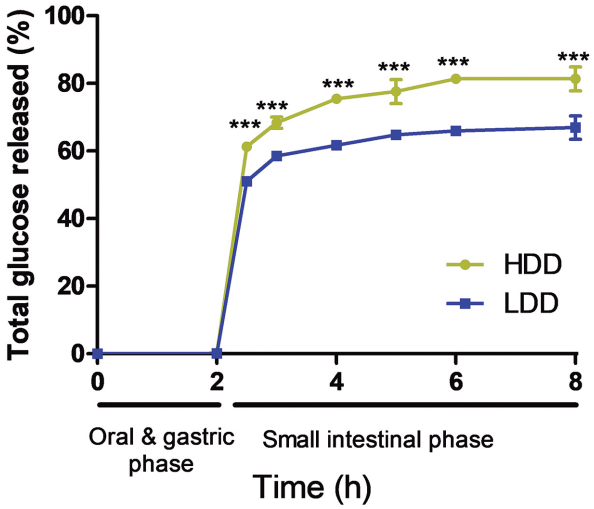


Figure 1. *In vitro* digestibility of starches in experimental diets. Triplicate samples of the lowly- and highly-digestible starch diets (LDD and HDD, respectively) were digested *in vitro*, and free glucose concentrations were determined at indicated time points. Statistical comparisons were made with two-way ANOVA with Bonferroni's post hoc test; ***$P \leq 0.001$. Values are plotted as mean ± s.d.

Measuring H_2 production in real time

Reduced digestibility likely also affects colonic fermentation, for which H_2 has been used as a marker in mice[20]. However, measurement of its continuous production in response to the diet has not yet been possible. We therefore adapted and extended an indirect calorimetry system to allow H_2 and CH_4 production to be studied in real time, by introducing the respective sensors in series with the O_2 and CO_2 sensors already present in the system (Fig. 2a). To determine if the small quantities of H_2 originating from microbial carbohydrate fermentation in mice could be detected by our system, we measured gas concentrations in cages with and without chow-fed mice over 24 h. Stable signals for all gases were seen in the absence of mice (Fig. 2b), and the concentrations were clearly decreased for O_2 and increased for CO_2 in mouse-occupied cages, as expected (Fig. 2c). H_2 increased (Fig. 2c), while CH_4 concentrations were not altered by the presence of a chow-fed mouse in the cage. The adapted indirect calorimetry system was therefore suitable for simultaneous respirometry and H_2 production measurements in real time, however under the conditions tested, CH_4 production appeared to be absent based on measured ambient levels well above the lower detection limit of the CH_4 sensor (Fig. 2b).

H_2 production indicates extent of carbohydrate digestibility

Since the contrasting digestibility of the experimental diets was expected to result in sustained differences in H_2 production as a consequence of fermentation in the large intestine, we fed female and male mice, as a proof-of-concept, either the HDD or the LDD for three weeks and measured H_2, CH_4, O_2, and CO_2 levels continuously during several days (Study 1). Calculation of energy expenditure, based on 24 h O_2 consumption and CO_2

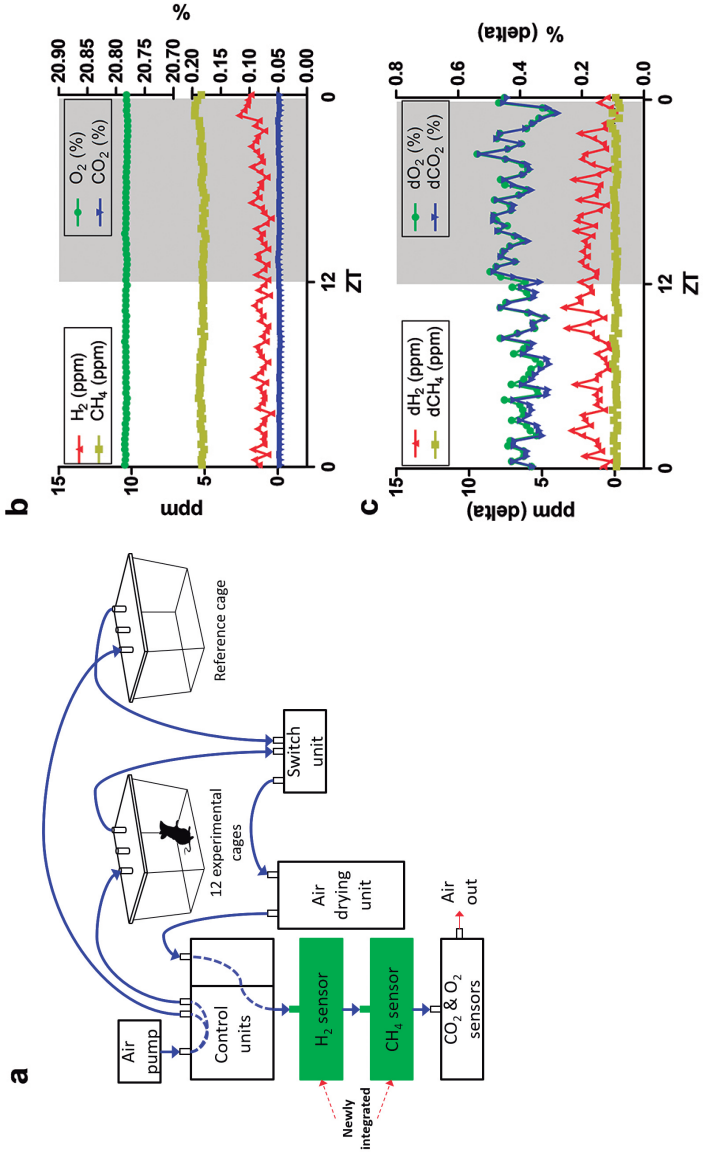


Figure 2. Real-time measurements of hydrogen (H_2) and methane (CH_4) production in mice within indirect calorimetry system. (**a**) Illustration of the indirect calorimetry system extended for H_2 and CH_4 measurements. Direction of air flow in the tubing is shown in blue, new gas sensors are shown in green. For clarity, tube lengths are not to scale (all equal) and food and drink containers with sensors are not shown, nor are the infrared beam bars for activity measurements. (**b**) Ambient concentrations of H_2 and CH_4 (left y-axis, ppm) and O_2 and CO_2 (right y-axis, %) were recorded in an empty (reference) cage at 20 min intervals for 24 h. (**c**) Gas concentrations in a cage occupied by a chow-fed female adult mouse were measured and compared to the corresponding concentrations in the reference cage and expressed as delta values. White and grey areas in panels **b** and **c** represent the inactive light and active dark phase for the animal, respectively. ZT, Zeitgeber time.

production, revealed no differences between dietary groups (females 1.59 ± 0.08 *vs* 1.63 ± 0.08 kJ h^{-1} in HDD and LDD respectively, $P = 0.2094$; males 1.80 ± 0.13 *vs* 1.76 ± 0.13 kJ h^{-1} in HDD and LDD respectively, $P = 0.5470$). However, 24 h mean respiratory exchange ratio (RER) was lower in LDD- *vs* HDD-fed male mice (0.85 ± 0.03 *vs* 0.88 ± 0.03 respectively, $P = 0.0097$), indicating higher fat oxidation and lower carbohydrate oxidation in LDD mice. Overall, these observations agree with indirect calorimetry data reported for mice fed diets containing carbohydrates similar to the carbohydrates used here[40].

Both LDD-fed females (Fig. 3a,b) and males (Fig. 3d,e) constantly produced more H_2 than HDD-fed mice. A distinct pattern of H_2 production became apparent in LDD-fed mice, with H_2 levels being higher in the active dark phase and lower, but still clearly present, in the inactive light phase (Fig. 3a,b,d,e). This was fully consistent with the circadian food and starch intake (Fig. 3c,f). Importantly, the difference in H_2 production between HDD- and LDD-fed mice was explained by the type of starch rather than the amount of starches ingested, as cumulative starch consumption was similar between the groups (Fig. 3c,f). Together, this data provides proof-of-concept for measuring H_2 production in real time as an indicator of carbohydrate digestibility.

H_2 evolution reflects adaptation to dietary carbohydrates

As we could show that H_2 production can be sensitively and continuously measured, we next questioned whether it would be possible to measure adaptation to the diet *in vivo* in real time.

For this, we provided HDD or LDD to mice that had no previous exposure to these diets and we followed H_2 production continuously. We introduced the new diets in one of two conditions; the first condition was as a single meal challenge given to fasted mice, followed by *ad libitum* access to the diet the next day, as a second fasting-refeeding challenge (Study 2, Fig. 4a). The second condition was by replacing the standard chow diet directly with HDD or LDD *ad libitum* (Study 3, Fig. 4b). H_2 production was significantly increased in LDD- compared to HDD-fed mice as early as 4 h after fasted mice gained *ad libitum* access to the experimental diet (Fig. 4a). The direct switch from chow to HDD or LDD without fasting had similar results, with LDD-fed mice producing significantly more H_2 after 53 h of access to the LDD compared to mice receiving HDD (2-way ANOVA, Fig. 4b). In both conditions, *i.e.* fasted or directly switched to HDD or LDD, cumulative H_2 production became significantly higher already within 12 h upon access to LDD *vs* HDD (Fig. 4c,d), and H_2 production patterns in LDD-fed mice closely followed the patterns of LDD intake (Fig. S2). Interestingly, mice that continued on the chow diet after a period of food restriction exhibited a spike in H_2 production (Fig. 4a), while consuming similar amounts of starches compared to the HDD and the LDD groups. H_2 production in HDD-fed mice remained lower compared to mice on LDD or chow, as expected. Importantly, LDD-induced H_2 production increased gradually before reaching its maximal levels (up to 0.89 ml h^{-1}), revealing the process of adaptation to the lowly-digestible starch. LDD- *vs* HDD-fed mice thus showed a differential adaptation, likely in their microbiota, based on increased H_2 production.

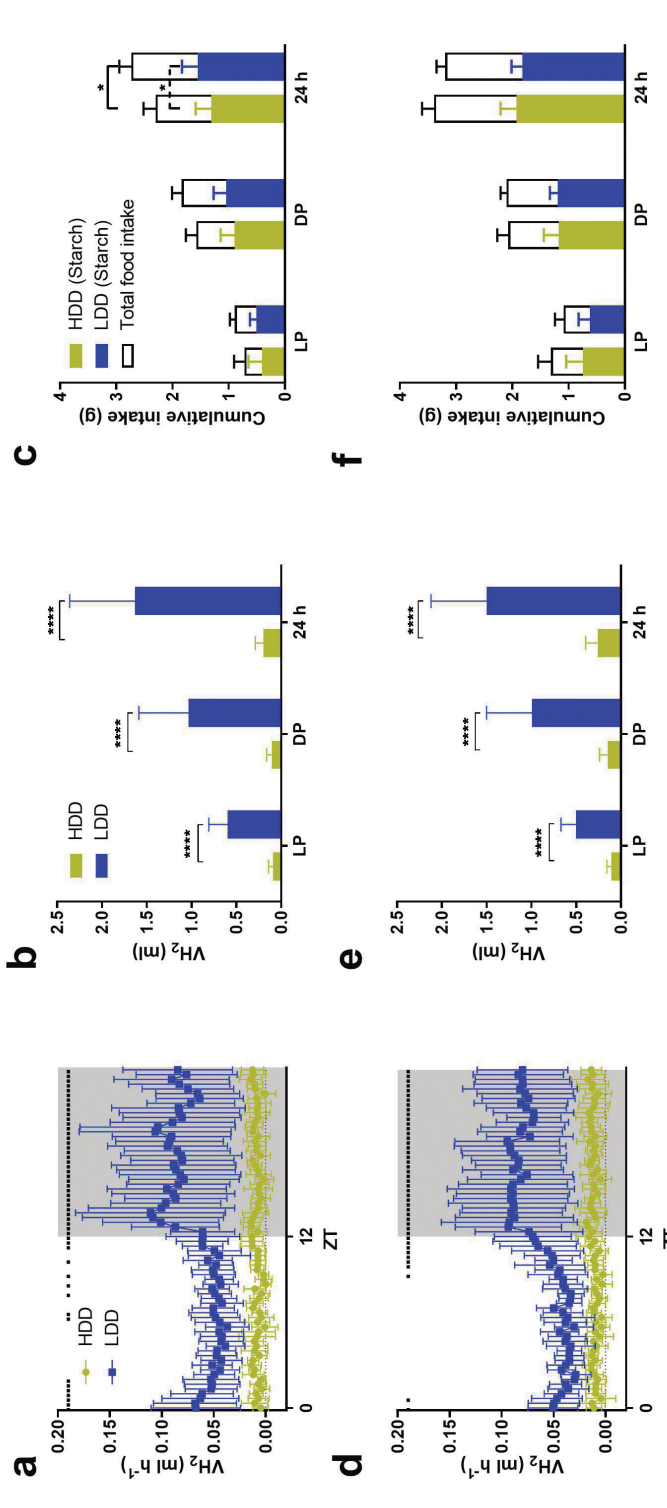


Figure 3. H_2 production in mice reflects starch digestibility. Female (**a**) and male (**d**) mice were fed either HDD or LDD for three weeks and volume of H_2 produced (VH_2) was recorded for 24 h in the adapted indirect calorimetry system. Cumulative H_2 production in females (**b**) and males (**e**) quantified during the 12 h light phase (LP), 12 h dark phase (DP) or the complete 24 h photoperiod. Cumulative starch and total food intake in females (**c**) and males (**f**) over the measuring period calculated from food intake records. White and grey areas represent the light and the dark phase, respectively. Time course data was analysed by repeated measures two-way ANOVA with Bonferroni's test for multiple comparisons and time points where $P < 0.05$ are indicated with black asterisks (panels **a** and **d**). Other statistical comparisons made by Student's *t*-test or Mann-Whitney *U* test; $^{*}P \leq 0.05$, $^{***}P < 0.0001$ (n=11 LDD females, n=12 remaining groups). Data shown as mean ± s.d. ZT, Zeitgeber time.

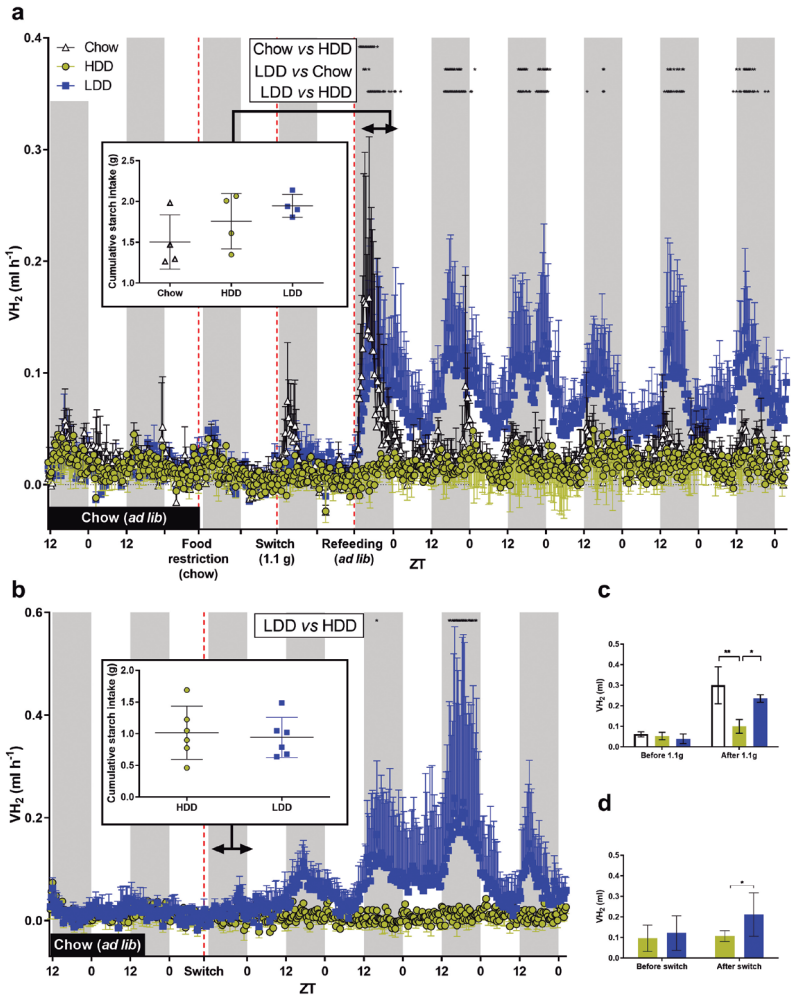


Figure 4. H_2 evolution upon first exposure to starches of different digestibility. (**a**) Standard chow-fed mice within indirect calorimetry were food-restricted leading to fasting (dotted line), which was followed by feeding 1.1g of chow (black), HDD (yellow), or LDD (blue; *n*=4 per group) prior to the dark phase as a single meal test (2nd dotted line). As a result, they were fasted the next day, and received prior to dark phase *ad libitum* access to the same diet (3rd dotted line) for an additional 5.5 days. Inset: First 12h cumulative starch-intake of *ad libitum* feeding with experimental diets. (**b**) Chow-fed mice (*n*=6 per group) were switched to LDD or HDD without prior food restriction and measurements continued for another 4.5 days. Inset: First 12h cumulative starch-intake after diet switch. (**c**) Cumulative H_2 production over 12h before (while food-restricted on chow) and after feeding 1.1g of chow, HDD, or LDD (*n*=4 per group). (**d**) Cumulative H_2 production over 12h before and after switching directly from chow to HDD or LDD (*n*=6 per group). All mice received no other diet than chow during their whole lifetime prior to these experiments and the dietary switch (black bar), but colour usage reflects subgroups after first exposure to new diets. White and grey areas represent light and dark phases, respectively. Time course data was analysed by repeated measures two-way ANOVA with Bonferroni's test for multiple comparisons (chow vs HDD, LDD vs chow, and LDD vs HDD), and time points where $P < 0.05$ are indicated with black stars (panels **a** and **b**). Cumulative data was statistically compared using unpaired two-tailed Student's *t*-test (between HDD and LDD) and one-way ANOVA with Bonferroni's multiple comparisons post hoc test (between chow, HDD, and LDD); $^*P \leq 0.05$, $^{**}P \leq 0.01$. Data is presented as mean ± s.d. For clarity, either upper or lower error bars are shown. ZT, Zeitgeber time.

Since the production of H_2 fully depends on intestinal microbial communities and their metabolism, we further investigated the changes in the microbiota induced by the LDD to validate our observations. As an additional parameter of fermentation, we first assessed SCFA levels in intestinal digesta after 3 weeks of exposure to the HDD or the LDD (Study 1). Total caecal SCFA levels were similar between LDD- and HDD-fed mice (35.6 ± 13.9 *vs* 34.9 ± 11.9 µmol g^{-1}, respectively), including valeric and isobutyric levels (data not shown), whereas total SCFA in colon were higher in LDD- compared to HDD-fed mice (25.6 ± 9.6 *vs* 9.6 ± 4.1 µmol g^{-1}, $P = 0.0059$). Acetic acid (Fig. 5a) and propionic acid (Fig. 5b) were the two most abundant SCFA, and both were significantly elevated in LDD-fed mice in colon and caecum contents, respectively. Butyric acid was the most differentially produced SCFA, enriched by 13.8-fold in LDD colon content (Fig. 5c). Finally, isovaleric acid, a product of microbial protein fermentation, was the least abundant of the measured SCFA in all groups and was significantly lower in caecum of LDD- *vs* HDD-fed mice (Fig. 5d).

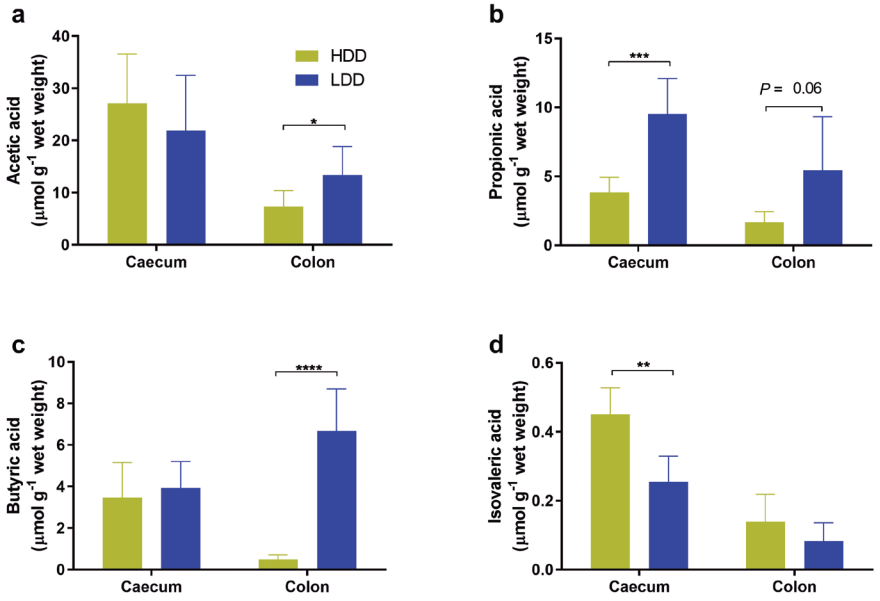


Figure 5. Short-chain fatty acids (SCFA) concentrations in intestinal digesta of mice fed starches of different digestibility. (**a**) Acetic acid, (**b**) propionic acid, (**c**) butyric acid and (**d**) isovaleric acid concentrations in mouse caecum (n=6 per group) and colon (n=5 HDD, n=7 LDD) contents obtained after three weeks of feeding HDD (yellow bars) or LDD (blue bars). Statistical comparisons were made using unpaired two-tailed Student's t-test; *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$, ****$P < 0.0001$. Data shown as mean ± s.d.

We next compared the overall changes in faecal microbiota communities induced by HDD or LDD after exposure to the diets for 3 weeks (Study 1) and 4.5 d (Study 3). Principal coordinates analysis (PCoA) using the UniFrac unweighted distance matrix revealed a clear separation between the two dietary groups (Fig. 6a). These observations were supported by Adonis analysis, using either weighted or unweighted UniFrac distances, as

diet explained a large part of the variation in microbiota composition (20% and 29%, respectively, $P < 0.001$, Table 3). H_2 volume was the second most important variable, followed by duration of intervention and age, and body weight, with minor but significant effects (Table 3). Of note, duration of intervention and age of mice are dependent variables due to study design. In order to control for the effects of duration of dietary exposure, we also analysed Studies 1 and 3 separately. After 3 weeks of intervention, diet and H_2 production were the only significant variables, with H_2 explaining up to 34% of the variation (Fig. 6b and Table 3). However, only diet contributed significantly to the variation after 4.5 d of intervention in adult mice (Fig. 6c and Table 3). Additionally, α-diversity appeared to decrease with duration of intervention irrespective of the dietary intervention, with no consistent effects of the diet (Fig. S3). This is in line with the differences in age of these mice, namely the young mice showing lower α-diversity.

We then aimed to identify which microbial taxa were significantly associated with the observed differences in β-diversity. The microbiota of mice fed LDD *vs* HDD for 3 weeks was enriched in *Bacteroides, Parasutterella, Roseburia,* and *Alloprevotella,* along with two other families (Fig. 7a and Fig. S4a). In comparison, *Lactobacillus, Rikenella, Odoribacter, Enterorhabdus,* and *Desulfovibrio* among others appeared enriched in HDD *vs* LDD-fed mice (Fig. 7a and Fig. S4b). Similar differences were seen after 4.5 d of exposure (Fig. 7b and Fig. S4c,d). While fewer taxa were affected by the short-term dietary intervention, changes in genus level were consistent for both groups (Fig. S5). Moreover, H_2 production was the only (environmental) variable that was significantly correlated with specific bacteria taxa after three weeks of intervention, with five genera correlating positively with H_2 production and eight genera showing a negative correlation (Fig. 8 and Fig. S6). Eleven of these 13 genera were also significantly influenced by diet (Fig. 7a and Fig. S4a,b). Finally, Archaea (some of which are CH_4 producers) could not be detected in any of the samples despite the use of primers targeting both bacterial and archaeal 16S rRNA genes equally well. This agrees with the absence of CH_4 detection in these mice and under these nutritional challenges.

Discussion

The goal of this study was to measure real-time interactions between diet, gut microbes, and the host. We implemented H_2 and CH_4 detection in an indirect calorimetry system to track fermentation continuously in mice. H_2 production revealed a time frame for microbiota adaptation to starch of low digestibility, which corresponded with shifts in microbial community composition induced by diet. Thus, measuring H_2 production allowed us to non-invasively study effects of the diet on the intestinal microbiota in real-time. The difference in starch digestibility as part of the experimental diets was confirmed both by *in vitro* and *in vivo* measurements, but did not significantly alter total intake of digestible energy (gross energy minus faecal energy losses) between dietary groups within a sex. The lower digestibility of the starch in the LDD thus suggests that partially undigested starch reached the large intestine which was subsequently partially fermented by the intestinal microbiota providing energy substrates, *e.g.* SCFA, to the host. Energy of undigested starch can be lost after fermentation in the form of products not utilizable by the host, such as H_2.

However, previous studies considered energy loss in the form of H_2 and CH_4 negligible, representing less than 0.2% of total energy expenditure in humans consuming non-starch polysacharides[41]. Studies in rats fed various types of resistant starch also indicated that energy loss through fermentation is minimal, although the actual H_2 output was not measured directly[42]. Here, our data show that H_2 is produced constantly on a lowly-digestible starch diet. Although the volume of H_2 produced by the mice in our study may be little in terms of energy loss, it is plausible that carbohydrates that give a higher level of fermentation could further increase the H_2 output, which might represent a significant factor to take into account over a lifetime.

H_2 production was detected in mice under all conditions tested, with the amounts produced clearly being influenced by the form of carbohydrate consumed. Mice fed moderately fermentable carbohydrates have been shown to produce H_2[20]. Even in conditions where little fermentation is expected, such as feeding corn starch-based chow[43] or pure sucrose[44], H_2 production has been seen in rats. In line with our data on three diets with a different carbohydrate profile, this illustrates that H_2 production can directly reflect subtle changes in carbohydrate fermentation. Interestingly, H_2 production was clearly associated with the food intake pattern. This is in contrast with data reported in humans, where H_2 and CH_4 peaked at rather unpredictable times after food intake despite the proper control of the meal schedule[41]. This might be due to e.g. differences in dietary meal composition, time resolution of sampling, intestinal transit time, or other differences in intestinal physiology between humans and mice. More recently, using gut capsule technology, a similar H_2 pattern as in our mice was also observed in a human pilot trial based on dietary fibre differences[45].

We demonstrated that real-time monitoring of H_2 production can be used to investigate transient effects of diet in time and explore the process of adaptation rather than the end stage only. So far, studies mainly investigated, by measuring H_2 and other fermentation parameters at selected time points, longer timeframes ranging from 1 day to several weeks[25,46-48]. In our study, significant differences in H_2 production appeared within 12 h upon access to LDD. This timeframe was clearly influenced by fasting and whether the diet was provided *ad libitum* or in a restricted amount. We speculate these early increases in H_2 output to reflect immediate effects of diet on microbial metabolism preceding changes in community structure. Another observation was that mice fed chow produced H_2, although at low levels. Real-time monitoring newly revealed that a period of food restriction followed by refeeding led to a marked and acute increase in H_2 production once chow became available again. A likely explanation is excessive eating after food deprivation, causing a larger amount of not fully digested chyme to enter the large intestine and thus increasing substrate availability to the microbiota. In addition, a 24 h fasting period alone has been shown to produce shifts in microbial diversity[49] and microbiota configuration[50]. Such changes could in turn alter fermentation stoichiometry and microbial function in response to the diet and ultimately lead to a higher H_2 output. Our analysis indicates (short-term) effects of fasting and refeeding on microbial activity, which should be careful taken into account in nutritional studies focussing on changes in microbiota composition and function.

Table 3. Faecal microbiota composition of mice fed HDD or LDD and other host and environmental variables.

	Weighted UniFrac		Unweighted UniFrac	
	R^2	*P* value	R^2	*P* value
	Studies 1 and 3 combined (long- and short-term exposures)			
Diet	0.198	0.001***	0.287	0.001***
Cumulative H_2 production	0.098	0.023*	0.173	0.001***
Duration of intervention*	0.094	0.015*	0.141	0.001***
Age*	0.094	0.02*	0.141	0.002**
Sex	0.057	0.12	0.05	0.018*
Body weight	0.08	0.042*	0.1	0.002**
Food intake	0.06	0.08	0.043	0.142
Starch intake	0.068	0.079	0.043	0.135
	Study 1 (long-term exposure, post-weaning, n=12)			
Diet	0.26	0.003***	0.4	0.001***
Cumulative H_2 production	0.198	0.005***	0.344	0.001***
Sex	0.062	0.195	0.03	0.658
Body weight	0.046	0.289	0.02	0.87
Food intake	0.099	0.062	0.049	0.3
Starch intake	0.1	0.077	0.05	0.279
	Study 3 (short-term exposure, adult, n=5)			
Diet	0.24	0.056	0.293	0.004***
Cumulative H_2 production	0.14	0.217	0.108	0.5
Body weight	0.08	0.594	0.137	0.228
Food intake	0.06	0.748	0.192	0.044*
Starch intake	0.06	0.761	0.192	0.05

Results are obtained using Adonis Permutational Multivariate Analysis of Variance. * Duration of intervention (short- and long-term) and age of animals (young *vs* adult) are not independent variables.

As could be expected, the driver of the experimental differences, the dietary starch digestibility, was the most important factor explaining the variation in microbiota, showing colinearity with our measured *in vivo* H_2 production. Although current knowledge of the dynamics of H_2 within the gastrointestinal tract is limited, it is well documented that H_2 is exclusively produced during fermentation by hydrogenogens[6]. Among the major hydrogenogenic bacteria are Bacteroidetes and clostridial members of Firmicutes[51]. In line with this, we observed that LDD, a source of carbohydrates for caecum and colon, stimulated the fermentative Bacteroidetes bacteria, more specifically the genus *Bacteroides*. This is consistent with the dose-dependent increase in caecal Bacteroidetes density in response to amylose[52] and similar findings for amylose on a high-fat background[53]. Here we extend these findings and show, for the first time *in vivo*, a positive correlation between *Bacteroides* and H_2 production in mice.

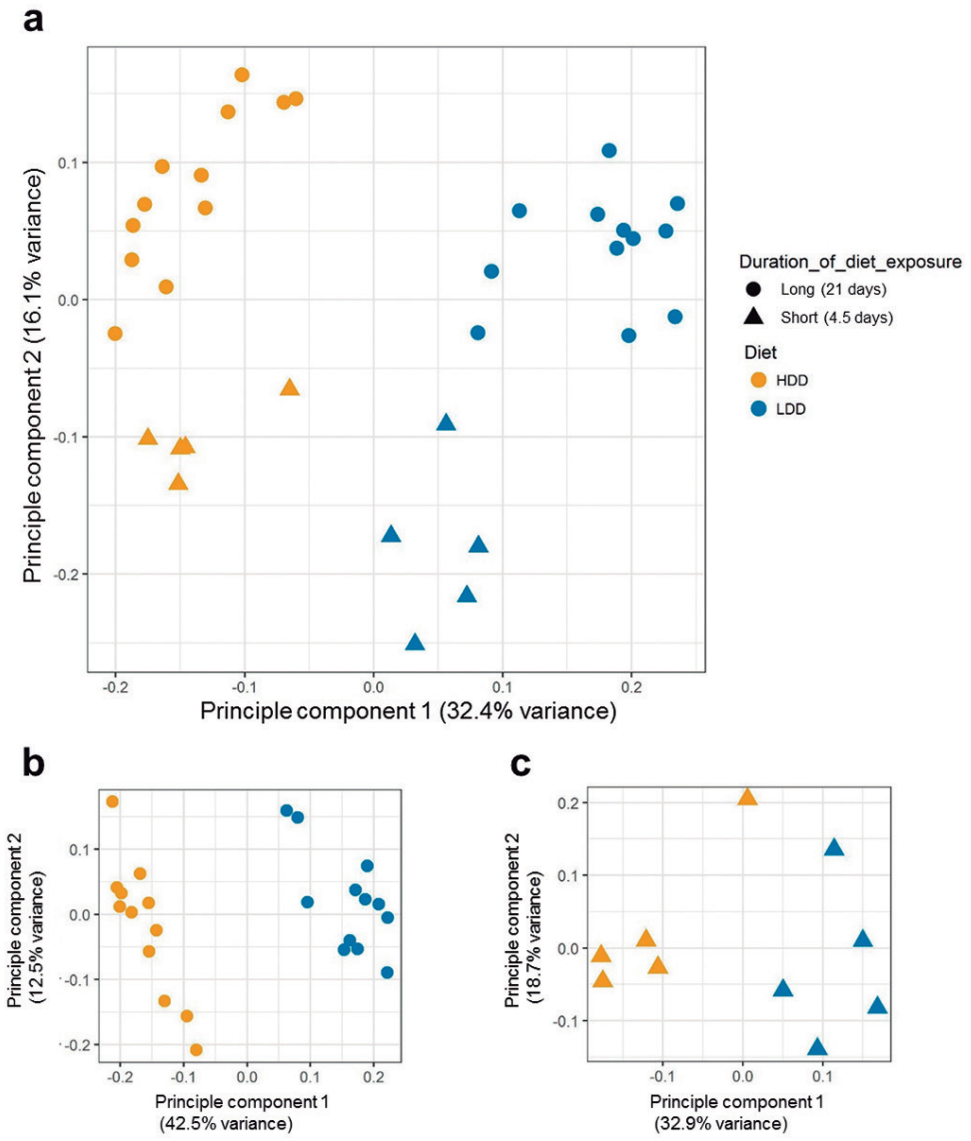


Figure 6. Starch digestibility primarily determines faecal microbiota composition. Principal coordinates analysis (PCoA) plot illustrating the unweighted UniFrac distances of the intestinal microbiota of mice after long- and short-term exposure to HDD and LDD (**a**, Studies 1 and 3 combined), and only long-term (**b**, Study 1) and short-term (**c**, Study 3) exposures. Each data point represents a sample of faecal pellets of one individual mouse (n=12 long-term exposure per diet, n=5 short-term exposure per diet).

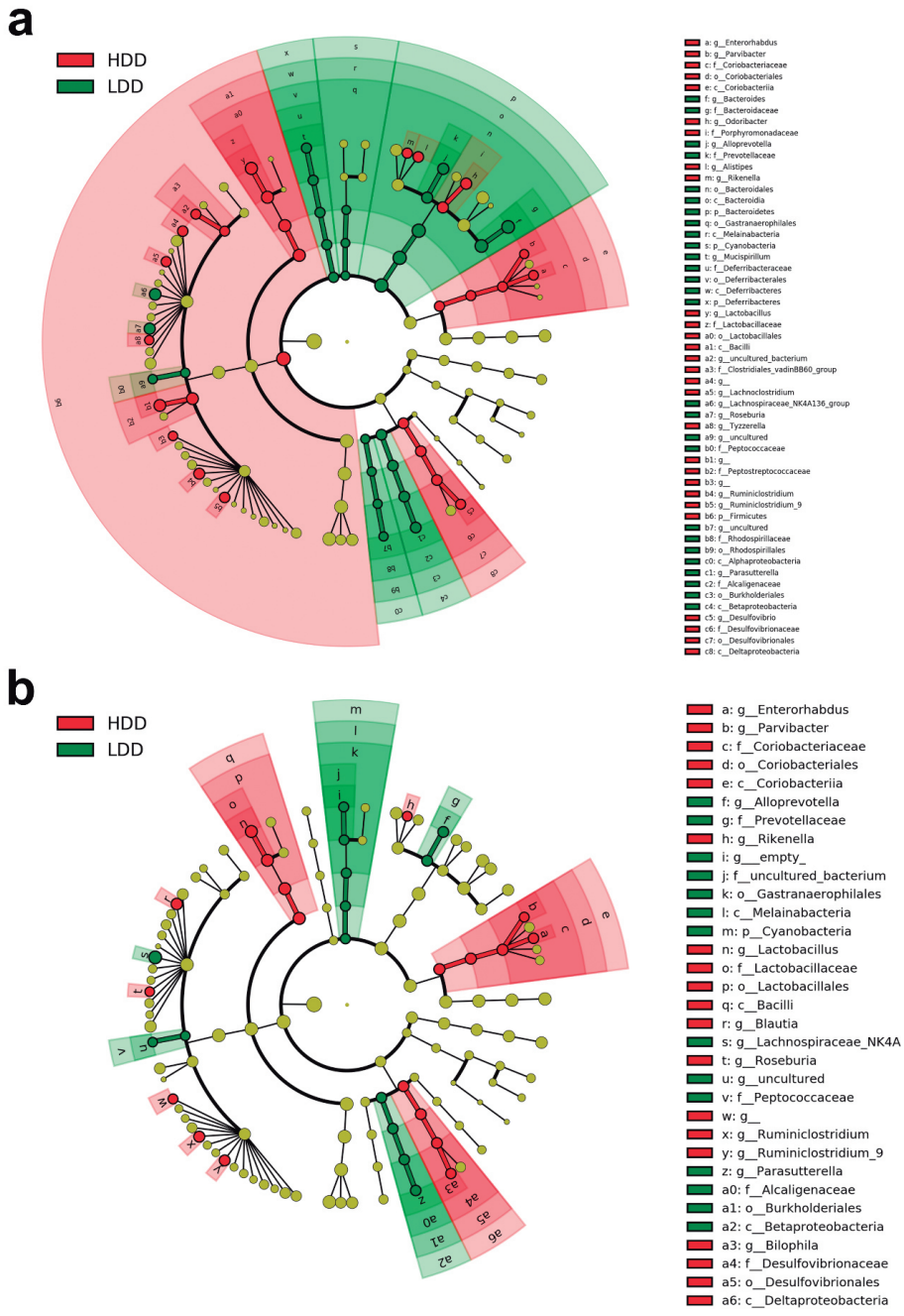


Figure 7. Exposure to starches of different digestibility induces distinct microbial taxa. (**a**) Cladogram representing bacteria genera that were significantly enriched by LDD or HDD after 3 weeks of exposure to the diets (*n*=12 per diet, Study 1). (**b**) Bacterial genera that were significantly increased by LDD or HDD after 4.5 d of exposure to the diets (*n*=5 per diet, Study 3). Comparisons were done using the linear discriminant analysis effect size (LEfSe) method. LDA scores are shown in Fig. S4e,f. Nomenclature of microbial strains is based on phylum, class, order, family, and genus.

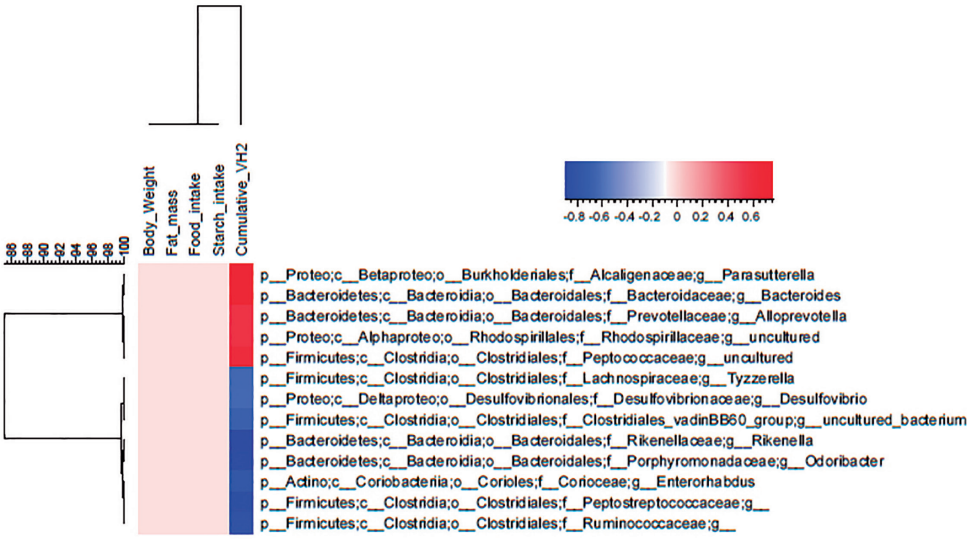


Figure 8. Specific bacterial genera correlate only with *in vivo* H_2 production. Spearman's rank correlation coefficients of faecal microbiota, H_2 production, food and starch intake, body weight, and fat mass of mice exposed to HDD or LDD for 3 weeks after weaning (n=12 per diet, Study 1). Non-red and non-blue cells all have a Spearman's correlation value of 0 with FDR P value > 0.13. Nomenclature of microbial strains is based on phylum, class, order, family, and genus.

Interestingly, after the short-term exposure to LDD in adult mice, Bacteroidetes were not significantly increased compared to the HDD group. This might be associated with the shorter duration of the treatment and possibly with more firmly established microbial communities in adulthood. However, most genera induced by diet in adult mice after 4.5 days correspond to those induced in mice after weaning, which were exposed for 3 weeks.

Another consistent shift in microbial community composition was the promotion of Deltaproteobacteria, particularly *Desulfovibrio* and *Bilophila*, in HDD-fed mice. Deltaproteobacteria are the major representatives of colonic sulphate reducing bacteria (SRB)[54] including *Desulfovibrio*. SRB along with methanogens and acetogenic bacteria are the only gut microbes able to use H_2 as an electron donor to produce H_2S and acetate. Although not a SRB itself, taurine-respiring *Bilophila* species can also produce H_2S. Additionally, there is evidence of CH_4 production in rats[26] and mice, with the presence of methanogens in humanized microbiota mouse models[55]and by high fat dietary feeding[56]. The fact that we neither detected CH_4 nor Archaea suggests that H_2 was preferentially used to produce H_2S in mice fed readily digestible starch. H_2S is a potentially toxic product of bacterial metabolism[57-59], and it has been implicated in human health and disease[19] and, more recently, thermogenesis[60]. Moreover, H_2S has been reported to inhibit the production of SCFA and specifically to impair butyrate oxidation, depriving colonic cells from their main energy source[58,61]. In line, we report a dramatic difference in colonic butyrate in HDD-fed mice. Apart from Deltaproteobacteria, we observed increased abundances of *Odoribacter*, a known H_2S producer[62], and *Rikenella*, a desulphatase-secreting bacterium[63], under HDD-

feeding. Members of the genus *Rikenella* are able to cleave sulphate from mucin glycans, making them available for microbial degradation[64] and potentially acting as a donor of sulphate to H_2S producers. Based on these facts we speculate that the lack of fermentable carbohydrates favoured the presence of hydrogenotrophs associated with the production of H_2S, which could have led to the decreased H_2 output and colonic SCFA levels that was observed in mice fed highly-digestible starch.

The major taxon increased in HDD-fed mice in our study belonged to the genus *Lactobacillus*. In contrast, diets supplemented with resistant starch tended to enrich the *Lactobacillus* population in mouse caecum, but much less at high doses of resistant starch[52]. Incidentally, hydrogenase genes, which encode enzymes for the reversible oxidation of H_2, were recently shown to be completely absent in Bacilli and bifidobacteria[51]. Considering the lack of a correlation between H_2 production and *Lactobacillus* in our study, new questions emerge about the ability of *Lactobacillus* to thrive in H_2-poor environments.

The increase in isovaleric acid, a product of branched-chain amino acid catabolism[65], in HDD-fed mice, suggests a shift of microbiota towards protein fermentation. Bacteria from the genera *Enterorhabdus*[66] and *Parvibacter*[67], both significantly induced by HDD-feeding, have the ability to ferment amino acids. Additionally *Olsenella*, only present in two samples in the HDD group, is documented to grow on tyrosine and produce *p*-cresol[68], supporting our hypothesis for a shift to protein fermentation. This might have important implications for the host, since products of protein fermentation such as phenols, ammonia, certain amines, and H_2S, are considered to play important roles in the initiation or progression of bowel diseases, inflammation, DNA damage, and cancer[69].

Altogether, our results emphasize H_2 as a key factor within the intestinal microenvironment and the usefulness of knowing its production dynamics to understand the interplay between host, diet, and the intestinal microbiota. At the same time, we are aware that our approach to study such interactions may have conceivable limitations. It has been argued that changes in gas evolution (and other indirect markers of fermentation) cannot accurately indicate changes in fermentation[70], and even "real-time", carefully controlled measurements have failed to show quantitative changes in H_2 and CH_4 production proportionally linked to consumption of fermentable carbohydrates[15,41]. We completely agree with these authors that the measured outcomes, H_2 and CH_4, not only reflect the type of carbohydrate consumed, but are the end result of a very complex fermentation stoichiometry that depends on the host's capacity to digest and absorb nutrients, the dominance and metabolic activity of microbial species, and their interactions. However, the conclusion that fermentation gases are extremely limited parameters to study carbohydrate fermentation is largely based on human data, where eating pattern, environment, genetic variation, and the gut microbe interact and ultimately determine an individual's response to the diet[71,72]. When these and other factors can be better controlled, as it is the case with animal models, the analysis of carbohydrate fermentation through H_2 and CH_4 quantification has much to offer. The fact that *in vitro* models to measure H_2 and CH_4 evolution are still developing and proposed as a tool to unravel the mechanisms behind the association between microbiota and host health[73] is encouraging.

Overall, the applications of gas analysis within an indirect calorimetry system go beyond the arena of carbohydrate quality and nutritional studies, and may be used as a diagnosis tool in clinical practice[19,74,75]. It opens up new avenues not only in preclinical research in rodents, but also has potential in human-diet-microbiota interaction studies if such sensor technology is incorporated into indirect calorimetry chambers or ventilated hood systems.

Conclusions

Using our customized indirect calorimetry system we were able to continuously quantify H_2 production in mice as a reflection of the starch digestibility of the diet. H_2 monitoring also allowed us to catch the earliest stages in the adaptation to carbohydrates of different digestibility, revealing a nuanced process with high inter-individual variation. Importantly, *in vivo* H_2 production was significantly correlated with specific microbial taxa, including *Bacteroides* and *Parasutterella*. The implemented H_2 and CH_4 sensor-technology described here opens yet unmet avenues to study the effects of nutrition on microbiota in real time, not only in rodents, but potentially also in humans.

Author Contributions

J.K. and E.M.v.S. conceived, designed, and supervised the project. J.M.S.F.-C., H.J.M.S., and L.M.S.B. conducted animal experiments. H.J.M.S. and E.M.v.S. integrated the H_2 and CH_4 sensors into the indirect calorimetry system. L.M.S.B., A.O., and H.S. provided input for experimental design and interpretation of results. N.B. and V.G.-C. analysed and interpreted *in vitro* digestion data. P.K. and H.S. carried out all microbiome analyses and interpreted the data. J.M.S.F.-C., P.K., E.M.v.S., J.K., and H.S. interpreted data and prepared the manuscript. All authors critically revised and approved the manuscript.

Acknowledgements

We acknowledge the help of the personnel at the CARUS animal facility. We also thank Inge van der Stelt and students Laura Dewitte and Lini Sholihah for their help in collecting data.

References

1 Englyst, K. N., Liu, S. & Englyst, H. N. Nutritional characterization and measurement of dietary carbohydrates. *Eur. J. Clin. Nutr.* **61 Suppl 1**, S19-39 (2007).

2 Elia, M. & Cummings, J. H. Physiological aspects of energy metabolism and gastrointestinal effects of carbohydrates. *Eur. J. Clin. Nutr.* **61 Suppl 1**, S40-74 (2007).

3 den Besten, G. *et al.* The role of short-chain fatty acids in the interplay between diet, gut microbiota, and host energy metabolism. *J. Lipid. Res.* **54**, 2325-2340 (2013).

4 Silk, D. B., Webb, J. P., Lane, A. E., Clark, M. L. & Dawson, A. M. Functional differentiation of human jejunum and ileum: a comparison of the handling of glucose, peptides, and amino acids. *Gut* **15**, 444-449 (1974).

5 Flint, H. J., Scott, K. P., Louis, P. & Duncan, S. H. The role of the gut microbiota in nutrition and health. *Nat. Rev. Gastroenterol. Hepatol.* **9**, 577-589 (2012).

6 Fischbach, M. A. & Sonnenburg, J. L. Eating for two: how metabolism establishes interspecies interactions in the gut. *Cell Host Microbe* **10**, 336-347 (2011).

7 Hooper, L. V. *et al.* Molecular analysis of commensal host-microbial relationships in the intestine. *Science* **291**, 881-884 (2001).

8 Cox, L. M. *et al.* Altering the intestinal microbiota during a critical developmental window has lasting metabolic consequences. *Cell* **158**, 705-721 (2014).

9 van Nood, E. *et al.* Duodenal infusion of donor feces for recurrent *Clostridium difficile*. *N. Engl. J. Med.* **368**, 407-415 (2013).

10 Martinez-Fernandez, G., Denman, S. E., Cheung, J. & McSweeney, C. S. Phloroglucinol degradation in the rumen promotes the capture of excess hydrogen generated from methanogenesis inhibition. *Front. Microbiol.* **8**, 1871 (2017).

11 Olijhoek, D. W. *et al.* Effect of dietary nitrate level on enteric methane production, hydrogen emission, rumen fermentation, and nutrient digestibility in dairy cows. *J. Dairy Sci.* **99**, 6191-6205 (2016).

12 Rooke, J. A. *et al.* Hydrogen and methane emissions from beef cattle and their rumen microbial community vary with diet, time after feeding and genotype. *Br. J. Nutr.* **112**, 398-407 (2014).

13 Sollinger, A. *et al.* Holistic assessment of rumen microbiome dynamics through quantitative metatranscriptomics reveals multifunctional redundancy during key steps of anaerobic feed degradation. *mSystems* **3**, e00038-18 (2018).

14 van Lingen, H. J. *et al.* Diurnal dynamics of gaseous and dissolved metabolites and microbiota composition in the bovine rumen. *Front. Microbiol.* **8**, 425 (2017).

15 Christl, S. U., Murgatroyd, P. R., Gibson, G. R. & Cummings, J. H. Production, metabolism, and excretion of hydrogen in the large intestine. *Gastroenterology* **102**, 1269-1277 (1992).

16 Symonds, E. L., Kritas, S., Omari, T. I. & Butler, R. N. A combined $^{13}CO_2/H_2$ breath test can be used to assess starch digestion and fermentation in humans. *J. Nutr.* **134**, 1193-1196 (2004).

17 Ong, D. K. *et al.* Manipulation of dietary short chain carbohydrates alters the pattern of gas production and genesis of symptoms in irritable bowel syndrome. *J. Gastroenterol. Hepatol.* **25**, 1366-1373 (2010).

18 Valeur, J., Puaschitz, N. G., Midtvedt, T. & Berstad, A. Oatmeal porridge: impact on microflora-associated characteristics in healthy subjects. *Br. J. Nutr.* **115**, 62-67 (2016).

19 Carbonero, F., Benefiel, A. C. & Gaskins, H. R. Contributions of the microbial hydrogen economy to colonic homeostasis. *Nat. Rev. Gastroenterol. Hepatol.* **9**, 504-518 (2012).

20 Isken, F., Klaus, S., Osterhoff, M., Pfeiffer, A. F. & Weickert, M. O. Effects of long-term soluble vs. insoluble dietary fiber intake on high-fat diet-induced obesity in C57BL/6J mice. *J. Nutr. Biochem.* **21**, 278-284 (2010).

21 Hartmann, L., Taras, D., Ramlage, B. & Blaut, M. A new technique to determine hydrogen excreted by gnotobiotic rats. *Lab. Anim.* **34**, 162-170 (2000).

22 Ostrander, C. R., Stevenson, D. R., Neu, J., Kerner, J. A. & Moses, S. W. A sensitive analytical apparatus for measuring hydrogen production rates. I. Application to studies in small animals. Evidence of the effects of an α -glucosidehydrolase inhibitor in the rat. *Anal. Biochem.* **119**, 378-386 (1982).

23 Dufourlescoat, C., Lecoz, Y., Andrieux, C. & Szylit, O. Effects of nature, size and level of incorporation of dietary fibers on colonic functions in germ-free rats and in heteroxenic rats inoculated with a human flora. *Food. Hydrocoll.* **9**, 9-15 (1995).

24 Rodkey, F. L., Collison, H. A. & O'Neal, J. D. Carbon monoxide and methane production in rats, guinea pigs, and germ-free rats. *J. Appl. Physiol.* **33**, 256-260 (1972).

25 Walter, D. J., Eastwood, M. A., Brydon, W. G. & Elton, R. A. An experimental design to study colonic fibre fermentation in the rat: the duration of feeding. *Br. J. Nutr.* **55**, 465-479 (1986).

26 Tuboly, E. *et al.* Determination of endogenous methane formation by photoacoustic spectroscopy. *J. Breath Res.* **7**, 046004 (2013).

27 Hoevenaars, F. P. *et al.* Effects of dietary history on energy metabolism and physiological parameters in C57BL/6J mice. *Exp. Physiol.* **98**, 1053-1062 (2013).

28 Duivenvoorde, L. P. *et al.* Oxygen restriction as challenge test reveals early high-fat-diet-induced changes in glucose and lipid metabolism. *Pflugers Arch.* **467**, 1179-1193 (2015).

29 Reeves, P. G., Nielsen, F. H. & Fahey, G. C., Jr. AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet. *J. Nutr.* **123**, 1939-1951 (1993).

30 Isken, F. *et al.* Impairment of fat oxidation under high- vs. low-glycemic index diet occurs before the development of an obese phenotype. *Am. J. Physiol. Endocrinol. Metab.* **298**, E287-295 (2010).

31 Garcia-Campayo, V., Han, S., Vercauteren, R. & Franck, A. Digestion of Food Ingredients and Food Using an In Vitro Model Integrating Intestinal Mucosal Enzymes. *Food and Nutrition Sciences* **09**, 711-734 (2018).

32 Versantvoort, C. H., Oomen, A. G., Van de Kamp, E., Rompelberg, C. J. & Sips, A. J. Applicability of an in vitro digestion model in assessing the bioaccessibility of mycotoxins from food. *Food Chem. Toxicol.* **43**, 31-40 (2005).

33 Morin, L. G. & Prox, J. Single glucose oxidase-peroxidase reagent for two-minute determination of serum glucose. *Clin. Chem.* **19**, 959-962 (1973).

34 Ladirat, S. E. *et al.* Impact of galacto-oligosaccharides on the gut microbiota composition and metabolic activity upon antibiotic treatment during in vitro fermentation. *FEMS Microbiol. Ecol.* **87**, 41-51 (2014).

35 Ramiro-Garcia, J. *et al.* NG-Tax, a highly accurate and validated pipeline for analysis of 16S rRNA amplicons from complex biomes. *F1000Res.* **5**, 1791 (2016).

36 Lahti, L. & Shetty, S. *Tools for microbiome analysis in R. Version 0.99.90.,* http://microbiome.github.io/microbiome/ (2017).

37 Segata, N. *et al.* Metagenomic biomarker discovery and explanation. *Genome Biol.* **12**, R60 (2011).

38 Caporaso, J. G. *et al.* QIIME allows analysis of high-throughput community sequencing data. *Nat. Methods* **7**, 335-336 (2010).

39 Duivenvoorde, L. P., van Schothorst, E. M., Swarts, H. J. & Keijer, J. Assessment of metabolic flexibility of old and adult mice using three noninvasive, indirect calorimetry-based treatments. *J. Gerontol. A Biol. Sci. Med. Sci.* **70**, 282-293 (2015).

40 Scribner, K. B., Pawlak, D. B., Aubin, C. M., Majzoub, J. A. & Ludwig, D. S. Long-term effects of dietary glycemic index on adiposity, energy metabolism, and physical activity in mice. *Am. J. Physiol. Endocrinol. Metab.* **295**, E1126-1131 (2008).

41 Poppitt, S. D. *et al.* Circadian patterns of total 24-h hydrogen and methane excretion in humans ingesting nonstarch polysaccharide (NSP) diets and the implications for indirect calorimetric and D_2 ^{18}O methodologies. *Eur. J. Clin. Nutr.* **50**, 524-534 (1996).

42 Tulley, R. T. *et al.* Comparative methodologies for measuring metabolizable energy of various types of resistant high amylose corn starch. *J. Agric. Food Chem.* **57**, 8474-8479 (2009).

43 Nishimura, N., Tanabe, H. & Yamamoto, T. Isomaltodextrin, a highly branched alpha-glucan, increases rat colonic H_2 production as well as indigestible dextrin. *Biosci. Biotechnol. Biochem.* **80**, 554-563 (2016).

44 Tonouchi, H. *et al.* Studies on absorption and metabolism of palatinose (isomaltulose) in rats. *Br. J. Nutr.* **105**, 10-14 (2011).

45 Kalantar-Zadeh, K. *et al.* A human pilot trial of ingestible electronic capsules capable of sensing different gases in the gut. *Nat. Electron.* **1**, 79-87 (2018).

46 Levrat, M. A., Remesy, C. & Demigne, C. Very acidic fermentations in the rat cecum during adaptation to a diet rich in amylase-resistant starch (crude potato starch). *J. Nutr. Biochem.* **2**, 31-36 (1991).

47 Key, F. B. & Mathers, J. C. Digestive adaptations of rats given white bread and cooked haricot beans (*Phaseolus vulgaris*): large-bowel fermentation and digestion of complex carbohydrates. *Br. J. Nutr.* **74**, 393-406 (1995).

48 Walker, A. W. *et al.* Dominant and diet-responsive groups of bacteria within the human colonic microbiota. *ISME J.* **5**, 220-230 (2011).

49 Kohl, K. D., Amaya, J., Passement, C. A., Dearing, M. D. & McCue, M. D. Unique and shared responses of the gut microbiota to prolonged fasting: a comparative study across five classes of vertebrate hosts. *FEMS Microbiol. Ecol.* **90**, 883-894 (2014).

50 Crawford, P. A. *et al.* Regulation of myocardial ketone body metabolism by the gut microbiota during nutrient deprivation. *Proc. Natl. Acad. Sci. USA* **106**, 11276-11281 (2009).

51 Wolf, P. G., Biswas, A., Morales, S. E., Greening, C. & Gaskins, H. R. H_2 metabolism is widespread and diverse among human colonic microbes. *Gut Microbes* **7**, 235-245 (2016).

52 Tachon, S., Zhou, J., Keenan, M., Martin, R. & Marco, M. L. The intestinal microbiota in aged mice is modulated by dietary resistant starch and correlated with improvements in host responses. *FEMS Microbiol. Ecol.* **83**, 299-309 (2013).

53 Barouei, J. *et al.* Microbiota, metabolome, and immune alterations in obese mice fed a high-fat diet containing type 2 resistant starch. *Mol. Nutr. Food Res.* **61**, 1700184 (2017).

54 Muyzer, G. & Stams, A. J. The ecology and biotechnology of sulphate-reducing bacteria. *Nat. Rev. Microbiol.* **6**, 441-454 (2008).

55 Turnbaugh, P. J. *et al.* An obesity-associated gut microbiome with increased capacity for energy harvest. *Nature* **444**, 1027-1031 (2006).

56 Laverdure, R., Mezouari, A., Carson, M. A., Basiliko, N. & Gagnon, J. A role for methanogens and methane in the regulation of GLP-1. *Endocrinol. Diabetes Metab.* **1** (2018).

57 Attene-Ramos, M. S., Wagner, E. D., Plewa, M. J. & Gaskins, H. R. Evidence that hydrogen sulfide is a genotoxic agent. *Mol. Cancer Res.* **4**, 9-14 (2006).

58 Roediger, W. E., Duncan, A., Kapaniris, O. & Millard, S. Sulphide impairment of substrate oxidation in rat colonocytes: a biochemical basis for ulcerative colitis? *Clin. Sci.* **85**, 623-627 (1993).

59 Attene-Ramos, M. S., Wagner, E. D., Gaskins, H. R. & Plewa, M. J. Hydrogen sulfide induces direct radical-associated DNA damage. *Mol. Cancer. Res.* **5**, 455-459 (2007).

60 Soriano, R. N. *et al.* Endogenous peripheral hydrogen sulfide is propyretic: its permissive role in brown adipose tissue thermogenesis in rats. *Exp. Physiol.* **103**, 397-407 (2018).

61 Segata, N. *et al.* Composition of the adult digestive tract bacterial microbiome based on seven mouth surfaces, tonsils, throat and stool samples. *Genome Biol.* **13**, R42 (2012).

62 Goker, M. *et al.* Complete genome sequence of *Odoribacter splanchnicus* type strain (1651/6^{T}). *Stand. Genomic. Sci.* **4**, 200-209 (2011).

63 Bomar, L., Maltz, M., Colston, S. & Graf, J. Directed culturing of microorganisms using metatranscriptomics. *MBio* **2**, e00012-00011 (2011).

64 Tsai, H. H., Hart, C. A. & Rhodes, J. M. Production of mucin degrading sulphatase and glycosidases by *Bacteroides thetaiotaomicron*. *Lett. Appl. Microbiol.* **13**, 97-101 (1991).

65 Dai, Z. L., Wu, G. Y. & Zhu, W. Y. Amino acid metabolism in intestinal bacteria: links between gut ecology and host health. *Front. Biosci. (Landmark Ed.)* **16**, 1768-1786 (2011).

66 Clavel, T. *et al.* Isolation of bacteria from the ileal mucosa of TNF^{deltaARE} mice and description of *Enterorhabdus mucosicola* gen. nov., sp. nov. *Int. J. Syst. Evol. Microbiol.* **59**, 1805-1812 (2009).

67 Clavel, T., Charrier, C., Wenning, M. & Haller, D. *Parvibacter caecicola* gen. nov., sp. nov., a bacterium of the family Coriobacteriaceae isolated from the caecum of a mouse. *Int. J. Syst. Evol. Microbiol.* **63**, 2642-2648 (2013).

68 Li, X., Jensen, R. L., Hojberg, O., Canibe, N. & Jensen, B. B. *Olsenella scatoligenes* sp. nov., a 3-methylindole- (skatole) and 4-methylphenol- (*p*-cresol) producing bacterium isolated from pig faeces. *Int. J. Syst. Evol. Microbiol.* **65**, 1227-1233 (2015).

69 Windey, K., De Preter, V. & Verbeke, K. Relevance of protein fermentation to gut health. *Mol. Nutr. Food. Res.* **56**, 184-196 (2012).

70 Topping, D. L. & Clifton, P. M. Short-chain fatty acids and human colonic function: roles of resistant starch and nonstarch polysaccharides. *Physiol. Rev.* **81**, 1031-1064 (2001).

71 Zoetendal, E. G., Akkermans, A. D. L., Akkermans-van Vliet, W. M., de Visser, J. A. G. M. & de Vos, W. M. The host genotype affects the bacterial community in the human gastronintestinal tract. *Microb. Ecol. Health Dis.* **13**, 129-134 (2009).

72 Zeevi, D. *et al.* Personalized nutrition by prediction of glycemic responses. *Cell* **163**, 1079-1094 (2015).

73 Carter, E. A. & Barr, R. G. Preliminary studies demonstrating acetoclastic methanogenesis in a rat colonic ring model. *J. Nutr. Metab.* **2013**, 540967 (2013).

74 Pimentel, M. Breath testing for small intestinal bacterial overgrowth: should we bother? *Am. J. Gastroenterol.* **111**, 307-308 (2016).

75 Pimentel, M., Mathur, R. & Chang, C. Gas and the microbiome. *Curr. Gastroenterol. Rep.* **15**, 356 (2013).

Supplementary Information

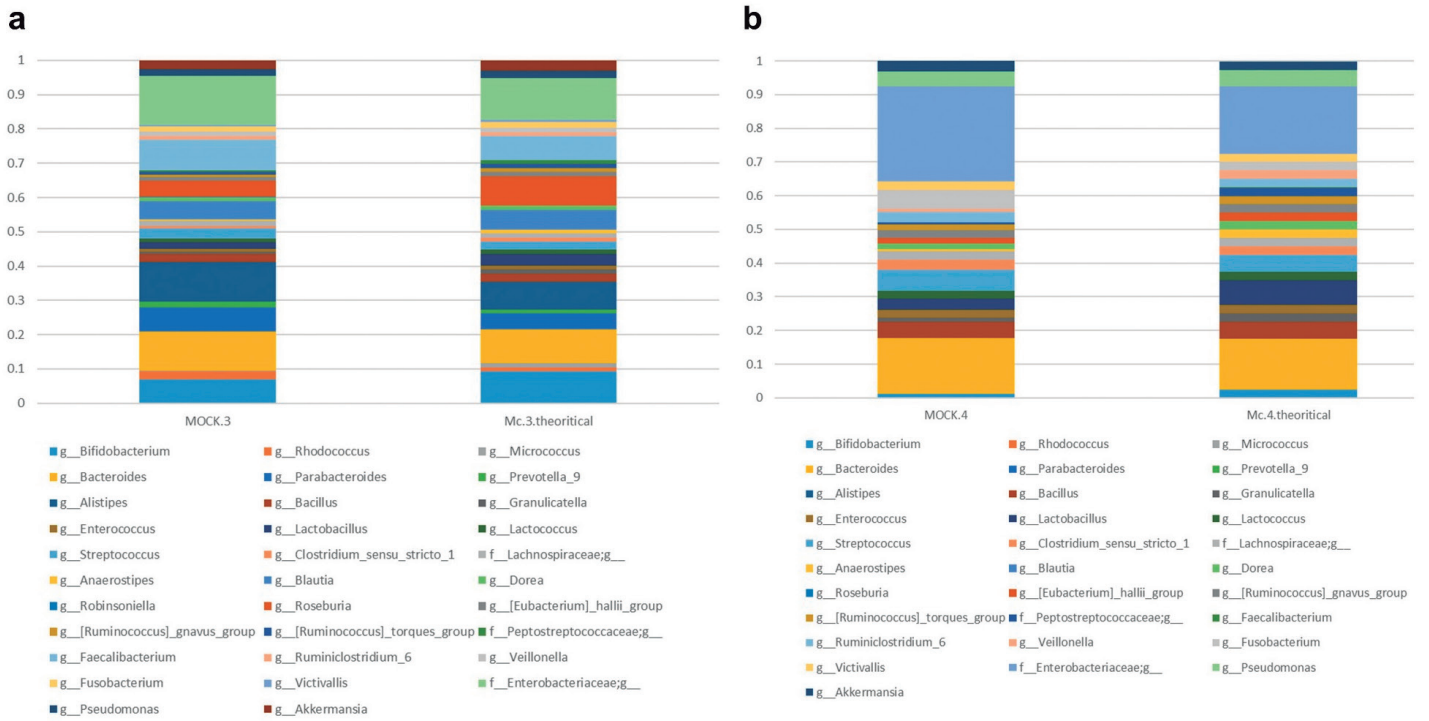


Figure S1. Bacterial composition of the two synthetic mock communities which were used as controls for sequencing run ("mock 3", a; "mock 4", b). Both mock communities were compared against their theoretical composition using non-parametric Pearson correlation showing high similarity. Correlation coefficients are 0.935 for mock 3 and 0.96 for mock 4, respectively.

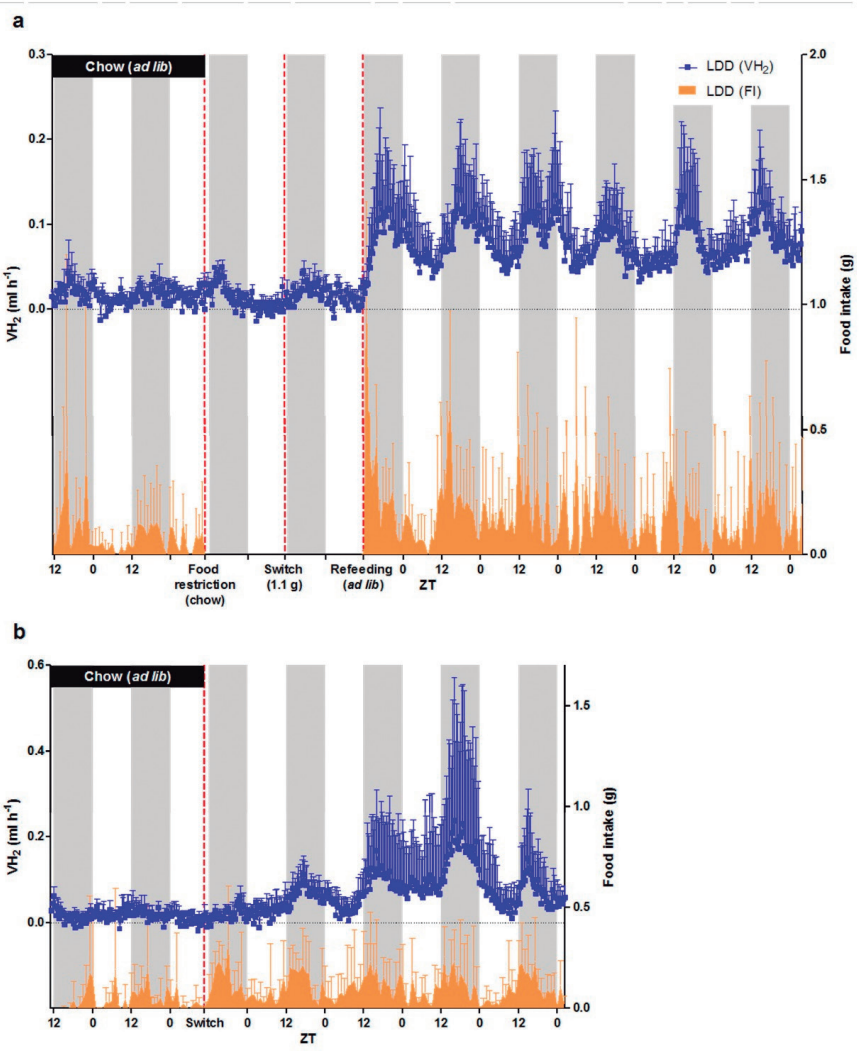


Figure S2. Hydrogen (H_2) production closely follows food intake (FI) patterns. (**a**) Mice within indirect calorimetry were fed standard chow, followed by chow restriction leading to a fasting state (left dotted line), which was subsequently followed by feeding 1.1 g of LDD (blue; *n*=4) prior to the dark phase as a single meal test (2nd dotted line). As a result, mice were fasted the next day, and received prior to next dark phase *ad libitum* access to LDD (3rd dotted line) for an additional 5.5 d. (**b**) Chow-fed mice (*n*=6) were switched to LDD without prior food restriction and measurements continued for another 4.5 d. All mice received no other diet than chow during their whole lifetime prior to these experiments until the dietary switch (black bar), but colour usage reflects exposure to new diets. Volume of H_2 produced (VH_2; blue squares, left y-axis, ml h-1) is plotted together with associated hourly food intake episodes (orange area, right y-axis, g), except when food was placed directly inside the cage and not in the food baskets (food restriction, panel **a**). White and grey areas represent light and dark phases, respectively. Data is presented as mean ± s.d. For clarity, only upper error bars are shown. ZT, Zeitgeber time.

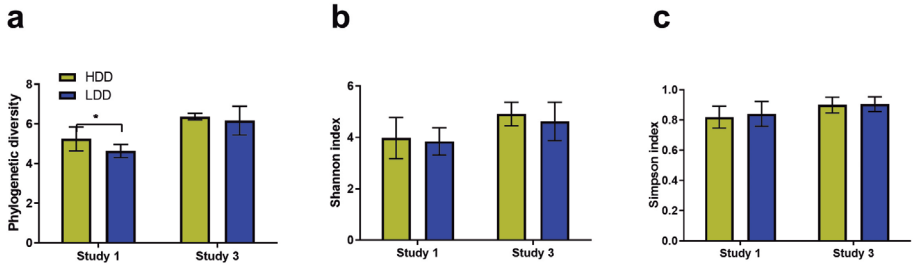
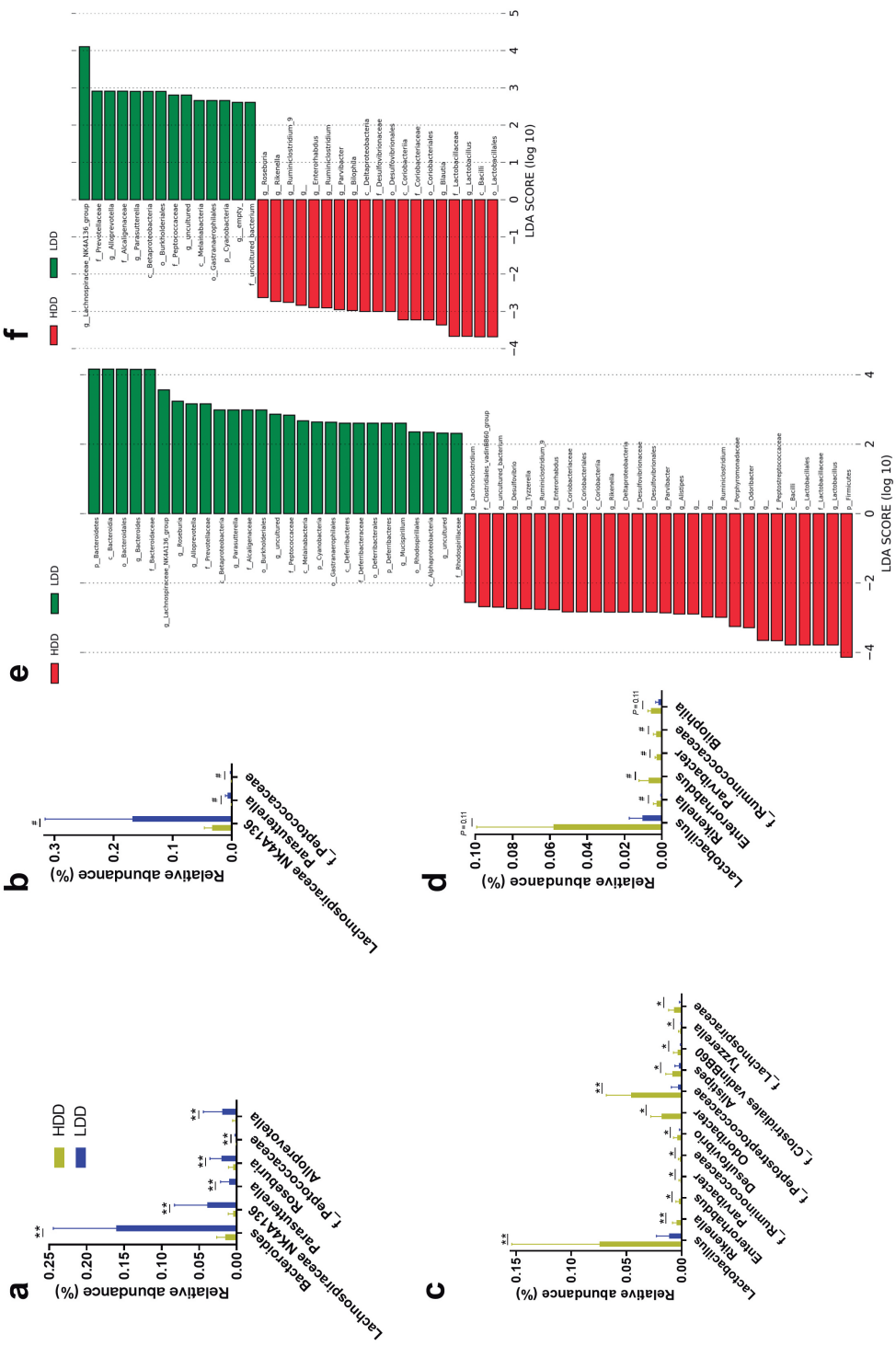


Figure S3. Metrics of α-diversity in faecal microbiota of HDD- or LDD-fed mice. Phylogenetic diversity (**a**), and Shannon (**b**) and Simpson (**c**) indexes of mice fed HDD or LDD for 3 weeks (n=12 per diet, Study 1) or 4.5 d (n=5 per diet, Study 3). Statistical comparisons were made using unpaired two-tailed Student's t-test; $^{*}P \leq 0.05$. Data shown as mean ± s.d.

Figure S4 (next page). Exposure to starches of different digestibility induces distinct microbial taxa. All bacterial genera that were significantly increased by LDD (**a**) or HDD (**c**) after 3 weeks of exposure to the diets (n=12 per diet, Study 1) are shown. All genera that were significantly increased by LDD (**b**) or HDD (**d**) after 4.5 d of exposure to the diets (n=5 per diet, Study 3) are also shown for comparison. Side-by-side boxes represent the $\log_{10}$ transformed LDA scores of bacterial taxa enriched in HDD- or LDD-fed mice in Study 1 (**e**) and Study 3 (**f**) analysed by LEfSe. For uncultured genera, family name is indicated (f_). Comparisons were done by non-parametric t-test followed by 999 permutations and P values were adjusted by False Discovery Rate (FDR) correction; $^{\#}P < 0.1$, $^{*}P \leq 0.05$, $^{**}P \leq 0.01$. Data is presented as mean ± s.d.


a
HDD
LDD
Relative abundance (%)
Bacteroides
Lachnospiraceae NK4A136
Parasutterella
Roseburia
f_Peptococcaceae
Alloprevotella
b
Relative abundance (%)
Lachnospiraceae NK4A136
Parasutterella
f_Peptococcaceae
c
Relative abundance (%)
Lactobacillus
Rikenella
Enterorhabdus
Parvibacter
f_Ruminococcaceae
Odoribacter
Desulfovibrio
f_Peptostreptococcaceae
Alistipes
YRC22
f_Clostridiales vadinBB60
f_Lachnospiraceae
d
Relative abundance (%)
Lactobacillus
Rikenella
Enterorhabdus
Parvibacter
f_Ruminococcaceae
Bilophila
e
HDD
LDD
LDA SCORE (log 10)
f
HDD
LDD
LDA SCORE (log 10)

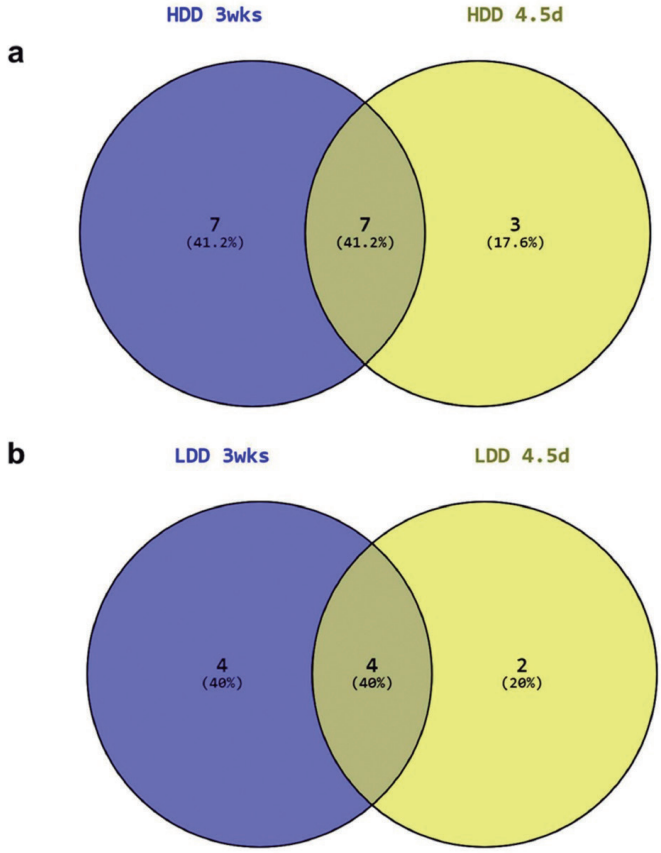
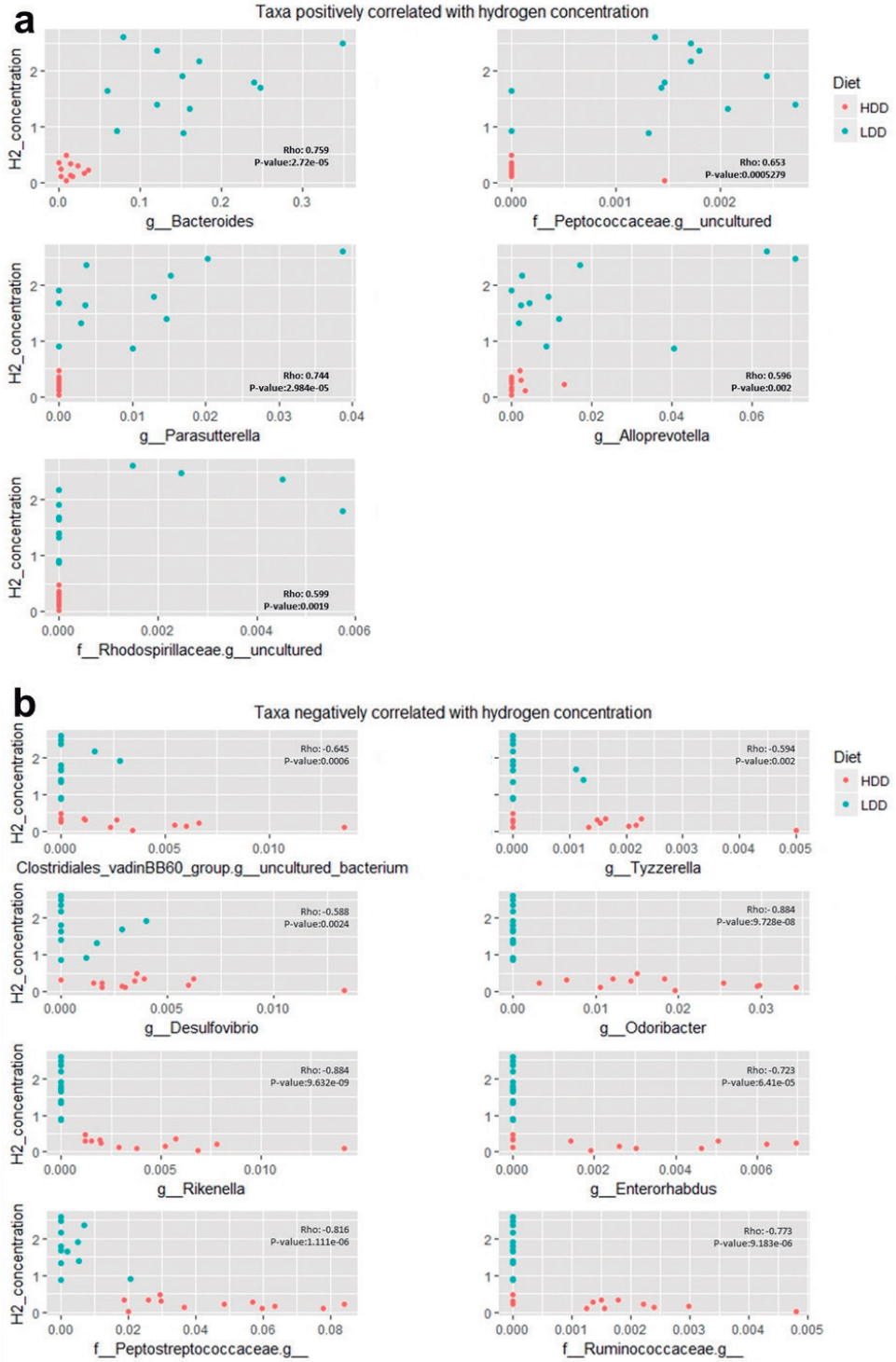


Figure S5. Venn diagrams showing the unique and shared genera which are significantly enriched as determined by the LefSe analysis between mice (**a**) exposed to HDD and (**b**) to LDD.

Figure S6 (next page). Specific bacterial genera correlating with *in vivo* H_2 production. (**a**) Positive and (**b**) negative Spearman's rank correlations of specific faecal bacterial genera and H_2 production of mice exposed to HDD or LDD for 3 weeks after weaning (n=12 per diet, Study 1); FDR threshold set to $P < 0.1$.


a
Taxa positively correlated with hydrogen concentration
H2_concentration
Rho: 0.759
P-value:2.72e-05
g__Bacteroides
Rho: 0.653
P-value:0.0005279
f__Peptococcaceae.g__uncultured
Rho: 0.744
P-value:2.984e-05
g__Parasutterella
Rho: 0.596
P-value:0.002
g__Alloprevotella
Rho: 0.599
P-value:0.0019
f__Rhodospirillaceae.g__uncultured
Diet
HDD
LDD
b
Taxa negatively correlated with hydrogen concentration
Rho: -0.645
P-value:0.0006
Clostridiales_vadinBB60_group.g__uncultured_bacterium
Rho: -0.594
P-value:0.002
g__Tyzzerella
Rho: -0.588
P-value:0.0024
g__Desulfovibrio
Rho: -0.884
P-value:9.728e-08
g__Odoribacter
Rho: -0.884
P-value:9.632e-09
g__Rikenella
Rho: -0.723
P-value:6.41e-05
g__Enterorhabdus
Rho: -0.816
P-value:1.111e-06
f__Peptostreptococcaceae.g__
Rho: -0.773
P-value:9.183e-06
f__Ruminococcaceae.g__

Chapter 3

Extended Indirect Calorimetry with Isotopic CO_2 Sensors for Prolonged and Continuous Quantification of Exogenous *vs.* Total Substrate Oxidation in Mice

José M. S. Fernández-Calleja

Lianne M. S. Bouwman

Hans J. M. Swarts

Annemarie Oosting

Jaap Keijer

Evert M. van Schothorst

Scientific Reports (2019) 9:11507 DOI: 10.1038/s41598-019-47977-w.

Abstract

Indirect calorimetry (InCa) estimates whole-body energy expenditure and total substrate oxidation based on O_2 consumption and CO_2 production, but does not allow to quantify oxidation of exogenous substrates with time. To achieve this, we here incorporated $^{13}CO_2$ and $^{12}CO_2$ gas sensors into a commercial InCa system and aimed to demonstrate their performance and added value. As performance indicator, we showed that discriminative oscillations in $^{13}CO_2$ enrichment associated with food intake in mice fed diets containing naturally low (wheat) *vs* high (maize) ^{13}C enrichment were measurable. To demonstrate the physiological added value, we quantified exogenous *vs* total carbohydrate and fat oxidation continuously, in real time in mice varying in fat mass. Diet-induced obese mice were fed a single liquid mixed meal containing ^{13}C-isotopic tracers of glucose or palmitate. Kinetics of immediate glucose and palmitate oxidation differed. Over 13 h, ~70% glucose and ~48% palmitate ingested were oxidised. Exogenous palmitate oxidation depended on body fat mass, while this was not the case for exogenous glucose oxidation. We conclude that extending an InCa system with $^{13}CO_2$ and $^{12}CO_2$ sensors provides an accessible and powerful technique for real-time continuous quantification of exogenous and whole-body substrate oxidation in mouse models of human metabolic physiology.

Keywords: Isotope labelling, C57BL mice, metabolic flexibility, obesity, metabolism, substrate oxidation.

Introduction

Indirect calorimetry (InCa) has been essential to understand human whole-body energy metabolism and fuel selection for more than a century[1]. It allows for the estimation of energy expenditure (EE) and of substrate utilisation by calculation of the respiratory exchange ratio (RER) from O_2 consumption and CO_2 production measurements. In the mouse, a commonly used model animal for human physiology, indirect calorimetry is the preferred technique to measure energy expenditure continuously[2]. However, estimations of RER represent only the whole-body net balance of substrates oxidised and do not distinguish between endogenous and exogenous (dietary) metabolic substrates. This knowledge can be obtained with the use of metabolic tracers, which are compounds that behave identically to the compound of interest, but can be analytically distinguished from them, for instance by mass difference.

The stable natural isotope ^{13}C has been widely used in metabolic studies for the past five decades[3]. Next to ^{12}C, ^{13}C is the second most abundant isotope of elemental carbon, representing about 1% of total terrestrial carbon[4]. Based on their distinct CO_2 fixation mechanisms, plants classified as C4 (*e.g.* maize or corn, and sugar cane) have a naturally higher ^{13}C abundance compared to C3 plants (*e.g.* wheat and sugar beet), and ingredients derived from C4 plants are often used as natural metabolic tracers[5].

Upon oxidation in the body, ^{13}C atoms in metabolic substrates are excreted as $^{13}CO_2$ which can be measured in breath samples and is usually given as atom % $^{13}CO_2$ enrichment[3]. While this provides a qualitative measurement of exogenous substrate oxidation, calculating actual substrate oxidation rates gives better insights into metabolic physiology and allows direct comparison between exogenous and endogenous substrates. Such quantitative measurements require knowledge of CO_2 production rates, and this has been regularly determined in human studies by InCa[3]. Only recently, livestock animal nutritionists and comparative biologists have also started to combine $^{13}CO_2$ enrichment analysis with InCa to calculate substrate oxidation rates[5-8]. Although informative, metabolic tracer research in animal models, like the mouse, rarely includes measurements of CO_2 production rates and the number of breath samples that can be collected and analysed for $^{13}CO_2$ enrichment is limited[9-13]. Continuous quantification of total CO_2 production together with $^{13}CO_2$ enrichment would allow precise measurement of responses to metabolic tracers in models of human physiology. To our knowledge, there have been only two attempts to obtain quantitative measurements of substrate oxidation in mice using $^{13}CO_2$ enrichment data in tandem with InCa over prolonged study times, and they involved expensive equipment[14] or labour-intensive sampling[15].

We have recently shown that a commercial InCa system can be successfully extended to incorporate analysis of other gases[16], in this case measurement of the gut microbiota fermentation gases. The inclusion of hydrogen (H_2) and methane (CH_4) sensors resulted in a system that offers a tool for more detailed phenotyping than conventional InCa for nutritional interventions in mice. Here, we have also incorporated $^{13}CO_2$ and $^{12}CO_2$ sensors into the same commercial InCa system and demonstrate their usefulness technically and

physiologically. The system was able to characterise changes in natural $^{13}CO_2$ enrichment based on 24 h feeding cycles and the consumption of diets with distinct ^{13}C signatures. Moreover, real-time $^{13}CO_2$ enrichment measurements linked to conventional InCa added value to current established metabolic phenotyping methodologies such as refeeding challenge tests, by not only quantifying exogenous and endogenous oxidation rates, but also quantifying the oxidative disposal of glucose and fat ingested with a meal in the context of diet-induced obesity.

Materials and Methods

Integration of $^{13}CO_2$ and $^{12}CO_2$ sensors into the indirect calorimetry (InCa) system

An Infrared Analyser Module URAS26 for separate analysis of $^{13}CO_2$ and $^{12}CO_2$ by nondispersive infrared absorption (ABB Automation, Frankfurt am Main, Germany) was incorporated into a 12-cage PhenoMaster InCa system (TSE Systems, Bad Homburg, Germany) in a closed circuit in series, upstream of the standard Siemens High-Speed Sensor Unit containing the standard O_2 and total CO_2 analysers (Fig. 1a). The integration of a methane (CH_4; ABB Automation) and a hydrogen (H_2; Honeywell Analytics, Hegnau, Switzerland) analyser into our InCa system has been reported previously[16]. The $^{12}CO_2$ sensor has a range of 0-6000 ppm which is appropriate in relation to total CO_2 exchange in mice, since total CO_2 ambient levels normally lie around 440 ppm and can raise up to about 5000 ppm for single-housed adult mice (based on previous observations in our laboratory). The $^{13}CO_2$ sensor has a measuring range of 0-150 ppm suitable to measure natural $^{13}CO_2$ concentrations, which are estimated to be 5.5 ppm in ambient air and 55 ppm in mouse cages. Calibration of the equipment was done routinely with three gas mixtures (Linde Gas Benelux, Dieren, The Netherlands): *zero* (20.947% O_2, in N_2, no other constituents), *span 1* (98.8 ppm H_2, in synthetic air), and *span 2* (0.521% total CO_2, 450 ppm CH_4, in N_2). The zero calibration point was performed by flushing the *zero* gas mixture through the system for 10 min and assigning ADC signals their corresponding gas concentration values. The same procedure was repeated for the span calibration points using gas mixtures *span 1* and *span 2*. The $^{12}CO_2$ and $^{13}CO_2$ span calibration points were set to 5153 and 57 ppm, respectively, based on the natural enrichment of atmospheric CO_2 (1.1 atom%)[3]. Cross-sensitivity between the $^{12}CO_2$ and $^{13}CO_2$ analysers is negligible. This calibration routine was performed before each experiment, with each experiment lasting for no more than a week, according to the stability of the $^{12}CO_2$ and $^{13}CO_2$ zero and span points of < 1% drift per week reported by the manufacturer. Raw data was acquired with a customised version of PhenoMaster software v.5.8.0 (TSE Systems), including $^{12}CO_2$ and $^{13}CO_2$ concentrations in ppm. Delta ppm values were obtained by subtracting reference cage values from mouse cage values at each time point, and these values were used for further calculations. Other operational settings and procedures have been described previously[17]. The overall performance of the newly extended system was first tested by measuring all gas concentrations over 5 d using empty cages.

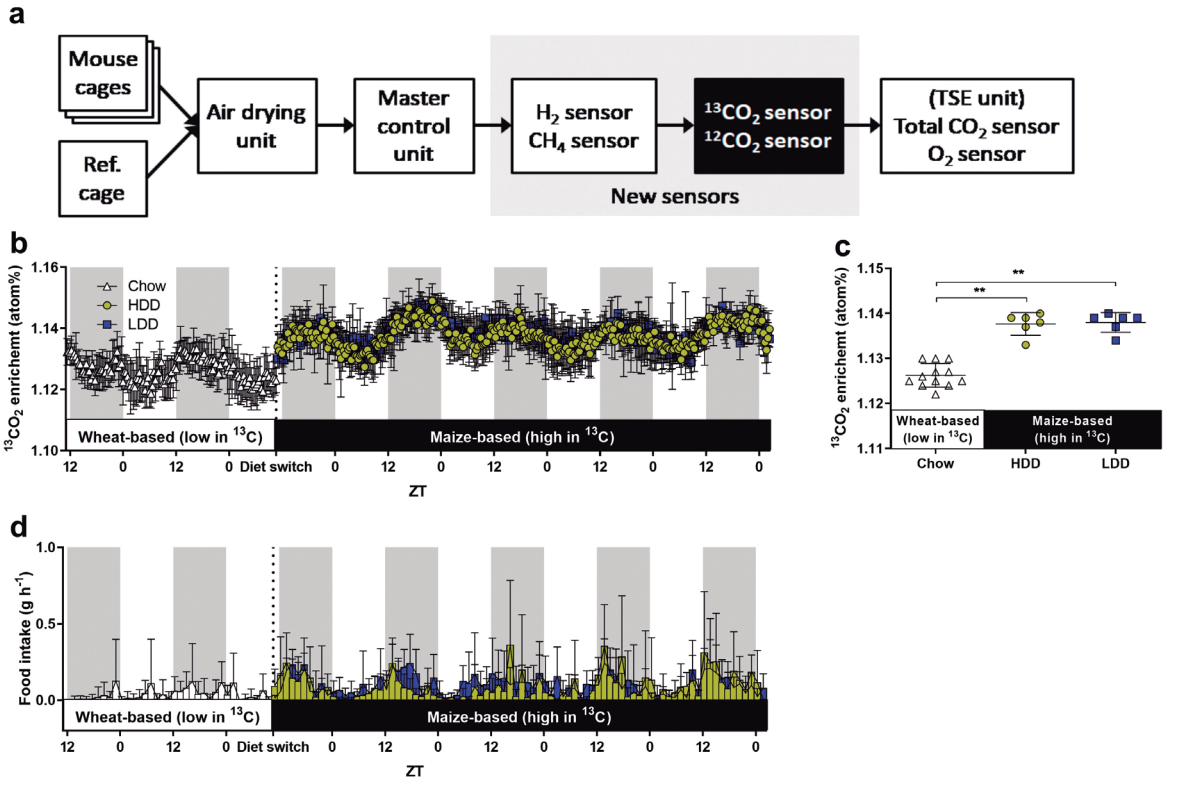


Figure 1. Extended InCa system with $^{13}CO_2$ and $^{12}CO_2$ gas analysers and technical validation with mice (first mouse study). (**a**) Simplified configuration of the system showing elements connected in parallel (12 mouse cages and one reference cage) and in in series (six gas sensors). Arrows indicate the direction of the airflow. (**b-d**) Female mice raised on wheat-based chow (n = 12) were acclimatised to the InCa system and then switched to one of two diets containing 57% w/w maize starch (HDD and LDD, both containing the same proportion of starch; n = 6 per diet). (**b**) $^{13}CO_2$ enrichment calculated from $^{13}CO_2$ and $^{12}CO_2$ concentrations were measured continuously for 6.5 d. (**c**) Mean $^{13}CO_2$ enrichment over the complete periods on wheat-based chow or maize-based diets. (**d**) Food intake was measured before and during HDD and LDD feeding. Shaded areas represent the dark phase. $^{**}P \leq 0.01$. Data is presented as mean ± SD. InCa, indirect calorimetry; Ref., reference cage; ZT, Zeitgeber time.

Composition of experimental diets

The highly digestible-starch diet (HDD) and the lowly digestible-starch diet (LDD) contained 20, 55, and 25 energy% protein, carbohydrate, and fat, respectively, and fulfilled the nutritional requirements for rodents according to AIN-93[18]. The starches in HDD and LDD (569 g kg^{-1} diet; Cargill, Sas van Gent, The Netherlands) were incorporated by Research Diet Services (Wijk bij Duurstede, The Netherlands) for the preparation of the pelleted diets. The high fat diet (HFD) contained 20, 40, and 40 energy% protein, carbohydrate, and fat, respectively. The exact composition of the experimental diets has been described in more detail elsewhere[19].

Elemental analysis isotope ratio mass spectrometry (EA-IRMS)

The ^{13}C enrichments of the chow diet and the HDD and LDD were measured by EA-IRMS as published[20]. Briefly, pulverized samples were combusted at 1020 °C in the presence of oxygen to convert carbon into CO_2, followed by separation for measurement of the $^{13}C/^{12}C$ ratio by EA-IRMS.

Mouse experiments

The experiments were approved by the Animal Experiment Committee of Wageningen University DEC2014085 and CCD/IvD 2017.w-0024.003, and performed in accordance with the European Union (EU) directives 86/609/EEC and 2010/63/EU, respectively. All mice (C57BL/6JRccHsd, Envigo, Horst, The Netherlands) were individually housed in Makrolon II cages with wood chips and enriched with wood shavings, at 23 ± 1 °C, 50 ± 5% humidity, on a 12 h light/dark cycle. Unless otherwise indicated, mice had *ad libitum* access to food and water.

Two mouse studies were conducted. The first study aimed to validate the newly incorporated $^{13}CO_2$ and $^{12}CO_2$ sensors using mice fed diets of variable natural ^{13}C enrichment. Ten-month old female mice (n = 12) raised on a chow diet (AM-II, AB Diets, Woerden, The Netherlands), with wheat as main ingredient and no declared content of C4 plant ingredients (1.078 atom% ^{13}C, EA-IRMS, see above), were weighed and acclimatised to the InCa environment for 48 h. Mice were then switched to one of two maize-based semi-purified diets with 57% w/w starch: HDD (1.085 atom% ^{13}C, EA-IRMS, see above) or LDD (1.085 atom% ^{13}C, EA-IRMS). The allocation of HDD and LDD was randomised and known to the experimenter, and the body weight (BW) of these groups was similar. Air measurements continued for another 4.5 d. Bedding volume was limited to approximately 200 ml during InCa measurements to facilitate detection of voluntary locomotion by infrared beam breaks in the horizontal plane. All gas concentrations were measured continuously. Other data obtained from the animals in this experiment (H_2 production and gut microbiota composition) has been reported previously[16].

The second study aimed at quantifying oxidation of ^{13}C-labelled exogenous metabolic substrates in diet-induced obesity. Mice on a chow diet (Teklad Global Diet 2920, Envigo) were time-mated and their offspring cross-fostered within 24-48 h after birth. Female

offspring (*n* = 48) were weaned at the end of postnatal week (PW) 3, stratified according to BW, and assigned to either HDD or LDD for 3 weeks; the experimenter was not blinded to these dietary treatments. Mice were then switched to a wheat-based HFD in PW 7 and continued on this diet until PW 15. Mice originally on HDD and LDD were initially treated as two experimental groups and, per group, were re-stratified by BW in PW 13 (prior to InCa and refeeding challenges in PW 14-15, see below) and again at the end of PW 15 before sacrifice in the fasted or postprandial condition (see below), to ensure the distribution of BW was similar across subgroups receiving the two differently labelled liquid mixed meals (*n* = 24 for either ^{13}C glucose or ^{13}C palmitate) and sacrificed in the two metabolic states (*n* = 24 fasted or postprandial). BW and food intake (FI) were determined weekly. Body composition (BC; EchoMRI 100V, EchoMedical Systems, Houston, Texas, USA) was determined weekly (PW 4-6) or biweekly (PW 7-15), and directly before and after InCa runs. The RER response to refeeding with a liquid mixed meal, circulating fasting and postprandial glucose and insulin levels, and 24 h EE (Table S1 and Fig. S1) were not different between the mice originally on HDD or LDD. These metabolically very similar mice were then pooled into a group of in total 48 mice with a widely different fat mass (FM), and this combined group was used to investigate the metabolic response to the exogenous substrates of the liquid mixed meals and its correlation with FM.

InCa and refeeding challenge tests with liquid mixed meals

Individually housed mice (in batches of 12 per InCa run) were acclimatised to the InCa environment for approximately 24 h. The following 24 h period was used for measurements of daily EE, RER, locomotor activity, and food and water intake. Sampling frequency for these basal gas measurements was every 20 min. On the third day, 6 mice per batch, to facilitate a higher gas sampling frequency, were restricted to 1.1 g of food (HFD) 1 h before the dark phase (DP; ZT = 11). The remaining 6 mice kept *ad libitum* access to food and water inside the InCa system. Twenty-four hours later (ZT = 11), the 6 cages with mice receiving restricted food were continuously measured at sample interval of 11 min, and these mice received a ^{13}C-labelled liquid mixed meal by oral gavage (0.4 ml per mouse, see below). The mice were continuously monitored in the extended InCa system, including $^{13}CO_2$ and $^{12}CO_2$ measurements, until the onset of the following light phase (LP; ZT = 0) for a total of 13 h upon ingestion of the liquid mixed meal, after which they regained *ad libitum* access to the HFD. On the fourth day, the same procedure (*i.e.* measurement following food restriction and a subsequent ^{13}C-labelled liquid mixed meal) was repeated with the remaining 6 mice per batch in the InCa system. All of the mice received the same dose of liquid mixed meal. The meal contained a mixture of glucose and fat, representing 16.3 ± 1.1% (mean ± SD) of daily energy expenditure, and contained either a ^{13}C glucose or a ^{13}C palmitate tracer (Table 1), allowing determination of glucose or palmitic acid oxidation specifically.

Table 1. Composition of the liquid mixed meals.

	^{13}C Glucose	^{13}C Palmitate
Unlabelled glucose (mg)	1358.4 ± 0.7	1401.3 ± 1.1
U-^{13}C Glucose (mg)	43.1 ± 0.4	0
Palm olein (mg)	977.5 ± 2.3	974.3 ± 2.0
Unlabelled sodium palmitate (mg)	23.9 ± 0.2	0
U-^{13}C Potassium palmitate (mg)	0	26.7 ± 0.2
Soy lecithin (mg)	8.1 ± 0.9	7.9 ± 0.6
Water (mg)	1501.7 ± 2.8	1501.0 ± 1.1
Density (g ml^{-1})	1.12 ± 0.01	1.11 ± 0.02
^{13}C content (μmol)[1]	157.2 ± 2.8	155.1 ± 2.5
Energy (kJ)[1]	7.19 ± 0.09	7.16 ± 0.12
Carbohydrate (energy%)	36.0 ± 0.1	36.0 ± 0.1
Fat (energy%)	64.0 ± 0.1	63.9 ± 0.1

[1]Amount per dose of 0.4 ml of liquid mixed meal given by oral gavage. Data is presented as mean ± SD.

Design and preparation of liquid mixed meals

The liquid mixed meals were based on a recently developed drink used to measure the metabolic response to refeeding and health in humans[21]. However, as the focus was exclusively on exogenous glucose and fat oxidation, and therefore included corresponding metabolic tracers (^{13}C glucose or ^{13}C palmitate), we omitted protein from the formulation. Palmitate was chosen as a fat tracer instead of labelled triglycerides or a mixture of fatty acids to circumvent possible fatty-acid-specific differences in absorption and oxidation. Sodium palmitate (Sigma-Aldrich, Missouri, State, USA) or D-glucose (Merck, Darmstadt, Germany) were partly replaced by either uniformly (U) ^{13}C-labelled potassium palmitate (98.8 atom%, 98% chemical purity; IsoLife, Wageningen, The Netherlands) or U-^{13}C D-glucose (99 atom%, 98.8% chemical purity; IsoLife), respectively, and mixed with soy lecithin (Emultop IP, Cargill, Hamburg, Germany) and ultrapure water. This mixture was then vortexed and microwaved until no visible lumps remained. Palm olein (Remia, Den Dolder, The Netherlands) was added and the aqueous and oily phases were integrated by vortexing and sonication until a homogenous emulsion was obtained. Fresh preparations were made 2 h before administration to the animals and remained stable. The overall composition of the labelled glucose and labelled palmitate liquid mixed meals is shown in Table 1.

Sacrifice in the fasted or postprandial state

Mice were food restricted (1.1 g HFD) for 16 h, starting at ZT = 11. Half of the mice was sacrificed in the post-absorptive state, and the other half was administered the liquid mixed meal with ^{13}C glucose by oral gavage and sacrificed after 45 min (postprandial state) by decapitation. Trunk blood was collected in MiniCollect serum tubes (Greiner Bio-One, Alphen aan de Rijn, The Netherlands). Serum was separated by centrifugation at 4 °C

for 10 min at 3000 × *g*, aliquoted, and stored at −80 °C. Glucose was measured in whole blood with a Freestyle glucose meter (Abbott Diabetes Care, Hoofddorp, The Netherlands) directly after sacrifice.

Serum insulin measurements

Fasting and postprandial serum insulin concentrations from animals sacrificed in PW 15 were determined with an Ultra-Sensitive Mouse Insulin ELISA Kit (ChrystalChem, Elk Grove Village, Illinois, USA) following the manufacturer's instructions. Samples were measured in duplicate.

Calculation of refeeding response (ΔRER), and total and exogenous substrate oxidation

The metabolic refeeding response (ΔRER) was determined per individual animal as the change in RER from the fasting post-absorptive state (baseline over 1 h) to postprandial 44 min after administering the liquid mixed meal, based on the median time when all mice achieved a postprandial RER peak. Total levels of glucose and fatty acid oxidation were calculated from VO_2 and VCO_2 obtained with the Siemens High-Speed Sensor Unit, using Péronnet & Massicotte's table of non-protein RER[22] and Weir's equation of EE[23], as follows. Individual values for glucose and fatty acid oxidation (as % of EE) were interpolated from the original table of Péronnet & Massicotte, ranging from RER 0.7036 to 0.996. These interpolated values of glucose or fatty acid utilisation (% of EE) and the EE data [also obtained from the TSE system, based on Weir's equation: EE = (3.941 × VO_2) + (1.106 × VCO_2); kJ min^{-1}], together with the energy equivalents of glucose and fatty acids (16.18 kJ g^{-1} and 40.76 kJ g^{-1}, respectively[22]), were then used to calculate the rates of total glucose oxidation (TGO) and total fatty acid oxidation (TFO) in mg min^{-1}, according to the following equations:

$$TGO = \frac{EE \times \%EE_{GO}}{16.18} \quad (1)$$

$$TFO = \frac{EE \times \%EE_{FO}}{40.76} \quad (2)$$

Rates of exogenous substrate oxidation (ESO; *i.e.* oxidation of ingested glucose and palmitate in the liquid mixed meal based on the ^{13}C tracers) were calculated using the following two equations:

$$at\%^{13}CO_2 = \frac{^{13}CO_2}{^{13}CO_2 + ^{12}CO_2} \times 100 \quad (3)$$

$$ESO(mg \times min^{-1}) = \frac{at\%^{13}CO_{2(t)} - at\%^{13}CO_{2(t0)}}{at\%^{13}C_S - at\%^{13}CO_{2(t0)}} \times \frac{VCO_{2(t)} \times MW_{tracee}}{22.2966 \times C_{tracer}} \quad (4)$$

In equations (3) and (4), at%$^{13}CO_2$ is the ^{13}C enrichment in expired CO_2 in atom% calculated from gas concentrations (delta ppm). In equation (4), time t_0 represents the baseline measurement over 1 h before administration of the liquid mixed meals and t represents any subsequent time point. The calculated ^{13}C enrichment of the whole

substrate pool ingested (either unlabelled glucose plus ^{13}C glucose, or unlabelled palmitate plus ^{13}C palmitate) is represented by at%$^{13}C_s$, assuming a natural terrestrial ^{13}C enrichment of 1 atom% (ref. 4), 100% chemical purity, and following the fatty acid composition of palm olein from literature[24]. VCO_2 is the production rate of CO_2 obtained using the summed concentrations of $^{13}CO_2$ and $^{12}CO_2$ measured by the URAS26 module multiplied by the air flow (constant). The molecular weight of the tracee (MW_{tracee}, glucose or palmitic acid) and the volume occupied by 1 mol of CO_2 in STPD (22.2966 l) are based on Péronnet & Massicotte[22]. The number of labelled carbons per mol of tracer (C_{tracer}) is 6 for U-^{13}C glucose and 16 for U-^{13}C palmitate. Of note, exogenous fat oxidation (EFO) represents only the oxidation of palmitic acid ingested (both labelled and unlabelled), thus oxidation of other fatty acids in the liquid mixed meal (mainly oleic acid and linoleic acid) is not accounted for.

Additionally, a dietary acetate recovery factor (dARF) was implemented in our calculations giving rise to an alternative ESO calculation, equation (5). The dARF is a factor suggested to be used to correct for ^{13}C sequestration based on studies in normal weight and obese humans[25,26], but has not been validated in mice. In detail, the animal with the highest FM and the animal with the lowest FM were assigned the dARF of obese (0.453) and normal weight humans (0.506), respectively, and the dARF of the remaining animals was interpolated by linear regression and applied to equation (4):

$$ESO(mg \times min^{-1}) = \frac{at\%^{13}CO_{2(t)} - at\%^{13}CO_{2(t0)}}{at\%^{13}C_S - at\%^{13}CO_{2(t0)}} \times \frac{VCO_{2(t)} \times MW_{tracee}}{22.2966 \times C_{tracer} \times dARF} \quad (5)$$

Statistical analysis

Each individual mouse was considered an experimental unit. Normal distribution of the data was tested with the D'Agostino and Pearson omnibus test; non-normally distributed data were log-transformed and retested for normality. The difference in natural ^{13}C enrichment on wheat- *vs* maize-based diets was analysed with the Kruskal-Wallis test with Dunn's multiple comparison test. Correlations were performed by Pearson correlation (normally-distributed data) or Spearman correlation (non-normally distributed data). Technical errors occurred in oral gavage in two animals, therefore this data was not included for analyses of postprandial metabolic outcomes. All statistical analyses and data visualisation were performed in Prism v.5.04 (GraphPad, San Diego, California, USA). Data is presented as mean ± SD (normally distributed data) or median and range (non-normally distributed data), and statistical significance was set at $P < 0.05$.

Results

Integration of $^{13}CO_2$ and $^{12}CO_2$ sensors into the InCa system and analysis of natural dietary ^{13}C enrichment

We first evaluated the overall performance of the newly integrated $^{12}CO_2$ and $^{13}CO_2$ sensors into the InCa system (Fig. 1a) with a 5 d test with empty cages to measure diurnal fluctuations in ambient air. Median ambient levels during this test were 5.30 ppm $^{13}CO_2$ (4.42, 6.08; range) and 462.8 ppm $^{12}CO_2$ (449.7, 535.2; range), respectively. This corresponded to a median of 1.130 atom% $^{13}CO_2$ (0.960, 1.159; range).

After these initial tests, female mice raised on wheat-based chow (a C3 plant, thus low in ^{13}C) were placed into the InCa system and $^{13}CO_2$, $^{12}CO_2$, and other gas concentrations were recorded continuously for 48 h (Fig. 1b). Once animals were acclimatised, the wheat-based chow was exchanged for one of two maize-based semi-purified diets, a highly digestible-starch diet (HDD) and a lowly digestible-starch diet (LDD), each containing the same amount of maize starch (a C4 plant, thus naturally high in ^{13}C). Oscillations in $^{13}CO_2$ enrichment followed a circadian pattern, reaching the lowest levels during the light phase (LP) and highest levels during the dark phase (DP; Fig. 1b); this pattern was similar to the food intake (FI) pattern (Fig. 1d). Upon the switch to a maize-based diet, overall $^{13}CO_2$ enrichment levels were significantly higher ($P = 0.0002$), independent of type of maize starch used, with a mean difference of 0.011 and 0.012 atom% for HDD and LDD, respectively, compared to chow (Fig. 1c). The ^{13}C enrichment of the diets measured by elemental analysis isotope ratio mass spectrometry (EA-IRMS) was 1.078 atom% for chow and 1.085 atom% for both HDD and LDD, thus a natural enrichment difference of 0.008 atom%, which is close to the observed $^{13}CO_2$ differences in the expired air. Additionally, analysis of a separate group of mice that remained on chow instead of switching to HDD or LDD confirmed that the increase in mean $^{13}CO_2$ enrichment was driven mainly by dietary ^{13}C enrichment and less so by increased FI (Fig. S2). Finally, to assess the functionality of the newly incorporated sensors as part of conventional respirometry, we recalculated EE by substituting total VCO_2 values obtained with a single sensor with the sum of $V^{13}CO_2$ and $V^{12}CO_2$ values obtained from two sensors. Recalculated and original 24 h EE values were almost identical (41.03 ± 0.78 *vs* 41.03 ± 0.77 kJ d^{-1}, respectively, Student's *t*-test $P = 0.994$; linear regression, $Y = 0.9993X + 0.0002$; $r^2 = 0.999$).

Refeeding metabolic response in diet-induced obesity

After 9 weeks on a high-fat-diet (HFD), mice that were previously fed LDD *vs* HDD for 3 weeks had a slightly higher fat mass (FM) and gained more FM over the period on HFD, however, BW was not significantly different between the groups (Table S1). The total group of 48 mice on HFD had a FM ranging from 5.52 g up to 15.88 g (288%; Table 2) and was metabolically fairly homogeneous, regardless of prior LDD or HDD feeding (Table S1 and Fig. S1). The metabolic response to refeeding was tested using ^{13}C-labelled liquid mixed meals, and the data derived from the $^{13}CO_2$ and $^{12}CO_2$ sensors allowed us to detect exogenous tracer (^{13}C) as a marker for the total specific substrate, simultaneously with overall substrate oxidation based on O_2 consumption and total CO_2 production. Baseline

RER values before administration of the liquid mixed meal were 0.70 ± 0.01 (mean ± SD; Fig. 2). The median peak RER (glucose oxidation) was achieved after 44 min (22, 88; range) upon feeding, and values declined thereafter until full fat oxidation[22] was reached again after approximately 140 min. The refeeding RER response, defined as the increase from baseline RER to RER at 44 min (ΔRER, Table 2), was negatively correlated to FM (measured directly prior to InCa measurements; Fig. 2c). In line, also BW (Spearman r = –0.386, P = 0.0074; n = 47) and LM (r^2 = 0.245, P = 0.0004; n = 47) were negatively correlated to ΔRER. Serum insulin levels 45 min post-prandially were also negatively correlated to ΔRER in a subgroup of animals that were fasted and challenged again with the liquid mixed meal before sacrifice (r^2 = 0.421, P = 0.0011; n = 22).

Table 2. Metabolic characteristics of the mice after HFD-feeding for nine weeks (second mouse study).

Parameter	Outcome
BW (g)[1]	31.57 (24.82, 38.22)
FM (g)[1]	10.21 (5.52, 15.88)
LM (g)[1]	19.89 (17.68, 21.64)
FM gain (g)[2]	8.66 (4.41, 14.06)
FI (g)[2]	188.38 (161.67, 226.33)
24 h EE (kJ d^{-1})[3]	44.24 (38.66, 51.45)
24 h RER[3]	0.81 (0.74, 0.89)
ΔRER ($RER_{44} - RER_0$)[3]	0.16 (0.10, 0.20)
Fasting glucose (mmol l^{-1})[1]	6.0 (4.9, 7.2)
Fasting insulin (ng ml^{-1})[1]	2.65 (0.85, 8.95)
Postprandial glucose (mmol l^{-1})[1]	6.9 (3.3, 9.1)
Postprandial insulin (ng ml^{-1})[1]	4.40 (1.47, 9.22)

[1]Measured in PW 15 (end of HFD). [2]Measured from PW 7 (start of HFD) to PW 15. [3]Measured in PW 14-15. HFD, high-fat diet; BW, body weight; FM, fat mass; LM, lean mass; FI, food intake; EE, energy expenditure; RER, respiratory exchange ratio (mean of 24 h). Data is presented as means and range, n = 48 (except ΔRER, n = 47; fasting glucose and insulin n = 24; and postprandial glucose and insulin, n = 23).

Oxidation of exogenous (tracer) glucose and fat

The liquid mixed meal was enriched with metabolic ^{13}C tracers for glucose or palmitate to investigate dietary fuel partitioning between storage and oxidation, continuously and in real time. Thus, all animals received a liquid mixed meal of identical macronutrient composition, but containing either ^{13}C glucose or ^{13}C palmitate, and exhaled $^{13}CO_2$ was measured to reflect the oxidation of tracer and total glucose and palmitate contained in

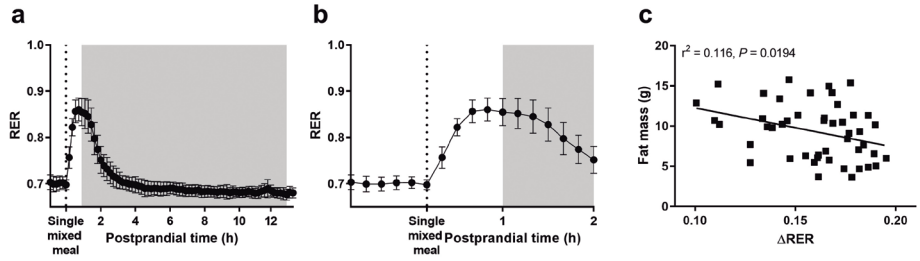


Figure 2. Refeeding metabolic response to a liquid mixed meal and its relation to body fat mass (FM) in mice fed a HFD for nine weeks (second mouse study). (**a**) Respiratory exchange ratio (RER) was measured in PW 14-15 after gavage of a single liquid mixed meal containing 36 energy% glucose and 64 energy% fat (n = 47). Data is obtained from conventional indirect calorimetry measurements (VO_2 and VCO_2). Data is presented as mean ± SD. Shaded areas represent the dark phase. (**b**) Data from panel (a), emphasizing the early postprandial 2 h period. (**c**) Refeeding metabolic response (ΔRER), measured as the increase from baseline RER to RER at 44 min postprandial, correlates negatively with FM. PW, postnatal week.

the meal. Concentrations of $^{13}CO_2$ and $^{12}CO_2$ were first computed as $^{13}CO_2$ enrichment to characterise exogenous fuel oxidation qualitatively (Fig. 3). The label in the ^{13}C glucose liquid mixed meal appeared in breath quickly in a single peak at 79 ± 12 min (mean ± SD), and $^{13}CO_2$ enrichment fell almost back to baseline by the end of the 13 h post-meal period (final enrichment 1.159 ± 0.012 atom%, mean ± SD; Fig. 3a). In contrast, the label in the ^{13}C palmitate liquid mixed meal appeared later and at a slower rate, generally peaking around 4 h (with individual animals showing multiple peaks), and continued to appear by the end of the 13 h period (final enrichment 1.242 ± 0.038 atom%, mean ± SD; Fig. 3c). The underlying concentrations of $^{13}CO_2$ and $^{12}CO_2$ used to calculate $^{13}CO_2$ enrichments are shown in Fig. 3b,d.

To quantify and dissect exogenous substrate oxidation rates, $^{13}CO_2$ enrichment data was combined with VCO_2 production rates and compared to total substrate oxidation, *i.e.* endogenous and exogenous, obtained from conventional InCa equations (Fig. 4). Since the substrate oxidation kinetics of HDD and LDD mice were almost identical (Fig. S1), the data was further analysed and plotted as one group. Exogenous glucose oxidation (EGO) and total glucose oxidation (TGO) rates followed in general similar kinetics (Fig. 4a,b). However, EGO achieved a later peak compared to TGO, with a median peak at 77 min (44, 132; range; n = 23) and 66 min (33, 99; range; n = 47), respectively (Mann Whitney, P = 0.0002; Fig. 4a,b). Maximal EGO and TGO were 0.934 ± 0.212 and 1.211 ± 0.213 mg min^{-1} (mean ± SD), respectively. Thereafter EGO and TGO started to decline until they became negligible, with EGO generally remaining slightly higher than TGO (Fig. 4a), likely due to underestimation of total glucose oxidation (see Discussion). In contrast to glucose oxidation, exogenous fat oxidation (EFO), accounting only for palmitic acid, and total fat oxidation (TFO) rates showed clearly different kinetics (Fig. 4c,d). Immediately after administration of the liquid mixed meal, EFO started to rise while TFO was abruptly suppressed (Fig. 4c,d). Later TFO returned back to baseline around 2 h and EFO peaked around 4 h postprandial (Fig. 4c,d).

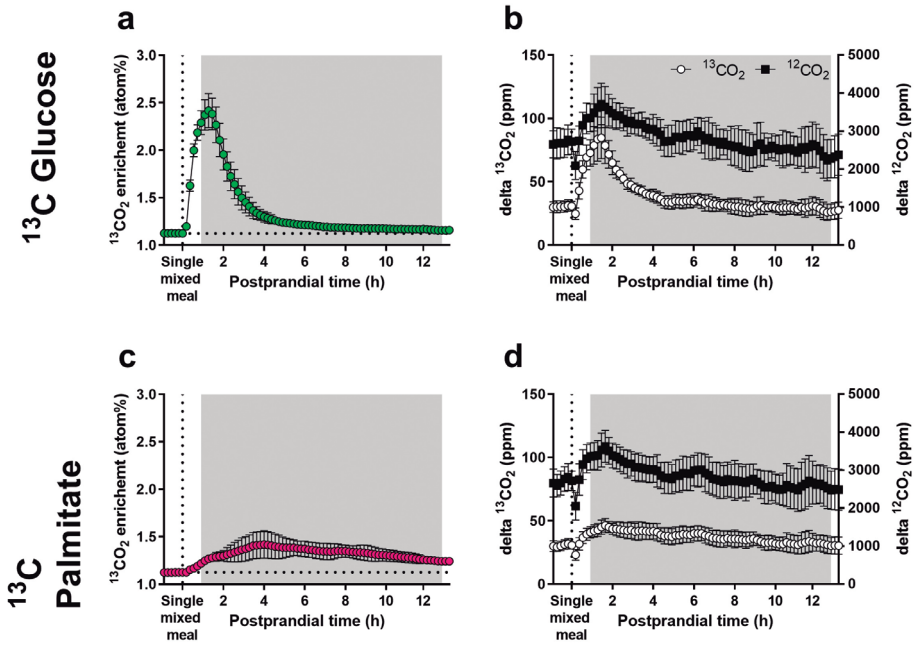


Figure 3. Kinetics of instantaneous $^{13}CO_2$ enrichment measured by extended InCa after gavage of a single liquid mixed meal containing ^{13}C-labelled tracers, in mice fed a HFD for nine weeks (second mouse study). (**a**) $^{13}CO_2$ enrichment after ingestion of the ^{13}C glucose liquid mixed meal followed for 13 h (n = 23), calculated from $^{13}CO_2$ and $^{12}CO_2$ concentrations. (**b**) $^{13}CO_2$ (left y-axis) and $^{12}CO_2$ (right y-axis) concentrations used to calculate $^{13}CO_2$ enrichments as shown in panel (**a**), expressed as the difference (delta) from gas concentrations measured in mouse-occupied cages minus gas concentrations in the reference cage (*i.e.* gas production). (**c**) $^{13}CO_2$ enrichment after ingestion of the ^{13}C palmitate liquid mixed meal (n = 24). (**d**) $^{13}CO_2$ (left y-axis) and $^{12}CO_2$ (right y-axis) concentrations used to calculate $^{13}CO_2$ enrichments as shown in panel (**c**). The horizontal dotted lines indicate baseline $^{13}CO_2$ enrichment. Shaded areas represent the dark phase. Data is presented as mean ± SD. PW, postnatal week.

Maximal EFO and TFO were 0.047 ± 0.013 and 0.936 ± 0.069 mg min^{-1} (mean ± SD), respectively. Both EFO and TFO tended to decrease towards the end of the measurements at 13 h but remained above the baseline (Fig. 4c).

Cumulatively, EGO was 111.7 mg (97.5, 122.8; median and range) and TGO 103.5 mg (68.5, 159; median and range) during the complete post-meal measurement period, from consumption of the liquid mixed meal 1 h before the DP and until the end of the DP (13 h in total). This amount of exogenous glucose oxidised corresponded to 69.6% (61.3, 76.6; median and range) of the total dose administered with the liquid mixed meal (Fig. 4e,f). In comparison, EFO was 16.4 mg (9.6, 18.7; median and range) and TFO 511.0 mg (404.2, 635.4; median and range) over the whole period. For exogenous palmitate oxidised, this amount corresponded to 47.6% (27.7, 52.6; median and range) of the oral dose (Fig. 4e,f). The percentage of dose oxidised was more variable for exogenous palmitate (CV = 13.4%) than for exogenous glucose (CV = 5.1%).

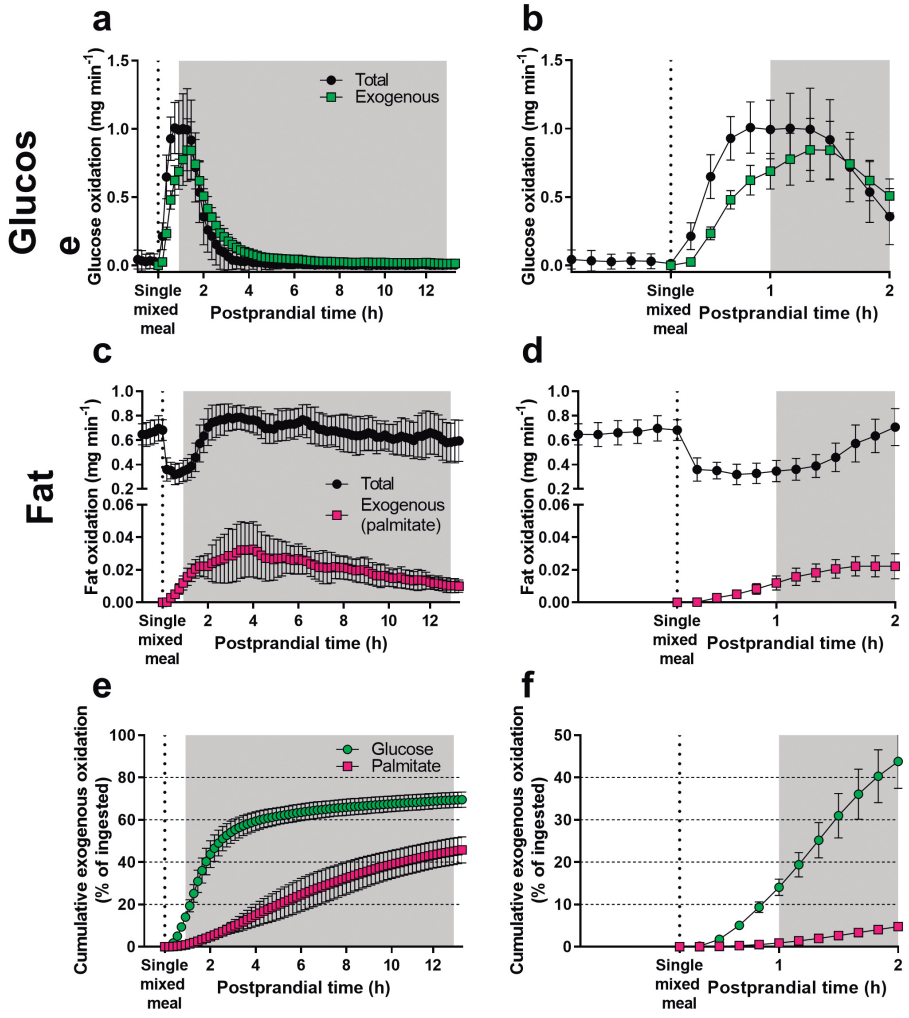


Figure 4. Kinetics of oxidation of total and exogenous metabolic substrates after gavage of a labelled liquid mixed meal, in mice fed a HFD for nine weeks (second mouse study). (**a**) Instantaneous total (n = 47) and exogenous (n = 23) glucose oxidation after administration of the ^{13}C glucose liquid mixed meal until the end of the measurements. (**b**) Data from panel (**a**), emphasizing the early 2 h postprandial period. (**c**) Instantaneous total fat oxidation (n = 47) and exogenous palmitate oxidation (n = 24) after administration of the ^{13}C palmitate liquid mixed meal. (**d**) Data from panel (**c**), emphasizing the early 2 h postprandial period. (**e**) Cumulative exogenous glucose (n = 23) and palmitate oxidation (n = 24) after administration of the labelled liquid mixed meals until the end of the measurements. (**f**) Data from panel (**e**), emphasizing the early 2 h postprandial period. Exogenous substrate oxidation data is calculated from conventional InCa data (VCO_2) and $^{13}CO_2$ and $^{12}CO_2$ data. Shaded areas represent the dark phase. Data is presented as mean ± SD. InCa, indirect calorimetry; PW, postnatal week.

Exogenous substrate oxidised in relation to body composition

We then attempted to explain the variation in exogenous fuel selection in diet-induced obese mice by correlating cumulative oxidation data with FM (determined directly before InCa). TFO was positively correlated to FM (0-2 h: $r^2 = 0.438$, $P < 0.0001$; 2-13 h: $r^2 = 0.467$, $P < 0.0001$; $n = 47$), while EGO, TGO, and EFO did not reach a statistically significant correlation, neither during the early postprandial period from 0-2 h, nor from 2-13 h after ingestion of the liquid mixed meal.

Although LM showed a narrow range of values (17.68, 21.64; Table 2), it correlated positively with FM ($r^2 = 0.349$, $P < 0.0001$; $n = 48$), and thus differences in total oxidative tissue may explain the correlations of substrate oxidation with FM. Therefore, oxidation data were also expressed relative to LM. Correlations of EGO with FM remained non-significant (Fig. 5a,b), and TGO correlated negatively with FM only during the early postprandial period (0-2 h: $r^2 = 0.101$, $P = 0.0296$; 2-13 h: $r^2 = 0.039$, $P = 0.1861$; $n = 47$). Positive correlations of TFO with FM (0-2 h: $r^2 = 0.355$, $P < 0.0001$; 2-13 h: $r^2 = 0.2838$, $P = 0.0001$; $n = 47$) remained significant. Moreover, after accounting for LM, EFO correlated negatively with FM (2-13 h: $r^2 = 0.165$, $P = 0.0488$; $n = 24$), only during the late postprandial and post-absorptive period (Fig. 5c,d).

Expressed relative to TFO, EFO was negatively correlated to FM (0-2 h: $r^2 = 0.262$, $P = 0.0105$; 2-13 h: $r^2 = 0.350$, $P = 0.0023$; $n = 24$; Fig. 5e,f). Additionally, EFO relative to TFO was positively correlated with ΔRER (0-2 h $r^2 = 0.225$, $P = 0.0192$; 2-13 h: $r^2 = 0.224$, $P = 0.0194$; $n = 24$; Fig. 5g,h), an unsuspected outcome since the refeeding response is generally qualified by a switch to glucose oxidation and concomitant suppression of fat oxidation.

In metabolic tracer studies with humans, particularly when using isotopically labelled fatty acids, it is important to account for label sequestration in the body bicarbonate pool and in exchange reactions in the TCA cycle, and this can be achieved by applying an acetate correction factor to the oxidation calculations[27]. When the labelled fatty acids are ingested, this factor is called the dietary acetate recovery factor (dARF) and its value depends on whole-body adiposity and fasting insulin levels[25]. To our knowledge, a dARF is yet to be determined for mice. Since ^{13}C label sequestration from palmitate could also depend on FM in mice and lead to potentially wrong interpretations of exogenous oxidation data, we tested the potential consequences of applying this factor. In view of a lack of dARF in mice, we applied the dARF known for normal-weight and obese humans (13.3, 31.3 kg FM; range) to individual mice, assuming a linear relationship between FM and the dARF. Including a dARF in the calculations of EFO led to much higher label recoveries, with a median of 99.0% (59.2, 116.0; range) exogenous palmitate oxidation of dose administered. After accounting for label sequestration in this way, EFO relative to LM was no longer significantly correlated to FM. However, EFO relative to TFO remained negatively correlated to FM (0-2 h: $r^2 = 0.188$, $P = 0.0345$; 2-13 h: $r^2 = 0.224$, $P = 0.0194$; $n = 24$), and also remained positively correlated to ΔRER (0-2 h: $r^2 = 0.211$, $P = 0.0241$; 2-13 h: $r^2 = 0.195$, $P = 0.0306$; $n = 24$).

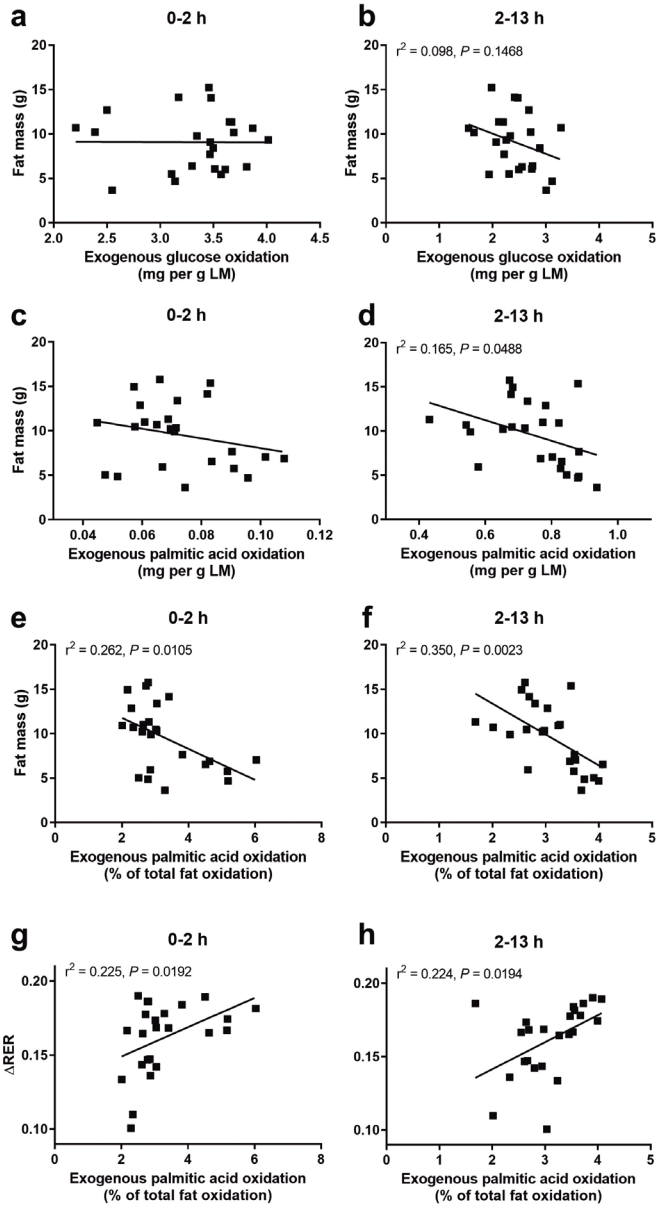


Figure 5. Correlation analysis between cumulative exogenous glucose or fat oxidation after gavage of a labelled liquid mixed meal and body fat mass and ΔRER, in mice fed a HFD for nine weeks (second mouse study). Exogenous glucose oxidation from 0-2 h (**a**) or 2-13 h postprandial (**b**) expressed per unit of LM is not significantly correlated to FM. Exogenous palmitic acid oxidation from 0-2 h postprandial expressed per unit of LM is not significantly correlated to FM (**c**), but it is negatively correlated to FM 2-13 h after ingestion of the liquid mixed meal (**d**). Exogenous palmitic acid oxidation expressed as a percentage of total fat oxidation correlates negatively with FM, both from 0-2 h postprandial (**e**) and from 2-13 h after ingestion of the liquid mixed meal (**f**). Exogenous palmitic acid oxidation relative to total fat oxidation correlates positively with ΔRER, both from 0-2 h postprandial (**g**) and from 2-13 h after ingestion of the liquid mixed meal (**h**). *n* = 24. PW, postnatal week.

Discussion

We have incorporated two new gas sensors, for $^{13}CO_2$ and $^{12}CO_2$, into a commercial indirect calorimetry (InCa) system for mice. Using this extended InCa system, we were able to detect long-term continuous changes in $^{13}CO_2$ enrichment based on the natural ^{13}C content of the diet (wheat-based *vs* maize-based diets) and to analyse and dissect the use of exogenous or endogenous body fuels (feeding *vs* fasting conditions). Furthermore, the extended system was particularly suitable for real-time and continuous quantification of exogenous substrate oxidation using ^{13}C-labelled ingredients. Combined with conventional InCa, providing mice a liquid mixed tracer meal revealed that with increased FM a blunted switch to glucose oxidation was accompanied with a decreased utilisation of exogenous dietary palmitate. Thus, extended InCa with $^{13}CO_2$ and $^{12}CO_2$ sensors provided a deeper understanding of metabolic phenotypes.

Quantifying the oxidative disposal of exogenous metabolic substrates has been used in many human metabolic studies[3]. However in mice, to the best of our knowledge, only one published study quantified oxidation of glucose and fat ingested in a meal using stable isotopic tracers[14]. Due to the choice of different tracers (^{13}C glucose replacing sucrose, and ^{13}C trioleate replacing soybean oil) and the lack of data on dose recovery percentages[14], it is not possible to compare our conclusions regarding quantification of oxidised substrates. Comparison with other species and protocols is not easy, given the diversity in metabolic rates, doses, tracers, study durations, and other experimental conditions. Nevertheless, our results are consistent with the general notion that ingested fats are less readily oxidised than carbohydrates[5], and with the observation that maximal oxidation rates of ingested palmitate were one order of magnitude smaller than for glucose, as was investigated in another rodent, *Phodopus sungorus*[8]. At the same time, the cumulative oxidation of ingested palmitate was highly variable compared to that of ingested glucose (CV = 13.4% *vs* 5.1% for palmitate and glucose, respectively). Together, these data reinforce the concept that glucose balance is more tightly controlled than fat balance, at least in the short-term[28,29]. Arguably, glucose oxidation is the most straightforward postprandial oxidation fuel strategy, as fat oxidation has been shown to depend on chain length and degree of saturation[30-33] and even physical structure[11,34].

Using $^{13}CO_2$ and $^{12}CO_2$ sensors in combination with InCa allows for a more refined interpretation of the capacity to adjust substrate oxidation to its availability, which is also referred to as metabolic flexibility[35]. We[17,19,36-38] and others[9,39] have used this concept as a strategy for metabolic phenotyping in mice by measuring the RER. However, it is unclear to what extent this adaptation to available substrates involves the oxidation of exogenous substrates *per se*, since this question cannot be answered calculating RER alone. As could be expected as part of an obese metabolic phenotype, a blunted RER response to refeeding was correlated to increased body fat mass in our mice (Fig. 2c). Remarkably, this was only reflected in the total amount of glucose oxidised in the early postprandial period, while the total amount of ingested glucose that became oxidised was not related to fat mass. This could suggest that hepatic uptake and regulation of ingested glucose remained unaffected,

while meal-induced glucose oxidation may have been impaired at the systemic level by competition with circulating fatty acids in obese mice.

The ability to study exogenous fat oxidation with isotopic sensor extended InCa provided renewed insights to interpret the fasting-and-refeeding challenge as a test of metabolic flexibility. Remarkably, exogenous palmitate was oxidised directly after administration of the meal, a time when fat oxidation was suppressed at the whole body level attributable to increased circulating insulin levels[40]. Likely, intestinal enterocytes were the first site of oxidation of exogenous palmitate directly after consumption of the meal, which is supported by human data showing that orally administered trioleate is oxidised more readily than intravenous trioleate[32], and that intestinal mucosa can readily oxidise about 17% of luminal palmitate in rats[41]. In line, fish omega-3 fatty acids *vs* plant omega-3 fatty acids were shown to induce fatty acid oxidation rates in the small intestines of mice[42]. Similar to what can be derived from RER data, our findings seem to indicate that oxidation of exogenous fatty acids is not a feature of metabolic flexibility in the early postprandial period. However, we found that exogenous palmitic acid oxidation correlated negatively with body fat mass in the late postprandial and post-absorptive periods (defined in this study as 2-13 h after ingestion of the liquid mixed meal; Fig. 5d). Moreover, the proportion of exogenous palmitic acid oxidised relative to total fatty acid oxidation not only correlated negatively with fat mass (Fig. 5e,f), but also correlated positively with ΔRER (Fig. 5g,h). Although speculative, it is possible that a blunted exogenous fatty acid oxidation is an unrecognised feature of metabolic inflexibility in response to a mixed meal.

Our observed ambient $^{13}CO_2$ enrichment levels (1.130 atom%) were not completely consistent with literature values, which are reported to vary between 1.100 to 1.103 atom% (refs. 3,43,44). This may be due to the ventilation characteristics of the animal room, because the magnitude of the difference in $^{13}CO_2$ enrichment between wheat- and maize-based diets (0.012 atom%) was close to the measured values of the diets using EA-IRMS (0.008 atom%). Moreover, the circadian variation in exhaled $^{13}CO_2$ enrichment of mice on non-labelled diets was as anticipated[5,45]. We also observed during the late postprandial and post-absorptive period following the ^{13}C-labelled meals, that exogenous glucose oxidation exceeded total glucose. This likely is a direct consequence of the fact that a large part of the RER values fell below 0.704. Below this value, net glucose utilisation is considered to be zero (ref. 22). More precisely, glucose oxidation can continue to take place, with ketogenesis and gluconeogenesis from amino acids contributing to a decrease in RER and effectively leading to an underestimation of glucose oxidation[1,22]. In our study, such processes could be expected as the mice were fasted for 24 h prior to the food restriction, which was with a high fat (*i.e.* a relative ketogenic) diet and returned to short-term negative energy balance already 4 h after ingesting the single mixed meal. The higher exogenous glucose oxidation compared to total glucose oxidation could also be an indirect consequence of the delayed appearance of the ^{13}C label in $^{13}CO_2$, a known phenomenon due to label retention in the bicarbonate pool in human studies[46,47] and in agreement with the half-life of CO_2 of about 15 min in mice[48]. In humans, label sequestration in *de novo* synthesized glucose, glutamine, and glutamate in fatty acid tracer studies likely pertains also to glucose tracers[49], although this is yet to be investigated in mice. Together with the

unrealistically high ^{13}C label recoveries we obtained after applying a human dietary acetate recovery factor (dARF) to the situation in mice, our data illustrates the need for a species-specific correction factor.

Integrating InCa with $^{13}CO_2$ and $^{12}CO_2$ sensors will improve the interpretation of mouse metabolic studies and provide crucial quantitative data. In addition, it will allow a wide variety of specific substrates to be studied, including metabolic substrates with highly variable or prolonged oxidation kinetics. Substrate-specificity could be further aided by the inclusion of other gas sensors. Moreover, a similar sensor technology would obviate some experimental challenges that remain in metabolic research with stable isotopes in humans, like the necessity to interrupt InCa sessions to obtain separate samples for $^{13}CO_2$ analysis.

In conclusion, the analysis of $^{13}CO_2$ enrichment coupled to conventional InCa is a powerful and targeted tool to quantify the kinetics of exogenous substrate oxidation. We have incorporated $^{13}CO_2$ and $^{12}CO_2$ sensors into a commercial InCa system for use in the mouse, a human-relevant model organism, and demonstrated its value to study fuel use strategies in physiological conditions, non-invasively, and continuously over long experimental periods.

Author Contributions

J.M.S.F.-C., A.O., J.K., and E.M.v.S. designed the work. J.M.S.F.-C., L.M.S.B., H.J.M.S., and E.M.v.S. acquired the data. J.M.S.F.-C., J.K., and E.M.v.S. analysed and interpreted the data. J.M.S.F.-C. drafted the manuscript and L.M.S.B., A.O., J.K., and E.M.v.S. revised it critically for important intellectual content. All authors critically revised and approved the manuscript.

Acknowledgements

We thank Remia (Den Dolder, The Netherlands) for their gift of palm olein, Cargill R&D Centre Europe (Vilvoorde, Belgium) for providing the starches and a free sample of Emultop IP, and Ries de Visser (IsoLife, Wageningen, The Netherlands) for fruitful discussions on tracer studies. We also thank the personnel at the CARUS animal facility for their assistance in mouse care-taking.

References

1 Ferrannini, E. The theoretical bases of indirect calorimetry: A review. *Metabolism* **37**, 287-301 (1988).

2 Speakman, J. R. Measuring energy metabolism in the mouse - theoretical, practical, and analytical considerations. *Front. Physiol.* **4**, 34 (2013).

3 Welch, K. C., Jr., Peronnet, F., Hatch, K. A., Voigt, C. C. & McCue, M. D. Carbon stable-isotope tracking in breath for comparative studies of fuel use. *Ann. N. Y. Acad. Sci.* **1365**, 15-32 (2016).

4 de Laeter, J. R. *et al.* Atomic weights of the elements. Review 2000 (IUPAC Technical Report). *Pure Appl. Chem.* **75**, 683-800 (2003).

5 McCue, M. D. & Welch, K. C. ^{13}C-Breath testing in animals: theory, applications, and future directions. *J. Comp. Physiol. B* **186**, 265-285 (2015).

6 van den Borne, J. J. *et al.* Body fat deposition does not originate from carbohydrates in milk-fed calves. *J. Nutr.* **137**, 2234-2241 (2007).

7 Bruininx, E. *et al.* Oxidation of dietary stearic, oleic, and linoleic acids in growing pigs follows a biphasic pattern. *J. Nutr.* **141**, 1657-1663 (2011).

8 McCue, M. D., Voigt, C. C., Jefimow, M. & Wojciechowski, M. S. Thermal acclimation and nutritional history affect the oxidation of different classes of exogenous nutrients in Siberian hamsters, *Phodopus sungorus*. *J. Exp. Zool. A Ecol. Genet. Physiol.* **321**, 503-514 (2014).

9 Isken, F. *et al.* Impairment of fat oxidation under high- vs. low-glycemic index diet occurs before the development of an obese phenotype. *Am. J. Physiol. Endocrinol. Metab.* **298**, E287-295 (2010).

10 Friedrich, M., Petzke, K. J., Raederstorff, D., Wolfram, S. & Klaus, S. Acute effects of epigallocatechin gallate from green tea on oxidation and tissue incorporation of dietary lipids in mice fed a high-fat diet. *Int. J. Obes.* **36**, 735-743 (2012).

11 Michalski, M. C., Briard, V., Desage, M. & Geloen, A. The dispersion state of milk fat influences triglyceride metabolism in the rat--a $^{13}CO_2$ breath test study. *Eur. J. Nutr.* **44**, 436-444 (2005).

12 Butts, C. A. *et al.* The fate of ^{13}C-labelled and non-labelled inulin predisposed to large bowel fermentation in rats. *Food. Funct.* **7**, 1825-1832 (2016).

13 Fromentin, C. *et al.* The postprandial use of dietary amino acids as an energy substrate is delayed after the deamination process in rats adapted for 2 weeks to a high protein diet. *Amino acids* **40**, 1461-1472 (2011).

14 Ishihara, K. *et al.* A soybean peptide isolate diet promotes postprandial carbohydrate oxidation and energy expenditure in type II diabetic mice. *J. Nutr.* **133**, 752-757 (2003).

15 McCue, M. D. & Pollock, E. D. Measurements of substrate oxidation using $^{13}CO_2$-breath testing reveals shifts in fuel mix during starvation. *J. Comp. Physiol. B.* **183**, 1039-1052 (2013).

16 Fernández-Calleja, J. M. S. *et al.* Non-invasive continuous real-time *in vivo* analysis of microbial hydrogen production shows adaptation to fermentable carbohydrates in mice. *Sci. Rep.* **8**, 15351 (2018).

17 Hoevenaars, F. P. *et al.* Effects of dietary history on energy metabolism and physiological parameters in C57BL/6J mice. *Exp. Physiol.* **98**, 1053-1062 (2013).

18 Reeves, P. G., Nielsen, F. H. & Fahey, G. C., Jr. AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet. *J. Nutr.* **123**, 1939-1951 (1993).

19 Fernandez-Calleja, J. M. S. *et al.* Direct and long-term metabolic consequences of lowly vs. highly-digestible starch in the early post-weaning diet of mice. *Nutrients* **10**, 1788 (2018).

20 Warner, D., Ferreira, L. M., Breuer, M. J., Dijkstra, J. & Pellikaan, W. F. Stable isotope labeled n-alkanes to assess digesta passage kinetics through the digestive tract of ruminants. *PLoS One* **8**, e75496 (2013).

21 Wopereis, S. *et al.* Multi-parameter comparison of a standardized mixed meal tolerance test in healthy and type 2 diabetic subjects: the PhenFlex challenge. *Genes Nutr.* **12**, 21 (2017).

22 Peronnet, F. & Massicotte, D. Table of nonprotein respiratory quotient: an update. *Can. J. Sport Sci.* **16**, 23-29 (1991).

23 Weir, J. B. d. V. New methods for calculating metabolic rate with special reference to protein metabolism. *J. Physiol.* **109**, 1-9 (1949).

24 Gold, I. L., Ukhun, M. E. & Akoh, C. C. Characteristics of eutectic compositions of restructured palm oil olein, palm kernel oil and their mixtures. *J. Am. Oil Chem. Soc.* **88**, 1659-1667 (2011).

25 Antoun, E. *et al.* The [1-^{13}C]acetate recovery factor to correct tracer-derived dietary fat oxidation is lower in overweight insulin-resistant subjects. *E Spen Eur. E J. Clin. Nutr. Metab.* **5**, e173-e179 (2010).

26 Bergouignan, A., Schoeller, D. A., Votruba, S., Simon, C. & Blanc, S. The acetate recovery factor to correct tracer-derived dietary fat oxidation in humans. *Am. J. Physiol. Endocrinol. Metab.* **294**, E645-653 (2008).

27 Sidossis, L. S., Coggan, A. R., Gastaldelli, A. & Wolfe, R. R. A new correction factor for use in tracer estimations of plasma fatty-acid oxidation. *Am. J. Physiol. Endocrinol. Metab.* **269**, E649-E656 (1995).

28 Bessesen, D. H., Bull, S. & Cornier, M. A. Trafficking of dietary fat and resistance to obesity. *Physiol. Behav.* **94**, 681-688 (2008).

29 Flatt, J. P., Ravussin, E., Acheson, K. J. & Jequier, E. Effects of dietary fat on postprandial substrate oxidation and on carbohydrate and fat balances. *J. Clin. Invest.* **76**, 1019-1024 (1985).

30 Bessesen, D. H., Vensor, S. H. & Jackman, M. R. Trafficking of dietary oleic, linolenic, and stearic acids in fasted or fed lean rats. *Am. J. Physiol. Endocrinol. Metab.* **278**, E1124-1132 (2000).

31 Leyton, J., Drury, P. J. & Crawford, M. A. Differential oxidation of saturated and unsaturated fatty acids in vivo in the rat. *Brit. J. Nutr.* **57**, 383-393 (1987).

32 Metges, C. C. & Wolfram, G. Medium- and long-chain triglycerides labeled with ^{13}C: a comparison of oxidation after oral or parenteral administration in humans. *J. Nutr.* **121**, 31-36 (1991).

33 Binnert, C. *et al.* Influence of human obesity on the metabolic fate of dietary long- and medium-chain triacylglycerols. *Am. J. Clin. Nutr.* **67**, 595-601 (1998).

34 Vors, C. *et al.* Modulating absorption and postprandial handling of dietary fatty acids by structuring fat in the meal: a randomized crossover clinical trial. *Am. J. Clin. Nutr.* **97**, 23-36 (2013).

35 Galgani, J. E., Moro, C. & Ravussin, E. Metabolic flexibility and insulin resistance. *Am. J. Physiol. Endocrinol. Metab.* **295**, E1009-1017 (2008).

36 Duivenvoorde, L. P., van Schothorst, E. M., Swarts, H. J. & Keijer, J. Assessment of metabolic flexibility of old and adult mice using three noninvasive, indirect calorimetry-based treatments. *J. Gerontol. A Biol. Sci. Med. Sci.* **70**, 282-293 (2015).

37 Bouwman, L. M. S. *et al.* No adverse programming by post-weaning dietary fructose of body weight, adiposity, glucose tolerance, or metabolic flexibility. *Mol. Nutr. Food Res.* **62**, 1700315 (2018).

38 Horakova, O. *et al.* Preservation of metabolic flexibility in skeletal muscle by a combined use of n-3 PUFA and rosiglitazone in dietary obese mice. *PLoS One* **7**, e43764 (2012).

39 Asterholm, I. W. & Scherer, P. E. Enhanced metabolic flexibility associated with elevated adiponectin levels. *Am. J. Pathol.* **176**, 1364-1376 (2010).

40 Randle, P. J., Garland, P. B., Hales, C. N. & Newsholme, E. A. The glucose fatty-acid cycle its role in insulin sensitivity and the metabolic disturbances of diabetes mellitus. *Lancet* **281**, 785-789 (1963).

41 Gangl, A. & Ockner, R. K. Intestinal metabolism of plasma free fatty acids. Intracellular compartmentation and mechanisms of control. *J. Clin. Invest.* **55**, 803-813 (1975).

42 van Schothorst, E. M. *et al.* Induction of lipid oxidation by polyunsaturated fatty acids of marine origin in small intestine of mice fed a high-fat diet. *BMC genomics* **10**, 110 (2009).

43 Kuc, T. *et al.* Two decades of regular observations of $^{14}CO_2$ and $^{13}CO_2$ content in atmospheric carbon dioxide in central Europe: long-term changes of regional anthropogenic fossil CO_2 emissions. *Radiocarbon* **49**, 807-816 (2016).

44 Keeling, C. D. *et al.* in *A History of Atmospheric CO_2 and Its Effects on Plants, Animals, and Ecosystems* (eds I. T. Baldwin *et al.*) 83-113 (Springer, New York, 2005).

45 Wolfe, R. R., Shaw, J. H., Nadel, E. R. & Wolfe, M. H. Effect of substrate intake and physiological state on background $^{13}CO_2$ enrichment. *J. Appl. Physiol. Respir. Environ. Exerc. Physiol.* **56**, 230-234 (1984).

46 Ravussin, E. *et al.* Carbohydrate utilization in obese subjects after an oral load of 100 g naturally-labelled ^{13}C glucose. *Br. J. Nutr.* **43**, 281-288 (1980).

47 Daly, M. E. *et al.* Acute fuel selection in response to high-sucrose and high-starch meals in healthy men. *Am. J. Clin. Nutr.* **71**, 1516-1524 (2000).

48 Speakman, J. R. & Thomson, S. C. Validation of the labeled bicarbonate technique for measurement of short-term energy expenditure in the mouse. *Z. Ernahrungswiss* **36**, 273-277 (1997).

49 Schrauwen, P. *et al.* Validation of the [1,2-^{13}C]acetate recovery factor for correction of [U-^{13}C] palmitate oxidation rates in humans. *J. Physiol.* **513**, 215-223 (1998).

Supplementary Information

Table S1. Metabolic characteristics of the female C57BL/6JRccHsd mice fed HDD and LDD from postnatal week (PW) 4 to PW 6, and HFD from PW 7 to PW 15 (second mouse study).

Parameter	PW 6			PW 15		
	HDD	LDD	*P*-value	HDD	LDD	*P*-value
BW (g)	17.02 (15.40, 19.28)	17.42 (15.35, 18.49)	0.0266	30.69 ± 3.00	32.44 ± 3.63	0.0744
FM (g)	1.51 (0.76, 2.83)	1.47 (0.82, 2.65)	0.8902	9.38 ± 2.47	11.04 ± 3.06	0.0442
LM (g)	14.56 ± 0.70	14.83 ± 0.67	0.1794	19.87 ± 0.91	19.90 ± 0.69	0.8744
FM gain (g)[1]	0.63 (0.02, 1.96)	0.66 (-0.07, 1.80)	0.9425	7.85 ± 2.29	9.48 ± 2.96	0.0381
dEI (MJ)[1]	0.88 (0.73, 1.05)	0.97 (0.86, 1.04)	< 0.0001	3.67 ± 0.30	3.71 ± 0.25	0.5839
24 h EE (kJ d^{-1})[2]	ND	ND	-	43.93 ± 2.91	44.55 ± 2.99	0.4745
24 h RER[3]	ND	ND	-	0.82 ± 0.04	0.80 ± 0.03	0.0698
ΔRER ($RER_{44} - RER_0$)[2]	ND	ND	-	0.17 (0.10, 0.19)	0.16 (0.11, 0.20)	0.4436
Fasting glucose (mmol l^{-1})	ND	ND	-	5.8 (4.9, 7.2)	6.0 (5.2, 7.1)	0.5245
Fasting insulin (ng ml^{-1})	ND	ND	-	2.72 (0.85, 8.95)	2.31 (1.06, 3.45)	0.6049
Postprandial glucose (mmol l^{-1})	ND	ND	-	7.1 ± 1.3	6.8 ± 1.7	0.6811
Postprandial insulin (ng ml^{-1})	ND	ND	-	4.31 ± 2.85	4.48 ± 1.42	0.8622

[1]Measured from PW 4 to PW 6 (post-weaning period on HDD or LDD), or from PW 7 to PW 15 (HFD-feeding). [2]Measured in PW 14-15. HFD, high-fat diet; HDD, highly-digestible starch diet; LDD, lowly-digestible starch diet; BW, body weight; dEI, digestible energy intake; FM, fat mass; LM, lean mass; EE, energy expenditure; ND, not determined; RER, respiratory exchange ratio (mean of 24 h). Data was analysed separately for PW 6 and PW 15 time points using a Student's *t*-test (normally distributed data) or a Mann Whitney test (non-normally distributed data). Data is presented as median and range (BW PW 6, FM PW 6, FM gain PW 6, dEI PW 6, ΔRER, fasting blood glucose, fasting blood insulin) or otherwise as mean ± SD, $n = 24$ per group (except ΔRER, HDD: $n = 24$, LDD: $n = 23$; fasting glucose and insulin, $n = 12$ per group; and postprandial glucose and insulin, HDD: $n = 11$, LDD: $n = 12$).

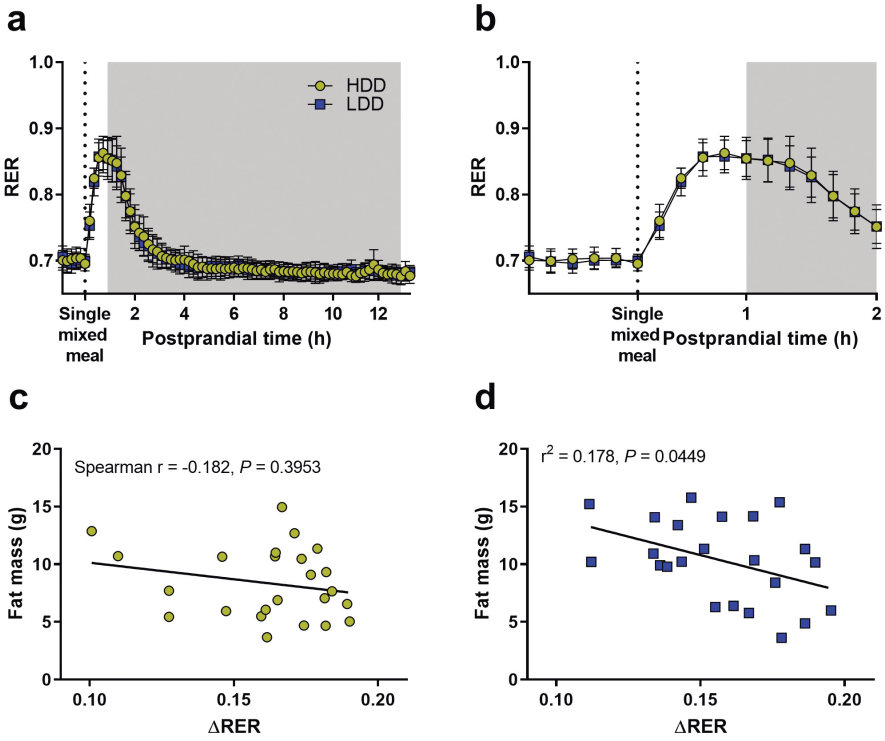
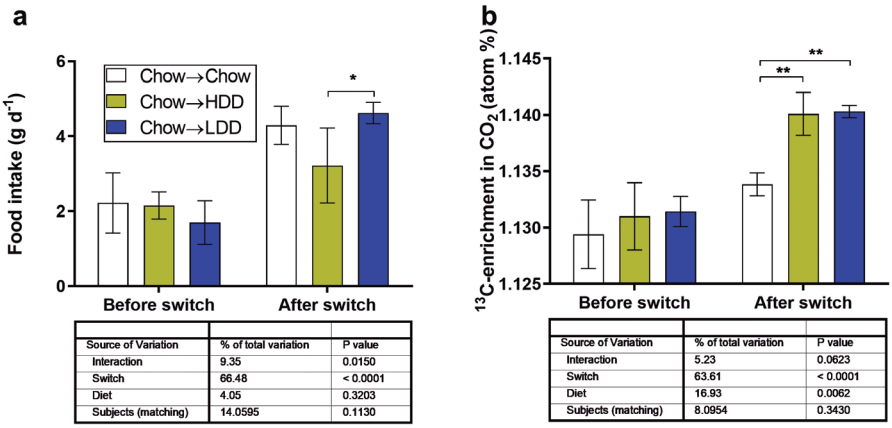


Figure S1. Refeeding metabolic response to a liquid mixed meal and its relation to body fat mass (FM) in mice fed a HFD for nine weeks (second mouse study). (**a**) Respiratory exchange ratio (RER) was measured in PW 14-15 after gavage of a single liquid mixed meal containing 36 energy% glucose and 64 energy% fat in mice fed the HDD (*n* = 24) or the LDD (*n* = 23) during early life and subsequently fed a HFD for nine weeks. Data was analysed with repeated-measures 2-way analysis of variance to directly test the hypothesis that RER response is influenced by the post-weaning diet. There were no significant effects of the post-weaning diet or its interaction with time. Data is presented as mean ± SD. Shaded areas represent the dark phase. (**b**) Data from panel (**a**), emphasizing the early postprandial 2 h period. Correlation analysis between FM and the re-feeding response (ΔRER), measured as the increase from baseline RER to RER at 44 min postprandial, in mice originally fed HDD (**c**) or LDD (**d**) during early post-weaning. HDD, highly digestible starch-diet; LDD, lowly digestible starch-diet.



Source of Variation	% of total variation	P value
Interaction	9.35	0.0150
Switch	66.48	< 0.0001
Diet	4.05	0.3203
Subjects (matching)	14.0595	0.1130

Source of Variation	% of total variation	P value
Interaction	5.23	0.0623
Switch	63.61	< 0.0001
Diet	16.93	0.0062
Subjects (matching)	8.0954	0.3430

Figure S2. Changes in food intake and $^{13}CO_2$ enrichment in mice fed chow and then switched HDD or LDD, or remaining on chow. Chow-fed mice (n = 12) were adapted to indirect calorimetry cages for 2 d, after which mice were allowed a restricted amount (1.1 g) of chow 1 h before the onset of the dark phase. At the end of the following light phase, mice were re-fed with a restricted amount (1.1 g) of chow, or HDD or LDD (n = 4). Before the following dark phase mice received *ad libitum* access to the same diet they were allocated the day before, and measurements continued for 5.5 d (see **Chapter 2** for details). (**a**) 24 h cumulative food intake and (**b**) 24 h mean ^{13}C enrichment immediately before food restriction and the day after *ad libitum* re-feeding. Tables show statistical output of repeated-measures 2-way analysis of variance. *P < 0.05, **P < 0.01, Bonferroni multiple comparisons. Data is presented as mean ± SD. HDD, highly digestible starch-diet; LDD, lowly digestible starch-diet.

Chapter 4

A Lowly Digestible-Starch Diet After Weaning Enhances Exogenous Glucose Oxidation Rate in Female, but Not in Male, Mice

José M. S. Fernández-Calleja

Lianne M. S. Bouwman

Hans J. M. Swarts

Nils Billecke

Annemarie Oosting

Jaap Keijer

Evert M. van Schothorst

Nutrients (2019) 11:2242 DOI: 10.3390/nu11092242

Abstract

Starches of low digestibility, compared to highly digestible starches, are associated with improved glucose metabolism in rodents. Most studies have been conducted in males, but available evidence suggests that the metabolic effects of starch digestibility are sex-dependent. Here, we test the hypothesis that a lowly digestible starch-diet (LDD) *vs* a highly digestible starch diet (HDD) improves the capacity to oxidise the starch molecule, and that this effect is stronger in females than in males. Female and male mice were fed an LDD or an HDD for 3 weeks directly after weaning. Body weight (BW), body composition (BC), and digestible energy intake (dEI) were determined weekly. At the end of the intervention period, whole-body energy expenditure (EE), respiratory exchange ratio (RER), hydrogen (H_2) production, and the oxidation of an oral ^{13}C-labelled starch bolus were measured by extended indirect calorimetry. Additionally, pancreatic amylase activity and total ^{13}C hepatic enrichment were determined in females sacrificed immediately before and 4 h after administration of the starch bolus. For both sexes, BW, BC, and basal EE and RER were not affected by the type of starch, but dEI and H_2 production were increased by the LDD. Only in females, total carbohydrate oxidation and starch-derived glucose oxidation in response to the starch bolus were higher in LDD *vs* HDD mice, and this was not accompanied by differences in amylase activity or partitioning of the ^{13}C label toward the liver. These results show that starch digestibility impacts glucose metabolism differently in females compared to males.

Keywords: Indirect calorimetry, C57BL mice, glucose oxidation, ^{13}C-starch, amylose, amylopectin, glycaemic index, amylase.

Introduction

The digestibility of dietary starch can influence metabolic health. This has been extensively studied in rodents, where lowly digestible starches has led to lower body weight (BW) and adiposity and normal glucose homeostasis compared to highly digestible starches[1]. In humans, lowly digestible starches have been shown to improve insulin sensitivity and other metabolic endpoints[2-5], although more evidence is needed to establish how this type of starches should be included in an individual's diet to bring about their beneficial effects. For instance, while a lower glycaemic response is achieved by replacing glycaemic carbohydrates with lowly digestible starches, it is not clear whether this is also the case when available carbohydrates remain constant[6], or whether factors like differences in gut microbiota composition could change how lowly digestible starches affect specific subgroups of people[7]{Robertson, 2012 #1011;Peterson, 2018 #1414;Peterson, 2018 #1414}. A previous study showed higher postprandial exogenous and total glucose oxidation in women compared to men. which was associated with intrinsic differences in insulin sensitivity and body composition[8]. Whether or not the metabolic impact of starches of different digestibility also depends on sex is currently unknown.

A recent meta-analysis of rodent studies showed that consuming lowly digestible starches results in similar BW and fat mass (FM) compared to highly digestible starch feeding in females[1]. In contrast, we have observed that feeding a lowly digestible starch diet (LDD) *vs* a highly digestible starch diet (HDD) for three weeks directly after weaning resulted in smaller adipocytes and less crown-like structures in gonadal white adipose tissue (WAT) despite similar BW and body composition (BC)[9]. After exposure to a high-fat diet in adulthood, these females developed a higher metabolic flexibility to a starch-containing meal, which was not observed in male mice[9]. Some effects of starch digestibility on metabolic health have been shown to precede alterations in BW or adiposity in mice. Adult males on a highly digestible starch for three weeks showed lower exogenous fat oxidation compared to those fed lowly digestible starch, prior to developing an obese phenotype [10]. This raises the question whether exogenous substrate oxidation is also influenced by exposure to starches of different digestibility in females of similar BW and BC.

In this study, we tested the hypothesis that exposure to an LDD compared to an HDD during the immediate post-weaning period leads to a better capacity to oxidise dietary carbohydrates, specifically the starch molecule, in female but not in male mice. Additionally, we explore whether small intestinal amylase levels or the partitioning of starch-derived glucose to the liver compartment could explain any potential sex differences in starch oxidation.

Materials and Methods

Mouse experiment

All mice (C57BL/6JRccHsd, Envigo, Horst, The Netherlands) were individually housed in Makrolon II cages with wood chips and wood shavings, at 23 ± 1 °C, 50 ± 5% humidity, on a

12 h light/dark cycle (6.00 AM lights on). Unless otherwise indicated, mice had *ad libitum* access to food and water.

Adult mice (9-23 weeks old) on a chow diet (Teklad Global Diet 2920, Envigo) were time-mated and their offspring cross-fostered within 24-48 h postnatally to produce standardized litters of 6 pups and a sex ratio of 3:3 or 4:2. At the end of postnatal week (PW) 3, male and female offspring were weaned and individually housed and stratified by BW to receive either an LDD (Research Diet Services, Wijk bij Duurstede, The Netherlands; n = 15 females, n = 8 males) or an HDD (Research Diet Services; n = 15 females, n = 9 males) for the rest of the study. These two diets only differed in the type of starch included. The experimenter was not blinded to the experimental diets. Welfare was monitored daily by visual inspection. BW, body composition (BC; EchoMRI 100V, EchoMedical Systems, Houston, Texas, USA), and food intake (FI) were determined weekly. At the end of PW 6 (for females) or during PW 7 (for males), mice were re-stratified by BW and assigned to be sacrificed in either the fasting state or after ingestion of a ^{13}C-labelled starch bolus (described below). Additionally, a subgroup of the mice assigned the ^{13}C starch bolus was transferred to an extended indirect calorimetry (InCa) system for measurements of whole-body metabolism and total and starch-derived glucose oxidation (described below). The study was approved by the CCD/IvD 2017.W-0024.002 and performed in accordance with European Union (EU) directive 2010/63/EU.

Diet composition

The chow diet for breeding and lactating dams consisted of 24, 60, and 16 energy% protein, carbohydrate, and fat, respectively. The semi-purified LDD and HDD contained 20, 55, and 25 energy% protein, carbohydrate, and fat, respectively, and were based on the BIOCLAIMS standard diet[11]. The starches in the LDD and HDD (569 g kg^{-1} diet; Cargill, Sas van Gent, The Netherlands) were incorporated by Research Diet Services. The exact composition and digestible energy density of the diets are shown in Table 1.

Preparation and administration of the ^{13}C-labelled starch bolus

A 20 mg ml^{-1} mixture of uniformly ^{13}C-labelled potato starch (98.2 atom% ^{13}C, > 98% dry w/w glucan chemical purity; IsoLife, Wageningen, The Netherlands) in phosphate buffered saline (PBS) was heated to 90 °C for 15 min and added to a 120 mg ml^{-1} suspension of non-labelled amylopectin maize starch (C*Gel 04201, Cargill) in PBS, to achieve a total starch concentration of 80 mg ml^{-1}. Preparations were made fresh on the day of use and kept under constant stirring before administration to the mice.

On the day prior to the administration of the ^{13}C starch bolus, mice (including the subgroup studied in InCa) were food-restricted approximately 2 h before the dark phase by receiving 1.21 (SD 0.02) g of their corresponding post-weaning diet (LDD or HDD). The day after, approximately 2 h into the light phase (LP) when the mice were fasted, each mouse was administered 0.5 ml of the ^{13}C starch preparation by oral gavage, representing a dose of 40 mg of total starch per mouse (0.6 kJ).

Table 1. Composition of the experimental diets.

	HDD	LDD
Casein (g kg^{-1})	212.2	212.0
L-Cysteine (g kg^{-1})	3.0	3.0
Amylose mix (AmyloGel 03003) (g kg^{-1})†	0.0	568.6
Amylopectin (C*Gel 04201) (g kg^{-1})‡	568.6	0.0
Coconut oil (g kg^{-1})	21.4	21.4
Sunflower oil (g kg^{-1})	83.1	83.1
Flaxseed oil (g kg^{-1})	14.2	14.2
Cholesterol (g kg^{-1})	0.03	0.03
Cellulose (g kg^{-1})	50.0	50.0
Mineral mix (AIN-93G-MX) (g kg^{-1})	35.0	35.0
Vitamin mix (AIN-93-VX) (g kg^{-1})	10.0	10.0
Choline bitartrate (g kg^{-1})	2.5	2.5
Calculated energy density (kJ g^{-1})§	17.9	17.9
Gross energy density (kJ g^{-1})‖	18.9	19.5
Digestible energy density (kJ g^{-1})¶	17.6	17.0
Protein (energy%)	20.1	20.1
Carbohydrate (energy%)	54.9	54.9
Fat (energy%)	25.0	25.0

HDD, highly digestible-starch diet; LDD, lowly digestible-starch diet. † 60% amylose, 40% amylopectin (Cargill). ‡ 100% amylopectin (Cargill). § Calculated based on Atwater's nutritional values. || Determined by bomb calorimetry. ¶ Determined by bomb calorimetry and faecal output in an independent experiment[12].

Extended indirect calorimetry (InCa)

Animals were acclimatised to the extended InCa system (PhenoMaster, TSE Systems, Bad Homburg, Germany) for approximately 36 h. The following 24 h period was used for measurements of O_2 consumption and CO_2 production from which daily energy expenditure (EE) and respiratory exchange ratio (RER) were calculated. Locomotor activity, and food and water intake were also measured. Recently, we incorporated hydrogen (H_2) and methane (CH_4) sensors for gut microbiota activity measurements in the InCa system[12]. After basal measurements, mice received a limited amount of food (~1.2 g) and were then administered the ^{13}C starch bolus in the fasted state, as described above, and InCa measurements continued for the next 24 h. For logistical reasons, gas sampling frequency was set at 20 min for males and 11 min for females. LDD and HDD animals were equally represented in each InCa measurement. Bedding was reduced to about 200 ml during InCa measurements to facilitate detection of voluntary locomotion by infrared beam breaks in the horizontal plane. BW and BC were determined directly before and after InCa measurement. The technological extensions of the InCa system and other operational settings and procedures have been described previously[12,13].

Total glucose oxidation (TGO) rates were calculated from VO_2 and VCO_2, based on Péronnet's table of non-protein RER[14]. Starch-derived glucose oxidation (SGO) rates were calculated using the following two formulas:

$$at\%^{13}CO_2 = \frac{^{13}CO_2}{^{13}CO_2 + ^{12}CO_2} \times 100 \qquad (1)$$

$$SGO\ (mg\ min^{-1}) = \frac{at\%^{13}CO_{2(t)} - at\%^{13}CO_{2(t0)}}{at\%^{13}C_S - at\%^{13}CO_{2(t0)}} \times \frac{VCO_{2(t)} \times 180.16}{22.29 \times 6} \qquad (2)$$

The at%$^{13}CO_2$ (formulas 1 and 2) is the ^{13}C enrichment in expired CO_2 in atom% calculated from $^{13}CO_2$ and $^{12}CO2$ gas concentrations (delta ppm). In formula 2 (based on[8]), the time t_0 represents the baseline measurement over approximately 1 h before administration of the ^{13}C starch bolus, and t represents any subsequent time point. The calculated total ^{13}C enrichment in the starch bolus (^{13}C-labelled plus non-labelled starch) is represented by at%$^{13}C_s$ and has a value of approximately 8.4 atom%, according to the declared isotopic and dry chemical purity of the ^{13}C-labelled starch, and assuming a ^{13}C enrichment of 1.1 atom% of the non-labelled maize starch based on measured values of maize-derived fructose[15]. VCO_2 is the production rate of total CO_2 obtained using the summed concentrations of $^{13}CO_2$ and $^{12}CO_2$ multiplied by the constant air flow. The molecular weight of glucose is 180.16 g mol^{-1}, the volume occupied by 1 mol of CO_2 in STPD is 22.29 l, and 6 carbons per mol of starch-derived glucose were ^{13}C-labelled. To express SGO rates as percentage of the dose of starch-derived glucose administered, the declared moisture content in the ^{13}C-labelled and non-labelled starches and the release of 1.1 g of glucose per 1 g of dry starch[16] were taken into account.

Dissection

Mice were food-restricted by receiving about 1.2 g of their corresponding post-weaning diet (LDD or HDD) 2 h before the dark phase, as described above. On the following day, mice were decapitated either in the fasted state (~3 h into the LP), or exactly 4 h after administration of a ^{13}C starch bolus (~5 h into the LP). No anaesthesia was used, to prevent changes in glucose levels. Glycaemia was determined in whole blood directly after sacrifice using a Freestyle glucose meter (Abbott Diabetes Care, Hoofddorp, The Netherlands). The liver was excised, weighed, separated into lobes, and snap-frozen in liquid N_2. The luminal content of the small intestine was gently pressed out, weighed, and snap-frozen. Samples were then stored at −80 °C.

Amylase activity

Amylase activity in small intestinal contents was measured with a colorimetric method (Amylase Activity Assay Kit, MAK009, Sigma-Aldrich, Zwijndrecht, The Netherlands) according to the manufacturer's instructions. The intra-assay and inter-assay CVs were 2.2 and 14.8%, respectively.

Elemental analysis isotope ratio mass spectrometry (EA-IRMS)

We focussed on the partitioning of ^{13}C starch into liver because this organ extracts about 30% of an enteral glucose load, particularly in fasting conditions[17,18]. Liver total ^{13}C enrichment was measured by EA-IRMS as previously published[19]. Briefly, a sample of the right lobe was freeze-dried and combusted at 1020 °C in the presence of oxygen to convert carbon into CO_2, followed by separation for measurement of the $^{13}C/^{12}C$ ratio by EA-IRMS. Results were expressed as delta over baseline (DOB) atom%, calculated from the algebraic difference between atom% ^{13}C from individual mice that received a ^{13}C starch bolus and the mean atom% ^{13}C of mice on LDD or HDD that did not receive the ^{13}C starch bolus and were sacrificed in the fasted state. Label recovery in the liver compartment was calculated from the dry weight of whole liver and the total C and ^{13}C content in the samples.

Statistical analysis

Sample size (n = 6) was calculated to detect a difference of 0.0466 mg min^{-1} in maximal exogenous glucose oxidation with 80% statistical power at a two-sided significance level of 0.05 (G*Power v. 3.1.9.3)[20], based on preliminary data with obese and lean mice in our extended InCa system. A single pup was considered one experimental unit. One HDD male mouse was excluded from all data analyses due to incisor malocclusion detected at the end of the study. Normal distribution of the data was tested with the D'Agostino and Pearson omnibus test; non-normally distributed data were log-transformed and retested for normality. When $n \leq 6$, a normal distribution was assumed. Equality of variances was tested with an F-test. Differences between LDD *vs* HDD mice in single outcomes at the end of the study (*e.g.* BW) were determined separately per sex with a two-tailed unpaired Student's t-test (normally-distributed data) with Welch's correction when variances were significantly different, or with a two-tailed Mann-Whitney test (non-normally distributed data). Time course data (*e.g.* SGO) was analysed separately per sex by a two-tailed unpaired Student's t-test on the incremental area under the curve (iAUC), taking as baseline the average RER over approximately 1 h before administration of the ^{13}C starch bolus. To locate specific time points showing statistically significant differences, time course data was analysed by repeated-measures (RM) 2-way ANOVA and, when the interaction between time and post-weaning diet was significant, followed by Bonferroni's *post hoc* test for multiple comparisons. Sphericity was assumed. Two RER values that were not recorded due to opening of the cage were replaced by the average of the previous and next recordings. To compare the cumulative SGO in males *vs* females on LDD or HDD, and to compare parameters measured in LDD *vs* HDD females in the fasted state or 4 h after receiving the ^{13}C starch bolus, a 2-way ANOVA was used. Statistical analyses, iAUC calculations, and data visualisation were performed in Prism v.5.04 (GraphPad, San Diego, California, USA). Statistical significance was set at $P < 0.05$.

Results

General metabolic phenotype

Both in males and females, BW, FM, lean mass, and fasting blood glucose concentrations were not different after three weeks of consuming the post-weaning LDD *vs* HDD (Table 2). Similarly, 24 h whole-body energy expenditure, respiratory exchange ratio (RER), and locomotor activity were not different in LDD *vs* HDD mice (data not shown). However, cumulative digestible energy intake over the complete post-weaning intervention was about 13% higher in LDD compared to HDD mice (Table 2), and cumulative hydrogen (H_2) production over 24 h was approximately 2.9-fold higher in LDD *vs* HDD mice (Figure 1). Methane (CH_4) production was undetectable.

Table 2. Body weight, body composition, and cumulative energy intake at the end of the post-weaning nutritional intervention.

	Males				Females			
	HDD		LDD		HDD		LDD	
	Mean or median	SD or range	Mean or median	SD or range	Mean or median	SD or range	Mean or median	SD or range
Body weight (g)	21.23	1.13	21.53	2.08	16.60	14.06, 18.54	16.72	14.01, 17.55
Fat mass (g)	2.14	0.43	1.74	0.52	1.69	0.60	1.43	0.33
Lean mass (g)	18.18	0.96	18.81	1.49	14.28	1.06	14.17	0.88
Fasting blood glucose (mmol l^{-1})	4.9	0.6	5.4	0.5	4.4	0.8	4.7	0.8
Digestible energy intake (MJ)†	1.15	1.12, 1.26	1.30***	1.20, 1.46	0.88	0.61, 0.93	0.99****	0.95, 1.06

HDD, highly digestible-starch diet; LDD, lowly digestible-starch diet. † Cumulative digestible energy intake from introduction of post-weaning diet until the end of the study (not including the starch bolus). Data is presented as mean and SD, except for body weight (females) and energy intake (both sexes), where median and range are shown. Males: n = 8-9, females: n = 9-15. ***P < 0.001, ****P < 0.0001.

Food restriction and substrate switching in response to the ^{13}C starch bolus

For both sexes, LDD mice showed a lower RER compared to HDD mice from the time of food restriction up until the moment before administration of the ^{13}C starch bolus (Figure S1). Fasted (~17 h after food restriction) LDD males had a higher RER (0.725 (SD 0.015)) compared to HDD males (0.697 (SD 0.009); P < 0.01). In contrast, fasting RER was similar between LDD and HDD females (0.678 (SD 0.016) and 0.677 (SD 0.024) respectively).

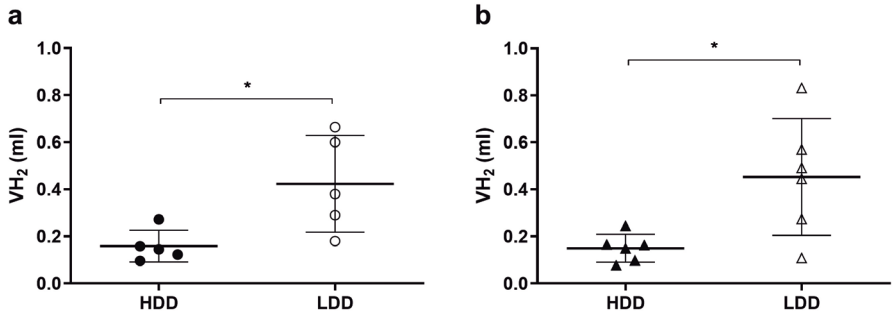


Figure 1. Cumulative 24 h H_2 production on HDD or LDD. (**a**) Males (*n* = 5; PW 4), (**b**) females (*n* = 6; PW 3). Data is presented as mean and SD. Student's *t*-test, **P* < 0.05. HDD, highly digestible-starch diet; LDD, lowly digestible-starch diet; PW, post-weaning week.

In response to administration of the ^{13}C starch bolus, LDD and HDD males showed a similar increase in RER (Figure 2a,b), but RER in LDD males remained higher throughout the 4 h postprandial period (Figure 2c). LDD females had an initially higher increase in RER compared to HDD females (Figure 2d,e), while both groups maintained a generally similar RER throughout the starch bolus challenge (Figure 2f).

Starch-derived and total glucose oxidation in response to the ^{13}C starch bolus

Analysis of ^{13}C enrichment in expired CO_2, as a qualitative indication of the oxidation of exogenous ^{13}C starch, showed a trend for a diet × time interaction in males (*P* = 0.095), and a main effect of the post-weaning diet (*P* < 0.05; Figure S2a). Comparing rates of starch-derived glucose oxidation, calculated from $^{13}CO_2$ and total CO_2 production values, showed no diet × time interaction in males, with a trend towards an effect of the post-weaning diet with seemingly higher values in LDD males (*P* = 0.083; Figure 3a). In addition, energy expenditure upon the administration of the starch bolus was initially higher in HDD males (post-weaning diet × time interaction, *P* = 0.045; Figure S3a). Consistent with the RER response (Figure 2a), total glucose oxidation (thus including exogenous starch-derived and endogenous glucose) showed no diet × time interaction, but showed a significant main effect of the post-weaning diet with higher values in LDD males (*P* < 0.01; Figure 3b). Thus, although LDD males exhibited generally higher glucose oxidation rates, the exogenous glucose oxidation response with time was similar in LDD and HDD males.

In contrast to males, the increase in $^{13}CO_2$ enrichment was greater in LDD compared to HDD females (diet × time interaction, *P* < 0.0001; Figure S2b). Similarly, oxidation rates of exogenous starch-derived glucose were also influenced by the post-weaning diet (diet × time interaction , *P* < 0.05), being significantly higher in LDD females at 33 and 44 min upon administration of the ^{13}C starch bolus (Figure 3d). Energy expenditure after consumption of the starch bolus was not affected by the post-weaning diet (Figure S3b).

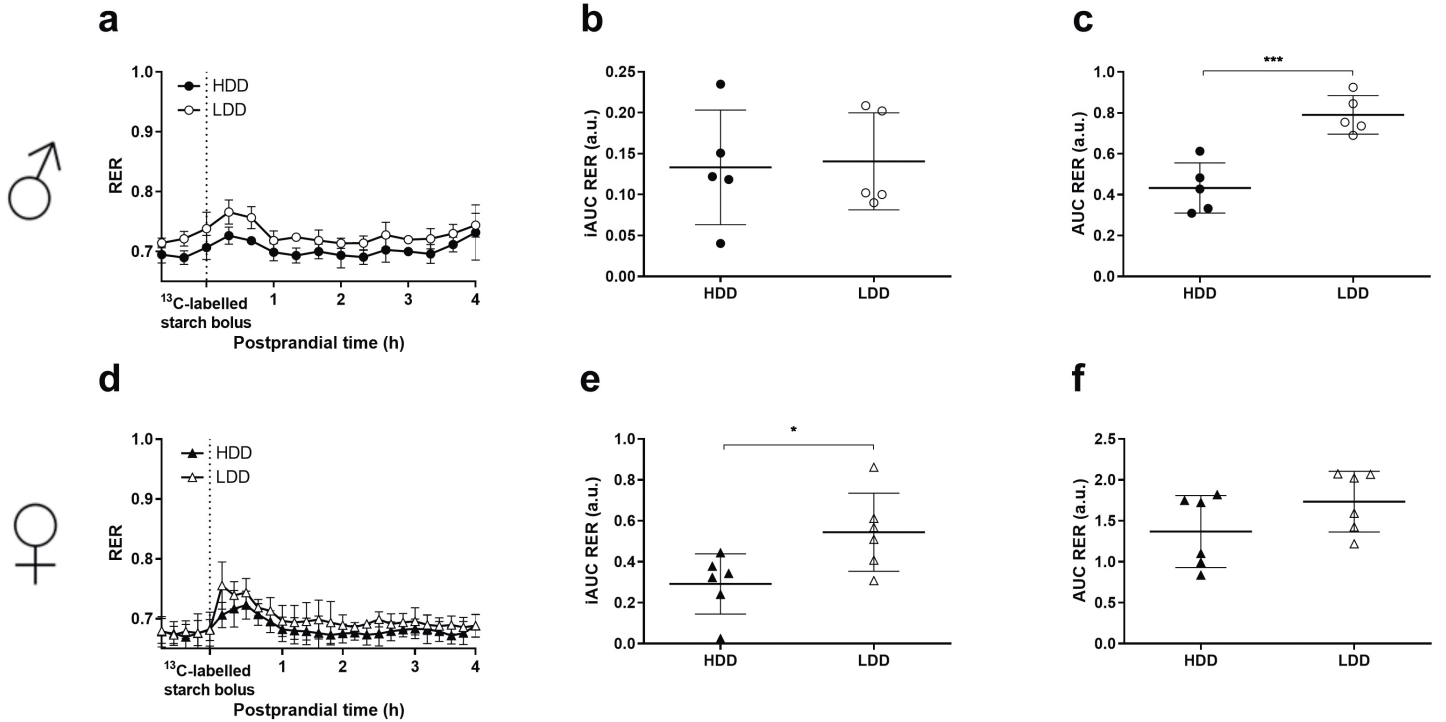


Figure 2. Whole-body substrate oxidation after oral administration of a ^{13}C-labelled starch bolus to HDD and LDD mice in PW 4. (**a, d**) RER evolution after administration of the starch bolus. (**b, e**) iAUC and (**c, f**) AUC of the RER response over 4 hours from administration of the bolus. (**a-c**) Males (n = 5), (**d-f**) females (n = 6). Data is presented as mean and SD. Student's t-test, $^{*}P < 0.05$, $^{***}P < 0.001$. HDD, highly digestible-starch diet; iAUC; incremental area under the curve; LDD, lowly digestible-starch diet; PW, post-weaning week; RER, respiratory exchange ratio.

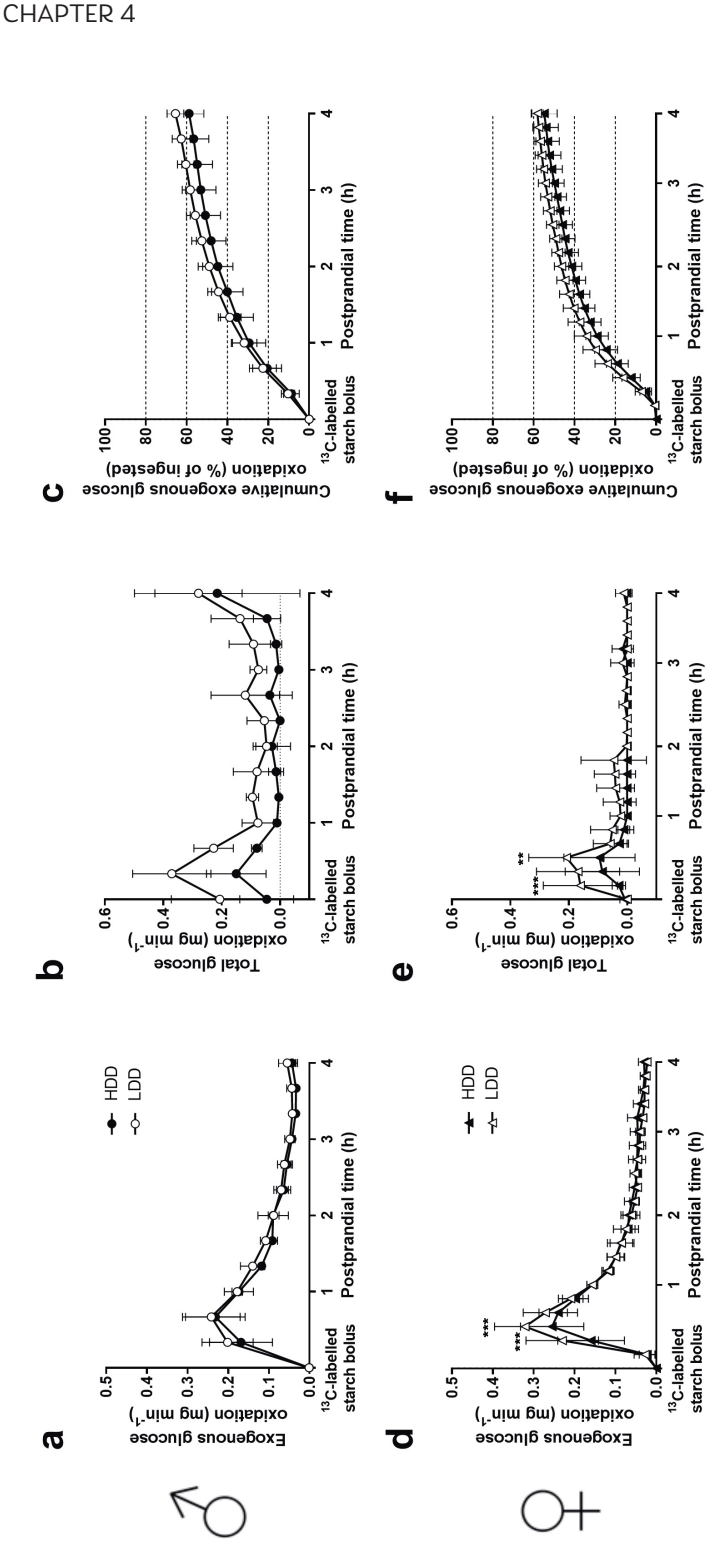


Figure 3. Glucose oxidation kinetics after oral administration of a ^{13}C-labelled starch bolus to HDD and LDD mice in PW 4. (**a, d**) Instantaneous starch-derived glucose oxidation rate. (**b, e**) Total glucose oxidation rate. (**c, f**) Cumulative starch-derived glucose oxidation. (**a-c**) Males (n = 5), (**d-f**) females (n = 6). Data is presented as mean and SD. Bonferroni's *post hoc* test for multiple comparisons, $^{**}P < 0.01$, $^{***}P < 0.001$. HDD, highly digestible-starch diet; LDD, lowly digestible-starch diet; PW, post-weaning week.

Finally, total glucose oxidation rates were initially higher in LDD females (diet × time interaction, $P < 0.05$; post-weaning diet, $P < 0.05$; Figure 3e). These data indicate that LDD females oxidised starch-derived glucose significantly faster than HDD females in the early postprandial phase on consumption of the starch bolus.

Regarding the cumulative amounts of starch-derived glucose oxidised throughout the 4 h after administration of the ^{13}C-starch bolus, LDD males oxidised more starch over time than HDD males (diet × time interaction, $P < 0.01$; Figure 3c), while this effect was not observed in females (Figure 3f). A comparison of the total amount oxidised at 4 h (as percentage of dose administered) across sexes and experimental diets showed significant main effects of sex ($P < 0.05$) and diet ($P < 0.05$), indicating that males oxidised starch-derived glucose more extensively than females and that overall LDD mice oxidised more exogenous glucose than HDD mice.

Intestinal amylase activity and hepatic ^{13}C label deposition in females

Two additional analyses were done in the females. First, we tested carbohydrate digestion capacity as a tentative explanation for the higher starch-derived glucose oxidation rates of LDD females. Amylase levels in small intestinal contents collected immediately before and 4 h after administration of the ^{13}C starch bolus showed that LDD mice had lower pancreatic amylase levels compared to HDD mice, as indicated by an overall effect of diet ($P < 0.001$; Figure 4a). However, after accounting for the larger amount of small intestinal contents in LDD mice (diet, $P < 0.05$; Figure 4b), there were not clear differences in pancreatic amylase levels between LDD and HDD females (Figure 4c). Secondly, we examined whether an increased flux of starch-derived glucose to liver could explain the higher oxidation rates seen in LDD females. Before ingestion of the ^{13}C starch bolus, total ^{13}C enrichment in the liver was marginally higher in LDD compared to HDD females [1.0852 (SD 0.0001) *vs* 1.0845 (SD 0.0004) atom%, respectively; $P = 0.053$]. Four hours after administration of the starch bolus, LDD females tended to have a higher deposition of the ^{13}C label in liver above baseline compared to HDD females ($P = 0.09$, Figure 5). This represented 6.5 (SD 1.9) and 5.1 (SD 2.2) % of the ^{13}C label administered to LDD and HDD females, respectively.

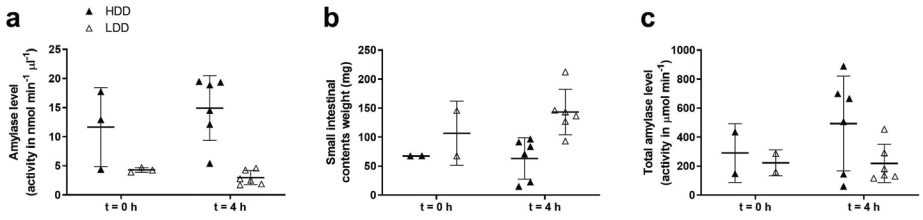


Figure 4. (**a**) Amylase levels, assayed as activity, per unit of small intestinal contents from HDD and LDD females in PW 4, immediately before (t = 0 h; $n = 3$) and 4 h after (t = 4 h; $n = 6$) oral administration of a ^{13}C-labelled starch bolus. (**b**) Total weight of small intestinal contents from females in panel (**a**); note 1 missing value in each group (t = 0 h) that failed to be recorded. (**c**) Estimated total amylase activity in the entirety of the small intestinal contents, based on data from panels (**a**) and (**b**). Data is presented as mean and SD. Student's *t*-test. HDD, highly digestible-starch diet; LDD, lowly digestible-starch diet; PW, post-weaning week.

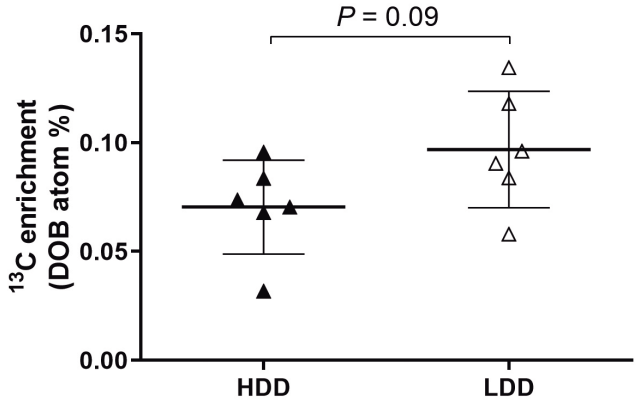


Figure 5. Total ^{13}C enrichment in liver tissue from HDD and LDD females 4 h after oral administration of a ^{13}C-labelled starch bolus in PW 4. Data (n = 6) is expressed relative to females that did not receive the ^{13}C starch bolus (n = 3) and presented as mean and SD. Student's t-test. DOB, delta over baseline; HDD, highly digestible-starch diet; LDD, lowly digestible-starch diet; PW, post-weaning week.

Discussion

We show that female mice exposed for three weeks to an LDD in the immediate post-weaning period developed an increased capacity to oxidise exogenous starch-derived glucose compared to females who consumed an HDD, even in light of the unaffected BW and BC by the type of starch. Moreover, the effect of LDD on starch-derived glucose oxidation was less pronounced in male mice, confirming that males and females respond differently to dietary starches.

An important advantage in our study was the use of $^{13}CO_2$ enrichment analysis combined with conventional indirect calorimetry (InCa). It has been shown previously that adult male mice fed a low glycaemic index diet for three weeks have a better capacity only for oxidisation of ingested fat compared to mice on a high glycaemic index diet[10]. The authors concluded that exogenous glucose oxidation is unaffected by prior exposure to starches of different digestibility, but this was based on a challenge with pure glucose, assessed only qualitatively (by measurement of only $^{13}CO_2$ enrichment, but not of total CO_2 production volumes), and the study did not include female mice. We therefore considered it important to focus on the oxidation of the starch molecule in both sexes using a quantitative method. While RER data alone is a well-founded approach to substrate oxidation analysis, the precise distinction of exogenous *vs* endogenous fuels can only be achieved by including $^{13}CO_2$ analysis together with total CO_2 determination. This method was particularly useful given the uncertainty about the true nature of the fuels oxidised in the fasted state prior to the administration of the ^{13}C starch bolus, as discussed below. Furthermore, complementing the analysis of exogenous glucose oxidation rates with O_2 measurements revealed that the starch bolus affected metabolic rate in LDD and HDD males differently (Figure S3a), offsetting the marginally higher $^{13}CO_2$ enrichment in LDD males (Figure S2a) and ultimately explaining the similar exogenous glucose oxidation rates in LDD *vs* HDD males (Figure 3a). Additionally, it was now possible to quantify the total amounts of starch

oxidised over the four-hour postprandial period, and this showed that post-weaning LDD increased the oxidative disposal of starch in both sexes. Thus, we verified that the three-week exposure to LDD and HDD in males had only a minor impact on the oxidation kinetics of ingested starch, despite total carbohydrate oxidation (calculated from RER) remaining higher throughout the starch bolus challenge and the final quantities of starch oxidised being higher in LDD *vs* HDD males.

We confirmed that consuming lowly digestible starches gives lower RER responses compared to highly digestible starches, as was previously seen in mice[21,22] and similar to the response to a low glycaemic index meal in humans[23,24]. A lower RER is generally interpreted as higher fat oxidation, a process that can be stimulated in skeletal muscle and liver by short-chain fatty acids (SCFA) produced from microbial carbohydrate fermentation[2,25]. Oxidation of SCFA could explain why LDD *vs* HDD males in our study oxidised remarkably different fuel mixtures despite both groups being in the fasted state, when whole body maximal FA oxidation would be indicated by lowest RER levels. However, it is somewhat surprising that LDD and HDD females had a similar fasting RER. Caecal and colonic digesta weights before the administration of the starch bolus where higher in LDD mice irrespective of sex (data not shown), but the quantities and fluxes of SCFA were not determined in this study. Speculatively, a similar fasting RER in LDD and HDD females, but not in males, could be attributed to the sexually dimorphic response to short-term fasting, with females favouring lipogenesis from amino acids[26]. Thus, lipogenesis from protein (respiratory quotient = 1.20)[27] could mask the influences of SCFA oxidation or signalling on RER and result in a similar fasting RER in LDD and HDD females.

An increased exogenous starch-derived oxidation could reflect a higher capacity for carbohydrate digestion, with the potential to cause obesity[28]. This is not a likely implication from our study, since luminal amylase levels were not higher in LDD *vs* HDD females, while exogenous glucose oxidation rates were higher in LDD females. Further, the cause of the increased starch derived oxidation remains unclear. About 57% of the ^{13}C label was recovered as $^{13}CO_2$ by 4 h after ingesting the starch bolus, and only 6% was recovered in liver tissue. We assumed the $^{13}CO_2$ to reflect direct splanchnic oxidation of glucose released from digested starch. However, a fraction of the starch may have also been fermented, producing $^{13}CO_2$ from the fermentation itself[29], or from oxidation of ^{13}C-labelled SCFA by the host. This, however, is considered unlikely, because the starch bolus was gelatinised by heating in water, a process that facilitates digestion by amylase[30], and because H_2 production did not accompany $^{13}CO_2$ appearance (as observed in humans consuming resistant starch[31]) in our study. Since liver lipid content is especially susceptible to interventions with starches[10,32,33], differences in hepatic deposition of the ^{13}C label in LDD vs HDD females could have provided an alternative explanation. Although we observed a trend in LDD females to have a higher total ^{13}C enrichment, the amount of label recovered in the liver was only 6%. Further interpretation is subject to knowing the exact metabolites that are enriched in this compartment, which could be mainly glycogen or, alternatively, triglycerides synthesised from starch-derived glucose. An explanation may also be provided by the fate of the remaining 37% of the ^{13}C label. This may be differently distributed in other organs in LDD and HDD mice. For instance, higher insulin-stimulated

glucose uptake[32], and higher glucose oxidation and lower synthesis of lipids from glucose[34], have all been observed in primary adipocytes isolated from epididymal WAT after dietary interventions with lowly *vs* highly digestible starches.

Shared responses to LDD *vs* HDD in both females and males included increased energy intake and higher H_2 production (Table 2 and Figure 1) by LDD, confirming previous findings of H_2 production in a similar setting[12]. Moreover, it confirmed that the digestible energy density between the LDD and the HDD was different and that a larger proportion of the carbohydrate fraction in the diet was utilised by the gut microbiota upon LDD feeding. In addition, we have shown that the changes in bacterial community structure after three weeks on LDD *vs* HDD were not significantly affected by sex[12]. These common responses between sexes are in stark contrast with the higher capacity to oxidize exogenous starch seen in females LDD *vs* HDD. It is tempting to speculate that this sexually dimorphic response to starch may be associated with the higher insulin sensitivity of females[35,36] and perhaps mediated by the gut microbiota, since some gut microbial metabolites may be processed differently by females and males[37].

All in all, the use of InCa with additional gas sensors ($^{13}CO_2$, $^{12}CO_2$, and H_2) helped us recognise important effects of starch digestibility shared by females and males and those that are sex-dependent, and is testament to the value of extended InCa systems for the refinement of animal research. Further, the absence of significant effects of LDD *vs* HDD on BW and BC, and on H_2 production, amylase levels, and total ^{13}C label deposition in liver after ingestion of a starch bolus, suggested that the higher capacity of LDD females to oxidise starch stems from differences in hepatocellular metabolism or may lie in other organ systems.

Conclusions

Female mice fed a lowly digestible starch post-weaning diet developed a better capacity to oxidise starch-derived glucose compared to females on a highly digestible starch diet. This effect was only marginal in male mice. Our results suggest that starch digestibility could have different consequences for metabolic health in females and males and should be taken into account when formulating health recommendations for carbohydrate quality.

Author Contributions

J.M.S.F.-C., J.K., and E.M.v.S. conceptualised the work and designed the studies. J.M.S.F.-C. and E.M.v.S. obtained ethical approval for the mouse experiment. L.M.S.B. and H.J.M.S. contributed to the planning and preparation of the mouse experiment. N.B. contributed to the design of the ^{13}C starch bolus. J.M.S.F.-C. acquired the data. J.M.S.F.-C., J.K., and E.M.v.S. analysed and interpreted the data. J.M.S.F.-C. drafted the manuscript and L.M.S.B., N.B., A.O., J.K., and E.M.v.S. revised it critically for important intellectual content. All authors approved the final version of the manuscript.

Acknowledgements

We thank Ries de Visser (IsoLife) for his technical advice regarding the use of ^{13}C-labelled starch. We also thank MSc student Farya Mubarik and the personnel at the CARUS animal facility for their assistance in mouse care-taking.

References

1 Campbell, G. J., Senior, A. M. & Bell-Anderson, K. S. Metabolic effects of high glycaemic index diets: a systematic review and meta-analysis of feeding studies in mice and rats. *Nutrients* **9**, 646 (2017).

2 Higgins, J. A. *et al.* Resistant starch consumption promotes lipid oxidation. *Nutr. Metab.* **1**, 8 (2004).

3 Johnston, K. L., Thomas, E. L., Bell, J. D., Frost, G. S. & Robertson, M. D. Resistant starch improves insulin sensitivity in metabolic syndrome. *Diabet. Med.* **27**, 391-397 (2010).

4 Robertson, M. D., Bickerton, A. S., Dennis, A. L., Vidal, H. & Frayn, K. N. Insulin-sensitizing effects of dietary resistant starch and effects on skeletal muscle and adipose tissue metabolism. *Am. J. Clin. Nutr.* **82**, 559-567 (2005).

5 Zhang, L. *et al.* Metabolic phenotypes and the gut microbiota in response to dietary resistant starch type 2 in normal-weight subjects: a randomized crossover trial. *Sci. Rep.* **9**, 4736 (2019).

6 Robertson, M. D. Dietary-resistant starch and glucose metabolism. *Curr. Opin. Clin. Nutr. Metab. Care* **15**, 362-367 (2012).

7 Peterson, C. M. *et al.* Effect of 12 wk of resistant starch supplementation on cardiometabolic risk factors in adults with prediabetes: a randomized controlled trial. *Am. J. Clin. Nutr.* **108**, 492-501 (2018).

8 Robertson, M. D., Livesey, G. & Mathers, J. C. Quantitative kinetics of glucose appearance and disposal following a C-labelled starch-rich meal: comparison of male and female subjects. *Brit. J. Nutr.* **87**, 569 (2007).

9 Fernandez-Calleja, J. M. S. *et al.* Direct and long-term metabolic consequences of lowly vs. highly-digestible starch in the early post-weaning diet of mice. *Nutrients* **10**, 1788 (2018).

10 Isken, F. *et al.* Impairment of fat oxidation under high- vs. low-glycemic index diet occurs before the development of an obese phenotype. *Am. J. Physiol. Endocrinol. Metab.* **298**, E287-295 (2010).

11 Hoevenaars, F. P. *et al.* BIOCLAIMS standard diet (BIOsd): a reference diet for nutritional physiology. *Genes Nutr.* **7**, 399-404 (2012).

12 Fernández-Calleja, J. M. S. *et al.* Non-invasive continuous real-time in vivo analysis of microbial hydrogen production shows adaptation to fermentable carbohydrates in mice. *Sci. Rep.* **8**, 15351 (2018).

13 Duivenvoorde, L. P. *et al.* A difference in fatty acid composition of isocaloric high-fat diets alters metabolic flexibility in male C57BL/6JOlaHsd mice. *PLoS One* **10**, e0128515 (2015).

14 Peronnet, F. & Massicotte, D. Table of nonprotein respiratory quotient: an update. *Can. J. Sport Sci.* **16**, 23-29 (1991).

15 Delarue, J. *et al.* The contribution of naturally labelled ^{13}C fructose to glucose appearance in humans. *Diabetologia* **36**, 338-345 (1993).

16 Englyst, H. N., Kingman, S. M. & Cummings, J. H. Classification and measurement of nutritionally important starch fractions. *Eur. J. Clin. Nutr.* **46 Suppl 2**, S33-50 (1992).

17 Petersen, M. C., Vatner, D. F. & Shulman, G. I. Regulation of hepatic glucose metabolism in health and disease. *Nat. Rev. Endocrinol.* **13**, 572-587 (2017).

18 Casado, J., Fernandez-Lopez, J. A., Argiles, M. J. & Alemany, M. Role of the rat liver in the disposal of a glucose gavage. *Mol. Cell. Biochem.* **113**, 33-41 (1992).

19 Warner, D., Ferreira, L. M., Breuer, M. J., Dijkstra, J. & Pellikaan, W. F. Stable isotope labeled *n*-alkanes to assess digesta passage kinetics through the digestive tract of ruminants. *PLoS One* **8**, e75496 (2013).

20 Faul, F., Erdfelder, E., Lang, A.-G. & Buchner, A. G*Power 3: a flexible statistical power analysis program for the social, behavioral, and biomedical sciences. *Behav. Res. Methods* **39**, 175-191 (2007).

21 Scribner, K. B., Pawlak, D. B., Aubin, C. M., Majzoub, J. A. & Ludwig, D. S. Long-term effects of dietary glycemic index on adiposity, energy metabolism, and physical activity in mice. *Am. J. Physiol. Endocrinol. Metab.* **295**, E1126-1131 (2008).

22 Zhou, J. *et al.* Failure to ferment dietary resistant starch in specific mouse models of obesity results in no body fat loss. *J. Agric. Food Chem.* **57**, 8844-8851 (2009).

23 Clapp, J. F. & Lopez, B. Low-versus high-glycemic index diets in women: effects on caloric requirement, substrate utilization and insulin sensitivity. *Metab. Syndr. Relat. Disord.* **5**, 231-242 (2007).

24 Kaur, B., Quek Yu Chin, K., Camps, S. & Henry, C. J. The impact of a low glycaemic index (GI) diet on simultaneous measurements of blood glucose and fat oxidation: a whole body calorimetric study. *J. Clin. Transl. Endocrinol.* **4**, 45-52 (2016).

25 Canfora, E. E., Meex, K. C. R., Venema, K. & Blaak, E. E. Gut microbial metabolites in obesity, NAFLD and T2DM. *Nat. Rev. Endocrinol.* **15**, 261-273 (2019).

26 Della Torre, S. *et al.* Short-term fasting reveals amino acid metabolism as a major sex-discriminating factor in the liver. *Cell Metab.* **28**, 256-267 e255 (2018).

27 Ferrannini, E. The theoretical bases of indirect calorimetry: A review. *Metabolism* **37**, 287-301 (1988).

28 Azzout-Marniche, D. *et al.* High pancreatic amylase expression promotes adiposity in obesity-prone carbohydrate-sensitive rats. *J. Nutr.* (2019).

29 Miller, T. L. & Wolin, M. J. Fermentations by saccharolytic intestinal bacteria. *Am. J. Clin. Nutr.* **32**, 164-172 (1979).

30 Englyst, K. N. & Englyst, H. N. Carbohydrate bioavailability. *Brit. J. Nutr.* **94**, 1 (2007).

31 Symonds, E. L., Kritas, S., Omari, T. I. & Butler, R. N. A combined $^{13}CO_2/H_2$ breath test can be used to assess starch digestion and fermentation in humans. *J. Nutr.* **134**, 1193-1196 (2004).

32 So, P. W. *et al.* Impact of resistant starch on body fat patterning and central appetite regulation. *PLoS One* **2**, e1309 (2007).

33 van Schothorst, E. M., Bunschoten, A., Schrauwen, P., Mensink, R. P. & Keijer, J. Effects of a high-fat, low- versus high-glycemic index diet: retardation of insulin resistance involves adipose tissue modulation. *FASEB J.* **23**, 1092-1101 (2009).

34 Kabir, M. *et al.* Dietary amylose-amylopectin starch content affects glucose and lipid metabolism in adipocytes of normal and diabetic rats. *J. Nutr.* **128**, 35-43 (1998).

35 Ingvorsen, C., Karp, N. A. & Lelliott, C. J. The role of sex and body weight on the metabolic effects of high-fat diet in C57BL/6N mice. *Nutr. Diabetes* **7**, e261 (2017).

36 Chen, X., McClusky, R., Itoh, Y., Reue, K. & Arnold, A. P. X and Y chromosome complement influence adiposity and metabolism in mice. *Endocrinology* **154**, 1092-1104 (2013).

37 Link, J. C. & Reue, K. Genetic basis for sex differences in obesity and lipid metabolism. *Annu. Rev. Nutr.* **37**, 225-245 (2017).

Supplementary Information

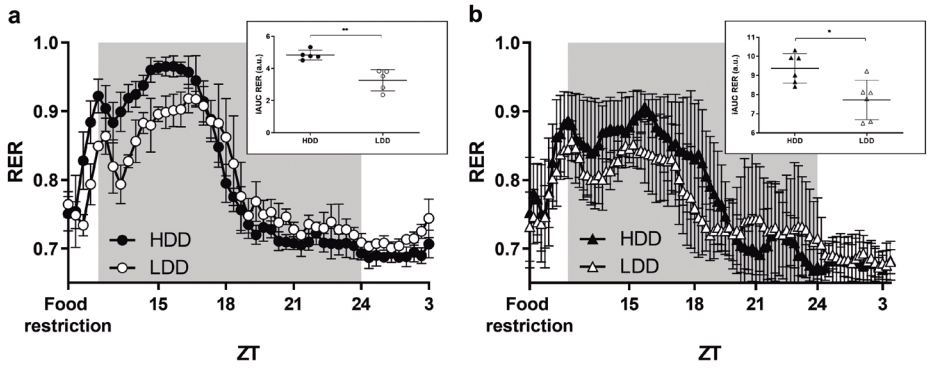


Figure S1. Whole-body substrate oxidation during food restriction in HDD and LDD mice in PW 4. (**a**) Males (*n* = 5), (**b**) females (*n* = 6). Approximately 2 h before DP, all mice received a single 1.2 g pellet of HDD or LDD, respectively ("food restriction"). Shaded areas represent the dark phase. Insets in panels (**a**) and (**b**) show the iAUC of the RER response from the moment of food restriction until just before the administration of the ^{13}C starch bolus. Data is presented as mean and SD. Student's *t*-test, $^{*}P < 0.05$, $^{**}P < 0.005$. DP, dark phase; HDD, highly digestible-starch diet; iAUC; incremental area under the curve; LDD, lowly digestible-starch diet; PW, post-weaning-week; ZT, Zeitgeber time.

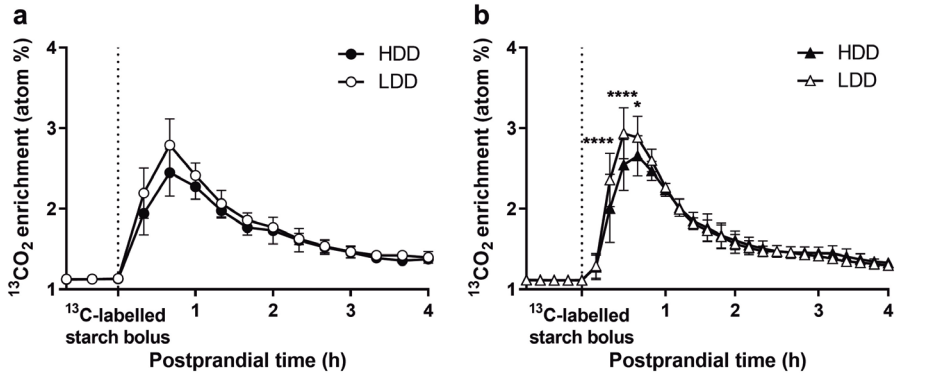


Figure S2. Instantaneous recovery of ^{13}C label as $^{13}CO_2$ after oral administration of a ^{13}C-labelled starch bolus to HDD and LDD mice in PW 4. (a) Males (*n* = 5), (b) females (*n* = 6). Data is presented as mean and SD. Bonferroni's *post hoc* test for multiple comparisons, $^{*}P < 0.05$, $^{****}P < 0.0001$. HDD, highly digestible-starch diet; LDD, lowly digestible-starch diet; PW, post-weaning week.

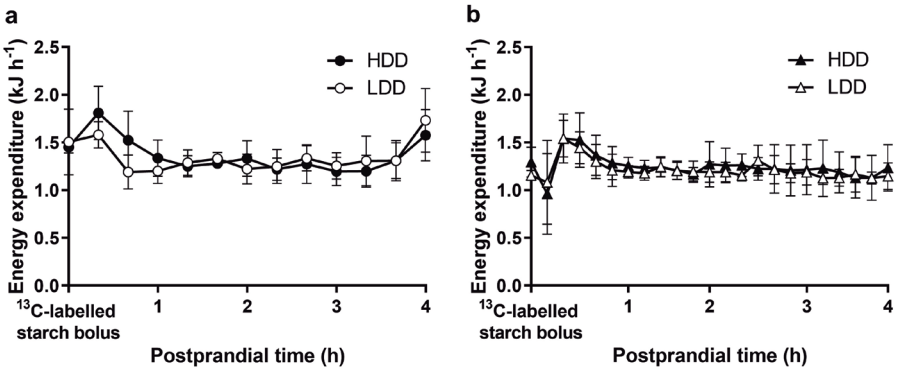


Figure S3. Energy expenditure after oral administration of a ^{13}C-labelled starch bolus to HDD and LDD mice in PW 4. (a) Males (n = 5), (b) females (n = 6). Data is presented as mean and SD. HDD, highly digestible-starch diet; LDD, lowly digestible-starch diet; PW, post-weaning week.

Chapter 5

Direct and Long-Term Metabolic Consequences of Lowly vs. Highly-Digestible Starch in the Early Post-Weaning Diet of Mice

José M. S. Fernández-Calleja

Lianne M. S. Bouwman

Hans J. M. Swarts

Annemarie Oosting

Jaap Keijer

Evert M. van Schothorst

Nutrients (2018) 10:1788 DOI: 10.3390/nu10111788

Abstract

Starches of low and high digestibility have different metabolic effects. Here, we examined whether this gives differential metabolic programming when fed in the immediate post-weaning period. Chow-fed mice were time-mated, and their nests were standardized and cross-fostered at postnatal days 1–2. After postnatal week (PW) 3, individually housed female and male offspring were switched to a lowly-digestible (LDD) or highly-digestible starch diet (HDD) for three weeks. All of the mice received the same high-fat diet (HFD) for nine weeks thereafter. Energy and substrate metabolism and carbohydrate fermentation were studied at the end of the HDD/LDD and HFD periods by extended indirect calorimetry. Glucose tolerance (PW 11) and metabolic flexibility (PW14) were analyzed. Directly in response to the LDD versus the HDD, females showed smaller adipocytes with less crown-like structures in gonadal white adipose tissue, while males had a lower fat mass and higher whole body fat oxidation levels. Both LDD-fed females and males showed an enlarged intestinal tract. Although most of the phenotypical differences disappeared in adulthood in both sexes, females exposed to LDD versus HDD in the early post-weaning period showed improved metabolic flexibility in adulthood. Cumulatively, these results suggest that the type of starch introduced after weaning could, at least in females, program later-life health.

Keywords: Glycemic index, nutrition, amylose, amylopectin, carbohydrates, C57BL mice, sexual dimorphism, indirect calorimetry, adipose tissue, metabolic flexibility.

Introduction

Early life experiences in critical periods during prenatal and postnatal development have the potential to program metabolic health later in life. While early-life nutrition has been identified as a major environmental condition inducing long-lasting effects in the organism, the optimal diet to promote a healthy life from conception to adulthood is still ill-defined. Much emphasis has been placed on nutritional interventions prenatally and during infancy, since this is considered the period of maximal developmental plasticity. However, it is recognized that the critical development period extends after infancy in some organs and systems[1].

Early life exposure to different qualities and quantities of protein and lipids has been shown to have a lasting impact on adult metabolic health[2-5]. Dietary carbohydrates may also have a role in programming of later-life metabolic health, as both quality and quantity could provide cues for disease development, treatment, and management. A high intake of low glycemic index (GI) foods is associated with improved health outcomes in both adults and children[6,7]. Using highly defined diets, with only the type of starch being different, we have previously shown that a low versus high GI diet delayed obesity-associated disease development in adult mice[8]. Moreover, the low versus high GI diet induced intestinal microbiota hydrogen production in young and adult mice[9]. Thus, the digestibility of starches provides them with different nutritional properties for both the host and the intestinal microbiota through fermentation[10].

The introduction of solid foods to gradually replace breast milk—or weaning—is a crucial period in the life course. In humans, this period also represents the transition from a high-fat to a high-carbohydrate content in the diet[11]. Importantly, it is during this transition that decisive interactions between the organism and the gut microbiota are being established[12]. Current evidence-based recommendations for complementary feeding are mainly focused on the time of introduction of allergenic foods and solids, with particular attention to protein and fat[13]. However, the rationale for choosing carbohydrates in complementary foods is only based on the development of taste preferences and the prevention of caries[13]. Clearly, carbohydrate intake during early life should also be examined from a metabolic health perspective[14].

The recommendations of the joint Food and Agriculture Organization of the United Nations and the World Health Organization (FAO/WHO) expert consultation for carbohydrate intake are virtually the same for all individuals over two years of age{, #1267;Food, 1998 #1349}[15], and did not substantially change over the course of 10 years due to limited new data. Rodent models are instrumental in developmental programming research due to short gestation and maturity periods and the possibility of exploring molecular mechanisms in ways that would be impractical or unethical in humans[16].

The strongest evidence for programming by early-life carbohydrates has been obtained from studies in rats fed a high-carbohydrate milk formula during the suckling period[17]. Another stepping stone in this area is the work of Gugusheff *et al.*[18], which suggested that starches of different digestibility consumed by dams during the perinatal period as well as their

offspring until early adulthood had long-term consequences for metabolic health. However, due to the study design, it was impossible to distinguish between the direct effects and metabolic programming effects, as offspring consumed these intervention diets until the end of the study.

Several rodent studies incorporating the post-weaning period of growth and development into the programming model have demonstrated metabolic programming by dietary lipids[19,20], protein[21], calcium[22], and the fat:carbohydrate ratio[23], and a lack of differential programming effects in the case of glucose versus fructose[24].

In this study, we investigated the long-term effects of starches of different digestibility consumed only during the specific window from weaning until mid-adolescence, on adult metabolic health. We focused on the general aspects of the resulting phenotypes, with an emphasis on metabolic function, *e.g.*, body composition development and whole body metabolism, in both female and male mice. We hypothesized that a lowly-digestible starch post-weaning diet would be protective against the metabolic impairment induced by a high-fat diet during adulthood. We concluded that the early post-weaning period is indeed amenable to metabolic programming by dietary starches, since females consuming a lowly-digestible versus highly-digestible starch diet in the early post-weaning period had a better metabolic flexibility in adulthood.

Materials and Methods

Animal model

The study was approved by the Animal Experiment Committee of Wageningen University (DEC 2014085) and performed in accordance to European Union (EU) directives 86/609/EEC and 2010/63/EU. All of the mice (C57BL/6JRccHsd; Harlan Laboratories BV, Horst, The Netherlands) were housed in polycarbonate type II cages enriched with wood chips and wood shavings, with free access to drinking water and food, at 23 ± 1 °C, 50 ± 5% humidity, on a 12-h light/dark cycle. A schematic overview of the study design is shown in Figure 1. Female and male mice (17–19 weeks old) were fed standard rodent chow (26% w/w protein, 38.8% w/w starch, 4.6% w/w sugar, 6.5% w/w fat; AM-II, AB Diets, Woerden, The Netherlands) and time-mated. At postnatal days 1–2, offspring were redistributed across foster dams to produce standardized litters of six pups and a sex ratio of 3:3 or 4:2. At the end of postnatal week (PW 3), all of the mice were housed individually and assigned either a highly-digestible starch diet (HDD; Research Diet Services, Wijk bij Duurstede, The Netherlands; details are described below) or a lowly-digestible starch diet (LDD; Research Diet Services) stratified according to body weight (BW; n = 24 per sex and diet; one male on LDD was excluded due to incisor malocclusion). At the end of PW 6, a subgroup of mice of each sex and dietary group was sacrificed and white adipose tissue (WAT) from gonadal and mesenteric origin, liver, pancreas, and intestine and its contents, were dissected, snap-frozen in liquid nitrogen, and stored at −80 °C until further analysis. The remaining mice (n = 12 per sex and diet) were switched to a high-fat diet (HFD, Research Diet Services BV) and continued on this diet until sacrifice in PW 15. Food intake (FI) was determined weekly. BW and body composition (BC; EchoMRI 100V, EchoMedical Systems, Houston,

TX, USA) were determined weekly from PW 4–6 and biweekly from PW 7–15. Two indirect calorimetry (InCa) measurements (PW 6 and PW 14) and an oral glucose tolerance test (OGTT; PW 11) were carried out as described below.

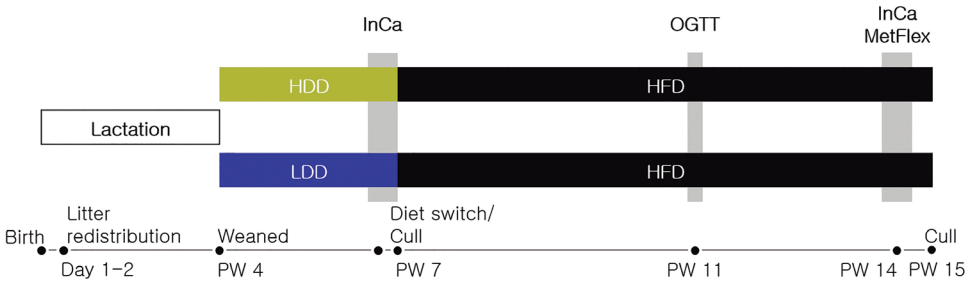


Figure 1. Experimental design. Female and male mice fed standard rodent chow were time-mated and their offspring were redistributed at postnatal day 1–2 to standardized nests. At the end of postnatal week (PW) 3, after weaning, the pups were individually housed and fed either a highly-digestible starch diet (HDD) or a lowly-digestible starch diet (LDD). In PW 6, all of the mice had their basal metabolic phenotype assessed by indirect calorimetry (InCa). A subgroup of animals of each diet and sex was dissected at the end of PW 6 to further assess the direct effects of the post-weaning dietary intervention. The remaining mice were switched to a high-fat diet (HFD) from PW 7 onwards to study metabolic programming in an obesogenic environment. In PW 11, mice underwent an oral glucose tolerance test (OGTT). At the end of HFD-feeding, basal metabolism was measured, and metabolic flexibility (MetFlex) was assessed by InCa. Mice were culled at the end of PW 15, and their blood and tissues were harvested for further analysis.

Experimental diets

All of the experimental diets were based on the BIOCLAIMS standard diet[25]. Both HDD and LDD contained 20 energy percentage (en%) protein, 55 en% carbohydrates, and 25 en% fat, with highly-digestible or lowly-digestible starches as the sole difference and source of available carbohydrate (Cargill, Sas van Gent, The Netherlands; incorporated into pelleted diets by Research Diet Services), as published[9]. The HFD contained 20 en% protein, 40 en% carbohydrates, and 40 en% fat[26]. Detailed diet formulations are shown in Table 1.

Oral glucose tolerance test

An OGTT was performed five hours after food withdrawal in PW 11 by the administration of glucose (2 g kg^{-1} BW) by oral gavage as published[24].

Indirect calorimetry (InCa) and metabolic flexibility

The general procedure for indirect calorimetry measurements has been described previously[24], with minor adjustments. After an 18-h adaptation period, the energy expenditure (EE), respiratory exchange ratio (RER), locomotor activity, and food intake were measured in a PhenoMaster indirect calorimetry system (TSE Systems GmbH, Bad Homburg, Germany), which was extended with hydrogen (H_2) and methane sensors for real-time measurements of intestinal microbial fermentation[9]. Uncorrected EE values were

used, since lean mass (LM) was not significantly different between dietary groups directly before or after each InCa period. To assess metabolic flexibility (PW 14), mice were fed a restricted amount of HFD (1.1 g, which is equivalent to about 55% of average food intake during the dark phase) two hours prior to the dark phase to induce a fasting state the next morning. Approximately one hour before the following dark phase, all of the mice were given a meal challenge (HDD) *ad libitum*, and measurements continued until the following light phase. The switch from predominantly fat oxidation (RER = 0.7) toward net carbohydrate oxidation (RER = 1.0) upon refeeding was used as a measure of metabolic flexibility[27]. A selection of data obtained from animals at PW 6 has been previously reported: EE, RER, and H_2 production[9].

Table 1. Composition of the experimental diets.

	HDD	LDD	HFD
Casein	212.2	212.0	233.5
L-Cysteine	3.0	3.0	3.0
Amylose mix (AmyloGel 03003) [1]	0.0	568.6	0.0
Amylopectin (C*Gel 04201) [2]	568.6	0.0	0.0
Wheat starch	0.0	0.0	285.6
Maltodextrin	0.0	0.0	100.0
Glucose	0.0	0.0	70.0
Coconut oil	21.4	21.4	0.0
Sunflower oil	83.1	83.1	0.0
Flaxseed oil	14.2	14.2	4.0
Palm oil	0.0	0.0	206.3
Cholesterol	0.03	0.03	0.097
Cellulose	50.0	50.0	50.0
Mineral mix (AIN-93G-MX)	35.0	35.0	35.0
Vitamin mix (AIN-93-VX)	10.0	10.0	10.0
Choline bitartrate	2.5	2.5	2.5
Total (g)	1000.0	1000.0	1000.0
Gross energy density (kJ g^{1}) [3]	18.9	19.5	20.8
Calculated energy density (kJ g^{-1}) [4]	17.9	17.9	19.8
Protein (energy%)	20.1	20.1	20.0
Carbohydrate (energy%)	54.9	54.9	40.0
Fat (energy%)	25.0	25.0	40.0

All values are in g kg^{-1} unless indicated. [1] 60% amylose, 40% amylopectin (Cargill). [2] 100% amylopectin (Cargill BV). [3] Determined by bomb calorimetry. [4] Calculated based on Atwater's nutritional values. HDD: highly-digestible starch diet; HFD: high-fat diet; LDD: lowly-digestible starch diet.

Sacrifice

At the end of PW 6 and PW 15, mice were deprived of food at the onset of the light phase and decapitated two to six hours thereafter. Blood glucose was measured in duplicate with

a Freestyle glucose meter (Abbott Diabetes Care, Hoofddorp, The Netherlands). Whole blood was collected in chilled MiniCollect serum tubes (Greiner Bio-One BV, Alphen aan de Rijn, The Netherlands), spun down at 4 °C for 10 min at 3000× *g*, and the resulting serum aliquoted and stored at −80 °C. Liver, mesenteric white adipose tissue (mWAT), and pancreas were weighed and snap-frozen in liquid nitrogen. A ~2 g clip was attached to the distal end of the small intestine and hung next to a ruler to determine the length of the small intestine. Thereafter, the small intestine and colon were each cut longitudinally, rinsed in ice-cold RNase-free phosphate-buffered saline to remove their contents, and weighed separately. Caecum contents were extracted, weighed, and snap-frozen. One pad of gonadal white adipose tissue (gWAT) was snap-frozen; the other pad was weighed, fixated in 4% paraformaldehyde overnight, and embedded in paraffin. Samples were stored at −80 °C until further analysis.

Serum measurements

Serum levels of insulin, leptin, and adiponectin were determined as described using commercial kits[24].

Hepatic triglycerides and glycogen content

Hepatic triglycerides were determined using a commercial kit as described[24]. Part of the same liver lobe was used for glycogen determination based on published protocol[28] with the following minor adaptations: protein-free and lipid-free extracts were obtained by homogenization of ~100 mg of liver tissue in cold 7% $HClO_4$, centrifugation at 4 °C for 15 min at 1500× *g*, and further extraction with petroleum ether. Glycogen concentration in the extracts was determined in triplicate by adding iodine–iodide solution in the presence of $CaCl_2$ (260 µL of reagent added to 10 µL of sample), and measuring absorbance at 460 nm.

Gonadal white adipose tissue (gWAT) histology

The paraffin-embedded gWAT pads of six mice per experimental group were selected to represent the average fat mass (FM) and gWAT weight of the complete group. Paraffin blocks were cut into 5-µm thick slices with 150-µm separation in between sections to ensure different areas within the tissue could be studied. Four to five sections per animal were used to determine the adipocyte area by hematoxylin–eosin (HE) staining, and the number of macrophages and crown-like structures (CLS, MAC-2 staining), as published[26,29]. All of the parameters were based on 1000 intact adipocytes per animal. The fluorescence of eosin resulting from HE staining was used to visualize adipocytes with a Leica DM6B microscope equipped with a DFC365FX camera (Leica Microsystems, Wetzlar, Germany), and fluorescent photographs were analyzed using CellProfiler software v. 2.1.1 using the adipocyte pipeline by the Rodeheffer Laboratory to measure cell area[30,31]. Adipocyte diameter was calculated from its area based on a circular shape.

Quantitative real-time reverse-transcription polymerase chain reaction (RT-qPCR)

Total RNA was isolated from gWAT using TRIzol reagent (Invitrogen, Breda, The Netherlands) as described[32], and cDNA was synthesized with the iScript cDNA synthesis kit (Bio-Rad Laboratories, Veenendaal, The Netherlands). The expressions of genes involved in macrophage infiltration (chemokine (C–C motif) ligand 2, *Ccl2*; lectin, galactose binding, soluble 3, *Lgals3*; S100 calcium binding protein A8, *S100a8*), insulin signalling (insulin receptor substrate 2, *Irs2*), and lipid metabolism (fatty acid binding protein 4, *Fabp4*) were analyzed in duplicate by RT-qPCR with iQ SYBR Green Supermix (Bio-Rad). Primers were designed to span exon–exon junctions to prevent the amplification of genomic DNA using the NCBI Primer BLAST tool, and PCR products were run on a gel to confirm amplicon sizes when necessary. Standard curves were constructed with cDNA pooled from all samples, a control containing no cDNA, a negative RT control, and a melt curve at the end of the each run, and included for quality control. In the case of the lowly-expressed *Ccl2*, *Lgals3*, and *S100a8* transcripts, cDNA was pre-amplified for 10 cycles with SsoAdvanced PreAmp Supermix (Bio-Rad) and the corresponding primers, according to the manufacturer's instructions. Full details of all of the primers can be found in Table S1. Normalized gene expression levels were computed with CFX Manager software, v. 3.1. (Bio-Rad) and used for statistical comparisons.

Data analysis

Statistical analyses were performed in GraphPad Prism 5.04 (GraphPad, San Diego, CA, USA), and female and male data were analyzed separately. Data was tested using the D'Agostino and Pearson omnibus for normality. Non-normally distributed data was log-transformed and re-tested for normality. Two-tailed comparisons between two groups were made using unpaired Student's *t*-tests or Mann–Whitney *U*-tests for normally and non-normally distributed data, respectively. Other group comparisons were tested with two-way ANOVA (adipocyte size frequency distribution and macrophage infiltration), with repeated measurements for matched time course data (OGTT, RER, and carbohydrate intake during InCa) and Bonferroni's post hoc test. Correlations analyses were performed using Pearson correlation on normally distributed data and Spearman correlation for non-normally distributed data. The incremental area under the curve (iAUC) of glucose during OGTT was also calculated in Prism. Statistical significance was set at $p < 0.05$ for all of the comparisons.

Results

Direct and long-term effects on body weight and body composition by post-weaning starches

Directly after three weeks of consumption of HDD or LDD (PW 4–6), there was no difference in body weight or lean mass between the two groups (Figure 2a,c; Figure S1a,c,d,f). However, males on the HDD developed more fat mass compared to those fed the LDD in this period (Figure 2c; Figure S1e). This was not seen in female mice (Figure 2a; Figure S1b).

Following the intervention period, all of the mice received nine weeks of HFD feeding. At 15 weeks of age, there were no significant differences seen in body weight or body composition in neither females nor males (Figure 2b,d).

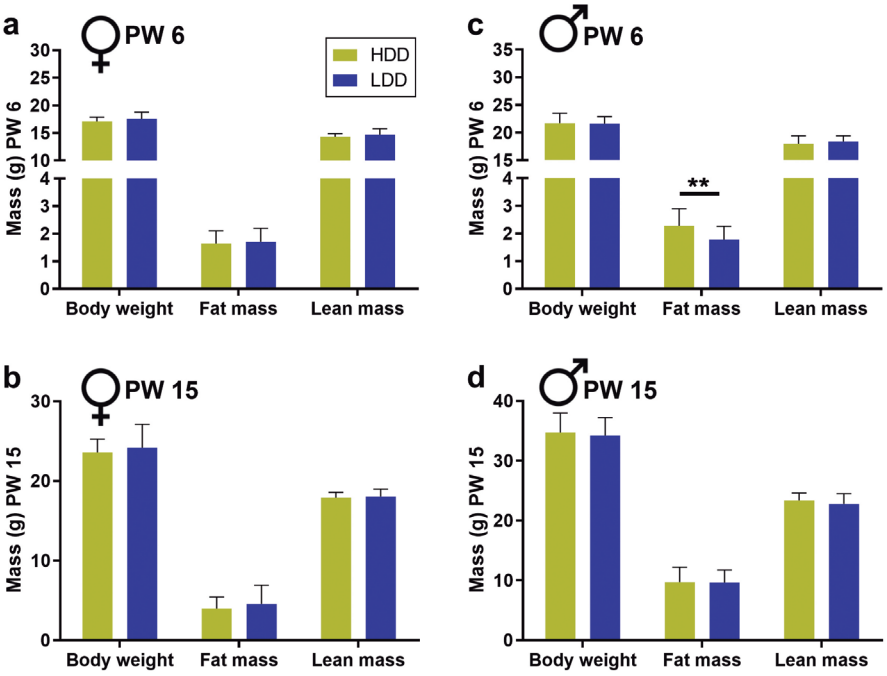


Figure 2. Direct and long-term effects of the type of starch consumed in the early post-weaning period on body weight (BW) and body composition. BW, fat mass (FM), and lean mass (LM) of females directly after exposure to HDD or LDD (**a**; PW 6, *n* = 24 per group), and after nine weeks on a HFD (**b**; PW 15, *n* = 12 per group). The BW, FM, and LM of males after exposure to HDD or LDD (**c**; PW 6, *n* = 24 for HDD and *n* = 23 for LDD), and after nine weeks on a HFD (**d**; PW 15, *n* = 12 per group). Note truncated x-axis in panels **a** and **c** to enhance visualization. Data shown as mean ± standard deviation (s.d.). Statistical differences denoted as ** $p \leq 0.01$.

Direct and long-term effects on basal metabolism

Energy expenditure and locomotor activity were not affected by the type of starch neither at the end of the intervention nor upon HFD feeding, for both females and males (Table 2). However, males consuming the LDD showed a lower RER compared to males fed the HDD (Table 2), indicating increased fat over carbohydrate oxidation. This difference in substrate utilization was completely absent in the females. The effects on basal RER that were seen in males disappeared, and thus were not metabolically programmed at the end of the HFD period (Table 2). This cumulatively suggests that basal metabolism, including fuel utilization, is not programmed by the type of starch consumed in the early post-weaning period either in females or males.

A novel parameter that can be measured using our extended indirect calorimetry system is production of the fermentation gases hydrogen (H_2) and methane[9]. H_2 is exclusively formed by gut microbes as a product of carbohydrate fermentation[33], and as such represents a

Table 2. Basal indirect calorimetry parameters measured at the end of the post-weaning intervention (PW 6) and subsquently eight weeks into HFD feeding (PW 14).

	Females				Males			
	PW 6		PW 14		PW 6		PW 14	
Parameter	HDD	LDD	HDD	LDD	HDD	LDD	HDD	LDD
EE (24 h, kJ h^{-1})	1.60 ± 0.08	1.65 ± 0.09	1.90 ± 0.12	1.90 ± 0.12	1.80 ± 0.12	1.77 ± 0.11	2.11 ± 0.25	2.15 ± 0.25
EE (LP, kJ h^{-1})	1.46 ± 0.08	1.52 ± 0.11	1.78 ± 0.13	1.79 ± 0.11	1.66 ± 0.13	1.61 ± 0.12	2.01 ± 0.23	2.04 ± 0.24
EE (DP, kJ h^{-1})	1.75 ± 0.09	1.78 ± 0.09	2.03 ± 0.13	2.02 ± 0.14	1.95 ± 0.12	1.93 ± 0.10	2.21 ± 0.27	2.27 ± 0.26
RER (24 h)	0.84 ± 0.04	0.84 ± 0.04	0.86 ± 0.04	0.86 ± 0.04	0.88 ± 0.03	0.85 ± 0.03 #	0.85 ± 0.02	0.85 ± 0.02
RER (LP)	0.81 ± 0.05	0.82 ± 0.05	0.86 ± 0.04	0.86 ± 0.04	0.85 ± 0.03	0.82 ± 0.04 *	0.85 ± 0.03	0.86 ± 0.02
RER (DP)	0.87 ± 0.05	0.87 ± 0.04	0.86 ± 0.05	0.86 ± 0.04	0.91 ± 0.03	0.88 ± 0.02 *	0.84 ± 0.02	0.84 ± 0.02
Activity (24 h, counts × 10^4)	6.93 (6.13, 7.45)	6.55 (5.88, 6.72)	5.07 (3.89, 7.07)	4.56 (3.85, 6.23)	4.95 (4.13, 6.06)	5.43 (4.43, 5.70)	2.93 (2.31, 3.81)	2.98 (2.37, 3.51)
Activity (LP, counts × 10^4)	1.46 (1.35, 1.83)	1.46 (1.27, 1.75)	1.36 (1.02, 1.89)	1.03 (0.84, 1.71)	1.4 (1.06, 1.74)	1.10 (0.97, 1.28)	0.80 (0.66, 1.39)	0.84 (0.71, 1.00)
Activity (DP, counts × 10^4)	5.45 (4.52, 5.57)	4.47 (4.32, 5.06)	3.42 (2.81, 5.24)	3.35 (2.80, 4.73)	3.65 (2.99, 4.28)	4.31 (3.37, 4.33)	1.88 (1.56, 2.50)	2.07 (1.64, 2.54)
H_2 (24 h, mL)	0.18 (0.14, 0.26)	1.64 (1.14, 2.12) §	0.24 (0.21, 0.32)	0.34 (0.28, 0.39)	0.24 (0.18, 0.35)	1.47 (1.11, 1.90) §	0.46 (0.36, 0.75)	0.38 (0.32, 0.72)
H_2 (LP, mL)	0.08 (0.06, 0.13)	0.60 (0.46, 0.74) §	0.10 (0.09, 0.16)	0.19 (0.14, 0.20)	0.10 (0.08, 0.14)	0.52 (0.40, 0.61) §	0.21 (0.16, 0.35)	0.18 (0.15, 0.36)
H_2 (DP, mL)	0.11 (0.07, 0.14)	1.07 (0.66, 1.41) §	0.14 (0.11, 0.17)	0.15 (0.14, 0.20)	0.14 (0.09, 0.21)	0.99 (0.67, 1.32) §	0.25 (0.20, 0.40)	0.21 (0.17, 0.36)

For EE and RER, data is presented as mean ± s.d. For activity and H_2, data is shown as median (95% CI of mean), since these values often did not follow a normal distribution. Statistically significant differences compared to the HDD for mice of the same age and sex denoted as * $p \leq 0.05$, # $p \leq 0.01$, and § $p < 0.0001$. DP: dark phase; EE: energy expenditure (averaged per period); LP: light phase; RER: respiratory exchange ratio (average per period); H_2: hydrogen (cumulative volume produced per period).

convenient marker for gut microbiota activity. In line with the known differences in digestibility of 40% amylose and 60% amylopectin compared with 100% amylopectin within the food matrix in vitro and in vivo[9], there were significant differences in H_2 output between mice consuming HDD or LDD in both females and males, with LDD mice producing approximately eight times more H_2 than HDD mice over 24 h (Table 2, ref. [9]). H_2 production was relatively low and similar for all of the mice consuming the HFD (Table 2). At the same time, absolute methane levels being at ambient levels indicated an absence of methane production at both the end of the intervention and HFD-feeding periods in any group.

Direct and long-term effects on other physiological parameters at sacrifice

There were no obvious differences in the weight of metabolic organs and circulating parameters after the early post-weaning intervention or at the end of the HFD period (Table 3). An important exception was the elevated serum leptin concentrations in HDD males in PW 6, which is consistent with the increased fat mass. There were significant differences in the gross energy intake during HDD and LDD feeding, with mice cumulatively ingesting about 0.1 MJ more on the LDD (Table 3); however, this did not lead to a significant difference in body weight or lean mass (Figure 2a,c).

The most remarkable finding in the overall phenotype at sacrifice was the direct effect of the type of starch on intestinal tract morphology. Both the weight and length of the small intestine, and colon weight were increased in females and males consuming the LDD (Table 3). In addition, despite being in the post-absorptive state, cecum contents were increased in females and males fed the LDD (Table 3). At the end of the HFD period, these differences were normalized to values similar to the LDD-fed mice in early life (Table 3).

Long-term effects on glucose tolerance

The perturbation of homeostasis may allow the detection of subtle or early differences in metabolic phenotypes, particularly those induced by nutritional interventions[34]. We first employed an OGTT to challenge glucose homeostasis in PW 11, when mice had been consuming a HFD for five weeks. No differences were seen at baseline in glycemia (Figure 3a,c) or insulinemia (females: 0.7 ± 0.1 ng mL^{-1} versus 0.7 ± 0.2 ng mL^{-1}; males: 1.6 ± 0.3 ng mL^{-1} versus 1.5 ± 0.4 ng mL^{-1}; mean ± s.d., HDD and LDD respectively, n = 12 per group). The glycemic response to the glucose bolus was also similar between groups, with only a trend for a lower iAUC in the LDD females (Figure 3b,d).

Long-term effects on metabolic flexibility

In contrast to the OGTT, which focuses on glucose metabolism, we also performed a nutritional challenge test that can impact a larger array of metabolic processes[35], which has been previously used for the detection of nutritionally-induced metabolic differences[26,36]. A fasting–refeeding challenge was performed in indirect calorimetry after eight weeks of HFD feeding (PW 14), using the HDD as the *ad libitum* refeeding meal. The diet provides a rapid influx of glucose into the bloodstream, competing with protein and fat as additional substrates. The highly coordinated response of the organism to switch from predominantly

Table 3. Organ weights and other physiological parameters at the end of the post-weaning intervention (PW 6) and at end of HFD feeding (PW 15).

	Females				Males			
	PW 6		PW 15		PW 6		PW 15	
Parameter	HDD	LDD	HDD	LDD	HDD	LDD	HDD	LDD
Cumulative GE intake (MJ)	0.90 (0.87, 0.91)	1.01 (0.98, 1.03)§	3.36 (3.26, 3.51)	3.24 (3.12, 3.50)	1.00 (0.97, 1.03)	1.13 (1.10, 1.15)§	3.81 (3.61, 4.09)	3.73 (3.58, 4.06)
gWAT (mg)	65 (58, 83)	65 (48, 77)	197 (178, 297)	240 (181, 422)	140 (122, 168)	117 (91, 145)	667 (568, 758)	796 (592, 885)
mWAT (mg)	104 (83, 139)	108 (81, 114)	202 (183, 303)	244 (180, 383)	176 (150, 200)	140 (117, 173)	562 (474, 797)	627 (523, 806)
Liver (g)	0.72 (0.66, 0.77)	0.67 (0.62, 0.75)	0.89 (0.85, 0.95)	0.89 (0.83, 1.01)	1.01 (0.93, 1.06)	1.05 (0.96, 1.11)	1.20 (1.08, 1.52)	1.23 (1.11, 1.49)
Liver (g g^{-1} body weight)	0.042 (0.039, 0.044)	0.039 (0.036, 0.042)	0.039 (0.037, 0.039)	0.039 (0.036, 0.040)	0.048 (0.044, 0.049)	0.049 (0.045, 0.051)	0.036 (0.033, 0.042)	0.036 (0.034, 0.041)
Liver TG (mg g^{-1} wet tissue)	n.m.	n.m.	36.1 ± 13.9	37.6 ± 14.2	n.m.	n.m.	n.m.	n.m.
Liver glycogen (mg g^{-1} wet tissue)	n.m.	n.m.	53.3 ± 24.0	68.7 ± 39.2	n.m.	n.m.	n.m.	n.m.
Pancreas (mg)	235 (200, 262)	255 (228, 296)	322 (299, 347)	319 (281, 380)	261 (254, 287)	282 (248, 303)	383 (344, 534)	363 (326, 489)
Small intestine (cm)	31.9 (30.8, 32.2)	33.8 (32.8, 36.3)$^{\#}$	33.3 (32.5, 33.7)	33.6 (32.9, 34.8)	33.3 (32.8, 34.0)	35.4 (35.0, 37.2)§	35.0 (34.4, 36.8)	35.7 (34.3, 37.4)
Small intestine (g)	0.60 (0.56, 0.61)	0.72 (0.67, 0.84)$^{¥}$	0.73 (0.69, 0.74)	0.72 (0.68, 0.79)	0.68 (0.65, 0.70)	0.78 (0.74, 0.89)§	0.84 (0.80, 0.94)	0.84 (0.79, 0.94)
Cecum contents (mg)	108 (90, 116)	225 (204, 295)§	104 (91, 125)	108 (87, 143)	152 (121, 162)	273 (250, 359)§	196 (164, 203)	140 (123, 171)*
Colon (mg)	92 (87, 97)	135 (126, 152)§	118 (109, 122)	112 (109, 123)	104 (97, 109)	153 (141, 166)§	139 (126, 151)	136 (129, 159)
Blood glucose (mmol L^{-1})	5.0 ± 0.7	4.7 ± 1.0	4.9 ± 0.5	5.3 ± 0.9	5.8 ± 0.9	6.2 ± 0.7	5.6 ± 0.9	5.8 ± 0.9
Serum insulin (ng mL^{-1})	0.73 (0.56, 0.87)	0.60 (0.48, 0.72)	0.62 (0.58, 1.11)	0.83 (0.65, 1.60)	1.03 (0.90, 1.12)	0.83 (0.72, 1.04)	2.09 (1.71, 3.20)	2.22 (1.93, 2.61)
Serum leptin (ng mL^{-1})	1.6 (1.2, 2.7)	1.3 (0.9, 2.5)	3.9 (2.9, 8,0)	5.0 (2.8, 13.6)	2.9 (2.1, 3.3)	1.8 (1.3, 2.1)$^{\#}$	52.1 (22.2, 125.9)	65.3 (41.6, 101.6)
Serum adiponectin (µg mL^{-1})	n.m.	n.m.	12.3 ± 1.1	12.2 ± 1.5	n.m.	n.m.	n.m.	n.m.

Liver TG, liver glycogen, blood glucose, and serum adiponectin are presented as mean ± s.d. All other data is shown as median (95% CI of mean) since values often did not follow a normal distribution. Statistically significant differences compared to HDD for mice of the same age and sex denoted as * $p \leq 0.05$, $^{\#}$ $p \leq 0.01$, $^{¥}$ $p \leq 0.001$ and § $p < 0.0001$. GE: gross energy; gWAT: gonadal white adipose tissue; mWAT: mesenteric white adipose tissue; n.m.: not measured; TG: triglycerides.

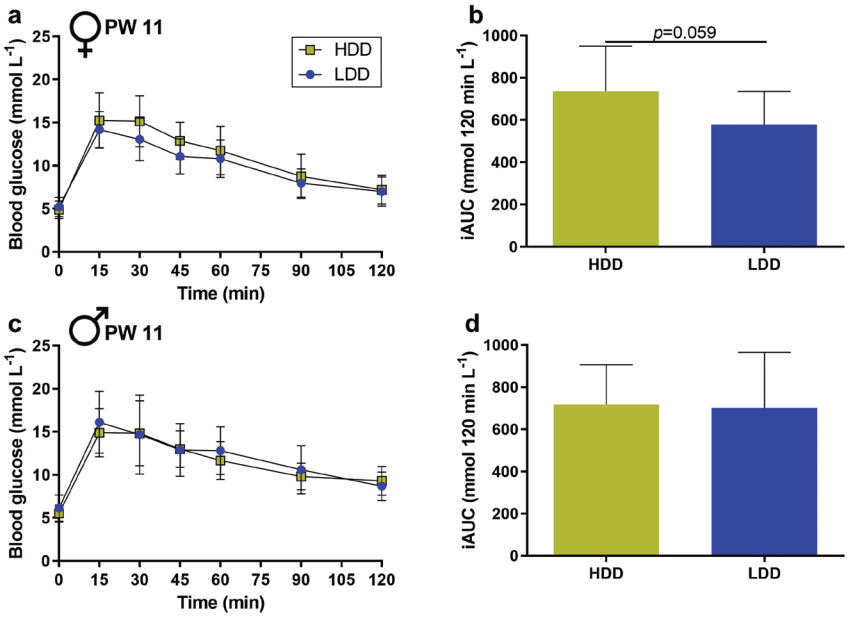


Figure 3. Glucose tolerance after five weeks of HFD feeding (PW 11). Plasma glucose concentrations measured directly before (0 min) and after oral administration of a glucose bolus (2 g kg^{-1} BW) in female (**a**, *n* = 12 for HDD and *n* = 11 for LDD) and male (**c**, *n* = 11 for HDD and *n* = 12 for LDD) mice. Incremental area under the curve (iAUC) for blood glucose over the 120min period for females (**b**) and males (**d**). Data shown as mean ± s.d.

fat oxidation (low RER) to glucose oxidation (high RER) was quantified as an indicator of metabolic flexibility[27,36]. Since the potential programming of glucose metabolism was hinted at only in females (Figure 3), we next focused primarily on females, presenting male data when available. The decline in RER after food restriction evolved in a virtually identical manner between mice fed HDD or LDD in the early post-weaning period, for both females and males, which also ensured that all of the mice were equally fasted before regaining access to food. Upon refeeding and particularly after the first hour of refeeding, the RER in females followed different trajectories, with a significant interaction between time and the post-weaning diet ($p < 0.0001$, Figure 4a). LDD females constantly had a numerically higher RER and reached a statistically significant higher RER at about six hours after access to food (Figure 4a). Similarly, the peak RER values that were reached within the refeeding period were higher in the LDD females (Figure 4b). The response in both the HDD and LDD males within three hours of refeeding was similar to that of the HDD females (Figure S2a), and the peak RER values that were reached upon refeeding were not significantly different between the male groups (Figure S2b).

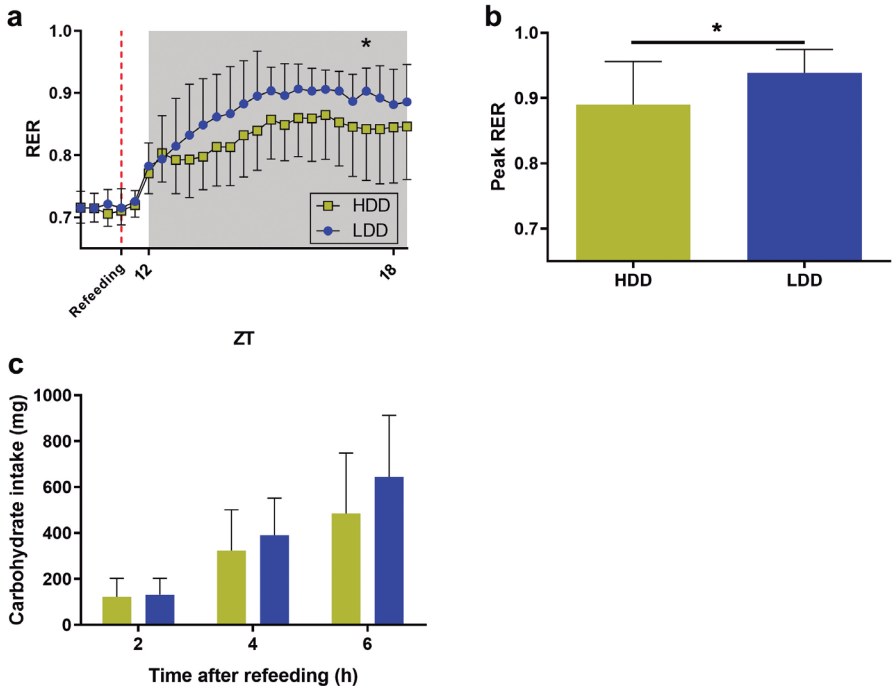


Figure 4. Metabolic flexibility of female mice after eight weeks of HFD feeding (PW 14). (**a**) RER evolution one hour before refeeding until seven hours upon *ad libitum* refeeding with a high carbohydrate diet (HDD). Statistical comparison was performed on all of the data points from the moment of food restriction (additional data points not shown to enhance visualization). (**b**) Mean peak RER values achieved within seven hours after refeeding. (**c**) Cumulative carbohydrate intake calculated from the automatic records of food intake after access to the refeeding diet. n = 13 for HDD and n = 11 for LDD. Data shown as mean ± s.d. Statistical difference denoted as * $p \leq 0.05$.

To corroborate equal food intake during the challenge, we analyzed automatic food intake following access to food. There were no statistical differences in food intake between groups, neither in females (Figure 4c) nor in males (Figure S2c). Thus, our data points toward an improved capacity of LDD females to adapt fuel utilization to fuel availability, i.e., a better metabolic flexibility.

Direct and long-term effects on adipose tissue in females

While mean adipocyte size in gonadal white adipose tissue (gWAT) was not different between groups (PW 6: 35.4 ± 4.5 µm versus 31.6 ± 4.0 µm; PW 15: 48.2 ± 5.4 µm versus 44.5 ± 6.4 µm; mean ± s.d., HDD and LDD respectively, representative pictures in Figure 5a–d), the distribution of adipocyte diameter in mice directly exposed to LDD was shifted toward smaller adipocytes compared to the HDD intervention (interaction between post-weaning diet and diameter bin: p = 0.0016, Figure 5e). Nonetheless, the statistical differences in adipocyte size distribution disappeared after nine weeks of HFD feeding, although they were still bearing some visual resemblance to the distribution at PW 6 (Figure 5f).

Adipocyte size has been linked to macrophage infiltration and inflammation[29], which in turn has been linked to metabolic flexibility[37]. We therefore characterized macrophage and CLS abundance in gWAT depots. Both directly after the early post-weaning intervention and at the end of the HFD period, the gWAT pads of HDD mice harbored higher numbers of macrophages and CLS; however, only CLS in PW 6 and macrophages in PW 15 achieved statistical significance (Figure 5g,h). Interestingly, the mRNA levels in the gWAT pads in PW 15 of three macrophage markers, *Lgals3*, *Ccl2*, and *S1008a*—the latter a key gene associated with M1 macrophages—as well as two other genes linked to adipose tissue function (*Irs2* and *Fabp4*), revealed no significant differences between groups (Figure S3). On the other hand, the histological data was consistent with the long-established association of macrophage abundance and CLS formation with adipocyte size in rodents[29,38] (Figure 5i,j). Collectively, this data indicates that the type of starch had clear direct effects on adipose tissue morphology in females with associated differences in inflammation markers. These differences did not persist later in life.

Discussion

The direct differential effects of lowly-digestible versus highly-digestible-starch diets were seen in females for gWAT morphology and CLS abundance, and in males for whole body substrate metabolism and fat mass gain, with robust effects in the gut physiology in both sexes. Female mice that were subsequently fed a HFD into adulthood showed an improved capacity to adapt energy substrate utilization to substrate availability at the whole body level; however, this effect was not seen in males. This shows that metabolic flexibility in later life can be programmed by the type of starch in the early post-weaning diet in a sex-dependent manner.

The direct metabolic effects by differences in starch digestibility can be due to two main factors: postprandial glycemia and gut microbiota. A highly-digestible starch will be more readily absorbed in the small intestine and stimulate insulin secretion more pronouncedly than a lowly-digestible starch, whereas a fraction of lowly-digestible starch will reach the cecum and colon and interact with gut microbiota. Both hyperglycemia and hyperinsulinemia could independently explain adipose tissue macrophage (ATM) homing and adipose tissue inflammation[39]. At the same time, gut microbiota is able to influence host health through multiple mechanisms[40]. Short-chain fatty acids (SCFA) derived from microbial fermentation have anti-inflammatory and other properties, directly inhibiting lipid storage via free fatty acid receptor 2 and indirectly increasing glucose uptake in adipocytes via the insulin-reinforcing action of gut peptide YY (PYY)[41]. The inhibition of fat storage and increased glucose disposal to adipose tissue would promote fatty acid utilization in other tissues, which is consistent with the lower RER seen in males consuming LDD. Moreover, as Zeevi *et al.*[42] demonstrated, postprandial glycemic responses to the same meal depend partly on microbiota features. This, together with the observational evidence that a microbiota composition with a higher capacity for carbohydrate fermentation dampens weight loss in obese adults[43], suggests a particularly important interaction between the two main factors that are involved in our study (postprandial glycemia and gut microbiota). Interestingly, our post-weaning LDD intervention led to a dramatically different fecal microbiota composition

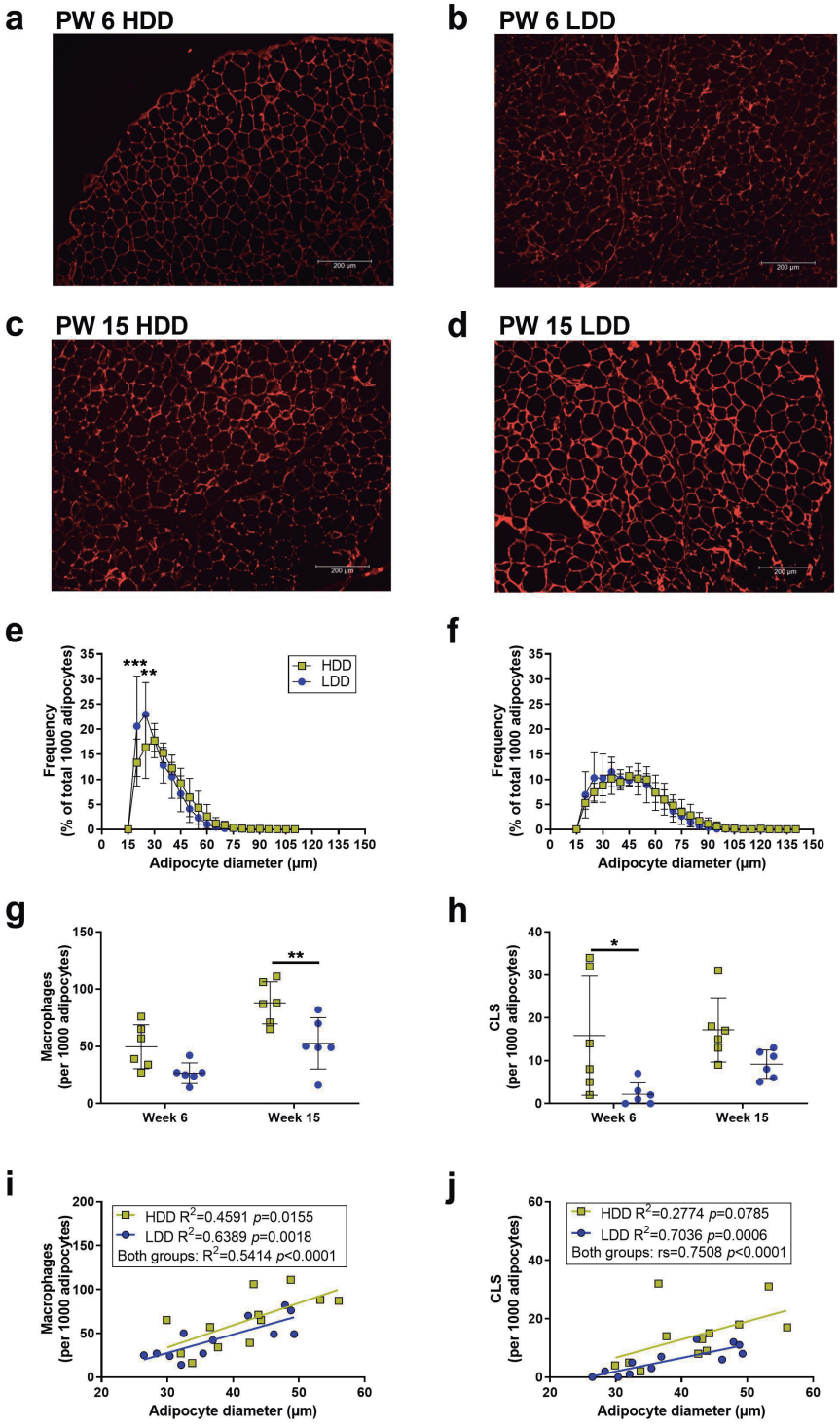

a
PW 6 HDD
b
PW 6 LDD
c
PW 15 HDD
d
PW 15 LDD
e
Frequency (% of total 1000 adipocytes)
HDD
LDD
***
**
Adipocyte diameter (µm)
f
Frequency (% of total 1000 adipocytes)
Adipocyte diameter (µm)
g
Macrophages (per 1000 adipocytes)
**
Week 6
Week 15
h
CLS (per 1000 adipocytes)
*
Week 6
Week 15
i
Macrophages (per 1000 adipocytes)
HDD R2=0.4591 p=0.0155
LDD R2=0.6389 p=0.0018
Both groups: R2=0.5414 p<0.0001
Adipocyte diameter (µm)
j
CLS (per 1000 adipocytes)
HDD R2=0.2774 p=0.0785
LDD R2=0.7036 p=0.0006
Both groups: rs=0.7508 p<0.0001
Adipocyte diameter (µm)

Figure 5 (previous page). Adipose tissue histology, adipocyte size distribution, and macrophage infiltration markers in the gWAT of females at the end of the post-weaning intervention (PW 6) and after the HFD feeding period (PW 15). Representative histological pictures of gWAT using hematoxylin–eosin (HE) staining in HDD-fed (**a**) and LDD-fed mice (**b**) at the end of the post-weaning intervention (PW 6), and HDD-fed (**c**) and LDD-fed mice (**d**) after the HFD feeding period (PW 15). Photos were made using fluorescent microscopy. Distribution of adipocyte diameter (5 µm bins) in gWAT pads collected in PW 6 (**e**) and PW 15 (**f**). Total count of macrophages (**g**) and crown-like structures (CLS, **h**) identified in the same areas using MAC-2 immunohistochemical staining. Correlation plots of adipocyte diameters with (**i**) macrophage and (**j**) CLS counts for all of the animals in each group. All of the coefficients were obtained with Pearson correlation except for the CLS of the combined HDD and LDD groups (rs = Spearman correlation coefficient). *n* = 12 per dietary group, *n* = six per age (PW 6 or PW 15). Data shown as mean ± s.d. Statistical differences denoted as ** $p \leq 0.01$ and *** $p \leq 0.001$.

in both sexes versus HDD-fed mice, with the increase in *Parasutterella*, *Bacteroides*, and *Alloprevotella* abundance after three weeks of LDD feeding strongly correlating only with H_2 production, but not with body weight, fat mass, or food intake[9]. It is most likely that a combination of host and microbiota-mediated mechanisms explains the direct phenotypes of HDD and LDD mice. Although it has been demonstrated that some of the metabolic effects of resistant starches appear to be independent of the presence of a gut microbiota[44], the starch in our LDD cannot quite be considered a resistant starch per se[45].

The observed direct impact of starch digestibility on metabolic health in the present study is in accordance with recent findings on dietary GI-induced effects in rodent models. Particularly, the increased fat mass in males fed a HDD is consistent with a recent meta-analysis of murine GI studies showing that males benefit more from a low GI diet compared to females for several metabolic outcomes, including adiposity[46]. The lower RER observed in LDD males is also consistent with previous studies in rodents[47]. Our data do not show effects on BW and fasting glycemia, which may be due to the duration of dietary exposure[46]. A key observation in this study is the sex-specific effect of the type of starch on substrate utilization. This merits further investigation, especially because of the lack of experimental work in female models of nutrition, including starches of different digestibility[46]. It is unclear whether these differences are due to the effects of circulating sex hormones, which have been shown to be able to protect females from HFD-induced obesity and inflammation[48], or alternatively, to developmental differences in metabolic regulation[49].

Perhaps the strongest direct exposure effects were the enlargement of the lower intestinal tract in the LDD-fed female and male mice, supporting previous data on starches of low digestibility[18,50]. This enhanced growth of the intestine is in line with the trophic effects of SCFA[51]. Indeed, we observed increased SCFA in the cecum and colon upon LDD versus HDD feeding, as well as increased H_2 production[9]. Yet another possibility is an effect of energy dilution due to the lower digestibility of the LDD. It has been demonstrated that mice invest in the growth of the stomach, ileum, cecum, and colon over three months of calorie restriction, at the same time preferentially utilizing WAT depots[52]. This investment in the alimentary tract was associated with a parallel increase in the assimilation efficiency of the diet[52]. In light of this evidence, it might be speculated that gut microbiota and host interact to maximize energy harvest in response to the lower nutrient availability in the LDD.

The observed acute responses could have potential lifelong programming consequences. Indeed, after a nine-week period of the HFD, females fed the LDD showed a better metabolic flexibility in adulthood, supporting the potential of the early post-weaning diet to program metabolic health. In this sense, it is somewhat surprising that most of the other phenotypic parameters that were measured were similar, irrespective of the early post-weaning diet, and existing differences disappeared. Even the strong effects of the type of starch on the intestinal parameters seen in both sexes appeared to be absent later in life.

Sexual dimorphism in animal models of metabolic programming has been repeatedly observed, not only upon prenatal, but also postnatal exposure[53]. With regard to dietary carbohydrates, together with the study of Gugusheff *et al.*[18], our work provides evidence that females are more susceptible to the long-term effects of particular types of starch on metabolic health. This is interesting given that, in line with our data shown here, the direct effects of starch digestibility are only seen in male rodents[46]. Surprisingly, males seemed largely unaffected over the long term, although we cannot exclude the possibility of programming other physiological outcomes. Moreover, sexual dimorphism in response to fasting has been only recently understood in great detail, with females actively promoting lipogenesis from amino acids, and males generally toning down anabolic pathways[54], which could have major implications for metabolic flexibility. Additional studies would be needed to clarify whether females and males have indeed different developmental windows that are amenable to metabolic programming by starches.

On the whole, the metabolic consequences of early post-weaning starches were mild. This might be due to the physiologically relevant dietary levels of starches during the intervention, as well as the fat content during the period thereafter. Programming effects may have been more apparent using *e.g.*, a 60 en% fat HFD, which was required to reach maximal body weight and adiposity in a recent 'wild-type' mouse study with 29 diets varying in macronutrient proportions[55]. Alternatively, the mice we used are too healthy, which is supported by the OGTT responses. The usage of relevant disease models, such as mice with an impaired redox homeostasis (C57BL/6J mice from Jackson Laboratory in fact, as they have a mutated non-functional *Nnt* gene, in contrast to C57BL/6JRccHsd strain we used here, which has a functional *Nnt* gene) might provide opportunities. The strongest consequences of early post-weaning starches could also have been delayed, with aging being an important factor in the development of metabolic disease. Nevertheless, within nearly the same time span and study design, beneficial metabolic programming effects have been observed using specific lipids in the early post-weaning diet[56,57].

On the question of what mechanisms could be responsible for the long-term effects of starches in the early post-weaning period, epigenetic processes are thought to underlie a considerable amount of programming phenomena, along with changes in tissue structure and accelerated cellular aging[58,59]. Neither we nor others[17] have attempted to unravel epigenetic mechanisms specifically in the context of programming by carbohydrates, although there is a strong possibility that such mechanisms take place. For instance, key components of the machinery governing metabolic flexibility can be programmed by maternal nutrient and protein restriction, and are susceptible to epigenetic changes[60-62].

Moreover, some tissues retain plasticity to epigenetic modifications through early adulthood, as is the case for the brain and the colonic mucosa[63,64], with butyrate—a SCFA—being able to cause epigenetic changes in the intestinal epithelium[40]. It is therefore conceivable that highly-digestible and lowly-digestible starches induce cellular biochemical changes that in turn cause epigenetic changes. Exactly which tissues are targeted is unclear.

We see value in placing the early post-weaning window that was chosen in this study within the current evolutionary paradigms of the Developmental Origins of Health and Disease (DOHaD). One of the fundamental premises of the DOHaD framework is that phenotypic adaptations in response to early-life environmental cues, including nutrition, can be predictive of future environments[65]. When the anticipated environment does not match the actual conditions encountered later in life, such early phenotypic responses can become maladaptive and increase disease vulnerability. It is then conceivable that some of the programming effects that we report are not only determined by the post-weaning diet in itself, but also by earlier nutritional cues. In this way, the hormonal and metabolic environment promoted by carbohydrates during the suckling period could be very different to that encountered in the post-weaning period, depending on the carbohydrates introduced. There is at least one report arguing for this kind of carbohydrate mismatch[18]. Human breast milk is considered a low GI food[66]{Wright, 2015 #804;Wright, 2015 #804}, and it also contains indigestible oligosaccharides that can influence the gut microbiome and SCFA profile[67]. Seen in this way, a lowly-digestible starch post-weaning diet could have produced a similar physiological environment as predicted during suckling, whereas a highly-digestible starch diet could fail to match the forecasted conditions and put the organism on course for disease.

Conclusions

Although the differences in the programmed adult phenotypes that we observed were subtle, our findings substantiate the notion that vulnerability to an obesogenic environment could partly depend on carbohydrate quality in early life. In line with the view that disease prevention must start with optimal nutrition early in life, our results need to be considered for the post-weaning diets as well as for products that target this period of growth and development.

Author Contributions

Conceptualisation, J.M.S.F.-C., L.M.S.B., A.O., J.K. and E.M.v.S.; Methodology, J.M.S.F.-C., H.J.M.S. and E.M.v.S.; Validation, J.M.S.F.-C. and E.M.v.S.; Formal Analysis, J.M.S.F.-C., and L.M.S.B.; Investigation, J.M.S.F.-C., L.M.S.B. and H.J.M.S.; Resources, H.J.M.S.; Writing-Original Draft Preparation, J.M.S.F.-C.; Writing-Review & Editing, J.M.S.F.-C., L.M.S.B., H.J.M.S., A.O., J.K. and E.M.v.S.; Visualisation, J.M.S.F.-C.; Supervision, J.K. and E.M.v.S.; Project Administration, J.M.S.F.-C., J.K. and E.M.v.S.; Funding Acquisition, A.O., J.K. and E.M.v.S.

Acknowledgments

We would like to thank MSc students Yurike Widya-Inrumitha, Laura Dewitte, and Lini Sholihah and the personnel at the CARUS animal facility for their support. We also thank Inge van der Stelt for her excellent technical assistance. We thank Nils Billecke (Cargill R&D Centre Europe, Belgium) for his contribution to data interpretation and thorough review of the manuscript.

References

1 Langley-Evans, S. C. Nutrition in early life and the programming of adult disease: a review. *J. Hum. Nutr. Diet.* **28 Suppl 1**, 1-14 (2015).

2 Koletzko, B., Demmelmair, H., Grote, V., Prell, C. & Weber, M. High protein intake in young children and increased weight gain and obesity risk. *Am. J. Clin. Nutr.* **103**, 303-304 (2016).

3 Tang, M. Protein intake during the first two years of life and its association with growth and risk of overweight. *Int. J. Environ. Res. Public Health* **15**, 1742 (2018).

4 Muhlhausler, B. S. & Ailhaud, G. P. Omega-6 polyunsaturated fatty acids and the early origins of obesity. *Curr. Opin. Endocrinol. Diabetes Obes.* **20**, 56-61 (2013).

5 Muhlhausler, B. S., Gibson, R. A. & Makrides, M. Effect of long-chain polyunsaturated fatty acid supplementation during pregnancy or lactation on infant and child body composition: a systematic review. *Am. J. Clin. Nutr.* **92**, 857-863 (2010).

6 Ludwig, D. S., Hu, F. B., Tappy, L. & Brand-Miller, J. Dietary carbohydrates: role of quality and quantity in chronic disease. *BMJ* **361**, k2340 (2018).

7 Schwingshackl, L., Hobl, L. P. & Hoffmann, G. Effects of low glycaemic index/low glycaemic load vs. high glycaemic index/ high glycaemic load diets on overweight/obesity and associated risk factors in children and adolescents: a systematic review and meta-analysis. *Nutr. J.* **14**, 87 (2015).

8 van Schothorst, E. M., Bunschoten, A., Schrauwen, P., Mensink, R. P. & Keijer, J. Effects of a high-fat, low- versus high-glycemic index diet: retardation of insulin resistance involves adipose tissue modulation. *FASEB J.* **23**, 1092-1101 (2009).

9 Fernández-Calleja, J. M. S. *et al.* Non-invasive continuous real-time *in vivo* analysis of microbial hydrogen production shows adaptation to fermentable carbohydrates in mice. *Sci. Rep.* **8**, 15351 (2018).

10 Elia, M. & Cummings, J. H. Physiological aspects of energy metabolism and gastrointestinal effects of carbohydrates. *Eur. J. Clin. Nutr.* **61 Suppl 1**, S40-74 (2007).

11 Shaoul, R., Tiosano, D. & Hochberg, Z. Evo-devo of child growth: the role of weaning in the transition from infancy to childhood. *Crit. Rev. Food Sci. Nutr.* **56**, 887-895 (2016).

12 Wopereis, H., Oozeer, R., Knipping, K., Belzer, C. & Knol, J. The first thousand days - intestinal microbiology of early life: establishing a symbiosis. *Pediatr. Allergy Immunol.* **25**, 428-438 (2014).

13 Fewtrell, M. *et al.* Complementary feeding: a position paper by the European Society for Paediatric Gastroenterology, Hepatology, and Nutrition (ESPGHAN) Committee on Nutrition. *J. Pediatr. Gastroenterol. Nutr.* **64**, 119-132 (2017).

14 Woo Baidal, J. A. *et al.* Risk factors for childhood obesity in the first 1,000 days: a systematic review. *Am. J. Prev. Med.* **50**, 761-779 (2016).

15 Food and Agriculture Organization of the United Nations. *Carbohydrates in Human Nutrition: Report of a Joint FAO/WHO Expert Consultation, Rome, 14-18 April 1997*. (FAO, Rome, 1998).

16 Penfold, N. C. & Ozanne, S. E. Developmental programming by maternal obesity in 2015: Outcomes, mechanisms, and potential interventions. *Horm. Behav.* **76**, 143-152 (2015).

17 Patel, M. S. & Srinivasan, M. Metabolic programming in the immediate postnatal life. *Ann. Nutr. Metab.* **58 Suppl 2**, 18-28 (2011).

18 Gugusheff, J. *et al.* The effect of maternal and post-weaning low and high glycaemic index diets on glucose tolerance, fat deposition and hepatic function in rat offspring. *J. Dev. Orig. Health Dis.* **7**, 320-329 (2015).

19 Oosting, A. *et al.* Effect of dietary lipid structure in early postnatal life on mouse adipose tissue development and function in adulthood. *Br. J. Nutr.* **111**, 215-226 (2014).

20 Wielinga, P. Y. *et al.* Arachidonic acid/docosahexaenoic acid-supplemented diet in early life reduces body weight gain, plasma lipids, and adiposity in later life in ApoE*3Leiden mice. *Mol. Nutr. Food Res.* **56**, 1081-1089 (2012).

21 Minana-Solis Mdel, C. & Escobar, C. Post-weaning protein malnutrition in the rat produces short and long term metabolic impairment, in contrast to earlier and later periods. *Int. J. Biol. Sci.* **4**, 422-432 (2008).

22 He, Y. H. *et al.* Postweaning low-calcium diet promotes later-life obesity induced by a high-fat diet. *J. Nutr. Biochem.* **23**, 1238-1244 (2012).

23 Shahkhalili, Y., Mace, K., Moulin, J., Zbinden, I. & Acheson, K. J. The fat:carbohydrate energy ratio of the weaning diet programs later susceptibility to obesity in male sprague dawley rats. *J. Nutr.* **141**, 81-86 (2011).

24 Bouwman, L. M. S. *et al.* No adverse programming by post-weaning dietary fructose of body weight, adiposity, glucose tolerance, or metabolic flexibility. *Mol. Nutr. Food Res.* **62**, 1700315 (2018).

25 Hoevenaars, F. P. *et al.* BIOCLAIMS standard diet (BIOsd): a reference diet for nutritional physiology. *Genes Nutr.* **7**, 399-404 (2012).

26 Duivenvoorde, L. P. *et al.* A difference in fatty acid composition of isocaloric high-fat diets alters metabolic flexibility in male C57BL/6JOlaHsd Mice. *PLoS One* **10**, e0128515 (2015).

27 Muoio, D. M. Metabolic inflexibility: when mitochondrial indecision leads to metabolic gridlock. *Cell* **159**, 1253-1262 (2014).

28 Krisman, C. R. A method for the colorimetric estimation of glycogen with iodine. *Anal. Biochem.* **4**, 17-23 (1962).

29 Cinti, S. *et al.* Adipocyte death defines macrophage localization and function in adipose tissue of obese mice and humans. *J. Lipid Res.* **46**, 2347-2355 (2005).

30 Carpenter, A. E. *et al.* CellProfiler: image analysis software for identifying and quantifying cell phenotypes. *Genome Biol.* **7**, R100 (2006).

31 *CellProfiler,* http://forum.cellprofiler.org/t/adipocyte-h-e-cell-profiler-pipeline/1460 (2012)

32 Van Schothorst, E. M. *et al.* Adipose gene expression patterns of weight gain suggest counteracting steroid hormone synthesis. *Obes. Res.* **13**, 1031-1041 (2005).

33 Fischbach, M. A. & Sonnenburg, J. L. Eating for two: how metabolism establishes interspecies interactions in the gut. *Cell Host Microbe* **10**, 336-347 (2011).

34 van Ommen, B., Keijer, J., Heil, S. G. & Kaput, J. Challenging homeostasis to define biomarkers for nutrition related health. *Mol. Nutr. Food Res.* **53**, 795-804 (2009).

35 Stroeve, J. H., van Wietmarschen, H., Kremer, B. H., van Ommen, B. & Wopereis, S. Phenotypic flexibility as a measure of health: the optimal nutritional stress response test. *Genes Nutr.* **10**, 459 (2015).

36 Horakova, O. *et al.* Preservation of metabolic flexibility in skeletal muscle by a combined use of n-3 PUFA and rosiglitazone in dietary obese mice. *PLoS One* **7**, e43764 (2012).

37 Sparks, L. M. *et al.* Relation of adipose tissue to metabolic flexibility. *Diabetes Res. Clin. Pract.* **83**, 32-43 (2009).

38 Weisberg, S. P. *et al.* Obesity is associated with macrophage accumulation in adipose tissue. *J. Clin. Invest.* **112**, 1796-1808 (2003).

39 Boutens, L. & Stienstra, R. Adipose tissue macrophages: going off track during obesity. *Diabetologia* **59**, 879-894 (2016).

40 Tilg, H. & Moschen, A. R. Food, immunity, and the microbiome. *Gastroenterology* **148**, 1107-1119 (2015).

41 den Besten, G. *et al.* The role of short-chain fatty acids in the interplay between diet, gut microbiota, and host energy metabolism. *J. Lipid. Res.* **54**, 2325-2340 (2013).

42 Zeevi, D. *et al.* Personalized nutrition by prediction of glycemic responses. *Cell* **163**, 1079-1094 (2015).

43 Muñiz Pedrogo, D. A. *et al.* Gut microbial carbohydrate metabolism hinders weight loss in overweight adults undergoing lifestyle intervention with a volumetric diet. *Mayo Clin. Proc.* **93**, 1104-1110 (2018).

44 Bindels, L. B. *et al.* Resistant starch can improve insulin sensitivity independently of the gut microbiota. *Microbiome* **5**, 12 (2017).

45 Yang, X. *et al.* Resistant starch regulates gut microbiota: structure, biochemistry and cell signalling. *Cell. Physiol. Biochem.* **42**, 306-318 (2017).

46 Campbell, G. J., Senior, A. M. & Bell-Anderson, K. S. Metabolic effects of high glycaemic index diets: a systematic review and meta-analysis of feeding studies in mice and rats. *Nutrients* **9**, 646 (2017).

47 Higgins, J. A. Resistant starch and energy balance: impact on weight loss and maintenance. *Crit. Rev. Food Sci. Nutr.* **54**, 1158-1166 (2014).

48 Grove, K. L., Fried, S. K., Greenberg, A. S., Xiao, X. Q. & Clegg, D. J. A microarray analysis of sexual dimorphism of adipose tissues in high-fat-diet-induced obese mice. *Int. J. Obes.* **34**, 989-1000 (2010).

49 Karastergiou, K. & Fried, S. K. Cellular mechanisms driving sex differences in adipose tissue biology and body shape in humans and mouse models. *Adv. Exp. Med. Biol.* **1043**, 29-51 (2017).

50 Charrier, J. A. *et al.* High fat diet partially attenuates fermentation responses in rats fed resistant starch from high-amylose maize. *Obesity* **21**, 2350-2355 (2013).

51 Topping, D. L. & Clifton, P. M. Short-chain fatty acids and human colonic function: roles of resistant starch and nonstarch polysaccharides. *Physiol. Rev.* **81**, 1031-1064 (2001).

52 Mitchell, S. E. *et al.* The effects of graded levels of calorie restriction: I. impact of short term calorie and protein restriction on body composition in the C57BL/6 mouse. *Oncotarget* **6**, 15902-15930 (2015).

53 Aiken, C. E. & Ozanne, S. E. Sex differences in developmental programming models. *Reproduction* **145**, R1-13 (2013).

54 Della Torre, S. *et al.* Short-term fasting reveals amino acid metabolism as a major sex-discriminating factor in the liver. *Cell Metab.* **28**, 256-267 e255 (2018).

55 Hu, S. *et al.* Dietary fat, but not protein or carbohydrate, regulates energy intake and causes adiposity in mice. *Cell Metab.* **28**, 415-431 e414 (2018).

56 Oosting, A. *et al.* Size and phospholipid coating of lipid droplets in the diet of young mice modify body fat accumulation in adulthood. *Pediatr. Res.* **72**, 362-369 (2012).

57 Baars, A. *et al.* Milk fat globule membrane coating of large lipid droplets in the diet of young mice prevents body fat accumulation in adulthood. *Br. J. Nutr.* **115**, 1930-1937 (2016).

58 Vickers, M. H. Early life nutrition, epigenetics and programming of later life disease. *Nutrients* **6**, 2165-2178 (2014).

59 Reichetzeder, C., Dwi Putra, S. E., Li, J. & Hocher, B. Developmental origins of disease - crisis precipitates change. *Cell. Physiol. Biochem.* **39**, 919-938 (2016).

60 Miles, J. L., Huber, K., Thompson, N. M., Davison, M. & Breier, B. H. Moderate daily exercise activates metabolic flexibility to prevent prenatally induced obesity. *Endocrinology* **150**, 179-186 (2009).

61 Holness, M. J., Zariwala, G., Walker, C. G. & Sugden, M. C. Adipocyte pyruvate dehydrogenase kinase 4 expression is associated with augmented PPARgamma upregulation in early-life programming of later obesity. *FEBS Open Bio* **2**, 32-36 (2012).

62 Smith, R. L., Soeters, M. R., Wust, R. C. I. & Houtkooper, R. H. Metabolic flexibility as an adaptation to energy resources and requirements in health and disease. *Endocr. Rev.* **39**, 489-517 (2018).

63 Marco, A., Kisliouk, T., Weller, A. & Meiri, N. High fat diet induces hypermethylation of the hypothalamic Pomc promoter and obesity in post-weaning rats. *Psychoneuroendocrinology* **38**, 2844-2853 (2013).

64 Kellermayer, R. *et al.* Epigenetic maturation in colonic mucosa continues beyond infancy in mice. *Hum. Mol. Genet.* **19**, 2168-2176 (2010).

65 Gluckman, P. D., Hanson, M. A. & Buklijas, T. A conceptual framework for the developmental origins of health and disease. *J. Dev. Orig. Health Dis.* **1**, 6-18 (2010).

66 Wright, C. J., Atkinson, F. S., Ramalingam, N., Buyken, A. E. & Brand-Miller, J. C. Effects of human milk and formula on postprandial glycaemia and insulinaemia. *Eur. J. Clin. Nutr.* **69**, 939-943 (2015).

67 Le Huerou-Luron, I., Blat, S. & Boudry, G. Breast- v. formula-feeding: impacts on the digestive tract and immediate and long-term health effects. *Nutr. Res. Rev.* **23**, 23-36 (2010).

Supplementary Information

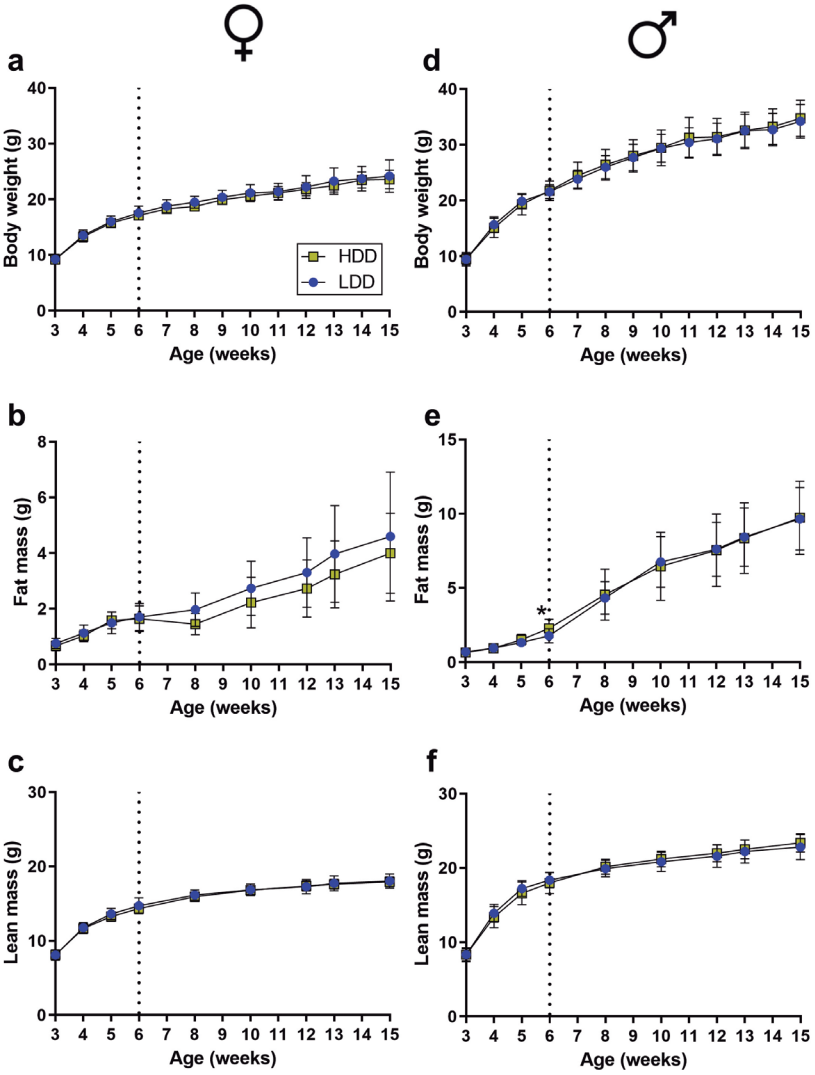


Figure S1. Body weight and body composition development. (**a**) Body weight (BW), (**b**) fat mass (FM), and (**c**) lean mass (LM) of females from weaning at postnatal week (PW) 3 until the end of the study (n = 24 per group from PW 3 through 6; n = 12 per group from PW 7 through 15). (**d**) BW, (**e**) FM, and (**f**) LM of males from weaning at PW 3 until the end of the study (n = 24 for HDD and n = 23 for LDD from PW 3 through 6; n = 12 per group from PW 7 through 15). To test for nutritional programming effects independently of initial differences at the start of HFD feeding, data corresponding to the starch intervention period (n = 23-24 per group and sex) and the HFD period (n = 12 per group and sex) were analysed separately by repeated measurements two-way ANOVA with Bonferroni's *post hoc* test. In both sexes and periods, both HDD and LDD gained BW, FM, and LM over time (p<0.0001). No difference was seen between the two dietary groups, except for the increase in FM in males during the intervention period (F=4.8, p=0.0344). However, males fed LDD *vs* HDD during the intervention period gained BW (F=2.9, p=0.0355) and FM (F=8.5, p<0.0001) at a lower rate, reaching a significant difference in FM at PW 6 (p<0.0001). Data shown as mean ± s.d. The dotted line indicates the start of HFD feeding.

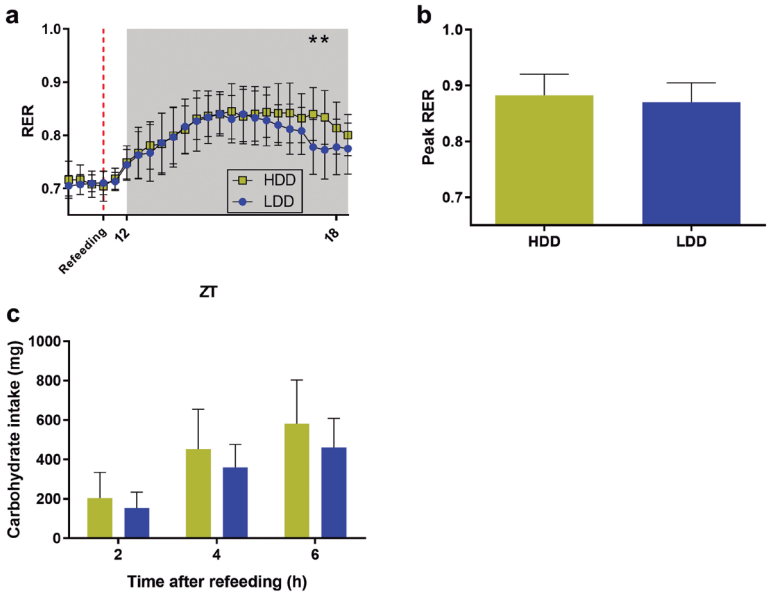


Figure S2. Metabolic flexibility of male mice after 8 weeks of HFD feeding (PW 14). (**a**) RER evolution 1 h before refeeding until 7 h upon *ad libitum* refeeding with a high carbohydrate diet (HDD). Statistical comparison was performed on all data points from the moment of food restriction (additional data points not shown to enhance visualisation). There was a significant interaction of time x post-weaning diet (p=0.0163). (**b**) Mean peak RER values achieved within 7 h after refeeding. (**c**) Cumulative carbohydrate intake calculated from automatic records of food intake after access to refeeding diet. n = 12 per group. Data shown as mean ± s.d. Statistical differences denoted as * p≤0.001.

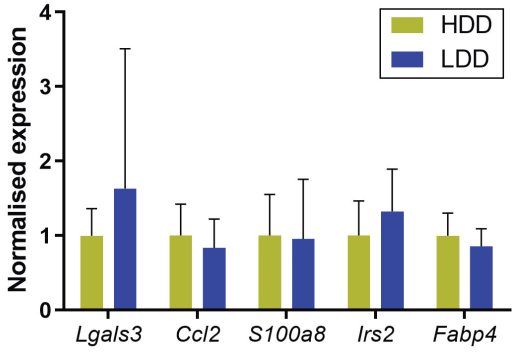


Figure S3. Gene expression in gWAT of females after the HFD feeding period (PW 15). n = 11 for HDD and n = 10 for LDD. Values are given relative to the mean normalised gene expression of the HDD group for each gene and plotted as mean ± s.d. *Lgals3*: lectin, galactose binding, soluble 3; *Ccl2*: chemokine (C-C motif) ligand 2; *S100a8*: S100 calcium binding protein A8 (calgranulin A); *Irs2*: insulin receptor substrate 2; *Fabp4*: fatty acid binding protein 4, adipocyte.

Table S1. Primers used for gWAT gene expression analysis.

Gene symbol	Official Name	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')	Annealing temperature (°C)
Ccl2	chemokine (C-C motif) ligand 2	TTAAAAACCTGGATCGGAACCAA	GCATTAGCTTCAGATTTACGGGT	58.0
Lgals3	lectin, galactose binding, soluble 3	TAATCAGGTGAGCGGCACAG	GCTAAGGCATCGTTAAGCGAAA	58.0
S100a8	S100 calcium binding protein A8 (calgranulin A)	ACTTCGAGGAGTTCCTTGCG	TGCTACTCCTTGTGGCTGTC	60.0
Irs2	insulin receptor substrate 2	GCACCTATGCAAGCATCGAC	GCGCTTCACTCTTTCACGAC	60.0
Fabp4	fatty acid binding protein 4, adipocyte	AATCACCGCAGACGACAGGAAG	TGCCCTTTCATAAACTCTTGTGGAAG	60.0
B2m	beta-2 microglobulin	CCCCACTGAGACTGATACATACGC	AGAAACTGGATTTGTAATTAAGCAGGTTC	60.0
Rps15	ribosomal protein S15	CGGAGATGGTGGGTAGCATGG	ACGGGTTTGTAGGTGATGGAGAAC	60.0

Chapter 6

General Discussion

The major aims of this thesis were 1) to develop and show the added value of an extended mouse metabolic phenotyping tool based on indirect calorimetry (InCa) for the real-time study of microbiota activity and the oxidation of exogenous *vs* endogenous substrates; and 2) to apply this tool to study the direct effects and metabolic programming effects of starches consumed during the early post-weaning period.

In **Chapter 2**, we developed an extended InCa (eInCa) system by incorporating hydrogen (H_2) and methane (CH_4) sensors. Gut microbial fermentation, measured as H_2 production, was higher in mice fed a lowly digestible starch diet (LDD) compared to a highly digestible starch diet (HDD) and exhibited a circadian pattern. Short and long-term consumption of LDD compared to HDD produced consistent changes in faecal bacterial taxa, and several of these bacteria were positively or negatively correlated to H_2 production. CH_4 was not produced at detectable concentrations on any diet, consistent with the absence of faecal methanogens. We further extended the eInCa system with $^{13}CO_2$ and $^{12}CO_2$ sensors in **Chapter 3**, allowing us to obtain real-time $^{13}CO_2$ enrichment data. The eInCa system detected circadian patterns in $^{13}CO_2$ enrichment corresponding to the natural isotopic enrichments of dietary ingredients and endogenous fuels. In addition, by enabling the separate quantification of the rates of exogenous *vs* endogenous fuels, eInCa detected an impairment of dietary fat oxidation in obese mice. The value of eInCa for metabolic phenotyping studies was further shown in **Chapter 4**, where we tested the direct effects of a 3 wk intervention with LDD *vs* HDD in weanling females and males. Daily H_2 production and the cumulative oxidation of a ^{13}C-labelled starch bolus were higher in LDD mice, but only LDD females showed significantly faster starch-derived glucose oxidation kinetics within 45 min after receiving the bolus compared to HDD females. Finally, both the direct and metabolic programming effects of post-weaning LDD *vs* HDD were tested in **Chapter 5**. In both sexes, H_2 production was confirmed to be directly increased by LDD. Females on LDD had a higher frequency of small adipocytes and abundance of crown-like structures (CLS) in gonadal white adipose tissue (gWAT) at the end of the post-weaning diet compared to females on HDD, but LDD males only had an increased fat mass (FM) compared to HDD males. After a period on a high-fat diet (HFD), adult females previously exposed to LDD had a better metabolic flexibility and lower macrophage infiltration in gWAT compared to HDD. The postweaning starches did not show a metabolic programming effect in males.

Until the studies completed in this work, there were no suitable methods to continuously study mouse EE, gut microbiota activity, and exogenous *vs* endogenous substrate oxidation rates in a single phenotyping test. Also, there were few studies comparing the direct metabolic effects of lowly *vs* highly digestible starches in both females and males, and there were no studies of the long-term metabolic consequences of starches consumed in early life. In this chapter, I will discuss what the above findings could mean for future technological developments in rodent studies, the potential implications of starch digestibility in early life for human health, and recommendations to approach animal experiments on nutritional programming.

Extended Indirect Calorimetry (eInCa)

Hydrogen (H_2) and methane (CH_4) sensors

We measured H_2 as an indicator of gut microbial activity. As we have seen in **Chapter 2**, H_2 production depended on the digestibility of the diet, followed a circadian pattern, and was strongly linked to specific changes in faecal microbiota taxa. But what does H_2 really indicate? Carbohydrates consumed but not assimilated by the host can be anaerobically catabolised in a series of redox reactions that provide energy to microbes. This process is called fermentation and results in the production of H_2, CO_2, and SCFAs. The H_2 generated by fermentative bacteria must be kept below a certain threshold, because a high H_2 partial pressure will inhibit bacterial growth and slow the rate of fermentation[1]. Disposing of H_2 can be accomplished in two ways. One way is by excretion of H_2 in flatus and breath (after absorption into the circulation), the latter known to account for up to 21 % of the H_2 produced in the human intestine[2]. The other, by utilisation of H_2 by hydrogenotrophic bacteria to obtain energy, mainly through oxidation of H_2 into H_2S and CH_4 by sulfate reducing bacteria and methanogens, respectively[3]. This implies that high levels of H_2 detected in the air in mouse cages reflect the disposal of excessive gas and an abundance of fermentative substrates. Thus, H_2 as an indicator of availability of substrates for fermentation explains the higher H_2 levels in LDD- *vs* HDD-fed mice in **Chapters 2**, **4**, and **5**. A pulsed supply of nutrients to the gut microbiota also explains the circadian patterns in H_2 levels and their similarity to food intake (FI) events observed in **Chapter 2**. In fact, other fermentation markers, *i.e.* caecal SCFAs, also show a circadian pattern[4]. The circadian pattern of fermentation is likely driven solely by FI, as we found no clear association between locomotor activity and H_2 production. However, the relationship between microbial activity and FI may be bi-directional. An emerging model of host-microbiota appetite regulation proposes that gut microbial activity stimulates intestinal hormone release and satiety pathways in the host, and that these pathways are less stimulated as microbial populations decrease (as would be the case in the postabsorptive state)[5].

By including H_2 analysis in real time, eInCa offers an attractive tool to study the flexibility of the microbiota to adapt to substrate availability in the short and the long term. The kinetics of faecal microbial community and caecal SCFA changes in response to fermentable carbohydrates have only been studied at intervals no shorter than one day[6-8]. These studies have thought us that the complete adaptation to increased availability of fermentable substrates is a long and nuanced process. The earliest changes in microbial community structures have been seen after 1-4 d in humans, and some of these changes are transient while others are sustained[7,9]. The extent and types of caecal fermentation during this process also vary, with lactic acid fermentations predominantly occurring within the first 5 d of a LDD in rats, and SCFA production being increasingly favoured during the following weeks[8]. Within 5 d, the early changes in microbial fermentation are concomitant to host anatomical and physiological adaptations like caecum enlargement and increased nitrogen fluxes[8]. In **Chapter 2**, we have indeed observed that half of the faecal bacterial taxa increased by 3 wk on LDD were also increased after 4.5 d of LDD feeding, and that the small and large intestines were enlarged in LDD *vs* HDD mice

after 3 wk (regrettably we were unable to measure this parameter also at 4.5 d because it required sacrificing an additional group of mice). Nocturnal H_2 production was already increased by ~700% in LDD mice by 4.5 d after switching from the chow diet, compared to the ~850% increase in H_2 output in mice adapted to LDD *vs* HDD for 3 wk, consistent with the known progressive increase in microbial fermentation mentioned above. Here, the added value of H_2 measurements gave insight into the very early stages (< 1 d) of adaptation to fermentable carbohydrates non-invasively, as we were able to observe that cumulative H_2 production was already doubled during the first 12 h after switching mice from chow to LDD *vs* HDD. This shows that the gut microbiota are more responsive to substrate availability in the very short term, much shorter than previously known, and aligns with the transit time of 6-8 h reported in mice[10].

Besides H_2, other fermentation gases like CH_4 should be taken into account to get a more complete picture of the gut microbial activity occurring *in vivo,* which as we show here, can be done without additional animal discomfort. This could be especially useful if H_2 excretion equals or approaches zero, when all H_2 produced could be disposed of by hydrogenotrophs. In this study, for the diets and mouse strains used and metabolic states (fasted *vs* fed) investigated we could not detect CH_4 production (**Chapters 2**-**5**), and this was consistent with the absence of methanogenic archaea in faecal samples (**Chapter 2)**. CH_4 excretion has been measured in rats and mice[11-13], and methanogens have been detected in mouse caecum[14] and faeces[15]. Likely, the H_2 produced by fermentative bacteria in fasted and HDD mice in this study was utilised by sulfate reducing bacteria instead. Especially when the supply of dietary carbohydrates is limited, microbes also ferment dietary proteins and endogenous nutrients (*e.g.* glycoproteins secreted by goblet cells, or mucins), leading to the production of H_2, branched chain fatty acids (BCFAs), phenols, and H_2S (ref. 16). Indeed, the reliance of the gut microbiota on protein and mucin glycans was suggested in **Chapter 2** by the higher abundance of caecal isovaleric acid (a BCFA) and faecal *Desulfovibrio* (a sulfate reducer) in HDD *vs* LDD mice. Thus, incorporating a H_2S sensor could offer an additional and non-invasive way to probe into these hydrogenotrophic processes. Currently, most of our understanding of the interactions between H_2-producing fermentative bacteria and H_2-consuming hydrogenotrophs is theoretical and based on *in vitro* evidence[17]. A rodent eInCa system enabling H_2, CH_4, and H_2S analysis in real-time could be an excellent next step to study how microbial communities compete for H_2 in the gut and how this influences host health.

Monitoring host metabolism and microbial activity simultaneously promises to complement the phenotypic assessment of physiological and pathophysiological conditions. Fermentation gases have been implicated in health and disease states. For instance, H_2 is related to intestinal bloating and discomfort in humans, but at the same time H_2 could be of therapeutic value because of the antioxidative, antiapoptotic, and anti-inflammatory activities of exogenous H_2 as demonstrated in both *in vitro* and *in vivo* models of reperfusion injury, liver inflammation, drug-induced nephrotoxicity, allergy, Parkinson's disease, and colitis[18]. There is also some evidence for an LDL cholesterol-lowering and improved glucose tolerance effect of supplemental H_2 in drinking water in humans at risk of metabolic syndrome[19,20]. Thus, it is possible that the availability of endogenous H_2 to the host may be beneficial for metabolic health. Furthermore, increased utilisation of fermentation H_2

by sulfate reducing bacteria leads to the production of cytotoxic and pro-inflammatory metabolites like p-Cresol and H_2S, which have been linked to colorectal cancer[21,22]. H_2S has also other physiological effects as a gasotransmitter similar to nitric oxide (NO) and carbon monoxide (CO)[23], underscoring the potential of microbial H_2 metabolism to influence the overall health status of the host. Fermentation gases could also impact the host's energy balance. On the one hand, a positive energy balance could be favoured by utilisation of H_2 by hydrogenotrophs, which increases the efficiency of fermentation and yields more SCFAs to the host that can in turn oxidise them to obtain energy[1]. In addition, mitochondrial H_2S oxidation at low concentrations can be a source of electrons for the electron transport chain, indicating that H_2S can also provide energy to the host[24]. On the other hand, a negative energy balance could be promoted by H_2S too, as this gas has been shown to modulate thermogenesis in brown adipose tissue, allowing the dissipation of energy as heat[25]. Some of the energy content of the diet is also lost to fermentation gases. In ruminants, who rely heavily on microbial fermentation, CH_4 excretion is a crucial component of energy metabolism[26]. The loss of energy to H_2 and CH_4 produced by humans consuming non-digestible polysaccharides has been considered negligible, representing 0.2% of daily EE[27]. This has not been directly determined in rodents, but energy lost to H_2 has also been deemed unimportant in one study of the effects of lowly digestible starch on energy balance in rats[28]. We have seen that the fraction of daily energy expenditure (EE) lost to H_2 in LDD-fed mice is 0.02% (**Chapter 2**). Assuming a constant metabolic rate of 44.2 kJ d^{-1} (11 kcal d^{-1}), this would represent a loss of 7.4 kJ (2 kcal, or about 220 mg of fat tissue; for comparison, we have seen up to 8 g of fat tissue in middle-aged chow-fed males) over the entire adulthood and postreproductive stages of a C57BL/6J male mouse (841 d, ref. 29). Although fermentation gases did not lead to sizeable differences in EE in our mice, it is possible that larger amounts of energy are lost as excreted H_2 or CH_4 at higher quantities or with other types of fermentable carbohydrates in the diet. Moreover, behavioural factors may decrease the digestibility of the food and increase the availability of substrates to the gut microbiota. In **Chapter 2**, after a period of food restriction, 2 out of 4 mice produced more than double the volume of H_2 per g of chow consumed compared to their production before food restriction. Hydrogen excretion in these mice peaked at 1.7 h after regaining access to food. This is considerably earlier than the 3 h it would take food to reach the caecum, based on a mouse study of transit time using a radiolabelled activated charcoal bolus[10]. Possibly, those mice in **Chapter 2** could have ingested food very rapidly, limiting chewing and resulting in larger particles in the chyme that may be difficult to digest, thus forming substrates for fermentation. Chow contains whole grains, and it is known that starches in whole grains are entrapped in a non-digestible matrix that can become physically accessible if this matrix is broken down mechanically[30]. Alternatively, gastric emptying could have been accelerated by increased ghrelin levels[31]. The possibility that fasting and then refeeding leads to a less efficient nutrient assimilation should be considered in mouse and human studies, as fasting-refeeding is the basis of various metabolic phenotyping tests[32]. Thus, accurately quantifying the excretion of fermentation gases in tandem with host metabolic gas exchange introduces a new dimension to the phenotyping power of rodent InCa and adds microbial activity to the standard components of EE like basal metabolic rate and physical activity.

$^{13}CO_2$ and $^{12}CO_2$ sensors

The combination of $^{13}CO_2$ enrichment analysis and respirometry is rare in rodent studies, while there are many studies using either technique separately. Why is it important to integrate both techniques? Enabling $^{13}CO_2$ enrichment analysis in tandem with O_2 and CO_2 analysis yields *quantitative* data: not only do we know the proportion of $^{13}CO_2$ content in mouse cages, but also the rate at which CO_2 is being produced. Early since stable isotopes were used in human research, the technique has been combined with quantitative respirometry in humans, and in 1976 the first study applied both techniques to calculate the amount of a dose of glucose that became oxidised over 7 h (ref. 33). Only decades later, this combination started to be implemented in non-model species[34]. In mice, however, the only two instances where quantitative $^{13}CO_2$ analysis has been achieved are the studies of Ishihara *et. al*[35] and McCue *et. al*[36]. Ishihara *et al.* developed a combination of mass spectrometry and O_2 and CO_2 analysers[37] and soon applied it to dissect the oxidation of exogenous *vs* endogenous glucose and fat in diabetic mice fed casein- or soy protein-based diets[35]. This system seemed promising because of its online analysis mode and high temporal resolution (< 1 h, although this is not consistently reported in their studies). Surprisingly, the investigators have reported the use of the system only once more, when they used $^{13}CO_2$ from ^{13}C acetate as a marker of gastric emptying (thus, only using $^{13}CO_2$ enrichment data, not respirometry data)[38], nor have others constructed a similar system. By painstakingly collecting air samples from metabolic cages every hour, McCue *et al.* obtained quantitative data of endogenous carbohydrate, lipid, and protein oxidation in mice raised on ^{13}C labelled tracers and deprived of food for 72 h. In this study, substrate partitioning could not be reliably interpreted from RER data only because of the high intra- and interindividual variation in RER values and the changes accompanying acid-base balance occurring during prolonged fasting[36]. Because the body carbohydrate, lipid, and protein stores were respectively labelled by ^{13}C glucose, ^{13}C palmitate, or ^{13}C leucine, the authors were able to calculate well-defined shifts in fuel oxidation in the progression of fasting. Carbohydrate oxidation was rapidly minimised but briefly increased around 12 h after food deprivation, lipids became the most important fuel from 2 h onwards, and protein was initially spared (8% EE between 8 and 12 h), but then became increasingly important (24% at 72 h)[36]. These results showed that the three phases of fuel use during fasting and starvation (oxidation of carbohydrates, then lipids, then proteins) are not as straightforward as typically stated. The combination of $^{13}CO_2$ analysis and respirometry was key to these conclusions: absolute protein oxidation was constant after the initial protein sparing stage, but its relative contribution to EE increased as fasting progressed[36]. Moreover, the duration and magnitude of the protein-sparing phase could not have been identified by relying on urinary N content, because those measurements are not possible at such short intervals (1 h). The study in **Chapter 4** also illustrates how both isotopic enrichment *and* respirometry data must be interpreted together. Our question was whether the oxidation kinetics of a single starch bolus fed to young mice that had been on an LDD *vs* HDD for 3 wk would be different and sex-dependent. Early upon administration of the starch bolus, $^{13}CO_2$ enrichment tended to increase faster in LDD *vs* HDD males (**Chapter 4 Figure S2a**, 2-way RM ANOVA, time × diet interaction $P = 0.095$; Bonferroni multiple comparisons $P < 0.05$ at t = 20 and 40 min). When $^{13}CO_2$ enrichment data and the rate of

total CO_2 production (VCO_2) were used to calculate the true oxidation rates of the starch bolus, an effect of LDD *vs* HDD was not apparent anymore (**Chapter 4 Figure 3a**, time × diet interaction $P = 0.999$). After receiving the bolus, HDD mice had a burst in activity (data not shown) and increased their metabolic rate more than LDD males (**Chapter 4 Figure S3a**). In other words, the proportion of $^{13}CO_2$ to $^{12}CO_2$ was indeed higher for LDD males, but the absolute quantity of $^{13}CO_2$ produced (reflecting the quantity of starch oxidised) was similar, offsetting the difference in the oxidation kinetics between LDD and HDD males. In **Chapter 4** we did not include behavioural aspects *a priori* (other than FI), but this result might give us a lead to investigate, for instance, whether the sudden activity burst of HDD mice could be due to lower feelings of satiety. Incidentally, rodents fed lowly digestible starch have persistent and elevated circulating GLP-1 (glucagon-like peptide 1, a satiety hormone), even when they tend to consume more food than those fed highly digestible starch[39].

I propose more emphasis should be put on *quantifying* oxidation data in mouse experiments that use isotopic tracers. There are excellent examples of well-designed metabolic tracer studies that could have been refined by including quantitative respirometry. Friedrich *et al*. showed that epigallocatechin gallate (EGCG), a bioactive compound in green tea, increased exogenous fat oxidation in BL/6N mice[40]. The authors had to rely on $^{13}CO_2$ enrichment data only (measured in air samples from mice placed inside a 140 ml syringe) to conclude that EGCG promotes the oxidation of a dose of ^{13}C palmitate. This data was backed by their previous study using InCa, where oral administration of EGCG before the dark phase (DP) decreased RER in *ad libitum* fed NZB mice, and no effects of EGCG on EE were apparent[41]. Because of the different mouse strain used by Friedrich *et al.*, it cannot be confidently excluded that the postprandial metabolic rate of EGCG-treated mice is higher than control mice, leading to a higher proportion of $^{13}CO_2$ inside the syringe. This could imply that EGCG might not specifically enhance the oxidation of recently ingested fat. Actually, the data supports that dietary fat is not absorbed as efficiently in EGCC mice, as shown by the increased ^{13}C enrichment in faeces and decreased expression of fatty acid transporters in intestinal mucosa[40]. A combined isotopic enrichment-InCa approach could have provided stronger evidence in a single experiment, integrating the true oxidation rates of ingested fat with metabolic rate and meal events. The use of this combined approach is a rare find in literature, and previous attempts at it have been either expensive or laborious. Our eInCa provides a way forward.

The data in **Chapters 3** and **4** suggested that part of the ^{13}C label orally administered was likely sequestered in TCA cycle metabolites, as we have seen in the delayed exogenous substrate *vs* total substrate oxidation curves. This effect is known to occur in humans[42] and is corrected for in metabolic studies with ingested fatty acid tracers by means of a dietary acetate recovery factor (dARF)[43]. Including a dARF in exogenous substrate oxidation calculations could impact the conclusions of metabolic tracer studies, since the dARF is lower for overweight *vs* lean humans[44]. There might be a similar relationship between adiposity and label sequestration in mice. In **Chapter 4**, the negative correlation between body FM and the oxidation of palmitate ingested in a mixed meal became less strong after applying the dARF of humans according to their FM. We assumed human dARFs would be

valid for mice because, at present, the data necessary to calculate a dARF for mice is not available. The correlation in **Chapter 4** hinted at an impairment in nutrient sensing in the obese state, and the results fit the hypothesis that the capacity to store fat is enhanced in obesity. This hypothesis has been around for some time and is supported by radioactive tracer studies in the rat, with increased adipose tissue LPL activity and altered hypothalamic sensing of fatty acids as potential mechanisms[45,46]. The relation between nutrient sensing and obesity may be causal in this model[47], but this has been recently challenged in humans. In an ongoing cohort of self-reported obesity-prone and obesity-resistant humans, there were no differences in their ability to couple fat intake to fat oxidation upon consumption of a labelled mixed meal after acute overfeeding or eucaloric feeding[48]. If an inverse relationship between the dARF and adiposity is also present in rodents, failing to apply a correction in the exogenous substrate oxidation formulas could overestimate dietary fatty acid oxidation in obese mice and rats, explaining the discrepancy in these models. At the time we obtained the ethical approval for the experiments compiled in this thesis, we were only aware that the ^{13}C label could be temporarily sequestered in the bicarbonate pool, and that this factor was negligible due to the short half-life of ^{13}C enriched bicarbonate in mice (~15 min)[49], but a new mouse study with orally administered ^{13}C acetate could be performed in eInCa at low cost and with minimal invasiveness to determine a mouse-specific dARF.

Widespread application of eInCa can result in greater insights into metabolism. Functional metabolic challenges, like fasting and refeeding challenge tests, can be refined with the use of eInCa. We have seen in **Chapter 3** that exogenous glucose oxidation was not part of the metabolically inflexible phenotype of obese mice, but rather total glucose oxidation and exogenous fat oxidation were impaired. Impaired total glucose oxidation is indicative for impaired insulin action, which operates on whole body level, not on exogenous glucose only. This only became apparent after complementing the standard RER-based metabolic flexibility test with $^{13}CO_2$ enrichment analysis. Furthermore, interpreting the oxidation of a two-fuel mixture (glucose and fatty acids) based on RER can sometimes be misleading. In certain situations, RER values can drop below 0.704, the oft-quoted value indicative of maximal net fat oxidation with respect to net glucose oxidation. We have observed RER < 0.704 in fasted animals in Chapters **3** and **4**, and in previous experiments in our laboratory[50]. While low RER values can result from erroneous O_2 and CO_2 measurements[51], these values were likely physiological as we optimized measurements by regular calibration and sufficient sample intervals between measurements of mouse and reference cages. During prolonged fasting, gluconeogenesis from protein and ketogenesis in excess of ketone body oxidation can become important and can lower RER to values below 0.704, overestimating fat oxidation and underestimating glucose utilisation[52,53]. The assessment of perturbations of substrate use by refeeding challenges is complicated by the fact that, due to the limited gastric volumes of mice, refeeding is ethically done with small amounts of food. Because RER measurements are not very sensitive to small changes in substrate flux[54], this would in turn produce relatively small and transient increases in RER. In our hands, a glucose dose of ~70 mg (2 g kg^{-1} body weight, BW) produced maximal RER responses of about 0.80 (ref. 50), and a 40 mg starch bolus elicited similar maximal RER responses of 0.81 (**Chapter 4**). Others have found higher RER increments using a larger

glucose dose: 225 mg per mouse (8.8 g kg BW^{-1}) produced an RER peak of 0.91 (ref. 55). This dose however, is far higher than commonly used 1 or 2 g kg^{-1} BW (ref. 56) and may not be physiological. In **Chapter 4**, RER fell after 45 min on administration of the starch bolus, often below 0.704. Under these conditions, net glucose oxidation would have been interpreted as zero, but the ongoing oxidation of the starch bolus was quantifiable thanks to the $^{13}CO_2$ and $^{12}CO_2$ analysers. Although less common, pathophysiological conditions can also invalidate the standard assumptions of substrate use based on RER. Shinozaki *et al.* have shown that changes in VO_2 are not linked to changes in VCO_2 in the post-cardiac arrest rat model[57]. After resuscitation of the rat and O_2 inhalation, the cellular utilization of O_2 increased disproportionally to the production of CO_2 after resuscitation, producing RER values lower than 0.7 (with inhalation of 30% O_2) or even below 0.6 (with inhalation of 100% O_2), the latter value outside the physiological range[57]. In this study, loading the body carbohydrate, fat, or protein pools with ^{13}C labelled substrates and measuring $^{13}CO_2$ during the post-cardiac arrest period would have allowed for a correct interpretation of substrate utilisation.

The above examples of eInCa as a deep phenotyping tool for rodents opens new possibilities for future extensions. Incorporating a sensor for volatile organic compounds (VOCs) would be of great interest. VOCs are increasingly used to study metabolic responses in humans and have diagnostic potential[58,59]. In this sense, excreted acetone could be measured and used to quantitatively correct for ketogenesis in the substrate oxidation formulas, going beyond the oxidative fate of nutrients and into biosynthetic processes. Other biomarkers could be further exploited or validated. Isoprene has been proposed as an indicator of the rates of cholesterol production[58], although it is also likely to respond to physiological challenges like exercise[60]. More broadly, other compounds like pentane (a marker of lipid peroxidation and reperfusion injury[61]) and toluene (a marker of lung cancer[62]) could be informative in mouse models of human pathophysiology. For these and the sensors described in this thesis, future technological challenges lie ahead. It will be important to establish, for instance, if the responsiveness of the $^{13}CO_2$ and $^{12}CO_2$ sensors to natural isotopic enrichments, as seen in the mice fed wheat-based and maize-based ingredients in **Chapter 3**, can also be used to *quantify* substrate oxidation rates. Although our experiments with non-labelled diets were not designed to quantify substrate oxidation rates, we concluded that the sensors are at least sensitive to the very small differences in $^{13}CO_2$ enrichment usually detected by mass spectroscopy. Other challenges will be to make affordable the incorporation of a sensor for every mouse cage to allow second-to-second resolution without compromising accuracy (already possible for the conventional O_2 and CO_2 sensors[63]), and to properly filter out other possible sources of gases and VOCs, like bedding and food pellets.

Conclusions

Our studies demonstrate how InCa can be extended with additional gas sensors and applied directly to areas of metabolic research where rodent models are essential. By simultaneously providing quantitative information on energy expenditure, microbiota activity, and oxidation of exogenous *vs* endogenous substrates, eInCa maximised the value of a single *in vivo* phenotypic assessment. Decidedly, getting more data out a

single phenotyping assessment contributes to the ethical use (or the three Rs: reduction, refinement, and replacement) of animals in experimental research.

Metabolic Consequences of Consuming Lowly *vs* Highly Digestible Starches

In parallel with the technological innovations discussed in the previous section, the research questions addressed in this thesis were initially conceived in the context of metabolic programming by lowly *vs* highly digestible starches. The mouse model used allowed for the distinction between the *direct* effects of starches during the post-weaning period and their *programming* effects onto adult life, *i.e.* long after the exposure of starches of different digestibility. This means that, while **Chapters 4** and **5** primarily focussed on the direct and programming effects of starches, respectively, there is considerable overlap with the study designs across all Chapters. Therefore, data from other Chapters will be integrated when appropriate. The reader is first presented with a discussion on the findings regarding the direct effects of LDD *vs* HDD. This will aid in the interpretation of the metabolic programming outcomes, discussed later. I will then briefly reflect on the translational value of our findings.

Sex-dependant direct effects of weaning starch on metabolism

As we have seen in **Chapters 4** and **5**, the metabolic outcomes in mice directly exposed to LDD were generally beneficial compared to mice exposed to HDD and consistent with what is reported in the meta-analysis of Campbell *et al.* for low glycaemic index (GI) diets in rodent studies, which were mostly designed as lowly digestible starch diets based on amylose content[64]. In our studies, however, fasting glucose and insulin concentrations were not affected by starch digestibility. The 3 wk duration of the starch interventions was short compared to the median duration of 10 wk in the studies analysed by Campbell *et al*, likely explaining why these static metabolic parameters (as opposed to functional ones, like those measured by challenge tests) were unchanged in our studies. However, we also observed that the oxidative disposal of a starch bolus was enhanced in LDD mice, demonstrating that carbohydrate metabolism was affected by starch digestibility in the short term. Although we found this effect to be stronger in females, the improved starch oxidation in males seem to contradict the observations of Isken *et al.*, who found no effects of starch digestibility on the oxidation of an oral glucose bolus in adult male mice after a 3 wk intervention[65]. This may be related to the age of the mice at the start of the experiment, but may also be due to the form of glucose used to test carbohydrate oxidation, since we used starch instead of pure glucose. A more extensive oxidation of the starch bolus could be a consequence of enhanced digestion. We have investigated this possibility, but amylase levels in the small intestine were not different in LDD compared to HDD mice. The trend towards higher deposition of the ^{13}C label in the LDD liver was also not sufficient to explain the differences in starch oxidation. The preferential oxidation of starch-derived glucose in LDD mice could result from a decreased glucose partitioning to *de novo* lipogenesis. Others have shown that consumption of lowly digestible starch led to smaller adipocytes in rat epididymal adipose tissue and higher insulin-stimulated ^{14}C glucose oxidation *ex vivo*[66].

In accordance, we found a higher frequency of small adipocytes in gWAT of LDD *vs* HDD females in **Chapter 5**. However, FM was lower in LDD *vs* HDD males (a bigger effect compared to females) despite their more similar exogenous glucose oxidation (a smaller effect compared to females), which could be the primary reason for smaller adipocytes. More research is needed to understand how and why consuming lowly *vs* highly digestible starches causes the same starch molecule to be handled differently.

A recurrent observation in this thesis was the sexually dimorphic response to starch digestibility. As an explicit question from the outset, this was reassuring, yet the mechanisms behind the phenomenon remain elusive. Inherent differences between females and males, namely gonadal hormones and the sex chromosomes, may shape the metabolic responses to nutritional factors and lead to sexually dimorphic phenotypes. For lack of a mechanistic explanation, I will explore a few potential avenues for further research.

There are a number of sex dimorphisms in body composition, insulin sensitivity, relative blood volume and distribution, temperature and pain perception, and depot-specific metabolism, cellularity, and growth of adipose tissue[67-69]. Some of our findings, like the more complete oxidation of starch in males *vs* females (**Chapter 4**), may be easily explained by the higher lean mass and lower adiposity of males. A contribution of sexual maturity may also be involved. At least for exercising humans, it is known that exogenous glucose oxidation depends on pubertal stage, with children being more "oxidative" than adults[70], and that this may be related to a different capacity to load or tap into endogenous glycogen stores[71]. The onset of puberty occurs later in male than in female mice[72]. In this sense, a more oxidative phenotype of sexually immature males would be consistent with the overall more extensive oxidation of the starch bolus in males *vs* females in **Chapter 4**.

The tougher question here is, exactly how could any of the above factors account for the discrepant female-male HDD and LDD phenotypes? Further targeted experimentation is needed before we can propose a detailed mechanism for the sex-specific metabolic response to starches of different digestibility, but the gut microbiome might be involved. Sex is known to influence microbiota communities in mice, and these in turn influence sex hormone levels and host metabolism[73]. Although we found that sex did not significantly contribute to faecal microbiota composition after 3 wk on LDD *vs* HDD (**Chapter 2**), it is still possible that the metabolic pathways of female *vs* male microbiota were differently activated as we did not analyse the metatranscriptome. This is conceivable because, despite the profound sequencing depth used for our microbial taxonomy analysis (*i.e.* genus level), there are structural variants in the genomes of otherwise identical microbial strains that could confer them different functions[74]. Even if the composition and metabolic activity of the gut microbiota were identical, gut microbial metabolites can be differently handled by females and males. By way of example, gut microbes can convert dietary choline to trimethylamine, which in turn is metabolised into trimethylamine-N-oxide (TMNO) by the hepatic enzyme flavin monooxygenase 3 (ref. 75). This hepatic enzyme is overexpressed (about 100 fold higher) in females *vs* males, leading to higher plasma levels of TMNO in females, and at the same time TMNO explained 11% of the variation in atherosclerotic lesions in a panel of 100 inbred mouse strains[76]. This opens the possibility that gut microbial metabolites

commonly derived from LDD *vs* HDD in both sexes had a different impact on the host's metabolic phenotype depending on sex.

Potentially interesting here is the sexual differentiation of liver metabolism, which starts before puberty. In the female liver, but not the male liver, the response to short-term fasting is to catabolise amino acids to synthesise lipids (to keep up building energy stores for the completion of ovulation) and glucose (both to maintain euglycaemia and to provide NADPH for lipogenesis, through the pentose phosphate pathway)[77]. This may be especially relevant for nutrient challenge tests conducted in the fasted state when there is additional supply of nutrients derived from fermentation (*i.e.* SCFAs). We have seen larger caecum contents in fasted LDD females and males, suggesting that SCFA may be available to the host (**Chapter 4**). Accordingly, fasting RER was higher in LDD (~0.73) *vs* HDD (~0.70) males, suggesting that LDD males oxidised the available SCFA, leading to a higher RER (**Chapter 4**). In females, fasting RER was lower than in males and not different between LDD and HDD females (~0.68; **Chapter 4**). The default switch of the liver to gluconeogenesis from amino acids during short fasting may partly explain a RER lower than 0.7 in females, but not the similar RER between HDD and LDD females. Speculatively, SCFA could have been used by LDD females as an additional substrate for lipogenesis instead of being oxidised.

It is thus not very clear what makes females respond differently to starch digestibility than males. Whatever the mechanism, it may also induce different phenotypes in the long run. As we will see shortly, there was indeed sexual dimorphism in the metabolic programming effects of starches. Sex dimorphisms in metabolic programming are well known, but a common drawback is that if these effects are measured too early they may reflect pubertal status rather than primarily sex differences[78]. However, we have measured effects both before complete sexual maturation and in mature animals, allowing us to interpret the sex-dependent metabolic programming effects more cleanly.

Metabolic programming effects of lowly vs highly digestible starches

The metabolic programming effects we observed were modest and only evident in females. In **Chapter 5**, LDD *vs* HDD females tended to have a better oral glucose tolerance when assessed 6 wk after introduction of the HFD. Later, these LDD females showed an improved metabolic flexibility (or the adaptation of fuel utilisation to fuel availability) 9 wk into the HFD regime (**Chapter 5**). At the end of the study one week later, LDD females exhibited a significantly lower absolute macrophage count in gWAT [LDD, 52.7 (22.5); HDD, 88.0 (18.3); mean and SD, $P < 0.01$; **Chapter 5**]. However, because LDD females started off with lower macrophage infiltration, the estimated number of macrophages per 1000 adipocytes accumulated during the HFD period was not significantly different [LDD, 26.2 (22.6); HDD, 38.3 (18.3); mean and SD, Student's *t* test $P = 0.329$]. The female study in **Chapter 3** was designed as an exact replication of **Chapter 5** (with the exception of an InCa test in early life and an oral glucose tolerance test only conducted in **Chapter 5**), and there we found no programming of metabolic flexibility, but a significantly higher increase in adiposity (3.3% or 1.7 g FM) in adult LDD compared to HDD females (**Chapter 3 Table S1**). Having seen that the programming evidence in **Chapters 5** and **3** does not support each other, what can be concluded? At least for female BW and body composition data, the

data from both Chapters can be analysed together with the help of a linear mixed model, taking into account breeding batch as a random factor, while maximising the power to detect differences (n = 36 females). This statistical approach is appropriate for unbalanced designs and allows us to estimate the effects of post-weaning diet after adjusting for the random effects affecting different animal batches. The analysis indicates that there was no programming of BW, FM, or LM by LDD *vs* HDD. With respect to metabolic flexibility, this direct comparison cannot be made directly because the challenge tests used to measure this parameter were different. In **Chapter 5**, we assessed metabolic flexibility as the RER increase in fasted mice after voluntary consumption of a solid food pellet containing starch (as the sole carbohydrate source), fat, and protein. In **Chapter 3**, a liquid mixed meal containing glucose and fat was administered by oral gavage instead. One could argue that the former type of challenge is more prone to chance findings because of its higher intersubject coefficient of variation (Chapter 5: 26%; Chapter 3: 11%; CV for RER), but both tests had similar intrasubject CV (Chapter 5: 7.1%; Chapter 3: 7.4%). We explicitly designed the nutritional challenge in **Chapter 3** to contain only glucose and fat because the direct effects of lowly *vs* highly digestible starches are known to target carbohydrate and fat metabolism[64,65,79], and so indicate parameters that may be amenable to programming. However, the inclusion of protein in nutritional stress challenges in human studies has been proposed because of its potential to modulate metabolic processes (*e.g.* suppression of lipolysis) related to metabolic health[80]. It is therefore possible that one of the key processes behind programming of metabolic flexibility by LDD *vs* HDD in **Chapter 5** was related to protein metabolism, and that this difference was not apparent in **Chapter 3** because protein was omitted in the challenge. The *form* of glucose included in the challenge might also be consequential. As discussed above for the direct effects of starch digestibility, dietary glucose oxidation was not influenced after 3 wk of intervention with lowly *vs* highly digestible starch in male mice[65], while we found a difference in dietary *starch* oxidation in **Chapter 4**. Our results remain inconclusive for metabolic flexibility in general, but the data suggests that LDD-programmed females are particularly efficient at adapting their fuel use strategy to glucose availability when provided as starch.

The consequences of early post-weaning LDD *vs* HDD on adult metabolic phenotypes have not been investigated previously. Gugusheff *et al.* investigated the effects of maternal intake of a lowly *vs* a highly digestible starch diet during pregnancy and lactation in rats. The findings were generally restricted to metabolic outcomes at weaning, showing lower visceral adiposity, lower hepatic lipid content, and improved glucose tolerance[81]. Only young adult female, but not male, offspring of dams fed a lowly digestible starch diet had a reduced hepatic expression of PI3K-p85 mRNA, speculated by the authors to eventually curb hepatic fat storage[81]. However, the experiment of Gugusheff *et al.* was not designed to test the direct and long-term effects of post-weaning starches separately, as offspring also had access to the mother's diet during weaning and the starch intervention continued until the end of the experiment, very early into adulthood. In support that the *form* of carbohydrates could program health outcomes much later in life, are the differences in longevity and reproductive success of female, but not male, offspring found when dams were fed a combination of glucose and fructose *vs* sucrose[82]. When understood as proof-of-concept studies that the *form* of carbohydrate (linear *vs* branched glucose polymers,

monosaccharides *vs* disaccharides) can programme later life health, and that this can be sex-dependent, these two studies are compatible with our conclusions.

Although there is little evidence to directly compare our results, the outcomes we found to be susceptible to metabolic programming by the post-weaning diet (FM and metabolic flexibility), have been shown to be programmed by perinatal nutrition in rodent models[83,84]. The notion that early life influences can alter disease risk, or the developmental origins of health and disease (DOHaD) paradigm[85], is increasingly applied to the post-weaning period[86]. There is substantial evidence for programing by nutritional and non-nutritional insults during the immediate post-weaning and adolescent stages on behavioural, cognitive, and neuro-endocrinological outcomes[87-89], and the evidence for obesity and related metabolic outcomes is also accumulating[90-93]. Within the DOHaD field, it is widely accepted that similar programming outcomes induced by different insults may reflect shared mechanisms[94]. These mechanisms can be conveniently divided into proximate (or physiological, those that operate in the recent past, *e.g.* epigenetic mechanisms) and ultimate (or the processes that explain how the DOHaD phenomenon was selected for during evolution) [95]. Next to epigenetic programming of gene expression and accelerated cellular aging, tissue structure changes have been demonstrated as proximate mechanisms for metabolic programming[96-99]. Changes in tissue structure may be especially important in programming by starches. One of the strongest direct effects of LDD was to increase small intestinal length and weight in both sexes (**Chapter 5**), perhaps as a consequence of relative caloric restriction with the less energy dense LDD[100], or the trophic effects of SCFAs derived from LDD[101]. Although the length and weight of the small intestine were not significantly different between LDD and HDD adult mice at the end of the HFD period, there seems to be some relationship between small intestinal weight and FM gain on HFD. LDD females that gained more FM during HFD feeding also had a higher small intestinal weight after HFD feeding (Pearson correlation, $r^2 = 0.69$, $P < 0.001$, $n = 12$); this correlation was less significant in HDD females ($r^2 = 0.43$, $P < 0.05$, $n = 12$) and was absent in males. Small intestinal weight of adult HDD females was on average 23% higher compared to the group culled at the end on the post-weaning intervention, whereas adult LDD females had a 3% lighter small intestine compared to the young LDD group. This suggests that LDD females had a full-grown intestine already before the HFD, compared to HDD females where this organ continued to grow. If the earlier maturation of the small intestine by LDD *vs* HDD led to enhanced nutrient absorption, this could partly explain the higher FM of adult LDD females in **Chapter 3**. Accordingly, FI completely equalised during the 1st week of HFD feeding and remained similar between groups, showing that the changes in FM were at least not mediated by an altered ingestive behaviour.

Regarding the "mismatch" hypothesis of DOHaD, that states that differences between the environmental factors to which an organism is adapted and the environment extant later can affect fitness, two possibilities come to mind. Firstly, a mismatch between the anatomical and physiological adaptations to the foetal or suckling environments (*e.g.* influenced by the fermentable carbohydrates or the GI of chow consumed by the dam or the dam's milk) could have made such changes maladaptive when the offspring were switched to the HDD (with no fermentable carbohydrates and possibly of high GI). This type of

developmental mismatch has also been proposed by Gugusheff *et al*.[81], and I would add that it is also a case of evolutionary mismatch. Second, if developmental plasticity in mice is still operative up till mid-adolescence, the mismatch may have happened between the phenotype adapted to LDD (less energy dense, possibly of low GI) during post-weaning and the later HFD (rich in energy and with highly digestible carbohydrates). This could explain why HDD females resisted body fat deposition on a HFD relative to LDD females in **Chapter 3**. Although this kind of developmental mismatch is mostly discussed in the context of foetal undernutrition and postnatal overnutrition in the DOHaD literature[86,102,103], the mismatch hypothesis may operate at later nutritional transitions as long as there is some degree of developmental plasticity.

We are only beginning to understand the potential of starches to shape the developmental trajectory of an organism. In this thesis, different phenotypic outcomes in adult mice who consumed LDD *vs* HDD from weaning to mid-adolescence, and then switched to a HFD until 15 wk of age, were considered as evidence of metabolic programming. We focussed on WAT based on previous observations in our laboratory showing strong direct effects of LDD *vs* HDD on this organ[79,104]. But is that all there is to programming by starches? By no means. Firstly, there may be deeper or more nuanced effects in the organs that we found to be targeted by starches. Adipogenesis in rodents can continue at least until puberty[105,106], and acetate, a SCFA increased directly by LDD, might induce adipogenesis[107]. This calls for measurements of cellularity in WAT, which requires complex techniques. The implications of macrophage infiltration in gWAT also await further exploration. Although we tested a selection of molecular markers in gWAT (**Chapter 5**), this type of analysis did not suggest that macrophages in this tissue in adult HDD females were pro-inflammatory (a more powerful approach could be, for instance, flow cytometry of macrophages isolated from fresh tissue). Nevertheless, at least one study suggests that eWAT macrophages in mice directly fed a lowly digestible starch may be pro-inflammatory[108]. Secondly, other organs and tissues (*i.e.* cardiovascular, kidney, liver, brain, pancreas, skeletal muscle[109]) are known to be susceptible to metabolic programming. For instance, the pancreas undergoes remodelling until four weeks after birth in rats[110], β-cell mass is maintained by SCFA (directly increased by LDD) through inhibition of apoptosis[107], and brain and colon can undergo epigenetic modifications up until early adulthood[111,112].

Our findings that the metabolic flexibility and adiposity of the adult are influenced by the digestibility of starches consumed earlier in life thus raise new questions: Could other organs and tissues be programmed by starches? Could stronger effects of post-weaning diet be observed when the animals are followed into middle age? Could effects on other health outcomes be observed in males? The way forward in answering these questions becomes easier when one has identified clear direct effects in both sexes, and then follows those leads in longer studies. A more detailed approach is offered in the closing section of this Discussion.

Implications of starch digestibility for human health

Metabolic flexibility, as tested by refeeding challenges, is considered a signature of metabolic health. This measurement integrates the digestion, absorption, distribution, peripheral

uptake, and ultimately the oxidation of the nutrients contained in a meal. In the transition from net fat oxidation during fasting to net glucose oxidation after a high carbohydrate meal, large flexibility may indicate optimal insulin sensitivity[113], while decreased flexibility may precede the development of glucose intolerance[114]. Further, being metabolically flexible is a component of phenotypic flexibility, or the capacity of an organism to recover homeostasis in the face of metabolic challenges[32], and is closely linked to WAT health[115]. On the other hand, body fat mass might be one of the factors contributing to cardiometabolic risk[116]. The programmed increase in FM by LDD was not significant when taking all the data together however, and some have argued that lower macrophage infiltration in WAT is a feature of metabolically healthy obesity[117]. Taken together, our data would seem to support the view that LDD-programmed females were metabolically fitter.

To what extent could we extrapolate our findings in this mouse model to humans? To appreciate the translational value of our findings we should consider two things: first, how well the developing mouse resembles human ontogeny, and second, how is the exposure to lowly or highly digestible starches in young humans. The ontogenetic window of WAT, the organ we mostly focussed on in this thesis, is different in mice and humans. Mice have an underdeveloped adipose tissue at birth (2.1% fat), while humans are born with a more developed adipose tissue (15% fat)[118]. However, mouse WAT development resembles that of humans in that both retain the capacity to increase adipocyte number during childhood and adolescence[119,120]. To the extent that the ontogenetic windows of mice and humans allow us to make a proper comparison, the beginning of our dietary starch intervention in post-weaning mice could represent an earlier developmental period in humans, possibly covering the very early postnatal period known as the first 1000 days of life, and extend until puberty, at least with regard to WAT development.

Since it is possible that the digestibility of the first starches consumed in life influences health in the long run also in humans, how does that fit into the current recommendations for carbohydrate intake for the developing human? Surprisingly, not at all. Currently, there are no science-based recommendations for starch intake for infants and toddlers, and it is assumed that carbohydrate intake after 2 years of age should follow to the recommendations for adults[121]. The current advice of WHO/FAO is to limit added sugars to <10 % of total energy intake (based on risk of developing caries and taste preferences)[121,122] and, if anything, we know that these recommendations are not always met[122,123]. The closest thing we have to proper recommendations for starch intake is the recently issued Early Nutrition Project consensus, stating that there is no evidence for non-digestible carbohydrates added to infant formula to be protective against later obesity risk[124]. The lack of recommendations may in part be because data on starch intake by infants and toddlers is extremely limited. We only know that European infants younger than 1 year of age consume about 15% of their energy intake as starch, and children older than two years 23%, but what type of starches these are is much less clear[122]. For babies, these are likely highly digestible, because only precooked and gelatinised starch can be included in infant formulas according to current EU Directives, but the types of starches included in complementary foods are not restricted[122]. This is compounded by the fact that the digestibility of starches largely depends on their origin and processing methods, such

as cooking and cooling, making it very difficult to precisely estimate the exposure of the toddlers and children to lowly and highly digestible starches. As an alternative way to compare our model to human feeding practices early in life, the post-weaning HDD could mimic the premature weaning of a baby onto a high carbohydrate diet because of its purported high GI. This comparison is not sufficient however, as it does not take the effects of fermentation into account, and because GI is a poor indicator of carbohydrate quality compared to dietary fibre[125]. Clearly, there is ample room to expand the recommendations for carbohydrate intake for babies and children. These recommendations need be informed by empirical evidence and will require a better recognition of digestibility as a measure of carbohydrate quality.

Conclusions

Females and males are affected differently by starches both directly and in the long-term. Direct exposure to LDD *vs* HDD leads to lower inflammation and smaller adipocyte sizes in the gonadal adipose depot in females, and to decreased fat mass in males. In the long run, LDD consumed from weaning until mid-adolescence may program the female organism for a better metabolic flexibility or for higher adiposity during adulthood compared to HDD. The programming effects we observed were subtle, and further mechanistic studies are needed to substantiate them. Finally, our results strengthen the evidence that childhood and adolescence are important periods of development during which metabolic programming can take place.

An Experimental Approach for Future Studies on Metabolic Programming

While the importance of childhood and adolescence as critical periods of development are increasingly recognised in the DOHaD field[126-128], most of the research efforts in experimental models have been focussed on the perinatal period. This choice may be conscious and very logical because, let it be said, intervening early on likely works much better to prevent disease risk[129]. However, I argue that another reason why metabolic programming studies do not commonly target phases of development later than weaning is because DOHaD hypotheses are difficult to study after this stage. I can offer two possible sources where this difficulty comes from. First, environmental cues become integrated as development progresses, and the resulting phenotype is influenced by each one of these cues. This was demonstrated by Gluckman *et al.*, when they showed that the hepatic gene expression response to leptin administration in the neonatal rat is directionally dependent on the *in utero* nutritional status (*ad libitum vs* undernourished dams)[130]. Because environmental exposures during highly plastic windows of development can determine the organismal response to future cues, this implies that even minor alterations in the experimental conditions before weaning can lead to different conclusions about metabolic programming by the post-weaning diet. We have seen indications of this phenomenon in other experiments conducted in our laboratory[90] and also the ones reported in this thesis (**Chapters 3** and **5**). Despite the study in **Chapter 3** was designed as an exact replication of **Chapter 5** (with the exception of an InCa and an oral glucose tolerance test only conducted

in Chapter 5), FM was programmed only in **Chapter 3** (LDD > HDD). Moreover, the same HFD generally induced obesity more in **Chapter 3** females [Chapter 3, 32 (22, 42); Chapter 5, 16 (9, 31)% fat, median and range; Mann Whitney test, $P < 0.0001$], indicating that mice in **Chapter 3** were more susceptible to the obesogenic environment. We know that at least two experimental variables may have affected different batches of mice at different stages of development: the breeding chow and the photoperiod. Since the chow provider stopped their services in the period between the studies reported in **Chapters 5** and **3**, we were forced to switch to another provider and thus chow composition. Although the chow diet of dams in all of our studies contained about 25% energy as protein, we later realized that the protein in the breeding chow in **Chapter 3** was mainly of meat and dairy origin, while that in **Chapter 5** came exclusively from grains. Also, the energy density of the breeding chow in **Chapter 3** was considerably lower than that in **Chapter 5** (13 *vs* 17.3 kJ g^{-1}). This is relevant because protein (of which almost only casein has been studied[94]) and energy restriction at various levels during gestation or lactation are known to affect metabolic outcomes like body weight and food intake in the offspring[131]. The chow in **Chapter 5** also had a slightly lower methionine content (4 g kg^{-1} *vs* 5 g kg^{-1}), and methionine levels in the maternal diet might impact hepatic gene methylation in the foetus, which could in turn affect gene expression during organogenesis and eventually the phenotype of the offspring[94]. In addition, the chow used in **Chapter 3** contained less fibre (2.7 %, from wheat and corn) compared to the chow in **Chapter 5** (3.5%, from wheat, oats, and alfalfa), which also suggests a very different fibre profile in terms of soluble and insoluble fibre, with potential consequences for the gut microbiome. Finally, some bioactive ingredients like phytoestrogens from alfalfa or linseed[132,133] in **Chapter 5's** chow could have had metabolic effects in the dams and their offspring. The other environmental factor that could have influenced our results was an unfortunate malfunction of the light schedule during the study in **Chapter 3**, when a 3 h extension of the light regime happened during the first week of suckling and around conception, potentially misaligning the circadian system of dams and pups. Whether the 3 extra hours of light exposure were sufficient to disrupt the circadian system is difficult to estimate and rather debatable, but any deviations in the light regime are always strictly advised against in animal husbandry since light is a powerful entrainer of biological rhythms. The effects of chronodisruption during the perinatal period can compromise maternal care[134], and circadian disruption during pregnancy is also known to affect foetal adrenal development which in turn may affect disease risk later in life[135]. Indeed, the statistical conclusions depended on the animal breeding batch between and within Chapters, and this factor accounted for 62% of the variance of the random effects in the whole data set. The bottom line is, even when we included littermate controls and further stratified mice by BW at weaning, if these environmental exposures had the power to change the response to later cues (LDD, HDD, and HFD) then these measures were likely ineffective.

The other reason why DOHaD hypotheses may be difficult to test in childhood and adolescence may be because these developmental windows are not very well defined in animal models. Mice do not have a childhood per se[29]. Apart from the generally straightforward considerations of sexual maturity and although adolescence is better defined in rodents than in other mammal models[136], adolescence in rodents is defined by

neurobehavioural characteristics[136], while adolescence in humans is characterised by both psychological and metabolic aspects[128]. Moreover, the precise periods of developmental plasticity have not been mapped for all organ systems. This implies that further mapping of developmental plasticity and comparable assessment, in mouse and humans, should be part of programming studies in mice. This will inform our interpretations of effects in model organisms and strengthen the value of DOHaD as a theory.

I would like to propose a two-step experimental approach to make further progress in metabolic programming studies based on post-weaning nutrition, including the exploration of mechanisms. One strategy is to continue to conduct reductive, proof-of-concept studies in both sexes, like in this thesis. A useful first step is to characterise the direct effects of nutritional insults, although some may escape phenotyping if they are subtle and only become obvious in extreme environments. These direct effects may result from predictive or immediate adaptive responses of the organism to the environment, and may give a hint about what systems may become programmed. It will be important to consistently target the intended developmental window of intervention in these studies which could imply, for example, the separation of the dam's diet from the pups during lactation by means of special cages (where pups and dams can be fed separately at least until 18 days postnatal), making sure that pups are weaned only onto the intervention diet and not onto chow[137]. These experiments should be followed by longer mechanistic studies that include attempts to revert the purported programmed outcomes. Additionally, this would clarify whether "permanence" of effects defines metabolic programming as proposed by some[99,138], but not fully supported by evidence[139], and improve our understanding of the programming mechanisms that are at play as developmental plasticity declines. To effectively and efficiently establish programming effects by post-weaning nutrition, all of these experiments should be highly controlled and standardised and ensure adequate statistical power, not necessarily by using more animals, but by using precise and integrative phenotyping tools.

At a later stage, one could release the control of some variables, attempt to resemble human diets more closely, and test the established programming effects in more ecologically relevant conditions. Such experiments could be conducted in semi-natural enclosures[82], making the timing of weaning more natural[140,141] and mimicking the baby-led weaning practice recommended in humans[142]. These less controlled but more realistic conditions would likely highlight the most consequential nutritional cues that can program metabolism after weaning. More importantly, it would also allow us to evaluate how nutritional cues play out under the evolutionary dogmas of DOHaD, *e.g.* developmental adaptations hypothesised to preserve survival up until reproductive age could be tested directly. Ultimately, this take on developmental programming would help achieve the holistic approach to nutritional science advocated by some scholars[143].

Conclusions

In 2015, the year when this PhD project started, the Sustainable Development Goals (SDGs) were launched. Together with our work, future studies trying to navigate the complexity of nutritional programming phenomena will hopefully contribute to reach SDG #3: "Good health and well-being for people". Nutrition during childhood and adolescence

may strongly impact later life health, but experimental evidence for the programming effects of nutrition during these stages is in short supply. Likely, this is because DOHaD hypotheses are difficult to test once the responses to all previous environmental cues have already been integrated. We should however embrace this complexity and keep on researching, because what this complexity is really telling us is that there are many ways to intervene.

General Conclusions

We have shown that a mouse indirect calorimetry system extended with additional gas analysers can be used to study gut microbial activity (H_2 and CH_4) and the oxidation of exogenous vs endogenous substrates ($^{13}CO_2$ and $^{12}CO_2$). Our studies also showed that, although the effects of a lowly digestible starch diet are generally beneficial, female and male mice are differently affected by dietary starch digestibility. Finally, dietary starch digestibility during the early post-weaning period seem to program metabolic health later in life especially in females, although the observed effects were subtle and need to be confirmed and substantiated mechanistically.

References

1 Smith, N. W., Shorten, P. R., Altermann, E. H., Roy, N. C. & McNabb, W. C. Hydrogen cross-feeders of the human gastrointestinal tract. *Gut microbes*, 1-19 (2018).

2 Levitt, M. D. Production and excretion of hydrogen gas in man. *N. Engl. J. Med.* **281**, 122-127 (1969).

3 Carbonero, F., Benefiel, A. C. & Gaskins, H. R. Contributions of the microbial hydrogen economy to colonic homeostasis. *Nat. Rev. Gastroenterol. Hepatol.* **9**, 504-518 (2012).

4 Andrieux, C., Pacheco, E. D., Bouchet, B., Gallant, D. & Szylit, O. Contribution of the digestive tract microflora to amylomaize starch degradation in the rat. *Brit. J. Nutr.* **67**, 489-99 (1992).

5 Fetissov, S. O. Role of the gut microbiota in host appetite control: bacterial growth to animal feeding behaviour. *Nat. Rev. Endocrinol.* **13**, 11-25 (2017).

6 Key, F. B. & Mathers, J. C. Digestive adaptations of rats given white bread and cooked haricot beans (*Phaseolus vulgaris*): large-bowel fermentation and digestion of complex carbohydrates. *Br. J. Nutr.* **74**, 393-406 (1995).

7 Walker, A. W. *et al.* Dominant and diet-responsive groups of bacteria within the human colonic microbiota. *ISME J.* **5**, 220-230 (2011).

8 Levrat, M. A., Remesy, C. & Demigne, C. Very acidic fermentations in the rat cecum during adaptation to a diet rich in amylase-resistant starch (crude potato starch). *J. Nutr. Biochem.* **2**, 31-36 (1991).

9 David, L. A. *et al.* Host lifestyle affects human microbiota on daily timescales. *Genome biology* **15**, R89 (2014).

10 Padmanabhan, P., Grosse, J., Asad, A. B., Radda, G. K. & Golay, X. Gastrointestinal transit measurements in mice with ^{99m}Tc-DTPA-labeled activated charcoal using NanoSPECT-CT. *EJNMMI Res.* **3**, 60 (2013).

11 Tuboly, E. *et al.* Excessive alcohol consumption induces methane production in humans and rats. *Sci. Rep.* **7**, 7329 (2017).

12 Tuboly, E. *et al.* Determination of endogenous methane formation by photoacoustic spectroscopy. *J. Breath Res.* **7**, 046004 (2013).

13 Tuboly, E. *et al.* Methane biogenesis during sodium azide-induced chemical hypoxia in rats. *Am. J. Physiol., Cell Physiol.* **304**, C207-214 (2013).

14 Swanson, K. S. *et al.* Phylogenetic and gene-centric metagenomics of the canine intestinal microbiome reveals similarities with humans and mice. *ISME J.* **5**, 639-649 (2011).

15 Laverdure, R., Mezouari, A., Carson, M. A., Basiliko, N. & Gagnon, J. A role for methanogens and methane in the regulation of GLP-1. *Endocrinol. Diabetes Metab.* **1**, e00006 (2018).

16 Hamer, H. M., De Preter, V., Windey, K. & Verbeke, K. Functional analysis of colonic bacterial metabolism: relevant to health? *Am. J. Physiol. Gastrointest. Liver Physiol.* **302**, G1-9 (2012).

17 Nakamura, N., Lin, H. C., McSweeney, C. S., Mackie, R. I. & Gaskins, H. R. Mechanisms of microbial hydrogen disposal in the human colon and implications for health and disease. *Annu. Rev. Food Sci. Technol.* **1**, 363-395 (2010).

18 Zheng, X. F., Sun, X. J. & Xia, Z. F. Hydrogen resuscitation, a new cytoprotective approach. *Clin. Exp. Pharmacol. Physiol.* **38**, 155-163 (2011).

19 Nakao, A., Toyoda, Y., Sharma, P., Evans, M. & Guthrie, N. Effectiveness of hydrogen rich water on antioxidant status of subjects with potential metabolic syndrome-an open label pilot study. *J. Clin. Biochem. Nutr.* **46**, 140-149 (2010).

20 Kajiyama, S. *et al.* Supplementation of hydrogen-rich water improves lipid and glucose metabolism in patients with type 2 diabetes or impaired glucose tolerance. *Nutr. Res.* **28**, 137-143 (2008).

21 Makki, K., Deehan, E. C., Walter, J. & Backhed, F. The impact of dietary fiber on gut microbiota in host health and disease. *Cell Host Microbe* **23**, 705-715 (2018).

22 Attene-Ramos, M. S., Wagner, E. D., Plewa, M. J. & Gaskins, H. R. Evidence that hydrogen sulfide is a genotoxic agent. *Mol. Cancer Res.* **4**, 9-14 (2006).

23 Wang, R. Two's company, three's a crowd: can H_2S be the third endogenous gaseous transmitter? *FASEB J.* **16**, 1792-1798 (2002).

24 Goubern, M., Andriamihaja, M., Nubel, T., Blachier, F. & Bouillaud, F. Sulfide, the first inorganic substrate for human cells. *FASEB J.* **21**, 1699-1706 (2007).

25 Soriano, R. N. *et al.* Endogenous peripheral hydrogen sulfide is propyretic: its permissive role in brown adipose tissue thermogenesis in rats. *Exp. Physiol.* **103**, 397-407 (2018).

26 Gerrits, W. J. J., Borne, J. J. G. C. v. d. & Labussière, E. in *Indirect Calorimetry* Ch. 1, 19-34 (Wageningen Academic Publishers, Wageningen, 2015).

27 Poppitt, S. D. *et al.* Circadian patterns of total 24-h hydrogen and methane excretion in humans ingesting nonstarch polysaccharide (NSP) diets and the implications for indirect calorimetric and $D_2{}^{18}O$ methodologies. *Eur. J. Clin. Nutr.* **50**, 524-534 (1996).

28 Tulley, R. T. *et al.* Comparative methodologies for measuring metabolizable energy of various types of resistant high amylose corn starch. *J. Agric. Food Chem.* **57**, 8474-8479 (2009).

29 Brust, V., Schindler, P. M. & Lewejohann, L. Lifetime development of behavioural phenotype in the house mouse (*Mus musculus*). *Front. Zool.* **12 Suppl 1**, S17 (2015).

30 Keenan, M. J. *et al.* Role of resistant starch in improving gut health, adiposity, and insulin resistance. *Adv. Nutr.* **6**, 198-205 (2015).

31 Mushref, M. A. & Srinivasan, S. Effect of high fat-diet and obesity on gastrointestinal motility. *Ann. Transl. Med.* **1**, 14 (2013).

32 Picó, Serra, Rodríguez, Keijer & Palou. Biomarkers of nutrition and health: new tools for new approaches. *Nutrients* **11**, 1092 (2019).

33 Mosora, F. *et al.* Quantitative evaluation of the oxidation of an exogenous glucose load using naturally labeled ^{13}C-glucose. *Metabolism* **25**, 1575-1582 (1976).

34 Welch, K. C., Jr., Peronnet, F., Hatch, K. A., Voigt, C. C. & McCue, M. D. Carbon stable-isotope tracking in breath for comparative studies of fuel use. *Ann. N. Y. Acad. Sci.* **1365**, 15-32 (2016).

35 Ishihara, K. *et al.* A soybean peptide isolate diet promotes postprandial carbohydrate oxidation and energy expenditure in type II diabetic mice. *J. Nutr.* **133**, 752-757 (2003).

36 McCue, M. D. & Pollock, E. D. Measurements of substrate oxidation using $^{13}CO_2$-breath testing reveals shifts in fuel mix during starvation. *J. Comp. Physiol. B.* **183**, 1039-1052 (2013).

37 Ishihara, K. *et al.* Use of ^{13}C-labeled glucose for measuring exogenous glucose oxidation in mice. *Biosci. Biotechnol. Biochem.* **66**, 426-429 (2014).

38 Ishihara, K. *et al.* Electrolyte-free milk protein solution influences sodium and fluid retention in rats. *J. Nutr. Sci.* **2**, e8 (2013).

39 Zhou, J. *et al.* Dietary resistant starch upregulates total GLP-1 and PYY in a sustained day-long manner through fermentation in rodents. *Am. J. Physiol. Endocrinol. Metab.* **295**, E1160-1166 (2008).

40 Friedrich, M., Petzke, K. J., Raederstorff, D., Wolfram, S. & Klaus, S. Acute effects of epigallocatechin gallate from green tea on oxidation and tissue incorporation of dietary lipids in mice fed a high-fat diet. *Int. J. Obes.* **36**, 735-743 (2012).

41 Klaus, S., Pultz, S., Thone-Reineke, C. & Wolfram, S. Epigallocatechin gallate attenuates diet-induced obesity in mice by decreasing energy absorption and increasing fat oxidation. *Int. J. Obes.* **29**, 615-623 (2005).

42 Schrauwen, P. *et al.* Validation of the [1,2-^{13}C]acetate recovery factor for correction of [U-^{13}C] palmitate oxidation rates in humans. *J. Physiol.* **513**, 215-223 (1998).

43 Bergouignan, A., Schoeller, D. A., Votruba, S., Simon, C. & Blanc, S. The acetate recovery factor to correct tracer-derived dietary fat oxidation in humans. *Am. J. Physiol. Endocrinol. Metab.* **294**, E645-653 (2008).

44 Antoun, E. *et al.* The [1-^{13}C]acetate recovery factor to correct tracer-derived dietary fat oxidation is lower in overweight insulin-resistant subjects. *E Spen Eur. E J. Clin. Nutr. Metab.* **5**, e173-e179 (2010).

45 Bessesen, D. H., Rupp, C. L. & Eckel, R. H. Dietary-fat is shunted away from oxidation, toward storage in obese Zucker rats. *Obes. Res.* **3**, 179-189 (1995).

46 Bessesen, D. H., Bull, S. & Cornier, M. A. Trafficking of dietary fat and resistance to obesity. *Physiol. Behav.* **94**, 681-688 (2008).

47 Jackman, M. R., Kramer, R. E., MacLean, P. S. & Bessesen, D. H. Trafficking of dietary fat in obesity-prone and obesity-resistant rats. *Am. J. Physiol. Endocrinol. Metab.* **291**, E1083-1091 (2006).

48 Rynders, C. A., Pereira, R. I., Bergouignan, A., Kealey, E. H. & Bessesen, D. H. Associations among dietary fat oxidation responses to overfeeding and weight gain in obesity-prone and resistant adults. *Obesity* **26**, 1758-1766 (2018).

49 Speakman, J. R. & Thomson, S. C. Validation of the labeled bicarbonate technique for measurement of short-term energy expenditure in the mouse. *Z. Ernahrungswiss* **36**, 273-277 (1997).

50 Duivenvoorde, L. P., van Schothorst, E. M., Swarts, H. J. & Keijer, J. Assessment of metabolic flexibility of old and adult mice using three noninvasive, indirect calorimetry-based treatments. *J. Gerontol. A Biol. Sci. Med. Sci.* **70**, 282-293 (2015).

51 Elia, M. & Livesey, G. Energy expenditure and fuel selection in biological systems: the theory and practice of calculations based on indirect calorimetry and tracer methods. *World Rev. Nutr. Diet* **70**, 68-131 (1992).

52 Schutz, Y. & Ravussin, E. Respiratory quotients lower than 0.70 in ketogenic diets. *Am. J. Clin. Nutr.* **33**, 1317-1319 (1980).

53 Ferrannini, E. The theoretical bases of indirect calorimetry: q review. *Metabolism* **37**, 287-301 (1988).

54 Sonko, B. J., Prentice, A. M., Coward, W. A., Murgatroyd, P. R. & Goldberg, G. R. Dose-response relationship between fat ingestion and oxidation: quantitative estimation using whole-body calorimetry and ^{13}C isotope ratio mass spectrometry. *Eur. J. Clin. Nutr.* **55**, 10-18 (2001).

55 Flachs, P. *et al.* Synergistic induction of lipid catabolism and anti-inflammatory lipids in white fat of dietary obese mice in response to calorie restriction and n-3 fatty acids. *Diabetologia* **54**, 2626-2638 (2011).

56 Bowe, J. E. *et al.* Metabolic phenotyping guidelines: assessing glucose homeostasis in rodent models. *J. Endocrinol.* **222**, G13-25 (2014).

57 Shinozaki, K. *et al.* Dissociated oxygen consumption and carbon dioxide production in the post-cardiac arrest rat: a novel metabolic phenotype. *J. Am. Heart Assoc.* **7**, e007721 (2018).

58 Pereira, J. *et al.* Breath analysis as a potential and non-invasive frontier in disease diagnosis: an overview. *Metabolites* **5**, 3-55 (2015).

59 Buszewski, B., Kesy, M., Ligor, T. & Amann, A. Human exhaled air analytics: biomarkers of diseases. *Biomed. Chromatogr.* **21**, 553-566 (2007).

60 Karl, T. *et al.* Human breath isoprene and its relation to blood cholesterol levels: new measurements and modeling. *J. Appl. Physiol. (1985)* **91**, 762-770 (2001).

61 Weitz, Z. W., Birnbaum, A. J., Sobotka, P. A., Zarling, E. J. & Skosey, J. L. High breath pentane concentrations during acute myocardial infarction. *Lancet* **337**, 933-935 (1991).

62 Peng, G. *et al.* Diagnosing lung cancer in exhaled breath using gold nanoparticles. *Nat. Nanotechnol.* **4**, 669-673 (2009)

63 Speakman, J. R. Measuring energy metabolism in the mouse - theoretical, practical, and analytical considerations. *Front. Physiol.* **4**, 34 (2013).

64 Campbell, G. J., Senior, A. M. & Bell-Anderson, K. S. Metabolic effects of high glycaemic index diets: a systematic review and meta-analysis of feeding studies in mice and rats. *Nutrients* **9**, 646 (2017).

65 Isken, F. *et al.* Impairment of fat oxidation under high- vs. low-glycemic index diet occurs before the development of an obese phenotype. *Am. J. Physiol. Endocrinol. Metab.* **298**, E287-295 (2010).

66 Kabir, M. *et al.* Dietary amylose-amylopectin starch content affects glucose and lipid metabolism in adipocytes of normal and diabetic rats. *J. Nutr.* **128**, 35-43 (1998).

67 Karastergiou, K. & Fried, S. K. Cellular mechanisms driving sex differences in adipose tissue biology and body shape in humans and mouse Models. *Adv. Exp. Med. Biol.* **1043**, 29-51 (2017).

68 Chen, X., McClusky, R., Itoh, Y., Reue, K. & Arnold, A. P. X and Y chromosome complement influence adiposity and metabolism in mice. *Endocrinology* **154**, 1092-1104 (2013).

69 Ingvorsen, C., Karp, N. A. & Lelliott, C. J. The role of sex and body weight on the metabolic effects of high-fat diet in C57BL/6N mice. *Nutr. Diabetes* **7**, e261 (2017).

70 Riddell, M. C. The endocrine response and substrate utilization during exercise in children and adolescents. *J. Appl. Physiol. (1985)* **105**, 725-733 (2008).

71 Tarnopolsky, M. A. Gender differences in substrate metabolism during endurance exercise. *Can. J. Appl. Physiol.* **25**, 312-327 (2000).

72 Falconer, D. S. Weight and age at puberty in female and male mice of strains selected for large and small body size. *Genet. Res.* **44**, 47-72 (1984).

73 Link, J. C. & Reue, K. Genetic basis for sex differences in obesity and lipid metabolism. *Annu. Rev. Nutr.* **37**, 225-245 (2017).

74 Zeevi, D. *et al.* Structural variation in the gut microbiome associates with host health. *Nature* **568**, 43-48 (2019).

75 Tang, W. H. & Hazen, S. L. Microbiome, trimethylamine *N*-oxide, and cardiometabolic disease. *Transl. Res.* **179**, 108-115 (2017).

76 Bennett, B. J. *et al.* Trimethylamine-*N*-oxide, a metabolite associated with atherosclerosis, exhibits complex genetic and dietary regulation. *Cell Metab.* **17**, 49-60 (2013).

77 Della Torre, S. *et al.* Short-term fasting reveals amino acid metabolism as a major sex-discriminating factor in the liver. *Cell Metab.* **28**, 256-267 e255 (2018).

78 Aiken, C. E. & Ozanne, S. E. Sex differences in developmental programming models. *Reproduction* **145**, R1-13 (2013).

79 van Schothorst, E. M., Bunschoten, A., Schrauwen, P., Mensink, R. P. & Keijer, J. Effects of a high-fat, low- versus high-glycemic index diet: retardation of insulin resistance involves adipose tissue modulation. *FASEB J.* **23**, 1092-1101 (2009).

80 Stroeve, J. H., van Wietmarschen, H., Kremer, B. H., van Ommen, B. & Wopereis, S. Phenotypic flexibility as a measure of health: the optimal nutritional stress response test. *Genes Nutr.* **10**, 459 (2015).

81 Gugusheff, J. *et al.* The effect of maternal and post-weaning low and high glycaemic index diets on glucose tolerance, fat deposition and hepatic function in rat offspring. *J. Dev. Orig. Health Dis.*, 1-10 (2015).

82 Ruff, J. S. *et al.* Compared to sucrose, previous consumption of fructose and glucose monosaccharides reduces survival and fitness of female mice. *J. Nutr.* **145**, 434-441 (2015).

83 Huber, M. *et al.* How should we define health? *BMJ* **343**, d4163 (2011).

84 Jousse, C. *et al.* Perinatal protein malnutrition affects mitochondrial function in adult and results in a resistance to high fat diet-induced obesity. *PLoS One* **9**, e104896 (2014).

85 Gluckman, P. D. *et al.* Metabolic plasticity during mammalian development is directionally dependent on early nutritional status. *Proc. Natl. Acad. Sci. USA* **104**, 12796-12800 (2007).

86 Koletzko, B. *et al.* The Power of Programming and the EarlyNutrition project: opportunities for health promotion by nutrition during the first thousand days of life and beyond. *Ann. Nutr. Metab.* **64**, 187-196 (2014).

87 Schmidt, M. V. Molecular mechanisms of early life stress--lessons from mouse models. *Neurosci. Biobehav. Rev.* **34**, 845-852 (2010).

88 Alamy, M. & Bengelloun, W. A. Malnutrition and brain development: an analysis of the effects of inadequate diet during different stages of life in rat. *Neurosci. Biobehav. Rev.* **36**, 1463-1480 (2012).

89 Boukouvalas, G., Antoniou, K., Papalexi, E. & Kitraki, E. Post weaning high fat feeding affects rats' behavior and hypothalamic pituitary adrenal axis at the onset of puberty in a sexually dimorphic manner. *Neuroscience* **153**, 373-382 (2008).

90 Bouwman, L. M. S. *et al.* Replacing part of glucose with galactose in the postweaning diet protects female but not male mice from high-fat diet-induced adiposity in later life. *J Nutr.* nxz028 (2019).

91 Oosting, A. *et al.* Effect of dietary lipid structure in early postnatal life on mouse adipose tissue development and function in adulthood. *Br. J. Nutr.* **111**, 215-226 (2014).

92 He, Y. H. *et al.* Postweaning low-calcium diet promotes later-life obesity induced by a high-fat diet. *J. Nutr. Biochem.* **23**, 1238-1244 (2012).

93 Minana-Solis Mdel, C. & Escobar, C. Post-weaning protein malnutrition in the rat produces short and long term metabolic impairment, in contrast to earlier and later periods. *Int. J. Biol. Sci.* **4**, 422-432 (2008).

94 Armitage, J. A., Khan, I. Y., Taylor, P. D., Nathanielsz, P. W. & Poston, L. Developmental programming of the metabolic syndrome by maternal nutritional imbalance: how strong is the evidence from experimental models in mammals? *J. Physiol.* **561**, 355-377 (2004).

95 Gluckman, P. D., Hanson, M. A. & Buklijas, T. A conceptual framework for the developmental origins of health and disease. *J. Dev. Orig. Health Dis.* **1**, 6-18 (2010).

96 Vickers, M. H. Early life nutrition, epigenetics and programming of later life disease. *Nutrients* **6**, 2165-2178 (2014).

97 Reichetzeder, C., Dwi Putra, S. E., Li, J. & Hocher, B. Developmental origins of disease - crisis precipitates change. *Cell. Physiol. Biochem.* **39**, 919-938 (2016).

98 Sutton, E. F. *et al.* Developmental programming: state-of-the-science and future directions - summary from a Pennington Biomedical symposium. *Obesity* **24**, 1018-1026 (2016).

99 Langley-Evans, S. C. Nutrition in early life and the programming of adult disease: a review. *J. Hum. Nutr. Diet.* **28 Suppl 1**, 1-14 (2015).

100 Mitchell, S. E. *et al.* The effects of graded levels of calorie restriction: I. impact of short term calorie and protein restriction on body composition in the C57BL/6 mouse. *Oncotarget* **6**, 15902-15930 (2015).

101 Bird, A. R., Brown, I. L. & Topping, D. L. Starches, resistant starches, the gut microflora and human health. *Curr. Issues Intest. Microbiol.* **1**, 25-37 (2000).

102 Hanson, M. A. & Gluckman, P. D. Early developmental conditioning of later health and disease: physiology or pathophysiology? *Physiol. Rev.* **94**, 1027-1076 (2014).

103 Gluckman, P. D., Hanson, M. A. & Beedle, A. S. Early life events and their consequences for later disease: a life history and evolutionary perspective. *Am. J. Hum. Biol.* **19**, 1-19 (2007).

104 van Schothorst, E. M., Bunschoten, A., Verlinde, E., Schrauwen, P. & Keijer, J. Glycemic index differences of high-fat diets modulate primarily lipid metabolism in murine adipose tissue. *Physiol. Genomics* **43**, 942-949 (2011).

105 Faust, I. M., Johnson, P. R. & Hirsch, J. Long-term effects of early nutritional experience on the development of obesity in the rat. *J. Nutr.* **110**, 2027-2034 (1980).

106 Greenwood, M. R. & Hirsch, J. Postnatal development of adipocyte cellularity in the normal rat. *J. Lipid Res.* **15**, 474-483 (1974).

107 Canfora, E. E., Meex, R. C. R., Venema, K. & Blaak, E. E. Gut microbial metabolites in obesity, NAFLD and T2DM. *Nat. Rev. Endocrinol.* **15**, 261-273 (2019).

108 Barouei, J. *et al.* Microbiota, metabolome, and immune alterations in obese mice fed a high-fat diet containing type 2 resistant starch. *Mol. Nutr. Food Res.* **61** (2017).

109 McMillen, I. C. & Robinson, J. S. Developmental origins of the metabolic syndrome: prediction, plasticity, and programming. *Physiol. Rev.* **85**, 571-633 (2005).

110 Fowden, A. L. & Hill, D. J. Intra-uterine programming of the endocrine pancreas. *Br. Med. Bull.* **60**, 123-142 (2001).

111 Marco, A., Kisliouk, T., Weller, A. & Meiri, N. High fat diet induces hypermethylation of the hypothalamic Pomc promoter and obesity in post-weaning rats. *Psychoneuroendocrinology* **38**, 2844-2853 (2013).

112 Kellermayer, R. *et al.* Epigenetic maturation in colonic mucosa continues beyond infancy in mice. *Hum. Mol. Genet.* **19**, 2168-2176 (2010).

113 Galgani, J. E., Moro, C. & Ravussin, E. Metabolic flexibility and insulin resistance. *Am. J. Physiol. Endocrinol. Metab.* **295**, E1009-1017 (2008).

114 Muoio, D. M. Metabolic inflexibility: when mitochondrial indecision leads to metabolic gridlock. *Cell* **159**, 1253-1262 (2014).

115 Sparks, L. M. *et al.* Relation of adipose tissue to metabolic flexibility. *Diabetes Res. Clin. Pract.* **83**, 32-43 (2009).

116 Romero-Corral, A. *et al.* Normal weight obesity: a risk factor for cardiometabolic dysregulation and cardiovascular mortality. *Eur. Heart. J.* **31**, 737-746 (2010).

117 Vegiopoulos, A., Rohm, M. & Herzig, S. Adipose tissue: between the extremes. *EMBO J.* **36**, 1999-2017 (2017).

118 Kuzawa, C. W. Adipose tissue in human infancy and childhood: an evolutionary perspective. *Am. J. Phys. Anthropol.* **Suppl 27**, 177-209 (1998).

119 Spalding, K. L. *et al.* Dynamics of fat cell turnover in humans. *Nature* **453**, 783-787 (2008).

120 Salans, L. B., Cushman, S. W. & Weismann, R. E. Studies of human adipose tissue. Adipose cell size and number in nonobese and obese patients. *J. Clin. Invest.* **52**, 929-941 (1973).

121 Food and Agriculture Organization of the United Nations. *Carbohydrates in Human Nutrition: Report of a Joint FAO/WHO Expert Consultation, Rome, 14-18 April 1997*. (FAO, Rome, 1998).

122 Stephen, A. *et al.* The role and requirements of digestible dietary carbohydrates in infants and toddlers. *Eur. J. Clin. Nutr.* **66**, 765-779 (2012).

123 Wang, Y., Guglielmo, D. & Welsh, J. A. Consumption of sugars, saturated fat, and sodium among US children from infancy through preschool age, NHANES 2009-2014. *Am. J. Clin. Nutr.* **108**, 868-877 (2018).

124 Koletzko, B. *et al.* Nutrition during pregnancy, lactation and early childhood and its implications for maternal and long-term child health: the Early Nutrition Project recommendations. *Ann. Nutr. Metab.* **74**, 93-106 (2019).

125 Reynolds, A. *et al.* Carbohydrate quality and human health: a series of systematic reviews and meta-analyses. *Lancet* **393**, 434-445 (2019).

126 Azzopardi, P. S. *et al.* Progress in adolescent health and wellbeing: tracking 12 headline indicators for 195 countries and territories, 1990–2016. *Lancet* **393**, 1101-1118 (2019).f

127 Woods-Townsend, K. *et al.* LifeLab Southampton: a programme to engage adolescents with DOHaD concepts as a tool for increasing health literacy in teenagers -a pilot cluster-randomized control trial. *J. Dev. Orig. Health Dis.* **9**, 475-480 (2018).

128 Alberga, A. S., Sigal, R. J., Goldfield, G., Prud'homme, D. & Kenny, G. P. Overweight and obese teenagers: why is adolescence a critical period? *Pediatr. Obes.* **7**, 261-273 (2012).

129 Godfrey, K. M., Gluckman, P. D. & Hanson, M. A. Developmental origins of metabolic disease: life course and intergenerational perspectives. *Trends Endocrinol. Metab.* **21**, 199-205 (2010).

130 Gluckman, P. D. *et al.* Metabolic plasticity during mammalian development is directionally dependent on early nutritional status. *P. Natl. Acad. Sci. USA* **104**, 12796-12800 (2007).

131 Pico, C., Palou, M., Priego, T., Sanchez, J. & Palou, A. Metabolic programming of obesity by energy restriction during the perinatal period: different outcomes depending on gender and period, type and severity of restriction. *Front. Physiol.* **3**, 436 (2012).

132 Hall, C., Tulbek, M. C. & Xu, Y. Flaxseed. *Adv. Food Nutr. Res.* **51**, 1-97 (2006).

133 Pellizzon, M. A. & Ricci, M. R. The common use of improper control diets in diet-induced metabolic disease research confounds data interpretation: the fiber factor. *Nutr. Metab.* **15**, 3 (2018).

134 Olejnikova, L., Polidarova, L., Behuliak, M., Sladek, M. & Sumova, A. Circadian alignment in a foster mother improves the offspring's pathological phenotype. *J. Physiol. (Lond.)* **596**, 5757-5775 (2018).

135 Salazar, E. R. *et al.* Gestational chronodisruption leads to persistent changes in the rat fetal and adult adrenal clock and function. *J. Physiol. (Lond.)* **596**, 5839-5857 (2018).

136 Spear, L. P. The adolescent brain and age-related behavioral manifestations. *Neurosci. Biobehav. Rev.* **24**, 417-463 (2000).

137 Koopman, J. P., Scholten, P. M. & Beynen, A. C. Hardness of diet pellets and growth of pre-weaned mice: separation of direct effects on the young and indirect effects mediated by the lactating females. *Z. Versuchstierkd.* **32**, 257-260 (1989).

138 Li, M., Reynolds, C. M., Segovia, S. A., Gray, C. & Vickers, M. H. Developmental programming of nonalcoholic fatty liver disease: the effect of early life nutrition on susceptibility and disease severity in later life. *Biomed Res. Int.* **2015**, 437107 (2015).

139 Kasch, J. *et al.* Insulin sensitivity linked skeletal muscle Nr4a1 DNA methylation is programmed by the maternal diet and modulated by voluntary exercise in mice. *J. Nutr. Biochem.* (2018).

140 Hahn, P. Effect of premature weaning to different diets on the subsequent response to a dietary change. *Biol. Neonate* **32**, 275-279 (1977).

141 Richter, S. H., Kastner, N., Loddenkemper, D. H., Kaiser, S. & Sachser, N. A time to wean? Impact of weaning age on anxiety-like behaviour and stability of behavioural traits in full adulthood. *PLoS One* **11**, e0167652 (2016).

142 Moorcroft, K. E., Marshall, J. L. & McCormick, F. M. Association between timing of introducing solid foods and obesity in infancy and childhood: a systematic review. *Matern. Child Nutr.* **7**, 3-26 (2011).

143 Penders, B. *et al.* Capable and credible? Challenging nutrition science. *Eur. J. Nutr.* **56**, 2009-2012 (2017).

Summary

Indirect calorimetry (InCa) is an essential tool for human and animal studies of energy metabolism. InCa measures the metabolic gases consumed (oxygen, O_2) and produced (carbon dioxide, CO_2) by the organism to calculate energy expenditure (EE). The ratio of CO_2 produced to O_2 consumed, or the respiratory exchange ratio (RER), indicates substrate utilisation (carbohydrate, fat, and protein) at the whole body level. Significant improvements in commercial InCa systems for rodents have increased its accuracy and resolution, yet there have been few attempts to extend the technique by measuring other physiological gases. These gases can be of microbial origin, like hydrogen (H_2) and methane (CH_4), which result from fermentation by the gut microbiome. Furthermore, conventional InCa cannot distinguish between the oxidation of exogenous (*e.g.* dietary) and endogenous (stored in the body) substrates. Stable isotopic tracers, like ^{13}C-enriched nutrients, make this distinction possible by analysis of the ratio of $^{13}CO_2/^{12}CO_2$ in expired air. These gases (H_2, CH_4, $^{13}CO_2$, $^{12}CO_2$) are rarely measured in mouse studies and, when they are, they need expensive equipment and considerable time and effort.

Dietary starches can be lowly or highly digestible. Lowly digestible starches generally produce lower glycaemic responses, provide fermentative substrates for the intestinal microbiota, and are thought to prevent excessive adiposity and favour metabolic health. There are some indications that starch digestibility has different metabolic effects in females and males. The evidence for this is very limited and, where both sexes have been studied, little attention has been paid to other aspects of carbohydrate metabolism, besides oral glucose tolerance tests and static tests for glycaemia. Moreover, despite the potential of starches to impact metabolism, there are no evidence-based recommendations for starch intake for young children. This is important because early life nutrition has the power to condition the metabolic function of the organism in the long term, a phenomenon known as metabolic programming. It is not known whether starch digestibility can program the organism metabolically, neither whether programming by starches can occur in the post-weaning period, a period where the organism faces a dramatic change in dietary macronutrient composition and density.

This thesis aimed to 1) develop an show the added value of an extended mouse metabolic phenotyping tool based on InCa for the real-time study of microbiota activity and the oxidation of exogenous *vs* endogenous substrates; and 2) to apply this tool to study the direct and metabolic programming effects of starches consumed during the early post-weaning period.

In **Chapter 2**, we aimed to examine whether we would be able to study microbiota activity non-invasively, continuously and in real time by extending a commercial InCa system (eInCa) with H_2 and CH_4 sensors (eInCa). Hydrogen production was circadian and depended on food intake and starch digestibility, as tested in mice fed a lowly (LDD) or a highly digestible-starch diet (HDD). Hydrogen production explained ~20% of the variation

in faecal bacterial composition, and correlated with specific bacterial genera known to produce H_2 *in vitro*. No CH_4 production could be measured, and this was consistent with the absence of faecal methanogenic archaea. We concluded that eInCa is a useful tool to study diet-microbiota-host interactions in real time.

We then incorporated $^{13}CO_2$ and $^{12}CO_2$ sensors into the same eInCa system and aimed to demonstrate their added value in **Chapter 3**. The system detected to differences in $^{13}CO_2$ enrichment based on the natural ^{13}C enrichment of the diet and daily food intake patterns. By combining $^{13}CO_2$ enrichment and conventional InCa data, we were able to quantify the oxidation rates of ^{13}C glucose or ^{13}C palmitate ingested by lean and obese mice, separately from total (exogenous + endogenous) glucose and fat oxidation rates. The oxidation of ingested palmitate was negatively correlated to the animal's fat mass and positively correlated to metabolic flexibility. We concluded that enabling $^{13}CO_2$ enrichment analysis in eInCa makes it a powerful tool for the quantification of specific substrate oxidation in physiological and pathophysiological conditions .

In **Chapter 4**, we aimed to test whether the capacity to oxidise the starch molecule is influenced by prior exposure to an LDD or an HDD, and if this effect is similar for both females and males. The oxidation of a highly digestible starch bolus was higher in mice that consumed LDD for 3 weeks prior. This effect was larger in females than in males, as shown by the faster starch oxidation kinetics of LDD *vs* HDD females during the early postprandial period, not present in males. However, LDD males exhibited a constantly higher RER, both in fasting conditions and during the postprandial period after consumption of the starch bolus. Small intestinal amylase levels did not explain the higher starch oxidation of LDD *vs* HDD females. From these data we concluded that short-term consumption of lowly digestible starch enhances the oxidation of the starch molecule, especially in females.

In **Chapter 5**, we aimed to retest some of the direct effects of starch digestibility and to test whether LDD *vs* HDD consumed in early post-weaning can programme metabolism in the long-term in both sexes. Direct exposure to LDD led to smaller adipocyte sizes and lower inflammation in the gonadal adipose compartment of females, and decreased body fat mass in males. In both sexes, digestible energy intake and H_2 production were directly increased by LDD. Adult females on a HFD that were exposed to LDD during post-weaning had a better metabolic flexibility and lower macrophage infiltration in the gonadal adipose depot. In males, no programming effects on metabolic flexibility and other metabolic outcomes like body composition and fasting glucose, insulin, and adipokine levels were observed. We concluded that the metabolic programming effects of starches consumed in the post-weaning period are subtle and sexually dimorphic.

I discuss the findings of my thesis and provide implications and suggestions for further research in **Chapter 6**.

To conclude, we have shown the added value of integrating new gas sensors into a commercial InCa system (**Chapters 2** and **3**). This technological extension yields real-time, continuous, and automated data that can inform microbiology, nutritional, and other physiological studies. By providing additional information with minimal discomfort

to the animal, eInCa can contribute to the replacement, reduction, and refinement (3 Rs) of animal experimentation. Furthermore, the data collected in **Chapters 4** and **5** indicates that lowly digestible starches consumed after lactation help to maintain metabolic health during the life course, especially in females. These findings underscore the importance of examining both sexes in nutritional studies, and may contribute to improve the formulation of food products for infants and young children.

Resumen

La calorimetría indirecta (CI) es una herramienta esencial para el estudio del metabolismo energético en humanos y roedores. La CI mide los gases metabólicos consumidos (oxígeno, O_2) y producidos (CO_2) por el organismo, lo que se utiliza para calcular el gasto energético (GE). La proporción entre CO_2 producido y O_2 consumido, o la relación de intercambio respiratorio (RIR), nos indica el tipo de sustrato (carbohidrato, grasa y proteína) utilizado por el animal entero. La precisión y resolución de los sistemas comerciales de CI para roedores han mejorado significativamente, aunque poco se ha hecho para ampliar esta técnica para la medición de otros gases fisiológicos. Algunos de estos gases son originados por la microbiota intestinal, como el hidrógeno (H_2) y el metano (CH_4) generados durante la fermentación. Por otra parte, la CI convencional no permite distinguir entre la oxidación de sustratos exógenos (*e.g.* provenientes de la dieta) y endógenos (provenientes de reservas corporales). Para hacer esta distinción se pueden emplear marcadores isotópicos estables, por ejemplo nutrientes marcados con carbono 13 (^{13}C), y calcular la proporción de $^{13}CO_2/^{12}CO_2$ en el aire exhalado. Es muy poco común que estos gases (H_2, CH_4, $^{13}CO_2$, $^{12}CO_2$) se midan en estudios con ratones y, en los casos en que se ha hecho, esto ha sido bastante costoso y laborioso.

Los almidones que se consumen en la dieta pueden ser altamente o poco digeribles. Los almidones poco digeribles producen en general una menor respuesta glicémica, proporcionan sustratos para la fermentación intestinal y se cree que previenen la acumulación de tejido adiposo y favorecen la salud metabólica. Hay indicios de que la digestibilidad del almidón afecta de manera distinta al sexo femenino y masculino. La evidencia a este respecto es poca y los estudios realizados en ambos sexos se han enfocado sólo en algunos aspectos del metabolismo de carbohidratos, como pruebas de tolerancia oral a la glucosa y mediciones de glicemia en el estado de ayuno. Así mismo, a pesar de su potencial impacto metabólico, actualmente no existen recomendaciones basadas en evidencia científica sobre la ingesta de almidones para bebés y niños. Esto es importe porque la nutrición en etapas tempranas del desarrollo tiene el poder de condicionar la función metabólica del organismo a largo plazo, un fenómeno conocido como programación metabólica. No se sabe si la digestibilidad del almidón puede programar al organismo metabólicamente, ni si esto puede ocurrir después de la ablactación, cuando el organismo enfrenta un cambio dramático en la composición y densidad de macronutrientes de la dieta.

El presente trabajo tuvo por objetivos 1) desarrollar y demostrar el valor añadido de una herramienta para el fenotipado del ratón basada en la CI, para el estudio de la actividad de la microbiota intestinal y la oxidación de sustratos exógenos *vs* endógenos en tiempo real, y 2) aplicar esta herramienta al estudio de los efectos directos y a largo plazo (programación metabólica) de los almidones consumidos durante el periodo inmediato tras la ablactación.

En el **Capítulo 2**, nos propusimos estudiar la actividad de la microbiota intestinal de manera no invasiva, continua y en tiempo real, mediante un sistema de CI aumentado (CIA) con

sensores de H_2 y CH_4. En ratones alimentados con una dieta basada en almidón poco digerible (APD) o almidón altamente digerible (AAD), la producción de H_2 mostró un ritmo circadiano dependiente del consumo de alimento y de la digestibilidad del almidón. La cantidad de H_2 producido fue suficiente para explicar el 20% de la variación taxonómica en bacterias fecales y se correlacionó con géneros específicos de bacterias que se sabe son productoras de H_2 *in vitro*. No se detectó producción de CH_4, lo que concuerda con la ausencia de arqueas metanógenas en heces. Concluimos que la CIA es una herramienta útil para el estudio en tiempo real de las interacciones entre la dieta, la microbiota intestinal y el huésped.

Posteriormente incorporamos sensores de $^{13}CO_2$ y $^{12}CO_2$ en el mismo sistema de CIA. El **Capítulo 3** tuvo por objetivo demostrar el valor añadido de estos sensores. Con este sistema fue posible detectar diferencias en el enriquecimiento en $^{13}CO_2$ correspondientes al enriquecimiento natural en ^{13}C de la dieta y los patrones de alimentación. Usando los datos combinados de enriquecimiento isotópico y CI convencional, pudimos cuantificar la tasa de oxidación de una dosis ^{13}C glucosa o ^{13}C palmitato administrada a ratones con diferente masa grasa corporal, independientemente de las tasas de oxidación de glucosa y grasa en el animal completo (la suma de sustratos exógenos y endógenos). La oxidación del palmitato ingerido se correlacionó negativamente con la masa grasa y positivamente con la flexibilidad metabólica del animal. Concluimos que habilitar el análisis de enriquecimiento en $^{13}CO_2$ como parte de la CIA la convierte en una poderosa herramienta para estudiar la oxidación de sustratos específicos cuantitativamente en condiciones fisiológicas y patofisiológicas.

En el **Capítulo 4** nos propusimos probar la hipótesis de que la exposición a una dieta basada en APD o AAD influye en la capacidad para oxidar la molécula de almidón, e investigar si estos efectos son similares entre hembras y machos. Los animales que consumieron APD durante 3 semanas oxidaron una mayor proporción de una dosis de almidón gelatinizado (altamente digerible) que aquellos que consumieron AAD previamente. Este efecto fue mayor en las hembras que en los machos, puesto que la cinética de oxidación del almidón fue más acelerada durante la etapa postprandial temprana en las hembras alimentadas con APD *vs* AAD, lo que no se observó en los machos. Sin embargo, los machos alimentados con APD sostuvieron una RIR más alta, tanto en condiciones de ayuno como durante el periodo postprandial después de consumir la dosis de almidón. La oxidación más completa de esta dosis de almidón en hembras alimentadas con APD no pareció obedecer a diferentes niveles de amilasa intestinal. Con base en estos datos hemos concluido que el consumo de almidón poco digerible aumenta la capacidad para oxidar la molécula de almidón a corto plazo, especialmente en las hembras.

Nuestro objetivo en el **Capítulo 5** fue demostrar nuevamente algunos de los efectos directos de la digestibilidad del almidón así como investigar si una dieta basada en APD o AAD inmediatamente después de la ablactación puede programar el metabolismo de machos y hembras a largo plazo. En el corto plazo, las hembras que consumieron APD desarrollaron adipocitos de menor tamaño y menor inflamación en el depósito adiposo gonadal, mientras que los machos alimentados con APD mostraron una menor masa

grasa corporal que aquellos que consumieron AAD. Así mismo, el consumo directo de APC llevó a una mayor ingesta de energía digerible y una mayor producción de H_2 en ambos sexos. En la etapa adulta y durante el consumo de una dieta alta en grasa, las hembras que estuvieron expuestas a la dieta APD después de la ablactación mostraron mejor flexibilidad metabólica y menor infiltración de macrófagos en el tejido adiposo gonadal. La flexibilidad metabólica de los machos no fue programada, ni tampoco otros parámetros ligados a la salud metabólica como la composición corporal o las concentraciones en sangre de glucosa, insulina y adipoquinas en el estado de ayuno. Concluimos que la capacidad de los almidones consumidos inmediatamente después de la ablactación para programar el metabolismo es sutil y sexualmente dimórfica.

En el **Capítulo 6** discuto los hallazgos de esta tesis y sus implicaciones y también ofrezco algunas sugerencias para futuras investigaciones en el campo de la programación metabólica.

En conclusión, hemos demostrado que integrar nuevos sensores de gases en un sistema comercial de CI añade valor a esta técnica (**Capítulos 2** y **3**). Esta extensión de la tecnología proporciona información en tiempo real, continua y automáticamente, que puede ser valiosa en estudios microbiológicos, nutricionales y en otras áreas de la fisiología. Al proporcionar información adicional con mínimas molestias para el animal, la CIA pude contribuir al reemplazo, reducción y refinamiento (principio de las 3Rs) en la investigación con animales. Así mismo, los datos reunidos en los **Capítulos 4** y **5** sugieren que los almidones poco digeribles consumidos después de la lactancia ayudan a preservar la salud metabólica a lo largo de la vida, en especial para las hembras. Estas observaciones subrayan la importancia de incluir ambos sexos en estudios nutricionales, y podrían contribuir a mejorar la formulación de los productos alimenticios destinados a bebés y niños.

Acknowledgements

At long last! At some point I thought I wouldn't make it. But here it is, the finished book, a few papers, and 100s of lessons learnt. Some of those lessons were rather painful, and some were unexpected. But ALL of them I appreciate now, along with every meaningful thing that people have said or done to make me happy during my PhD... Here's a list, feel free to skip right to your name:

- Kim: Gracias x esperarme hasta el momento en que empezarrá mi verdadero despegue como adulto. Gracias x ser tú en lo que pienso cuando "tengo" que poner una sonrisa en mi cara (tú sabes a qué me refiero). Te dedico esta tesis.
- Jaap: I think I'm only beginning to see what you tried to do with me, and I'm grateful in every way. All your comments, from the many times you criticised the way I delivered my presentations, to those other times when you interrupted my sentences, to the awkward moments when you spotted my lousy (sic.) arguments... All of these are now gold to me. These difficult interactions showed me how to use my presentation skills effectively, and what then felt like underappreciation now became part of my strengths as a scientist. And let's not think about where I'd be now had you always let me carry on talking, because I realise how much I really needed to listen back then. I actually owe a great deal of my scientific output to knowing to keep quiet and trust you (that H_2 paper and some data in the $^{13}CO_2$ paper were almost serendipitous, and I'd have missed the opportunity if I hadn't had your experience on my side). From you I learnt how to read a paper in 5 minutes and how to think in terms of "research questions". One thing I didn't quite learn, I see, is to keep it simple. So this list could go on ad infinitum but I'd better stop right here.
- Evert: where to start? The first thing that comes to mind is: thank you for taking me soooo seriously. There was not a single moment when what I did or say was indifferent to you. You supervised me closely and carefully, and I know not many PhD candidates can get that much attention from their supervisors. Of course, we had a few disagreements and I got mad at you, but all the time you were "only" doing your job. No matter how stubborn I was, you were always patient and polite, and you were more than ready to provide feedback. Thanks to your feedback my writing is much better and scientific, and my plots are always on point. More than once I got more feedback than I asked for, and I consider myself lucky that you took the time to prepare it for me as part of my scientific training. A good example is the way you helped me see the value of my work and be very confident in it. I'm especially grateful because under your academic wing I felt safe to make mistakes and grow as a scientist, because first and foremost you saw me as a human being. And thank you for lending me a handful of research questions from which to start off my academic journey (it took me some time to welcome them, but I learnt my lesson!). Evert, if there was only 1 thing I could thank you for, that'd be the way you never gave me orders, but instead encouraged me to think critically. At times it felt like every step was a huge deal: nothing could be done or even written without prior consultation. But it is because you took me so seriously that now I'm as rigorous and sceptical as a true scientist should be. Maybe you didn't even plan to be such a teacher to me, but I find it beauti-

ful that I keep learning from you to this day (I think the last thing was about multiple testing...). Thank you for shaping my identity as an academic, and know that I'll always see a role model in you. Funny thing is, this is the only part of this thesis that you cannot edit, but something tells me you'd approve :)

- Jaap and Evert, to both of you: I treasure every moment when I could clearly see your passion for the truth, when I could see that I meant much more to you than a way to boost your h-indices. I lived for the times when I saw you marvel at nature and the understanding of the Universe. Thank you for this, and for staying late at many of our work discussions. This was to me, in a way, a "critical period" of my scientific development. You've shaped me, you've "programmed" me, and I'm grateful for your influence on my "academic phenotype." You were my mentors, and I hope you continue to be a source of wisdom and support for the years to come.
- Annemarie: A lot of what I said to Evert and Jaap applies to you. You've also been a major "environmental cue" in my development, a brilliant one at that. Thank you for being the strong, yet sensitive voice of reason. Your feedback never failed to refresh me, always direct but positive and constructive. I really enjoyed when you came to our meetings, those days were exciting and filled me with energy. Why? I dunno, I suppose that's your super power. But you really gave me comfort, and I'm sure you know what I mean. Thank you!! :)
- Lianne: I really value having worked in the same project together. We had our differences, but I learnt a ton from them. I appreciate how you were sensitive to my fears, like the many times I felt stupid in the lab. Or how you were really proactive in the decisions we had to make, even when they concerned my "own" studies. You may not know it, but I'm very grateful for your attitude during the dissections. You commanded us around with such confidence. I really needed you to be strong, and you picked up on it immediately... I especially remember one time when Melissa was late and you saved the day. Thank you! And also thank you for reading my drafts so very carefully. You spotted things that not even the native Dutch among us did. And you helped me overcome some of my abuse of the article "the". Lianne, thank you for being yourself. I admire you and I miss you. That's why I sometimes say to myself "WWLD?" when I need to feel like a boss in the lab. Thank you so much for being my inspiration.
- Wenbiao: It was always good to talk to you. I appreciate our discussions about science, academia, and our life goals. You always seemed to be in a good mood. Your ever-smiling face gave me a warm feeling that it is possible to do your PhD without losing your mind. Thank you for making time for helping me with my mice even when you really didn't have it, and for thinking along with me about my data. I now see you as someone with quite some experience, and I thank you for being so ready to share it with us who still have a lot to learn. I am honored to have been your paranymph, because I could actually do something to make your celebration happen. A celebration of the efforts we "young" scientists do to take a place in this whimsical system. I respect you Wenbiao, and I hope we can keep building our friendship within or outside academia.

• Arjan, Ruud, Harry, Henry, Nils, Bent and Jörg: thank you for contributing your insights during our TTW meetings, and also outside these meetings. I think you represented much more than the institutions/companies you work(ed) for, because you really showed a genuine interest in our project and the development of me and Zi-anne as scientists. Thank you for making our project possible and for making every meeting a rather enjoyable one. I hope our efforts continue to pay off and benefit science & society.

• Ries: You're part of the paragraph above, but I think you really went the extra mile. I think we matched very nicely at some point and I really enjoyed talking to you and sharing our first results. You know how excited I was about the whole iso-tope thing, and it was nice to have someone share that feeling. Thank you for trying to make my PhD as smooth as possible by pointing me in the right direction and even giving me some "people advices". I really hope I can do something with IsoLife in the future!

• Vincent: we didn't talk much, but when we did it was refreshing. When you came to the group I think you brought a new dynamic. I felt you were a truly impartial, experienced scientist who could criticize my work. I look forward in particular to hearing precisely why you question the concept of "programming" in my work. I also thank you for introducing a bit of politeness to the group, at least the kind I'm more used to. As much as I love a direct challenge, sometimes one really needs to hear a positive comment when receiving feedback. Thank you for being that person, Vincent.

• Jeske: Your self-confidence and carefree approach to life was a reminder to me that things don't have to be terrible all the time. It's mainly how we see them. I'll always remember that nice flowchart you had on your wall about worrying. Thank you for buying me coffee sometimes and for encouraging me to join the coffee breaks ☺

• Lonneke: You were also very helpful. I really liked having around someone so chatty and sociable. I really liked the time you invited me to your place while I was doing the laundry. And I'm grateful for the advice you gave me when I asked you about getting an extension for my PhD.

• Natasja: You're another person I admire. Thank you for sharing statistical tips & tricks with me, and for all the times you helped with our discussions.

At times you were not so sociable, but whenever you were, you always made me laugh. I realize we have a few things in common, something in our sense of humour perhaps. Our our love of pigs and cats, and our dislike of spiders. Anyhow, I believe you'll be a great scientist and I hope you have many students whom ya can teach how to do good science. ~~(Also, you're really smart and I'm jealous).~~

- Taolin: Thank you for being kind and helpful. I see you work very hard and I admire you. And I must say having you in our group helped me relax a little and come to the coffeebreaks more often. I liked how sweet & cute you're at times, because that made me feel good :)
- Anna: Another "refreshing" person. You're cool, smart, and sociable. While I'm writing these acknowledgements I realise how much I really appreciate having you guys around. Somehow you and the other PhDers have qualities I don't, and that really made the group a very nice place to work. I think you represent this feeling of mine, the gratitude to have a diverse group of peers to which I can compare myself with and think in ways I can improve as a person. Thank you Anna, and thank you all of the other PhDers: Li, Marjanne, Bart & Joëlle.
- Shujin: During your time with us I learnt a lot about myself just by talking to you. You'll probably won't see this, but somehow I still need to thank you for giving me a few life lessons.
- Nana: ¡Amiga Nana querida! Fue un deleite tenerte en HAP y continuar nuestra amistad fuera del grupo. Recuerdo con cariño las veces que tomábamos un cafecito en Zodiac y me platicabas de tu historia de vida y las retribeciones. Eso me marcó. Te quiero mucho y te agradezco por tratarme con cariño, me hacía mucha falta en el momento en que llegaste. No sé a dónde nos llevará la ciencia (o la vida), pero ojalá que nuestros caminos se crucen felizmente.
- Marina: HAP tuvo el gusto de conocerte por relativamente poco tiempo, pero yo me quedo con muy gratos recuerdos de ti. Me gustó mucho cómo nos reunimos en tu casa para Eurovision, y las veces que platicamos de la vida y el trabajo. Gracias por darme un espacio para hablar en mi idioma e intercambiar impresiones. Te admiro mucho y espero que sigamos en contacto mucho tiempo más.
- Silvie, Dorien, Jessica, Marcel, Katja, Arie, Sander, Annet, Annika and Melissa: Thank you for being nice colleagues and helping me!!

• Tyabo: You, my friend, are special. I still can't think of a better example of a strong woman. Moving over here with a family to care for and a baby in your womb is really something else, and you had to face an entirely different culture and way of working all at the same time. Trying to do a PhD is hard enough. I am glad you realised you're very smart and capable, and that you've decided to stand up for yourself. Not every PhD candidate has the same privileges, and I will always respect you for having the courage to take on this challenge. Please don't ever let those barriers question your worth as a person. Thank you for being a very precious friend to me!!

• Hans: A lot has been said already when you retired, but I'll say it again. You are one-of-a-kind. Many times I lost it when we worked together, but looking back I see how much I learned from it. I still miss you where I am now. I need someone who can share with me everything that so many years of experience has taught him. I really liked how you always tried to make our science better. If I didn't see it back then now I do, and I thank ya for it! What's more, you are a kind and noble person, and I hope I have more colleagues like you in the future.

• Inge: I think you always had an optimistic mind. Or at least all of our interactions were like that, and I really appreciate it. Perhaps because of that attitude, more than once I came to you with my "$p >>> 0.05$" kind of data and you helped me see value in them all the same. I hope you continue to guide many scientists in training like me because you have a lot to teach them! Thank you!

• Annelies: I could write a whole chapter about you. We don't have that kind of budget, so I'll just say I'm grateful for all the times you came to "unglue" me from my computer to join the coffee breaks. And one more thing: that time I had a personal situation at home, you were the only one who cared to listen to me. I'll always remember this, and I thank you for showing me that you cared.

• Corine, Irene, Anneke & Annika: I think I wouldn't have been able to deal with all the paperwork and logistics involved in my PhD without

your professional help. You all made my life easier and I would not like to see it as a given, but as a gift from you to me. Thank you for being excellent and helping our group stay organised.

- Eva, Anke, Lini, Laura, Yunike, and Tanya: I learnt a lot from you. Being a supervisor can be really scary, but you made it fun. You all were really good at pointing out my deepest fears and weaknesses, although you didn't notice. So, besides thanking you for the hard work you put into our project (especially at CKP), I thank you for being my teachers.
- Yunike: I thank you for the hard work you did. Without it, I wouldn't have been able to finish everything in time. I wish I could show my gratitude in another way, but please know you've truly contributed to the programming field.
- Peter de Gijsel & Michel Brewer. You must be the most patient people in Holland. Thank you for talking me through all the intricate technicalities I needed to understand at some point.
- Prokopis: It was great to work with you and finally understand some really high-level concepts about data science thanks to your explanation. Your hard work added a very interesting perspective to our paper. I hope my future collaborations with other scientists could be like this one.
- Hauke: A lot of what I said to Prokopis applies to you as well, but I must say your willingness to take my ideas seriously was remarkable. Thank you for being an approachable professor.
- Wilma, Beat & René: The CKP was my second home, and you always made sure it was tidy. Even more so, our precious experimental units lived in a lux resort thanks to your care. On behalf of all of the mice and also of the ones I will keep for my future studies, thank you for helping me prioritise animal welfare above everything else.
- Arturo: Gracias por ayudarme a ver la realidad, como siempre.
- Rosa me: ¡Amiga querida! Gracias por ser mi mejor amiga hondureña y por comprender lo complicado que estuve durante el doctorado. Sabes que te quiero mucho y que estoy muy agradecido por que me acompañaste en todo momento. Pasaron muchas cosas en 4 años, unas muy difíciles, pero siempre estuvimos el uno para el otro. ¡Gracias, te quiero mucho!

• Eli y Adriancito: Ustedes fueron parte del proceso. Gracias por siempre estar súper listos para pasarnos la rico y disfrutar de las otras cosas de la vida, no sólo el trabajo.

• Maren y Joost: Gracias por apoyarme TANTO. Todas esas chanlas sobre las justicias e injusticias del mundo fueron un gran alivio para mí. Ustedes saben lo difícil que fue esto a ratos, y me tuvieron la paciencia para esperarme a que tuviera tiempo para salir. Su apoyo no sólo estuvo conmigo durante el doctorado sino desde antes de empezar la maestría. Gracias por hacerme un espacio en su casa y en sus almas. Y Joost, tú me inspiras a ser un científico más chingón. Cuando me dijiste que la ciencia avanza a solas, supe que tenemos mucho más en común de lo que pensé. Sigamos dejando que la gente se pelee por los galardones mientras descubrimos la naturaleza. Y Maren, tú has sido mi mejor mentora en este país sobre cómo defender mis derechos. Tú y Joost, sin duda, son personas que me han ayudado a crecer y ser un buen ser humano. ¡Los quiero mucho!

• Cristóbal/Ximena: ¿Qué decir? Sabes que esto me costó mucho. Me viste hacer entrampados con cada jalada que sucedió, y a pesar de mi agenda tan caprichosa ahí estuviste, siempre invitándome a fuay o todos esos lugares tan... peculiares. Desde la maestría has sido tú la razón por la que perdí la inocencia, pero gané muchas experiencias que no hubiera conocido sin ti. Gracias por todo tu cariño y ternura y por todas las risas y momentos "máximos" que hemos vivido. ¡Te Quiero mucho! ♡

• Jorch: La mamá de todxs aquí. Siempre fuiste muy lindo, pero durante mi doctorado sacaste tu verdadero yo. Una calidad humana como pocas, una manera de hacerme sentir tan pero tan querido... ¡No sé! No me explico cómo puede haber alguien como tú. Eres una persona muy bella Jorch. Siento que me quieres mucho y me entiendes muy bien. Gracias por todos tus consejos sobre cómo ser un adulto en Holanda. Gracias por tu amistad y por quererme tanto. ¡¡¡Yo también te quiero!!!

• Parranfas: Gracias x ser tan chingonas. Kthxbai. (si pudiera poner un "mishi triste" aquí, saben que lo haría.)

• Leo y Juve, C mamaron. Nadie logró llegar hasta acá, pero ustedes sí que hicieron el esfuerzo. Son unos reyes, y no por nada mis

mejores (y más viejos) amigos. Gracias por todo lo que hemos vivido juntos, y por apoyarme en mi decisión de viajar hasta acá para continuar con mis estudios. Quiero que sigamos haciéndolo todo para cultivar nuestra amistad y seguir siendo una bonita influencia sobre nuestras vidas, aunque sea desde México, Holanda o Estados Unidos. Los quiero mucho y no saben lo que significa para mí que hayan venido a celebrar conmigo. Este es el mejor regalo que me han hecho y mi corazón se llena de dicha de tenerlos aquí. ♥

• BRO: ¡Cómo olvidarlos! Gracias muchas veces a ustedes he podido reírme en momentos feos. Desde que me fui de nuestro país han querido seguir en contacto y, aunque no siempre le puede, seguimos con la tradición de reunirnos al menos una vez al año. Gracias Anis, Eves, Arru/fis, Gari y ~~Alfredo~~ Paola por ser tan chistosos y hacer de la vida algo más amena. Los extraño mucho y quisiera que se reunieran más y me invitaran, aunque fuera virtualmente. Y mis Kokonitos Guadalupe y Nadia, son ustedes una fuente de alegría y ternura. Los llevo siempre en mi corazón. Ya no se alejen tanto; podemos estar juntos desde la distancia. ¡Los amo de rato!

• Don Manuel, Sra. Rosalba, Srita. Deni, Marecita y Chris: Gracias por recibirme cada que voy a México. Son como mi 2ª familia y doy gracias a Logos porque Marolito es como es por haber crecido en un lugar donde hay tanto amor!!! Los quiero!!! ♡

• Dra. Rosario: ¡Maestra! Espanti que amo la ciencia y la programación metabólica. De ti fue quien escuché el término por primera vez. Todo, realmente todo, lo que aprendí contigo me ha ayudado en este doctorado y a lo largo de mi formación. Gracias por ser mi mentora y por transmitirme todo ese compromiso que tienes por ser una buena científica. Te quiero mucho.

• Jorge Maldenado: Otro de mis grandes mentores. Ojalá que te pueda entregar mi tesis en persona pronto. La parte de los isótopos te la debo en parte a ti: no me hubiera ido tan bien si no me hubieras enseñado lo que son. Muchos trucos los aprendí de ti, y te lo agradezco infinitamente. ¡Gracias por ser mi maestro!

• Familia: Mi apoyo desde siempre. Gracias a Dios y José por venir hasta acá. Es como si mi querida Andy estuviera aquí. Fue gracias a ella que nuestra familia se unió más. Estoy muy agradecido con ustedes por que gracias a su compañía mis papás no se quedaron solitos. Gracias por estar con nosotros y ser nuestra familia. Los quiero mucho y ojalá sigamos uniéndonos mucho más aún. Y un beso hasta el cielo para Andy. ♡ Y (mucho más que) un saludo a mi abuelita.

• Dadito del amor: Mew. Gracias por hacer este trabajo tan bonito. Nos hizo sufrir, pero quedó muy bonito. Gracias por ayudarme a transmitir mi manera de pensar en forma de este diseño tan poderoso. Admiro tu talento. Y sobre todo admiro tu manera de ser quien eres. Gracias por siempre darme amor y sabe que este es el más bello regalo que me pudiste dar. Gracias por ser mi hermanita y por darme tanto tanto amor. ¡Te quiero mucho!

• Pez y Monsi: Aww. No alcanzarían las páginas para agradecerles su amor y apoyo. Lo que soy se los debo a ustedes. Sin el hogar tan rico que hicieron para Yuyo y para mí no creo que hubiera tenido las ganas de lograr un sueño tan noble como este que es la ciencia. Doy gracias por cómo me lo dieron todo para ser un hombre que le puede aportar algo bueno al mundo. Los quiero mucho y creo que sólo me queda seguir cuidándolos y retribuyéndoles todo lo que me han regalado y, sobre todo, seguir disfrutándolos ojalá que por muchos años más. Los amo.

• P.S./Manolito: El último agradecimiento es para ti. Como en los artículos científicos, muchas veces el último coautor no ha hecho el trabajo rudo pero sí lo ha invertido todo en él. Tú apostaste todo por mí, me tuviste fe y me diste tu consejo en todo momento. Estuviste cada una de esas más de dos mil mañanas para darme tu amor desde México desde el primer día que me fui a vivir fuera. Gracias a ti nunca estuve realmente solo por lejos que estuviera. Gracias por cuidar a mis papitos y a Yuyo, y por compartirme de tu hermosa familia. Y sobre todo, gracias por ser un espejo de alma que me ayuda a practicar la virtud y llevar una vida "bien vivida". Ojalá que podamos seguir haciéndonos felices hasta el último aliento. "Te amo!!! ♡♡♡

About the author

Curriculum Vitae

José María Salvador Fernández Calleja was born on the 15th of June 1988 in Mexico City, Mexico. In 2006, he completed his pre-university education at Universidad La Salle in Mexico City. In the same year and at the same institution, he started a BSc programme in Food Chemistry. It was during his BSc thesis work at the Mexican Institute of Social Security, analysing the lipid quality of infant formulas under the supervision of his "academic parents" Dr. Rosario Ayala Moreno and the researcher Dr. Jorge Maldonado Hernández, that he first encountered the promising field of early life nutrition and metabolic programming. After obtaining his BSc degree and a short flirt with the pharmaceutical industry in 2012, José decided to continue his scientific education abroad. Thanks to a scholarship from the Consejo Nacional de Ciencia y Tecnología (CONACYT), he moved to the Netherlands to start the MSc programme Molecular Life Sciences at WUR on the same year. Home sickness ensued, but this was the intention. During his MSc studies, José was lucky to have his first serious attempt at molecular biology supervised by Prof. Sander Kersten of the Nutrition, Metabolism and Genomics group, where he investigated the role of a muscle-derived humoral factor in insulin resistance. Because he was already too comfortable among the Dutch, José then sought an MSc internship in Aberdeen, UK, where Dr. Perry Barrett trusted him with his all-important Siberian hamster brain samples to analyse the seasonal gene expression patterns using ~~dangerous~~ radioactive probes. This was excellent preparation for what was to come, as soon upon his arrival back in the Netherlands in 2014 he landed a PhD position at WUR's Human and Animal Physiology group. Molecular biology, rodent studies, and isotopes were all exciting parts of the game but, more than anything, José was enamoured of the metabolic programming focus of the proposal. His patient PhD supervisors and sparring partners, Prof. Jaap Keijer, Dr. Evert van Schothorst and Dr. Annemarie Oosting, taught him a lesson or two (actually, too many to name, all of them welcome) on physiology and how to conduct oneself as an ethical, truth-seeking scientist amidst the contemporary "publish or perish" academic culture. During his PhD José himself had six pupils who gave him a rewarding taste of the pleasures of teaching. After this critical period in his academic training, a sort of second adolescence, José came out as a young investigator and sooner than later other scientists too acknowledged his passion and skills to comprehend the Universe. José is currently enjoying his first postdoctoral position with Dr. Lucianne Groenink at Utrecht University's Pharmacology group, where he hopes to keep contributing to improve the developmental trajectories of young children through metabolic programming.

List of Publications

R. Ayala-Moreno, **J.M.S. Fernández-Calleja**, J. Maldonado-Hernández. Assessment of lipid quality and composition of commercial infant milk formulas in Mexico: Emphasis on *trans* fatty acid isomers. *Food and Nutrition Sciences* 2016; 07:273-283. DOI:10.4236/fns.2016.74029

I. Petri, V. Diedrich, D. Wilson, **J.M.S. Fernández-Calleja**, A. Herwig, S. Steinlechner, P. Barrett. Orchestration of gene expression across the seasons: hypothalamic gene expression in natural photoperiod throughout the year in the Siberian hamster. *Scientific Reports* 2016; 6:29689. DOI:10.1038/srep29689

J.M.S. Fernández-Calleja, L.M.S. Bouwman, H.J.M. Swarts, A. Oosting, J. Keijer & E.M. van Schothorst. Monosaccharides in post-weaning diet of young mice program body composition and feeding behaviour in adulthood. *Scripta Scientifica Pharmaceutica* 2017; DOI:10.14748/ssp.v4i1.3962

L.M.S. Bouwman, **J.M.S. Fernández-Calleja**, H.J.M. Swarts, I. van der Stelt, A. Oosting, J. Keijer, E.M. van Schothorst. No adverse programming by post-weaning dietary fructose of body weight, adiposity, glucose tolerance or metabolic flexibility. *Molecular Nutrition & Food Research* 2018; 62(2):1700315. DOI:10.1002/mnfr.201700315

J.M.S. Fernández-Calleja, P. Konstanti, H.J.M. Swarts, L.M.S. Bouwman, V. Garcia-Campayo, N. Billecke, A. Oosting, H. Smidt, J. Keijer & E.M. van Schothorst. Non-invasive continuous real-time *in vivo* analysis of microbial hydrogen production shows adaptation to fermentable carbohydrates in mice. *Scientific Reports* 2018; 8:15351. DOI:10.1038/s41598-018-33619-0

J.M.S. Fernández-Calleja, L.M.S. Bouwman, H.J. Swarts, A. Oosting, J. Keijer, E.M. van Schothorst. 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

L.M.S. Bouwman, **J.M.S. Fernández-Calleja**, I. van der Stelt, A. Oosting, J. Keijer, E.M. van Schothorst. 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. *The Journal of Nutrition* 2019; 148 DOI:10.1093/jn/nxz028.

> Accompanied by a commentary: J.S. Ruff. Galactose-mediated protection from metabolic disease: nutritional programming, parental effects, and the milky way forward. *The Journal of Nutrition* 2019; doi: https://doi.org/10.1093/jn/nxz080.

J.M.S. Fernández-Calleja, L.M.S. Bouwman, H.J.M. Swarts, A. Oosting, J. Keijer, E.M. van Schothorst. Extended indirect calorimetry with isotopic CO_2 sensors for prolonged and continuous quantification of exogenous *vs.* total substrate oxidation in mice. *Scientific Reports* 2019; 9:11507. DOI: 10.1038/s41598-019-47977-w.

J.M.S. Fernández-Calleja, L.M.S. Bouwman, H.J.M. Swarts, N. Billecke, A. Oosting, J. Keijer & E.M. van Schothorst. A lowly digestible-starch diet after weaning enhances exogenous glucose oxidation rate in female, but not in male, mice. *Submitted*.

Overview of Completed Training Activities

Discipline-specific activities

Course, Laboratory Animal Sciences	Utrecht, NL, 2015 (UU)
Course, Metabolic Phenotyping of Mice	Düsseldorf, DE, 2015 (EASD)
Conference & (3) oral presentations, Dutch Nutritional Science Days	Heeze, NL, 2016-2018 (NAV)
Symposium, Nutritech	Lisbon, PT, 2016 (NutriTech)
Course, Chemometrics	Wageningen, NL, 2016 (VLAG)
Course, Indirect Calorimetry	Cracow, PL, 2016 (WUR-INRA)
Conference & poster presentation, The Power of Programming	Munich, DE, 2016 (ENA)
Course & poster presentation, Intestinal Microbiome	Wageningen, NL, 2017 (VLAG)
Course & poster presentation, Epigenesis and Epigenetics	Wageningen, NL, 2017 (VLAG)
Conference, workshop, & poster presentation, DOHaD World Congress	Rotterdam, NL, 2017 (DOHaD society)
Conference, course, & oral presentation, NuGO Week	Varna, BG, 2017 (NuGO)

General courses

VLAG PhD Week	Baarlo, NL, 2015 (VLAG)
PhD Workshop Carousel	Wageningen, NL, 2015 (WGS)
Course, Teaching and Supervising Thesis Students	Wageningen, NL, 2016 (ESD)
Course, Scientific Publishing	Wageningen, NL, 2017 (WGS)
Course, Interpersonal Communication	Wageningen, NL, 2017 (WGS)
PhD Peer Consultation	Wageningen, NL, 2017-2018 (WGS)
Course, Philosophy and Ethics of Food Science and Technology	Wageningen, NL, 2018 (VLAG)

Optional activities

Preparation of Research Proposal	Wageningen, NL, 2015
HAP Weekly Group Meetings	Wageningen, NL, 2015-2018
HAP Journal Club	Wageningen, NL, 2016-2017

The research described in this thesis was financially supported by the Dutch Technology Foundation STW (13509), which is part of the Netherlands Organization for Scientific Research (NWO), and which is partly funded by the Ministry of Economic Affairs; and by the Consejo Nacional de Ciencia y Tecnología (CONACYT, 311576), Mexico.

Financial support from Human and Animal Physiology for printing this thesis is gratefully acknowledged.

Cover and graphic design by Giuliana Fernández-Calleja

Printed by Digiforce | ProefschritMaken