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Impact of the metabolic program of germline cells on feeding behaviour

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IMPACT OF THE METABOLIC PROGRAM OF GERMLINE CELLS ON FEEDING BEHAVIOR

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Abstract

Animals control how much and what they eat to ensure an optimal nutrient balance for organismal function. *Drosophila melanogaster* shows specific nutrient appetites depending on its internal nutrient and mating state. We hypothesize that the Central Nervous System (CNS) is able to read the nutritional requirements of several organs and adapt feeding behavior to maintain tissue nutrient homeostasis. Oogenesis is a highly metabolically demanding process, strongly responding to nutrient availability and a large part of carbohydrates ingested by females are used for egg production. Females without germline show a strong reduction in sugar appetite even when carbohydrate-deprived, suggesting that indeed the CNS can sense the nutrient requirements of this organ and instruct the animal to behave accordingly. We hypothesize that carbohydrate metabolism in the germline might underlie this modulation of sugar appetite. To address this hypothesis, we took advantage of *Drosophila melanogaster*'s vast array of genetic and molecular tools, together with a high precision quantitative assay for fly feeding behaviour (flyPAD) and a full synthetic diet that allows precise nutrient manipulations of the diet. We show that dietary sugar is key for maintaining optimal egg production, since dietary sucrose deprivation reduces egg-laying by 37%. Furthermore, we show that egg production is highly dependent on the Pentose Phosphate Pathway (PPP) as we show that down-regulating the levels of enzymes in this pathway leads to a drastic reduction in egg-laying. Finally, we also show that the PPP in the germline modulates sugar appetite.

Our data supports a model where the germline cellular metabolic program is surveyed by the CNS to modulate the uptake of carbohydrates in order to achieve high fertility. It will be interesting to explore if pathologies in which cellular metabolic programs are altered, such as in certain tumors, also impinge on appetites in order to obtain the required nutrients for disease progression.

Key-words: *Drosophila*, feeding, behaviour, metabolism, PPP

Resumo

Os animais controlam as suas escolhas alimentares de forma a manter níveis nutricionais adequados para suportar as diversas funções do organismo. A mosca da fruta, *Drosophila melanogaster*, modula o apetite por nutrientes específicos em função dos seus níveis nutricionais internos e do seu estado de acasalamento. Nesta tese é proposto que o Sistema Nervoso Central (SNC) avalia os requerimentos nutricionais dos diferentes órgãos adaptando as escolhas alimentares, de forma manter a homeostasia do organismo. A oogénese é um processo metabolicamente exigente e modulado pela dieta, sendo que grande parte dos carbohidratos ingeridos pela fêmea adulta são utilizados na produção de ovos. Fêmeas sem linha germinal apresentam uma forte redução no consumo de açúcar mesmo quando este é removido da dieta, sugerindo que o SNC identifica os requerimentos nutricionais dos ovários e modula as escolhas alimentares do animal. Colocámos a hipótese de que o metabolismo de carbohidratos na linha germinal fosse responsável pelas alterações comportamentais observadas. Para testar esta hipótese, recorremos ao extenso conjunto de ferramentas genéticas e moleculares disponíveis para *Drosophila melanogaster*, juntamente com um ensaio quantitativo de elevada precisão para avaliar escolhas alimentares da mosca (flyPAD) e uma dieta sintética que permite a manipulações precisas dos nutrientes. Nós demonstramos que o açúcar proveniente da dieta é essencial para manter a produção de ovos, pois a remoção de sucrose da dieta reduz drasticamente este processo. Adicionalmente demonstramos que a produção de ovos depende especificamente da via das pentoses-fosfato, pois redução dos níveis das enzimas desta via metabólica conduz à drástica diminuição do número de ovos produzidos. Por fim, é também demonstrado que a via das pentoses-fosfato na linha germinal modula o apetite por açúcar.

Os nossos resultados propõem um modelo em que o programa metabólico das células da linha germinativa é interpretado pelo SNC, modulando o consumo de açúcares de forma a optimizar a produção de progenia. Neste contexto, será interessante explorar se patologias com alterações semelhantes a nível metabólico, como diversos tumores, também afectam diferentes apetites de forma a assegurar os nutrientes necessários para a progressão e desenvolvimento da doença.

Palavras-chave: *Drosophila*, alimentação, comportamento, metabolismo, PPP

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List of Abbreviations

°C – Celsius degrees

d – day

hr – hour

g – grams

µL – microlitre

mL – millilitre

µM – micrometre

mM – millimetre

% – percent

min – minute(s)

rpm – rotations per minute

kb – kilobase

bp – base pairs

RT – room temperature

6PGDH – 6-Phosphogluconate dehydrogenase

6PGL – 6-Phosphogluconolactonase

AA – amino acid

Akh – adipokinetic hormone

AP – Alkaline phosphatase

ATP – adenosine-5'-triphosphate

bam – bag of marbles

CNS – central nervous system

COC – cumulus-oocyte complex

Dig – digoxygenin

Dilp – *Drosophila* insulin-like peptide

DNA – deoxyribonucleic acid

EAA – essential amino acid

EC – escort cells

ESCs – embryonic stem cells

F6P – fructose-6-phosphate

$FADH_2$ – flavine adenine dinucleotide

FB – fat body

FLIC - Fly Liquid-Food Interaction Counter

flyPAD – fly Proboscis and Activity Detector

FSC – follicular stem cells

G3P – glyceraldehyde-3-phosphate

G6P – Glucose-6-phosphate

G6PDH - Glucose-6-phosphate dehydrogenase

GFP – green fluorescent protein

GK – Glucokinase

GLUT – glucose transporters for facilitated diffusion

GSC – germline stem cells

GTP – guanosine-5'-triphosphate

Hex-A – Hexokinase-A

HK – Hexokinase

HM – holidic medium

KD – knockdown

LCHP – low-carbohydrate-high-protein

LDH – Lactate dehydrogenase

mRNA – messenger RNA

NADH – nicotinamide adenine dinucleotide

NADPH – nicotinamide adenine dinucleotide phosphate

NCDs – non-communicable diseases

nos – nanos

OE – overexpression

OXPHOS – oxidative phosphorylation

PBS – phosphate-buffered saline

PCOS – polycystic ovary syndrome

PFK – Phosphofructokinase

Pgd – Phosphogluconate dehydrogenase, gene encoding 6PGDH in Drosophila

Pgi – Phosphoglucose isomerase

PK – Pyruvate kinase

PPP – pentose phosphate pathway

R5P – ribose-5-phosphate

RNA – ribonucleic acid

RPE – Ribulose 5-phosphate epimerase

RPI – Ribose 5-phosphate isomerase

Ru5P – ribulose-5-phosphate

SGLT – sodium-dependent glucose transporters

shRNA – short hairpin RNA

TAL – Transaldolase

TFC – terminal filament cells

TKL – Transketolase

TOR – target of rapamycin

Tps1 – Trehalose-6-phosphate synthase

Treh – Trehalase

Tret-1/2 – Trehalose transporter 1/2

UAS – upstream activating sequence

UDP-glucose – uridine 5′-diphosphate-glucose

UGP – UDP-glucose pyrophosphorylase

XU5P – xylulose 5-phosphate

Zw – *Zwischenferment*, gene encoding G6PDH in *Drosophila*

CHAPTER 1

INTRODUCTION

Context and Motivation

Animals face constant environmental changes to which they must adapt to achieve health and well-being. Accordingly, animals can adapt their behavior in order to maintain homeostasis, conventionally described as a series of mechanisms that perpetuate the *status quo* for organismal physiology, promoting a stable and constant internal state (Torday, 2015). A major factor influencing health is the maintenance of adequate nutrient levels, or nutritional homeostasis, which is mainly achieved through diet (Leonard, 2012). In fact, diet has been long known to be key in human health and disease, influencing the development of several conditions including chronic noncommunicable diseases (NCDs), such as obesity, diabetes *mellitus*, cardiovascular disease, hypertension, chronic respiratory disease and some types of cancer (WHO, 2003). Accordingly, there has been an increased interest in understanding how specific nutrients are used in organism functions, from the cell to the organ perspective.

It is now known that animal tissues react differently to nutrient availability and have mechanisms to maintain nutritional homeostasis. However, how this information is computed and centralized at the level of the Central Nervous System (CNS) and possibly translated into a behavior output is still poorly understood. In this project, we aim at exploring whether peripheral organs are sensitive to nutritional deficits and to whether these nutritional requirements are reported to the CNS, modulating feeding behaviour to maintain tissue nutritional homeostasis. To do so, we resort to *Drosophila melanogaster*, in which changes in feeding behavior have been shown to occur as response to a nutritional deficit in order to re-establish homeostasis. For example, fruit flies display an increase of the intake of nutrients such as amino acids (AAs) and carbohydrates when these are lacking from the diet (Itskov *et al.*, 2014; Corrales-Carvajal, Faisal and Ribeiro, 2016; Leitão-Gonçalves *et al.*, 2017; Steck *et al.*, 2018). We focused on the female reproductive system to investigate our hypotheses, since this organ was already shown to be highly responsive to dietary changes, it is a non-essential organ and it is constantly active in the adult fly. Clarifying the mechanisms underlying these processes can help to a better

understanding on how diet might impact organ function and also the aforementioned medical conditions. Furthermore, it may also shed light onto potential treatments in the context of deficient organ-brain communication that may rely on specific dietary regimes.

State of the Art

1.1 Modulation of nutrient homeostasis and animal physiology by diet

While studies on the impact of food and nutrition on health have been presented for centuries, modern nutritional science only arose less than 100 years ago. The first steps in the development of this concept occurred with calorimetric studies at the end of the 18th century. Antoine Lavoisier and Pierre-Simon Laplace proposed an important link between physiology and metabolism, unraveling cellular respiration as combustion process (Karamanou and Androutsos, 2013). Subsequent studies characterized the different importance of protein and energy to the human organism (calories), so that in 1827, William Prout classified alimentary principles (foodstuffs) into “saccharinous” (carbohydrates), “oleaginous” (fats) and “albuminous” (proteins) (Rosenfeld, 2003). Later work from both Edith G. Willcock and Frederick Hopkins, and Stephen M. Babcock and Edwin B. Hart lead to the acknowledgment of other “factors” important for a complete diet (Willcock and Hopkins, 1902; Hopkins, 1912; Carpenter, 2003). The first half of the 20th century was thus marked with the identification of these key dietary components, coined vitamins (from “vital amines”) by Kazimierz Funk (Semba, 2012). Supplementation of these in the diet confirmed their suspected role in several diseases, such as scurvy, beriberi, pellagra, rickets, xerophthalmia and nutritional anemias (Piro *et al.*, 2010). In 1961, François Jacob and Jacques Monod made another link between nutrition and metabolism, proposing that gene expression could be influenced by nutrient availability and specific metabolites, studying how single-cell organisms adapt to alterations in sugar supply (Schvartzman, Thompson and Finley, 2018). These findings were strongly building up awareness on the impact of nutrient availability and metabolism on physiology.

At the same time, the great impact of diet related non-communicable diseases (NCDs) began to be recognised. Unbalanced diets can lead to failure of the organism to control nutritional homeostasis, resulting in the development of these medical conditions (Pang *et al.*, 2014). For instance, the worldwide incidence of obesity has nearly tripled since 1975, raising awareness as

this complex and multifactorial disease increases the risk for debilitating morbidity and mortality (**Figure 1.1**) (WHO, 2003; Hruby and Hu, 2015; Fontana and Della Torre, 2016). Several changes in human civilization have been associated with this phenomenon, such as economic growth, industrialization, mechanized transport, which led to an increased sedentary lifestyle combined with a nutritional transition to processed foods and high calorie diets (Hruby and Hu, 2015).

Due to the high incidence of such diseases in modern society, there has been a focus of research in understanding the role of dietary sugar and fat as underlying causes and in the development progression of obesity and diabetes (Mozaffarian, 2018). Different scientific approaches have been used to untangle this question: epidemiological analysis between the several NCDs and the intake of different nutrients and diet compositions; animal studies in which several constituents were manipulated in the diet (Simpson, Le Couteur and Raubenheimer, 2015). In humans, epidemiological studies show a significant positive relationship between the consumption of dietary fat and the proportion of the population that is overweight (Hariri and Thibault, 2010). Case control studies in humans have suggested that diabetic patients which display hyperglycaemia and hyperinsulinemia, had higher cancer risk and cancer mortality (Donaldson, 2004; Suh and Kim, 2011). It has even been proposed that foods which contribute to hyperinsulinemia, such as refined sugar, should be avoided and eliminated from a cancer protective diet (Donaldson, 2004). Animal models, on the other hand, can greatly mimic the variables observed in humans accompanying obesity, being extensively used to advance

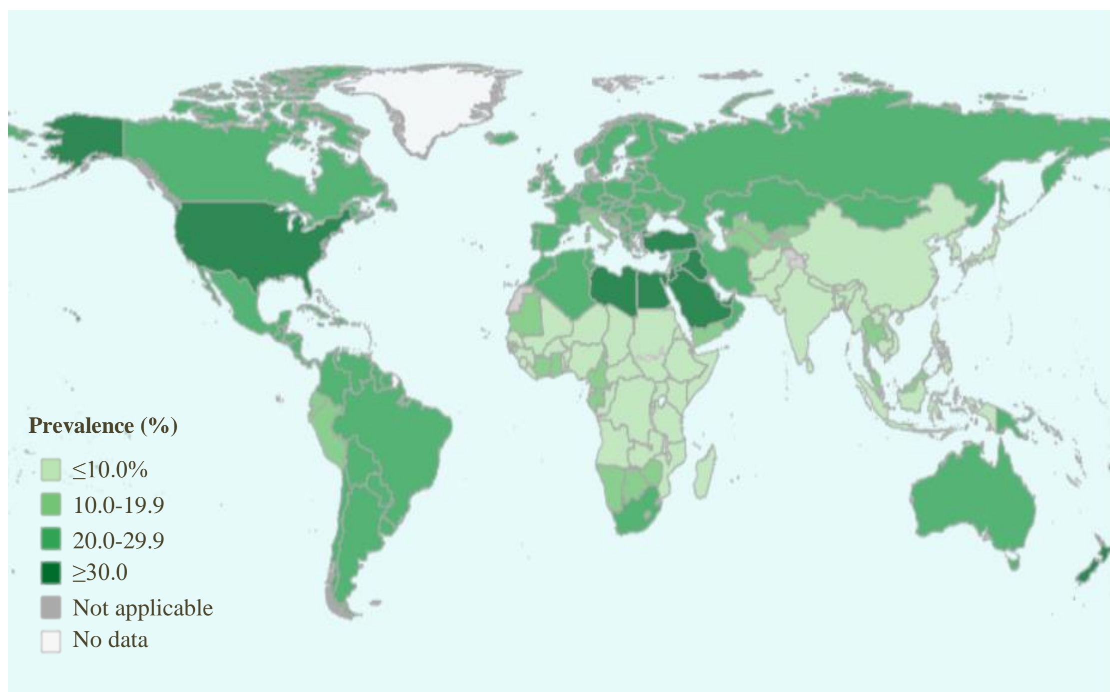

Figure 1.1 | Representation of worldwide prevalence (%) of obesity in 2016, measured as Body Mass Index ≥ 30 kg/m^2. *(Figure adapted from WHO, Obesity and Overweight 2016)*

knowledge in this area of research (Trinh and Boulianne, 2013; Musselman and Kuhnlein, 2018). For example, fat-rich diets have been shown to increase body weight and lead to diabetes and obesity in rodents (Sclafani, 1984; Buettner, Scholmerich and Bollheimer, 2007). In *Drosophila melanogaster*, inducing obesity with several diets (high fat, high sucrose) was also associated with many of the pathophysiological consequences found in humans, including hyperglycaemia, insulin resistance, cardiac arrhythmia, reduced longevity and alterations in gut microbiota (Conlon and Bird, 2015; Musselman and Kuhnlein, 2018). Furthermore, classical studies in mice and in fruit flies highlighted the importance of analysing not only the role of single nutrients, but rather the ratio between protein and carbohydrates, which impacts life traits such as fertility and lifespan (Lee *et al.*, 2008). Therefore, all these studies have further strengthened the idea that regulation of nutritional homeostasis is imperative to assure organism functions and a subsequent healthy status.

The communication between the different organ systems is key to maintain organismal functions and nutritional homeostasis. The function of different organ systems is based on the continuous supply of specific molecules which are constantly being transformed via different metabolic pathways into a variety of different compounds. Due to the implication of changes in glucose levels and its metabolism in several NCDs, one of the major goals of metabolism research is to understand how glucose affects organ functions and how it is used by different tissues. Glucose is also one of the dietary molecules under strict regulation in animals and defective communication between peripheral organs and the brain was shown to contribute to the development of obesity and type 2 diabetes (Kim, 2016). It is then valuable to understand how carbohydrates can be metabolized within cells, since they can serve many purposes, from being energy stores, fuels and metabolic intermediates, fulfilling the nutritional requirements and physiological functions of the animal (Berg, Tymoczko and Stryer, 2012).

1.1.1 Carbohydrate metabolism in cell function

Carbohydrates are a main source of energy for animals, maintaining several reactions and processes flowing inside cells. The most relevant carbohydrates in the diet are monosaccharides, such as the hexoses, glucose, fructose and galactose and the pentoses, xylose and arabinose (Maughan, 2013). Sugars are also commonly found in the form of disaccharides, such as: sucrose, a combination of glucose and fructose found in refined sugar; lactose, a combination of glucose and galactose, the main sugar found in milk; and maltose, the combination of two glucose

molecules, derived from hydrolysis of starch (Dashty, 2013). These complex molecules can be broken down via catabolic pathways, usually accompanied by the transfer of energy into higher energy compounds (cellular respiration), or used for the synthesis of new macromolecules, via anabolic pathways which require energy input. All of these carbohydrates can be converted into glucose which is the main source of energy in cells (Dashty, 2013). Glucose, as any hydrophilic molecule, is not able to permeate the plasma membrane and needs to be actively shuttled in and out of cells by transporters (Dupont and Scaramuzzi, 2016). After entering in the cell, glucose can enter three major pathways: glycogenesis, glycolysis and the pentose phosphate pathway (PPP). Different cells will display different activities in these metabolic pathways according to their current function (eg. energy storage or production of energy and building blocks for biosynthesis). The common substrate for these metabolic pathways, glucose-6-phosphate (Figure 1.2), results from immediate glucose phosphorylation by hexokinases (HKs) isoenzymes, which comprise a family of four isoforms in humans (John, Weiss and Ribalet, 2011; Adeva-Andany *et al.*, 2016). Hexokinases I, II, and III immediately phosphorylate glucose (or other hexose sugars) after entering the cell, thus driving glucose metabolism (Irwin and Tan, 2014). Phosphorylation of glucose has two purposes: it retains sugar in the cell, since this phosphorylated form cannot cross the membrane, and decreases the intracellular concentration of the unphosphorylated form of the sugar, thus creating a concentration gradient that drives the uptake of the sugar from the

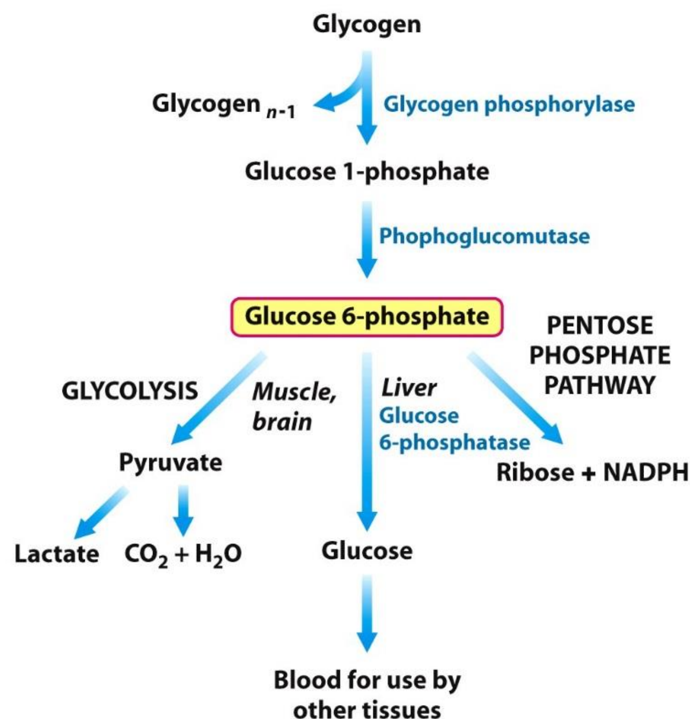

Figure 1.2| Main carbohydrate metabolic pathways. After directly entering the cell and being phosphorylated or being generated by the breakdown of other molecules (eg. glycogen), glucose-6-phosphate can be metabolized directly through glycolysis, the pentose phosphate pathway or glycogenesis (the reverse steps of glycogenolysis here represented). *(Figure 21.4 from Biochemistry © W.H.Freeman and Company, 2012)*

external environment (Irwin and Tan, 2014). HKI and HKII are the most abundant isoforms, the first ubiquitously present in most tissues, especially brain and red blood cells, and the second, found primarily in insulin-sensitive tissues such as adipocytes and adult skeletal and cardiac muscle (John, Weiss and Ribalet, 2011). Hexokinase IV, also known as glucokinase (GK), in contrast, has been adapted for a regulatory role and mutations in this gene can lead to diabetes (Irwin and Tan, 2014). Glucokinase is expressed in the liver, pancreatic islets and specific cells in the gut and the brain (Irwin and Tan, 2014). In the liver, glucokinase functions to regulate glucose metabolism, while in the pancreatic islets, intestinal cells and neurons it functions as a glucose sensor (Irwin and Tan, 2014).

- **Glycogenesis**

Glycogenesis converts glucose into glycogen which is an essential fuel reserve (Stryer, 1995). Glycogen is the main source of rapidly available glucose, being the most important compound for energy storage in animals. It is mainly stored in the liver and skeletal muscle, tissues that dephosphorylate glucose, supplying glucose to the blood stream during fasting periods and to the muscle cells during muscle contraction, respectively (Adeva-Andany *et al.*, 2016). Although it does not produce as much energy as fatty acids' metabolism, glycogen can provide energy in anaerobic conditions through its conversion to glucose (Stryer, 1995).

Storing phosphorylated glucose obtained from the diet as glycogen is initiated by the isomerization of glucose-6-phosphate to glucose-1-phosphate, by the action of phosphoglucomutase (Adeva-Andany *et al.*, 2016). The final reaction consists on the formation of uridine 5′-diphosphate-glucose (UDP-glucose), catalyzed by the enzyme UDP-glucose pyrophosphorylase (UGP), and UDP-glucose becomes the direct glucose donor for glycogen synthesis (Stryer, 1995; Adeva-Andany *et al.*, 2016). Then, a branching enzyme, glycogenin, catalyses the formation of a short glucose polymer that is later extended by the action of glycogen synthase (Adeva-Andany *et al.*, 2016). Both the synthesis and degradation of glycogen are complex processes that require the coordination of several enzymes. Congenital disorders of these processes can result in fasting hypoglycaemia and exercise intolerance (Adeva-Andany *et al.*, 2016). Glycogen storage diseases can display a broad clinical phenotype, impairing the nervous system, kidney and cardiac function (Adeva-Andany *et al.*, 2016).

- **Glycolysis**

Glycolysis occurs in the cytoplasm and is the metabolic pathway that converts glucose into two pyruvate molecules, producing energy in the form of adenosine-5'-triphosphate (ATP), as well as substrates for glycogenesis and lipogenesis (Guo *et al.*, 2012). This pathway is comprised by a series of ten chemical reactions, described in Figure 1.3, each catalysed by a specific enzyme. The first five reactions are endergonic, where energy is invested for the breakdown of glucose,

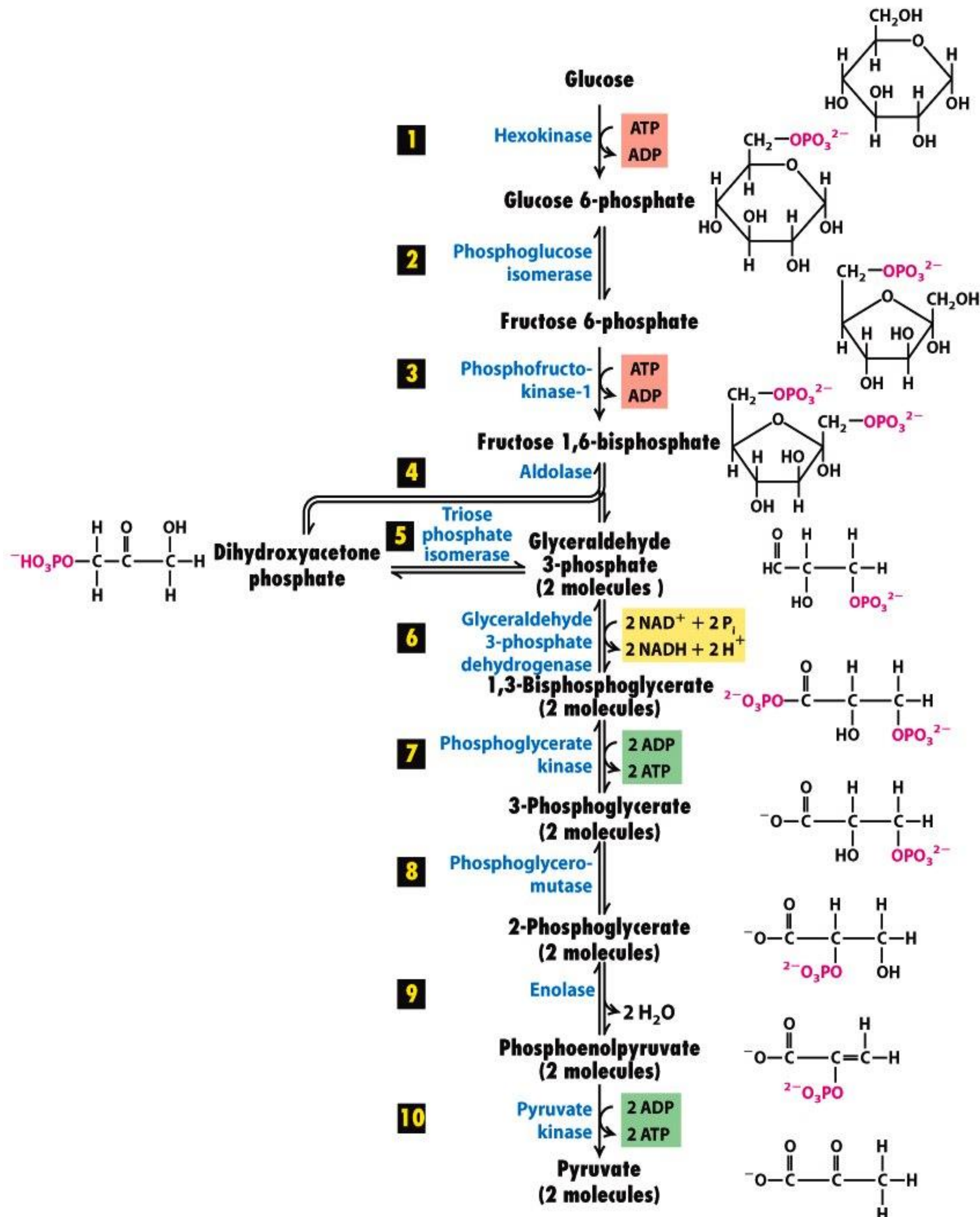

Figure 1.3| Glycolysis as the major pathway for energy production in the form of ATP. The three key steps are catalysed by Hexokinase (1), Phosphofructokinase-1 (3) and Pyruvate Kinase (10). (*Figure 12-3* from *Molecular Cell Biology © W.H. Freeman and Company)*

with reactions 1 and 3 requiring ATP molecules (Sadava *et al.*, 2006). The following reactions are exergonic, harvesting energy and thus generating ATP. These occur twice per glucose molecule, resulting in a positive net for ATP production (Sadava *et al.*, 2006). In aerobic conditions, cytosolic pyruvate is transported into the mitochondrial matrix, converted into acetyl coenzyme A by pyruvate dehydrogenase complex and incorporated into the citric acid cycle (McCommis and Finck, 2015). This cycle generates 32 molecules of ATP or guanosine-5′-triphosphate (GTP), as well as both reduced nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide ($FADH_2$) (Bonora *et al.*, 2012). Under low oxygen availability (anaerobic glycolysis) and in muscle during intense activity, glucose is only partially oxidized so that pyruvate is reduced into lactate, yielding 2 ATP molecules (Sadava *et al.*, 2006).

Glycolysis is regulated at the enzyme level at multiple points. It is dependent on glucose uptake mediated mainly by glucose transporters GLUT2 or GLUT4 (Guo *et al.*, 2012). The reactions catalysed by HKII or GK, phosphofructokinase (PFK) and pyruvate kinase (PK) are highly regulated, being key steps in this pathway as they are not reversible (Dashty, 2013). These can be regulated by both nutritional and hormonal signals at the levels of transcription, translation and post-translational modifications (Guo *et al.*, 2012).

Glycolysis flux varies widely, depending on cell status, function and energetic/metabolite requirements. For example, red blood cells lack mitochondria and are thus highly dependent on anaerobic glycolysis to generate energy (van Wijk and van Solinge, 2005). Therefore, deficiency of glycolytic enzymes, particularly PK, is specially critical for these cells, leading to haemolytic anaemia (Zimmermann, 2001). Cell division is also highly dependent on glycolysis, since it has high energy and biosynthetic demands when compared to nondividing cells (Burgess, Agathocleous and Morrison, 2014). Embryonic stem (ESCs) cells, for example, have a high rate of glycolytic lactate production compared with differentiated cells; differentiation of ESCs to neural stem cells is also accompanied by increased glycolysis and decreased oxidative phosphorylation (OXPHOS) (Burgess, Agathocleous and Morrison, 2014; Ito and Suda, 2014). During tumorigenesis, cells enter an aberrant state of proliferation displaying abnormally high division rates. Such cells also produce energy through high rates of glycolysis, followed by lactic acid fermentation in the cytosol, even in the presence of oxygen, a process also known as aerobic glycolysis or the Warburg effect (Li, Gu and Zhou, 2015). The three key enzymes of glycolysis, HK, PFK and PK together with lactate dehydrogenase (LDH) are overexpressed in some tumors, including in lung cancer, and can be regulated by many oncoproteins to promote tumor proliferation, migration and metastasis (Li, Gu and Zhou, 2015). This activation of glycolysis is

often accompanied by high Pentose Phosphate Pathway (PPP) activity, being the preferred metabolic state of rapidly proliferating cells, which together provide the cells not only with energy but also all intermediate metabolites required to generate all the building blocks for cells to divide and grow (Ito and Suda, 2014).

- **Pentose Phosphate Pathway**

The enzymatic reactions of the PPP, also known as the hexose monophosphate shunt, are subdivided into two biochemical branches, known as the oxidative (red contour in **Figure 1.4**) and

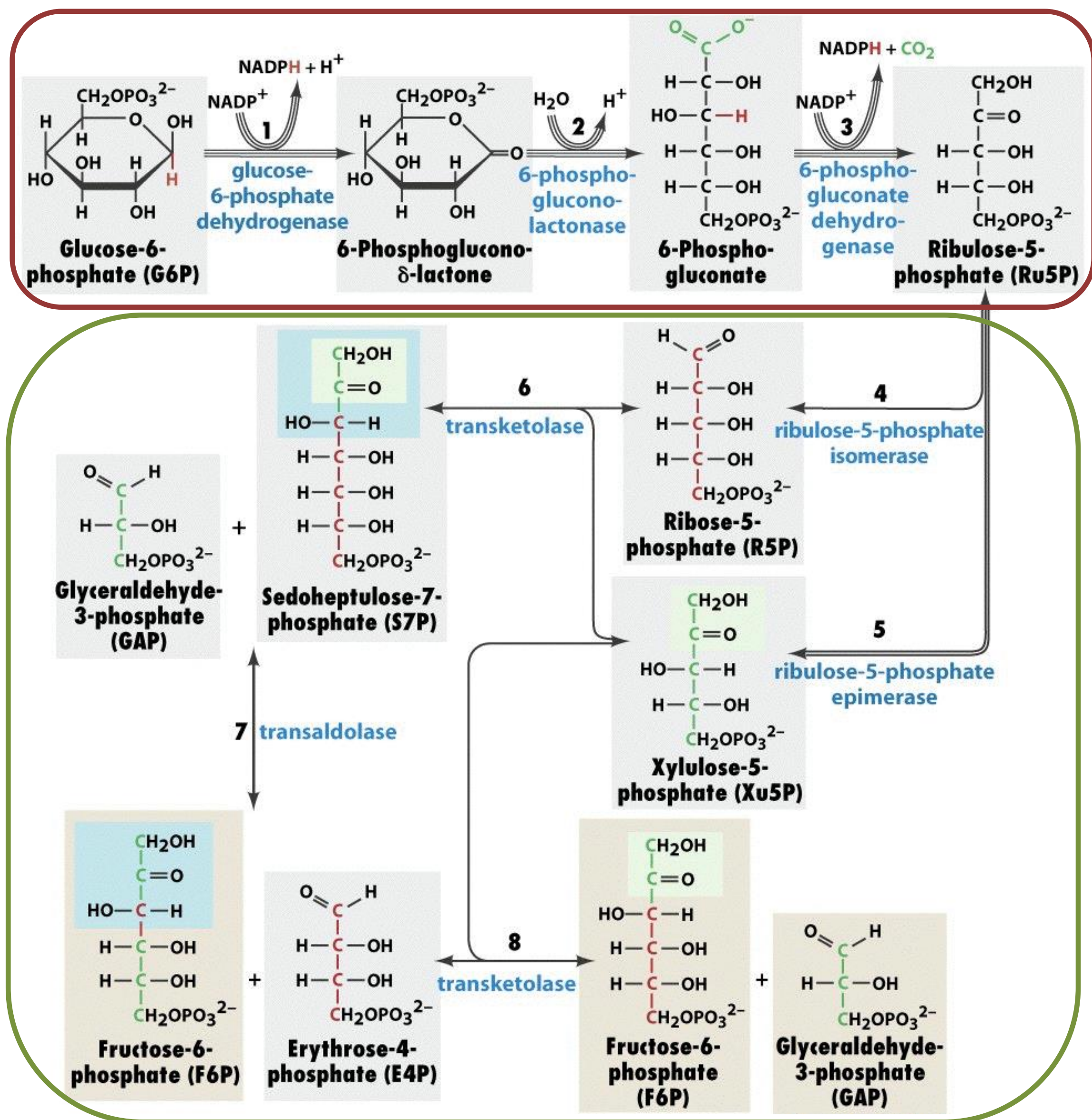

Figure 1.4| The Pentose Phosphate Pathway (PPP) is used for biosynthesis of several macromolecules, supporting growth and proliferation. The pathway consists of an oxidative phase (red contour), responsible for the final production of ribulose-5-phosphate (Ru5P), accompanied by NADPH release to use in reductive biosynthesis, and in a non-oxidative phase (green contour) that results in the interconversion of phosphorylated sugars. **(*Figure 14-30*** from *Fundamentals of Biochemistry ©John Wiley and Sons)*

non-oxidative (green contour in **Figure 1.4**). The first, occur ubiquitously and consists of three irreversible reactions that maintain a central metabolic role in generating nicotinamide adenine dinucleotide phosphate (NADPH) and ribonucleotides (Patra and Hay, 2014). There are two major drivers of the PPP flux: the rate of cell proliferation is linked with the high need of both ribose-5-phosphate (R5P) and NADPH, to synthesize deoxyribonucleic acid (DNA) and (ribonucleic acid) RNA; the maintenance of the redox homeostasis, where NADPH is used as a reducing agent in several synthetic steps of fatty acid, cholesterol and steroid hormones, and also in several detoxification reactions (Riganti *et al.*, 2012; Patra and Hay, 2014; Stincone *et al.*, 2015).

In the first step, glucose-6-phosphate (G6P) is dehydrogenated by glucose-6-phosphate dehydrogenase (G6PDH) to yield NADPH and 6-phosphogluconolactone. G6PDH catalyses the rate-limiting step, generating the first molecule of NADPH, being its expression and activity tightly regulated (Stincone *et al.*, 2015). This enzyme is expressed in many highly metabolically active tissues, including the liver, adipose tissue and mammary and adrenal glands (Patra and Hay, 2014; Stincone *et al.*, 2015). Subsequently, 6-phosphogluconolactone is hydrolysed by 6-phosphogluconolactonase (6PGL) into 6-phosphogluconate. This product goes through oxidative decarboxylation catalysed by 6-phosphogluconate dehydrogenase (6PGDH), producing a second NADPH and ribulose-5-phosphate (Ru5P). Next, the formed Ru5P enters the non-oxidative branch and can be converted either to R5P by ribose 5-phosphate isomerase (RPI) or to xylulose 5-phosphate (Xu5P) by ribulose 5-phosphate epimerase (RPE). R5P is essential for the synthesis of DNA and RNA backbone whereas Xu5P is directly converted to erythrose 4-phosphate, required for aromatic amino acid synthesis (Stincone *et al.*, 2015). These reactions produce the intermediates that are interconverted in different forms of monophosphate sugars (Stincone *et al.*, 2015). These interconversions also recruit glycolytic intermediates, such as fructose-6-phosphate (F6P) and glyceraldehyde-3-phosphate (G3P), which can be converted into pentose phosphates and vice-versa. These reactions are catalysed by the two enzymes, transketolase (TKL) and transaldolase (TAL), which are responsible for these relatively complex reactions at the core of the non-oxidative PPP (Stincone *et al.*, 2015).

Alterations in the flux of the PPP have been frequently implicated in several human diseases including metabolic syndrome, Alzheimer's disease, cardiovascular disease, parasite infections and cancer (Stincone *et al.*, 2015).

1.1.2 Carbohydrate homeostasis in organ and tissue function

There has been a strong focus on understanding the contribution of particular carbohydrates and their metabolism for correct organ functions and pathological states (Desvergne, Michalik and Wahli, 2006; Lushchak *et al.*, 2014). Glucose metabolism in humans, for example, is regulated by the interplay of several organs involved in nutrient homeostasis (Figure 1.5), mainly the pancreas, liver, muscle, adipose tissue and the brain (Aronoff *et al.*, 2004; Liangyou, 2014). Thus, the organ functions that highly depend on glucose should also be highly dependent on glucose homeostasis regulation.

- **Glucose absorption by the gut and hormonal control of glucose levels**

Glucose is supplied to the organism through intestinal absorption of dietary glucose or through its production in the liver from its precursors, for example, from other carbohydrates (glycogen, fructose, galactose) and amino acids. The absorptive process highly depends on the degree of systemic and gastrointestinal hormonal activity (Pang *et al.*, 2014). The gastrointestinal

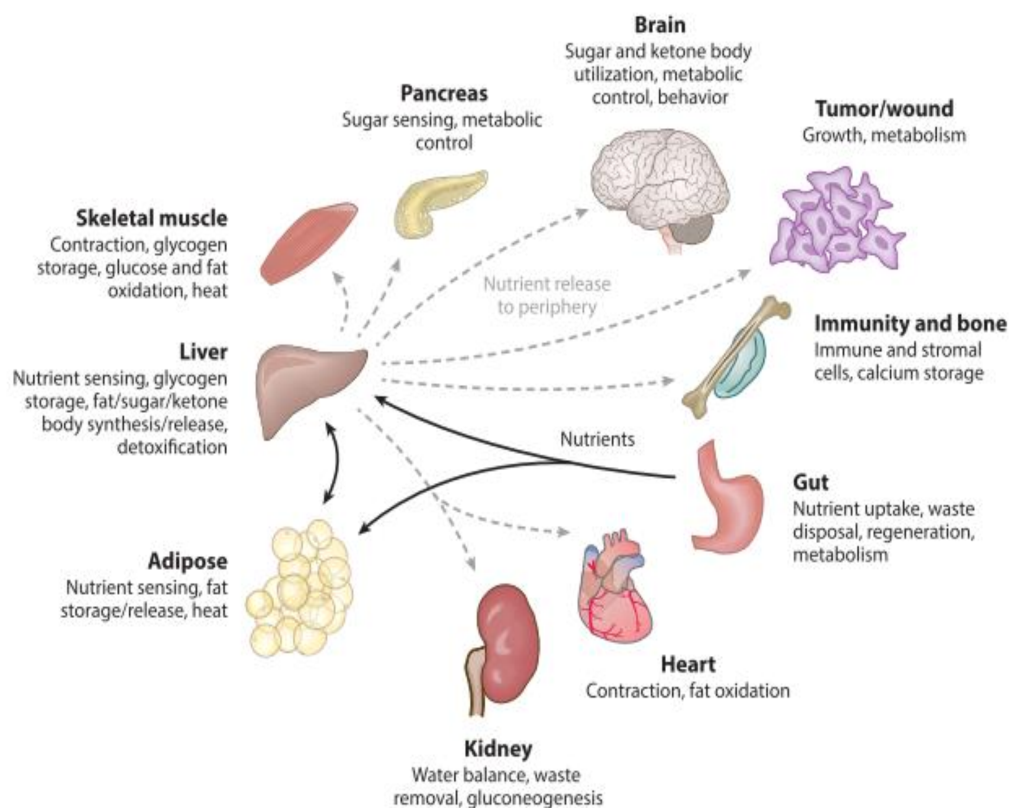

Figure 1.5| Interorgan specialization and communication in nutrient homeostasis. Nutrients are taken up by the gut and shuttled to the nutrient sensing organs, adipose tissue and liver (black arrows) where they are processed, stored and released to peripheral organs (grey dashed arrows) for different functions. *(Figure from* Droujinine and Perrimon, 2017)

mucus layer not only identifies the sugars in the ingested food but also regulates immune, metabolic and endocrine signals (cytokines and chemokines) which are transmitted to specific organs, tissues and cells through the circulatory system (Pang *et al.*, 2014).

The uptake of glucose is mediated by facilitated diffusion or Na^+ co-transport (through an electrochemical gradient across the membrane), respectively by facilitative glucose transporters (GLUTs) or sodium-dependent glucose transporters (SGLTs). Its absorption from the intestinal lumen into enterocytes occurs mainly through the Na^+/glucose cotransporter (SGLT1), but can also occur through glucose transporter type 2 (GLUT2) (Chen, Tuo and Dong, 2016). In humans, there are 14 GLUT proteins with different expression patterns and affinities (Mueckler and Thorens, 2013).

Maintaining blood glucose levels is of paramount importance. Low blood glucose levels can lead to seizures, coma and death, whereas conversely, prolonged elevated glucose levels, as in the diabetic state, can result in renal failure, neuropathy (associated with infection) and cardiovascular disease (Watson, Kanzaki and Pessin, 2004; Kawahito, Kitahata and Oshita, 2009). High glucose can also lead to insulin resistance, present in most of the mentioned medical conditions, which is characterized by the inability of insulin to promote normal glucose uptake by muscle and fat and to inhibit hepatic glucose production. This is one the hallmarks of type 2 diabetes and has also been linked to cancer development/progression (Wagner and Petruzzelli, 2015).

- **The Liver**

Following absorption, the liver occupies a central position in the regulation of circulating glucose concentration. Glucose uptake into human hepatocytes and pancreatic β-cells is performed by GLUT2 transporters (Adeva-Andany *et al.*, 2016). This transporter works as a sensor of circulating glucose levels as it has lower affinity for glucose compared to other GLUTs, thus it will only efficiently transport glucose across the plasma membrane when glycaemia is high (Efeyan, Comb and Sabatini, 2015).

Glucose homeostasis is primarily maintained by pancreatic islet' hormones, glucagon and insulin, both of which are regulated by nutrient levels and are modulated by the gastrointestinal hormones (Drucker, 2007). Insulin has the ability to lower blood glucose levels, whereas glucagon, growth hormones, cortisol and epinephrine have the opposite effect (Aronoff *et al.*, 2004; Chang, Chiang and Saltiel, 2004; Watson, Kanzaki and Pessin, 2004). In the fasted state, glucagon acts on

the liver to promote the release of glucose into circulation, through both breakdown of glycogen (glycogenolysis) and use of lactate, pyruvate, glycerol, and amino acids as sources for glucose synthesis (gluconeogenesis) (**Figure 1.6**). In the fed state, when glucose enters the liver, insulin will promote its conversion into glycogen (glycogenesis), fatty acids or amino acids and supress gluconeogenesis (Liangyou, 2014). Furthermore, the levels and activity of the glycolytic enzymes are modulated by the nutritional status, being lower in the fasted state and increased in the fed state (Liangyou, 2014). This organ takes up around two thirds of the circulating glucose and remaining monosaccharides (Berg, Tymoczko and Stryer, 2012). Liver glucose uptake is regulated by several factors, most of which ultimately relate to the rate of its utilization by the different organs mainly through the action of glucagon and insulin (Chang, Chiang and Saltiel, 2004).

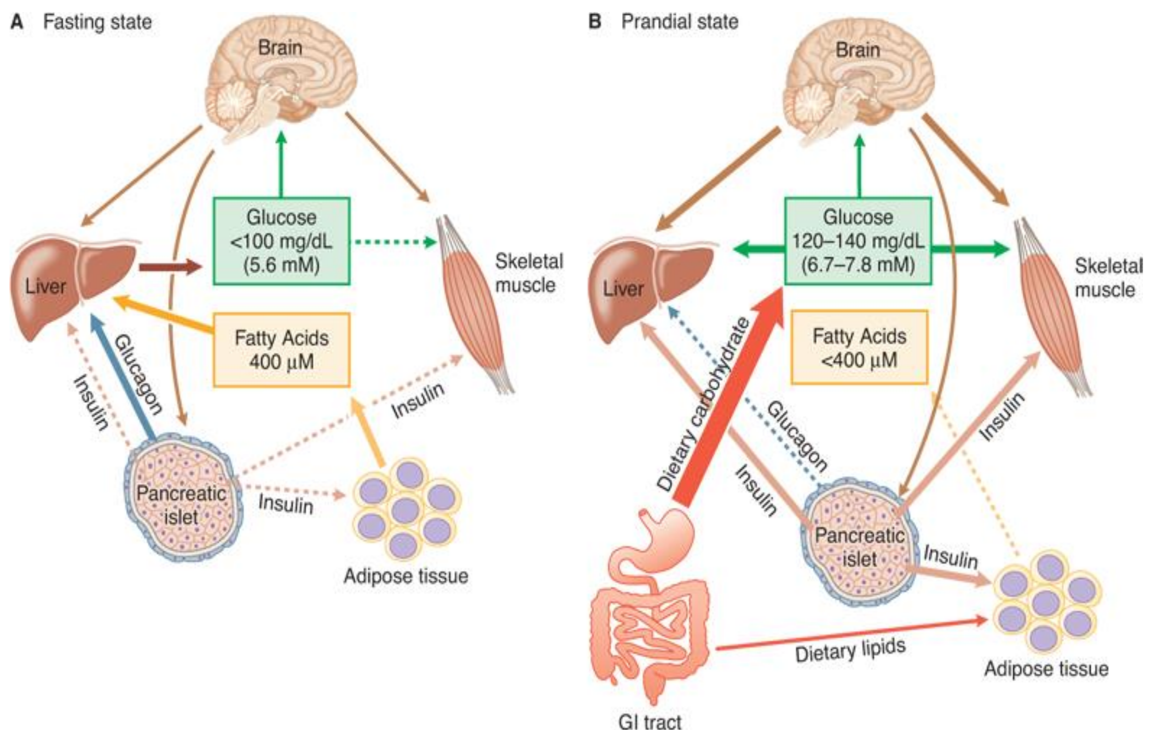

Figure 1.6| Glucose homeostasis is maintained and regulated through interorgan communication. (A) Fasting State – In the absence of nutrient supply, glucose and fatty acids are obtained primarily from the liver and adipose tissue, respectively; circulating insulin levels are low whereas plasma glucagon is elevated, contributing to increased hepatic glycogenolysis and gluconeogenesis and increased lipolysis in adipose tissue. **(B)** Prandial State – After a meal, nutrient absorption causes an increase in plasma glucose, promoting insulin secretion and inhibiting glucagon release from the pancreatic islet; glucose is actively taken up by the liver, skeletal muscle and adipose tissue; hepatic glucose production and lipolysis are inhibited, and total body glucose oxidation increases. The brain has mechanisms to sense circulating glucose concentrations to provide regulatory inputs to maintain homeostasis.
The boldness of the arrows reflects relative intensity of action; dashed line indicates low or no activity. (Figure from Goodman & Gilman's: The Pharmacological Basis of Therapeutics ©McGraw-Hill Education.

- **Skeletal Muscle and Adipose tissue**

The main fuels for muscle contractibility are glucose, fatty acids and ketone bodies (Berg, Tymoczko and Stryer, 2002). In human skeletal muscle, glucose uptake is carried out by two main transporters, GLUT1 and GLUT4. The first is present in the plasma membrane facilitating basal glucose transport into the muscle fiber, whereas the latter, GLUT4, resides inside intracellular storage vesicles being only translocated to the plasma membrane upon stimulation by muscle contraction or insulin (Adeva-Andany *et al.*, 2016). When the skeletal muscle' activity is induced, the rate of glycolysis surpasses that of the citric acid cycle, reducing most of the pyruvate to lactate, some of which will be mobilized to the liver where it is converted into glucose (Berg, Tymoczko and Stryer, 2012).

Adipose tissue secretes several peptides, proteins and hormones that are important regulators of glucose homeostasis (Rosen and Spiegelman, 2006). Glucose levels also impact the metabolism of these tissues through insulin signalling, which supresses muscle proteolysis and adipose tissue lipolysis, while stimulating glucose uptake and metabolism in these tissues (Chang, Chiang and Saltiel, 2004; Watson, Kanzaki and Pessin, 2004). Additionally, adipocytes were shown to modulate insulin signalling, since too much fat (obesity) and too little fat (lipodystrophy) have been both associated with insulin resistance and hyperglycaemia (Rosen and Spiegelman, 2006).

- **The Ovaries**

Glucose is an important supply of ATP for the metabolic and physiological functions of the ovary, one of the most dynamic of the endocrine organs (Dupont and Scaramuzzi, 2016). In humans, this sugar supports the development, the maturation and ovulation of the selected follicle(s) and subsequent formation and maintenance of the key endocrine gland, the corpus luteum (Baerwald, Adams and Pierson, 2005; Dupont and Scaramuzzi, 2016).

Various GLUTs are present in mammalian ovarian tissues, although there are differences between species. Studies in sheep, bovine and rat follicles and ovaries show the common expression of GLUTs 1, 3 and 4 (Dupont and Scaramuzzi, 2016). Throughout oocyte maturation, glucose is metabolized through glycolysis, providing substrates such as pyruvate for energy production, through both aerobic and anaerobic metabolism (Sutton-McDowall, Gilchrist and Thompson, 2010). Furthermore, it is also required as a substrate for many cellular functions during this process, including regulation of nuclear maturation and of the redox state via the PPP (Sutton-McDowall, Gilchrist and Thompson, 2010). Manipulation of the PPP is sufficient to alter

the developmental potential of porcine and bovine oocytes (Wang *et al.*, 2012). During follicle' development, the oocyte becomes surrounded by layers of granulosa cells that latter form the cumulus oocyte complex (COC), which were described to have specific mechanism of glucose transport to the oocyte (Eppig 1991, Albertini et al. 2001).

In humans, changes in insulin levels, which are influenced by dietary glucose, interfere with ovulation, sperm quality and the functioning of the hypothalamo–pituitary–gonadal axis (Dupont *et al.*, 2014). For example, in excess insulin conditions, the female egg or follicle may not completely mature, which could impair ovulation and increase ovarian cyst formation (Dupont *et al.*, 2014).

- **The Brain**

The nervous system depends on glucose as its main source of energy so that tight regulation of glucose metabolism is critical for brain physiology (Zhu *et al.*, 2012). GLUT1 and GLUT3 are the primary mediators of glucose transport across the endothelium of the blood–brain barrier, and the latter is mostly saturated, assuring the continuous supply of glucose to the brain (Berg, Tymoczko and Stryer, 2002). The relative energy consumption by the brain has increased during evolution. From humans to lower primates it reflects both the expansion in the relative size of the brain and an increase in the number of synapses per cortical neuron (Aiello and Wheeler, 1995; Harris, Jolivet and Attwell, 2012). Although it has high glucose requirements, the brain lacks fuel stores, hence requiring a continuous supply of this nutrient (Berg, Tymoczko and Stryer, 2002). It is estimated that most of the energy obtained by the brain is used for neuronal computations and information processing, such as: the generation of action potentials and postsynaptic potentials during synaptic events; the maintenance of ion gradients and neuronal resting potential; the biosynthesis of neurotransmitters (Berg, Tymoczko and Stryer, 2012; Zhu *et al.*, 2012).

In the brain, the activity of the different carbohydrate metabolic pathways has been shown to be cell and function specific (reviewed in (Magistretti and Allaman, 2018)). Astrocytes, on one hand, primarily function using ATP from glycolysis, producing and releasing lactate to the extracellular environment (Turner and Adamson, 2011). Neurons predominantly metabolize glucose through the PPP and use the lactate from astrocytes as a source for aerobic metabolism, after its conversion to pyruvate (Magistretti and Allaman, 2018). Contrary to neurons, astrocytes

can store energy in the form of glycogen, key for the glycolytic activity of these cells (Turner and Adamson, 2011).

The brain integrates multiple metabolic inputs from the periphery through nutrients, gut-derived satiety signals and adiposity-related hormones (Chambers, Sandoval and Seeley, 2013). The hypothalamus is crucial for the regulation of food intake, containing neurons that are responsible for glucose sensing and appetite (Roh, Song and Kim, 2016). Understanding how organs modulate glucose metabolism according to their function and how this aspect is communicated to the brain may provide crucial insights into the physiology of glucose homeostasis, which becomes impaired in several human diseases.

1.2 *Drosophila melanogaster* as a model to study carbohydrate homeostasis

Drosophila melanogaster, the common fruit fly, is a well-studied and highly tractable genetic model organism for understanding molecular mechanisms of human diseases. *Drosophila* is a member of the order *Diptera,* also known as the higher flies (McLaughlin and Bratu, 2015). As a holometabolous insect, *Drosophila* undergoes a complete metamorphosis (McLaughlin and Bratu, 2015). Its fast life cycle consisting of four stages allows for the generation of hundreds of genetically identical offspring within 10 to 12 days at 25°C, allowing for large scale studies (Pandey and Nichols, 2011).

There is a high degree of similarity among metabolic pathways and physiological responses to nutrients between mammals and the fruit fly, making *Drosophila* an ideal animal model to test the interactions between these processes (Graham and Pick, 2017). Humans and the fruit fly share homologs of a great number of genes required for a vast number of functions, including transcription factors, signalling molecules and most intermediates of the metabolic pathways described (Reyes-DelaTorre, Peña-Rangel and Riesgo-Escovar, 2012). Nearly 75% of human disease-causing genes are believed to have a functional homolog in the fly (Pandey and Nichols, 2011) and this organism has been successfully used as a model to study various human conditions, including metabolic disorders.

The fruit fly was one of the first multicellular organisms having its genome fully sequenced and annotated (Adams *et al.*, 2000). Several genetic tools have been developed for *Drosophila* that allow genetic manipulation in a tissue and temporal manner. Many of the tools available are based on the Gal4/UAS system, a system which was adapted from yeast (**Figure 1.7**). Gal4 expression can be controlled by defined promoters placed upstream of its sequence, which can

control the cell specificity of Gal4 action (Duffy, 2002). When Gal4 is expressed it binds an Upstream Activating Sequence (UAS) promoting the transcription of genes which are downstream of these sequences. The UAS sequences can be used to control the expression of gene coding sequences for overexpression (OE) experiments; short hairpin RNAs (shRNAs) for gene knock down (KD), etc. Each component is inserted in one parental strain, so that when mated, the F1 generation expresses the desired transgene in the tissue pattern defined by the Gal4 promoter (Duffy, 2002).

Cloning and functional analysis of genes in *Drosophila* led to the elaboration of vast libraries of different genetic strains, allowing the study of many cellular processes and extrapolation to the function of the ortholog gene in humans and other organisms (Reyes-DelaTorre, Peña-Rangel and Riesgo-Escovar, 2012). A large number of these stocks are available through the Bloomington Drosophila Stock Center and VDRC (Vienna Drosophila Resource Center).

Drosophila has recently emerged as a potent model organism to study the effects of diet in metabolism and physiology. Classically these studies were carried out using standard foods, using complex macromolecule composition, creating a problem of unification in terms of nutritional

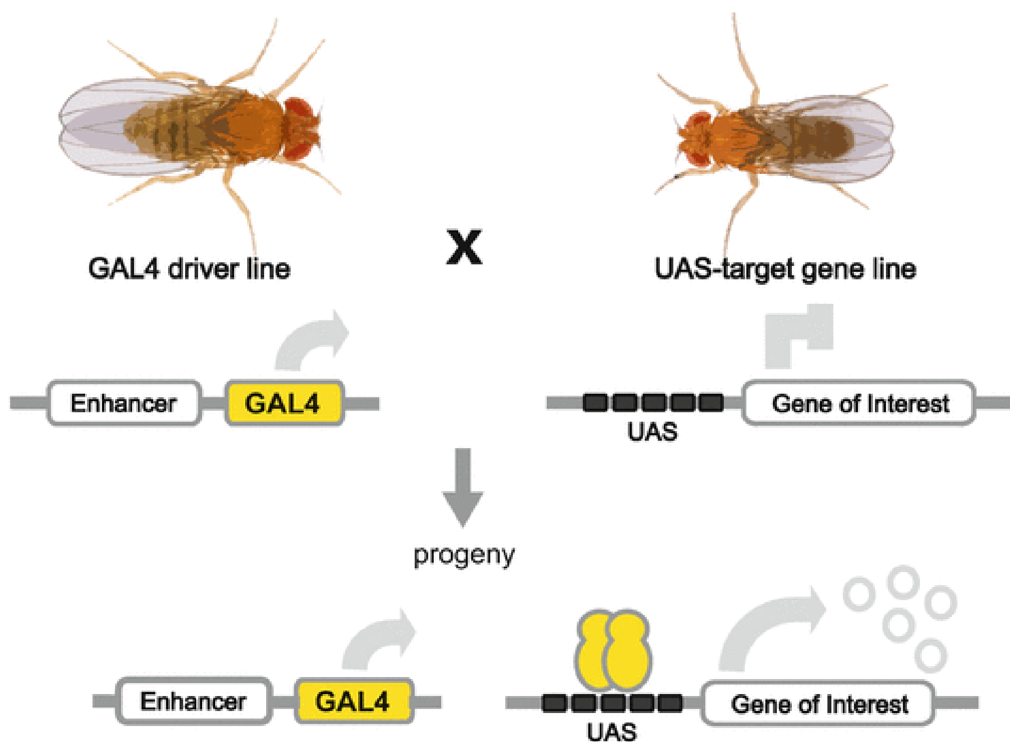

Figure 1.7| An overview of the Gal4/UAS system in *Drosophila melanogaster* for OE of a specific gene of interest. Two stocks are crossed, one carrying a Gal4 driver, which expresses Gal4 under a certain tissue specific promoter, and the other carrying the gene of interest which will be expressed under the control of a UAS sequence. *(Figure from Drosophila, Methods in Molecular Biology, ©Humana Press)*

content across studies. In order to overcome this problem and to explore the importance of specific dietary nutrients in animal physiology, Matt Piper and colleagues developed a full synthetic diet (Bass *et al.*, 2007; Piper *et al.*, 2014, 2017).

1.2.1 Cell metabolism and nutrient homeostasis

In the fruit fly, the type and concentration of carbohydrates in the diet, as well as its proportion to other dietary components affects lifespan (Lee, 2015; Bowman and Tatar, 2016). The study of how dietary carbohydrates impact *Drosophila's* physiology also allowed to elucidate the function of evolutionarily conserved signalling pathways, like the insulin/IGF (insulin growth factor) and Target of Rapamycin (TOR) pathway in controlling growth according to nutritional status (Reyes-DelaTorre, Peña-Rangel and Riesgo-Escovar, 2012). Carbohydrate metabolism in the fly has been linked to several physiological processes, such as circadian regulation and developmental transitions (Mattila and Hietakangas, 2017).

Sugars in fruits, the source of carbohydrates for many *Drosophila* species, comprise a mixture of fructose, glucose, sucrose, amongst others. As in mammals, fruit flies have all the metabolic pathways for glucose processing. *Drosophila melanogaster* has two glucose transporters, GLUT1 is expressed ubiquitously and GLUT3 only in testes (Niccoli *et al.*, 2016). In *Drosophila*, some enzymes of these metabolic pathways are also tissue specific. For instance, the fly expresses different hexokinase genes in different tissues, *Hex-A*, *-C*, *-t1* and *-t2*. *Hex-A* is expressed in nearly all tissues in the fly, *Hex-C* is selectively expressed in the brain, fat body and gut, and *Hex-t1* and *Hex-t2* are testis-specific hexokinases (Moser, Johnson and Lee, 1980; Duvernell and Eanes, 2000; Dus *et al.*, 2016).

Drosophila has two main circulating carbohydrates, glucose and trehalose (a non-reducing disaccharide of glucose). In insects, sugars in circulation are comprised mostly by trehalose, with glucose being maintained at low levels (Graham and Pick, 2017). The use of trehalose as a circulating sugar molecule compared to glucose has the advantage of protecting organisms against several environmental stressors, such as starvation, due to its inert chemical properties (Yasugi, Yamada and Nishimura, 2017). This high energy storage molecule, with low reactivity and high stability, can also stabilize and preserve other biomolecules: insect hemolymph has high amounts of peptides, free amino acids and proteins, all of which have amine and amino functional groups to which reducing sugars, such as glucose, could react (Reyes-DelaTorre, Peña-Rangel and Riesgo-Escovar, 2012). Thus, comparing to mammals, flies have, in addition, the machinery to metabolize trehalose.

The fly has two trehalose transporters, Tret1-1 and Tret1-2, expressed in both the fat body and in several peripheral tissues (Yoshida *et al.*, 2016). Trehalose synthesis and degradation occurs in the fat body, by Trehalose-6-phosphate synthase (Tps1) and by Trehalase (Treh), either using or producing glucose (Yasugi, Yamada and Nishimura, 2017). Tps1 has two functionally distinct catalytic domains: the N-terminus with a synthase domain produces trehalose-6-phosphate from glucose-6-phophate (G6P) and UDP-glucose; the C-terminus with a phosphatase domain produces trehalose from the UDP-glucose previously synthetized. UDP-glucose can also be stored as glycogen (Soulages, 2010). The *Drosophila* genome encodes two genes with catalytic activity for trehalose hydrolysis: *Treh* displays ubiquitous expression and *CG6262* is mainly expressed in the testis (Yoshida *et al.*, 2016). Loss of Trehalase activity prevents trehalose catabolism, leading to elevated circulating trehalose levels (Mattila and Hietakangas, 2017). In the CNS, most of Treh is found in the surface of glia, cells that form a barrier analogous to the blood-brain barrier in mammals. The local breakdown of trehalose followed by glycolysis in glia produces alanine and lactate, which are secreted and used for neuronal metabolism and function (Mattila and Hietakangas, 2017; Yasugi, Yamada and Nishimura, 2017).

The mobilization of hemolymph trehalose to glucose is critical for metabolic homeostasis (Matsuda *et al.*, 2015). *Drosophila* also regulates the concentration of circulating sugars, by controlling the release of insulin-like peptides (Dilps) and adipokinetic hormone (Akh), a glucagon-like molecule (Matsuda *et al.*, 2015).

1.2.2 Organ physiology

Many of the internal organ systems of *Drosophila melanogaster* are functionally analogous to those in vertebrates, including humans, with many of the molecular mechanisms that regulate development and drive physiological processes being conserved (Ugur, Chen and Bellen, 2016). *Drosophila* has functionally similar organ systems to the human liver/adipose tissues (fat bodies and oenocytes, respectively), gut, brain, muscle and gonads (**Figure 1.8**) (Droujinine and Perrimon, 2016).

Nutrients are first taken up through the gut, being sensed, metabolised, stored and release by the fat body (FB) and oenocytes for further utilization by the peripheral organs (Soulages, 2010). The FB in particular, plays critical roles in energy metabolism and storage of lipids and carbohydrates and responds to energy demands by regulating both glucose and trehalose supply to the hemolymph (Soulages, 2010). Nutrients are used by the muscles for contraction, by the

brain for neuronal function, by the gonads for reproduction and by developing organs (imaginal discs) and other tissues for growth (Droujinine and Perrimon, 2016).

1.2.3 Feeding Behavior

When compared to mice, studying feeding behavior in flies encompasses some challenges including the small size of the animal and low amounts of food ingestion. Assays mostly used to rely on the *post hoc* quantification of the ingested food using colorants or radioactive substances, proboscis extension assays scored manually and measurement of the volumetric change of food ingested from capillaries (Itskov and Ribeiro, 2013). However, these different methodologies have limitations and constraints that might add confounding effects to the analysis of feeding behaviour. The development of automated assays, namely the flyPAD (fly Proboscis and Activity Detector) and the FLIC (Fly Liquid-Food Interaction Counter) overcame this problem since they quantify the physical contact of flies' proboscis with the food surface by monitoring the changes in capacitance and conductance, respectively (Itskov *et al.*, 2014; Ro, Harvanek and Pletcher,

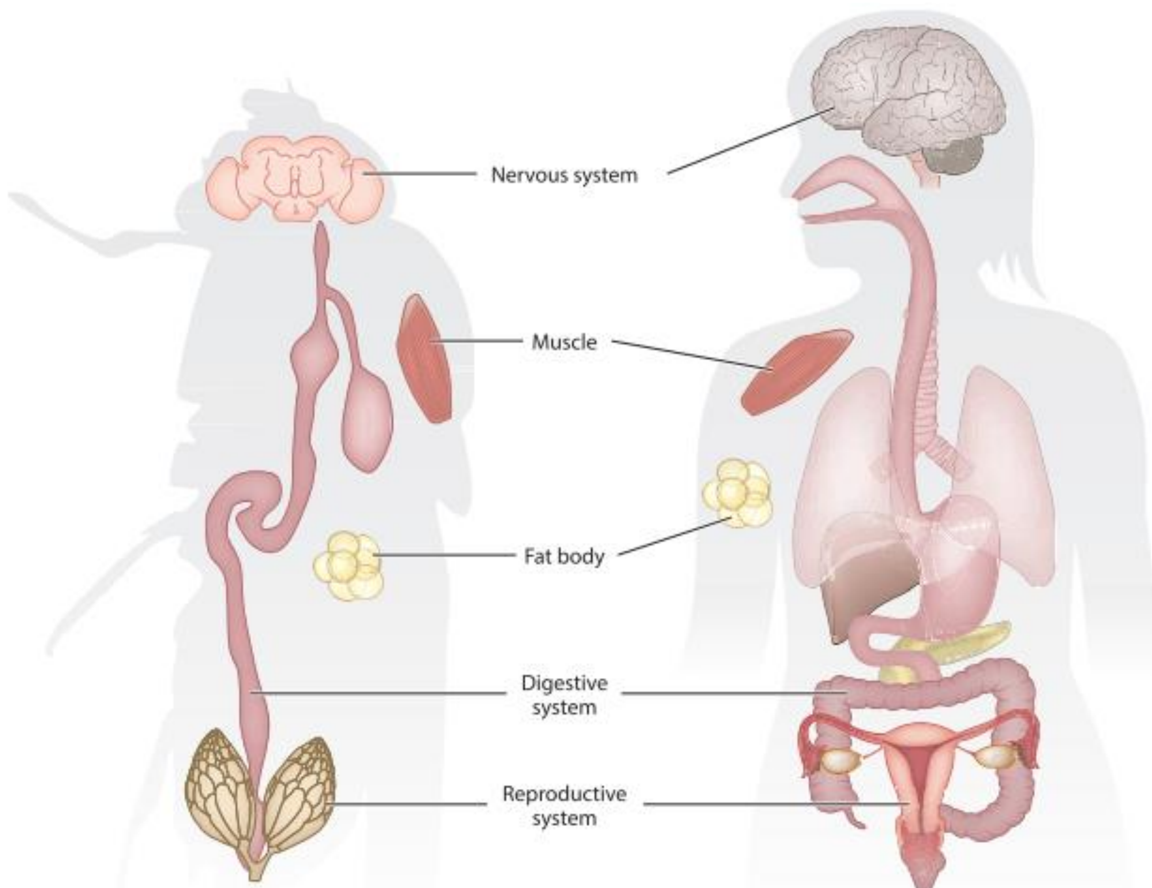

Figure 1.8| *Drosophila melanogaster* and humans share most organ systems as well as their function. Fruit fly and human share analogous nervous system, musculature, digestive and reproductive systems. The liver/adipose tissues in humans and the fruit flies are functionally represented by the fat body and oenocytes in *Drosophila*. *(Figure from Droujinine, I. A. and Perrimon, N., 2016).*

2014). The flyPAD allows the detailed, automated and high throughput quantification of feeding behaviour (Itskov *et al.*, 2014). This assay provides the fly's feeding motor program which can be defined by a range of parameters (**Figure 1.9**) (Itskov *et al.*, 2014). The parameter which best correlates with food intake is the number of sips (rhythmic interactions with food) (Itskov *et al.*, 2014; Walker, Corrales-Carvajal and Ribeiro, 2015).

Using the flyPAD, it has been shown that upon deprivation of specific nutrients (AAs, sucrose), flies increase the consumption of food sources that allow them to replenish these nutrients (Steck *et al.*, 2018). Behavioural assays combining the synthetic diet, the flyPAD, and tracking systems have shown that flies modulate their feeding behavior both according to their mating status and to their internal nutritional state (Ribeiro and Dickson, 2010; Itskov *et al.*, 2014; Piper *et al.*, 2014; Walker, Corrales-Carvajal and Ribeiro, 2015; Corrales-Carvajal, Faisal and Ribeiro, 2016; Piper *et al.*, 2017). *Drosophila* is therefore a powerful animal model to study organ-communication in the context of nutritional homeostasis, metabolism and feeding behavior.

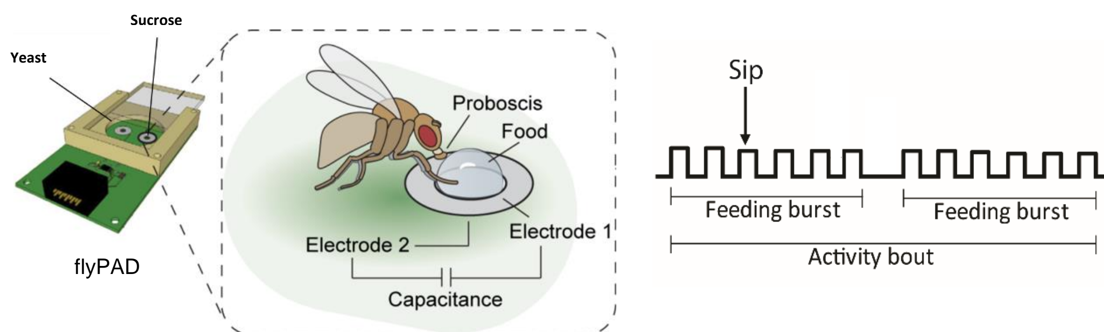

Figure 1.9| Schematic representation of the flyPAD setup and the feeding program obtained from the capacitance data. (*Figure adapted from Itskov et al., 2014)*

1.3 Oogenesis as a model system to explore the interaction between the diet, organ physiology and nutrient appetite

1.3.1 Overview of oogenesis

Drosophila has been used as a model to study nutrition and reproduction. In the flies, fecundity is highly modulated by diet: for example, female fruit flies reared in a yeast-rich diet lay an average of 80 eggs per day, whereas following yeast deprivation, their prime protein source, egg laying rate dramatically decreases to one or two eggs per day (Drummond-Barbosa and Spradling, 2001; Laws and Drummond-Barbosa, 2017). This response occurs within a day and is quickly reversible by protein supply, reflecting the tight regulation of the various processes coupling oogenesis and nutrient signaling (Drummond-Barbosa and Spradling, 2001; Laws and Drummond-Barbosa, 2017).

Ovaries are not vital organs, allowing for their manipulation without compromising the adult integrity (Bastock, 2008). The female reproductive system has been used to understand complex processes, such as the mechanisms underlying developmental processes and disease progression (Bastock, 2008; Becalska and Gavis, 2009). This system has also been used to understand how diet influences cell physiology. Both germline and somatic stem cells, as well as their differentiated counterparts, adjust their proliferation rates according to nutritional availability (Drummond-Barbosa and Spradling, 2001). Egg formation requires multiple dietary nutrients, which might be crucial for different stages of oogenesis (Piper *et al.*, 2014). For instance, availability of protein and essential amino acids (EAAs) is essential for maintaining integrity of ovary morphology and keeping high rates of egg production (Drummond-Barbosa and Spradling, 2001; Piper *et al.*, 2014). Furthermore, it was also shown that the levels of ecdysone, which in adult female fruit flies is mainly produced by the ovaries, reflect the amount of dietary protein ingested (Carvalho-Santos and Ribeiro, 2018).

Oogenesis is a highly metabolically demanding process that requires the coordination of several cellular components throughout the developmental stages. Female *Drosophila'* reproductive system is analogous to the female reproductive system in humans. It is composed of ovaries, uterus, oviducts and secretory glands (spermathecae and parovaria) (Sun and Spradling, 2013).

Female fruit flies have two ovaries, each composed of around 16-20 ovarioles (Figure 1.10), each of which contain all oocyte developmental stages (Rosales-Nieves and González-Reyes, 2014). At the most anterior part of each ovariole resides the germarium, divided into four regions (1, 2a, 2b, and 3), that contains the stem cell niche: around 2-4 **g**ermline **s**tem **c**ells (GSCs) and 2

follicle stem cells (FSCs), which collectively produce the nurse cells, oocyte and follicle cells of the mature egg chamber throughout the female's life (McLaughlin and Bratu, 2015). This niche also includes three differentiated somatic cell types: the terminal filament cells (TFCs) that form the terminal filament, connecting the germarium to the surrounding ovariole envelope and determining the orientation of ovariole development; cap cells, each in close contact with one GSC, maintaining stem cell pool and division; escort cells (ECs), which sheath the GSCs to avoid GSC-GSC contact (Rosales-Nieves and González-Reyes, 2014; McLaughlin and Bratu, 2015). The entire process of oogenesis comprises roughly a week and is divided into 14 morphologically distinct stages (Bastock, 2008). Oogenesis initiates in the region 1 of the germarium with an asymmetric division of GSCs, originating another stem cell and a daughter cell (Rosales-Nieves and González-Reyes, 2014). This daughter cell undergoes four mitotic divisions without cytokinesis and in region 2 becomes a cyst of 16 cells, interconnected by cytoplasmic bridges

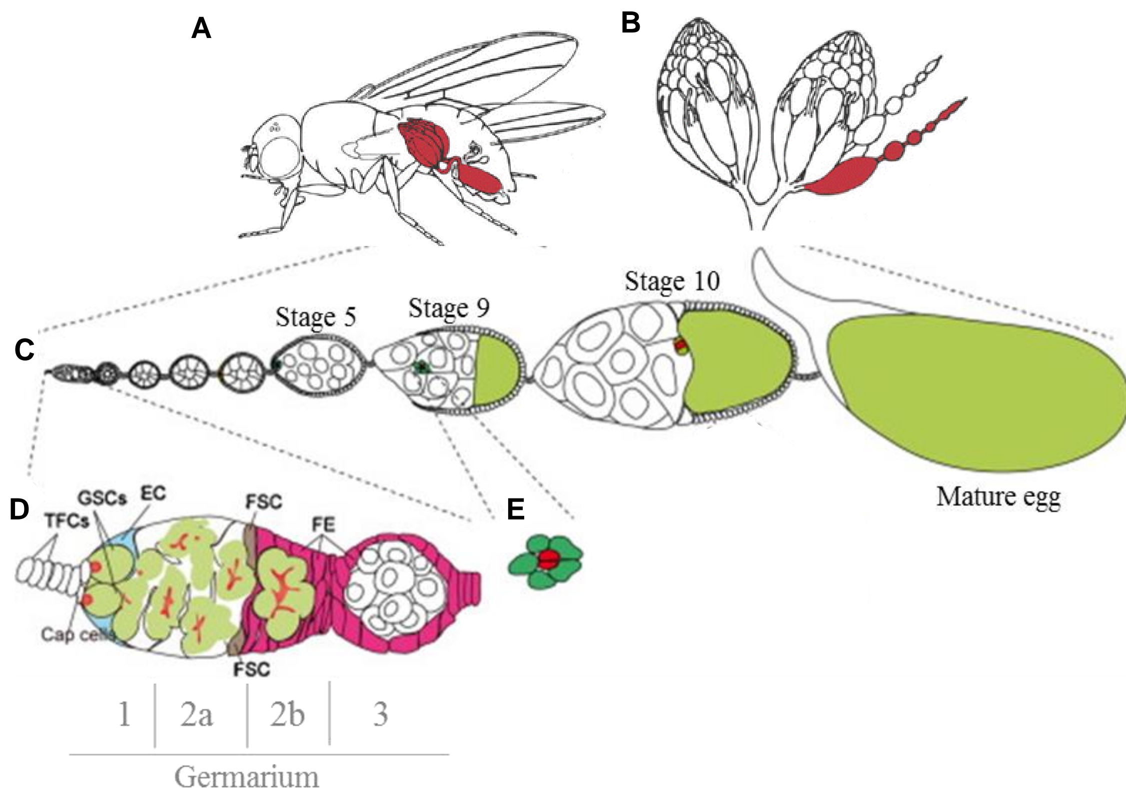

Figure 1.10| The female reproductive system of *Drosophila melanogaster*. Female reproductive system is composed by the ovaries, uterus, oviducts and secretory glands (spermathecae and parovaria), represented in red in **(A)**. Each female has two ovaries composed of multiple ovarioles, represented in red in **(B)**. **(C)** Schematic representation of oogenesis in *Drosophila melanogaster*. **(D)** Schematic representation of the germarium, the most anterior part of the ovariole. Oogenesis initiates in the germarium with the end production of a 16 germline cell-cyst in region 3; one cell differentiates and develops into the oocyte and the remaining cells differentiate into nurse cells; these cysts are surrounded by somatically derived follicle cells that later will form the monolayer follicular epithelium that surrounds the egg-chambers; **(E)** Border cell migration occurs between stage 9 and stage 10 required for sperm entry during fertilization. *(*Figure adapted *from* (Rosales-Nieves and González-Reyes, 2014) and (Jambor, Mejstrik and Tomancak, 2016).

known as ring canals (Rosales-Nieves and González-Reyes, 2014). As the germ cell cysts move from region 2a to 2b, the two FSCs that reside there produce prefollicle cells, which associate with the germ cells and begin to differentiate gradually (Huang *et al.*, 2014). In regions 2b and 3, prefollicle cells become differentiated to main follicle cells, forming the **f**ollicular **e**pithelium (FE), stalk cells (connecting adjacent follicle) and polar cells (providing positional cues) (Huang *et al.*, 2014). In region 3, the germ-line cyst, which is then surrounded by follicle cells, buds off from the germarium as an egg chamber (Drummond-Barbosa and Spradling, 2001). The germ-line cell located posteriorly becomes the oocyte which enter meiosis, whereas the remaining 15 cells differentiate to nurse cells (Bastock, 2008). Throughout oogenesis, nurse cells provide mRNAs, proteins and organelles to the developing oocyte via the ring canals (Peterson *et al.*, 2015). This is critical to development of the oocyte and the future embryo as the oocyte nucleus is also shown to undergo transcriptionally quiescence, between stages 5 and 8 (Navarro-Costa *et al.*, 2016). At stage 7, follicle cells stop mitotic divisions and undergo several rounds of endoreplication (Drummond-Barbosa and Spradling, 2001). Yolk uptake by the oocyte takes place at stage 8, establishing the onset of vitellogenesis (Drummond-Barbosa and Spradling, 2001). Border cell migration occurs between stage 9 and 10, with follicle cells migration to enclose the oocyte, secreting both the vitelline membrane and the chorion/egg shell to protect the maturing egg (Schonbaum, Perrino and Mahowald, 2000; Rosales-Nieves and González-Reyes, 2014). By the end of stage 10, the nurse cell cluster becomes similar to the oocyte's volume, thus starting contractions to release their contents into the oocyte and subsequently being eliminated by apoptosis (Bastock, 2008; Becalska and Gavis, 2009).

1.4 Unpublished Data

1.4.1 The germline and carbohydrate appetite

Drosophila oogenesis is therefore an ideal model to study if and how changes in availability of dietary sugars cause alterations on reproductive system' physiology. Furthermore, this is a great system to explore if these changes are surveyed by the brain and modulate feeding behaviour in order to achieve germline nutritional homeostasis to optimize egg production.

This hypothesis has been tested using available germline mutants or the Gal4/UAS system to knock down (KD) or overexpress (OE) certain genes, asking whether the reproductive system interacts with the brain to modulate nutrient appetite. These studies have shown that mutant flies carrying the ovo^{D1} allele, which have impaired oogenesis that halts at stage 4, have a lower

overall food intake (Carvalho-Santos and Ribeiro, 2018). Furthermore, mutant females for the ecdysone receptor, being ecdysone synthesized in the ovaries, have a significantly reduced feeding rate (Sieber and Spradling, 2015). Altogether, these studies suggest that indeed oogenesis might impact nutrient appetite.

Preliminary unpublished work for this thesis also uncovered a role for oogenesis in regulating fly's sugar appetite. In order to generate female flies which completely lack germline, the Gal4/UAS system was used to manipulate the gene *bag of marbles* (*bam*), a critical regulator of oogenesis. Its expression is both necessary and sufficient for GSC differentiation into cystoblasts (Dansereau and Lasko, 2008). During stem cell division, the daughter closest to the cap cell receives a signal which supresses *bam* expression, allowing this cell to remain within the niche in an undifferentiated state. The other daughter cell, farther from the niche, does not receive this signal and starts expressing *bam*, becoming a cystoblast.

The germline specific OE of *bam* thus promotes the differentiation of germline stem cells leading to a complete blockade in egg-formation (**Figure 1.11A**). These flies with impaired oogenesis were tested for alterations in feeding behaviour using the flyPAD. When given the choice to eat from a yeast or sucrose source, control sucrose deprived flies (*nos-Gal4>+* and *+>UAS-bam*) increased the intake of this nutrient when compared to fully fed flies, in order to achieve homeostasis (**Figure 1.11B**). However, germless sucrose deprived flies (*nos-Gal4>UAS-bam*) did not significantly increase the number of sips on sucrose when compared to the same flies in a fully fed state (**Figure 1.11B**). Thus, *bam* OE flies completely lack the ability to react to sugar deprivation. Because the germline is composed of many cell types, the first approach was to ask which of these could underly this behaviour phenotype, using KD for *bam* in the germline, leading to the generation of ovaries composed only of expanded germline stem cells (**Figure 1.11A**). *bam* KD in the germline (*nos-Gal4>bam shRNA*) also led to a complete lack of response to carbohydrate deprivation (**Figure 1.11C**), suggesting that the phenotype does not derive from these cells but from other downstream components in the oogenesis process, such as the nurse cells, follicle cells and the oocyte itself.

These results are the first to show that oogenesis modulates sugar feeding having a crucial role in the maintenance of carbohydrate homeostatic feeding. Carbohydrate metabolism is known to be required to support energetically demanding processes. The current working hypothesis is that oogenesis would be an example of such a process, being highly dependent on sugar availability and metabolism for oocyte development. If true, then oogenesis could be a major determinant of sugar appetite.

Oogenesis impairment could lead to a reduction in overall sugar requirements simply because of an accumulation of this nutrient that would not be used for egg production, thus, inhibiting feeding. However, measurements for circulating sugars revealed that this is not the

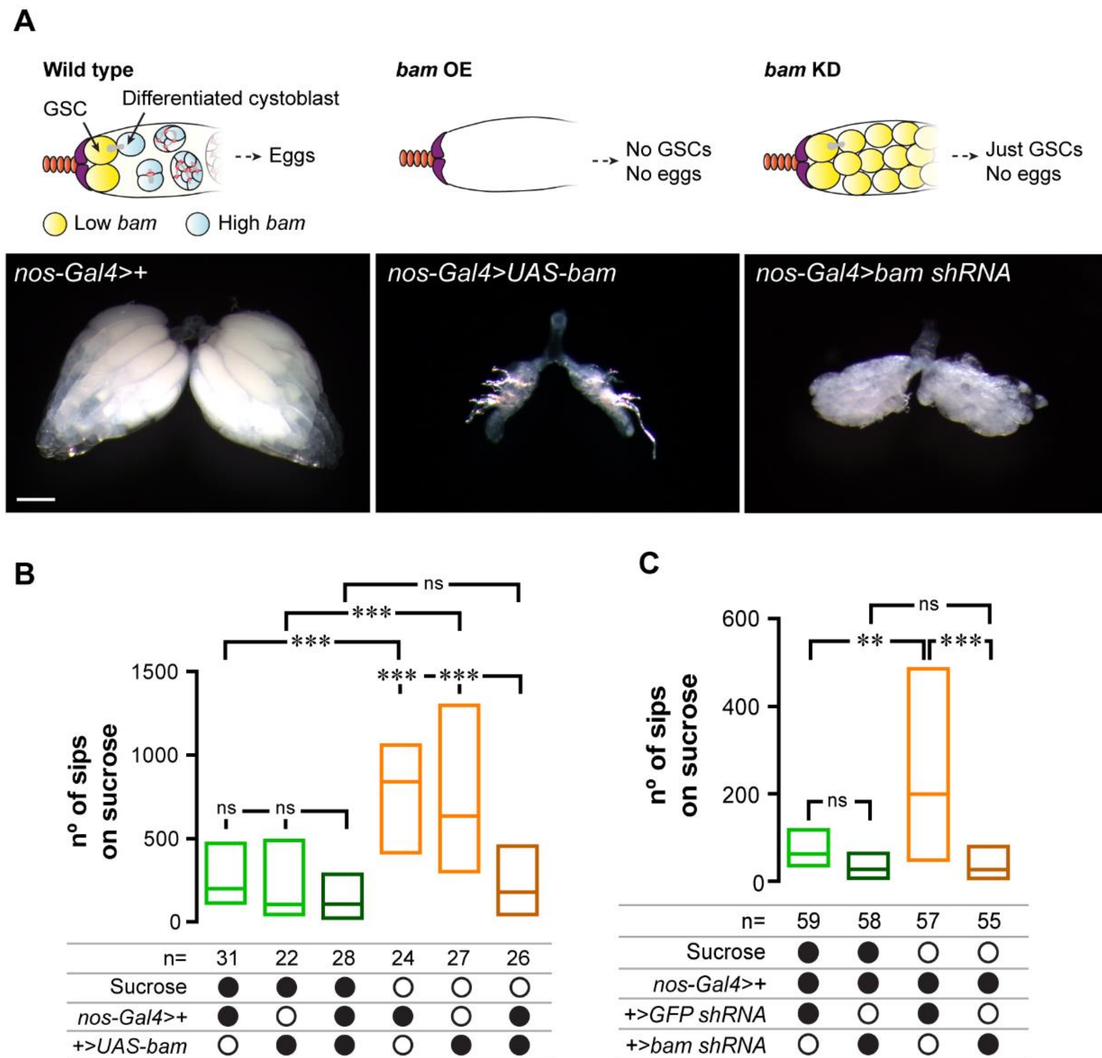

Figure 1.11| The germline modulates homeostatic carbohydrate feeding in *Drosophila melanogaster.* (A) Schematic representation of the effects of genetic manipulation of *bam* in the germline. Representative example of ovaries from females with OE of *bam* in the germline (*nos-Gal4>UAS-bam*), KD of *bam* in the germline (*nos-Gal4>bam shRNA*) and the genetic background control (*nos-Gal4>+*). *bam* OE leads to complete loss of germ cells and *bam* KD leads to germ stem cell overproliferation and the formation of ovaries bearing tumors. In both manipulations, no eggs are produced. Scale: 200 μM. **(B)** Number of sips on sucrose of flies prefed on holidic medium with and without sucrose for *bam* OE, using the flyPAD. OE of *bam* in the germline (*nos-Gal4>UAS-bam*) compared to the genetic background controls (*nos-Gal4>+* and *+>UAS-bam*). **(C)** Number of sips on sucrose of flies prefed on holidic medium with and without sucrose for *bam* KD, using the flyPAD. KD of *bam* in the germline (*nos-Gal4>bam shRNA*) compared to the genetic background control (*nos-Gal4>GFP shRNA*). **(B–C)** Boxes in green represent flies prefed the full diet, and in orange flies prefed a diet without sucrose, and the lighter colours represent the genetic background controls. Boxes represent upper and lower quartiles with the median. Filled black circles represent the presence of a given nutrient in the pre-treatment diet or the presence of a given transgene in the tested flies. *n represent the number of flies assayed in the flyPAD in each condition.* Significance was tested using the 1way ANOVA, Kruskal–Wallis test followed by Dunn's multiple comparison test. Not significant (ns) p > 0.05, * p < 0.05, ** p<0.01, *** p<0.001.

case (**Figure1.12**). These results raise several questions: *Which molecular and metabolic processes occurring in the germline impact feeding behavior? How do gonads communicate with the brain?* These questions were explored in this thesis.

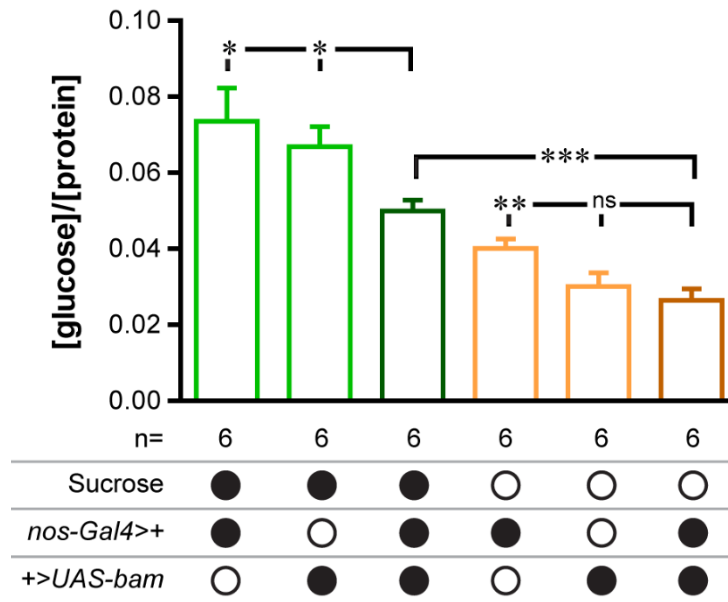

Figure 1.12| Glucose measurements of female flies with and without germline. Concentration of glucose normalized to the protein measured in the heads of flies prefed on holidic medium with and without sucrose. Columns in green represent flies prefed the full diet, and in orange flies prefed a diet without sucrose. The lighter colours represent the flies with germline, which are the genetic background controls (*nos*-Gal4>+ and +>UAS-*bam*), and the darker colours the flies without germline from *bam* OE in the germline (*nos*-Gal4>UAS-*bam*). *n represents the number of samples in each condition*. The columns represent the mean value and the error bars the standard error of the mean between samples. Significance was tested using the Mann–Whitney test. Not significant (ns) $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Project Aims and Hypothesis

The main aim of this thesis is to explore the mechanism by which organs regulate food choice, in order to maintain the intake of the nutrients required for their function. In order to do this, we took advantage of the reproductive system of the female fruit fly that unpublished work had already shown to modulate sugar appetite. Building on these data, we wanted to further explore the impact of dietary sugars on ovary physiology and egg production and to explore how the germline modulates feeding behavior. To do so, we addressed the following questions:

1) Are dietary carbohydrates required for egg production?

2) Is carbohydrate metabolism in the germline a key process in maintaining egg production?

3) Is carbohydrate metabolism in germline cells modulating sugar appetite?

CHAPTER 2

MATERIALS AND METHODS

2.1 Drosophila stocks and genetics

The experiments regarding dietary effects on egg-laying were performed with mated *w^{1118}* female flies. The genetic lines used in this study were obtained from the Bloomington Drosophila Stock Center and for the KD experiments, were obtained from the Transgenic RNAi Project (TRiP). The full genotypes of the lines used in this thesis are listed in ***Annex I, Table 1***.

2.2 Drosophila rearing and dietary conditions

Flies were reared at 25°C, 70% relative humidity on a 12 hour light-dark cycle (Aralab, FitoClima 60000EH). Flies were crossed and reared on yeast-based medium (YBM) with the following composition per liter of water: 8 g agar [NZYTech, PT], 80 g barley malt syrup [Próvida, PT], 22 g sugar beet syrup [Grafschafter, DE], 80 g corn flour [Próvida, PT], 10 g soya flour [A. *Centazzi*, PT], 18 g instant yeast [Saf-instant, Lesaffre], 8 mL propionic acid [Acros] and 12 mL nipagin [Tegospet, Dutscher, UK] 15% in 96% ethanol [AGA, PT], supplemented with instant yeast granules on the surface [Saf-instant, Lesaffre]. To control the density of offspring among experiments, fly cultures were set with 6 females and 3 males per vial and left to lay eggs for ¾ d, after which the parents were removed. Flies were reared in YBM until the adult stage, 14 d after the cross, in which 16 females of the desired genotype were sorted and put together with 6 Canton-S males. After the sorting, flies were kept on YBM for 3d, after which they were put on holidic media (HM) for 2-3 days, prepared as described previously (Piper *et al.*, 2014, 2017). The components of the HM from Sigma-Aldrich and Difco had high purity values and the remaining reagents were aliquoted and stored at -4°C.

2.3 Egg-laying assays

Groups of 16 females of a specific genotype and 6 Canton-S male flies were briefly anesthetized using light CO_2 exposure and transferred to cages with apple juice agar plates (per liter, 250 mL apple juice, 19,5 g agar, 20 g sugar, and 10 mL nipagin 10%), where they were allowed to lay eggs for 24 hr, at 25ºC. Flies were removed from the cages and the females were counted. The cages were put back at 25ºC for 48 hrs for the larvae to eclose. Afterwards, the cages were stored at 4ºC for later egg counting. Both eclosed and not eclosed larvae were counted. Egg laying rate per animal was calculated by dividing the number of eggs by the number of living females at the end of the assay. Fraction of viable eggs was obtained by dividing the eclosed larvae by the total number of eggs counted. The percentage values for changes in egg-laying were calculated using the *mean* values from a specific dietary/genotype condition and comparing to either a fully fed condition or the genetic background control.

2.4 Behavior assays using the flyPAD setup

The flyPAD (fly Proboscis and Activity Detector) assays were based on a protocol previously described (Itskov *et al.*, 2014). This is an automated behavioural monitoring system that uses capacitive-based measurements to detect the physical interaction of single flies with food. Each flyPAD setup has 32 arenas, each one with two wells, one containing 10% yeast whereas the other 20 mM sucrose, with their respective sensors **(Figure 1.9)**. All the food solutions used in the experiments were prepared by dissolving 1% agarose at 70°C in a water bath, adding the relative nutrient, dividing the solution into 2 mL aliquots and freezing them at -20°C. On the day of the experiment, aliquots were melted at 70°C in a heat block immediately before use. After loading each well with the respective medium, each fly was transferred to an arena by mouth aspiration for a duration of 1 hr at 25ºC, 70% relative humidity. The total number of sips on each well per fly was extracted using described flyPAD algorithms (Itskov *et al.*, 2014). Noneating flies (defined as having fewer than two activity bouts during the assay) were excluded from the analysis.

2.5 *In situ* hybridization in ovaries of the fruit fly

2.5.1 Preparation of RNA probes for in situ hybridization

- **Fly RNA Extraction**

15 anaesthetized wild type background flies were collected in an eppendorf and frozen on dry ice. Flies were ground in 100 µL of TRI REAGENT (SIGMA T9424) with disposable tissue grinder on ice followed by addition of 900 µL of TRI REAGENT and vortex for 15 sec. 200 µL of chloroform

were added to each sample and the tube was shaken vigorously for 15 sec. The sample was put on ice for 15 min, after which it was spun at 14000 rpm for 15 min at 4ºC (Eppendorf Centrifuge 5415 R). The aqueous phase was transferred to a fresh tube to precipitate the RNA by mixing with 500 µL of isopropanol. The samples were stored on ice for 10 min, after which they were spun at 13000 rpm for 10 min at 4ºC. The supernatant was removed, and the RNA pellet washed with 500 µL of 75% ethanol. This was followed by removal of ethanol to air-dry the pellet after which it was resuspended in 12 µL of RNase free water. The cDNA was synthesized according to the instructions in the Transcriptor high fidelity cDNA synthesis KIT (Roche cat. No. 05081955001). All the solutions used in this protocol were stored at 4ºC.

- **Amplification of the regions of interest by PCR and subsequent *in vitro* transcription**

The starting cDNA for in vitro transcription was amplified using primers designed in the Primer3 online software (http://bioinfo.ut.ee/primer3-0.4.0/), using the exonic sequences for *Hex-A* and *Pgd* retrieved from the flies genome in the Ensembl genome browser 93 (https://www.ensembl.org/index.html), for a final size around 1 kb. The Forward Primers include a SP6 RNA promotor sequence (ATTTAGGTGACACTATAG) and the reverse a T3 RNA promoter sequence (AATTAACCCTCACTAAAG):

Hex-A SP6 F – gactacATTTAGGTGACACTATAGAACTTCTAACGGACGAACAG,
Hex-A T3 R – gcaacgAATTAACCCTCACTAAAGGGGCATTGGTCAGACCGAGCT,
Pgd SP6 F – gactacATTTAGGTGACACTATAGAAGGCAACTCGGAGTATCAGGA,
Pgd T3 R – gcaacgAATTAACCCTCACTAAAGGGCGTAGAAGCTTAGGGCGGTA.

The PCR reaction was set with KOD hot start master mix (71842 Novagen), according to the kit instructions for a target size of 1000-3000 bp (see ***Annex II, Table 1***), and subsequently purified with Quiagen kit (Cat. No. 28706). Afterwards, the *in vivo* transcription reaction was set in a PCR tube for 4 h at 37ºC according to ***Annex II, Table 2***. After the synthesis, 1 µl of DNAseI (M0303S NEB) was added to the reaction for 15 min at 37ºC, to remove the template DNA. The probe was then hydrolyzed by adding 20 µl of carbonate buffer (2x) (see composition in ***Annex II***) for 20 min at 65ºC. After this period, 1.67 µl of Lithium Chloride (6M) and 120 µl of ethanol (100%) were added to the mixture and the RNA was left to precipitate overnight at -20ºC. The recuperation of the probe was carried out by centrifuging the samples for 30 min at 4ºC and maximum speed, removing the supernatant and then washing in 200 µl of 70% ethanol for a final centrifugation of 15 min at 4ºC. The pellet was resuspended (after drying) in 50 µl of hybridization buffer (see composition in ***Annex II***).

2.5.2 Ovary dissection and fixation

The ovaries were dissected on ice in 1x ice cold phosphate-buffered saline (PBS), under a dissection scope. The ovaries were fixed in 4% paraformaldehyde (PFA) in PBS + 0,1% Triton (X-100, Supelco) for 20 min at room temperature (RT). The ovaries were washed during 5 min 3x with 0,5 mL PBT (PBS + 0.3% Tween 20 (P9416 Sigma). All washes and incubations are performed with rotation at RT.

2.5.3 Ovary *in situ* hybridization

The PBT solution was removed and the ovaries were incubated for 1 hour at RT with 200 µl of pre-hybridization buffer (see composition in ***Annex II***). Then the solution was replaced by 200 µl of hybridization buffer and incubated for 1 hour at 55ºC. The probe was added at 1:100 dilution in hybridization buffer and incubate overnight (O/N) at 55ºC. Ovaries were next washed in pre-hybridization buffer for 30 min at 55ºC, followed by 5X in PBT (PBS + 1% Tween 20 for 20 min each at RT. After washes, ovaries were incubated for 30min in Roche blocking buffer (1:10 in PBT) at RT and O/N in Roche blocking buffer (1:10 in PBT) + mouse anti-DIG alkaline phosphatase (AP) antibody (11093274910 Roche) (1:2000) at 4ºC. The next day, they were washed 5X in PBT (PBS + 1% Tween 20) for 10 min each at RT, followed by rinsing in 1X AP buffer (see composition in ***Annex II***). Then, the NBT/BCIP stock solution (11681451001 Roche) was added to AP buffer in 1:50 proportion and mixed to stain in a multi-well plate which was regularly checked under the stereo microscope (Zeiss Stereo Discovery.V8), in parallel with control ovaries. The reaction was stopped after 1h by adding PBT, followed by washing 3X for 5 min in PBT, and mounted afterwards in Vectashield (H1000 Vector Labs). Images were obtained using Zeiss Axio Scan.Z1.
Assays in 2.5 were performed in collaboration with Ana Paula Elias, PhD.

2.6 Statistical analysis

Unpaired *t* test and 1way ANOVA, Kruskal-Wallis test were performed using GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA).

CHAPTER

3 RESULTS

3.1 Carbohydrate availability is key for fly fitness

Germline ablation in flies leads to a strong impairment in homeostatic carbohydrate feeding (**Figure 1.11**). This shows oogenesis as an important process controlling carbohydrate appetite. On the other hand, although nutrient availability has been shown to strongly impact reproduction (Lee *et al.*, 2008), we still know very little of how carbohydrates affect this process.

We hypothesized that the ovaries highly depend on dietary supply of carbohydrates to produce eggs. In order to test this hypothesis, we acutely manipulated dietary sugar availability to understand if and how it affected egg production. To do so, we deprived female flies from sucrose for 3 days using the synthetic diet previously mentioned (Piper *et al.*, 2014, 2017). After those 3 days, we scored the number of eggs produced during one day by females pre-treated with a diet containing all nutrients *versus* a diet without sucrose. We also deprived flies of amino acids as a positive control since this manipulation has been described to have a strong impact on egg production (Drummond-Barbosa and Spradling, 2001; Piper *et al.*, 2014). In accordance with what has been published, the removal of amino acids from the diet led to a strong decrease (81%) in the number of eggs laid per female when compared to females fed on a diet with all nutrients (**Figure 3.1A**). This was consistent with the morphology of the ovaries in these flies (**Figure 3.1C**) which are rudimentary, showing a drastic to complete reduction in the vitellogenic stages of oogenesis and with very few or no last stage oocytes. Similarly, we observed that removal of sucrose from the diet led to a 37% decrease in the number of eggs laid per female (**Figure 3.1A**) when compared to flies kept on a full diet. These results are also consistent with the morphology of the ovaries of sugar deprived flies (**Figure 3.1C**) which are smaller and have fewer last stage oocytes than the ovaries of flies in a fully fed state. Despite the fact that amino acid or sucrose deprivation leads to strong decrease in egg production, the quality of these eggs is not affected as the fraction of viable eggs is not changed if compared to the one from flies in a fully fed state (**Figures 3.1 B**). These results clearly show that dietary sucrose impacts ovary morphology and is hence essential for optimal egg production.

These findings are in accordance with studies showing that ovaries use a great portion of the carbohydrates provided by the diet in the adult stage for egg production (Min *et al.*, 2006) and with metabolomics analysis of this organ revealing it to be highly metabolically active (Chintapalli *et al.*, 2013). These results led us to next explore whether sugar metabolism in the germline could underlie these effects in egg production.

Main conclusions: These results confirm our hypothesis that sugar is necessary for the process of oogenesis, leading to a reduction of the number of eggs laid per female fly. Thus, dietary sugar is key to assure a regular reproductive output.

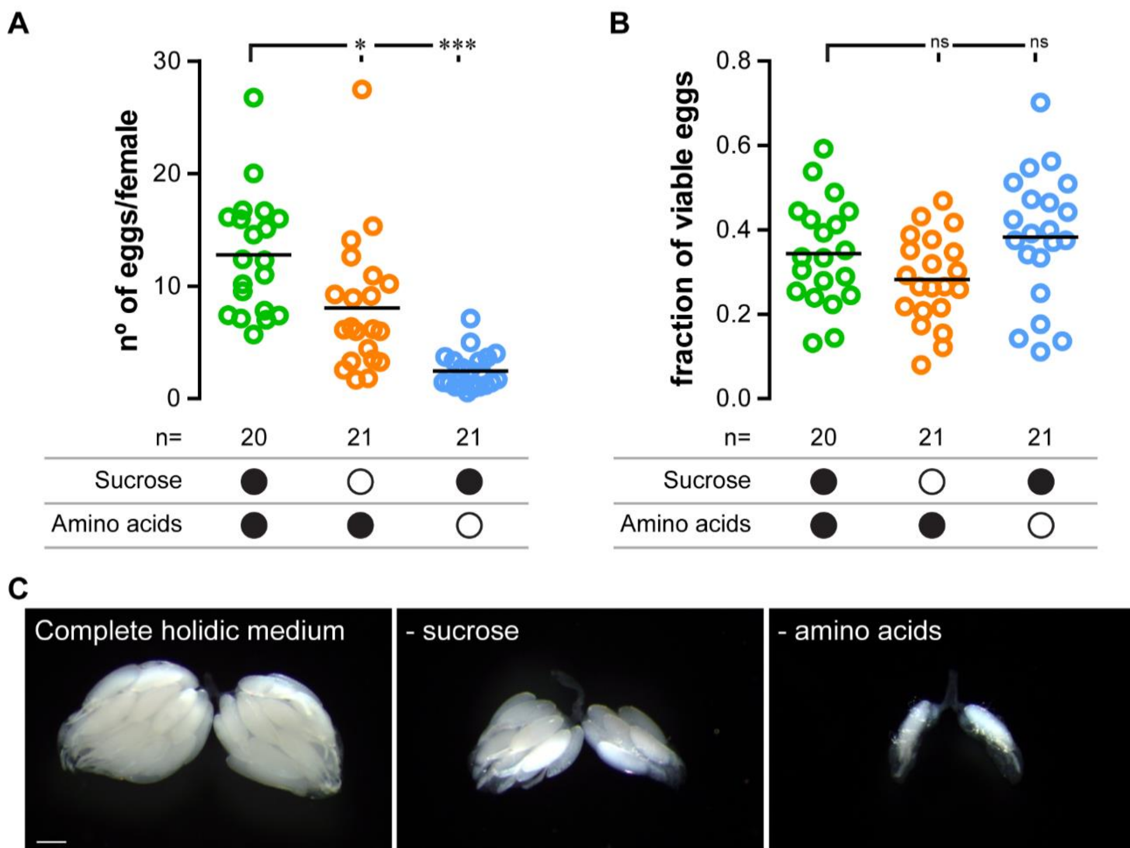

Figure 3.1| Dietary sugar and amino acids modulate oogenesis. (A) Number of eggs laid per female in 24 h of animals kept on full holidic diet (green), holidic diet without sucrose (orange) or without amino acids (blue). *n* represents the total coloured open circles per condition, and each of the circles represents eggs laid in single assays, with the line representing the mean. (B) Fraction of viable eggs (eclosed larvae) per female in 24 h of animals kept on full holidic diet (green), holidic diet without sucrose (orange) or without amino acids (blue). *n* represents the total coloured circles per condition, and each of the circles the fraction of viable eggs laid in single assays, with the line representing the mean. (A-B) Filled black circles represent the presence of a given nutrient in the pre-treatment diet. Statistics were performed using a 1way ANOVA, Kruskal-Wallis test, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ (C) Representative example of ovaries from females kept on full holidic diet, holidic diet without sucrose or without amino acids. Scale: 200 µM.

3.2 Carbohydrate metabolism in the germline is essential for egg production

3.2.1 PPP but not glycolysis activity in the germline sustains egg production

Carbohydrate metabolism regulation is highly dependent upon fluctuations in nutrient intake. In the first section, we show that dietary sugar availability is key for egg production, and further hypothesized that sugar metabolism in the germline could play an important role in this process.

After being transported inside the cell, glucose is immediately phosphorylated by hexokinases, a process that blocks its diffusion out of the cell. After being phosphorylated, it can be used as substrate for multiple metabolic pathways. In *Drosophila*, Hexokinase-A, Hex-A, is the main enzyme responsible for glucose phosphorylation in the adult stage (**Figure 3.2A**).

To assess if sugar metabolism impacts egg formation, we genetically manipulated this enzyme specifically in the germline, using the dual Gal4/UAS system. We combined a stock of flies which expressed Gal4 specifically in the germline, *MTD-Gal4*, with different fly stocks carrying shRNAs under the control of UAS sequences, which effectively silence target gene expression through RNAi interference in the target tissue (VALIUM 20 or 22 lines). It is of high importance to use the correct genetic background controls, as different fly stocks may have different baseline egg production. The genetic background controls used in these assays consists of expressing a shRNA that targets green fluorescent protein (*GFP*) gene, a gene that is not present in *Drosophila melanogaster's* genome and so should not impact flies' organ physiology and function. These flies will be labeled as *MTD-Gal4>GFP shRNA*.

When we knocked down *Hex-A* in the germline (*MTD-Gal4>Hex-A shRNA*), the number of eggs per female was strongly reduced by more than 73%, when compared to the genetic background control *(MTD-Gal4>GFP shRNA (I))*, revealing an essential role for *Hex-A* in egg production (**Figure 3.2B**). Surprisingly, despite the drastic reduction in egg laying rates the ovary morphology in *Hex-A* KD female flies was very similar to the one of control flies. This suggests that knocking down *Hex-A* in the germline strongly reduces the efficiency of the oogenesis process. This happens by either decreasing the rates of stem cell or cystoblast division, or slowing down the differentiation process, and/or leading to an increase in apoptosis of egg chambers in the different oogenesis checkpoints. We conclude from these experiments that blocking sugar usage as substrate for multiple metabolic pathways, by impeding its phosphorylation, leads to functional defects of the reproductive system ultimately leading to a decrease in female fertility.

Since glucose 6-phosphate can serve as substrate for multiple metabolic pathways we next wanted to explore which of these pathways was required for oogenesis progression (**Figure 3.3**). Glycolysis is responsible for generating energy in the form of ATP and for the generation of several metabolites that serve as substrates for other metabolic pathways, being usually active in cells that are undergoing proliferation (Heiden, Cantley and Thompson, 2009; Burgess, Agathocleous and Morrison, 2014). Since oogenesis fulfills these criteria we decided to test if glycolysis plays a role in egg production. In order to test this, we knocked down three genes for glycolysis enzymes downstream of *Hex-A*, phosphofructokinase (*Pfk)* (*MTD-Gal4>Pfk shRNA*), pyruvate kinase (*PyK)* (*MTD-Gal4>PyK shRNA*) and phosphoglucose isomerase (*Pgi*) (*MTD-Gal4>Pgi shRNA*) specifically

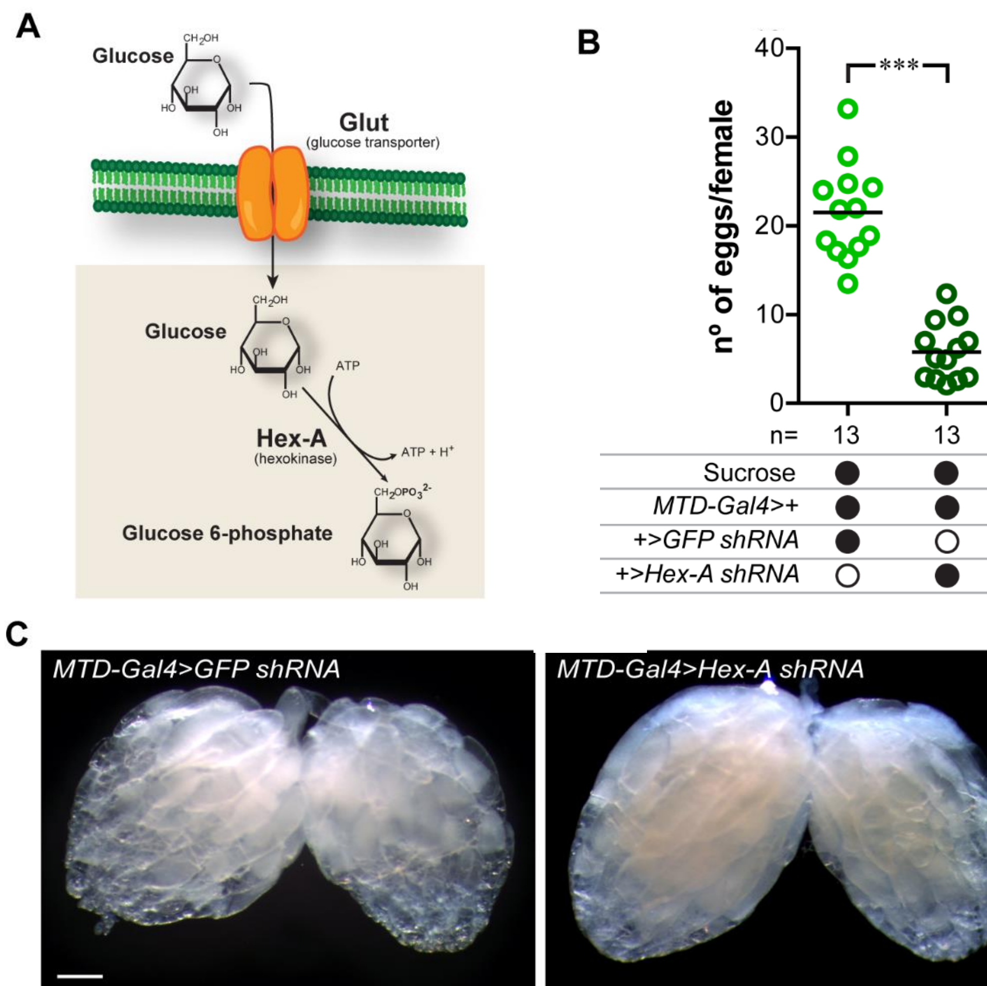

Figure 3.2| *Hex-A* is necessary to maintain egg-laying rates in *Drosophila melanogaster*. (A) Glucose is transported into the cells via GLUTs, where it is immediately phosphorylated through the action of *Hex-A*. **(B)** Number of eggs laid per female in 24 h of animals kept on full holidic diet, for *Hex-A* KD in the germline (*MTD-Gal4>Hex-A shRNA*) compared to its genetic background control (*MTD-Gal4>GFP shRNA (I)*). *n* represents the total coloured open circles per condition, and each of the circles represents eggs laid in single assays, with the line representing the mean. Filled black circles represent the presence of a given transgene in the tested flies. Statistics were performed using an unpaired *t* test, *P < 0.05, **P < 0.01, ***P < 0.001 **(C)** Representative examples of ovaries from females with *Hex-A* KD in the germline (*MTD-Gal4>Hex-A shRNA*) and respective genetic background control flies (*MTD-Gal4>GFP shRNA (I)*). Scale: 200 µM.

in the germline (**Figure 3.3**). To test for a role of glycolysis in oogenesis we tested the rates of egg laying of the flies knocked down for the different enzymes in the germline. Interestingly, the KD of any of the glycolytic enzymes tested, *Pfk*, *PyK* and *Pgi* (**Figure 3.4A**), does not lead to a noticeable reduction in egg production when compared to their respective genetic background controls (*MTD-Gal4> GFP shRNA(I) and (II))*. These results suggest that glucose metabolism through glycolysis does not play a major role in the process of oogenesis. Moreover, since the KD of *Hex-A* in the germline shows that this enzyme is clearly necessary for egg production, we speculated that another branch of carbohydrate metabolism might be involved in this process.

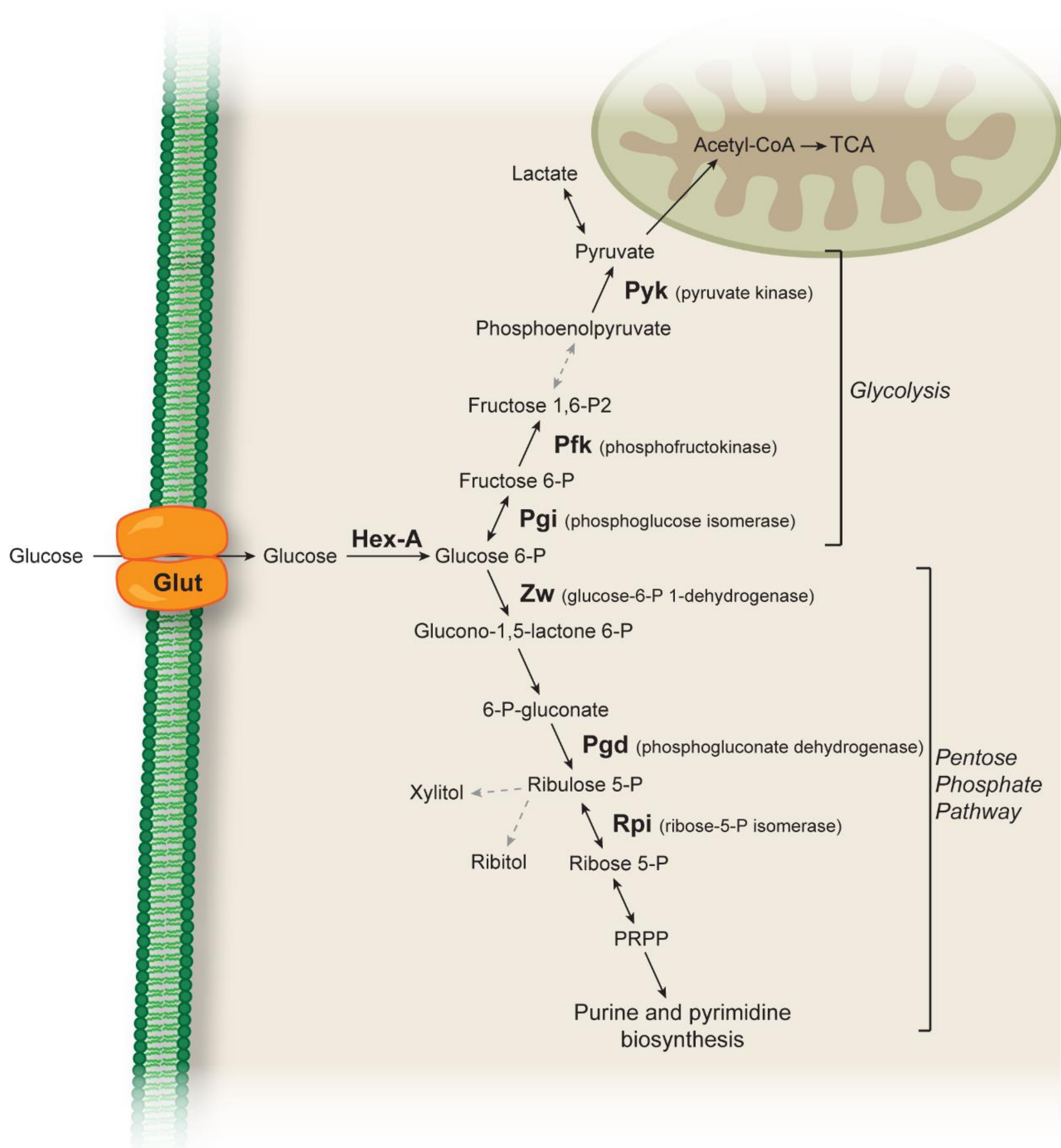

Figure 3.3| Simplified schematic of the carbohydrate metabolism pathways Glycolysis and PPP. Glucose-6-phosphate is the entry point for both Glycolysis and the Pentose Phosphate Pathway.

Another crucial pathway where glucose-6-phosphate enters as substrate is the Pentose Phosphate Pathway (PPP), which is required for the synthesis of ribonucleotides and is a major source of NADPH, supporting growth and proliferation (**Figure 3.3**). Thus, the same approach was used as for testing a role for glycolysis in oogenesis. Two enzymes of this pathway, the orthologues of human glucose-6-P-dehydrogenase (*Zw*) and 6-P-gluconate dehydrogenase (*Pgd*), were knocked down specifically in female germline. These flies were then assayed for their egg laying rates. When compared to its background control, *MTD-Gal4>GFP shRNA (III), Zw* KD *(MTD-*

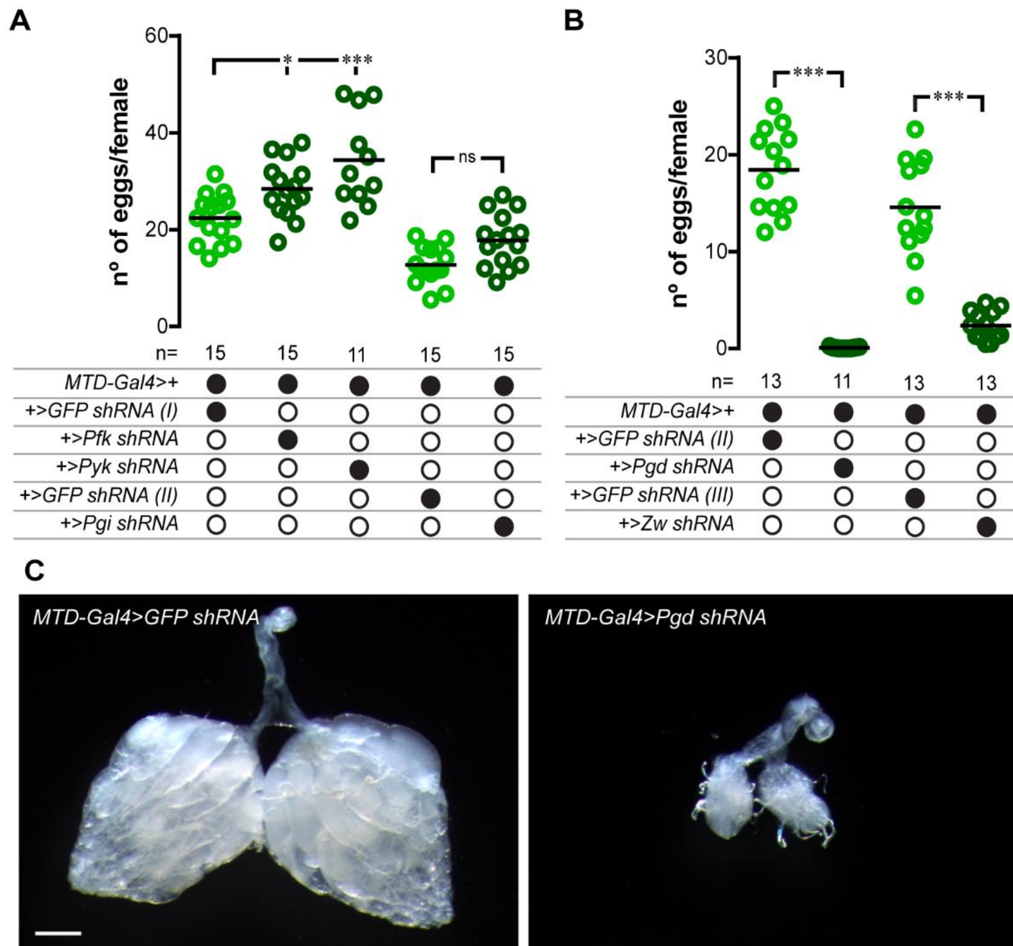

Figure 3.4| The activity of the PPP but not glycolysis in the germline modulates egg-laying. (A) Number of eggs laid per female in 24 h of animals kept on full holidic diet, for the KD glycolytic enzymes in the germline. *Pfk* KD (*MTD-Gal4>Pfk shRNA*) and *Pyk* KD (*MTD-Gal4>Pyk shRNA*) in the germline are compared to the same genetic background control (*MTD-Gal4>GFP shRNA (I))*. *Pgi* KD (*MTD-Gal4>Pgi shRNA*) is also compared to its genetic background control is (*MTD-Gal4>GFP shRNA (II))*. **(B)** Number of eggs laid per female in 24 h of animals kept on full holidic diet, for the KD PPP enzymes in the germline. *Pgd* KD (*MTD-Gal4>Pgd shRNA*) is compared to its genetic background control (*MTD-Gal4>GFP shRNA (II))* and *Zw* KD (*MTD-Gal4>Zw shRNA)* is also compared to its genetic background control (*MTD-Gal4>GFP shRNA (III)).* **(A-B)** *n* represents the total coloured open circles per condition, and each of the circles represents eggs laid in single assays, with the line representing the mean. Filled black circles represent the presence of a given transgene in the tested flies. Statistics were performed using a 1way ANOVA, Kruskal-Wallis test, *P < 0.05, **P < 0.01, ***P < 0.001 **(C)** Representative examples of ovaries from females with KD of *Pgd* in the germline (*MTD-Gal4>Pgd shRNA*) and respective genetic background control flies (*MTD-Gal4>GFP shRNA (II)).* Scale: 200 µM.

Gal4>Zw shRNA) leads to a reduction of approximately 84%, in the number of eggs laid per female. This is very similar to what we observed for *Hex-A* KD (**Figure 3.4B**). Similarly, *Pgd* KD in the female germline (*MTD-Gal4>Pgd shRNA*) strongly impairs oogenesis, leading to an almost complete sterility of females when compared to their genetic background control, *MTD-Gal4>GFP shRNA (II)* (**Figure 3.4B**). Furthermore, this manipulation leads to very small ovaries, with barely no observable late egg-chambers and vitellogenic states (**Figure 3.4C**). Altogether, these results show that the PPP is necessary for oogenesis progression, possibly using glucose as a metabolic precursor for cellular building blocks required for this process. PPP is also key in preventing oxidative stress, which is often observed in such metabolically active cells. Several processes during oogenesis are expected to require high levels of metabolite synthesis and conversion such as: in the germarium, during the mitotic divisions forming the cystoblast; in nurse cells, which undergo several endoreplication cycles to support oocyte development and growth; in stage 7, during follicle cell endoreplication to enable vitellogenesis. Therefore, this metabolic pathway could be required for one or several of these oogenesis' steps. Identifying the detailed effects of loss of PPP in oogenesis will be an important task for the future.

We next wanted to explore which cells or stages during the oogenesis process would be more dependent on this metabolic pathway. In order to tackle this question, we used *in situ* hybridization, to visualize the mRNA expression of PPP enzymes in different stages of oogenesis**.** This technique uses the binding of labelled RNA probes with the complementary target endogenous mRNA. This procedure has been used to analyze mRNA expression at cellular and subcellular levels in several different sample preparations – tissue sections, cultured cells or intact whole mounted tissues (Bell, Sánchez-Alvarez and Eberwine, 2008; Gall, 2016). Before hybridization, the probes were prepared with nucleotides that had been directly modified to contain digoxygenin (Dig), a steroid hapten, for a subsequent colored colorimetric detection (**Figure 3.5A**).

Whole ovaries were used for visualizing the mRNA of either *Hex-A* or *Pgd*. Sense probes are usually used to control for unspecific binding as these should not bind the target mRNAs. In contrast, the anti-sense probes bind mRNA of the target enzymes allowing for the detection of gene expression in a cell specific manner. Using this technique, we analyzed the expression of the two enzymes in the different regions of the ovariole. The *in situ* hybridization for both enzymes reveals a very similar expression pattern (**Figures 3.5B and 3.5C**). There is very low to no expression of these enzymes within the most anterior part of the germarium (marked with an asterisk) consisting in **regions 1 and 2a** (orange arrow). This region is where the germline stem cells, cap cells and escort cells are located. We started to detect expression of these enzymes in

the most posterior tip of the germarium, in **regions 2b** and **3,** where 16-cell cysts are already formed and are encapsulated by follicle cells to form egg chambers, although little to no signal is observed in the latter (**Figures 3.5B and 3.5C**). From this stage until the end of oogenesis, both the oocyte and the polyploid nurse cells show a high expression of these genes. The follicle cells

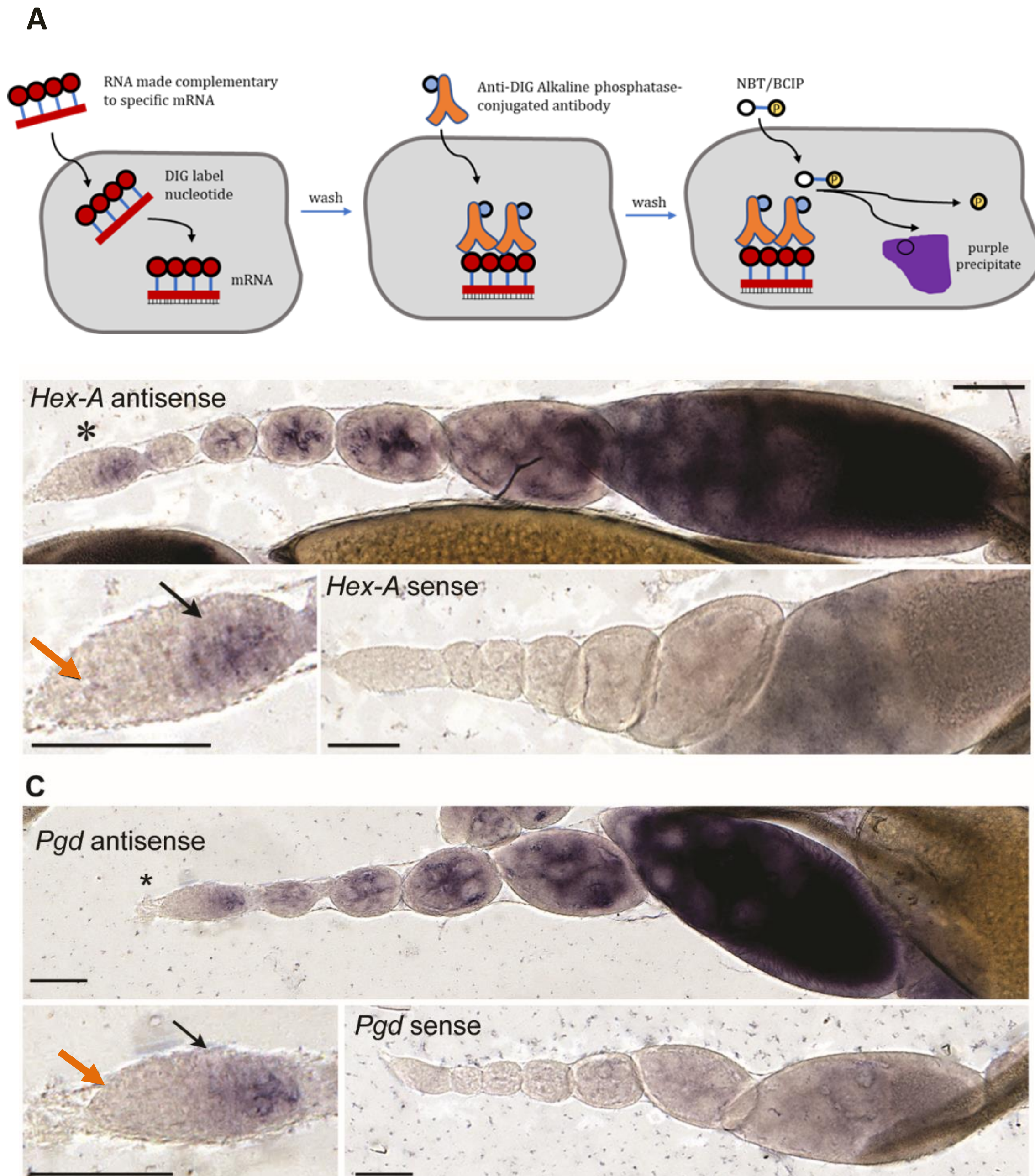

Figure 3.5| *In situ* hybridization reveals presence of mRNA for *Hex-A* and *Pgd* throughout oogenesis. (A) Schematic representation of the *in situ* hybridization technique using Dig-labeled RNA probes. **(B)** Representative example of ovarioles in the top panel after *in situ* hybridization technique for Hex-A, from control females with *GFP* KD in the germline (*MTD-Gal4>GFP shRNA (I)*) and **(C)** for Pgd, from control females with *GFP* KD in the germline (*MTD-Gal4>GFP shRNA (II)*), where the asterisk indicates the germarium. **(B-C)** The lower panel on the left depicts the germarium for both antisense probes, and within this compartment, the orange arrow depicts the anterior region and the black arrow the posterior region. The lower panel on the right corresponds to the control for the hybridization for each enzyme, using the respective sense probes. Scale: 50 µM.

that surround the egg chambers throughout oogenesis keep low expression for these enzymes. No signal was observed when controlling for non-specific hybridization with a sense mRNA (**Figure 3.5B** ***Hex-A sense*** and **Figure 3.5C** ***Pgd sense***).

These results reveal a non-uniform pattern of the PPP enzymes' expression within the different cell types and stages of oogenesis. These results suggest that the phenotypes in oogenesis observed when knocking down *Hex-A* and *Pgd* are likely not deriving from an effect on the stem cells and cystoblasts before they reach the 16-cell stage but affect oogenesis at later stages of proliferation.

Main conclusions: Carbohydrate metabolism, specifically the Pentose Phosphate Pathway is necessary for the process of oogenesis and to maintain the proper ovary morphology in *Drosophila melanogaster. In situ* hybridization reveals high PPP activity throughout oogenesis but not in the stem cell niche.

3.2.2 PPP activity in the germline modulates sugar appetite

Up to now we have shown that germless females have a drastic impairment in homeostatic carbohydrate feeding, that dietary sugars are required for egg production and that the activity of the PPP in the germline is essential for oogenesis. These data led us to the simple hypothesis that this metabolic process in the germline could be underlying a signaling mechanism that would act on the CNS, informing it on the current nutritional needs of the fly ovaries and modulating sugar appetite. Germless flies would lack these processes since the germline is completely absent, hence their inability to modulate sugar appetite. To test if this is the case, we knocked down the enzymes of the PPP in the germline and asked if these flies have an impairment in sugar appetite, as observed in flies without germline.

We started by testing the feeding behavior of flies where *Hex-A* was specifically knocked down in the germline. To do so, we used the flyPAD setups and analyzed the feeding program of single flies while given the choice to eat between a sucrose or a protein food source. We observed that sucrose deprived flies with *Hex-A* KD in the germline show a drastic decrease in sugar appetite when compared to control flies (*MTD-Gal4>GFP shRNA (I)*) (**Figure 3.6A**). In this nutritional state, control animals (*MTD-Gal4>GFP shRNA (I))* show a dramatic increase in sucrose intake when compared to animals that are fully fed. In contrast, the flies with *Hex-A* KD in the germline (*MTD-Gal4>Hex-A shRNA*) lack any feeding behavior response to the absence of sugar in the diet, maintaining a very low intake of sucrose although being sugar deprived. In the fully

fed state, female flies have a low baseline intake of sugar, which makes it very difficult to detect a further decrease. Although there is not a significant phenotype, even in this dietary condition there is a trend for a decrease in the sugar intake in flies with *Hex-A* KD in the germline (*MTD-Gal4>Hex-A shRNA*), when compared to the genetic background control flies (*MTD-Gal4>GFP shRNA (I)*) (Figure 3.6A). These results suggest that glucose availability in the ovaries and posterior metabolism is not just important for overall oogenesis but also to instruct the CNS regarding the

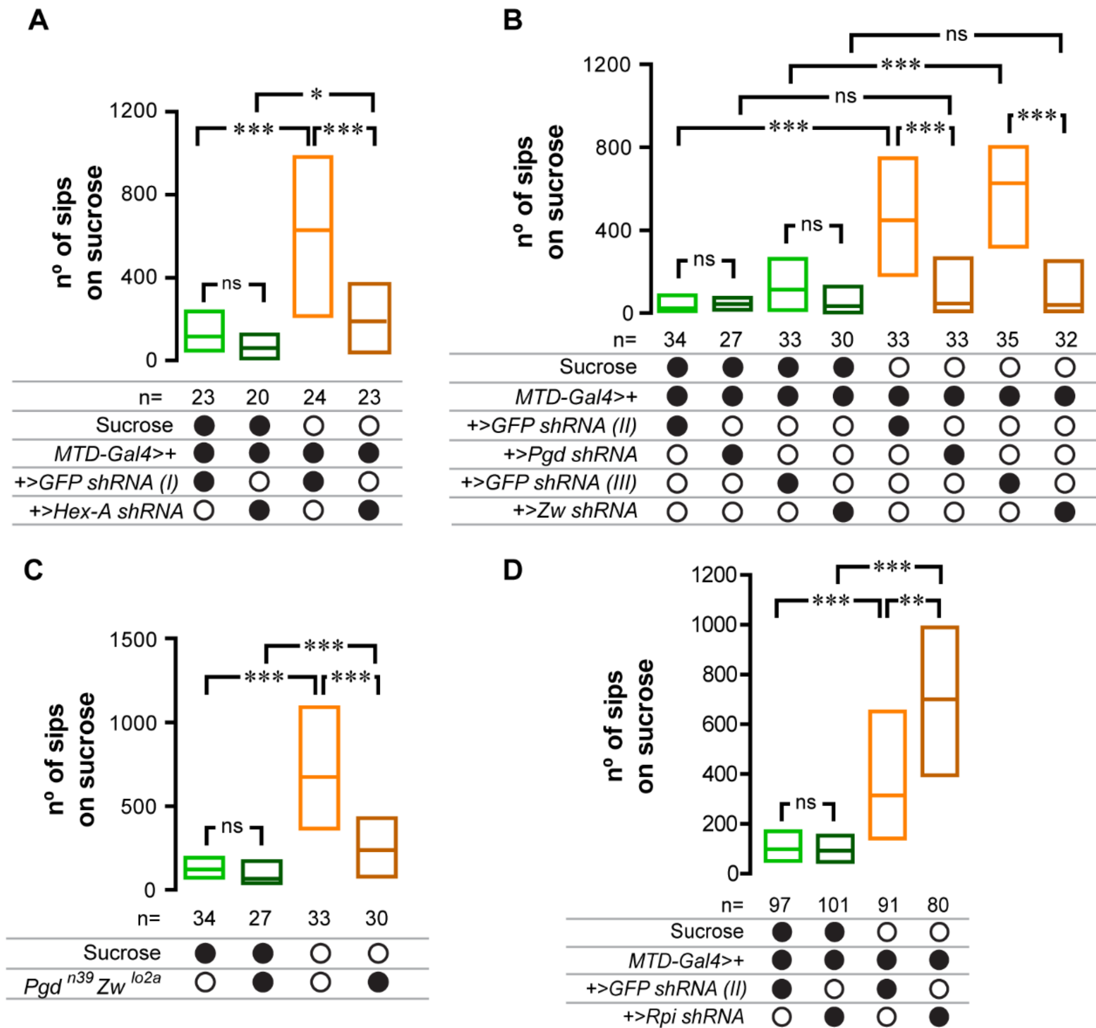

Figure 3.6| PPP activity in the germline modulates sugar appetite. Number of sips on sucrose of flies prefed on holidic medium with and without sucrose for the KD of different enzymes in the germline, using the flyPAD. **(A)** *Hex-A* KD in the germline (*MTD-Gal4>Hex-A shRNA*), is compared to its genetic background control (*MTD-Gal4>GFP shRNA (I)*). **(B)** *Pgd* and *Zw* KD in the germline (*MTD-Gal4>Pgd shRNA* and *MTD-Gal4>Zw shRNA*, respectively) each are compared to their respective genetic background controls (*MTD-Gal4>GFP shRNA (II)* and *MTD-Gal4>GFP shRNA (III)*, respectively). **(C)** *Pgd* and *Zw* double mutant ($Pgd^{n39}Zw^{lo2a}$) is compared to its genetic background control (heterozygous). **(D)** *Rpi* KD in the germline (*MTD-Gal4>Rpi shRNA*) is compared to its genetic background (*MTD-Gal4>GFP shRNA (II))*. **(A-D)** Boxes in green represent flies prefed the full diet, and in orange flies prefed a diet without sucrose, and the lighter colours represent the genetic background controls. Boxes represent upper and lower quartiles with the median. Filled black circles represent the presence of a given nutrient in the pre-treatment diet or the presence of a given transgene in the tested flies. *n* represents the number of flies assayed in the flyPAD in each condition. Significance was tested using the 1way ANOVA, Kruskal–Wallis test followed by Dunn's multiple comparison test. Not significant (ns) $p > 0.05$, * $p < 0.05$, ** $p<0.01$, *** $p<0.001$.

organ needs for this nutrient. It is important to note that *Hex-A* KD does not lead to a severely perturbed germline. These females are clearly different from the germline ablated animals. Therefore, the behavioral effect of *Hex-A* germline KD can not be explained as a consequence of the absence of a germline.

We next tested the effect on feeding behavior of knocking down other enzymes of the PPP that we had previously shown to impact oogenesis (**Figure 3.4.B**). When we tested the effect of either *Pgd* KD (*MTD-Gal4>Pgd shRNA*) or *Zw* KD (*MTD-Gal4>Zw shRNA*) in the germline and assayed the flies' feeding program in the flyPAD setups (**Figure 3.6 B**), we found phenotypes very similar to the ones described for *Hex-A* KD. Overall, these flies show a general decrease in the appetite for sugar. In order to confirm these results, we used a different approach to generate a loss of function situation of these genes and obtained a double mutant fly stock for *Pgd* and *Zw* ($Pgd^{n39}Zw^{lo2a}$). Consistent with the results obtained when knocking down *Pgd* or *Zw* in the germline using shRNA's, the mutant flies ($Pgd^{n39}Zw^{lo2a}$) show a decrease in sugar intake when compared to the heterozygous control flies (**Figure 3.6 C**). Again, this phenotype is very clear in flies deprived of sucrose prior the behavior assays than in the fully fed flies. However even fully fed flies show a non-significant trend for decrease in sugar intake. These results suggest that glucose metabolism specifically through the PPP is required for both egg production and to instruct the CNS to increase sugar intake likely by signaling the ovary nutritional needs. Accordingly, this feeding behavior phenotype resembles the one observed in germless flies without however always phenocopying the drastic loss of germline phenotype observed in the flies with an ablated germline.

A simple hypothesis of how the PPP would act on the CNS to modulate nutrient appetite would be through the direct action of an intermediate metabolite of the pathway that would diffuse from the ovaries and directly act on the neurons, modulating their activity. In order to test this hypothesis and trying to pinpoint specific metabolites that could be contributing to this behavior phenotype, we continued to KD more downstream enzymes of the PPP specifically in the germline. The step after ribulose-5-phosphate (Ru5P) synthesis through *Pgd*, is the entry in the non-oxidative phase, which converts Ru5P into ribose-5-phosphate (R5P) through **r**ibose-5-**p**hosphate **i**somerase (Rpi). Ribose-5-phosphate can then be used for nucleotide synthesis. We again analyzed the feeding program of *Rpi* germline KD flies (*MTD-Gal4>Rpi shRNA*), using the flyPAD technology (**Figure 3.6 D**). Contrary to what we found for *Hex-A*, *Pgd* or *Zw* germline KD flies, *Rpi* KD (*MTD-Gal4>Rpi shRNA*) leads to a significant increase in sucrose intake when compared to the corresponding genetic background controls (*MTD-Gal4>GFP shRNA (II)*). This gain in sugar appetite is only visible in flies which were sucrose deprived prior to the behavior

assays and no trend for an increase is observable in fully fed flies. This suggests that the mechanism that control sugar appetite is only able to alter behavior if sugar levels in the diet, and hence in the organism, are low. Intuitively, such a system would make sense as an animal should not increase sugar intake if this nutrient is available.

If analyzing the consequences of knocking down the different enzymes of the PPP at the level of the intermediate metabolites (**Figure 3.3**), we predict that all downstream metabolites will be either absent or found at low levels in the cell and the upstream components to be accumulated or shuffled into other metabolic pathways. The point in PPP where the behavior phenotype is opposite is in *Rpi* KD. We predict that this manipulation should lead to an accumulation of Ru5P. Ru5P is known to be metabolized into several sugar alcohols such as arabitol, xylitol and ribitol (Kordowska-Wiater, 2015). We thus hypothesize that in flies where we KD *Rpi* in the germline, there could be an accumulation of these metabolites within the ovary and at the level of the organism, through their diffusion to the hemolymph. Interestingly, it has been shown that a human patient with a mutation in this enzyme, shows a dramatic accumulation of these exact metabolites in the brain, further supporting this theory (Wamelink, Struys and Jakobs, 2008). Altogether these data suggest that one or several of these sugar alcohols could mediate the communication between the ovaries and the CNS, serving as an indicator of the PPP activity and thus of sugar availability in the organism. A key future step will be to manipulate enzymes producing these compounds and assess their effects on both fertility and feeding behaviour.

Main conclusions: The PPP in the germline modulates sugar appetite in *Drosophila melanogaster,* possibly through the synthesis of sugar alcohols.

CHAPTER

4 Discussion and Conclusions

Fruit flies have the ability to react to sugar deficits by changing their feeding behaviour to increase the intake of this nutrient. Moreover, a functional germline is an important driving organ of sucrose appetite in adult flies. Accordingly, germless flies show a drastic decrease in sugar appetite. We have thus used this organ as a model system to understand how this nutrient affects tissue physiology and metabolism, and in turn how these can signal to the CNS to modulate appetite and control homeostasis. Moreover, this organ being a model for proliferation and differentiation and being characterized by cells with high metabolic activity, we think this work may shed light on how human diseases affecting these cellular aspects may impact organism physiology and nutrient appetite.

4.1 Carbohydrates are essential for the function of highly metabolically active cells

We show that dietary sucrose deprivation leads to a significant reduction in the number of eggs laid per female fly which correlates with a reduction in ovary size (Figure 3.1). This result shows that this nutrient is essential for oogenesis to occur, further supporting our hypothesis that being a high metabolically active organ, the ovaries are sensitive to nutritional changes. These results are in accordance with a study showing that most of the dietary sugar is used for egg-formation in the adult female (Min *et al.*, 2006), further denoting the importance of sucrose for oogenesis. We also show that deprivation of amino acids leads to a much stronger effect on both egg production and ovary size when compared to sucrose deprived females. There are several hypotheses to explain the differences in the effects of removing these different nutrients from the diet on ovary function. One hypothesis is that the fruit fly maintains a bigger amount of sugar reserves, so that it still can provide the essential nutrients for oogenesis when sugar is absent from the diet. If this is the case, a prolonged deprivation of this nutrient could lead to a more drastic phenotype. Sugar and amino acids can be also interconverted: amino acids can be used to produce sugar through gluconeogenesis whereas glucose can be converted to amino acids, the

last step relying on transamination. Thus, when sugar is absent from the organism, protein can still be transformed into glucose; however, when amino acids are absent, since these are the major nitrogen pool of the organism, possibly glucose is not easily transformed into amino acids. Also, it is important to note that the animal fully depends on dietary uptake of essential amino acids as it can not synthetize them or only very inefficiently. This could explain why sugar removal from the diet is less drastic in terms of egg formation and impairing ovary morphology when compared to amino acid deprivation. Interestingly, a previous report by Piper, et al (2014) shows that removal of sucrose from the diet did not significantly affect cumulative lifetime fecundity, whereas removal of amino acids strongly did. However, our assays show the effect of acutely removing sucrose from the diet, which significantly affects egg-laying. THese results are not necessarily contradictory being in accordance with our hypothesis that the fruit flies maintain sugar reserves and possibly are able to compensate for the long-term lack of this nutrient. In this case in our assays, then possibly depriving flies from sucrose for longer periods might not exacerbate the decrease in egg-laying to an extent similar to AA deprivation.

The nutritional effects of sucrose on egg number per female and ovary morphology could be explained by decreased egg-formation rates at different levels of oogenesis (stem cell division, cystoblast division, follicle stem cell and follicle cell divisions, etc.) due to nutrient limitations; or it could be due to activation of a nutritional checkpoint which has been shown to exist for protein availability, leading to the apoptosis of cells and egg chambers at different stages of oogenesis (Drummond-Barbosa and Spradling, 2001). Studies in other animal models are in accordance with our results, having previously shown that glucose plays an important role in oogenesis. In female mice, it was shown that glucose within the oocyte regulates meiotic maturation and that it is also important for embryonic development (Dupont et al., 2014). Furthermore, it has been shown that the oocyte has a decreased ability to metabolize sugar, however, glucose is shown to be transported to the oocyte through the cumulus cells, where it is thought to play a role in ensuring oocyte quality (Dupont et al., 2014).

In humans, the role of sugars in female reproduction has been increasingly studied in the context of polycystic ovary syndrome (PCOS) development and progression. Obesity, a dietary related NCD, has been long linked to a diminished reproductive output. Even in ancient Greece Hippocrates noted that servant women, which were more fit, were also more fertile when compared to their overfed and sedentary employers (Dupont and Scaramuzzi, 2016). This suggests that the amount and quality of carbohydrates in the diet and related NCDs might influence reproductive functions in humans.

Glucose availability is also key for other highly proliferative states and has been proposed to modulate tumor formation and progression (Graham *et al.*, 2012). Contrary to normal cells, most malignant cells depend on steady glucose supply due to their high energetic and biosynthetic requirements and are not able to metabolize significant amounts of fatty acids or ketone bodies due to mitochondrial dysfunction (Klement and Kämmerer, 2011). These proliferating cells also require reduced carbon molecules for the biosynthesis of a diverse array of biomolecules, such as pentose and hexose sugar derivatives (Pavlova and Thompson, 2016). Although there is still not an obvious and clear causative/consequential relation between diet and cancer emergence, available data strongly suggests that high sugar levels, which can be obtained from the diet, can facilitate tumor progression. One of the major concerns is that the high insulin levels resulting from chronic ingestion of the carbohydrate-rich Western diet can facilitate tumor cell proliferation via the insulin signalling pathway (Klement and Kämmerer, 2011). This idea is further supported by studies showing that, ketone bodies, which are elevated when insulin and blood glucose levels are low, have been found to negatively affect proliferation of different malignant cells *in vitro*, probably because they are not usable by tumor cells to fuel their metabolic demands (Klement and Kämmerer, 2011). Similarly, there is increasing evidence that some diets increase the likelihood of recovery for cancer patients (Donaldson, 2004).

In a broader context, our results denote the importance and impact of carbohydrate availability on tissue homeostasis and function. We propose that the ovaries of the fruit fly are a powerful model to study and elucidate the mechanisms that might be underlying several human diseases that are highly correlated with an imbalanced intake of carbohydrates.

4.2 Carbohydrate metabolism is key for ovary function and fertility

The above described results allowed us to confirm that dietary sugar impacts oogenesis, further confirming oogenesis as a high nutritional demanding process. We next sought to understand if sugar metabolism in this organ could be underlying the observed decrease in egg production upon sucrose deprivation. We have shown that this is the case, and that the PPP is required for ovary function and high fertility (**Figure 3.4**). The process of oogenesis comprises several steps and several cell types that have been described to have different energetic requirements. Therefore, we tested the expression of the PPP throughout oogenesis, which revealed that this branch of carbohydrate metabolism starts to be expressed after the cell-cyst formation, therefore not being expressed in stem cells (either GSCs or FSCs) (**Figure 3.5**).

Our *in situ* hybridization results show that expression of PPP during oogenesis is strictly controlled and seems to continuously increase throughout progressive stages of oogenesis in both nurse cells and in the oocyte. The expression of this carbohydrate branch in these stages is much lower for the somatic follicle cells, which are required for proper axis specification of the oocyte and synthesis of yolk, vitelline membrane and chorion (Pritchett, Tanner and McCall, 2009). Both nurse cells and the oocyte have high metabolic requirements since they are continuously supporting oocyte development. The nurse cells are responsible to synthesize nutrients, proteins, mRNAs and organelles which are continuously provided to the developing oocyte; furthermore, during late oogenesis, around stage 11, these cells rapidly transfer their content to the oocyte in a process commonly called "dumping" (Pepling, 2016),(Peterson *et al.*, 2015). Throughout oogenesis nurse cells undergo endoreplication cycles, which have been suggested to maximize mRNA and protein synthesis probably to support the mentioned functions (Lee, Davidson and Duronio, 2009).

It should also be interesting to analyse the role of PPP independently in both these cell types. With our work we are not able to identify significant changes in expression for glucose metabolism (through *Hex-A* and *Pgd* expression) between the oocyte and the nurse cells. Studies in mammals have depicted metabolic differences between the oocyte and the cells that support its development, the granulosa cells that during ovulation form the cumulus-oocyte complex, providing most of its substrates for energy metabolism and biosynthesis. *In vitro* studies using mouse and bovine oocytes have shown that in the cumulus–oocyte complex (COC), glycolysis by the cumulus cells provides essential metabolites that support oocyte development (Sutton-McDowall, Gilchrist and Thompson, 2010). Furthermore, in the oocyte itself, a higher PPP activity was measured compared to cumulus cells, which has been linked with meiotic regulation (Sutton-McDowall, Gilchrist and Thompson, 2010). These studies have strengthened the idea that cells supporting oocyte development have higher carbohydrate metabolic rates compared to the oocyte itself. However, the PPP might display an important role in the oocyte probably due to the requirement of a very controlled oxidative environment, to prevent eventual damage to this unique cell within the 16-cell cyst and later on, egg-chamber. Furthermore, similar mechanisms have been shown in male gametogenesis. *In vitro* studies using rat cells have shown that Sertoli cells in males, which functionally resemble the COC in female mice and nurse cells in the female fruit fly, metabolize glucose mostly to lactate that can then be used by germ cells (Alves et al., 2013).

There is accumulating evidence that neoplastic lesions in cancer cells which are highly dependent of glucose, also increase the metabolic flux through the PPP. In cancer cells, this

pathway generates high NADPH levels which are key to avoid ROS damage to the cell, while simultaneously generating high levels of nucleotides for DNA synthesis and repair (Patra and Hay, 2014). Furthermore, studies have proposed targeting the PPP in cancer cells as a medical approach supporting chemotherapy. Some of these have specifically proposed the use of drugs that target specific enzymes of this pathway, such as G6PD, a gatekeeper of PPP, as potential therapeutics for human cancer (Cho et al., 2018). Therefore, these studies also strengthen the idea that manipulating this pathway in high metabolically dependent environments, could facilitate or impair proliferation processes.

We have shown that the activity of this pathway is essential for proper ovary development and egg formation, which is also in accordance with the above mentioned studies. The constant need of taking up nutrients to synthesize new eggs, could also lead to the need of the germline to control the enzymes of this pathway. Thus, it could be very interesting to explore the activity of this pathway in both men and women reproductive system in the context of infertility.

4.3 The PPP activity in germline cells modulates sugar appetite

We tested whether the nutritional status of the ovaries could be surveyed by the CNS to modulate appetite. We tested the feeding behavior of flies with different components of the PPP knocked down in the germline. We found that KD of *Hex-A*, *Pgd* or *Zw* leads to a dramatic reduction in sucrose appetite. In contrast, KD of *Rpi* in the germline leads to the opposite behavioral phenotype, meaning an increase in sugar intake.

Interestingly, *Rpi* deficiency in humans has been described in one rare individual, displaying an accumulation of sugar alcohols, ribitol and D-arabitol (M. Verhoeven and Jakobs, 2006). These sugar levels were changed in the periphery and also in the brain, which would be coherent with these functioning as possible behavior modulators. Our results together with this evidence suggest that depending on PPP activity, production of one or more of these metabolites in the germline can potentially lead to their diffusion to the hemolymph to then act on the CNS. These metabolites could modulate sugar appetite, assuring that the proper sugar levels reach the ovaries to maintain a high level of fertility, either by acting directly on the CNS or through a relay via other tissues.

A quite interesting yet concerning fact is that actually these pentitol sugars are used in the food industry as sweeteners, having a very low caloric content while tasting sweet. Studies in both animals and humans have suggested that consuming artificial sweeteners can modulate hunger and thus food intake (Blundell and Hill, 1986; Rogers and Blundell, 1989; Wang *et al.*, 2016). The use of these substances has caused a lot of controversy due to the possible detrimental effects on human health. In flies, chronic sweet/energy imbalance promotes a sustained increase in food and caloric intake, which is reversed upon removal of sucralose supplementation (Wang et al., 2016). Intake of commercial sweeteners in mice elicits changes in signalling pathways that have been related to the control of appetite and energy balance *in vivo*, which can impact the nutritional status and long term health (Barrios-Correa et al., 2018). A study in rats has also shown that the sweet taste induced an insulin response, which causes blood sugar uptake from tissues. However, since blood sugar is not increased with artificial sweeteners, this leads to hypoglycaemia and a subsequent increase in food/caloric intake (Swithers and Davidson, 2008; Tandel, 2011). The intake of these compounds has also been associated with increased body weight and increased adiposity (Swithers and Davidson, 2008). Additionally, these artificial sweeteners are also known to be metabolized by bacteria raising the hypothesis of them being metabolized by the gut microbiome, which has also been proposed as a modulator of feeding behavior (Kordowska-Wiater, 2015). Therefore, evidence points for both the modulation of these sugar alcohols levels when the PPP activity is changed, and for the potential role of these compounds in modulating appetite.

In a similar manner it is tempting to speculate that cells that also display a high level of PPP activity, such as tumor cells, could have similar mechanisms to signal their sugar requirements to the brain. Cancer cells display an advantageous competitive status compared to healthy surrounding cells. Therefore, it will also be interesting to analyse if these metabolites are expressed in these cells and in the brain of cancer individuals. These could signal the high nutritional requirement of tumors for sugars which promote these cells' survival and proliferation. Furthermore, there has been a lot of speculation regarding the role of sugar intake/appetite in cancer onset and progression (Klement and Kämmerer, 2011). However, most dietary changes in tumor-bearing individuals have mostly been assessed once cancer is diagnosed from tumors large enough to be detected and/or after treatments have started, so that the eventual changes in diet and appetite could either be causative or consequential of cancer progression or even both (Thomas et al., 2017). For instance, there is the well accepted idea that obesity and diabetes can increase the risk of developing certain types of cancer. This is supported by evidence that changes in blood insulin levels associated with glycaemic dysregulation appear

early during cancer development (Thomas et al., 2017). A promising hypothesis is that early changes in diet and modifications of metabolic parameters (blood glucose or lipids levels) may arise from the tumor modifying the host's metabolism to favour tumour/cancer development (Thomas et al., 2017). These are often, later on, associated with anorexia (reduced appetite), a general symptom observed in patients with advanced cancer, which also significantly contributes to cancer cachexia, a wasting process that results in a dramatic loss of muscle and adipose tissue mass (Thomas et al., 2017).

Our studies strongly support the idea that some highly metabolically active cells strongly depend on dietary sugar supply and have mechanisms to communicate their nutritional requirements to the brain, leading to a modulation of feeding behavior. We show that in the female germline of the fruit fly, dietary sugar and its metabolism through the PPP in the germline is essential for oogenesis, which in turn also uses this pathway for modulation of sugar feeding behavior. We hypothesize a model in which the activity of the PPP in the germline is necessary to inform the CNS on the nutritional carbohydrate status of the ovaries (**Figure 4.1**). In our feeding behavior paradigm, we show that flies either without a germline or with a loss of function in the PPP in this organ, behave like they are satiated, eating less sucrose even if carbohydrate deprived. We therefore propose that the PPP activity in the germline inhibits satiety and thereby promotes feeding on sucrose. This communication could be achieved through a direct interaction between the germline and the brain, for example through the release of the signal generated by the PPP to the hemolymph. Furthermore, it could also result through the relay of a signal by other organs, such as the fat body and the *corpus cardiacum*, two endocrine organs in *Drosophila* that

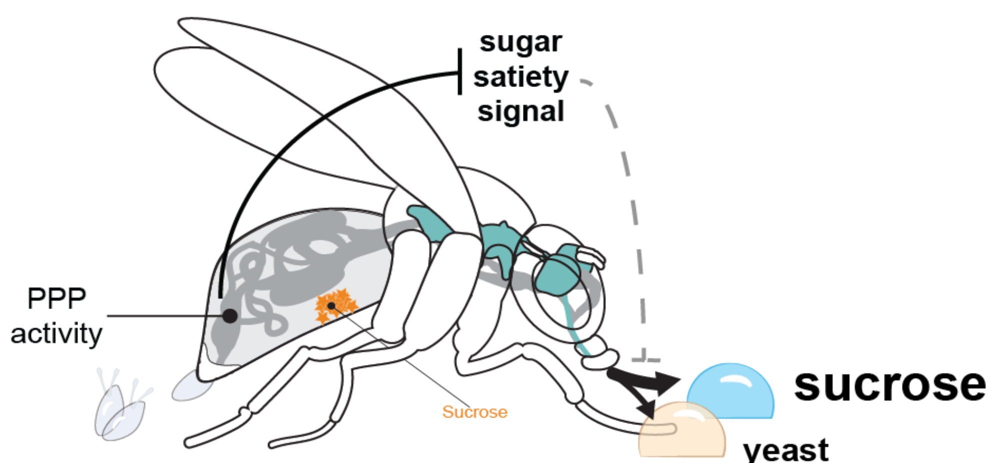

Figure 4.1| The PPP activity in the germline modulates nutrient appetite and feeding behavior in *Drosophila melanogaster*. Our current working hypothesis is that the PPP activity in the germline is a reporter of the sugar nutritional status of the ovaries of the flies, communicating these needs to the CNS, to modulate sugar intake. Our results made us predict that this pathway activity in the germline is necessary to inhibit satiety and promote sugar feeding.

coordinate metabolism and behavior with nutritional state by secreting systemic signals (Wang *et al.*, 2016). Altogether, our results highlight the role of cell metabolism in modulating feeding behavior to support optimal cell/organ function, raising a series of possibilities for therapeutical approaches in human diseases where metabolism and organ communication play a key role.

Future Work

This project has provided new knowledge regarding how organs can influence feeding decisions to adapt the behavior of the animal to specific nutritional requirements. As discussed in the chapter ***4.Discussion and Conclusions***, we hypothesize that signals from the PPP might act as modulators of feeding behavior. We shown that the metabolic activity within a specific organ is able to simultaneously modulate organ function and food choice. Interestingly, it was previously proposed that ecdysone regulation in female flies modulates both feeding behavior and lipid metabolism to support the biosynthetic requirements of oocyte production (Sieber and Spradling, 2015). To further confirm our hypothesis, further work is needed to understand how the communication between the germline and the brain is occurring. One hypothesis is that the sugar alcohols produced by the PPP activity in the germline are modulating feeding behavior. Thus, in light of our studies, one could measure these compounds both in the ovaries and in heads of the flies without germline and with manipulations of the PPP in this organ. Regarding the latter, it will be interesting to measure these metabolites in the context of *Hex-A*/*Pgd/Zw* and *Rpi KD* in the germline, since the behavioural phenotype is the opposite in these manipulations. This could allow us to understand if indeed the levels of these sugars are changed in these conditions and if they could be used to communicate the nutritional carbohydrate status of the ovaries in order to modulate feeding behavior.

The supplementation of these substances has already been shown to modulate the adipose tissue content, which both in mammals and flies, is a centre for hormone production key for regulating the reproductive system. These compounds could then be modulating both fertility and feeding behavior. Therefore, it should be very interesting to also analyse the impact of these compounds in female fertility, coupled with the analysis of their feeding behavior. This could be achieved through genetic manipulations of the enzymes producing these compounds or through supplementation of these metabolites in the diet, followed by egg-laying assays and feeding behaviour assays using the flyPAD. As *Hex-A*/*Pgd/Zw* KD in the germline leads to flies that behave as if they would be satiated, we hypothesize that the supplementation with sugar alcohols in the diet, would lead to an increase in sucrose feeding.

In this thesis, we have compared the metabolic reprogramming occurring during oogenesis to the metabolic changes occurring in tumor cells. It will also be interesting to use *Drosophila melanogaster* to further characterize if our hypothesis is also applicable to this

context, since tumor cells also have very high metabolic requirements, also displaying carbohydrate metabolism reprogramming. This could be achieved by generating flies with tumor models to further analyze their metabolic activity and their impact on nutrient specific appetites. These experiments could serve to expand the relevance of our findings to pathological situations as well as opening the opportunity to test if special metabolic programs in proliferating cells are able to also modify nutrient cravings in vertebrates.

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Appendix I - List of genotypes

Referred to as	***Detailed genotype***
control background	w^{1118}
MTD-Gal4>+	*P{w[+mC]=otu-GAL4::VP16.R}1, w[*]; P{w[+mC]=GAL4-nos.NGT}40; P{w[+mC]=GAL4::VP16-nos.UTR}CG6325[MVD1] (BL31777)*
+>GFP shRNA (I)	*y[1] sc[*] v[1]; P{y[+t7.7] v[+t1.8]=VALIUM22-EGFP.shRNA.1}attP2 (BL41558)*
+>GFP shRNA (II)	*y[1] sc[*] v[1]; P{y[+t7.7] v[+t1.8]=VALIUM20-EGFP.shRNA.4}attP40 (BL41552)*
+>GFP shRNA (III)	*y[1] sc[*] v[1]; P{y[+t7.7] v[+t1.8]=VALIUM20-EGFP.shRNA.4}attP2 (BL41553)*
+>Hex-A shRNA	*y[1] sc[*] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.GL00023}attP2 (BL35155)*
+>Pfk shRNA	*y[1] sc[*] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.GL00298}attP2 (BL36782)*
+>Pgi shRNA	*y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HMC03362}attP40 (BL51804)*
+>Pgd shRNA	*y[1] sc[*] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HMC05959}attP40 (BL65078)*
+>Zw shRNA	*y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HMC03068}attP2 (BL50667)*
Pgd^{n39} Zw^{lo2a}	*Pgd[n39] pn[1] Zw[lo2a] (BL6033)*
+>Rpi shRNA	*y[1] sc[*] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HMC05203}attP40 (BL62196)*

Table 1 | Detailed genotypes of the *Drosophila melanogaster* stocks used in this thesis.

Appendix II - *In situ* hybridization in ovaries of the fruit fly

1. Preparation of RNA probes for *in situ* hybridization

- **Amplification of the regions of interest by PCR and subsequent in vitro transcription**

	Hex-A	*Pgd*
Primer Forward (µL)	0.6	0.6
Primer Reverse (µL)	0.6	0.6
KOD (µL)	10	10
H_2O (µL)	8.3	8.3
cDNA (µL)	0.25	0.25
Annealing Temp.(ºC)	48	50
Extension (s)	30	30
Number of cycles	35	35
Expected size (bp)	1062	1214
[PCR product] (ng/µL)	92	55

Table 1 | PCR details for amplification of genes of interest

	Hex-A (µL)	*Pgd (µL)*
BSA (100x) (NEB)	2	2
Dig-labelled nucleotides (10x)	2	2
DTT (100 mM)	1	1
RNase inhibitor	0.5	0.5
RNApol buffer (10x)	2	2
RNA pol SP6/T3 (M0378S/M0207S NEB)	1	1
DNA (template PCR) for 1µg	10.8	18

Table 2 | Details for in vitro transcription of RNA probes for the genes of interest

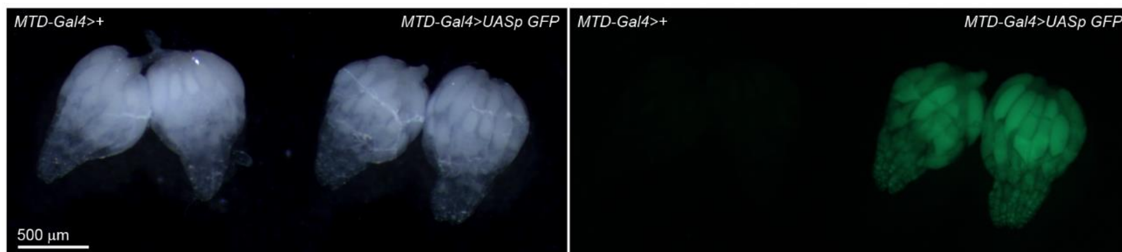

Figure 1 | *MTD-Gal4* drives the expression of a GFP reporter throughout all stages of oogenesis. Representative examples of ovaries from females driving the expression of a GFP reporter in the germline (*MTD-Gal4>UASp GFP*) compared to the genetic background control (*MTD-Gal4>+),* under light field (left panel) and bright field (right panel). Scale: 500 µM.

- **Stock solutions**

2x Carbonate buffer recipe:
Na_2CO_3: 120 mM
$NaHCO_3$: 80 mM
pH to 10.2 with NaOH

Alkaline phosphatase staining buffer (AP buffer)
5 M NaCl (H_2O MQ): 1 mL
1 M MgCl (H_2O MQ): 2,5 mL
1 M Tris pH 9.5 (H_2O MQ, keep at -20ºC): 5 mL
10% Tween 20: 0,5 mL
Water (H_2O MQ): 41mL
Final volume: 50 mL

Pre-hybridization buffer:
50% formamide: 20 mL
4x Saline sodium citrate (SSC) (20x): 8 mL
0.1% Tween20 (10%): 400 µL
Final volume: 40 mL

Hybridization buffer:
50% formamide:20 mL
5x Saline sodium citrate (SSC) (20x): 10 mL
100 µg/mL Heparin (50 mg/mL): 80 µL (Sigma H4784)
0.1% Tween20 (10%): 400 µL
100 µg/mL sonicated and boiled ssDNA (salmon sperm DNA): 400 µL (Sigma D7656)
Final volume: 40 mL