# Understanding the defects associated with High Sugar Diet in the midgut of *Drosophila*

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***A dissertation submitted for the partial fulfillment of BS-MS dual degree in Science***

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**Indian Institute of Science Education and Research Mohali**

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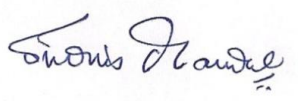
*Dedicated to my family*

# Certificate of Examination

This is to certify that the dissertation titled "**Understanding the defects associated with High Sugar Diet in the midgut of *Drosophila***" submitted by **Farzana N** (Reg. No. MS15145) for the partial fulfillment of B.S.-M.S. dual degree programme of the Institute, has been examined by the thesis committee duly appointed by Institute. The committee finds the work done by the candidate satisfactory and recommends that the report be accepted.



**Dr. Rhitoban Roy Choudhuri** **Dr. Lolitika Mandal** **Dr. Sudip Mandal (Supervisor)**

**Dated: June 13, 2020**

# Declaration

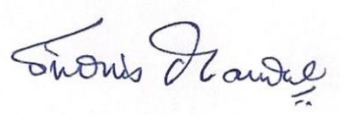
The work presented in this dissertation has been carried out by me under the supervision of Dr. Sudip Mandal at the Indian Institute of Science Education and Research Mohali. This work has not been submitted in part or in full for a degree, a diploma, or a fellowship to any other university or institute. Whenever contributions of others are involved, every effort is made to indicate this clearly, with due acknowledgment of collaborative research and discussions. This thesis is a bonafide record of original work done by me and all sources listed within have been detailed in the bibliography.

**Farzana N**

**(Candidate)**

**Dated: 13-06-2020**

In my capacity as a supervisor of the candidate's project work, I certify that the above statements by the candidate are true to the best of my knowledge.



**Dr. Sudip Mandal**

**(Supervisor)**

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I am grateful to my family for their support and love they gave me throughout my life. I am lucky to have parents who believe in me and support me to fulfil my dreams. My brother is my biggest strength and has been always there for me.

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## Abstract

Diet is one of the most important factors in the maintenance of an individual's good health. Altered food habit that includes excessive consumption of carbohydrate-rich diets, has contributed significantly to the global rise of patients with diet-induced diabetes. Considering the alarming effects of diet-induced diabetes on human health and physiology, it has become imperative to understand the molecular basis of the defects in order to design therapeutic strategies. The genetically amenable model organism, *Drosophila melanogaster*, has come up as a wonderful model organism for this study, primarily because of significant conservation of genes and signaling pathways between Drosophila and mammals. In this study, we tried to analyze the effects of high sugar diet on the midgut of *Drosophila* by rearing them on high sugar diet (1M), as opposed to rearing them on diet with normal sugar (0.1M). Our results show that with an increase in the number of days, flies reared on high sugar diet demonstrate a gradual reduction in the size of the midgut cells, with a concomitant increase in cell death and a decrease in the rate of cell proliferation as compared to normal feeding flies. We also studied the impact of high sugar diet on the carbohydrate metabolic pathways. We saw changes in the transcript levels of carbohydrate metabolic pathway enzymes. We looked into the transcript level of the glycolytic enzyme, Hexokinase, and Phosphofructokinase. The transcript level of Hexokinase decreases while there was no significant change in the transcript level of Phosphofructokinase.

Chapter 1

# Introduction and Objectives

## Introduction

An individual's health, growth, and development are largely defined by the diet one follows. From the studies conducted by the World Health Organisation on Diet, Nutrition and Prevention of Non-communicable Diseases (NCDs), it is known that the growing epidemic of chronic diseases is due to the dietary and lifestyle changes. Some of these chronic NCDs include obesity, diabetes mellitus, cardiovascular diseases, hypertension, stroke and cancer [1]. Since the rate of lifestyle diseases increased rapidly, it became quite important to study these diseases. One of the most common lifestyle diseases is Type-2 Diabetes. The number of people with diabetes increased from 153 million in 1980, to 347 million in 2008 [2]. It is important to understand how these disease conditions affect our physiology. Many studies are there using different model organisms. Recently *Drosophila melanogaster* has come up as a model organism to understand the effects of diet induced metabolic disorders. By feeding *Drosophila* on high sugar diet it demonstrates several characteristic features of type 2 diabetics in humans.

The gastrointestinal tract, one of the largest organs in the body, was earlier considered as a passive organ responsible for digestion and absorption. But now this view has changed and recently it is studied in multiple research fields such as stem cell biology, neurobiology, metabolism, immunity, development, and regeneration. It is the first line of defence against pathogens. Many studies in gut started since it has emerged as a major modulator of different biological processes like food intake, energy balance, insulin secretion, immunity, physiology, and behaviour [3][4].

## Diet and Diseases

Due to lifestyle and dietary changes the burden of chronic diseases has increased rapidly worldwide. In 2001 the global burden of NCDs was approximately 46%. The proportion of the burden of NCDs is expected to be 57% by the turn of 2020. Cardiovascular diseases, obesity and diabetes attribute to almost half of the total chronic disease deaths. This shows a worrying trend because they affect a large population as well as have started to appear earlier in life.[1]

Sugar is the main source of energy for all organisms and it is one of the main ingredients on diet in all parts of the world. As society and lifestyle changed, the eating habits of people also changed [5]. People started to consume more sugar-sweetened beverages,

grain-based desserts, fruit drinks, dairy desserts and different varieties of sweet dishes rather than traditional plant-based diets [6][7]. High intake of sugars leads to increased risk of dental caries and tooth demineralisation[8], overweight and obesity[5], hypertension and type 2 diabetics (T2D)[9], steatohepatitis[10], coronary heart diseases[11], cancer [12][13][14], kidney disease[2], dementia or impaired memory[15], gout[16] etc.

T2D is resistant to the hormone that regulates blood sugar levels. This hormone is insulin. Resistance to insulin causes blood sugar levels to rise and it strongly promotes the risk of diabetes. Insulin resistance develops as a result of many factors like genetic susceptibility, obesity, and diet which causes hyperglycemia. 55% of T2D patients are obese showing that it is the most obvious clinical risk factor for humans [17].

## *Drosophila* as a model system

*Drosophila melanogaster,* commonly known as the fruit fly, is a small insect of about 2-3mm long. It was introduced 100 years ago, and since then it is being used extensively as a model organism in biology [18][19]. It belongs to the family Drosophilidae, which comes under the order Diptera.

Use of *Drosophila* in labs was first documented by William Castle's group at Harvard in 1901, however, the "father" of *Drosophila* research is undeniably Thomas Hunt Morgan [20]. T. H. Morgan and his students A. H. Sturtevant, C. B. Bridges, and H. J. Mullar carried out various genetic analyses using *Drosophila* in the famous fly lab of Columbia University, which refined the theory of inheritance proposed by Mendel. Using *Drosophila* they defined genes and formulated the chromosome theory of heredity [18][20].

*Drosophila* can be used as a model system because the entire genome of this organism has been sequenced making it easy to study and manipulate the gene of interest. Besides, it has a short generation time and has cheap maintenance. It has served as a useful model system for studying various human diseases because the fly genome is 60% homologous to humans and also about 75% of the genes responsible for human diseases have a homolog in flies [21]. A large number of mutants have been generated over the years and are available in various stock centres around the world.

## Life Cycle of *Drosophila melanogaster*

*Drosophila melanogaster* is a holometabolous insect, progressing from egg to larva, to pupa, and then finally to adult flies. The life cycle of *Drosophila* lasts approximately 10 days at 25°C. The growth and development of this fly depend on the temperature it is reared. The maximum life span of these flies ranges from 60-80 days, depending on the food culture condition. *Drosophila* has internal fertilization. A single fertile female fly can lay hundreds of eggs and its embryogenesis lasts approximately 24hours. The larval stage can be divided into three different phases, first instar, second instar and third instar. The first instar larvae begin to feed immediately on the surface of the culture medium soon after hatching and passes through two molting stages. This feeding and growth phase will last for four days until the larvae stop feeding and leave the food in search of an area for pupariation. Second instar larvae burrow into the medium and third instar larvae after maturation, leaves the medium and wanders up the walls of the culture bottle, searching for a place to pupariate for 24-48 hours (**Figure 1**). During the pupariation, metamorphosis occurs for four days. Most larval tissues get degraded and adult organs develop from the imaginal discs, which are made up of diploid cells of the undifferentiated epithelium. After this adult flies eclose from the pupal case. Adult female flies are larger than male flies, with females weighing approximately 1.4mg and males 0.8mg. Females get ready to mate in less than 24hr [19][21]. (**Figure 2**)

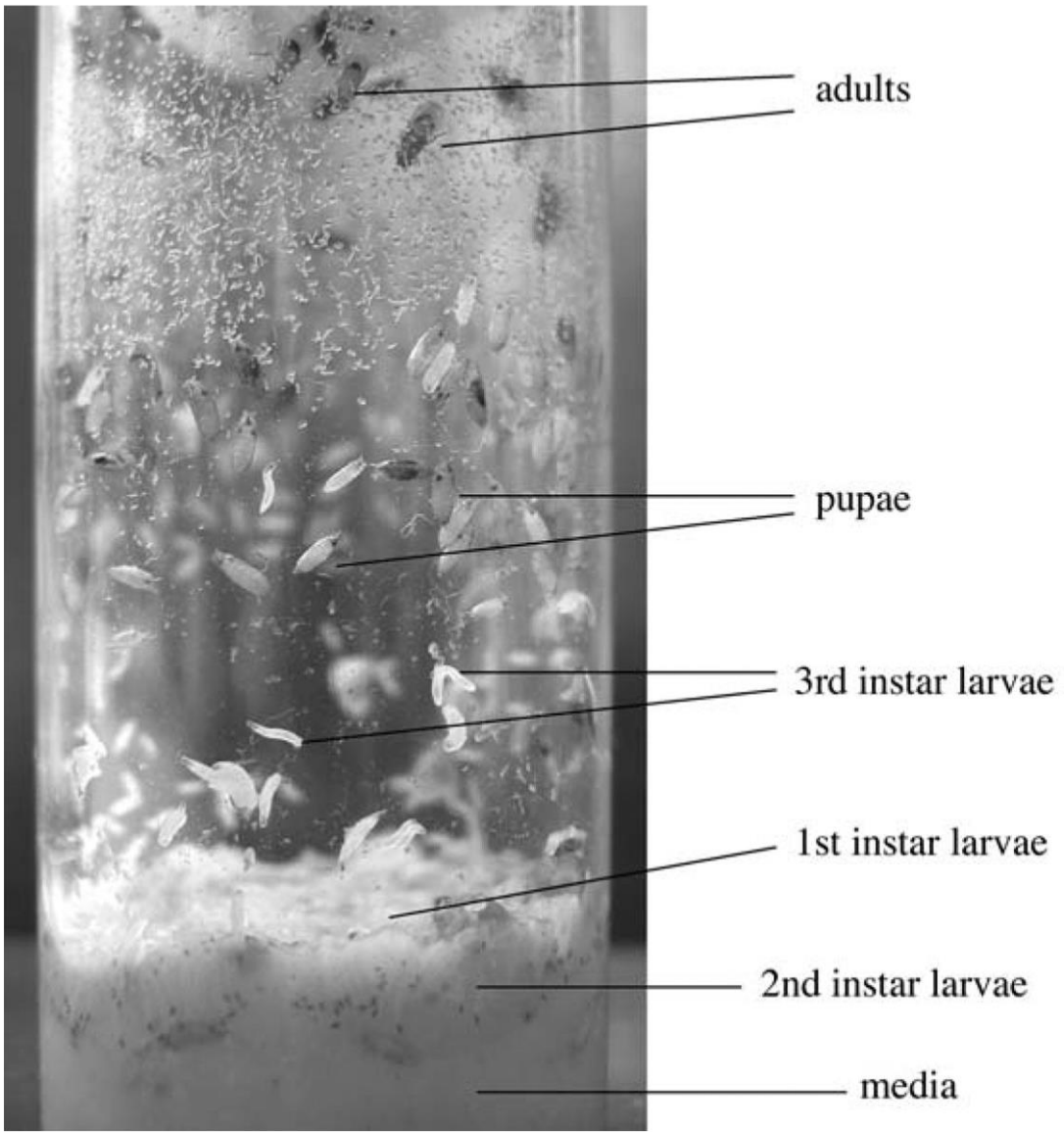



**Figure 1: Different stages of *Drosophila melanogaster* growing in a vial.**

First instar larvae feed on the surface of the medium. Second instar larvae burrow into the medium to feed (small black dots are the jaws of second instar larvae). Third instar larvae wander up the walls of the bottle, where they will pupariate. Adults are at the top of the bottle [18].

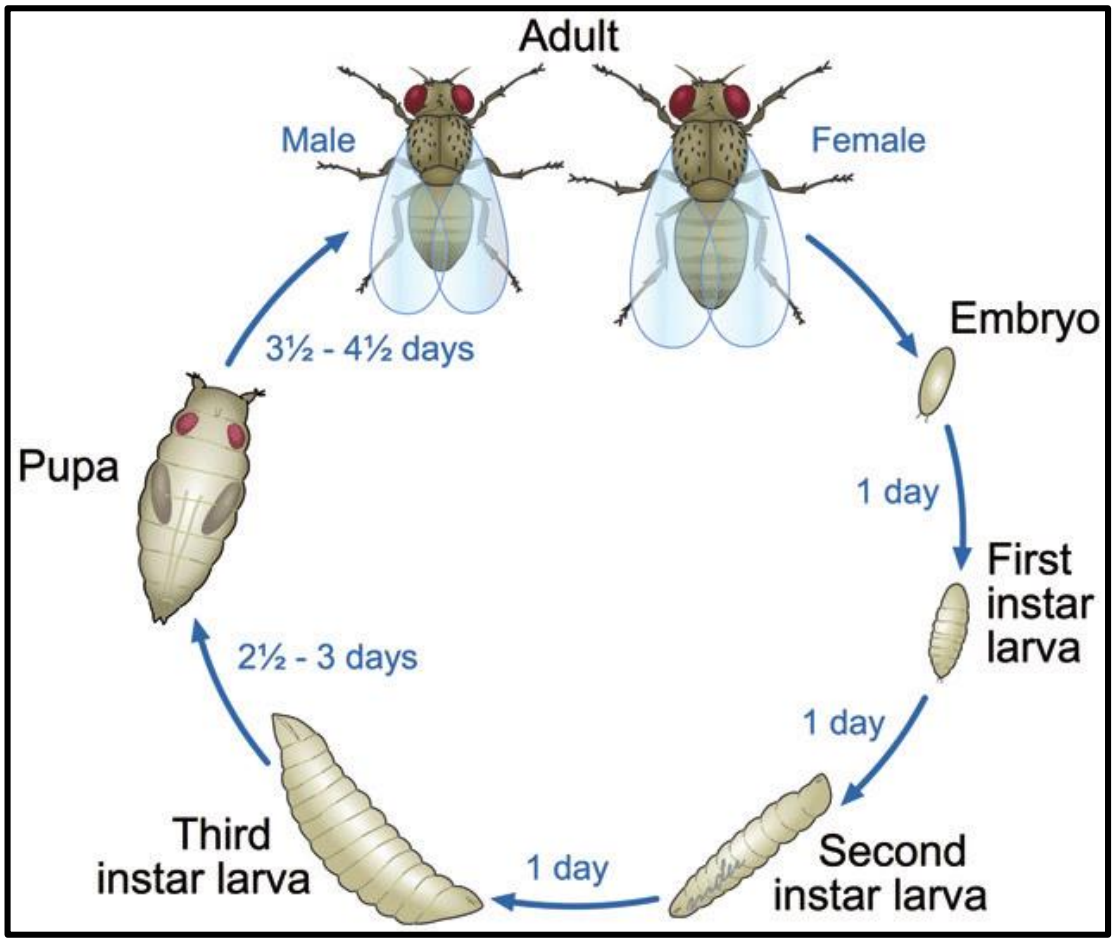



**Figure 2: Life Cycle of *Drosophila melanogaster***

Schematic representation of male and female fly and different stages of the life cycle of *Drosophila melanogaster*. *Drosophila* has four distinct developmental stages - embryos, larvae, pupae, and the adult fly. Larval stages are significantly different from adult stages. After growing and undergoing several molts, larvae become pupae. Pupae undergo metamorphosis and after 3-4 days, fly emerges out of the pupal case [19].

## *Drosophila* as a model for studying the gastrointestinal tract.

The gastrointestinal tract is an essential organ for absorption and whole body metabolism. The adult *Drosophila* midgut is an equivalent of the mammalian small intestine where food is digested and absorbed [22]. Both are structurally and functionally similar. In mammals, the food they eat passes through the esophagus to the stomach. There the food gets accumulated and digestion starts. It then moves to the small intestine for further digestion and absorption and then to the large intestine for absorption of nutrients.

Finally, it reaches rectum and waste is excreted out through the anus. Similarly, in *Drosophila*, the food passes through the foregut to crop, where it is temporarily stored, and then moves to the midgut. It passes through anterior midgut, where absorption starts. Then it passes through the middle midgut, which contains the iron and copper cells (Fe/Cu cells), a region of low pH, it transits through the posterior midgut for further absorption and the hindgut and rectum, and finally reaches the anus for excretion. The foregut of *Dro*sophila is analogous to the esophagus, crop to the stomach, midgut to small intestine and hindgut to the large intestine. The fly Fe/Cu cells, which are found in a region of low pH that seem to be functionally distinct from the low-pH stomach of mammals [23]. (**Figure 3**)

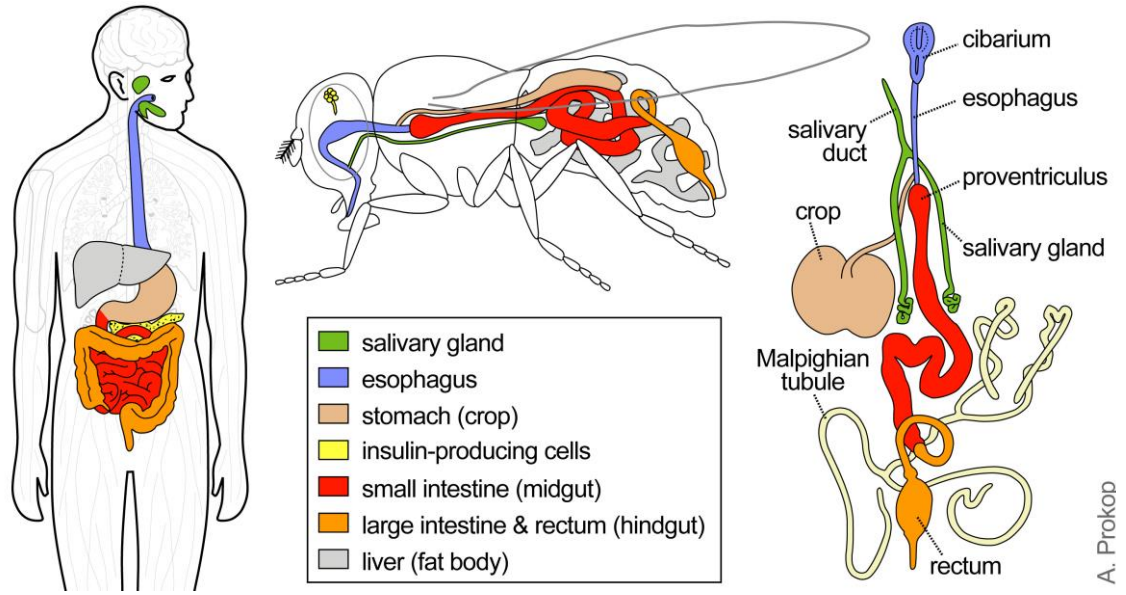



**Figure 3: Comparison of the alimentary canal of *Drosophila melanogaster* and Human.**

Colours indicate homologous and functionally related structures of the alimentary canal. Crop is similar to stomach, foregut to esophagus, midgut to small intestine and hindgut to large intestine & rectum.

droso4schools, online resource for school lessons using fruit fly

Fly gut and human intestine, both are of endothelial origin. They both comprise an epithelial monolayer of columnar or cuboidal cells called enterocytes [23]. Both harbour adult intestinal stem cells (ISCs) [24][25][26][27]. ISCs are multipotent and they are capable of giving rise to all the differentiated cell types of the intestinal epithelium. In

*Drosophila* ISCs divide to give rise to a progenitor cell type known as the enteroblast (EB). The enteroblast (EB) cell will differentiate into either an absorptive enterocyte (EC) or a hormone-producing enteroendocrine (EE) cell depending on the amount of notch signalling received [23][24]. Similarly, the mammalian intestinal epithelium is composed of absorptive cells (ECs) and secretory cells (EE and Goblet cells) [28]. (**Figure 4**)

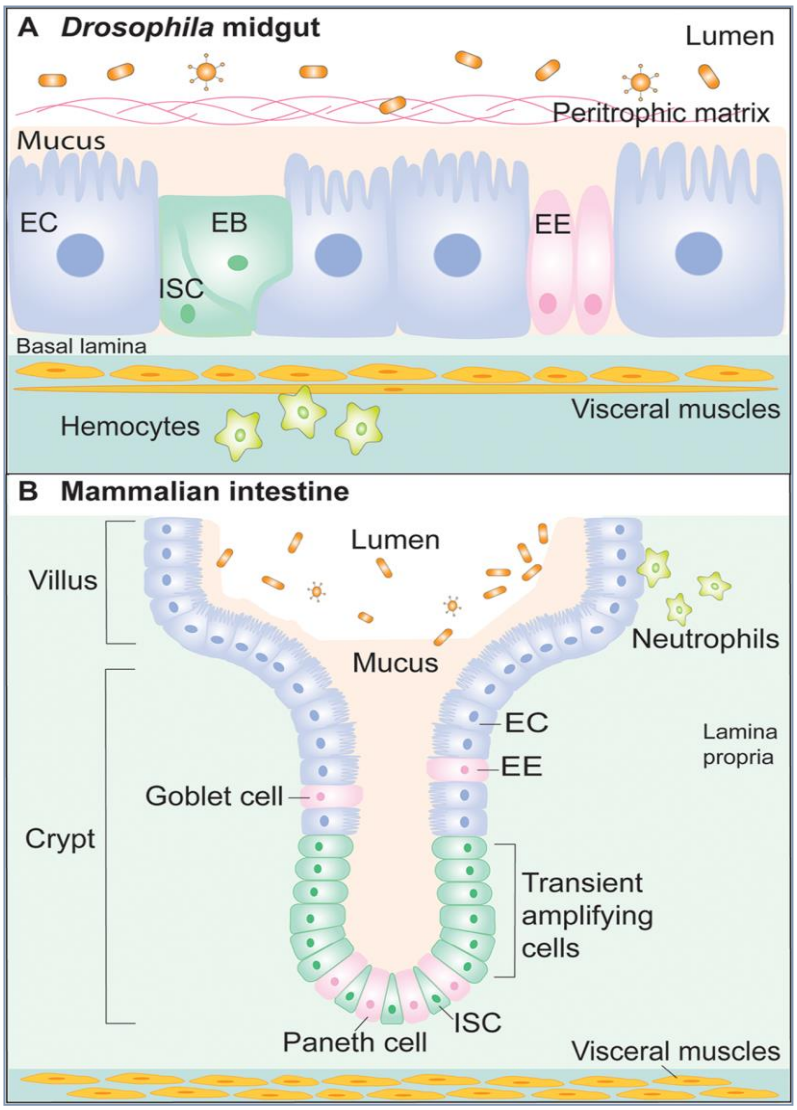



**Figure 4: Comparison between the gut epithelia of *Drosophila* and mammals [28].**

Significant homology exists between the signalling pathways that control the ISC proliferation of mammals and flies. *Drosophila* wingless (Wg) is required for the maintenance of ISCs and is secreted from muscle cells located adjacent to the basal

epithelium [29]. Wnt which is responsible for the maintenance of ISCs and TA cells in an undifferentiated state is its mammalian counterpart and is similarly active at the bottom of the crypts [30]. Other pathways that are involved in this ISC self-renewal and differentiation are also conserved. These include Notch, JNK, EGFR, and JAK/STAT to sustain gut homeostasis [23]. (**Figure 5**)

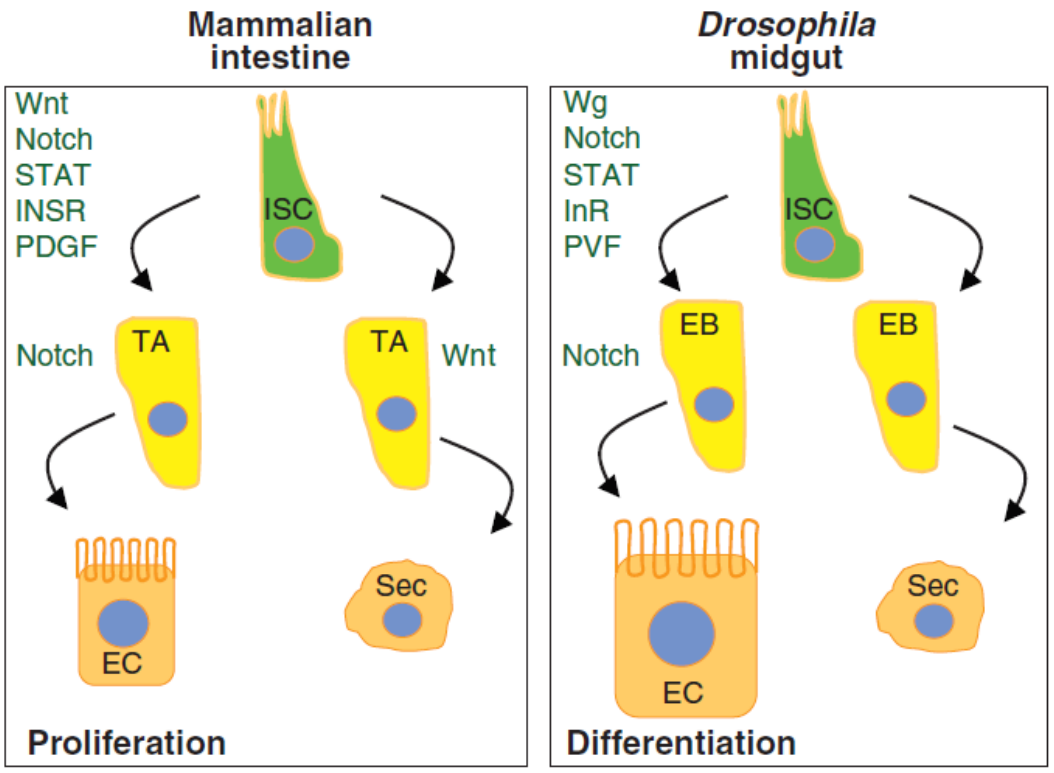



**Figure 5: ISC lineages of mammalian intestine and *Drosophila* midgut, and the common pathways that control them [23].**

Despite the physiological divergence between vertebrates and insects, there is a high degree of conservation between mammalian and *Drosophila* digestive tract. The anatomy, physiological function, tissue and signaling pathways that control intestinal development, regeneration and immune response strongly resemble that of mammals. This shows that flies can be used as a model to investigate basic mechanisms of mammalian intestinal epithelial development, homeostasis, regeneration and diseases.

## Carbohydrate metabolism

Carbohydrate metabolism is essential for all life and it is important for growth, reproduction, and organismal maintenance. Organisms have different cell types and developmental stages and have their metabolic requirements, together with the changing nutrient intake. This poses the need for constant regulation of carbohydrate metabolism [31]. Carbohydrate metabolism focuses on the synthesis and usage of glucose, a major fuel for most organisms. If the energy reserves in the cells are low, glucose is degraded by the glycolytic pathway. Depending on a cell's metabolic requirements, glucose can also be used to synthesize other monosaccharides, fatty acids, and certain amino acids [32]. (**Figure 6**)

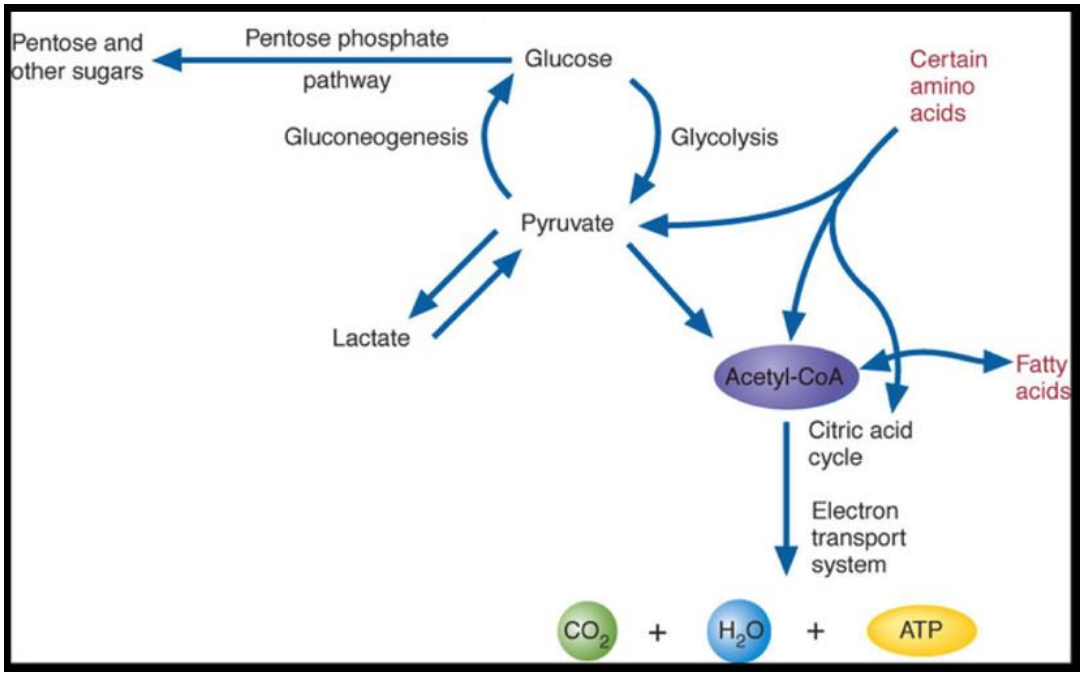


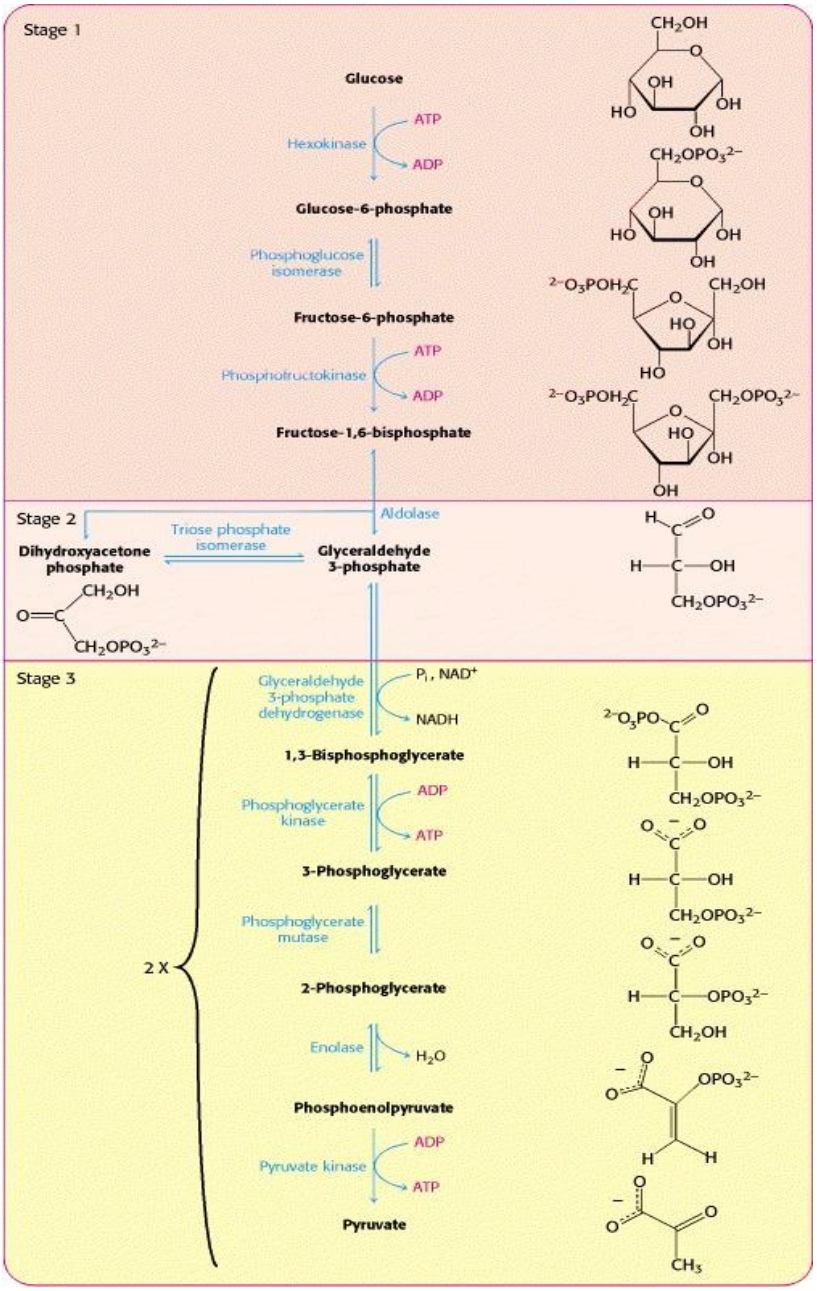

**Figure 6: Carbohydrate metabolic pathway**

The McGraw-Hill Companies, Inc.

## Glycolysis

Glycolysis takes place in almost every living cell. This series of reactions is believed to be among the oldest of all the biochemical pathways. The enzymes, as well as the number and mechanisms of the steps in this pathway, are highly conserved in prokaryotes and

eukaryotes. Glycolysis is also known as the Embden-Meyerhof-Parnas pathway. In this ten-step process, the glucose molecule is split and converted to two three-carbon units (pyruvate) by oxidizing several carbon atoms. The amount of energy captured during glycolytic reactions is stored temporarily in two molecules each of ATP and NADH (the reduced form of the coenzyme NAD+). The pyruvate produced is still an energy-rich molecule, which can yield a substantial amount of ATP. Under aerobic conditions, most cells in the body convert pyruvate into acetyl-CoA and further citric acid cycle takes place and last by electron transport system ATP is produced [32]. (**Figure 7**)



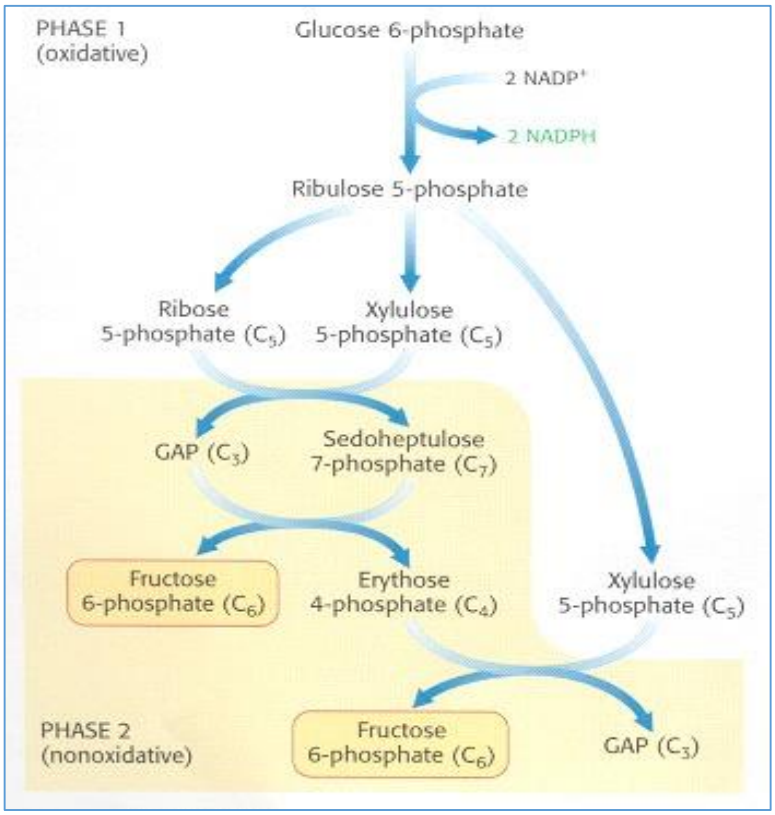

Schematic representation of glycolytic pathway. The glycolytic pathway can be divided into three stages: (1) glucose is trapped and destabilized; (2) two interconvertible three-carbon molecules are generated by cleavage of six-carbon fructose; and (3) ATP is generated.

W. H. Freeman and Company

**Figure 7: Glycolysis**

## Pentose phosphate pathway

The pentose phosphate pathway is an alternative metabolic pathway for glucose oxidation in which no ATP is generated. It produces ribose-5-phosphate, a structural component of nucleotides and nucleic acids along with NADPH, a reducing agent required in several anabolic processes. The PPP occurs in the cytoplasm in two phases: oxidative and nonoxidative. In the oxidative phase of the pathway, the conversion of glucose-6-phosphate to ribulose-5-phosphate is accompanied by the production of two molecules of NADPH. The nonoxidative phase involves the isomerization and condensation of several different sugar molecules. Three intermediates in this process that are useful in other pathways are ribose-5-phosphate, fructose-6-phosphate, and glyceraldehyde-3-phosphate [32]. These pentose and other sugars produced by PPP is used in the biosynthesis of new cellular building blocks like DNA and RNA. (**Figure 8**)




Schematic representation of Pentose Phosphate Pathway.
The pathway consists of (1) an oxidative phase that generates NADPH and (2) a nonoxidative phase that interconverts phosphorylated sugars.

W. H. Freeman and Company

**Figure 8 : Pentose Phosphate Pathway**

## Experimental Strategy

Several recent studies utilize the diet induced T2D (feeding organisms on high sugar diet) model to explore the important changes in metabolic homeostasis and its possible effects on the growth, development, and physiology of an organism. HSD has been linked to obesity, insulin resistance, and several cardiac disorders like cardiomyopathy [33][34][35].

The experimental strategy we followed as shown in (**Figure 9**), has been developed by Ms. Jayati Gera, one of the graduate students in our laboratory. The strategy is to feed the experimental flies (with 2:1 Females to Males ratio) on the High Sugar Diet (1M sugar) after two days of eclosion, while control flies reared on Normal Diet (0.15 M sugar). Two days after eclosion healthy flies were chosen to prepare both control and experimental batches. It has been already studied in our lab that this high sugar feeding fly model resembles the Type 2 Diabetic model from day 13 onwards.

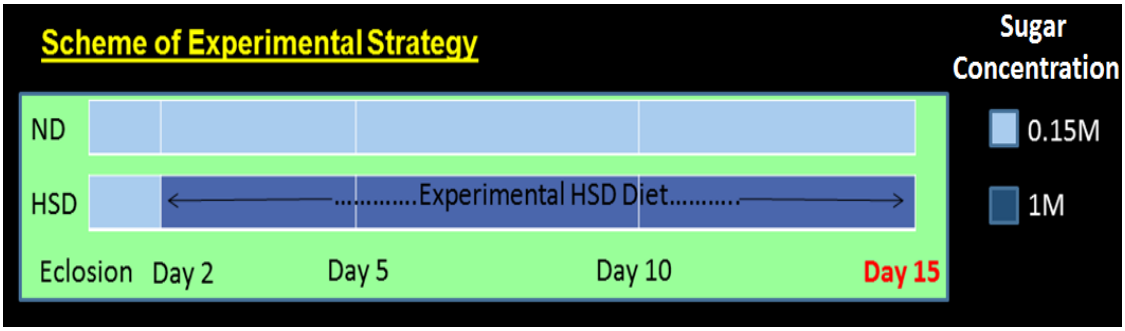



**Figure 9: Schematic Representation of experimental strategy**

High Sugar Diet (HSD) and Normal Diet (ND) batches were made on Day 2 after eclosion. Dissections were done on Day 5, Day 10 and, Day 15. In this diagram Light blue boxes represent Normal Diet and Dark blue boxes represent High Sugar Diet.

It shows T2DM characteristics such as:

1. The increased Glucose level in hemolymph (fluid equivalent to blood).
2. Increased Trehalose (sugar consisting of 2 molecules of Glucose, the principal source of energy in Fruitfly).
3. Tissues develop insulin resistance while insulin production is not affected.
4. With prolonged high sugar uptake, the levels of Triglycerides increase.

5. These files exhibit a shorter lifespan as compared to control flies.

This experimental model itself serves as a powerful tool to understand the effect of HSD in the gut and correlate the effects with T2D conditions in humans.

The previous studies done in our laboratory on the effect of high sugar diet on the gastrointestinal tract of *Drosophila* showed reduction in the size (both width and length) of gut upon feeding HSD. Smurf Assay, *Drosophila* feeding assay to test the feeding, aging, and permeability or leakage -functional defects suggested that there are no feeding defects till Day 15. High sugar diet also showed enhanced cell membrane integrity in the midgut. Parallel studies conducted to check the impact of HSD on the gut commensal bacteria showed a significant reduction in the amount of gut commensal bacteria in HSD fed flies. As a result, the related immune pathway also got affected (reduction in the expression levels of anti-microbial peptides in the midgut). Upon studying the signaling pathways involved in the homeostasis of the gut, it was found that high sugar diet upregulates Notch signaling and downregulates JAK-STAT signaling activity in the midgut. The transcript levels of *upd3* ligand in the midgut reduced on feeding flies on HSD which resulted in the downregulation of the JAK/STAT pathway. Also, earlier studies conducted in the lab showed that high sugar diet results in the depletion of the intestinal stem cell (ISC) population, enhance the absorptive Enterocyte population, and depletes the EE population of the midgut. In response to high sugar in the diet, the fly midgut cells also showed high uptake of glucose. Reduced mitochondrial activity was observed in the high sugar fed flies. Also, results showed the reduction of Fatty Acid Oxidation due to high sugar diet.

## Objective

My MS thesis was designed to address the following two objectives:

1. Rearing adult flies on high sugar diet affects their physiology and metabolism. Previous studies from our laboratory, have demonstrated a gradual shortening of the adult gut of high sugar diet (HSD) fed flies. However, the reason behind this process is not clear. Therefore, one of the objectives of my project was to decipher whether the shortening of the gut is due to reduction in size of individual gut cells or it's an outcome of increased cell death of gut cells or both.

2. Furthermore, previous results from our laboratory have demonstrated that midgut cells of HSD fed flies have increased Glucose uptake capacity. However, the metabolic impact of this process is not clear. Therefore the other objective of my project was to analyze the expression levels of the genes associated with carbohydrate metabolism pathways.

Chapter 2

# Materials and Methods

## Rearing of flies and maintenance

Most of the *Drosophila* stocks used for this study were obtained from The Bloomington *Drosophila* Stock Center. The flies were reared on standard food made from cornmeal, agar, yeast, and fungicides. The flies were maintained at 25ºC in culture bottles if not mentioned otherwise.

## Fly stocks and genotypes

Oregon$^{R}$ : Wild-type laboratory stock of *Drosophila*

## Experimental setup

Freshly eclosed flies (age 0) of the required genotype were collected and reared on normal diet for two days. After two days (age 2), the experimental flies were transferred to a high sugar diet (1M sugar) (2:1 Females to Males ratio) and control flies were transferred to normal diet(0.15 M sugar) ( 2:1 Females to Males ratio). Approximately 40 healthy female flies and 20 male flies were transferred to each bottle. The number of flies reared on each diet are kept equal. The experimental and control fly bottles were flipped every 3 days to maintain a healthy rearing environment. Female flies were dissected at 3 different time points namely, Day 5, Day 10 and Day 15 after eclosion.

## Immunohistochemistry

The guts of adult female *Drosophila* were dissected in 1X PBS followed by fixation in 5% paraformaldehyde for 60 minutes on shaker. After removing the fixative, three 0.3% PBT washes for 15 minutes each were given on 60 rpm shaker for permeabilization of cell membranes. Then gut samples were incubated in blocking solution, 10% NGS (Normal Goat Serum) for 1 hour on 60 rpm shaker. Once blocking is done, the samples were incubated with primary antibody for 18-20 hours at 4ºC in Nunc multiwell plates (All primary antibodies, source, and its dilution are mentioned below). Next day, samples were transferred back into cavity blocks. Then samples were washed three times using 0.3%PBT for 15 minutes each at room temperature on 60 rpm shaker. Before adding, secondary antibody guts were incubated in 10% NGS for 30 minutes (Secondary antibody, source, and its dilution mentioned below). Samples were incubated in

secondary antibody for 16-18 hours at 4ºC followed by three 0.3% PBT washes for 15minutes each. Then samples were incubated with DAPI (1:500 dilution) for 1 hour. After DAPI treatment, samples were washed in 1X PBS for two times of 5 minutes each and mounted in vectashield mounting medium.

## Antibodies used

Primary antibody

| Antibody | Developed in | Source | Antibody number | Dilution used |
|---|---|---|---|---|
| Anti-Dlg | Mouse | DSHB, Iowa | 4F3 | 1:5 |

Secondary antibody

| Antibody | Source | Details | Dilution used |
|---|---|---|---|
| CyTM3-Conjugated AffiniPure Goat Anti-Mouse IgG(H+L) | Jacksons Immuno Research laboratories Code-711-165-152 | Conjugated with cyanine CyTM3 dye (absorption maxima/ emission maxima is 550 nm/ 570 nm) | 1:500 |

## Stains used

DAPI (4',6-diamidino-2-phenylindole dihydrochloride). This is a blue fluorescent dye that binds to A-T rich region in double-stranded DNA. It is used to stain nuclei in live (less efficient) as well as fixed tissues (more efficient). Its absorption maxima/ emission maxima are 351nm/461nm. The working dilution is 1μg/ml.

## Imaging

Images of the mounted samples were taken by using the confocal microscope (Zeiss LSM 780) and processed using Fiji or Image J 9(NIH) software.

## TUNEL assay

Entire guts were dissected from the *Drosophila* adult flies in 1X PBS followed by 1 hour fixation in 5% paraformaldehyde on shaker. After removing the fixative, five times 0.4% PBT washes on a shaker (60 rpm) of 15 minutes each was given. The tissues were incubated in 100mM Sodium citrate solution for 50 minutes at 75°C. Then, the samples were kept at RT for 20 minutes and 3 PBS washes of 10 minutes each was given. Total volume -20 µl of Enzyme Solution to the remaining 180 µl Label Solution to obtain a 200 µl TUNEL reaction mixture was added. Tissues were incubated at 37°C for one and a half hours in the reaction mixture on Nunc multiwell plates. Then samples were washed three times for 10 minutes each using PBS at room temperature (60 rpm shaker) and incubated with DAPI for 1 hour. Samples were washed in 1X PBS for two times of 5 minutes each and mounted in vectashield.

## RNA Isolation

Approximately 20 female *Drosophila* adult guts were dissected out in 1XPBS. Then after centrifugation, the 1XPBS was removed and 200µl TRIzol was added, and the samples were kept in eppendorf tubes at -80°C overnight. The next day, the samples were thawed on ice. Using autoclaved pestles, the samples were homogenized. Then 800 µl of TRIzol was added to the samples and the following mixture was kept at room temperature for 30 min along with intermittent pipetting. After that, the samples were centrifuged at 10,000 rpm for 10 mins (4°C). The supernatants were collected to which 200 µl chloroform was added. The samples were shaken vigorously for 15 seconds at room temperature. Next, the samples were incubated at room temperature for 15 minutes. The resulting mixture was then centrifuged at 4°C for 15 minutes at 10,000 rpm. Three layers were separated, an uppermost aqueous layer containing RNA, a middle layer containing proteins and a lowermost organic layer containing DNA. The upper layer was carefully taken out into a separate RNase-free tube so that the middle layer remained unaffected. An equal volume of isopropanol was added to each sample and incubated overnight at -80°C. On the next day, the sample was incubated for 10 minutes at room temperature to precipitate out the RNA and then centrifuge at 10,000 rpm for 15minutes at 4 °C to pellet out the RNA. The supernatant was discarded without disturbing the pellet. Later the pellet was washed using 500 µl 70% chilled ethanol and the samples were centrifuged at 4°C, 13000rpm for 10

mins. The supernatant was discarded and the pellet was air-dried. Finally; the pellet was dissolved in 20µl autoclaved miliQ water. The amount of RNA was quantified using a NanoDrop spectrophotometer (Thermofisher).

## cDNA synthesis

cDNA synthesis was performed using the cDNA synthesis kit (Verso) using the protocol suggested.

| **Components** | **1X** |
|---|---|
| RNA + water | 10µl |
| cDNA mix | 7µl |
| RNA primer mix | 2µl |
| Verso Enzyme Mix | 1µl |

cDNA mix comprises of the following components:

| **Components** | **Ratio** |
|---|---|
| RT enhancer | 1 |
| dNTP mix | 2 |
| 5XcDNA synthesis buffer | 4 |

Primer mix comprises of the following components:

| **Components** | **Ratio** |
|---|---|
| Random hexamer | 1 |
| Oligod T | 1 |

The following PCR reaction was followed for the cDNA conversion:

| | **Temperature** | **Time** | **Number of cycles** |
|---|---|---|---|
| cDNA synthesis | 42°C | 30 min | 1 |
| Inactivation | 95°C | 2 min | 1 |
| Forever | 4°C | ∞ | - |

## Qualitative Polymerase Chain Reaction

The following reaction mixture was used to set up the PCR as shown below:

| **Components** | **1X** |
|---|---|
| 10X PCR Buffer | 2.5 µl |
| 5mM dNTPs | 0.5 µl |
| Forward Primer | 0.5 µl |
| Reverse Primer | 0.5 µl |
| Template DNA | 1 µl |
| Taq DNA polymerase | 0.1 µl |
| Water | 19.9 µl |

The following reaction setup was used to run the PCR:

| | **Temperature** | **Time** | **Number of cycles** |
|---|---|---|---|
| | 95°C | 3 minutes | 1 |
| Denaturation | 95°C | 30 seconds | 40 |
| Annealing | 55°C | 30 seconds | |
| Extension | 72°C | 1 minute | |
| | 72°C | 10 minutes | 1 |
| Forever | 4°C | ∞ | - |

## Gel electrophoresis

## Preparation of 1% Agarose Gel

To prepare 1% agarose gel**,** 1 g of agarose was weighed and added to the conical flask. Next; 100 ml of 1X Tris-acetate-EDTA buffer (1X TAE) was added to the flask. The following mixture was microwaved for 1-3 minutes until the agarose was completely dissolved (swirled the flask every 30 seconds). Then the gel was cooled down for 5 minutes (50 °C). Then 5 µl of ethidium bromide (10µg/ml) was added to the gel and gently mixed. The gel was then transferred to a casting tray with the well comb in place. The gel was then left to solidify at room temperature for 15-20 minutes.

## Loading samples and running an agarose gel

The solidified agarose gel was transferred to the electrophoresis unit. The gel box was then filled with 1X Tris-acetate-EDTA buffer until it completely submerged the gel. Then the 6x loading dye was added to each of the loading sample. Molecular ladder and 15 µl of each sample were added to the wells. The gel was run at 80 V, approximately for an hour, until the dye has reached 70% down the gel. The gel was then visualized using a Gel doc system.

## Real-Time Quantitative Polymerase Chain Reaction

The following components were used to set up the qPCR reaction as shown below:

| **Components** | **1X** |
|---|---|
| Sybr Green | 10µl |
| Forward Primer | 1µl |
| Reverse Primer | 1µl |
| Template DNA | 1µl |
| Water | 7µl |

The following reaction setup was used to run the qPCR reaction:

| Temperature | Time | Number of cycles |
|---|---|---|
| 95°C | 3 minutes | 1 |
| 95°C | 10 seconds | 40 |
| 55°C | 50 seconds | |
| 95°C | 30 seconds | 1 |
| 65°C | 5 seconds | 1 |
| 95°C | 5 seconds | 1 |
| 4°C | ∞ | - |

## Buffers and Reagents

- **10X PBS**: For a volume of 500ml, 40g NaCl, 1g KCl, 7.2g $Na_2HPO_4$ and 1.2g $K_2HPO_4$ were weighed and sequentially dissolved in.480 ml $dH_2O$ by constant stirring. Then the pH was adjusted to 7.2 and the volume was scaled to 500ml. Finally, the buffer was autoclaved.

- **0.1%PBT**: For a volume of 40ml, 40µl of 100% TritonX was added to 40ml 1XPBS. The solution was then kept on a roller mixer to completely dissolve the TritonX.

- **0.3% PBT**: For a volume of 40ml, 120µl of 100% TritonX was added to 40ml 1X PBS. The solution was then kept on a roller mixer to completely dissolve the TritonX.

- **0.4% PBT**: For a volume of 40ml, 160µl of 100% TritonX was added to 40ml 1X PBS (pH 7.2). The solution was then kept on a roller mixer to completely dissolve the TritonX.

- **Sodium citrate solution 100mM**: For a volume of 20ml, 588mg of tri-sodium citrate anhydrous was dissolved in 0.1% PBT.

- **10% PFA**: For a volume of 2ml, 0.2g of PFA was weighed and 2ml volume was made with 1X PBS. This was kept in waterbath(65°C) till it dissolves. While in waterbath every 5 minutes interval, for easy dissolving it was mixed by shaking. After dissolving it was kept for cooling down.

Chapter 3

# Results and Discussions

## High sugar diet results in decreased size of Midgut cells

Previous studies done in our laboratory demonstrated that there is a decrease in the gut size of *Drosophila melanogaster* due to feeding on high sugar diet. Importantly, this reduction in gut size begins on day5 aged flies, that are fed on HSD for 3 days and by day15 the gut samples demonstrate a drastic drop. In order to identify the cause of this size reduction, we analysed the size of individual gut cells on day5, day10, and day15 after hatching. For this purpose, I dissected out the guts of adult flies either fed on HSD or on normal diet (ND; that served as controls) and immunostained them with antibody against Disc large (Dlg). Disc large mark the cell boundaries.

As evident from **Figure 10A,C,E**, the midgut cells of adult flies on day5, day10 and day15 fed on normal diet demonstrated a gradual reduction. This gradual drop might be an age dependent reduction in cell size. Compared to them, on day5 a modest reduction in cell size is observed in the midgut of flies reared on HSD (**Figure 10B**). However, a significant reduction in midgut cell size is observed on day10 and day15 (**Figure 10D,F**) upon rearing the flies on HSD.

To have a quantitative estimate of this reduction, we measured the size of the individual cells. For this purpose, we measured the cell size of a randomly selected area of a midgut. From one sample 3 areas were selected randomly. Then using ImageJ average area per cell was calculated for the selected region. Similarly, areas were calculated for 5 samples each for ND and HSD fed flies on day5, day10 and day15.

Our results demonstrate that on day5 around 37% reduction in average cell size is observed in the midgut of HSD fed flies when compared to their age isogenised controls (**Graph 1A**). Subsequently, on day10 and day15 further reduction of 50% (**Graph 1B**) and 66% (**Graph 1C**) respectively in cell size is evident.

Taken together, these results clearly demonstrate that gradual reduction in size of individual midgut cells of HSD fed flies is a contributing factor for the observed reduction in the overall size of the midgut in these flies.

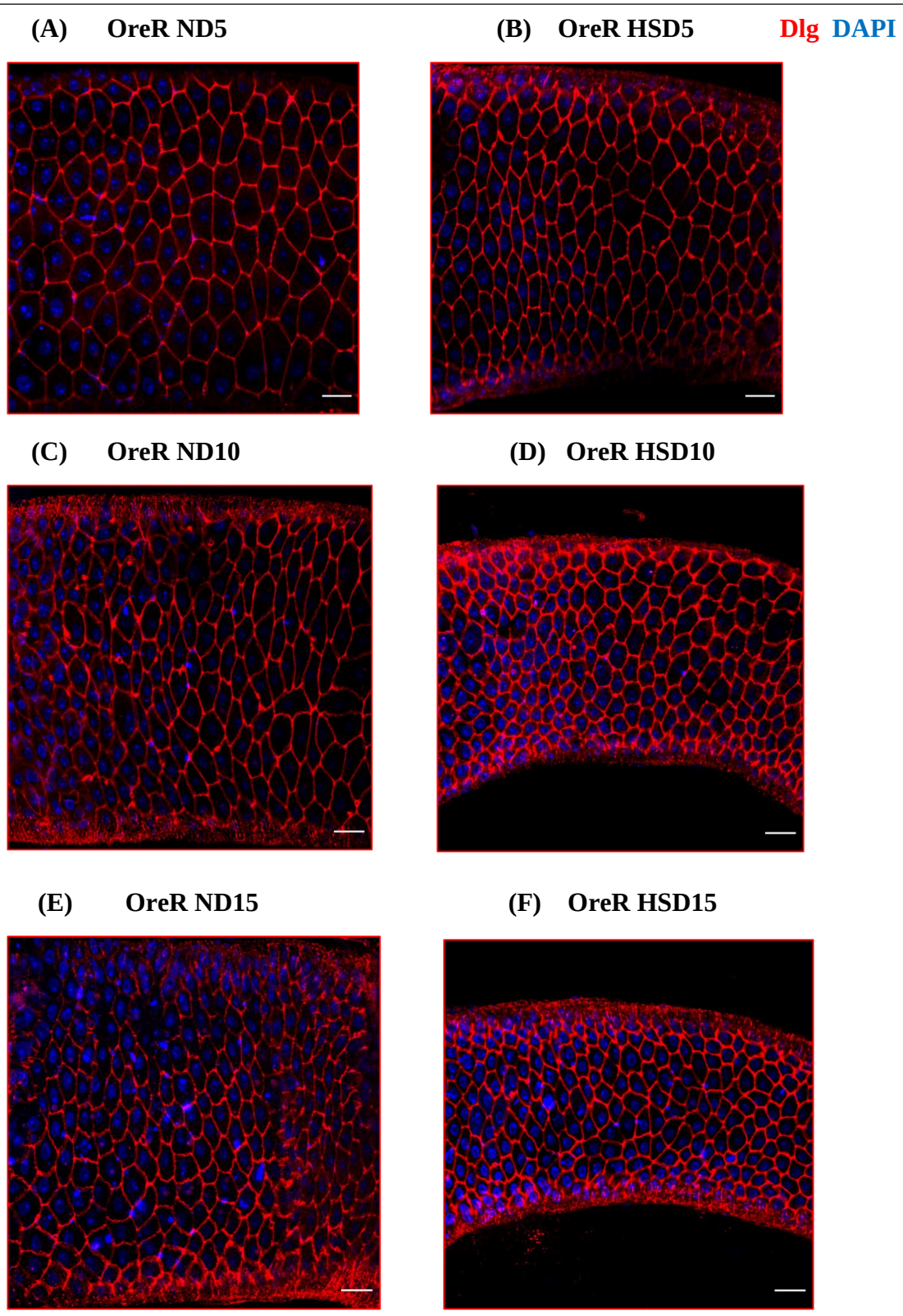
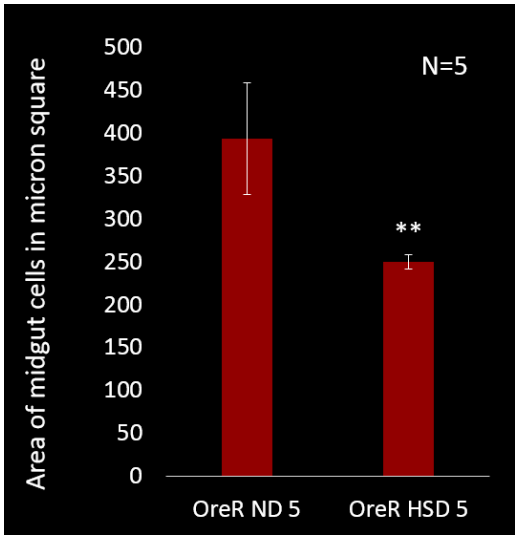


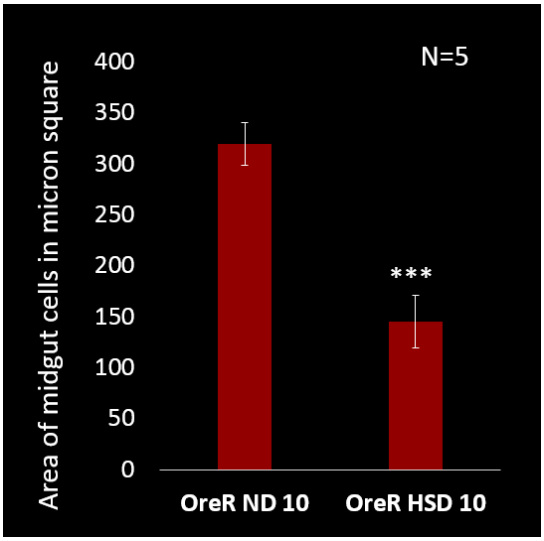

**Figure 10: Reduction in the size of Midgut cells**

Scale bar-20µm. Expression of cell membrane junction marker Dlg in ND and HSD midgut on Day 5 **(A,B)**, Day10 **(C,D)**, and Day 15 **(E,F)**. HSD midgut shows depletion in cell size.

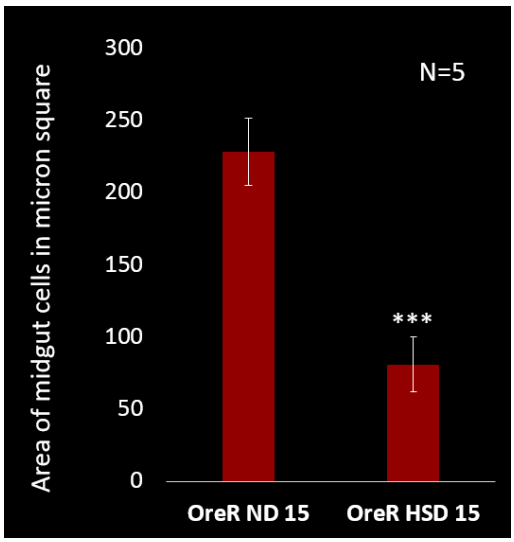
**(A)**




**(B)**




**(C)**




**Graph 1: Area of midgut cells in micron square**

Graph showing the comparison of Area of midgut cells in ND and HSD on Day 5 **(A)**, Day 10 **(B)** and Day 15 **(C).** Stars flag levels of significance. In graph **(A)**, p-value is less than 0.01 and in graph **(B)** and **(C)**, p-value is less than 0.001.

## High sugar diet results in increased cell death in Midgut

Using the cell membrane marker we found one of the reasons for gut size reduction in HSD flies is the reduction in size of individual cells. Another possibility is cell death. So to check the survivability of cells under HSD, we analysed the cell death in midgut using TUNEL Assay (Terminal deoxynucleotidyl transferase dUTP nick end labelling Assay). TUNEL Assay is used to detect the cells undergoing apoptosis, which is a form of programmed cell death. One of the hallmarks of apoptosis is the nuclear DNA fragmentation by nucleases. These enzymes are activated by caspases, the family of proteins that execute the cell death program. TUNEL assay is a method that takes advantage of this and detects apoptotic cells. In this Assay, an enzyme called terminal deoxynucleotidyl transferase catalyzes the addition of dUTP nucleotides to the free 3' ends of fragmented DNA. By using dUTPs that are labelled with chemical tags that can produce fluorescence, apoptotic cells can be specifically identified [36].

To study the apoptosis of midgut, I dissected out guts from *Drosophila* reared in ND (as controls) and HSD (as experimental). I conducted the Assay on day 5, day 10 and day 15 aged flies. On day5, there was no apoptosis observed in Normal Diet (ND) fed fly midguts as well as in HSD fed fly midguts (**Figure 11A,B**). On day 10 and day 15, the number of apoptotic cells were more in the midgut of HSD fed flies (**Figures 11C-F**). To quantify the results, I randomly selected 5 areas from one sample and counted the number of red (Tunel) positive cells and blue (DAPI) positive cells. Then I divided red positive cell number with blue positive cell number and took the average of all the ratios. I did this for 5 different samples for each day of ND and HSD. Finally plotted the graph with the data of these 5 samples.

On day 5, there was no apoptotic cells found in ND and HSD fed fly midguts. On day10, there was 7% increase in apoptosis in HSD fed fly midguts comparing ND fed flies (**Graph 2A**). And on day15, HSD fly midguts showed 20% more apoptosis than ND fed fly midguts (**Graph 2B**). As the age increases the cell death also increased. Also, cell death was more in the HSD fed fly midguts. From these results, it is clear that the increase in cell death of midgut cells of HSD fed flies is also a contributing factor for the observed reduction in the overall size of the midgut in these flies.

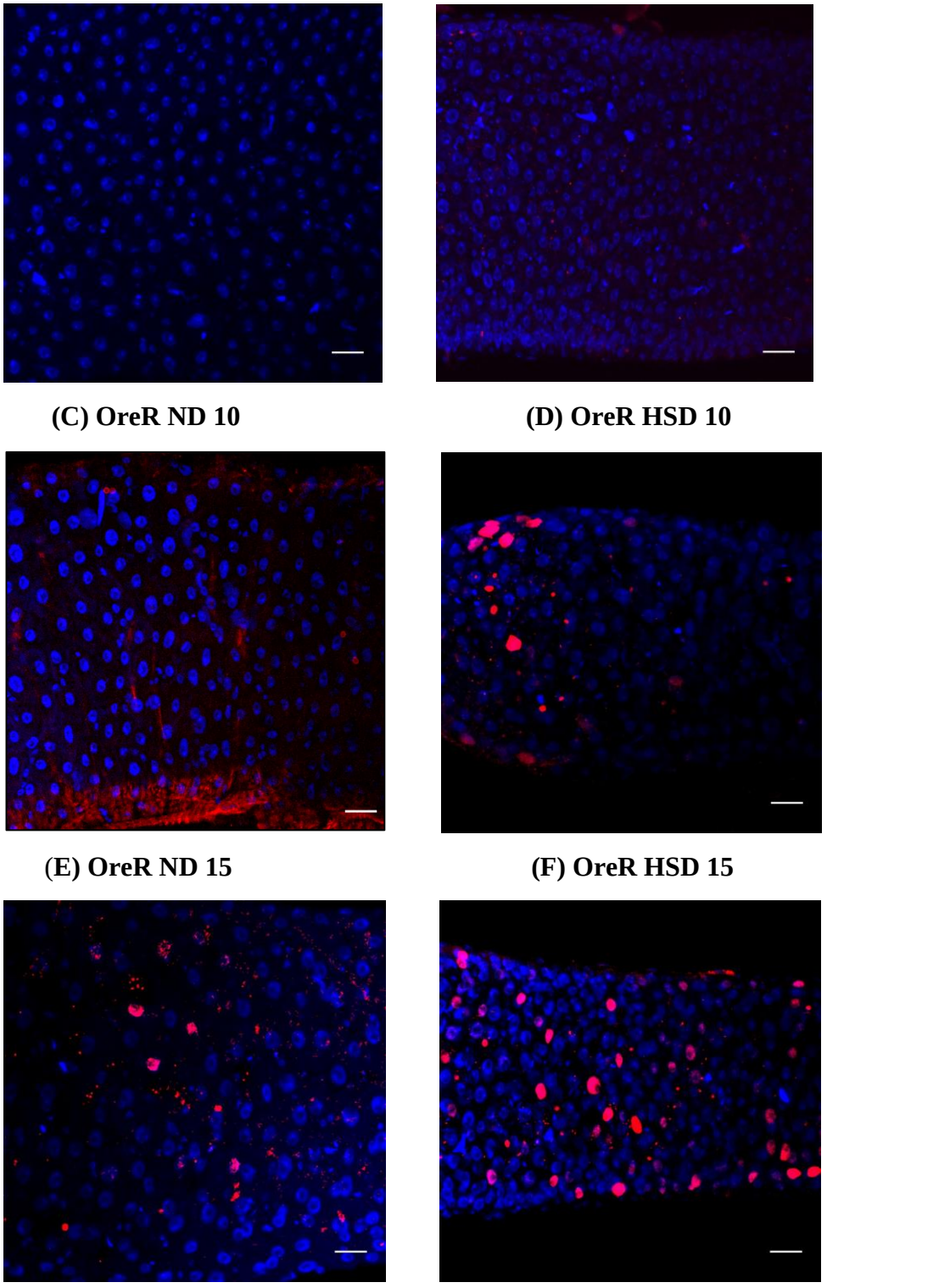
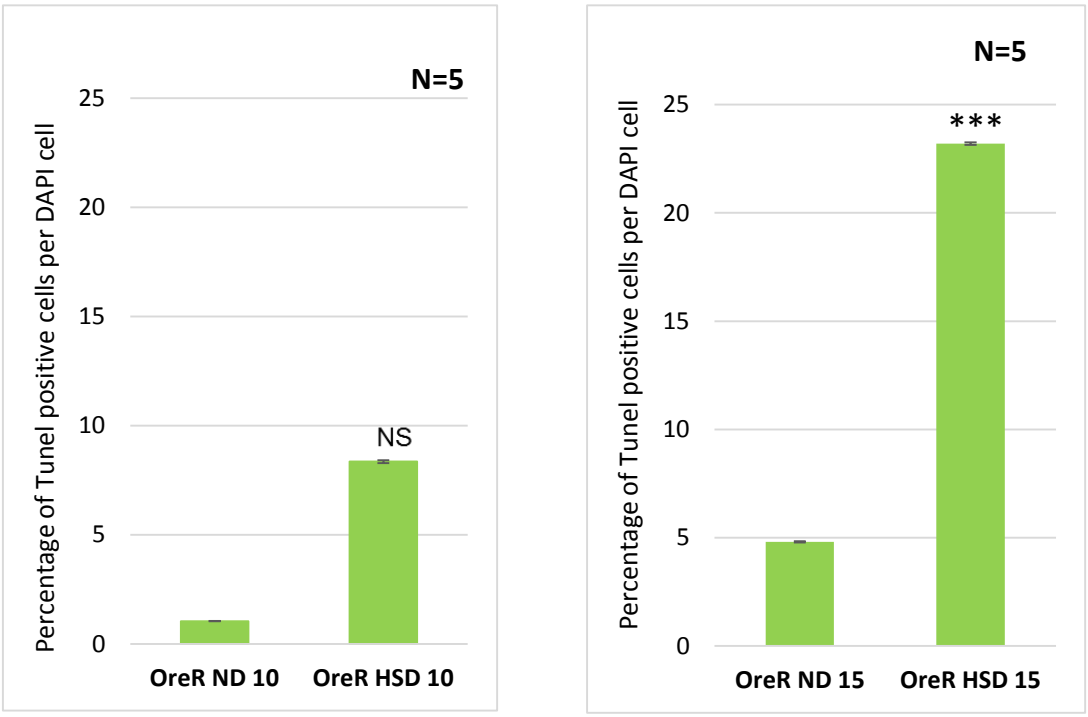



**Figure 11: Increase in the apoptosis of Midgut**

Scale bar-20µm. Apoptosis detected using TUNEL Assay. **(A),(B)** shows day 5, **(C),(D)** shows day10 and **(E),(F)** shows day 15 of ND and HSD respectively. HSD midgut shows increase in cell death.




**Graph 2: Percentage of apoptotic cells in the midgut.**

Graph showing the comparison of percentage of apoptotic cells in the midgut of ND and HSD fed flies on Day 10 **(A)** and Day 15 **(B)** . On Day 5 there was no apoptotic cells observed. The stars flag levels of significance. In garaph **(A)** the difference is not significant (NS) i.e the p-value is more than 0.05. In graph **(B)**, the p-value is less than 0.001.

## High sugar diet does not upregulate glycolysis

Previous studies in our laboratory demonstrated that HSD fly midguts are indeed high on sugar uptake. This was shown by one of my seniors by experimenting using 2-NBDG a fluorescence analogue of glucose and observed an increased uptake in day10 and day15 HSD flies. Quantification data suggested that there is an approximately two fold increase in the glucose uptake by the midgut cells on Day 10 and a significant four fold increase in the sugar uptake by the midgut cells on day 15. Also, there were earlier reports which suggested that administration of various glucose containing carbohydrates have shown to cause postprandial glycemia which triggers the release of insulin and subsequent glucose

uptake by numerous cell types [37]. To understand how high sugar uptake affects carbohydrate metabolic pathway, we did quantitative PCR to check the transcript levels of enzymes of glycolysis and pentose phosphate pathway.

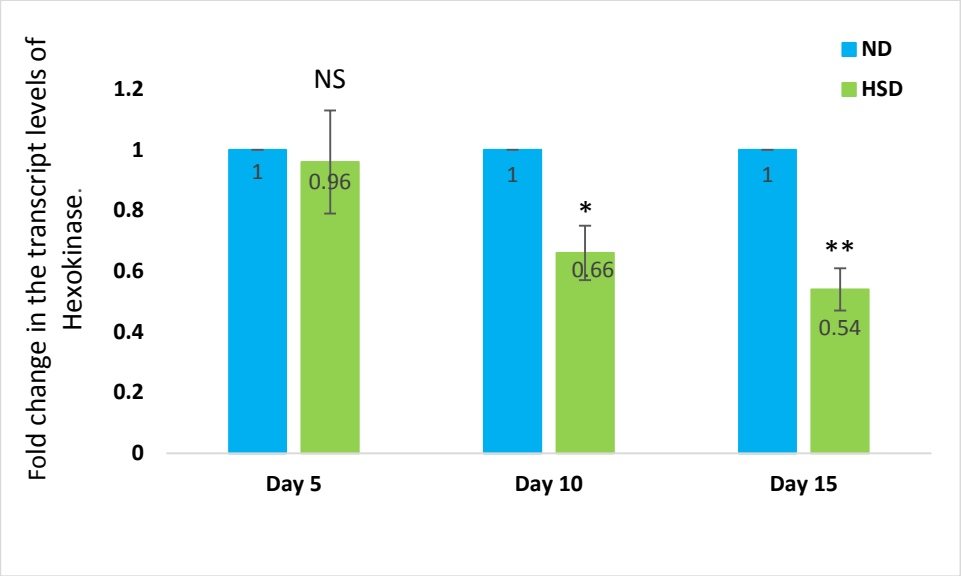
## The transcript level of glycolytic enzyme hexokinase decreases

To check the hexokinase transcript levels mRNA was specifically isolated from the midgut of day5, day10 and day15 aged flies fed on normal and high sugar diet. Then cDNA was synthesised using Verso cDNA synthesis kit. The hexokinase transcript levels were analysed by performing RT-PCR followed by gel electrophoresis. Reaction for each sample was also setup without reverse transcriptase to ensure that the RNA samples are devoid of any contamination. From the gel image, we couldn't show any upregulation of glycolysis.


**Figure 12 : Hexokinase transcript levels in the midgut**

Agarose gel image showing the levels of hexokinase transcript in the midgut of control and HSD flies on Day 5, Day 10 and Day 15 respectively. The plus (+) sign signifies the +RT reactions and the minus (-) sign signifies the –RT reactions

To quantitatively analyse the data we examined the hexokinase transcript levels using quantitative real-time PCR. The results show that there is no significant change in the hexokinase transcript level in day5 samples but in day10 and day15 samples, the hexokinase transcript level gets reduced by 34% and 46% respectively in HSD fly midgut.

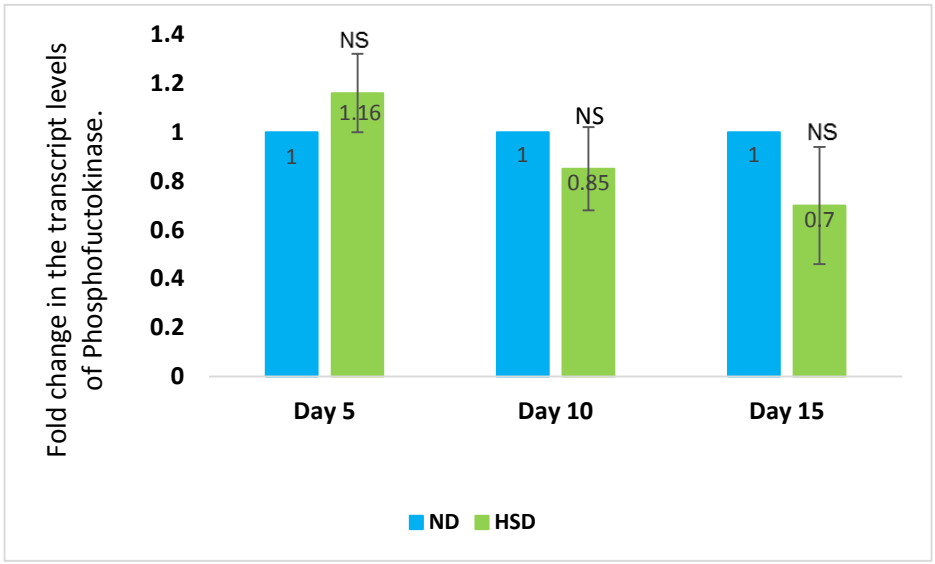



**Graph 3: Hexokinase transcript levels in the midgut**

Graphical representation of reduction in the expression of hexokinase enzyme transcript levels, of HSD fed fly midguts compared to control. The stars flag levels of significance. In this graph, on day5 the p-value is more than 0.05, on day10 the p-value is less than 0.05 and on day15 the p-value is less than 0.01.

## The transcript level of glycolytic enzyme phosphofructokinase does not show any significant change.

The transcript levels of phosphofructokinase were checked similarly as hexokinase by performing the quantitative real-time PCR using the same samples. We couldn't see any significant change in the transcript levels of phosphofructokinase in the HSD samples. This shows that even though there is a higher uptake of sugar, the glycolysis pathway is not upregulated.




**Graph 4: Phosphofructokinase transcript levels in the midgut.**

Graphical representation of the expression of Phosphofructokinase enzyme transcript levels, of HSD fed fly midguts compared to control. The p-value is more than 0.05 in day5, day10 and day15. Therefore it is not significant.

## Discussions

Diet and health are correlated. Changes in the diet have become an important factor for many lifestyle diseases. One such commonly seen disease is type2 diabetics, caused due to the high consumption of sugar. The gastrointestinal tract, one of the largest organs in the body is the first contact of food that an organism consumes. Earlier it was considered as a passive organ majorly responsible for digestion and absorption of nutrients, but this view was changed drastically since the gut has emerged as the major modulator of various other biological processes. The gastrointestinal tract was rarely discussed as an organ system of principal importance for metabolic control. Dysregulated metabolism is often instead attributed to disturbances in other metabolically active organs and tissues such as liver, pancreas, adipose tissue and musculature. Now a considerable amount of evidence exists demonstrating gut-initiated direct and anticipatory metabolic control of distant organs. The present overview connects various metabolic research lines into an integrative physiological context in which the gastrointestinal tract is included [38]. In fact, gastrointestinal surgery is now recommended as a treatment option for type 2 diabetes in obesity [39]. So it has become an important field to study. As energy homeostasis is a fundamental feature in evolution, mechanisms of action discovered in nonhuman models could certainly be of importance. In our study, we looked into the effects of high sugar diet on the midgut of *Drosophila melanogaster*.

Following the previous result of the gut being short and thin in high sugar fed flies, we looked at the possible reasons for this. We found that due to high sugar diet, the individual cell size of midgut decreases. Also, we saw an increased cell death in high sugar fed fly midguts. Parallel studies conducted by my colleague found out that there is a decrease in cell proliferation in HSD fly midguts. All these results give us an explanation for the reduction of gut size.

Even though flies are fed with high sugar diet, we needed to know whether the HSD flies are indeed high on sugar uptake. In this direction, one of my seniors did an experiment to understand the glucose uptake by the midgut cells of normal and high sugar fed flies. She used 2-NBDG a fluorescence analogue of glucose and observed a significant increase in uptake of sugar on day10 and day15 high sugar fed flies. So we checked how high sugar uptake affects the carbohydrate metabolic pathway.

The high glucose uptake can potentially be metabolized by glycolysis or by the pentose phosphate pathway. We looked into the transcript levels of glycolysis enzymes, hexokinase, and phosphofructokinase. We observed a decrease in the transcript levels of hexokinase on day10 and day15 HSD fly midguts. There was no significant change observed on day5. Moreover, we couldn't see any significant change in the phosphofructokinase transcript levels. Thus, this result shows that high sugar uptake is not upregulating glycolysis. In the meantime, my colleague checked the transcript levels of the Pentose phosphate pathway (PPP) enzymes, glucose-6-phosphate dehydrogenase, and gluconolactonase. Interestingly, there was a significant increase in the transcript levels of both the enzymes on day10 and day15. On day5 there is not much change observed. This suggests that most probably after day5, the glucose is shunted toward PPP. On day5 the flies are not much affected by the high sugar diet. But on day10 and day15 the metabolic pathways are getting affected.

From our studies, we were able to find the possible reasons for the gut size reduction in high sugar fed fies. Also, we found changes in the expression levels of the genes associated with carbohydrate metabolic pathways, in high sugar fed flies comparing normal diet fed flies. A deeper study in this work will help us to understand the molecular basis of the defects in order to design therapeutic strategies. Also, a deeper understanding of the role of gut physiology in metabolism will motivate effective preventive lifestyle measures both at the individual and society levels [38].

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