STUDYING THE EFFECTS OF HIGH SUGAR DIET ON THE MIDGUT OF *DROSOPHILAMELANOGASTER*

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

Department of Biological Sciences Indian Institute of Science Education and Research Mohali

Dedicated to my family

Certificate of Examination

This is to certify that the dissertation titled "**Studying the Effects of High Sugar Diet on the midgut of *Drosophila melanogaster***" submitted by Ms. **Ramsi Nilopher (Reg. No. MS15071)** for the partial fulfilment of BS-MS dual degree programme of the Institute, has been examined by the thesis committee duly appointed by the Institute. The committee finds the work done by the candidate satisfactory and recommends that the report be accepted.

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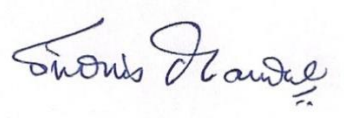


Declaration

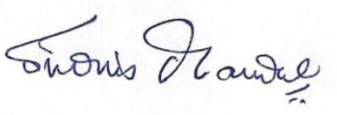
The work presented in this dissertation has been carried out by me under the guidance of Dr. Sudip Mandal at the Indian Institute of Science Education and Research Mohali.

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 contribution of others are involved, every effort is made to indicate this clearly, with due acknowledgement 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.

Ramsi Nilopher
(Candidate)
Dated: June 13,2020.

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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ABSTRACT

A healthy diet is very important for a person's well being. Many chronic diseases like diabetes and obesity are considered as diet-related diseases because of their association with altered food habit of individuals. Considering this fact, we are trying to study the effects of high sugar diet on the midgut using *Drosophila melanogaster* as the model organism. We chose *Drosophila* as our model organism because it has been extensively used as a genetic model to study various metabolic disorders and diseases for the past few years, primarily because of significant conservation of genes and signaling pathways between *Drosophila* and mammals. In this study, the experimental flies were reared on a diet supplemented with 1M sugar in contrast to the control flies that were reared on food supplemented with 0.1 M sugar. Our results demonstrate that high sugar diet does impact the size of individual gut cells, and also affects their proliferation. Furthermore, our analyses of the effects of high sugar diet on the carbohydrate metabolic pathways revealed that while the genes associated with pentose phosphate pathway are upregulated, the expression of key glycolytic genes are either unchanged or downregulated.

CHAPTER 1: INTRODUCTION AND OBJECTIVES

1 INTRODUCTION

The gastrointestinal tract has recently come to the forefront of multiple research fields [1]. It was considered as a passive organ until it has emerged as the major modulator of various biological processes including insulin secretion, energy balance, food intake, immunity, physiology, and behaviour[1[. As a result, what had historically been a relatively unnoticeable organ has now emerged as an important organ in research areas as diverse as stem cell biology, neurobiology, metabolism, and immunity [2]. The digestive system of *Drosophila* shares many similarities with the mammalian system in terms of structural, functional, cellular, and signalling aspects. Here, we are trying to study the impact of the High Sugar Diet (HSD) on the Gut using model organism *Drosophila melanogaster*.

1.1 Diet and Diseases

In today's world, lifestyle diseases are a major threat to human health. These are the disease conditions that arise due to the lifestyle we live, the food we consume, the kind of work we do, the kind of stress we go through, etc. Current lifestyle and dietary changes have led to the increased incidence of chronic diseases such as obesity, diabetes mellitus, cardiovascular disease (CVD), hypertension and stroke, and some types of cancer [3].

The most prominent of diet-based diseases are obesity and diabetes. Globally, sugar is one of the main ingredients in a carbohydrate-rich diet. The increased consumption of sugar-supplemented food is a habit of an unhealthy diet, which leads to unhealthy outcomes such as weight gain, type 2 diabetes mellitus, and CVD. [4]

There are two types of diabetes – Type 1 diabetes and type 2 diabetes. Type 1 diabetes is the condition of autoimmune destruction of pancreatic beta cells. While type 2 diabetes is the condition that occurs when the blood sugar level (glucose) is too high. In the case of type 2 diabetes, the body either can't produce enough insulin or resist insulin. Type 2 diabetes (T2D) is a chronic disease accompanied by various other diseases including obesity, cardiovascular disease, fatty liver, neuropathy, retinopathy, and nephropathy, etc [5].

So, in summary, High Sugar Diet could cause obesity and resistance to insulin which are risk factors for diabetes. Also, the high intake of sugar-based diet is associated with heart diseases [6], cancer [7], kidney disease, tooth demineralisation, etc.

1.2 *Drosophila melanogaster* as a model system

Animal models have historically played a crucial role in the study of diseases, and developing novel therapeutic agents and treatments [8]. Animal models are increasingly being used to study both type 1 and type 2 diabetes. One of the extensively used model organisms to study type 2 diabetes is mouse. However, recently, *Drosophila* has come up as an excellent model to study about type 2 diabetes.

Drosophila melanogaster, colloquially called fruit fly, belongs to the order Diptera, family *Drosophilidae*. For several decades, it has been extensively used as a potential model organism and lead to many milestone discoveries in various fields of biology. It also has many characteristics that make it an ideal organism for the study of animal development, behaviour, neurobiology, genetic diseases etc. The trait for an excellent model system is to share higher physiology within the molecular pathways with humans, and it is remarkable that around 75% of genes related with human diseases have *Drosophila* homologs and also have similarities in their functions [9] [10].

The first reported use of *Drosophila* within the laboratory was by William Castle's group at Harvard in 1901, although the “father” of *Drosophila* research is undeniably Thomas Hunt Morgan. The theory of inheritance first proposed by Gregor Mendel was refined by Morgan, by using *Drosophila* to define genes and establish that they were found within chromosomes [11].

Drosophila is relatively inexpensive and easy to keep. There are generally very rare constraints on their use in the laboratory as there is minimal safety and ethical issues [10].The small size and short lifecycle of *Drosophila* make it feasible for raising a large number of individuals for many generations. There are lots of mutants available in *Drosophila* which are affecting several metabolic pathways and different physiological processes. Also, there are a bunch of genetic tools available that can be employed to understand these processes. *Drosophila*'s

intermediate level of complexity, in combination with a sophisticated array of genetic and molecular tools, makes it as a potential model organism for the study of basic problems in metazoan biology [12].

1.3 Life Cycle of *Drosophila melanogaster*

The growth and development of *Drosophila* are dependent on the temperature at which it is reared. At 25°C the life cycle from fertilization to fly eclosion takes about ten days. The lifecycle of *Drosophila* consists of four stages - egg, larva, pupa, and fly (**Figure 1**). *Drosophila* has internal fertilization. After fertilisation females lay approximately 50- 100 eggs per day. The larval stage begins after the 24 hours of embryogenesis. There are three distinct phases in the larval stage- first instar, second instar, and third instar. Within 24 hours of egg laying, the embryos hatch into tiny first instar larvae. After 24 hours, it moults and develops into second instar and then into third instar larvae in the next 24 hours. Third instar larvae continue feeding for around 48 hours. Once the third instar larva is mature they climb upward and become sedentary and start preparing for the pupal stage. The larvae get pupated on the wall of the vial or bottle. During this pupal stage, adult structures replace the larval structures which are termed as metamorphosis. The maximum life span ranges between 60 to 80 days.

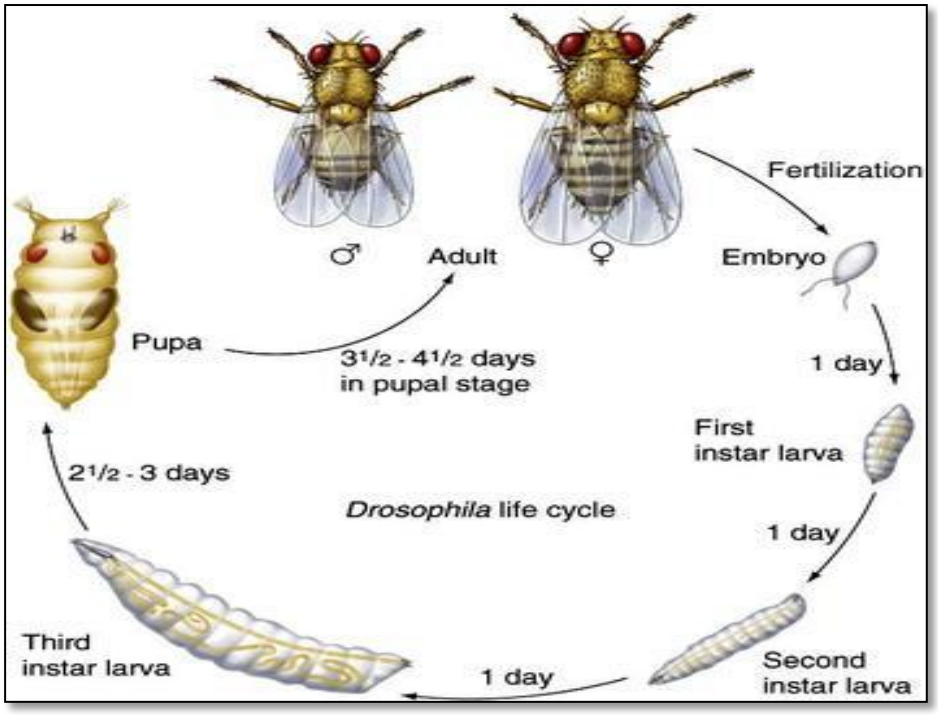


Figure 1Life cycle of *Drosophila* melanogaster
Drosophila has four distinct developmental stages-embryos, larvae, pupae, and the adult fly. There are three larval stages. After encapsulation of the 3d instar larva, pupal stage starts. Pupae undergo metamorphosis and after 3-4 days, fly emerges out of the pupal case. [45].

1.4 *Drosophila* as a model for studying alimentary canal

The gastrointestinal tract is one of the largest organs in the body cavity. The gastrointestinal tract is the first organ exposed to diet and has a critical role in the context of nutritional physiology. It is divided into three parts - foregut, midgut, and hindgut (**Figure 2**). The foregut and hindgut epithelium are of ectodermal and midgut epithelium is of endodermal origin. Compartmentalization of the digestive tract is a salient feature because it optimizes digestion by enabling sequential functions ranging from the uptake and processing of food to nutrient absorption and elimination of solid waste [13]. Many features of digestion and absorption appear to be conserved between flies and mammals. The functional complexity,

plasticity, and genetic amenability of the *Drosophila* gut allow us to study developmental as well as physiological questions in this interesting organ [2].

1.4.1 Structural and functional similarity

Drosophila adult midgut is the functional equivalent of the mammalian small intestine where food is digested and the majority of nutrients are absorbed [14]. In structural wise also there are a lot of similarities between *the Drosophila* intestinal system and the mammalian intestinal system (**Figure 3**). Most of the structures in the mammalian intestinal system have an analogous structure in the *Drosophila* intestinal system [15]. The stomach in the mammalian intestinal system is analogous to crop in *Drosophila* intestinal system. Likewise, foregut is analogous to the large intestine, the midgut is analogous to the small intestine, and hindgut is analogous to the rectum.

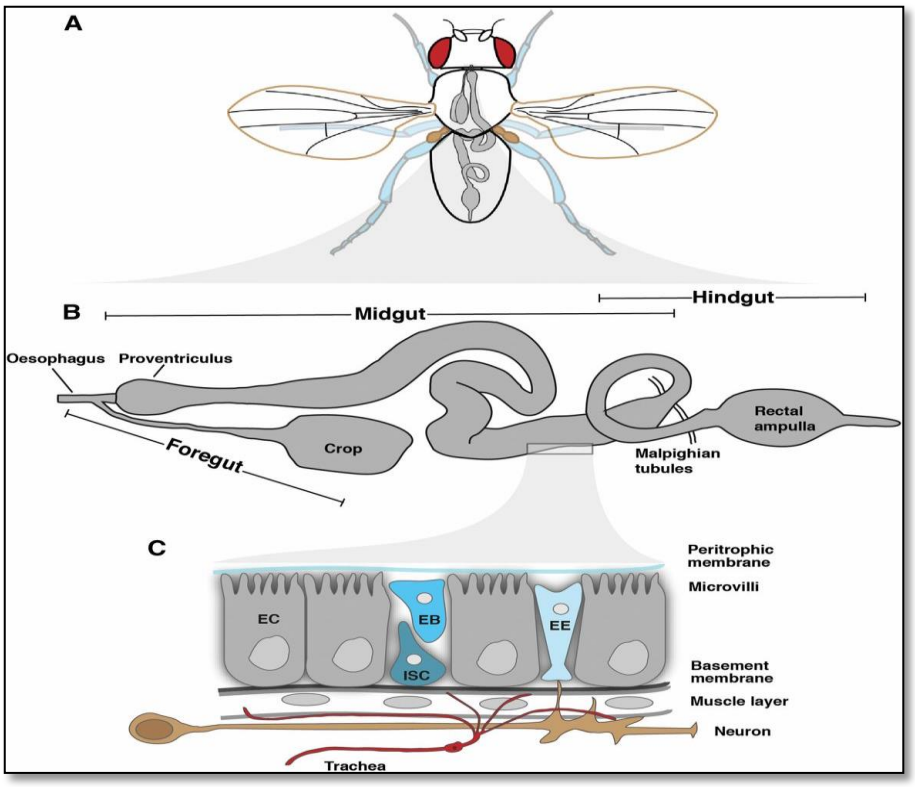


Figure 2 The adult intestine of *Drosophila* and its cell types.
(A) The digestive tract is highlighted in gray inside an adult fly. (B) Main anatomical features of the adult digestive tract. (C) General cellular composition of the digestive tract [1].

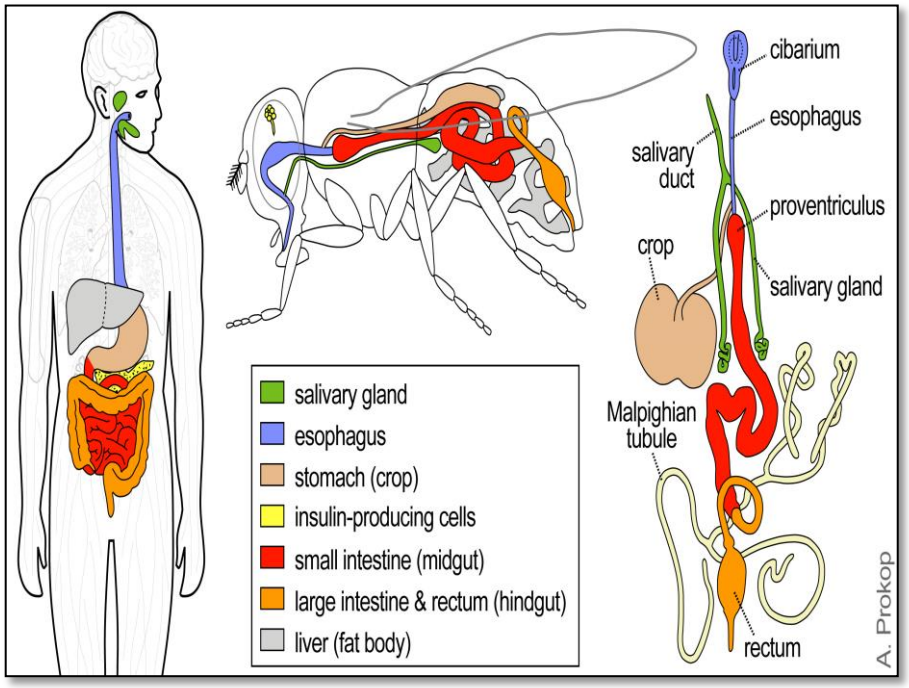


Figure 3 : Structural similarities between mammalian and *Drosophila* alimentary canals.
The alimentary canal of humans and flies has a similar organisation into foregut, stomach, mid- and hindgut.
droso4schools, online resource for school lessons using fruit

1.4.2 Cellular and Signalling level

The adult *Drosophila* midgut comprises of a simple, essentially monolayer cell epithelium enveloped by two layers of mesodermally derived visceral muscles [15]. Both fly and mammalian guts harbour adult intestinal stem cells [16]17] [18] [19]. ISCs undergo division to self-renew themselves and to give rise to committed progenitor cells, called enteroblasts (EBs). Enteroblasts directly differentiate into two conserved functional gut cell types, the absorptive EC (Enterocytes) and secretory (hormone producing) enteroendocrine cells (EEs) [15].Similar to the vertebrate gut, the *Drosophila* midgut is largely comprised of absorptive enterocytes (EC) with interspersed secretory enteroendocrine (EE) cells (**Figure 4**) [18].

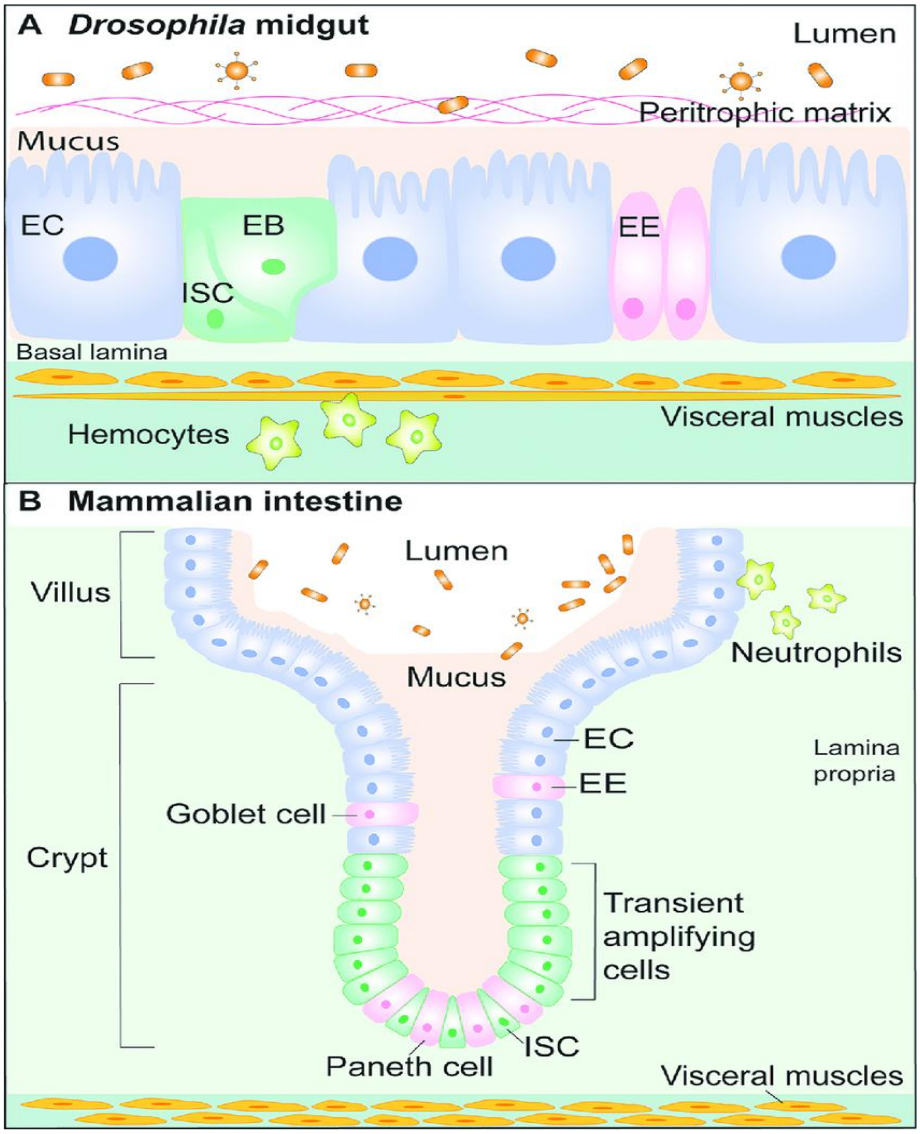


Figure 4: Similarities between the gut epithelia of Drosophila and mammals.(A)The fly midgut is composed of basally embedded intestinal stem cells (ISCs), which undergo differentiation to form absorptive enterocytes (ECs) and secretory enteroendocrine cells (EEs). Enteroblasts (EBs) are transient progenitors destined to differentiate into ECs. (B) Similarly, the mammalian intestinal epithelium is composed of progenitor and Paneth cells and absorptive cells (ECs) and secretory cells (EE and Goblet cells) [46].

Drosophila and mammals show similarity not only in morphology, but also in signalling pathways [20]. At the molecular level, various signalling pathways that are involved in the homeostasis, regeneration, and immune response are similar in mammals and *Drosophila* (**Figure 5**). There is a strong resemblance between *Drosophila* intestinal system and mammalian intestinal regeneration in the case of Wnt/Wg and K-Ras/Ras1 pathways in ISC homeostasis and tumour formation,

respectively [21]. In addition, the conserved innate immune pathways (such as the Imd and STAT pathways) and the production of AMPs and ROS as primary components of the intestinal resistance to infection make *Drosophila* an excellent model for studying basic mechanisms of mammalian intestinal epithelial homeostasis, regeneration and disease[21].

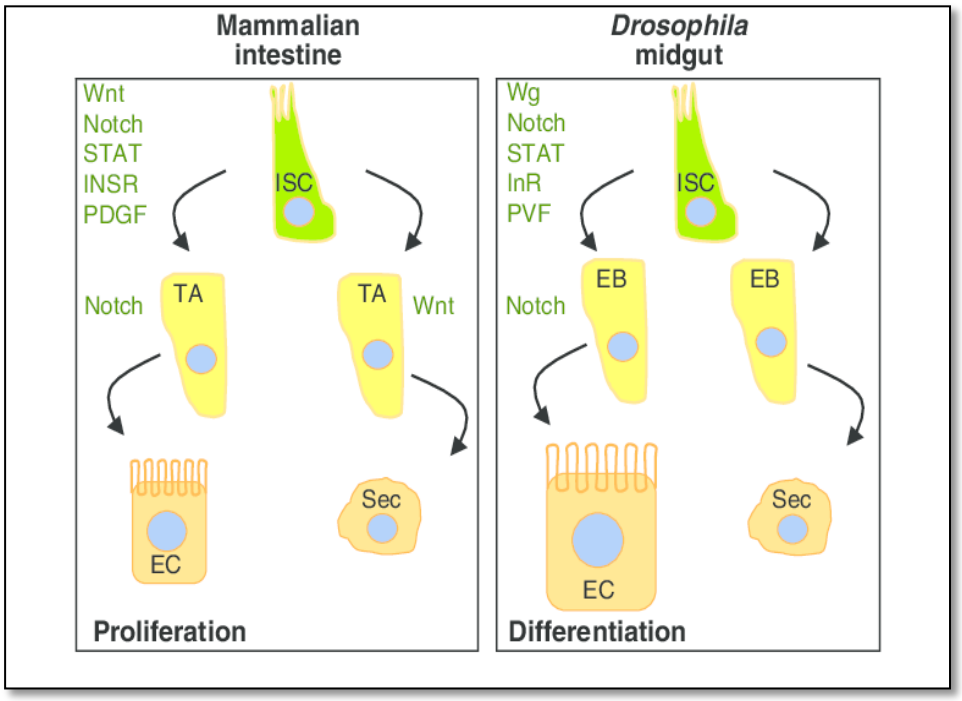


Figure 5: The common pathways that control ISC lineages of mammalian intestine and Drosophila midgut [21].

1.5 Metabolic homeostasis

Although *Drosophila melanogaster* has been used to study various fields of biology for decades, it has only been recently appreciated as a model for human metabolic disease and pathways [22]. Most of the basic metabolic functions found in vertebrates are found in *Drosophila* [23]. Considering this fact, *Drosophila* has been used as a potential model organism to study the fundamental aspects of metabolism during the past few years. Like vertebrates, fly maintains appropriate circulating sugar levels and stores excess energy in the forms of glycogen and lipids, which is reserved for future energy demand [22]. Also, researchers were

successful in generating diabetic and obese phenotypes of *Drosophila*. Hence, along with the availability of powerful genetic tools, *Drosophila* can offer new insights in better understanding of metabolism.

1.6 Regulation of carbohydrate metabolism in *Drosophila melanogaster*

Carbohydrate metabolism is a fundamental biochemical process that is important for maintaining cellular energy balance. The family of carbohydrates includes both simple sugars like glucose and complex sugars like glycogen. In almost all living cells, glucose is the most prominent energy source. To meet the specific metabolic needs of the body, carbohydrate metabolism occurs in an elaborate regulatory mechanism. Some of the important metabolic pathways are glycolysis, pentose phosphate pathway, glycogenolysis, and gluconeogenesis (**Figure 6**).

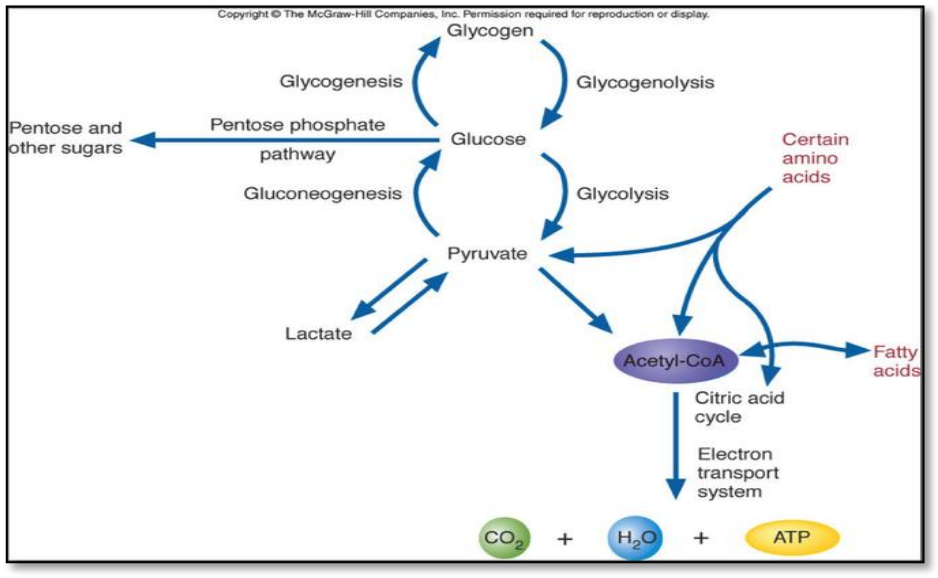


Figure 6: Carbohydrate metabolism.
Metabolism of carbohydrates includes the process where in complex carbohydrates broken down into simple mono- and disaccharides like glucose and sucrose which are important components of several metabolic pathways.

1.6.1 Glycolysis

Glycolysis is a simple pathway of glucose metabolism (**Figure 7**). Depending on cell types, rate of glycolysis is decided at various steps of the process that are

exposed to the control of key metabolic and regulatory enzymes, which include glucokinase, 6-phosphofructo-1-kinase, and 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase. These enzymes are regulated by both nutritional and hormonal signals at the levels of transcription, translation, and post-translational modifications [24].

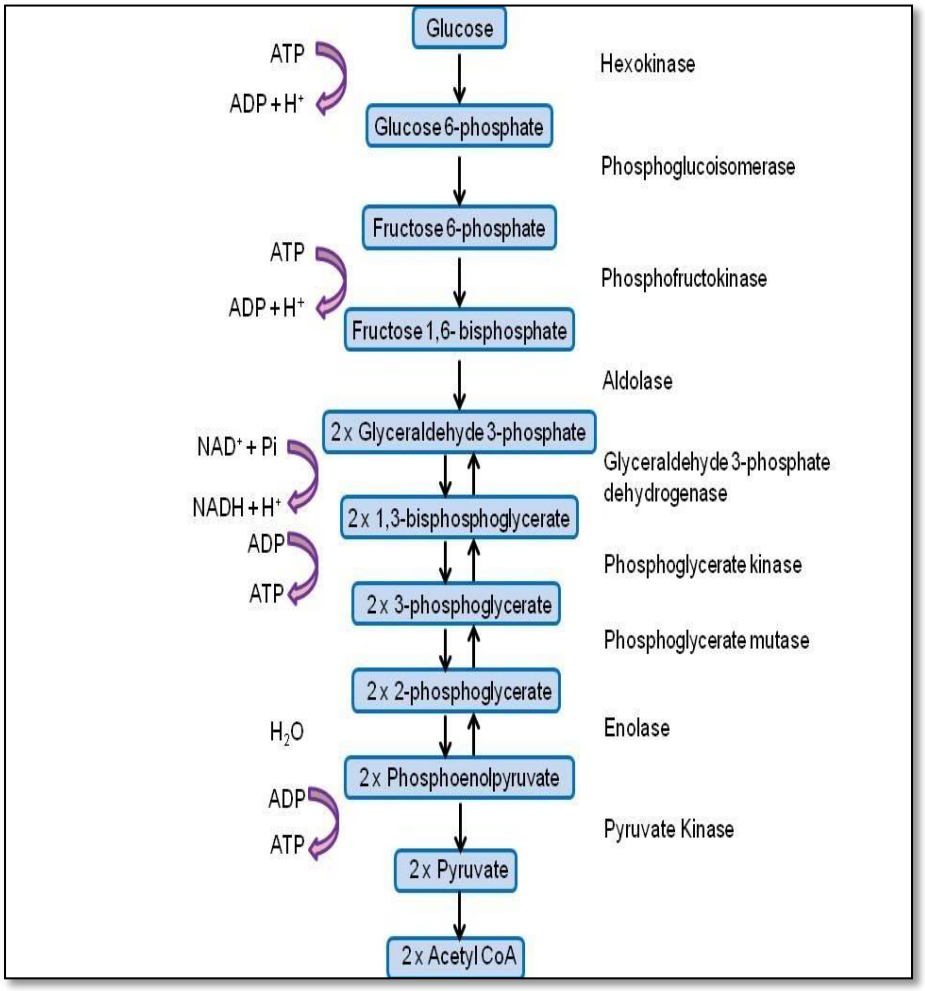


Figure 7: Steps in Glycolysis.
Glycolysis has ten steps. Each reaction in glycolysis is catalyzed by its own enzyme. Glycolysis converts one six-carbon molecule of glucose into two three-carbon molecules of pyruvate.

1.6.2 Pentose Phosphate pathway

The pentose phosphate pathway (PPP), a parallel metabolic pathway to glycolysis, is also a fundamental component of cellular metabolism [25]. The pentose phosphate pathway (PPP), branches from glycolysis at the first committed step of glucose metabolism is required for the synthesis of ribonucleotides and is a major source of NADPH (**Figure 8**). NADPH is required for and consumed during fatty acid synthesis and the scavenging of Reactive Oxygen Species (ROS) [26]. PPP has two phases – the oxidative phase and the non-oxidative phase. In the oxidative phase NADPH is generated and in the non-oxidative phase 5- carbon sugars are synthesized. The glucose-6-phosphate dehydrogenase (G6PD) is the rate-limiting enzyme of the PPP [27]. The PPP, one of the main antioxidant cellular defence systems, has been previously considered limited to its role as a provider of ribose phosphate to the cell. But recently, multiple roles have emerged for this metabolic cascade, involving the cell cycle, apoptosis, differentiation, motility, angiogenesis, and the response to anti-tumour therapy. [28]. All these studies reveal that PPP can provide important insights for a better understanding of diet-related metabolic disorders.

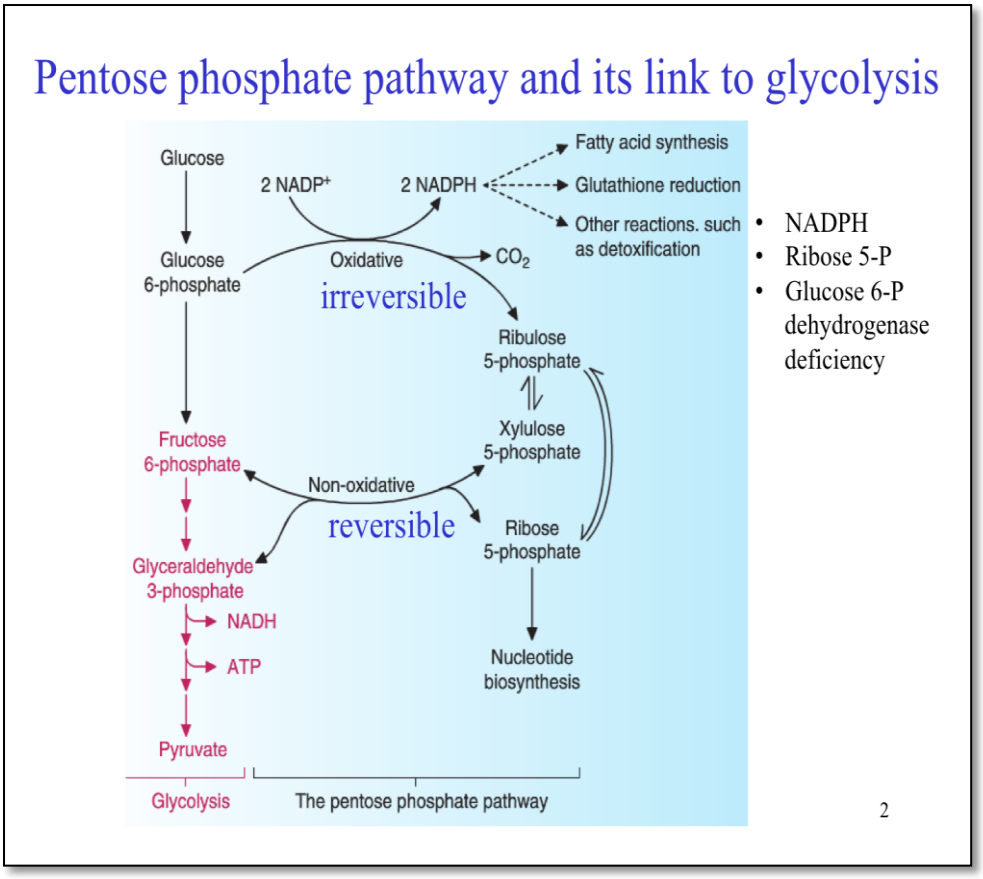


Figure 8 : Pentose Phosphate Pathway.
There are two phases for pentose phosphate pathway – Oxidative phase (Irreversible) and Non-oxidative phase (Reversible).

1.7 Significance of ROS

Gut act as the first line of defence against pathogens by producing Reactive Oxygen Species (ROS) and Antimicrobial Peptides (AMPs) [29]. Intracellular ROS exists primarily in three forms: superoxide anions (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals (OH^-). Alteration in the ROS level is one of the major ways in which metabolism can affect signalling pathways. ROS can directly react with various proteins, such as kinases, phosphatases, or transcription factors, to alter processes that regulate cell cycle progression, apoptosis, quiescence, or differentiation [30][31]. ROS can be considered as signalling molecules that take part in the crosstalk between metabolism and stem cell fate decisions. [30]. Also,

high ROS can trigger ISC proliferation. But the connection between oxidative stress and mitogenic signals in ISCs are still unclear [32].

1.8 Experimental Model.

***Drosophila* as a model system to study the effects of High Sugar Diet.**

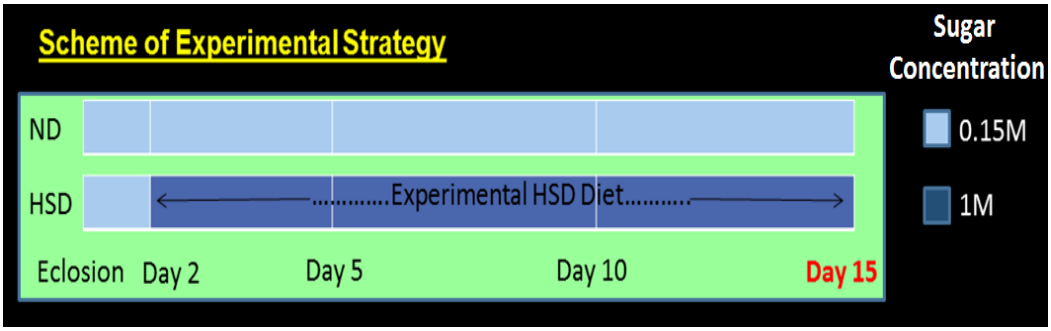


Figure 9: Schematic Representation of experimental strategy.
The experimental batches are made with high sugar concentration (1 M) and control batches are made with lower sugar concentration (0.15M).The batches were made on Day 2 after eclosion (Age 2). Dissections were done on Day 5, Day 10 and, Day 15.

In our laboratory, one of my colleagues, Jayati Gera (PhD scholar) has already designed an experimental model (**Figure 9**) for the study of the effects of high sugar diet (HSD) in *Drosophila*. For this, the flies were reared on high sugar medium (1M sugar). Studies done in our lab have reported that, this model provokes specific aspects of diet-induced diseases like type 2 diabetes. It displays T2DM characteristics such as increased Glucose level in hemolymph (fluid equivalent to blood), increased Trehalose (sugar consisting of 2 molecules of Glucose, the principal source of energy in Fruitfly), insulin resistance with unaffected insulin production from day 13 onwards and TAG accumulation with prolonged sugar uptake. This experimental model itself serves as a powerful tool to understand the effect of HSD in the gut and an excellent model to study about T2D condition in humans.

1.9 Results obtained so far

- Reduction in whole gut size (except crop size) observed in flies reared on HSD.
- HSD does not cause permeability and feeding defects until Day 15.
- HSD affects gut homeostasis by upregulating Notch and downregulating JAK/STAT signalling activity in the midgut.
- HSD reduces the transcript level of upd3 ligand in the midgut.
- HSD results in the depletion of intestinal stem cell (ISC) population.
- HSD diminishes the gut microbiota which results in the reduction of the expression levels of anti-microbial peptides (AMPs) in the midgut.
- Higher glucose uptake by the midgut cells in the high sugar fed flies.

OBJECTIVES

The main of objectives of my MS thesis are as follows:

1. Previous studies from our laboratory demonstrated that the gut of adult flies demonstrate gradual shortening, both in terms of width and length, upon rearing on High Sugar Diet (HSD). However, the reason behind this shortening is not evident. One objective of my study was to characterize the cellular basis of shortening of the gut cells. Specifically, I wanted to determine whether this shortening is due to change in cell shape or it is an outcome of reduced proliferation.

2. The other objective of my study was to perform initial analysis of metabolic activities of the gut cells of flies reared on HSD. Given the fact that midgut cells of HSD fed flies have reduced Glucose uptake capacity, I wanted to check the levels of ROS generated in these cells (readout for OXPHOS) and analyze the expression levels of the genes associated with carbohydrate metabolism pathways.

CHAPTER 2:
MATERIALS AND METHODS.

2 Materials and methods

2.1 Rearing of flies and maintenance

The flies were maintained at 25ºC in food bottles. The flies were reared on food made from cornmeal, agar, yeast, and fungicides. Freshly eclosed flies were collected in a bottle. After 2 days of eclosion (age 2), fly batches of Normal diet (control) and High sugar diet (experimental) were made with 2:1 female to male ratio). Approximately 40 healthy female flies and 20 male flies were transferred to each bottle. The concentration of sugar in control and experimental were 0.15 M and 1 M respectively. To maintain a healthy rearing environment, the experimental and control fly bottles were flipped every 3 days. We used only female flies for our experiments. They were dissected at 3-time points, Day 5, Day 10, and Day 15 after eclosion for most of the experiments.

2.2 Fly stocks and genotypes

Oregon R: Wild-type laboratory stock of *Drosophila* is the fly stock used for this thesis work.

2.3 Immunohistochemistry

Entire guts were dissected from the *Drosophila* adult flies in 1X PBS followed by fixation in 5% paraformaldehyde for 60 minutes on 60 rpm shaker. After removing the fixative, three times 0.3% PBT washes of 15 minutes each were given for permeabilization (60 rpm shaker). Gut samples incubated in blocking solution, 10% NGS (Normal Goat Serum) for 1 hour (60 rpm shaker). Once blocking is done, the sample was incubated with primary antibody for 18-20 hours at 4ºC in Nunc multiwell plates. After the primary antibody incubation samples were transferred back into cavity block. Then samples were washed three times for 15 minutes each using 0.3%PBT at room temperature (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

dilutions) for 1 hour. After DAPI and Phalloidin treatment, samples were washed in 1X PBS for two times of 5 minutes each and mounted (Bridge mounting) in vectashield mounting medium.

2.4 Antibodies used

The following primary antibodies were used for immunostaining.

Sl	Antibody	Raised in	Dilution Used	Source
1	Anti-Armadillo	Mouse	1:3	DSHB, Iowa
2	Anti-PH3	Rabbit	1:200	DSHB, Iowa

Armadillo in Drosophila is a homolog of beta-catenin in vertebrates. Arm antibody marks the adherent junctions at apical cell membranes. . PH3 antibody is a mitosis marker. It stains the condensed chromatin just before chromosomal segregation

For immunostaining the following secondary antibodies were used:

Sl	Antibody	Dilution Used	Source
1	Cy^{TM}3-Conjugated AffiniPure Goat Anti-Mouse IgG(H+L)	1:500	Jackson Immuno Research Laboratories Code-711-165-152
2	Cy^{TM}3-Conjugated AffiniPure Goat Anti-Rabbit IgG(H+L)	1:500	Jackson Immuno Research Laboratories Code-111-165-003

2.5 Stains used

- **DAPI**

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 18 live (less efficient) as well as fixed tissues (more efficient). Its absorption maxima/ emission maxima are 351nm/461nm. The working dilution is 1µg/ml.

- **DHE**

Dihydroethidium (DHE) is a fluorescent probe for the detection of ROS and is specific for superoxide and peroxide radicals.DHE has been shown to be oxidized by superoxide to form 2-hydroxyethidium or by non-specific oxidation by other sources of reactive oxygen species (ROS) to form ethidium.

2.6 Cell Proliferation Assay (Incorporation of EdU)

Gut tissues were quickly dissected in 1X PBS (maximum time: 20 minutes). After dissection, tissues were incubated in Edu solution (Component A – 1:1000 in 1X PBS) for 1 hour on 60 rpm shaker. Then Edu solution was removed and samples were directly fixed in 5% PFA for 1 hour. After fixation, in order to permeabilize the cells, the tissues were washed four times with 0.3% PBT of 10 minutes each (60 rpm shaker) followed by35 minutes of blocking with 10% NGS solution in 0.3% PBT. Then Edu staining solution was used to stain the samples for 45 minutes at room temperature in Nunc plate under dark conditions.

Sl No:	Components	For 100 µl
1	10 X click Reaction buffer	8.6
2	Copper sulphate	4
3	Alexa Fluor	0.24
4	10X Reaction buffer Additive	1
5	Water	86.16

After incubation with the Edu staining solution, two washes with 0.3% PBT for 10 minutes followed by one wash in 1X PBS were given under dark conditions. Then samples were incubated with DAPI for 1 hour. After DAPI staining, samples were washed in 1X PBS for two times of 5 minutes each. Finally, mounted in vectashield.

2.7 DHE Staining

Firstly, Gut tissues were quickly dissected in 1X PBS (maximum time of dissection should be20 minutes). After that tissues were incubated in DHE solution (1:500 in 1X PBS).Using needles dip all the samples properly in DHE

solution. The DHE staining was done for 15 minutes on rpm shaker Then DHE solution was removed and samples were fixed in 10% PFA. After fixation, two quick washes were given with 1X PBS. Finally, mounted in vectashield.All these steps, except dissection were done in complete dark conditions.

2.8 Imaging

Images of the mounted samples were taken by using confocal microscope (Zeiss LSM 780 and Leica SP8) and processed using Fiji or Image J 9(NIH) software.

2.9 RNA Isolation

Drosophila adult midguts were dissected out (~20) in 1XPBS. After centrifugation, 1X PBS was removed and 200µl of TRIzol solution was added in 1.5 ml centrifuge (Eppendorf) tubes kept on ice. Samples were then kept at -80°C overnight. The next day, samples were thawed on ice. Using autoclaved pestles, the samples were homogenized. 800 µl of TRIzol was added to the samples and the following mixture was kept at RT for 30 min along with intermittent pipetting. Then the samples were centrifuged at 10000 rpm for 10 minutes (4°C). The supernatants were collected to which 200µl chloroform was added. The samples were shaken vigorously for 15 seconds at room temperature. The samples were then incubated at room temperature for the next 15 minutes. The resulting mixture was then centrifuged at 4°C for 15 minutes at 13000 rpm. Three layers were separated out, the uppermost aqueous layer containing RNA, the middle layer containing proteins, and the 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 samples were incubated for 10 minutes at room temperature to precipitate out the RNA and then centrifuged at 10,000 rpm for 10 minutes at 4 °C to pellet out the RNA. The supernatant was discarded without disturbing the pellet. Later the pellet was washed using 500 µl of 70% chilled ethanol and the samples were centrifuged at 4°C, 13000rpm for 10 minutes. 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.

2.10 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 samples. 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.

cDNA synthesis

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

Sl	Components	1X
1	RNA + Water	10µl
2	cDNA Mix	7µl
3	RNA Primer Mix	2µl
4	Verso Enzyme Mix	1µl
TOTAL		20µl

The following reaction was followed for the cDNA synthesis**:**

SI	Event	Temperature	Time	No of cycles
1	cDNA synthesis	42°C	30 minutes	1
2	Inactivation	95°C	2 minutes	1
3	Hold	4°C	∞	-

Qualitative Polymerase Chain Reaction:

SI	Components	1X
1	10X PCR Buffer	2.5 µl
2	5mM dNTPs	0.5 µl
3	Forward Primer	0.5 µl
4	Reverse Primer	0.5 µl
5	Template DNA	1 µl
6	Taq DNA polymerase	0.1 µl
7	Water	19.9µl

The following reaction setup was used to run the PCR:

Event	Temperature	Time	No. 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	∞	-

Real-Time Quantitative Polymerase Chain Reaction

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

SI	Components	1X
1	Sybr Green	10µl
2	Forward Primer	1µl
3	Reverse Primer	1µl
4	Template DNA	1µl
5	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	∞	-

2.11 Buffers and Reagents

10X Phosphate Buffer Solution (PBS): For a volume of 500ml, 40g NaCl, 1g KCl, 7.2g Na2HPO4, and 1.2g K2HPO4 were weighed and subsequently dissolved in 490 ml dH_2O.Then the pH of the solution was adjusted to 7.2. The volume was scaled to 500ml. Finally the buffer was autoclaved.

10% Paraformaldehyde (PFA): For a volume of 2ml, 0.2g of PFA weighed and 2ml volume was made up with 1XPBS. The Eppendorf tubes were then sealed using parafilm and incubated at 65°C in a waterbath until the PFA was completely dissolved.

0.3% Phosphate Buffer TritonX (PBT): For a volume of 40ml, 120µl of 100% TritonX was added to 40ml 1X PBS. The solution was then kept on a shaker to completely dissolve the TritonX.

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.

CHAPTER 3:
RESULTS AND DISCUSSIONS

3 RESULTS

3.1 High sugar diet results in reduction in cell size in the midgut.

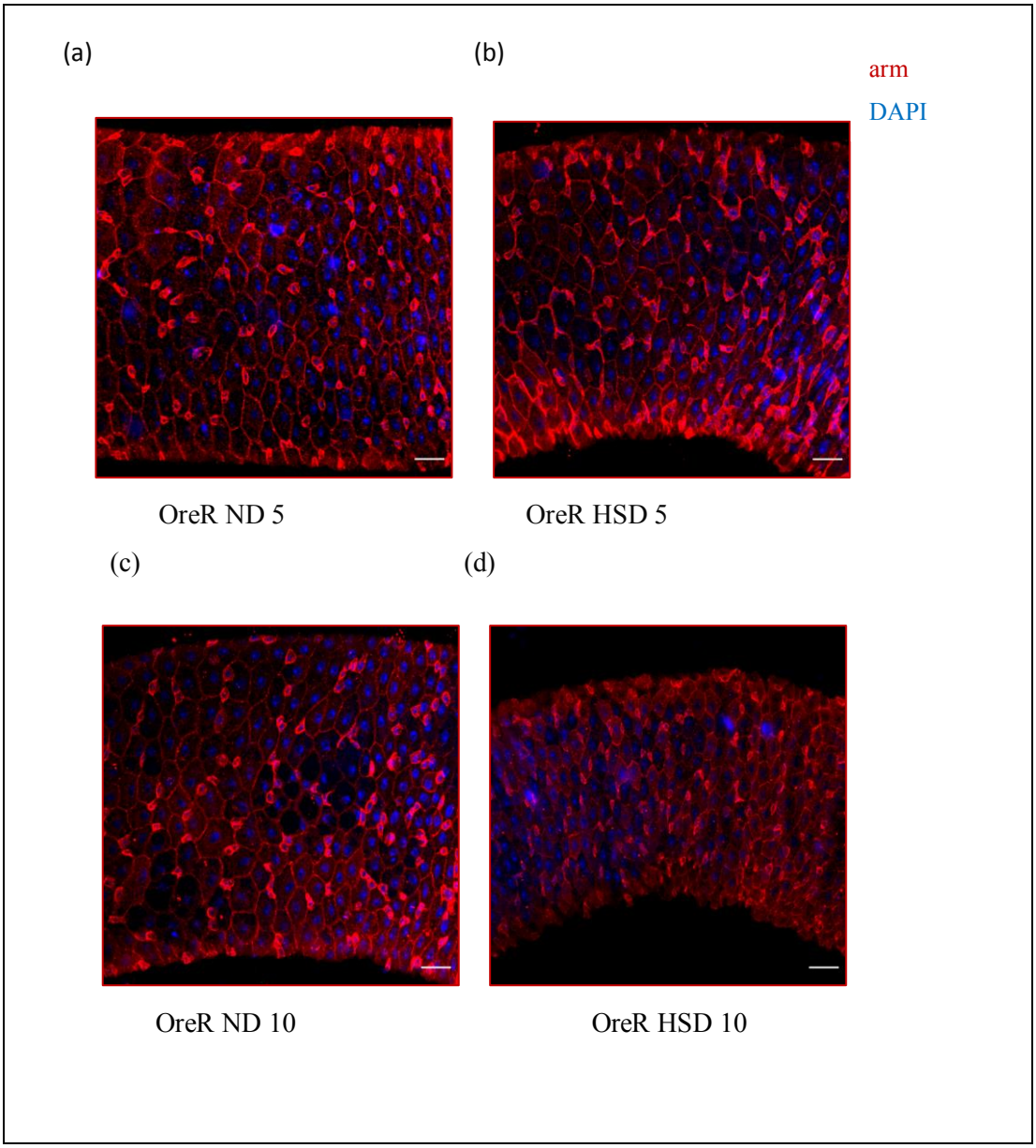
Previous studies from our laboratory demonstrated that upon feeding on high sugar diet, the gut of adult flies demonstrate a gradual shrinkage. This reduction in gut size begins on day 5 (that are fed on HSD for 3 days after 2 days of eclosion) and by day15 demonstrate a drastic drop. To find the reasons behind the gut size reduction, we wanted to analyze the effect at the cellular level on day5, day10, and day15 after eclosion. 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 Armadillo (Arm). The protein Armadillo is the Beta catenin homolog in flies and it forms a strong link between E-adhering and the acting cytoskeleton and is essential for epithelial integrity [33]. Therefore, Arm marks the adherent junctions at apical cell membranes.

As evident from **Figures 10 (a), (b) and (c)**, the midgut cells of adult flies on day5, day10 and day15 demonstrated a gradual reduction in cell size. Compared to them, on day5 a modest reduction in cell size is observed in the mid gut of flies reared on HSD (**Figure 10 (a)**). However, a gradual reduction in midgut cell size is observed on day10 and day15 (**Figure 10 (b) and(c)**) upon rearing the flies on HSD. Since there is a gradual reduction in cell size with age, this might be an age dependent reduction.

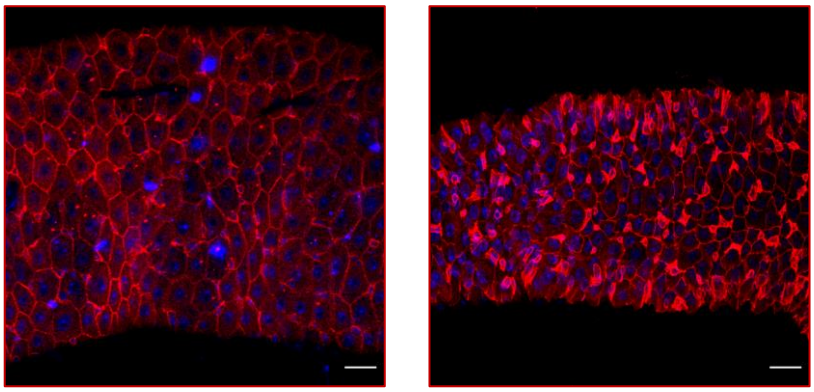
Next, I performed quantitative analyses of the reduction in cell size.To have a quantitative estimate of this reduction, we measured the size of the individual cells. For this purpose, we measured the cell size of randomly selected area of a midgut. From one sample, 3 areas were selected randomly. Then 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.Statistical analyses were performed using Microsoft Excel. For comparison of data groups, p values were determined by Student's two-tail t-test, where the p value was determined by two-tail type 3 t-test. Finally plotted the graph with the data of these 5 samples.

The results found demonstrate that on day5 around (22%) reduction in average cell size is observed in the midgut of HSD fed flies. Subsequently on day 10 and day 15, we observed a reduction in size by (52%) and (59%) respectively. Moreover, the guts cells of HSD fed d flies demonstrated more intense expression of Arm as compared to their controls.

Put together, these results demonstrate that gradual reduction in size of individual gut cells serves as a contributing factor for the overall reduction in gut size as observed in flies reared on HSD. In this context it is important to note that similar results were obtained upon analyses of the gut cells using an independent marker, Disclarge (Dlg).



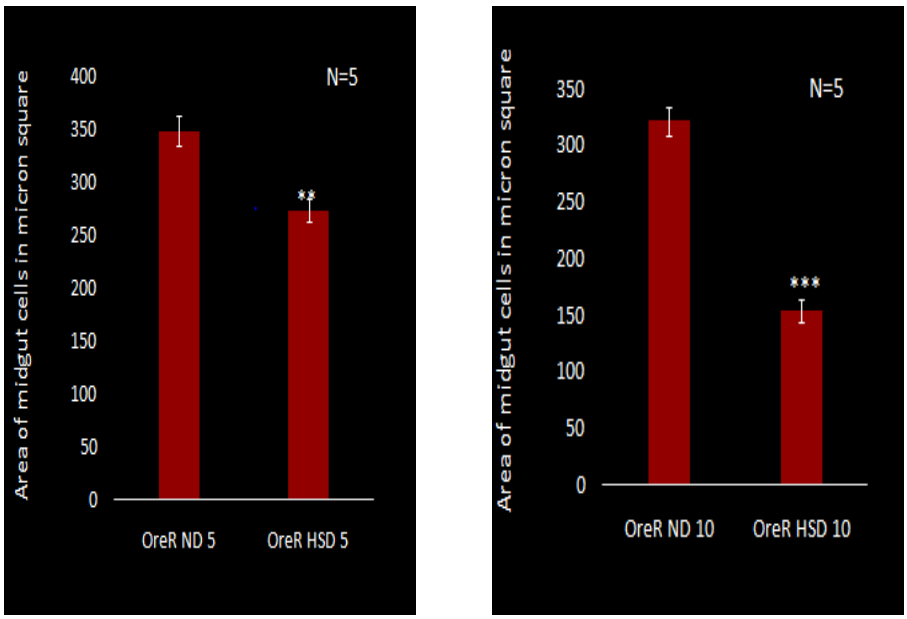
(e) (f)



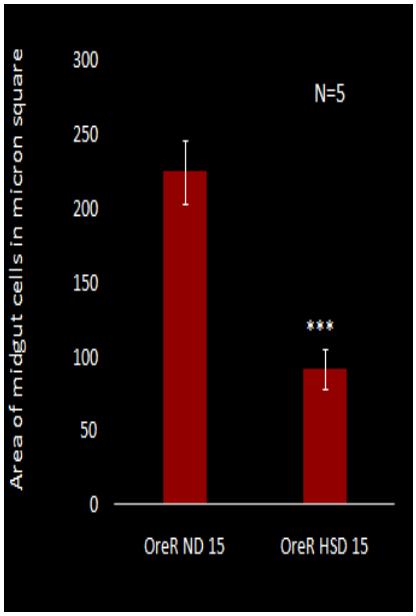
OreR ND 15 OreR HSD 15

Figure 10 : Cell size reduction in midgut cells.
Expression of cell membrane junction marker arm in ND and HSD midgut. Scale bar-20µm.

(a) (b)



(c)



Graph 1: Graph showing the comparison of Area of midgut cells in ND and HSD
(a) Day 5, (b) Day 10 and (c) Day 15.
The (*) value denotes the level of significance.

3.2 High sugar diet results in decreased cell proliferation in midgut – Edu Cell Proliferation Assay.

One of the hypothesized parameter for decreased cell gut size in HSD flies is decrease in cell proliferation. This is because Cell proliferation is the process that results in an increase of the number of cells. So altered cell proliferation can be a potential reason for overall reduction in gut size in HSD. The main problem of diabetes is that cells do not remove glucose from the blood efficiently and thereby leading to hyperglycemia. There are many experimental evidences which suggest that hyperglycemia will lead to disturbed cell cycle regulation and proliferations [34].

We performed Edu cell proliferation assay to check the cell proliferation. Edu cell proliferation assay analyzes cellular proliferation by measuring Edu (thymidine analog) incorporated into the DNA of cells using click chemistry. The incorporated Edu can be encountered through a reaction between ethynyl group of

Edu and a fluorescent azide. It allows sensitive and quantitative detection of proliferating cells and also continuous cell cycle assessment [36].

To study the cell proliferation 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. To quantify the results, I randomly selected 5 areas from one sample and counted the number of red (Edu) positive cells and blue (DAPI) positive cells using ImageJ. Then I took the ratio of red positive cell number and 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. Statistical analyses were performed using Microsoft Excel. For comparison of data groups, p values were determined by Student's two-tail t-test, where the p value was determined by two-tail type 3 t-test. Finally, plotted the graph with the data of these 5 samples.

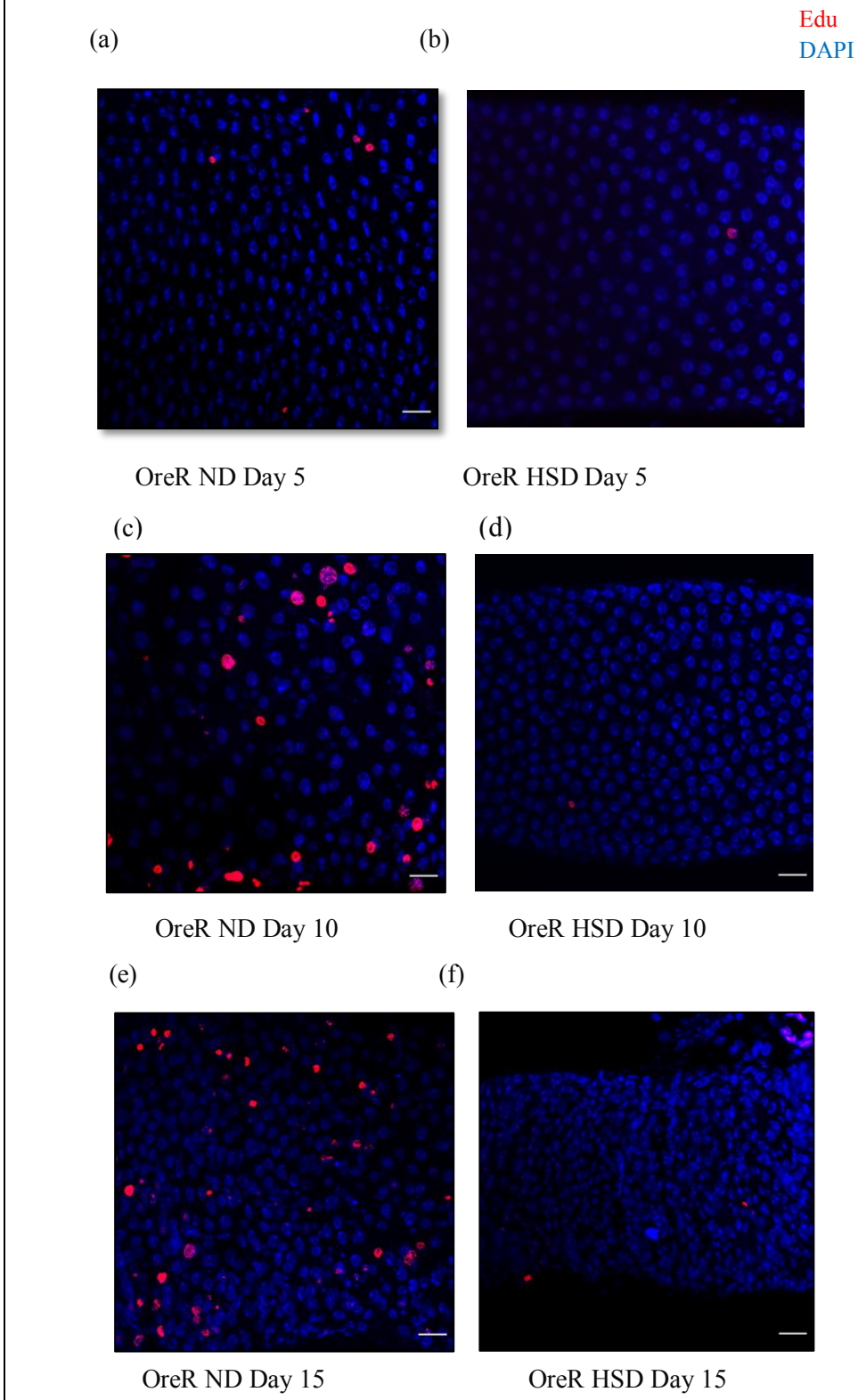
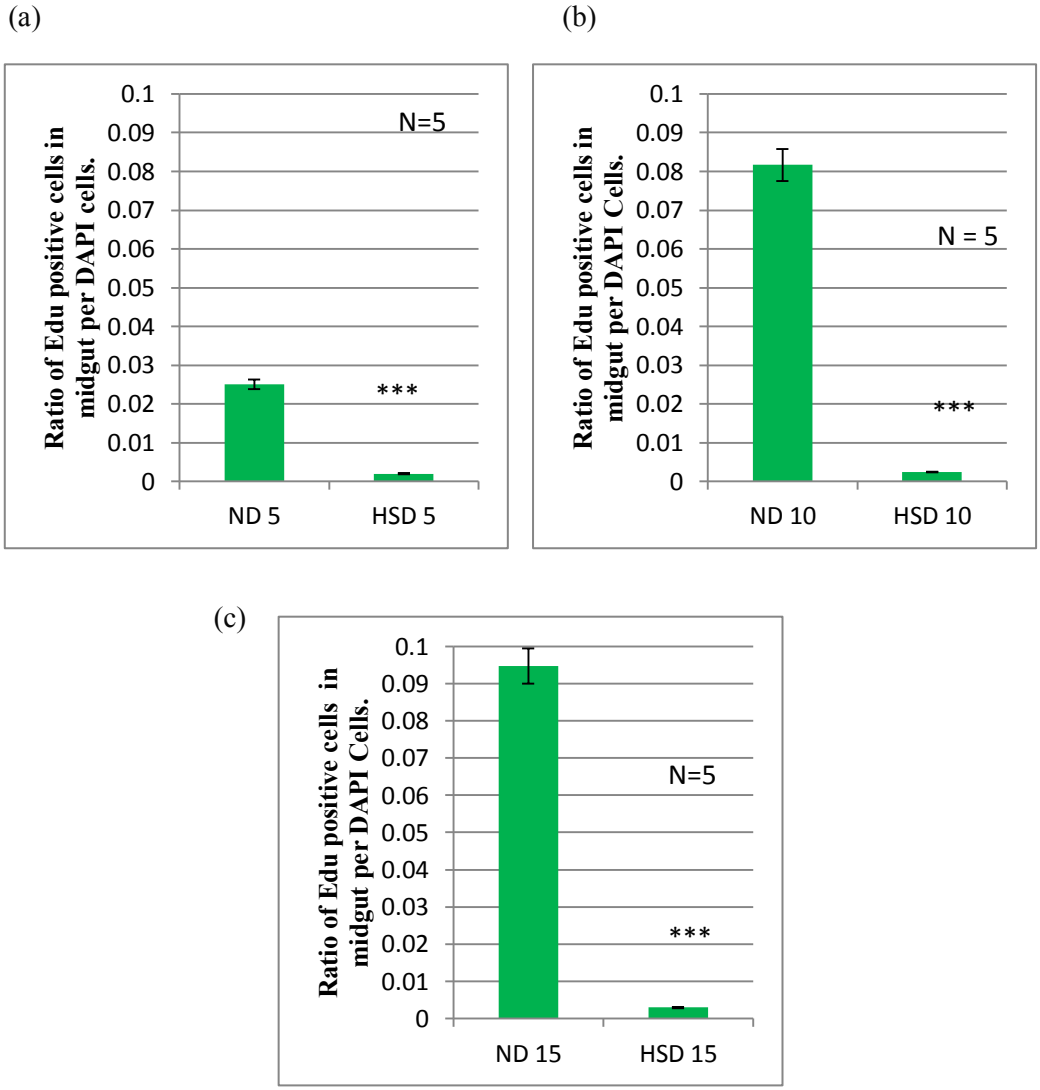


Figure 11 Figure 13 : Cell Proliferation in ND and HSD midguts.
Scale bar-20µm.

In the Edu cell proliferation assay, we observed less number of proliferating cells in high sugar diet midguts as compared to normal midgut (control) on Day 5, Day 10, and Day 15.As evident from **Figures 11 (a), (c) and (e),** the midgut cells of adult flies on day5, day10 and day15 in ND demonstrated an enormous increase in cell proliferation. Whereas in HSD, the cell proliferation is highly compromised. In case of HSD flies it remained almost same on Day 5(**Figure 11(b)**) and Day 10(**Figure 11(c)**). On Day 15(**Figure 11(f)**), we observed a slight increase in cell proliferation in HSD. Overall, as compared to ND midgut cells, the rate of cell proliferation in HSD midgut cells is very low.



Graph 2 : Ratio of Edu positive cells per DAPI cells.
(a) Day 5, (b) Day 10, (c) Day 15.
The (*) value denotes the levels of significance.

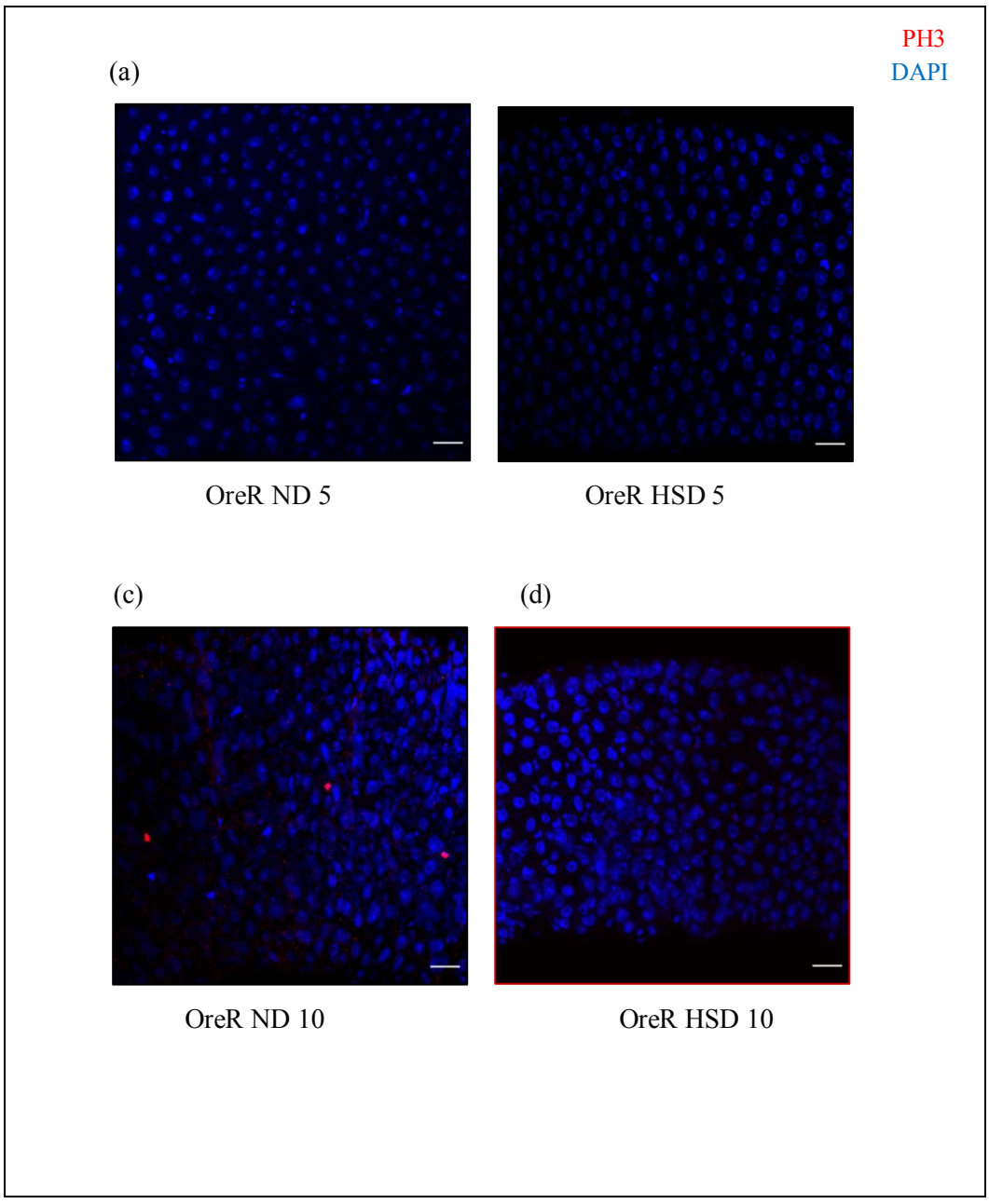
The quantitative data denotes that, there is around 4 fold increase in cell proliferation on Day 10 ND (**Graph 2(b)**) as compare to Day 5 ND (**Graph 2(a)**). And the cell proliferation has increased further by 16% on Day 15 HSD (**Graph2(c))**. But in HSD, although the number of proliferating cells are almost same in HSD day5 (Graph 2(a)), Day 10 ND (Graph 2(b)) and Day 15 HSD (Graph 2(c)), they are highly reduced in compared to their corresponding control couterparts.

3.3 Confirmation of decreased cell proliferation in HSD midguts using PH3 antibody staining.

Cell cycle is an ordered sequence of events that leads to cell division. The stages of the cell cycle are divided into two major phases: interphase and the mitotic (M) phase. The interface consists of G1 phase, S phase and G2 phase.
During interphase, the cell grows and makes a copy of its DNA. During the mitotic (M) phase, the cell separates its DNA into two sets and divides its cytoplasm, forming two new cells.
Edu marks the cells in the S phase. The Edu assay results have already showed reduced number of cells in S phase in HSD midgut cells as compared to control cells. So we decided to check whether the cell cycle is further getting affected at M phase. For this, we performed an immunostaining experiment using mitosis marker PH3.In mitosis, a specialized structure called microtubules are formed by the condensation of nuclear DNA of the cells into visible chromosomes and is pulled apart by the mitotic spindle.
Mitosis takes place in four stages: prophase metaphase, anaphase, and telophase Histone H3 which is a nuclear core histone protein of DNA chromatin plays an important role in chromosome condensation and cell-cycle progression during mitosis and meiosis after phosphorylation of serine-10 and serine-28 residues. Phosphorylation and dephosphorylation happens during late G2 to early prophase and from late anaphase to early telophase respectively. Therefore, histone H3 is in every case intensely phosphorylated and positive for PH3, though interphase doesn't or insignificantly expresses PH3 – a property that permits PH3 to mark just mitotically active cell [37]. PH3 counts should theoretically correlate with

mitotic counts and has emerged as a potential immunohistochemical marker of mitotic activity [38].

To study the cell proliferation of midgut using the mitosis marker pH3, 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. To quantify the results, I randomly selected 5 areas from one sample and counted the number of red (PH3) positive cells and blue (DAPI) positive cells using image. Then I took the ratio of 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.



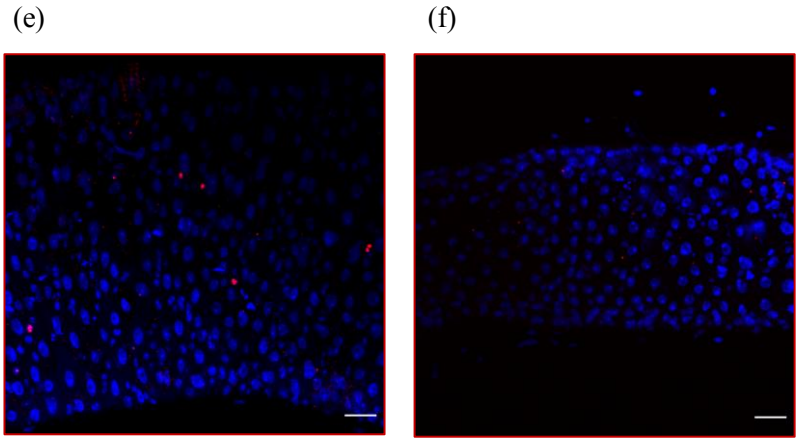


Figure 12: Proliferating cells in mitotic phase in ND and HSD.
Scale bar-20µm.

As expected, we found less number of mitotic cells in high sugar midguts as compared to normal midguts. The number of proliferating cells in mitotic phase in midgut cells of ND and HSD is very low as compared to proliferating cells in S phase in ND and HSD. Surprisingly, we hardly observed any mitotic cells in HSD guts. This result signifies that the cell proliferation is getting highly affected in HSD midguts (**Figure 12)**.

3.4 High sugar diet upregulates pentose phosphate pathway

Previous studies from our laboratory based on the in vivo assessment of glucose uptake by 2-NBDG assay revealed that there is a difference in the glucose uptake between the control and experimental gut. Previous studies in our lab has reported that there is approximately two fold increase in the glucose uptake by the midgut cells on Day 10.On day 15, there is a significant fourfold increase in the sugar uptake by the midgut cells in HSD. Since there is an increase in glucose uptake in HSD, we expected some glucose metabolic adaptations in HSD. Inorder to study about this, we decided to look at one of the major glucose metabolic pathways i.e. Pentose Phosphate Pathway (PPP).

The glucose metabolism consists of highly interconnected pathways. Upon entering cells, glucose is rapidly phosphorylated to G-6-P by hexokinase (HK), and is further metabolized toward glycolysis or the PPP [39]. Under stressed conditions like cancer, PPP plays a critical role in cell survival and growth by producing pentose phosphate for nucleic acid synthesis and providing nicotinamide-adenine dinucleotide phosphate (NADPH), which is required for fatty acid synthesis and cell survival. Therefore, the regulatory network of PPP flux serve as an important metabolic adaptation in stressed conditions [40].

To check the transcript levels of Glucose-6-phosphate dehydrogenase (G6pd) and glucanolactonase, mRNA was specifically isolated from the midgut of day5, day10and day15 aged flies fed on normal and high sugar diet. Then cDNA was synthesised using Verso cDNA synthesis kit. To ensure our samples are fine and devoid of contamination, we performed RT-PCR followed by gel electrophoresis by checking the transcript levels of Upd3 (already validated primer in our lab) in +RT and –RT reactions. Since there are no bands in –RT reactions, our samples are fine (**Figure 13).**

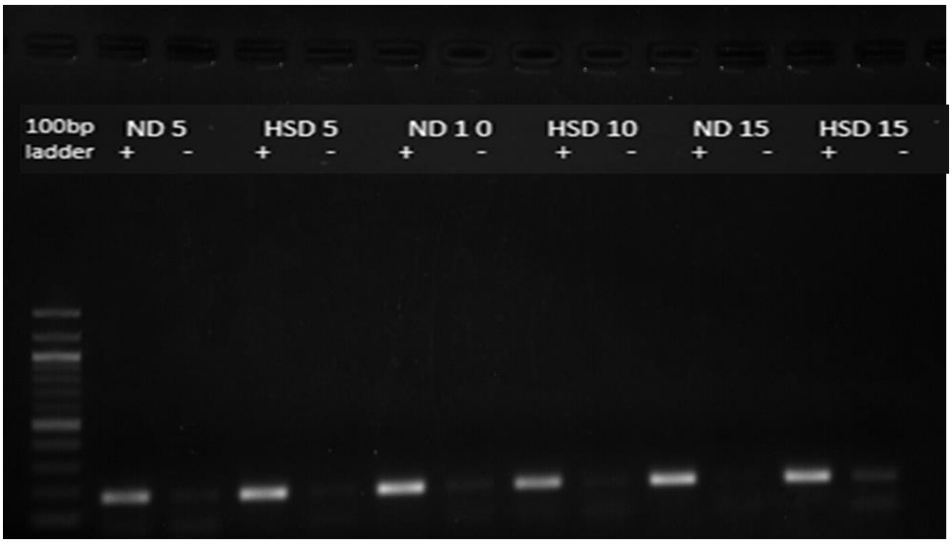


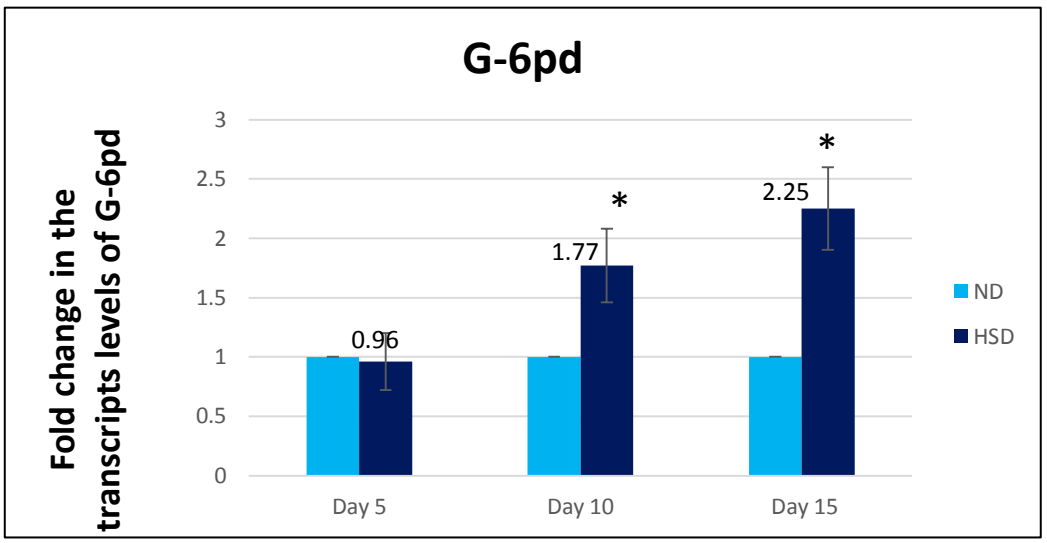
Figure 13: Upd3 transcript levels in the midgut.
Agarose gel image showing the levels of Upd3 in the midgut of control and HSD flies on Day 5, Day 10 and Day 15 respectively. The plus (+) sign signifies the +RT reactions and the minus (-) sign signifies the –RT reactions.

Inorder to quantitatively analyse the data we examined the G6pd and Glucanolactonase transcript levels using quantitative real-time PCR.

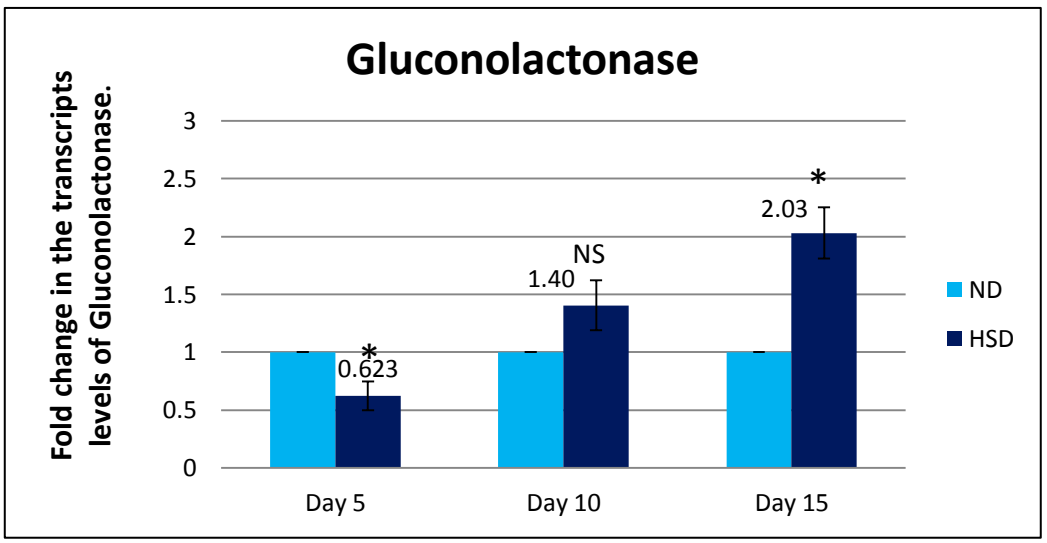
Statistical analyses were performed using Microsoft Excel. For comparison of data groups, p values were determined by Student's two-tail t-test, where the p value was determined by two-tail type 3 t-test. The results show that there is no significant change in the G6pd transcript level in day5 samples but in day10 and day15 samples, the G6pd transcript level gets increased by 77% and 125% respectively in HSD fly midgut (**Graph 3 (a)**).

The transcript level of Glucanolactonase enzyme reduced by around 38% in Day 5 samples. The results showed no significant change in Day 10 samples and in Day 15, the transcript levels in HSD almost doubled in comparison with ND (**Graph 3(b)**). Altogether, these results show that there is an upregulation in pentose phosphate pathway in the midgut cells of HSD fed flies by Day 15.

(a)



(b)



Graph 3 : Transcript levels of PPP enzymes in ND and HSD.
(a) G6pd , (b) Glucanolactonase
The (*) value denotes the levels of significance.
NS denotes not significant.

3.5 High Sugar Diet results in decreased ROS levels in the midgut.

From our so far experiments, we have already found that there is a drop in cell proliferation and observed changes in transcript levels of cellular metabolic pathways in HSD as compared to control.ROS are short-lived unstable and highly reactive molecules which are derived from partially reduced molecular

oxygen that are produced, transformed and eliminated in a variety of cellular processes including metabolism, proliferation, differentiation signal transduction, metabolism, growth, and apoptosis [41].Considering the critical role of Reactive Oxygen species in various cellular processes such as cell proliferation and cellular metabolism, we decided to check ROS levels in HSD midguts.

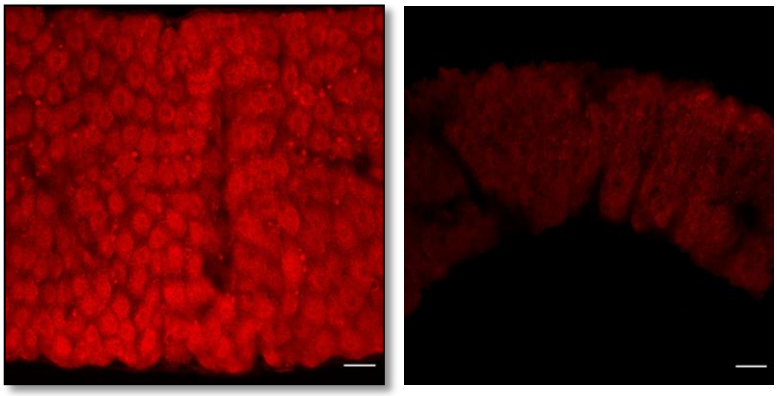
We performed DHE staining to check the ROS levels in the midgut. Dihydroethidium (DHE) measures ROS directly in live cells. The total DHE fluorescence represents the ROS level. A more intense DHE nuclear staining is observed in cells with a high ROS level and less intense staining is observed in cells with low ROS levels.

To study the ROS level in midgut, I dissected out guts from *Drosophila* reared in ND (as controls) and HSD (as experimental). I conducted the staining experiment in age 15 flies (after rearing). To quantify the results, I randomly selected 5 areas from one sample and noted the fluorescence intensity of red using Image. I did this for 5 different samples for each and HSD. Statistical analyses were performed using Microsoft Excel. For comparison of data groups, p values were determined by Student's two-tail t-test, where the p value was determined by two-tail type 3 t-test. Finally, plotted the graph with the data of these 5 samples.

When we performed DHE staining for control and experimental midguts of Day 15 flies, we observed a less intense expression of DHE in HSD midguts (**Figure14 (a), (b)**). From quantitative analysis of these results, we found that there is around 25% reduction in intensity of DHE expression in Day 15 samples (Graph **4**). This suggests that the ROS level in midguts is getting affected by High sugar diet.

DHE

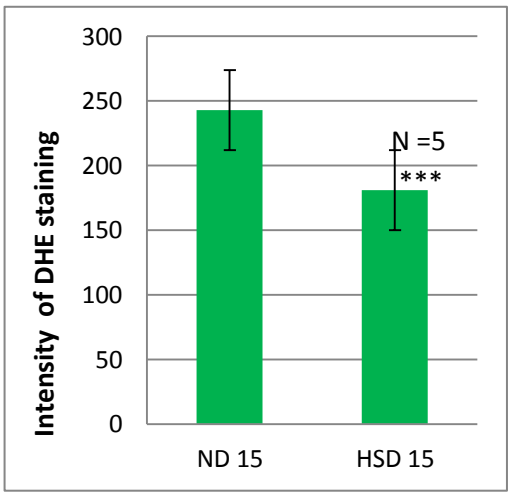
(a) (b)



ND Day 15 HSD Day 15

Figure 14 DHE Staining in Day 15 Samples.
Scale bar-20µm.

(c)



Graph 4 : Intensity of DHE staining in Day 15 samples.
The (*) value denotes the levels of significance.

3.6 DISCUSSIONS

Lifestyle and diet habits have a major impact on human health. Unfortunately, today's world has been adapted to an unhealthy diet which can cause several adverse effects on human health. High sugar intake is a major trend in today's diet habits. This can lead to chronic diseases like obesity and diabetes (T2D).

The gastrointestinal tract is one of the largest organs in the body cavity. The first contact of food happens in the gut. It was earlier considered as a passive organ for digestion and absorption until some remarkable discoveries which uncovered the role of gut in various biological processes

.In our study, we addressed the effects of high sugar diet on midgut using *Drosophila* as the model organism.

Following the observation of reduction in gut size, we found the possible reasons for this as reduced cell size and reduced cell proliferation. And my colleague, Farzana found that there is an increased cell death in midguts of High Sugar diet. Previous studies from our laboratory, by Greeshma and Ashitha, have evidenced that feeding flies on high sugar diet resulted in the depletion of intestinal stem cell (ISC) population and the down-regulated expression of Upd3, one of the ligands of the JAK-STAT signaling. In adult *Drosophila* midgut, the ISCs are the only cells that can undergo mitosis [42]. The cytokines (Upd/s) activate *Drosophila* Jak/Stat signaling in the midgut progenitors, including ISCs and EBs, and this promotes ISC proliferation [43]. Therefore, reduced proliferation might be due to depletion of ISC and downregulation of Jak /Stat. Hence we can say that the reduced cell proliferation in HSD is correlating with these previous results.

In HSD fed flies, it is obvious that there is high sugar intake. But we were curious about whether there is a high glucose uptake in HSD. So one of my colleagues checked the glucose uptake by the midgut cells of normal and high sugar fed flies. For this purpose, she performed 2-NBDG (a fluorescence analogue of glucose) assay and observed a significant increase in uptake of sugar on day10 and day15 high sugar fed flies. Quantification data suggested that there is an approximately two-fold increase in the glucose uptake by the midgut cells on Day as well as day 15.The enhanced glucose uptake can potentially be metabolized by glycolysis or by the pentose phosphate pathway. So we decided to focus on how high sugar

uptake affects the carbohydrate metabolic pathways like glycolysis and pentose phosphate pathway. We checked the transcript levels of pentose phosphate pathway enzymes - *G*lucose-6-phosphate dehydrogenase (G6pd) and glucanolactonase**.** We observed an upregulation of the pentose phosphate pathway in HSD. My colleague, Farzana, performed parallel experiments to check the transcript levels of two key enzymes of glycolysis – hexokinase and phosphofructokinase. From that, we found that the High Sugar Diet decreases the levels of expression of hexokinase transcripts in the midgut. And there is not much significant difference in the levels of expression of phosphofructokinase transcripts in the midgut by feeding HSD. Since we observed high glucose uptake in HSD, we expected an increase in the transcript level of the metabolic pathways in HSD midguts inorder to deal with the high glucose uptake. Surprisingly, we observed an increase in the transcript levels of the key enzymes of pentose phosphate pathway (PPP).Since the pentose phosphate pathway (PPP), branches from glycolysis at the first committed step of glucose metabolism, we can say that the high glucose level in HSD is shunted to pentose phosphate pathway from the first step of glycolysis.

The redox status of the environment regulates and modulates a variety of cellular activities including metabolism, growth, and death [44].Considering this crucial role of Reactive Oxygen species in various cellular processes , we checked the ROS levels in ND and HSD midguts by DHE staining experiment. We observed reduced ROS level in HSD midgut. The redox status of the environment regulates and modulates a variety of cellular activities including metabolism, growth, and death [44]. Hence the change in ROS level might be one of the factors for altered cellular activities in HSD.

From all these results, we found that HSD has great impacts on the midgut cells of *Drosophila melanogaster*. HSD can alter changes in cellular morphology, cell proliferation, cell death, cellular metabolism and ROS levels.

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