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Effect of high fat diet feeding on resistance to bacterial infection in *Drosophila melanogaster*

Bachelor Thesis

Laboratory of Molecular Integrative Physiology in Drosophila

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Annotation: The aim of this thesis was to identify the effect of high fat diet feeding on resistance to bacterial infection and to check the ability to fight the bacteria using *Drosophila melanogaster* model. This work is the basis for providing the methods to investigate the relationship of high fat diet and bacterial infection.

Affirmation: I hereby declare that I have worked on the submitted bachelor thesis independently. All additional sources are listed in the bibliography section. I hereby declare that, in accordance with Article 47b of Act No. 111/1998 in the valid wording, I agree with the publication of my bachelor thesis, in full form to be kept in the Faculty of Science archive, in electronic form in publicly accessible part of the STAG database operated by the University of South Bohemia in České Budějovice accessible through its web pages. Further, I agree to the electronic publication of the comments of my supervisor and thesis opponents and the record of the proceedings and results of the thesis defense in accordance with aforementioned Act No. 111/1998. I also agree to the comparison of the text of my thesis with the Theses.cz thesis database operated by the National Registry of University Theses and a plagiarism detection system.

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Abbreviations used in thesis

D. melanogaster	Drosophila melanogaster
HFD	High fat diet
Sp	*Streptococcus pneumoniae*
CFUs	Colony forming units
ImpL2	Ecdysone-inducible gene L2 (imaginal morphogenesis protein late 2)
AMPs	Antimicrobial peptides
ATP	Adenosine triphosphate
IR	Insulin resistance
UAS	Upstream activation sequence
RNAi	RNA interference
MPS	Mononuclear phagocyte system
FABP	Fatty-acid binding protein
ROS	Reactive oxygen species
Th1/2 Type	Type 1/2 helper
TSB	Tryptic Soy Broth
Hpi	Hours post infection
adgFA	Adenosine deaminase growth factor
eAdo	3′-C-ethynyladenosine
CDB6	Cellulose degrading bacteria
JNKinase	c-Jun N-terminal kinases
Hifla	Hypoxia inducible factor
NFkB	nuclear factor kappa-light-chain-enhancer of activated B cells
MME	Membrane Metalloendopeptidase
TNF	Tumor necrosis factor
Upd3	unpaired 3
FFA	free fatty acids
Il-1	Interleukin
RS	Reactive species
GHSR	Growth Hormone Secretagogue Receptor
LPS	Lipopolysaccharide

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1. Introduction

1.1 General introduction

It is generally accepted that high fat diet induces systemic metabolic changes mediated by immune cells. In case of gene specific knockdown in immune system or genetic depletion of immune cells, particularly macrophages, it can completely reverse pathological effects of high fat diet feeding. Common effects of HFD feeding are increased amount of circulating glucose (hyperglycemia), decreased sensitivity to insulin (insulin resistance) and starvation of insulin insensitive tissues (cachexia) (Matsuzawa-Nagata and Takamura *et al*., 2008; Asp *et al*., 2010).

All these three phenotypes are common with effects of bacterial infection and were shown to be essential for resistance to pathogen. Concurrently it was recently discovered that in early phases of HFD fed animals the immune cells induce mentioned metabolic phenotypes without production of pro-inflammatory cytokines and antimicrobial peptides (antibodies) (Morgantini et al). These are produced in later stages of HFD feeding (Woodcock *et al.* 2015). Involvement of immune cells in both of these processes (resistance to bacterial infection, response to high fat diet feeding) together with similarity of observed outcomes of their activation raises several interesting questions.

The general goal of this work is to investigate the relationship between high fat diet feeding and what is its effect on bacterial resistance. To target these complicated questions, I used the most well-established model's specie *Drosophila melanogaster*.

1.2 Effects of high fat diet on systemic immune response

The systemic inflammatory response is caused due to the effect of some external stressors and acts as a set of physiologic actions that have a mission to fight against this stressor. In addition, a low-grade chronic inflammation that is pertinacious, such those seen in diabetes, obesity and the metabolic syndrome can trigger serious health risks (Bistrian., 2007). As some studies showed, HFD-induced is predominant in contribution to diabetes and cardiovascular disease, however the genetic mechanisms behind it is poorly understood (Birse *et al*. 2010).

On the other side, there is growing body of evidences that immune cells undergo strong metabolic rearrangement during response to infection. Transcriptionally and epigenetically induced rewiring of crucial metabolic pathways is connected with changed requirement of sources such as glucose, glutamine and fatty acids. Dis-balance in intake of these essential energetic sources can significantly influence their accessibility and then immune cell function (Heinrichsen and Zhang *et a*l., 2014).

Particularly in HFD, the high amount of fat results in increase in circulation of fat molecules that are cleaned by macrophages. This effect leads to activation of pro-inflammatory phenotype and systemic hyperglycemia as well as insulin resistance, the processes observed in early stages of inflammation (Woodcock *et al. 2015)*. However, how the chronic state of these signaling pathways influences the resistance to pathogenic bacteria still remains unknown.

1.3 Effects of bacterial infection on *Drosophila's body*

During a potentially life-threatening infection, the body tries to fight back, usually by releasing some chemicals in order to response to that infection. When body is attacked by certain bacteria, the adipose tissue releases FFA when there is increased concentration of catecholamine. Furthermore, the liver uptake of FFA leaves the consequences by promoting the TGFA synthesis and output. This is the form of how to deliver not only FFA but also triglycerides (Spitzer et al., 1988).

Moreover, when there is limited amount of the plasma carrier albumin available, regulatory signals for increasing the release of FFA from adipose tissue can be balanced by the restriction of blood flow which are blocking the release of FFA. These conditions are causing arterial FFA concentration and peripheral FFA to change/decrease. On the other hand, metabolism of triglycerides is altered as well where plasma levels of cytokines are way up. Specifically, TNF and IL-1 cytokines decrease the rate of TGFA approval by smothering the synthesis of lipoprotein lipase. This is one of the possible ways how hypertriglyceridemia might be developed which further leads to cardiovascular diseases (Spitzer et al., 1988).

The level of cytokines needed to inhibit LPL and how often this process happens is not yet very well understood in humans. What is discovered so far is that during the bacterial infection, either by stimulation of mononuclear phagocytosis or release of cytokines, immune system is

activated. Increase of lactate production and glucose uptake by skeletal muscles results from utilization of glucose by immune tissues (Spitzer et al., 1988).

Nevertheless, *D. melanogaster* releases antimicrobial peptides which are essential to fight *Streptococcus*. Several phases can be differentiated during the infection response: Acute phase (until 24 hpi), plateau (24-120hpi) and resolution phase (120hpi and later). An interesting observation can be found in the research of Rolf and his co-workers where they proposed that the timing was actually crucial for AMP's to succeed in reducing the number of bacteria and thus minimizing the opportunity for resistance induction (Haine et al., 2008).

In the next paragraph we will see how the fat and glucose are utilized and distributed during the immune response to infections.

1.4 Selfish immune system theory

In "Selfish brain theory", that is established by Prof. Achim Peters, he argues that human brain carries this special characteristic of being "selfish" due to the most energy consumption. The brain does so by prioritizing the regulation of its own adenosine triphosphate (ATP). Moreover, it uses distinct substrates and it has ability to record and control information from peripheral organs. This places the brain high on hierarchical position and consequently peripheral energy supply is of a secondary importance (Peters et al., 2004). However, this behavior has been noticed in immune system as well.

There are two important sources from which fly can get its energy supply. Those are feeding on glucose which acts as a primary source and feeding on fat which is usually a secondary source but in the absence of glucose it acts as primary source where it produces ketone bodies. Upon the glucose consumption, the part of it is used as energy and the excess is stored in a form of glycogen. Glycogen acts as an energy reservoir (Arrese and Soulages, 2010). The key enzyme responsible for the conversion of glucose into glycogen is called glycogen synthase.

Furthermore, when body is exposed to chronic metabolic or psychological stress, infection, starvation, exercise, etc. where demand for energy is higher, glycogen converts back to glucose and trehalose via glycogen phosphorylase and can be a quick energy source to desired tissues (Reyes-DelaTorre et al., 2012). Trehalose is also well-known for its involvement in immune response as it plays a role in production of AMPs, mechanism of innate immunity (Govind, 2008).

Moreover, the "selfish behavior" of certain tissues is so important that it becomes necessary to have an increase in energy consumption for immune processes. That being said, the other non-immune tissues must decrease their energy consumption. Insulin resistance has been found to act as supportive mechanism in this energy consumption, since hemocytes induce insulin resistance of non-immune tissues. IR causes glucose to stay in hemolyph where it is available for the immune cells (Bajgar et al., 2015).

One of the very important molecules responsible for the reallocation of the energy, during the immune response, is found to be adenosine, purine nucleoside. This molecule usually has its role in development but Dolezal group found that inhibition of adenosine signaling minimize host resistance (Bajgar et al., 2015). Furthermore, Bajgar and his colleagues also showed that immune cells also express adgfA, under purpose of silencing the effect of eAdo. This effect follows immediately after acute phase of infection and is important to forestall wasting induced death (Bajgar et al., 2018).

Very recent research found another important molecule, ImpL2, that causes insulin resistance in *Drosophila* by binding insulin-like peptides. ImpL2 is produced by hemocytes, phagocytes of the immune system which are significant for infection survival (Sokcevicova, MSc. Thesis 2017 [in Czech]). Moreover, the reason why immune cells behave selfishly is most likely because of the Warburg effect, since while activated, immune cells prefer anaerobic glycolysis and production of lactate rather than mitochondrial TCA cycle and oxidative phosphorylation. This metabolic switch is beneficial, but only in the case of sufficient supplementation with sources (Krejcova, MSc. Thesis 2018 [in English]).

1.5 *D. melanogaster* as a model organism for study of bacterial resistance

Drosophila melanogaster is a very valuable model organism for modern science since it has many advantages and its application is highly found for humans. The first studies conducted on *Drosophila* as a model organism were performed by William Castle at Harvard University in 1901. Later on, it has been improved by Thomas Hunt Morgan who was the first in isolating *white* - naturally occurring *Drosophila* mutation. Together with three of his students (Sturtevant, Bridges and Muller) he showed that genes are carried on chromosomes.

Nonetheless, the actual establishment of *Drosophila* as a model organism occurred in 1995 when Nobel Prize was awarded to Lewis, Nuesslein-Volhard and Wieschaus for their work in genes controlling development (Morgan et al., 1920; Sturtevant et al., 1919). One of the advantages of *Drosophila* that makes it so important for research is found in its small size, they take limited space in lab, they are cheap, clean and causing no harm (some people may have allergic reaction).

Furthermore, males are distinguishable from females with the naked eye, they require only about 10 days at 25^0C for regeneration and lifespan can be manipulated by either raising or lowering the temperature since the length of the *Drosophila's* life cycle is temperature dependent. All those features allow researchers to conduct multiple independent experiments in a very short time scale. Moreover, by sequencing the entire genome of *Drosophila* model organism, it has been discovered that more than 90% of its genes are similar to mouse and human genes. *Drosophila* has only four chromosomes (2,3,4 and X/Y). This provides the scientists much easier way to conduct some experiments that can be hardly or even not at all performed on mammalian model (Adams et al., 2000).

In addition to that, one of the best things on *Drosophila* is collection of mutants for all the genes as well as RNAi lines and we can induce these RNAi constructs within any tissue and time frame by using UAS Gal4 system (Brand and Parrimon, 1993).

In the following figure (*Figure1*) different life cycles of *Drosophila* are depicted.

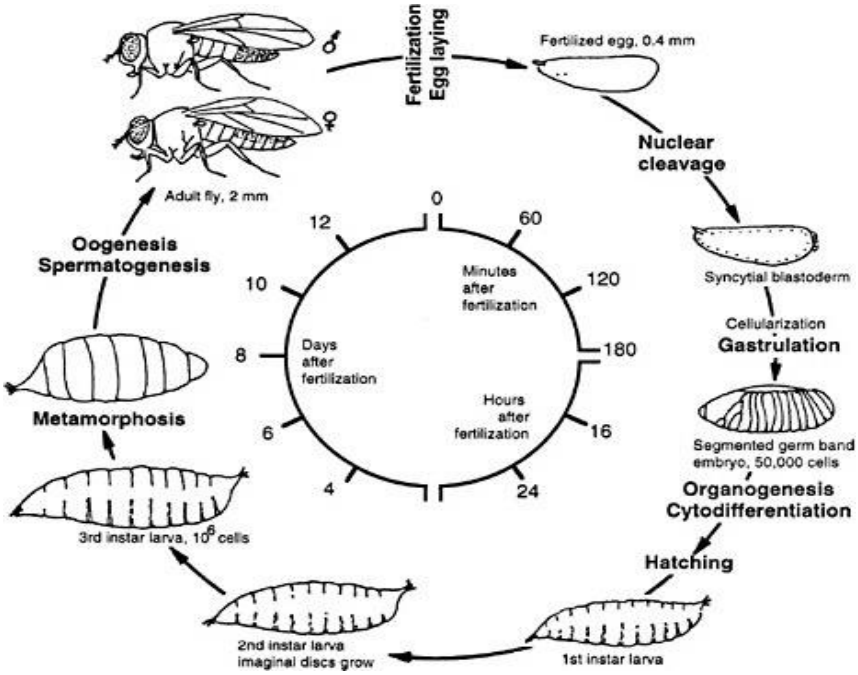

Figure1: Life cycle of Drosophila Melanogaster. Source: http://www.nap.edu/openbook.php?record_id=9871&page=162

Bacterial infection triggers an immune response in *Drosophila's* body and it may depend on many different factors including developmental stage, the tissue that has been affected and by which pathogen it has been attacked. These processes have to do with host survival of infection because, as explained in selfish immune theory, energy demand of an immune response is higher and therefore in competition with other important processes (McKean et al., 2008; Short and Lazzaro, 2013).

One of the typical ways to infect *Drosophila* in the lab is by poking the anaesthetized body into the thorax or abdomen using a thin needle which was previously dipped in a concentrated bacterial pellet (Tzou et al., 2002). During an infection process there has been found two options for *Drosophila* to address the pathogen. One is simply by clearing the pathogen from the host, called resistance and the other one is tolerance (Ayres and Schneider, 2012). By counting CFUs after the infection we can determine the efficiency of an immune response. In this thesis, the flies have been infected by penetration of *S. pneumoniae* and pathogen is killed by plasmatocytes phagocyte which actually serve the same function as M1 macrophages in mammalian organisms (Govind, 2008; Novak and Koh, 2013).

The experimental system done is that the flies were infected by injection of 20 000 bacteria into the abdomen thus making a septic state in these animals.

1.6 *D. melanogaster* as a model organism for high fat diet induced diseases

Macrophages occur in all animals and play a significant role in inflammation activities. They produce so called chemokines that have a function to recruit other cells to the site of infection (Tsou et al., 2007). However, the studies of macrophages in human models are highly complex and carry a lot of limitations for cell and tissue specificity. Thus, *Drosophila* makes it very attractive model for studying *in vivo* the role of different tissues for high fat induced diseases. The system that provides the fly services to study the effects of the given gene on a specific cell type but also including the importance that this gene can have somewhere else in the fly, is called the Gal4-UAS expression system (Brand and Perrimon, 1993).

Another advantage which makes *Drosophila* magnificent model organism is the existence of plentiful mutant fly lines, so the new mutations can be easily introduced. Moreover, RNA interference (RNAi) lines as well as library of fluorescent reporter fly lines are readily available too. Fluorescence allows the visualization of the path of certain genes during development or simply a response, *in vivo* (Beckingham et al., 2007).

Furthermore, the fat body is actually metabolic hub in the fly's body because it is responsible for the metabolism of lipids, carbohydrates, amino acids, nitrogen and protein synthesis as well (Arrese and Soulages, 2010). In mammalian's body this would be adipose tissue and liver. The fat body can be found throughout the whole *Drosophila* body like for example in lobes bathed by hemolymph which detects the changes in the levels of hormones (Arrese and Soulages, 2010). The second stage of fly development is called larva and here the fat body serves as an energy storage which provides support for further development into the third stage called pupa. After the eclosion, larval fat body cells are much smaller (Aguila et al., 2007). However, the emerging fat body tissue in adult flies serves again as energy reservoir (Hoshizaki et al., 1995).

Beside the action of the fat body in the fly there are also cells called oenocytes, which are hepatocyte-like cells, responsible for lipid metabolism and have an important role in regulating growth, development, detoxification and feeding behavior. Oenocytes are found in the abdominal integument and are lined up into clusters along the inner cuticular surface (Krupp and Levine, 2010). During the periods of starvation, oenocytes accumulate lipids from the fat

body tissue. Moreover, it became noticeable that fruit fly *D. melanogaster* shares many similar or, in some cases, even the same metabolic symptoms as mammalians do, upon exposure to high fat diet induced diseases. When there is an increase of triglycerides and FFA from adipose tissue, blood flow can get restricted in order to block the release of FFA. This eventually leads to hypertriglyceridemia which further causes cardiovascular disease.
All those similarities to mammalians put *Drosophila* high on the scale for the most researched model organism.

1.7 Effect of HFD feeding on lifespan and ability to orchestrate immune response

In many studies, it has been proven that diet composed of high fat leads to several diseases such as type II diabetes, atherosclerosis, metabolic syndrome and several others which eventually results in premature death (Taubes, 2001; Ford et al., 2004). On the other hand, the exact mechanisms of how that happens are not yet fully understood. Upon feeding with food high in fat, the levels of glucose and triglyceride increases which as a consequence develops insulin resistance. This has been also shown to occur in the fly body with HFD feeding (Birse et al., 2010, Eckel et al., 2005).

In addition to insulin resistance, *Drosophila* lifespan is significantly reduced on HFD and this turned out to be dose dependent (Driver and Cosopodiotis, 1979). Moreover, there has been noted some deviations in *Drosophila* behavior on HFD. The flies preferred to stay at the bottom of the vial which is not usual for them, their cardiac function was damaged as well as myofibrillar organization (Birse et al., 2010). In addition to that, it is very important to understand the complexity of the full immune network. We can differentiate two distinct categories of the immune response in *Drosophila*. The first one is fast-acting immune response in which phagocytosis and melanization are involved. Hemocytes are phagocytic cells that are found in a fly, concentrated in adherent groups on the dorsal side of the abdomen and located in the anterior abdominal segment of the heart in adult flies (Chambers et al., 2012).

The second category of the immune response is the induction of anti-microbial peptides (AMPs). The cluster action of several AMPs can provide a fly much powerful defense against harmful invaders, for instance bacteria (Bulet et al., 1999). Beside the AMPs one of the best

defenses characterized aspects of a fly immune response are synthesized by the fat body (Bulet et al., 1999).

1.8 Crucial role of macrophages in HFD induced systemic response

The macrophage reciprocation to chronic lipid exposure has been linked to many HFD induced diseases. A type of macrophages called foam cells clots the walls of blood vessels which can ultimately lead to a heart attack and stroke (Fan et al., 2019). They are also involved in early stages of atherosclerosis (Rahaman et al., 2006). Another research suggests that foam cells could be seen as an imbalance in cholesterol homeostasis (Linton and Fazio, 2003). Moreover, another finding propose that protein called fatty-acid binding protein (FABP), expressed by foam cells, is involved in regulating systemic insulin resistance in obesity regulation (Linton and Fazio, 2003). All these findings connote the link between macrophage unregulated lipid uptake and pathology seen in HFD induced diseases.

Furthermore, inflammation activities are by a great part influenced by mononuclear phagocyte system (MPS), that further splits into bone marrow precursors, circulating monocytes, resident macrophages and dendritic cells (Taylor and Gordon, 2003). Monocytes are producing cytokines and are located in bone marrow. They represent one of the most important roles during an immune trigger event (Serbina et al., 2008). Macrophages are crucial during an inflammation event since the cytokine IL-1β induces apoptosis of β-cells (Bendtzen et al., 1986) which further has an impact on type II diabetes (Donath, 2014). Feeding on HFD strongly correlates with obesity which further has an impact on insulin-resistance state in adipose tissue cells. As a consequence, insulin resistance contributes to the combination of insulin target cells and accumulation of macrophages.

However, on a molecular level the transition between M2 macrophage activation state, which is maintained by STAT6 and PPARs to M1 activation state, activated by NF-kB, AP1 has an enormous role in innate immunity (Olefsky and Glass, 2009). Macrophages recognize circulating lipids by surface scavenger receptors CD36 leading to their internalization, which is followed by activation of JNKinase signaling pathway and by Hif1a stabilization. Another way of how circulating lipids influence macrophage biology is by activation surface TOL like receptor4, further leading to NFkB signaling pathway stimulation and metabolically active

phenotype MME, in case of chronic stimulation developing into proinflammatory M1 and release of proinflammatory cytokines Il1, Il6, TNFa (in *Drosophila* Upd3, Impl2). These circulating signaling factors cause systemic insulin resistance (Dongsheng et al., 2005).

1.9 Crucial role of macrophages in regulation of systemic metabolism during bacterial infection

Metabolism and immune system are connected in such way that immune cells called macrophages contribute to metabolic homeostasis (Chawla et al., 2011). There are two types of macrophages, one responsible for healing noted as M2 and the other M1 known for its ability to inhibit pathogens. M1 type macrophage has its role to secrete cytokines which inhibit proliferation and damage of surrounding cells and to produce ROS for microbial killing by a process called respiratory burst (Wang et al., 2014; West et al., 2011). On the other hand, M2 type is responsible for tissue repair and to maintain homeostasis. Both types of macrophages act as mirrors to Th1 and Th2 in T-cells and are T-cell dependent (Mills and Ley, 2014).

Macrophages also possess the ability to intrude pathogens to T-cells and in that way activate them (Mills et al., 2015). Furthermore, chemokines are produced by macrophages and particularly the chemokine CCL2 (C-C motif ligand 2) recruit monocytes to the site of inflammation (Tsou et al., 2007). During an infection roughly two phases can be differentiated: inflammatory and recovery phase. Macrophages has many varieties of functions during inflammatory phase and if it is not properly regulated it can cause harm not only to pathogen but to the host as well (Nathan, 2002). The M1 bactericidal macrophages are known to accumulate increased amount of lipid droplets to supplement their function, on the other hand macrophages accumulate high amount of lipid droplets in adipose tissue and also polarize into the M1, but their bactericidal function is probably lower than in the case of the previous situation. The difference can be explained maybe by the case of temporal and chronic exposition of macrophages to the increase of lipids in the circulation (Prieur et al., 2011).

2.0 Polarization of macrophages and accumulation of lipid droplets in macrophages – its reasoning and effect on systemic signaling

Macrophages can undergo various forms of activation at specific points in response to external factors. This is referred as macrophage polarization and it is not a fixed process since macrophages combine multiple signals (Murray, 2017). There are three pathways that control polarization: the tissue microenvironment, cell survival pathways and epigenetic and extrinsic factors which include for example cytokine production during inflammation (Murray, 2017). Various diseases and inflammatory conditions are related to the dysregulation of M1-M2 macrophage polarization. For example, it has been found that in type II diabetes and obesity, M1 macrophages are increased compared to M2 (Kraakman et al., 2014). Chronic inflammation has been also found in patients who suffer from chronic venous ulcerus due to the failure in switch between M1 macrophage to M2 phenotype (Sindrilaru et al., 2011). Hence, broadening our knowledge of macrophage polarization has a significant importance.

Furthermore, accumulation of lipid droplets is most often seen phenotype in infections and inflammation conditions (Bozza and Viola, 2010). There is an evidence indicating that macrophage infiltration of white adipose tissue (WAT) is related to the metabolic consequences in obesity (Cinti et al., 2005). The fusion between dead adipocytes and syncytia sequester and remove remaining "free" adipocyte lipid droplet and as a consequence form multinucleate giant cells which is a designation of chronic inflammation (Cinti et al., 2005). The manipulation of M1/M2 macrophages would significantly improve our understanding of systemic signaling on HFD if there would be proofs that the switch can be applied on *Drosophila* macrophages as well. Moreover, we have right now many of unpublished observations on macrophages infiltrating the adipose tissue in *Drosophila* in response to HFD feeding (personal communication with Bajgar Adam, 2019)

3. Aims of the thesis

In order to obtain further knowledge in effects of high fat diet on resistance to bacterial infection, the following aims will be discussed:

- Identification of effects of HFD feeding on resistance to bacterial infection
- Describing the effect of HFD feeding on ability to fight the bacteria
- Comparison of the effect of HFD feeding on level of free metabolites (glucose, glycogen and lipids)
- Analysis of triglyceride amounts in the whole body of *Drosophila* and hemocytes particularly

4. Materials and methods

4.1 Drosophila diet composition

Flies in stocks were raised in glass vials with cotton plugs on the two different diets, control (regular) Tab. 1 and high fat diet shown in Tab. 2. Furthermore, flies were kept in incubators at 25 ºC with 70 % humidity with 12/12 hours light/dark cycle. For high fat diet treatment, the flies were held on the diet for at least 10 days. They were selected early after their emergence and then transported to high fat diet or regular diet. Only males were used for the analysis because the females could be different a lot due to the ovary development and the phase of their reproductive cycle.

The composition of diet was counted to have the same energetic content per gram of the food. Experimental individuals were moved on fresh diet every second day and were not overcrowded in vials (30 individuals average). Only males were used for quantification of metabolites. Infected males that were selected for survival were kept in plastic vials on a high fat diet experiment (Tab. 2). Infected flies were kept in incubators at 29 ºC due to the temperature sensitivity of *Streptococcus pneumoiae*.

Table 1: Control diet

Water	1000 mL
Cornmeal	8 %
Yeast	4 %
Agar (Ambresco)	0,62 %
Glucose	5 %
Cook for 12 min at 100 ºC, then 50 min at 90 ºC, then cool to 60 ºC	

Table 2: High fat diet

Water	1000 mL
Cornmeal	1 %
Yeast	2,5 %
Glucose	5 %
Agar (Ambresco)	0,62 %
Fat (lard)	10 %
Cook for 12 min at 100 °C, then 50 min at 90 °C, then cool to 60 °C	

4.2 Bacterial infection and survival analysis

Streptococcus pneumoniae (EJ1 strain, referred as *Sp*) was stored in microtubes in Tryptic Soy Broth media (TSB) (Sigma) with 16% glycerol at -80 °C. By using a disposable inoculation loop (Biologix) the upper layer was scraped off and spreaded on a Petri dish prepared from the following table:

Table 3: Ingredients for bacterial infection procedure

dH_2O	400 mL
Tryptic Soy Broth (Sigma) 3 %	12 g
Agar (Amresco) 1,5 %	6 g
Boil for 1 min in microwave oven. Autoclave for 20 min at 121 °C, let it cool down to 50 °C.	
Streptomycin sulfate salt (Sigma) 0,0075 %	0,03 g

After this, Petri dish was left to incubate at 37 °C, (5 % CO_2) overnight. TSB liquid media is also prepared in a following way:

Table 4: Ingredients for bacterial infection procedure

dH_2O	100 mL
Tryptic Soy Broth (Sigma) 3 %	3 g
Agar (Amresco) 1,5 %	6 g
Boil for 1 min in microwave oven. Autoclave for 20 min at 121 °C, let it cool down to 50 °C.	At the end, bacterial filter (∅ 0.20 um) was used

For further experiment, three glass tubes were used with TBS liquid media. First one is used for *S. pneumoniae* inoculation from plate, second one for *S. pneumoniae* inoculation to fresh media in the next day and third one served as a blank (shown in Fig. 7). In each glass tube 3 mL of TBS liquid media, 100 µL of streptomycin (Sigma) and 100 µL of catalase (Sigma) was added. In the second day, one colony from the Petri dish was placed in the first glass tube. This tube was then incubated at 37 °C, 5% CO_2 for 24h. Afterwards, 100 µL from this tube was pipetted into the second glass tube. By using this approach, at the time of infections, the growth of *S. pneumoniae* was in exponential phase.

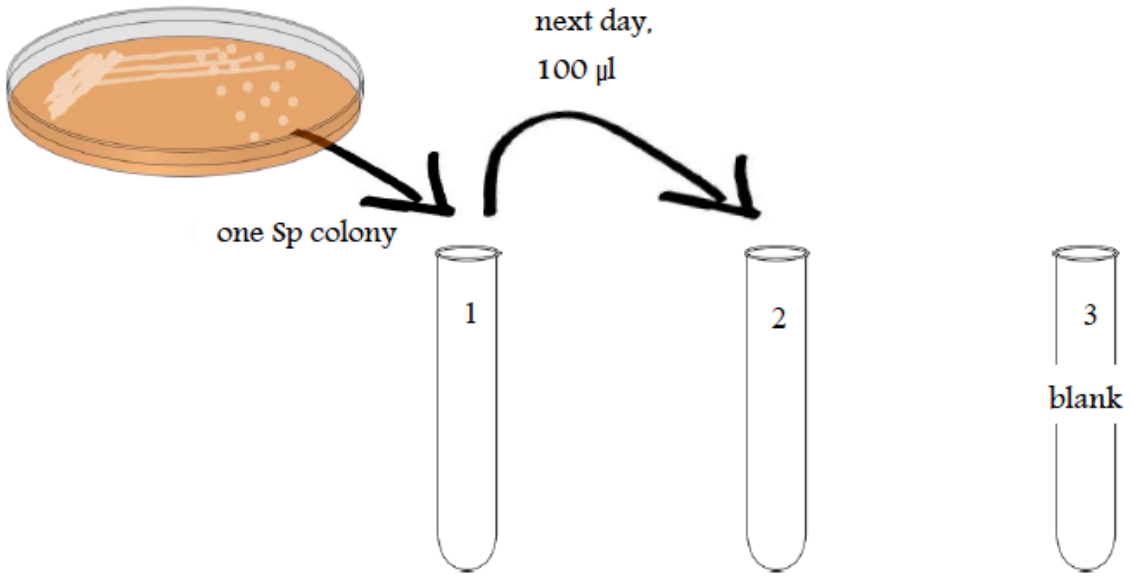

Figure 2: Schematic representation of S. pneumoniae preparation for infections [Krejcova, 2018]

In order to inject the flies, they were first anaesthetized with carbon dioxide (CO_2). After that, the Eppendorf Femtojet microinjector and a drawn glass needle were used to inject precisely 50 nL of bacteria or buffer into the fly. The fly is injected at the cuticle on the ventrolateral

side of the abdomen. After injection, it was checked if all the flies were awake and in the case of those who were not, they were discarded from the experiment. Infected flies were kept in incubators at 29 ºC due to the temperature sensitivity of *Streptococcus pneumoiae.* Every second day, flies were transferred on a fresh diet and the number of the dead flies was recorded.

Flies were transferred without using CO_2 since it could affect their lifespan. On each vial, number of dead flies was recorded so it was not counted twice. This was done until all flies from the vial were dead. These data were statistically processed using standard survival analysis.

4.3 Colony-forming units

To estimate the number of viable bacteria cells in *Drosophila*, method of colony-forming units (CFUs) is performed, at 18 and 24 hours post infection (hpi). In order to make sure that distribution of bacteria among individuals was even, the number of bacteria per fly was also evaluated immediately after infection (0hpi). Motorized plastic pestle (VWR) is used to homogenize single flies in 200 μL of PBS using 1,5 mL tubes. All microtubes were kept on ice. Bacteria were plated in spots onto TSB (*S.pneumoniae*) agar plates containing streptomycin (20 μL of this homogenized solution and 180 μL of PBS). It was done in serial dilutions (with dilution factor $1/10^{-4}$) and was incubated overnight at 37ºC before manual counting was performed. Pathogen loads of 16 flies were determined in each experiment, for each genotype and treatment. Three independent infection experiments were conducted at least, and results were combined into one graph. This was suitable because individual experiments, in all introduced cases, showed comparable results.

To evaluate the results of the previous, values were transformed to logarithmic values since they followed lognormal distribution. Values were also compared using unpaired t-tests corrected for multiple comparisons using the Holm-Sidak method in the Graphpad Prism software.

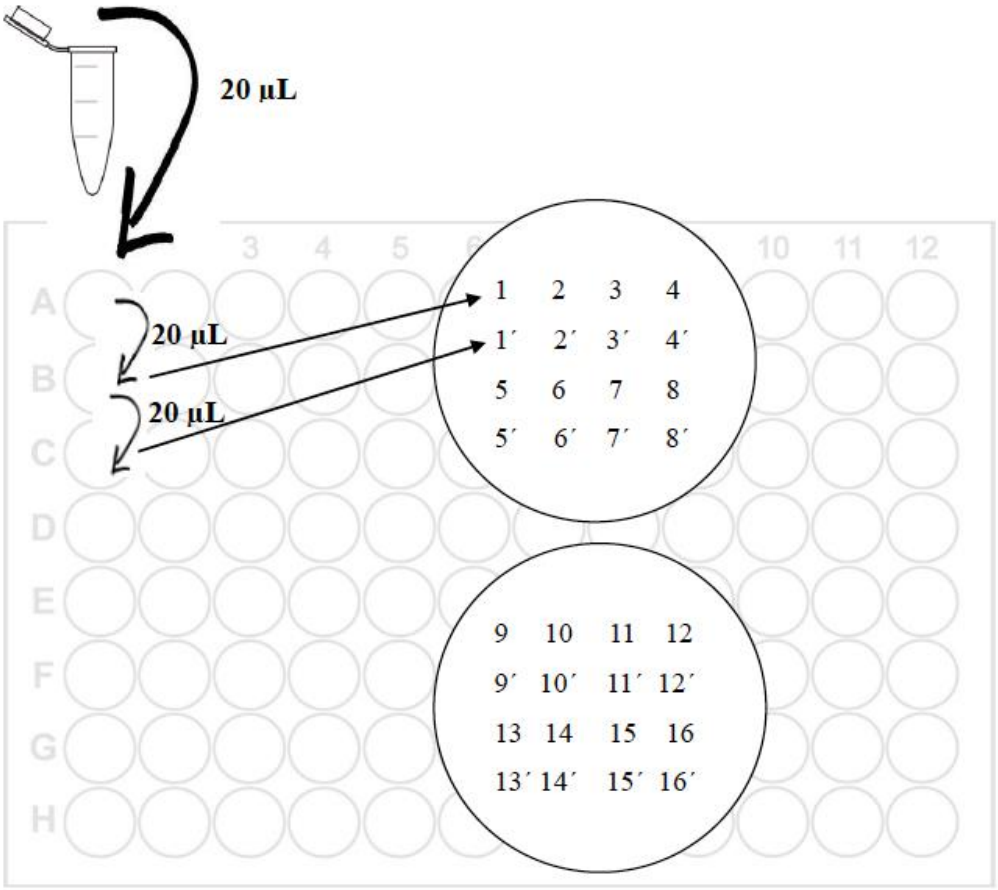

Figure3: Schematic representation of CFUs [Krejcova, 2018]

4.4 Quantification of free glucose

In order to prepare the samples, five flies were brought in one microtube where they were homogenized in 200 µL of PBS and then centrifuged for 5 minutes at 8000rpm. The supernatant was transported into the fresh Ependorf tube. Half of the lysate was separated and freezed at -80 °C for later quantification of proteins. The second half was heated to 75 °C for 15 minutes to denature the proteins and to stop chemical reactions. The samples were held on ice as much as possible, centrifugation was carried at 4°C.

The Bradford Protein Assay was used for protein quantification. Samples were homogenized, and proteins were dissolved in 1xPBS. A 10 µL of protein sample was mixed with 100 µL of Bradford solution. The following amounts were used:

Table 3: Bradford measurement

Briliant Comassive Blue	100 mg
Ethanol (95%)	50 mL
Phosphoric acid (85%) per 1L	100 mL
Sample 10 uL	Bradford 100 uL

Protein concentration was deduced from absorption at 595 nm (Sunrise-Absorbance reader, Tecan). Values were compared by multiple unpaired t-tests using the Graphpad Prism Software. Glucose was determined by GAGO-20 kit (Sigma). 100 µL of Assay reagent (glucose oxidase-peroxidase reagent + o-dianisidine) was mixed with 45 µL of sample solution. The mixed solution was incubated for 30 minutes at 37 °C. Afterwards, 100 µL of 12N H_2SO_4 was added to intermit the reaction. Absorption was measured at 540nm and standard curve of specific range (0; 0,03 mg/mL; 0,067 mg/mL; 0,125 mg/mL; 0,5 mg/mL; 1 mg/mL) was obtained using Glucose (Sigma).

4.5 Quantification of glycogen

For quantification of glycogen, 5 µL of sample is first treated with 5 µL of amyloglucosidase reaction solution (Sigma), 20 µL PBS and 100 µL of Assay reagent (glucose oxidase-peroxidase reagent + o-dianisidine, Sigma). The solution was incubated for 30 minutes at 37 °C. Again, as for glucose measurement, 100 µL of H_2SO_4 was added to intermit the reaction. By this process, glycogen is cleaved into glucose and absorption was measured at 540 nm. For evaluation, the same standard curve as for glucose was used. To determine the exact amount of glucose resulted from the cleavage of glycogen, the measured glucose amount was subtracted from the total amount.

4.6 Quantification of lipid content

Quantification of lipid content is determined by using triglyceride quantification kit (Sigma). For colorimetric detection, 40 µL of the 1 mM Triglyceride Standard is diluted with 160 µL of Triglyceride Assay Buffer to get the final 0.2 mM standard solution. Afterwards, the 0.2 mM

standard solution is added into a 96 well plate (0,10,20,30,40 and 50 μL) generating 0 (blank),2,4,6,8 and 10 nmole well standards. For Fluorometric detection, Triglyceride standard is prepared in the same way as for colorimetric. 20 μL of this was then diluted with 180 μL of the triglyceride assay buffer to get final concentration of the triglyceride standard solution, 0.02 mM. The same procedure is done for a 96 well plate but generating 0 (blank), 0.2,0.4,0.6,0.8 and 1.0 nmole well standards.

Finally, to bring the volume up to 50 μL, triglyceride assay buffer is added to each well. For sample preparation, 100 mg of tissue is homogenized in 1 mL solution of 5 % Nonidet P40 Substitute (Catalog Number 74385) and water. It is then slowly heated to 90 ^{o}C in a water bath for 5 minutes and cooled to room temperature. The heating was repeated once more to solubilize all triglycerides. It was then centrifuged for 2 minutes at top speed for removing insoluble material.

Afterwards, 2-50 μL of samples were added into duplicate wells of a 96 well plate. Final volume was brought up to 50 μL with triglyceride assay buffer. To convert triglyceride into glycerol and fatty acid, 2 μL of lipase was added to each sample standard reaction (well). It was mixed well and incubated for 20 minutes at room temperature. 50 μL of master reaction mix was added to each sample and standard well and is prepared from the following table:

Table 4: Quantification of lipid content using the following ingredients:

Reagent	Samples, Standards
Triglyceride Assay Buffer	46 uL
Triglyceride probe	2 uL
Triglyceride Enzyme Mix	2 uL

This was mixed well and incubated for 40 minutes at room temperature. The plate is protected from light during the incubation time. For colorimetric assays, the absorbance was measured at 570 nm while for fluorometric intensity λ_{ex} = 535/λ_{em} = 590 nm.

The standard curve is obtained and the amount of triglyceride present in the samples is calculated using the formula:

$$\frac{S_a}{S_v} = C$$

S_a – amount of triglyceride in the unknown sample (nmole) from standard curve

S_v – sample volume in μL added into the wells

C – concentration of triglyceride in sample

4.7 Oil red o staining

The stock stain is prepared using the data from the following table:

Table 5: Stock stain solution

Oil red O stock stain	
Oil red O (Cl 26125)	0.5 g
Isopropanol	100.0 mL
Dissolve the dye in the isopropanol using the very gentle heat of a water bath.	

For Oil Red O working solution preparation:

30 mL of the stock stain is diluted with 20 mL of distilled water, it was allowed to stand for 10 minutes and later was filtered into a Coplin jar, covered immediately. Since the stain does not keep it was always made up fresh from the stock solution each time.

4.8 Microscopy analysis (Confocal microscopy)

Flies were rinsed in 75 % EtOH for a few seconds and then dissected on ice in 4 % paraformaldehyde. Samples were washed three times using PBS, 10 minutes each washing. Dorsal part of the abdomen (containing heart, fat body and immune cells) were opened and then rinsed by 60 % isopropanol and stained using Oil Red O solution, for 5 minutes. The unbound staining was washed out by using 60 % isopropanol and the tissue samples were

mounted on the microscopic slide. The picture of macrophages on the dorsal part of the body was taken by using confocal microscopy (Olympus FluoView 1000) and picture was analyzed using Fiji software.

4.9 Software used and statistical analysis

The data analysis for metabolites, survival as well as statistics were visualized using GraphPad Prism 7. Survival curves were estimated using Gehan-Breslow-Wilcoxon test. Fiji software was used to analyze the picture, from confocal microscopy, of macrophages on the dorsal part of the fly body. Two-way ANOVA with multiple comparisons – Tukey multiple comparisons test was used for metabolites and colony forming units. Sidak's multiple comparison test was made for corrections due to the multiple comparisons of the data.

5. Results

5.1 HFD feeding affects lifespan and has detrimental effect on resistance to *Streptococcal* infection

In order to evaluate the hypothesis that during the resistance to bacterial infection the lifespan of a model *D. melanogaster* is negatively affected in a great way, survival experiment is conducted. The obtained data were statistically processed using standard survival analysis.

As we can see from the graph below, the red lines represent Sp infected male adults which show a major change between normal diet and HFD. Furthermore, in the first 5 days of Sp infection the flies that were fed a normal diet all survived whereas flies fed an HFD had a significant decrease in their lifespan. From a period of 5 to 20 days flies on HFD kept dying in a rapid way compared to flies on a normal diet. After about 24 days all flies were dead on both diets. HFD fed flies have showed to be more vulnerable in response to infection.

The source of fat used in the experiment was lard which was based on previously tested experiments in studies done by Driver and Cosopodiotis (1979). The main reason that lard was chosen for this experiment was its ability to stay solid and make food consistency more stable compared to olive oil which is liquid at room temperature and flies could be caught in oil which would not give us reliable outcomes. A detailed information about fat composition and diet content can be seen in Table 1 and 2 (Control and HFD respectively).

A huge distinction can be seen between control group and the flies who were infected by *Streptococcus* and were fed an HFD. We can conclude based on the results that after only 5 days, more than 50% of the flies were dying in a very rapid way whereas in a control group the lifespan line rather was decreasing in a steady motion.

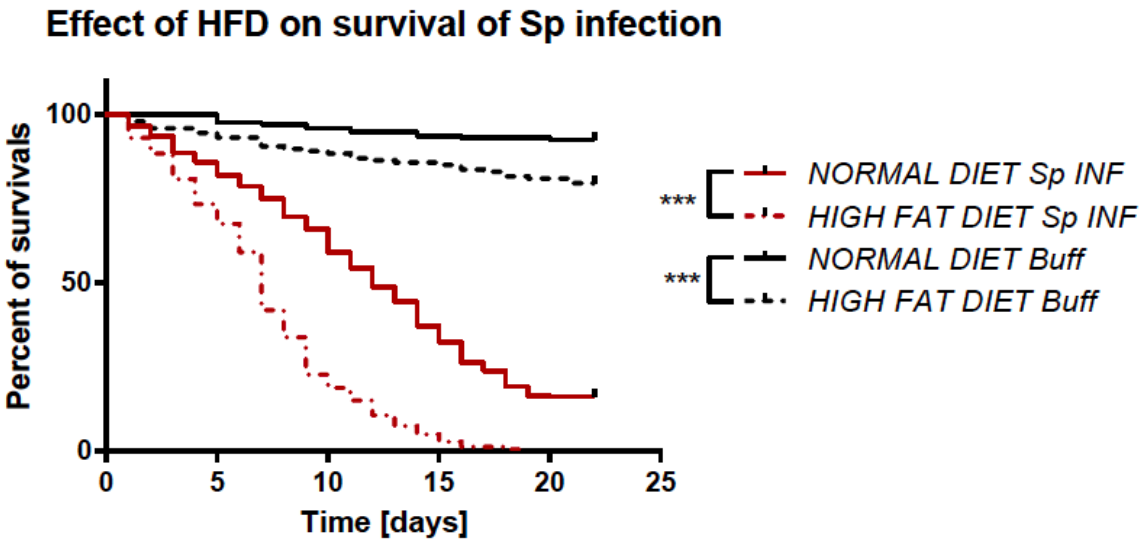

Figure 4: Survival of Streptococcus infection during HFD feeding process of male adult flies, each day counted in the period of 25 days. Maximum 30 flies per vial were used for the experiment. The graph shows data combined from four independent experiments. A detailed information about fat composition and diet content can be seen in *Table 1* and *2* (Control and HFD respectively). Survival experiments data were statistically processed using standard survival analysis. Survival curves were estimated using Gehan-Breslow-Wilcoxon test.

5.2 HFD feeding decreases the ability of macrophages to fight the pathogens resulting in an increased pathogen load

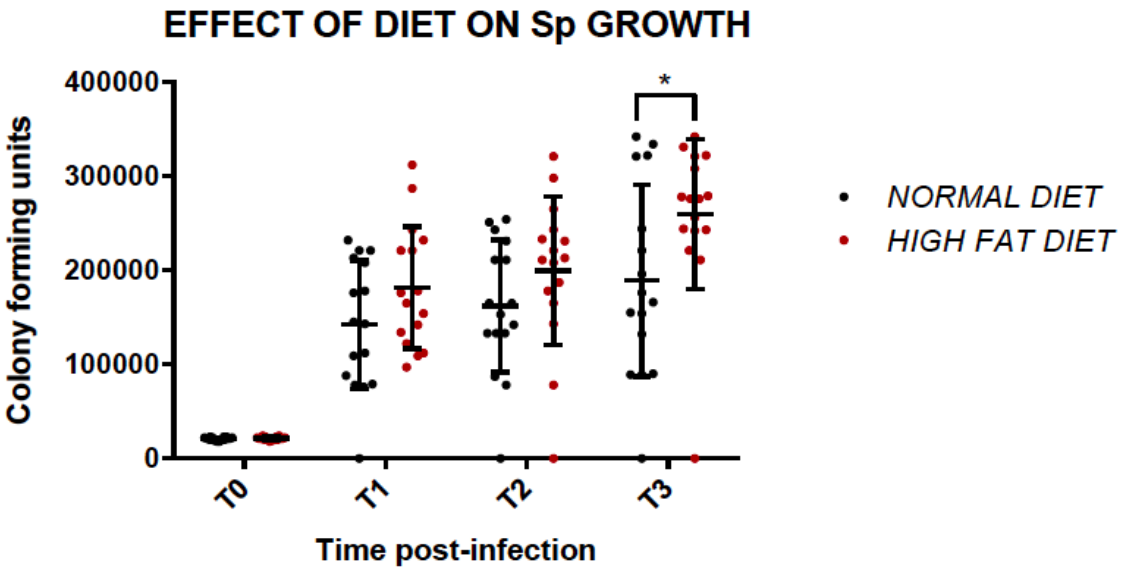

Figure5: Y-axis represent the number of viable bacteria cells in *Drosophila* whereas x-axis represent the post-infection time. Pathogen loads of S. pneumoniae in colony forming units (CFU) per fly were determined over a span of 3 days. Each dot represents a load in one fly in linear scale (multiplied by thousand). Zero levels represent flies that cleared infection. Pathogen loads of 16 flies were determined in each experiment, for each genotype and treatment. Individual experiments were combined into one graph coming from two different diet styles, control and HFD. CFUs is performed at 24, 48 and 72 hpi. Values were also compared using unpaired t-tests corrected for multiple comparisons using the Holm-Sidak method in the Graphpad Prism software. Error bars represents standard deviation and p-value (0.05) was determined using Two-Way ANOVA (Sidak's multiple comparison test).

Since we received negative results on resistance to bacteria upon HFD feeding we were further interested in how this way of feeding affects the macrophages ability to respond to a certain pathogen. As from the previous studies, we have discovered that immune response urges a vast amount of energy and getting this energy sources is of a crucial step to fight pathogens hence the type of a diet will have a tremendous impact.

From the results obtained, we can see that HFD led to an increased pathogen load which might indicate that energy needed to fight the pathogen was actually limited. Figure 7 shows that *S. pneumoniae* load increased during first and second day up to 300 000 units CFUs per fly. After the 48 hpi it increased even more. We can also observe that more of the pathogen load was presented in HFD fed flies.

The findings suggest us that macrophages probably had hard time fighting the pathogen since the energy sources were 'reserved' and not enough nutrients were provided which led to severe problems with recovery from infection therefore increasing pathogen load. As seen in the previous experiment, HFD flies were dying faster and the explanation could be hidden in the information that macrophages are phagocyting less effectively. It can be presumably caused by the capacity of macrophages to phagocyte since their phagolysosomal capacity is somehow occupied by lipids, metabolized by lysosomal lipolysis.

However, it is important to note that more samples and of a longer duration are required for collecting better results.

5.3 HFD as well as bacterial infection increase level of free glucose in *D. melanogaster* but has different effect on amount of glycogen stores

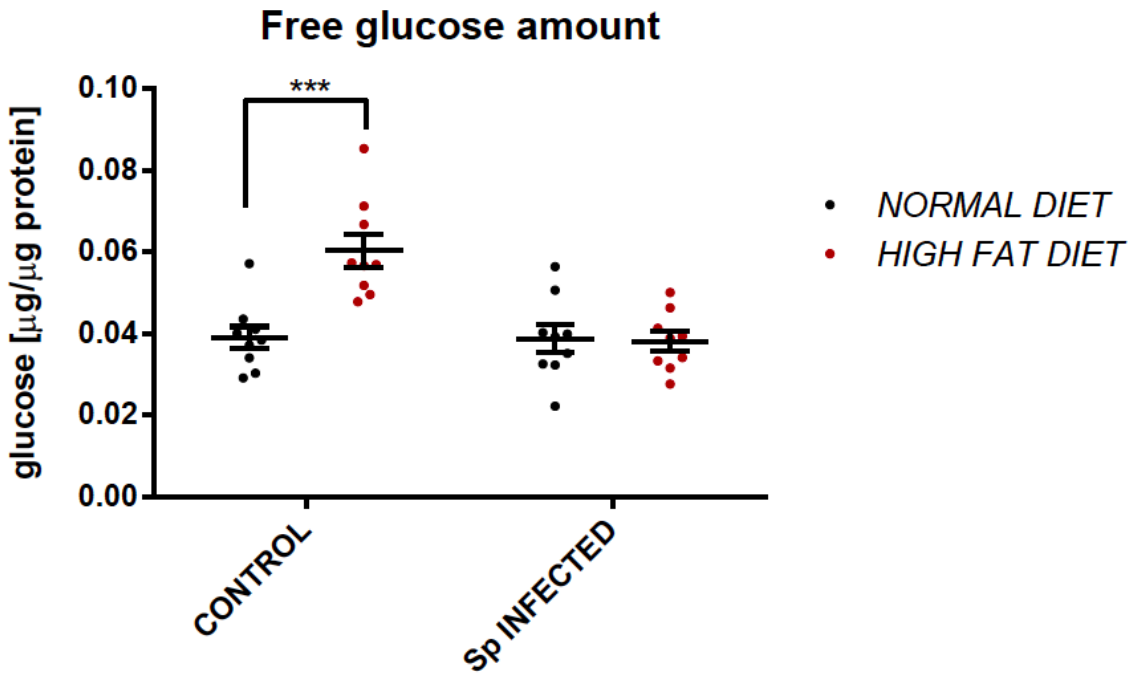

Figure 6: The concentration of free glucose per μg of protein in control and Sp infected flies on a normal and HFD respectively. The graph shows data combined from independent experiments. Five flies were used per microtube during analysis. Values were compared by multiple unpaired t-tests using the Graphpad Prism Software. Glucose was determined by GAGO-20 kit (Sigma). Black dots represent flies on a control diet, while red dots represent flies on an HFD. Each dot represents a load in one fly in linear scale (multiplied by thousand). Error bars represents standard deviation and p-value (0.001) was determined using Two-Way ANOVA (Sidak's multiple comparison test).

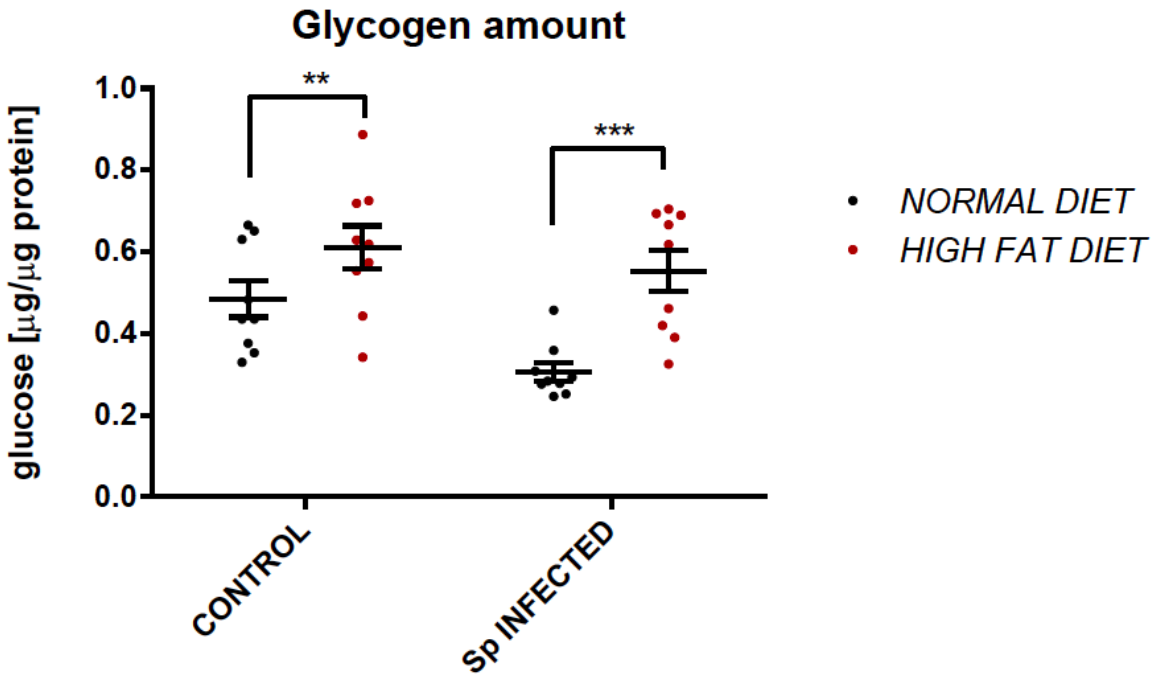

Figure 7: The concentration of glycogen per μg of protein in control and Sp infected flies on a normal and HFD respectively. The graph shows data combined from independent experiments. Five flies were used per microtube during analysis. Values were compared by multiple unpaired t-tests using the Graphpad Prism Software. Glucose was determined by GAGO-20 kit (Sigma). Error bars represents standard deviation and p-value (0.01 and 0.001) was determined using Two-Way ANOVA (Sidak's multiple comparison test). Black dots represent flies on a control diet, while red dots represent flies on an HFD. Each dot represents a load in one fly in linear scale (multiplied by thousand).

One of the aims of this thesis was to check the effect of free metabolites such as glucose during HFD feeding and its response to infection as well. We observed some interesting findings from the experiment and that is that in a control group the HFD has a higher intake of glucose compared to the normal diet. On contrary, the infected flies showed almost no significant difference between those two diets. In fact, the infection usually leads to release of free glucose into the circulation as a response to that infection, but it is not so significant as in the case of HFD. The increase in HFD is normal and suggests that individuals have problems with insulin insensitivity. Quite surprisingly is that in the case of infection this effect is not visible. Considering the treatments consequence, though flies were fed first by HFD and were expected to have increased glucose concentration in circulation, after the infection their glucose level resulted in a decrease. The questions that should be raised here are: Where is that glucose? Why there is not free glucose anymore? If it is consumed by macrophages, why they are rather worse in the response to bacterial infection?

On the other hand, in the case of the concentration of glycogen, both control and HFD can be distinguished in the presence and the absence of an infection. In the case of the absence of Sp infection, there is an increased amount of glycogen in the case of HFD. In a control diet an increase is observable too but not as high as in HFD. However, the concentration is increased in HFD in comparison to control diet during a Sp infection. In an HFD the absence of Sp infection causes the higher increase in glycogen concentration compared to HFD.

This data might suggest us that maybe upon Sp infection, flies were not able to metabolize efficiently glycogen but neither flies on a control diet during an infection. This might also tell us that effects in a long term (HFD non-infected flies have shorter lifespan) of HFD induced effects together with infection are even worse and in short term induced metabolic changes are essential for bacterial resistance, based on findings so far.

Nevertheless, further experiments would need to be conducted to determine if the outcomes are the same in case of chronic infection in comparison to acute, as it is the case of Sp done in our experiments.

5.4 HFD increases triglyceride amounts in *Drosophila's* body

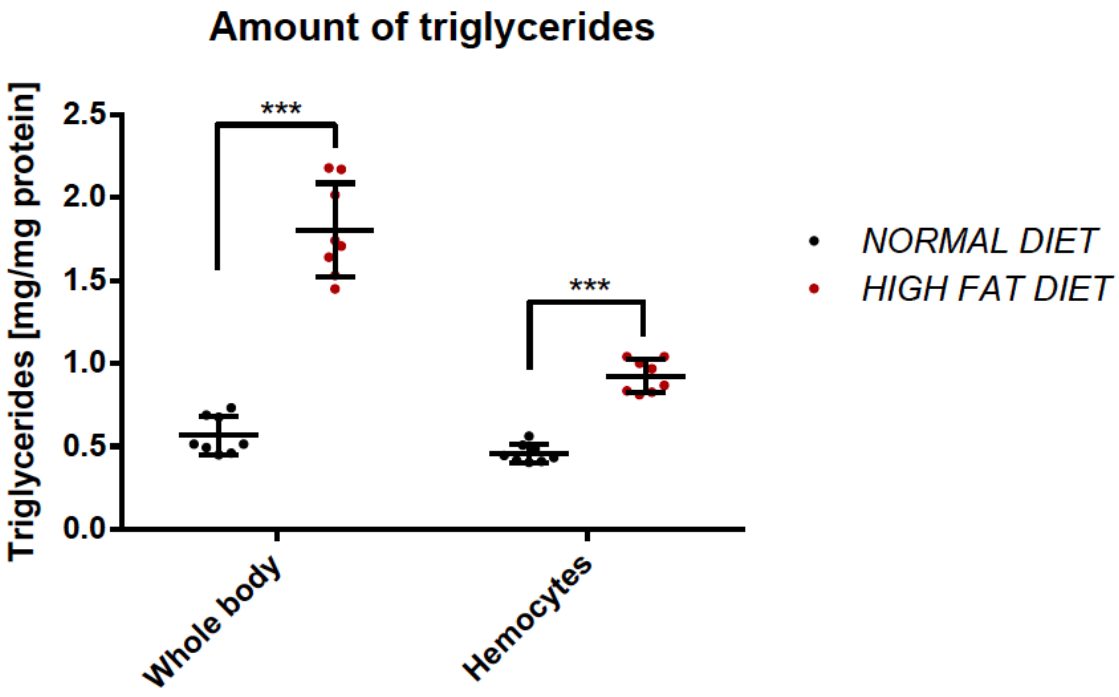

Figure 8: The concentration of triglycerides per mg of protein in the whole body (left) and in hemocytes specifically (right). Quantification of lipid content is determined by using triglyceride quantification kit (Sigma).

Our previous discovery revealed that macrophages are poor in killing the bacteria on an HFD but how exactly is this affected by the triglyceride amounts in the body of *Drosophila*, triggered our curiosity. The experiments have been done considering the whole *Drosophila's* body and taking only hemocytes for observation. On the left side of a graph, we can notice that in the case of the whole-body, triglycerides are massively increased on an HFD compared to normal (control) diet. On the right side, the triglyceride amounts in hemocytes is found to be increased as well, much more in HFD compared to normal diet. The accumulation of triglycerides in milligrams for a normal diet has found to be around 0.5 mg/mg of protein whereas for HFD it goes up to 2.5 mg/mg of protein.

Since hemocytes serve a protection role in *Drosophila's* body and are essential for fighting infections and play an important role during immune response, these outcomes affect the overall ability to fight the pathogen in a negative way.

The question that has not been answered in my thesis but can serve for future experiments is: Can accumulation of lipids be bad as well in the case of HFD feeding during an infection?

5.5 HFD as well as bacterial infection induce accumulation of lipid droplets in macrophages

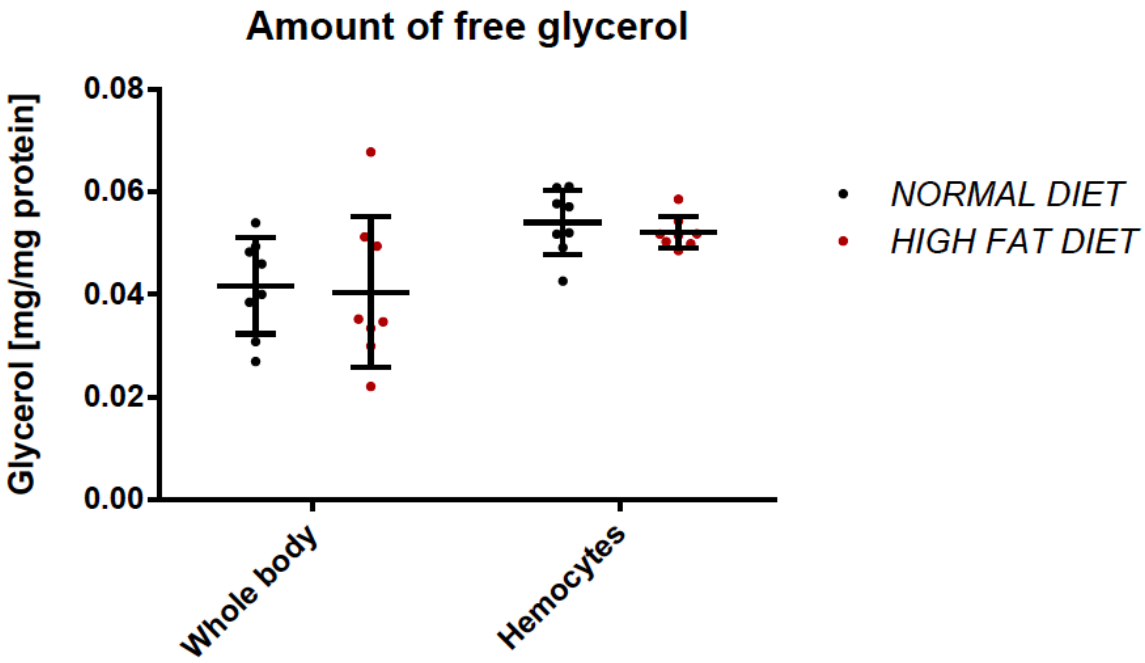

Figure 9: The concentration of glycerol per mg of protein in the whole body (left) and in hemocytes specifically (right). Quantification of lipid content is determined by using triglyceride quantification kit (Sigma).

Our further experiments on lipid content gives us interesting outcomes as well. Amount of free glycerol ranges from 0.02 mg/mg up to 0.06 mg/mg in both cases. Considering the whole body of *Drosophila* there is a higher increase of free glycerol found on an HFD feeding in comparison to a normal diet. However, in the case of hemocyte observations, there has been found a slightly higher increase in a normal diet compared to HFD.

One of the reasons why there has been more glycerol found compared to triglycerides is that they are able to pass the cell membrane unlike triglycerides. Glycerol molecules are, however, mainly responsible for maintaining the physical structure of the cell whereas fatty acids serve as an extensive amount of energy source.

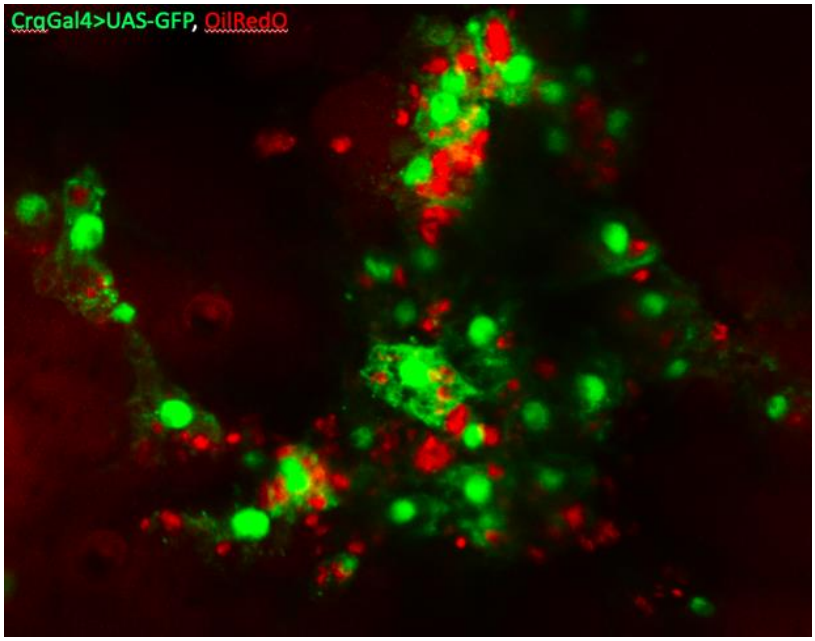

Figure 10: Representative confocal image of dissected abdomen with macrophages (green) containing significant amount of lipid droplets (red), the image was made by composition of 10 layers of both fluorescent channels by confocal microscope (FluoView 1000).

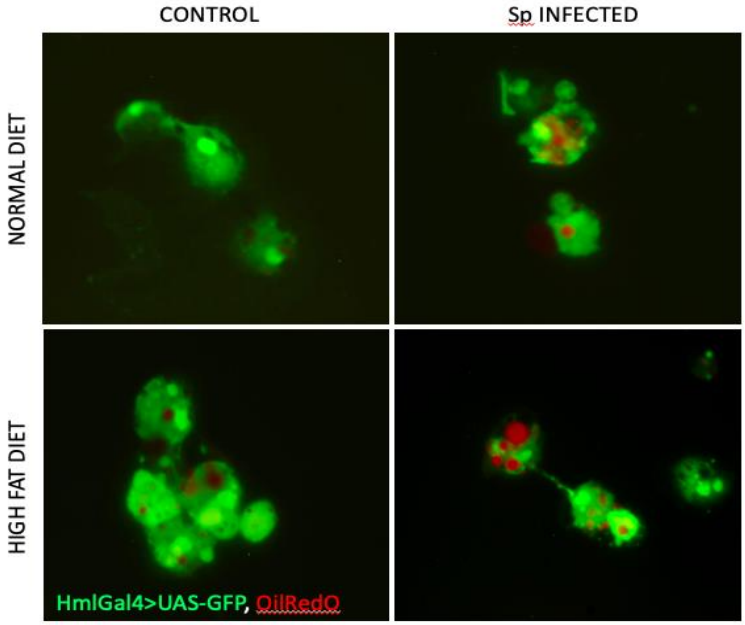

Figure 11: OilRedO staining in control (left) and Sp infected (right) on a normal diet (above/upper part) and normal diet (below/lower part). Picture made on inverted microscope.

6. Discussion

The aim of this thesis was to identify the effects of HFD feeding on resistance to bacterial infection, particularly *S. pneumoniae*, and to check the organism's model (*Drosophila melanogaster*) ability to fight the bacteria. This work has provided some basis methods to investigate the relationship of high fat diet and bacterial infection. Moreover, even though obesity and immune response are both frequently targeted in studies, their interaction or better say reciprocal relationship is not studied very often and drosophila opens this possibility. The question is – if both bacterial infection and HFD feeding are accompanied by similar effects on systemic metabolism such as hyperglycemia and insulin resistance why isn't HFD beneficial for individuals treated by bacterial infection? Even though answer to this question is highly challenging we can at least try to dissect some basic mechanisms and relationship.

The obtained results clearly showed that upon HFD feeding the hemocytes of *D. melanogaster* undergo a significant change. In the case of triglycerides, the increase has been found higher upon HFD feeding compared to control diet. However, in the case of glycerol the increase is observed in both diets with slightly higher lead of a control diet. Hemocytes are cells that play an important role in immune system defense and participate in phagocytosis upon an intruder attack (Lemaitre and Hoffmann, 2007). Hemocytes are also found to be successful in producing antimicrobial peptides (AMPs) which are very powerful defense towards harmful invaders such as bacteria (Bultet et al., 1999). Not only that, but other researches have suggested that other very intense help can come from a fly's immune response synthesized by the fat body (Bultet et al., 1999). Interested research has been conducted by Ramond and his colleagues, where they discovered that the adipokine NimB5 is produced by fat body in the event of nutrition scarcity. NimB5 then binds to hemocyte which further enhances their proliferation (Ramond et al., 2019). Therefore, the combination of a poor diet (insufficient nutrients) with excessive number of hemocytes can lead to lethality (Ramond et al., 2019).

We showed that there are also significant differences found in regulation of glucose metabolism in a control and Sp infected group. Free glucose is importantly increased on a control diet whereas upon Sp infection there is almost no distinct difference between control and HFD. In fact, there is a decrease in glucose amount which triggers our interest for where

that glucose can be found then? One research has been suggesting that metabolic syndrome in a fly, insulin resistance and cardiac diseases were related to TOR-insulin pathway interaction, responsible for tissue growth, stress response, reaction to starvation and aging (Birse et al., 2010). This pathway allows opportunities to study more in depth the causes of several diseases such are diabetes, cancer and obesity (Grewal., 2009). Another study proposes that availability of increased glucose levels could lead to metabolic reprogramming and therefore modulate the macrophages inflammatory response (Freemerman et al., 2014). In addition, an interesting finding have been offered by Pavlou et al. where they stated that having increased glucose in a long run can sensitize macrophages to cytokine stimulation and by that reduce phagocytosis and nitric oxide production which can be linked to damaged glycolytic capacity (Pavlou et al., 2018). What we can draw from those findings is that developments of induced pathologies caused by HFD in *Drosophila* are in a great way similar to what can be observed in mammalians, therefore findings performed using fly as a model can improve our understandings behind the HFD aroused diseases.

In order to evaluate the effects that HFD feeding has on *D. melanogaster* lifespan during a Sp infection, survival experiments were carried out, and to understand more how diet rich in lipid content has adverse effects on *Drosophila* survival. The data from the experiment confirmed what has been previously showed in experiment conducted by Driver and Cosopodiotis (1979), lifespan of *D. melanogaster* is significantly reduced upon feeding on a diet that is rich in lipid content in comparison to a normal diet. Since we know, based on findings, that HFD feeding is bad for resistance to bacterial infection the main questions we should ask is, why? May it be due to triggering another response with just similar outcomes? One study suggests that HFD increases the Acetyl-CoA synthetase enzyme, which increases amount of acetate available and there are many studies supporting the idea that acetate metabolism plays a significant role in aging since it involves NAD^+ dependent protein deacetylases which has one of the main roles in aging (Trinidade de Paula et al., 2016). Furthermore, the research also proposed that enzymes ACSL1 and ACeCS1 are increased on a HFD which are responsible for mitochondrial dysfunction, consequently oxidative damage is caused, increased RS generation and decreased ATP production and cell viability (Trinidade de Paula et al., 2016).

Last but not least, the experiments showed that macrophages are poor in bacterial killing and that is probably due to macrophages capacity to phagocyte. This might be due to the fact

that phagolysosomal capacity of macrophages is occupied by lipids which are metabolized by lysosomal lipolysis. It has been found that in type II diabetes and obesity, M1 macrophages are dominating over M2 macrophages (Kraakman et al., 2014) and failure of M1 to switch to M2 can also lead to chronic inflammation (Sindrilaru et al., 2011). On contrary, another research suggests that acyl ghrelin could potentially promote polarization to M1 under inflammatory state *in vitro* while on the other hand deletion of GHSR would cancel this effect and promote the polarization of M2 phenotype which will as a consequence, improve insulin sensitivity (Yuan et al., 2018). An interesting point comes from the work of Komegae and his colleagues where they found that macrophages located in lung alveoli were not responsive to LPS conditioned in obesity, however macrophages located in adipose tissue and peritoneum did have response to LPS, referring to the early stage of cytokine secretion (Komegae et al., 2019).

Overall, the link between HFD feeding and various diseases can be found controversial, with both favorable and unfavorable outcomes coming from vast number of researches. Maybe explanation can be found in length of the time these effects are induced. In short term it might be beneficial while in a long run can be detrimental. Outcomes might be of an opposite nature in the case of chronic and acute infections. All this suggests us that further insights into the experiments and much more detail and advanced material and methods are required for deeper intellection.

7. Literature

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