

**Using of fruit fly, *Drosophila melanogaster* for
study some physiological and molecular aspects
of human fatty liver disease**

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List of abbreviations

aa	amino acid
ANOVA	Analysis of variance
AKH	Adipokinetic Hormone
ATP	Adenosine triphosphate
<i>bmm</i>	brummer lipase
BODIPY	4,4-difluoro-1,3,5,7,8-pentamethyl-4-bora-3a,4a-diaza-s-indacen
bp	base pairs
bPAC	photoactivated cyclase from <i>Beggiatoa SP.</i>
cAMP	cyclic adenosine monophosphate
CCA	Coupled Colorimetric Assay
cDNA	Complementary deoxyribonucleic acid
D	Day
DA	Dopamine
DAPI	4',6-diamidino-2-phenylindole
<i>dilp6</i>	drosophila insulin like peptid-6
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	Deoxyribonucleotide triphosphate
et al.	et alumni
EtOH	Ethanol
FB	Fat body
Fig.	Figure
FoxO	Forkhead box subgroup O
G	Gram
GFP	Green Fluorescent Protein
H	Hour
5HT	5-hydroxytryptamine
IHC	Immunohistochemistry

IIS	Insulin/IGF1-Signalling
ILP	Insulin- like peptide
JAK-STAT	Janus kinase signal transducers and activators of transcription
mg	Milligram
min	Minute
ml	milliliter
μl	microliter
mRNA	messenger ribonucleic acid
NGS	Normal goat serum
OA	Octopamine
Oe	Oenocyte
PBS	Phosphate buffer saline
PBT	PBS-triton-X 100
PCR	polymerase chain reaction
PFA	Paraformaldehyde
qRT-PCR	quantitative Real-Time Polymerase Chain Reaction
RNA	Ribonucleic acid
RNase	Ribonuclease
RNAi	RNA interference
Rpm	rounds per minute
Rpl32	Ribosomal protein 32
RT	Room Temperature
RT-PCR	Reverse Transcription PCR
sec	Second
S.E.M	Standard Error of the Mean
SOCS	Suppressor of Cytokine Signaling
TA	Tyramine
TAG	Triacylglycerin
UAS	Upstream Activating Sequence
upd	unpaired

1. Introduction

1.1 Overview and epidemiology of fatty liver diseases

Fatty liver diseases are classified as either alcohol and nonalcohol related. While alcohol is a direct toxin to the liver cells and may also cause inflammation, nonalcoholic fatty liver disease (NAFLD) is obviously a different pathology and there are many possible causes that are linked to fat aggregation in the liver. Some of these causes involve consumption of high caloric diets, obesity, type II diabetes, insulin resistance, high blood triglyceride levels, other types of malnutrition, genetics factors (e.g. Patatin-like phospholipase domain-containing protein 3 'PNPLA3' variants) and medications with e.g. tamoxifen, methotrexate, and amiodarone (Sayiner et al., 2016; Younossi et al., 2018).

NAFLD comprises non-alcoholic fatty liver (NAFL) and non-alcoholic steatohepatitis (NASH). NAFL is characterized by steatosis of the liver with no evidence of hepatocyte injury, while NASH is defined as the liver cells become injured in a background of steatosis (Kanwar and Kowdley, 2016; Brown and Kleiner, 2016).

In terms of epidemiology, several reports have highlighted the highly increasing prevalence in all main world regions. Highest rates were reported from Middle East (32%) and South America (31%), followed by Asia (27%), the USA (24%) and Europe (23%), whereas NAFLD appears to be less common in Africa (14%) (Fig. 1) (Younossi et al., 2016). Using cohort data from the USA, it was shown that the incidence of NAFLD was 10% higher in overweight individuals compared to skinny persons (Younossi et al., 2012). In fact, during the next twenty years, NAFLD is expected to become the major cause of liver related morbidity and mortality as well as a main cause for liver transplantation (Calzadilla and Adams, 2016). Currently, NAFLD represents the second most common reason to be recorded for a liver transplant. Additionally, NAFLD affects not only on the medical system and its resources, it's also associated with a high percentage chance of dying within the next 15 years when compared with the general population (Patel et al., 2016).

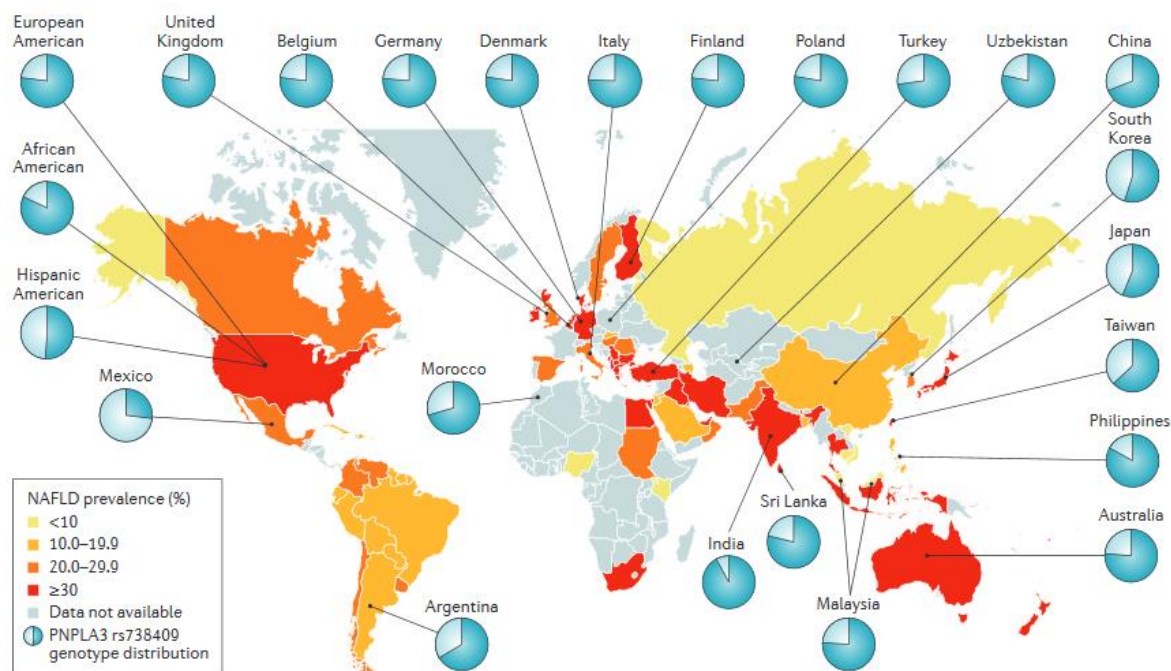


Figure 1: Worldwide estimated prevalence of NAFLD and distribution of PNPLA3 genotype.

(Younossi et al., 2018).

1.2 Pathophysiology and histopathology of fatty liver

Present concepts refer to insulin resistance as the primary metabolic defect leading to NAFLD. Due to the failure of insulin to suppress the hormone-sensitive lipase, causing more FFA to be released from the adipose tissue, insulin resistance leads to an increased influx of free fatty acids (FFA) (Bugianesi et al., 2010). Moreover, hyperinsulinemia and insulin resistance promote continuous synthesis of triglycerides in the liver. These two sources of triglycerides result in the accumulation of fats in liver cells, the hepatocytes, causing macrovesicular hepatic steatosis (Schild and Guy, 2018). Free fatty acids and elevated insulin levels stimulate lipid peroxidation and the release of hydroxyl radicals, directly injuring hepatocytes by recruiting neuroinflammatory mediators (Guilherme et al., 2008). Chronic liver injury will also cause stimulation of stellate cells, creating a potential for the development of hepatic fibrosis (Federico et al., 2016).

Histologically, non-alcoholic fatty liver disease shows a wide range of histologic appearances, which can range from a very mild steatosis to more offensive pathological forms showing

lobular and/or portal inflammation, balloon hepatocytes, fibrosis, and eventually liver cirrhosis. However, less than 5% of steatosis is not considered to be clinically significant (Sanyal et al., 2011). In very young subadult patients, the periportal or panacinar patterns are more likely seen, whereas in adults, steatosis typically affects the centrilobular hepatocytes first (Yeh and Brunt 2014). The term of macrovesicular steatosis is used when large lipid droplets inhabit the cytoplasm and push the nucleus to the periphery of the cell (Bedossa, 2016). Nevertheless, macrovesicular steatosis also include small lipid droplets and hepatocyte nuclei remaining essentially in the same original location (Bedossa, 2016; Burt et al., 2015). Lipid is a dynamic and metabolically active substance and the same applies for fatty lipid droplets in the liver. Triacylglycerols with or without cholesterol esters and a peripheral monolayer of phospholipids are the main components of fat droplets (Sahini and Borlak, 2014). The inactive Patatin-like phospholipase domain-containing protein 3 (PNPLA3) gene has been shown to accumulate on the surface of lipid droplets and is linked to an increase in macrovesicular steatosis (Smagris et al., 2015). In the late stage of fatty liver there is bridging fibrosis formed from extensions of the pericellular sinusoidal fibrosis between adjacent vascular structures (Petts et al., 2014).

1.3 Lipid accumulation in liver-induced starvation

Metazoans have to coordinate the mobilization of glycogen, lipids, and protein, to maintain metabolic homeostasis during fasting periods, thus ensuring an adequate energy supply across tissues. Endocrine signals are critical components of the fasting response in addition to tissue-autonomous metabolic adjustments (Chatterjee et al., 2014). In mammals, the liver plays a key role in regulating the intermediary metabolism during periods of malnutrition. Starvation stimulates lipid accumulation in hepatocytes, which oxidize these lipids to provide energy in the form of ketone bodies for other tissues. As a response to starvation and to some endocrine signals such as insulin and glucagon, hepatocytes regulate the expression of enzymes involved in gluconeogenesis and glycogenolysis, lipolysis, fatty acid oxidation, and ketogenesis, respectively, all regulated by a battery of transcription factors that include hepatocyte nuclear factor 4a (HNF4a), peroxisome proliferator-activated receptor (PPAR), forkhead box O (Foxo), cAMP-response element binding (CREB), and coactivator 1 alpha

(PGC1a) (Altarejos and Montminy, 2011). Additionally, hepatocytes integrate a suite of endocrine signals which are derived from major energy storing and consuming tissues, such as the adipose tissue, the muscle, and the brain, (Saltiel and Kahn, 2001). Any defect in these processes can lead to the development of the metabolic syndrome including diabetes, insulin resistance and obesity, which are major causes of hepatic steatosis (Sayiner et al., 2016; Calzadilla and Adams, 2016; Saltiel and Kahn, 2001).

1.4 Advantage of *Drosophila* as a model organism

The fruit fly, *Drosophila melanogaster* has been used for over a century as the most suitable model organism for genetics research, but also for diverse studies in the fields of metabolic, behavioral, neuronal, microbial and immunological related disorders (Roberts, 2006). Compared with other model organisms, it has a great number of advantages. *Drosophila* has a very short life cycle, approximately 10-12 days at 25°C and an average life span of 50 to 60 days and an inexpensive husbandry. Additionally, *Drosophila* offers a great number of highly relevant genetic tools and techniques for experimental design that are not found in other animal studies and that can be used for genetic manipulation in the target tissue. Moreover, the *Drosophila* genome involves about 14,000 genes that share a large proportion of homology with the corresponding human genes (Chen et al., 2008). During the past century, a large collection of *Drosophila* stocks including P-element insertions (Bellen et al., 2004), induced mutations and RNA interference (RNAi) mediated gene-specific knockdown lines (Dietzl et al., 2007) and genomic gene-defined deficiencies (Parks et al., 2004) have been generated. These lines are mostly available from public repositories such as the Vienna *Drosophila* Resource Centre (VDRC) or the Bloomington Stock Centre. In addition, more information could be obtained online by the most important bioinformatics database for *Drosophila*, namely Flybase.

One of the most important advantage for using *Drosophila* as a research model is the availability of binary GAL4/UAS-systems invented more than 20 years ago (Brand and Perrimon, 1993). In brief, it is a bipartite transcription activation system that relies on two essential components, GAL4 and UAS which are inserted separately into two transgenic fly lines, called driver and responder lines, respectively. Crossing these two lines leads to

activation of this system in the F1 generation (Duffy, 2002). Both factors, GAL4 and the UAS enhancer come together to direct expression of the target gene in the F1 generation by the expression pattern of the GAL4 line, the gene of interest is introduced under the control of upstream activating sequence (UAS). Thereby, the target gene is only expressed in a cell- or tissue-specific pattern in their progeny. This system is ideally suited for overexpression or silencing (RNAi) of a target gene in a specific organ [Fig. 2]. Due to these advantages, *Drosophila* has been studied extensively regarding biological processes and investigating developmental aspects, but also processes such as growth, or the molecular framework underlying metabolic disorders, in order to increase our understanding of the corresponding processes in humans.

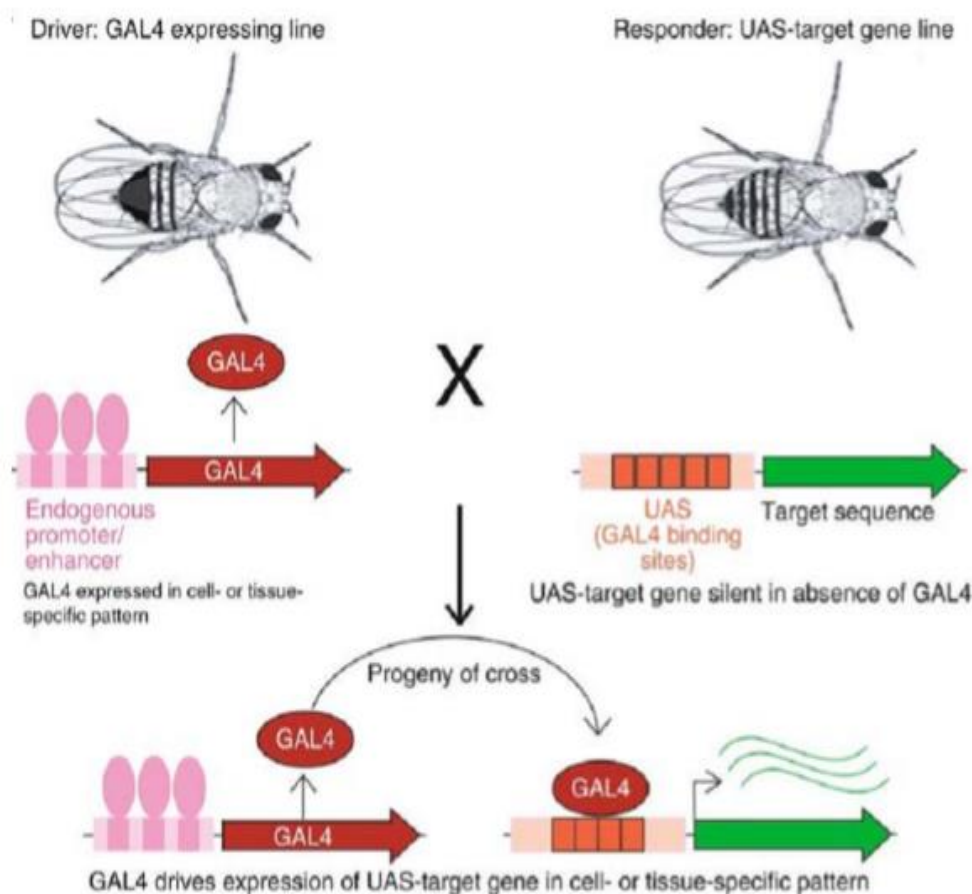


Figure 2: Schematic description of the GAL4/UAS system.

While the GAL4 driver line is crossed with the UAS responder line, the target gene is only expressed in a cell- or tissue-specific pattern in their F1 progeny. Adopted from (Elliott and Brand, 2008).

1.5 *Drosophila melanogaster* as a model to study fatty liver disease

Drosophila has emerged as a prolific model organism to describe and evaluate the endocrine regulation of metabolic adaptation (Roberts, 2006; Younossi et al., 2012). The central function of the liver in metabolic adaptation of flies is shared by the fat body and by the so-called oenocytes (Ugur, 2016). Whereas the fat body serves as an endocrine organ to coordinate metabolic homeostasis, it is also an important organ for glycogen and fat storage in flies (Patel et al. 2016; Apidianakis and Rahme, 2011.) Similar to mammalian hepatocytes, oenocytes have a critical role in lipid mobilization and turnover, which comprises to accumulate lipids during starvation and expressing genes with homology to liver-specific enzymes in mammals (Ryu et al., 2008; Makki et al., 2014). Oenocytes accumulate lipid droplets, a process that is required for mobilization of lipids from the fat body, suggesting a close interaction between fat body and oenocytes. However, and according to their response to starvation, several studies have shown that oenocytes are more similar to the mammalian hepatocytes than fat body cells are. Furthermore, oenocytes express 22 homologs of human fat-metabolizing genes expressed in hepatocytes, and also express genes related with hepatocyte differentiation, including COUP-transcription factor (COUP-TF) and hepatocyte nuclear factor 4-a (Hnf4-a) (Gutierrez et al., 2007).

1.6 Oenocytes: Liver-like cells of *Drosophila*

Oenocytes are mysterious cells were first discovered in 1856. They have been identified as unicellular glands, respiration cells and intermingled cells. The currently accepted term, oenocytes, was adopted in 1886 according to the wine yellow color of these cells (Makki et al., 2014). Oenocytes are secretory cells that are found in most of pterygote insects, display obvious evolutionary variations in number, size and anatomical location across larva and adult flies. In larval *D. melanogaster*, oenocytes are arranged in lateral and subepidermal clusters of 6-8 cells per abdominal hemisegment, whereas the adult oenocytes are located within the abdominal cuticle, and are arrayed in dorsal ribbons, radiating along the inner cuticular

surface and ventral clusters in each abdominal segment [Fig. 3] (Makki et al., 2014; Krupp and Levine, 2010).

Indeed, by using neutral lipid specific dyes such as Oil Red O and Bodipy, scientists demonstrated that the larval oenocytes of *D. melanogaster* are unusual cells because they specifically accumulate fat droplets as a normal response to starvation, unlike most other tissues, which tend to lose them under these physiological conditions (Makki et al., 2014). Lipid droplets are the major intracellular structure for the storage and lipolysis of neutral lipids such as triglycerides and cholesterol esters [Fig. 4] (Ugur et al., 2016; Krupp and Levine, 2010).

Oenocytes play a key role in the growth and developmental progression during both larval and pupal stages, and regulate the body fat content and lipid homeostasis. These cells are critical for the synthesis of several long chain hydrocarbons based sex pheromones and other cuticle components. Analogous to the mammalian adipose–liver axis, several studies suggest the presence of marked metabolic coupling between the fat body and oenocytes (Gutierrez et al., 2007; Chatterjee et al., 2014).

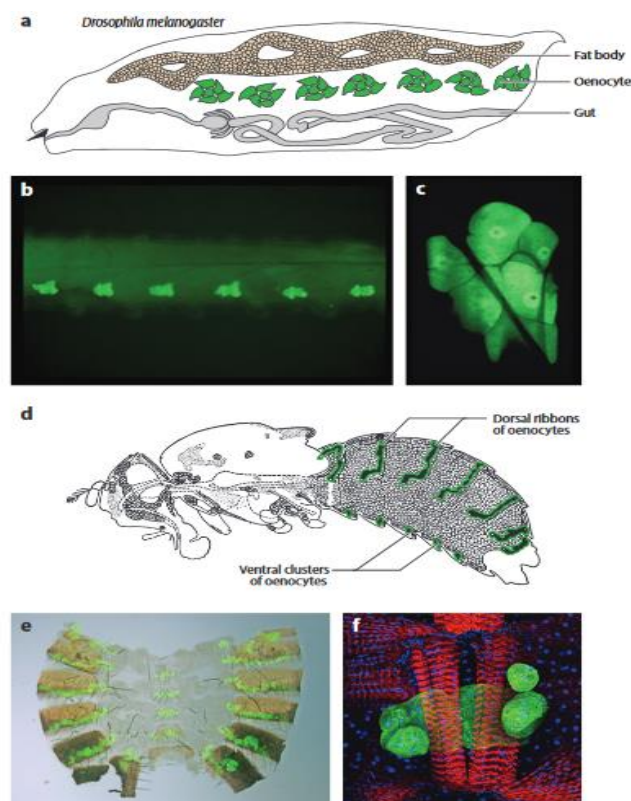


Figure 3: Larval and adult oenocytes in *Drosophila melanogaster*.

(a) Drawing of a larva showing the site of oenocytes, the fat body, and the gut. (b) Lateral view of a larva showing oenocyte clusters (green). (c) Section of one larval oenocyte cluster of six cells. (d) Drawing of adult fly showing the abdominal dorsal ribbons and ventral clusters of oenocytes (green). (e) A filleted adult abdomen showing the segmental dorsal stripes and ventral clusters of oenocytes (green). (f) Confocal image of a ventral adult oenocyte cluster (green) connected with muscles (red) also shown are the nuclei (blue) (Makki et al., 2014).

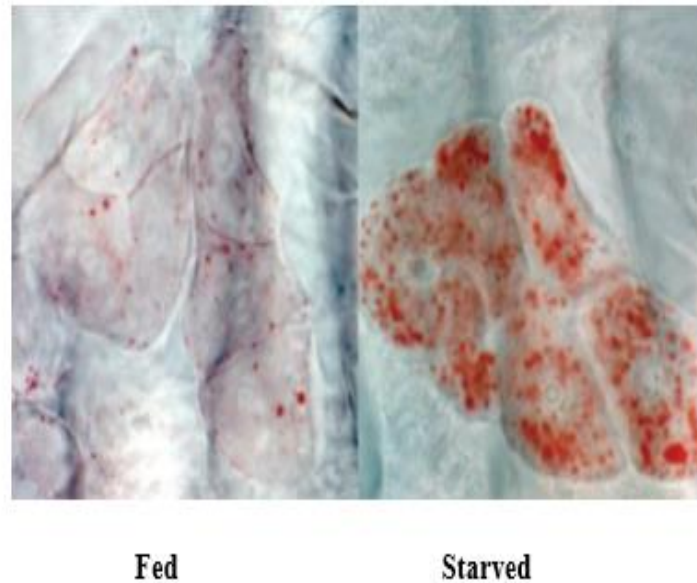


Figure 4: Oenocytes accumulate lipids during fasting.

Staining of L3 larval oenocytes under fed and starved conditions using Oil Red O, showing that oenocytes take up neutral lipids (red) during fasting (Gutierrez et al. 2007).

1.7 Optogenetic tools to manipulate second messengers in target tissues of *Drosophila*

Studies performed by Earl W. Sutherland Jr. and Theodore W. Rall in 1956, found cyclic adenosine 3',5'-monophosphate (cAMP) as the first intracellular second messenger to be identified (Sutherland and Rall, 1958). cAMP is a nucleotide that regulates diverse cellular functions, including cell growth and differentiation, gene transcription and protein expression (Yan et al., 2016). Pathways converging on the production of cAMP are most commonly initiated following the binding of the first messengers, which is a neurotransmitter, hormone,

or modulator. Subsequently, the corresponding G protein-coupled receptors (GPCRs) are activated and the Gs alpha subunit of the stimulated G protein complex exchanges GDP by GTP and is released from the complex, binds to and activates an enzyme on the cell membrane named adenylyl cyclase, which in turn, stimulates the conversion of ATP into cyclic adenosine monophosphate (cAMP). cAMP acts as a second messenger that can diffuse freely throughout the cytosol. High concentrations of the second messenger cAMP may lead to the activation of a cytoplasmic enzyme called protein kinase A (PKA), this enzyme has a key role as it catalyzes phosphorylation and therewith regulation of a number of other proteins, thus ultimately causing a cellular response [Fig. 5] (Sassone-Corsi, 2012).

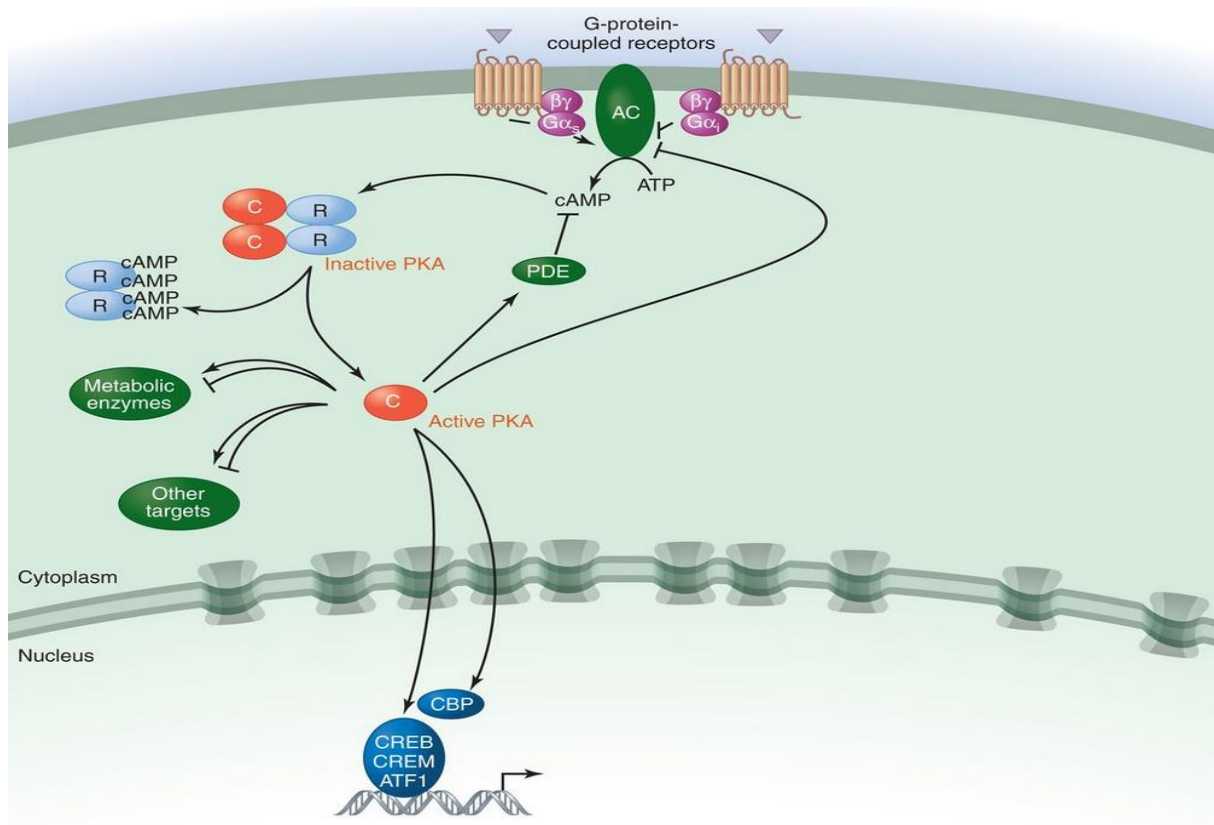


Figure 5: The cAMP signaling pathway.

The cAMP signaling pathway is one of the most important pathways in the liver and it plays an important role in gene regulation via activate transcriptional activators such as cAMP response element-binding protein (CREB). It binds to certain DNA sequences called cAMP response elements (CRE), thereby decreasing or increasing the genes transcription, these

events proceed from hepatocyte surface to nucleus through a specific enzyme such as protein kinases A (PKA) (Molina et al., 1993; Della et al., 1997).

Recently, optogenetic tools have been developed that allow to precisely manipulate second messenger systems. Second messengers are intracellular signaling molecules released in the cell in response to exposure to extracellular signaling molecules—the first messengers. Second messengers trigger physiological changes such as proliferation, differentiation, migration, survival, and apoptosis. They are one of the triggers of intracellular signal transduction cascades. Examples of second messenger molecules include cyclic AMP, cyclic GMP and calcium (Kerr et al., 2004). Moreover, these tools have also been adapted to *Drosophila* genetics, which helps to open a new epoch in research to easily activate the second messengers in the living animal. *In vivo*, the optogenetic process for cAMP manipulation can be applied by using a small bacterial photoactivated adenylyl cyclase (bPAC), of the soil bacterium *Beggiatoa*, which have a BLUF (blue light receptor using FAD) domain (Stierl et al., 2011; Penzkofer et al., 2014). Thus, utilizing Gal4 driven UAS-bPAC effector lines can be useful tools to study blue-light dependent cAMP increase in the oenocyte of *Drosophila* to resolve its physiological role [Fig. 6].

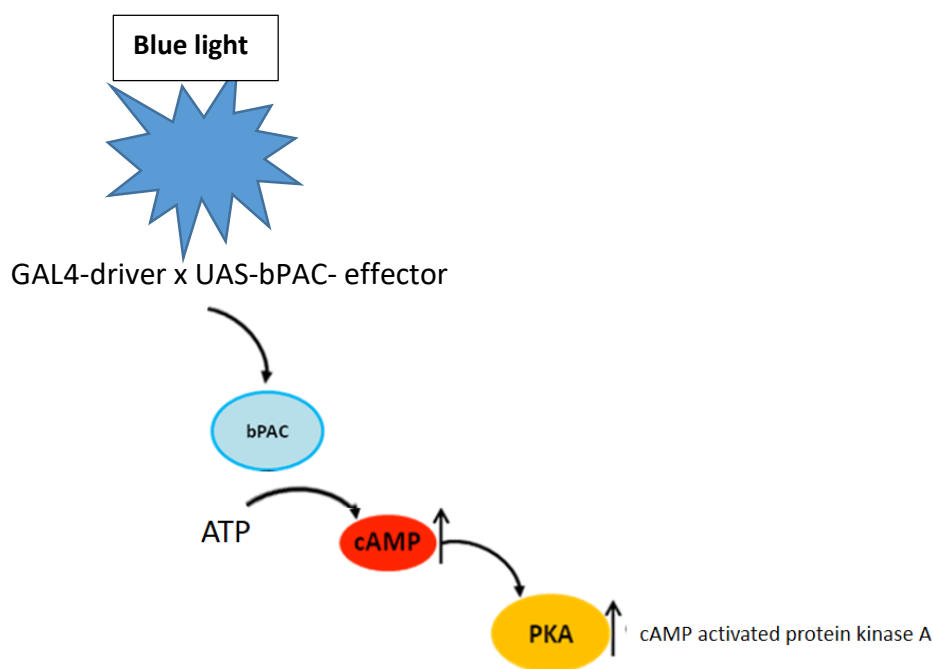


Figure 6: Utilization of photoactivated adenylyl cyclase (bPAC), of the soil bacterium *Beggiatoa* to modulate cAMP in *D. melanogaster*.

1.8 JAK-STAT pathway in *Drosophila*

From insects to mammals, the Janus Kinase-Signal Transducer and Activator of Transcription (JAK-STAT) pathway is very conserved among all these animal species. JAK-STAT signaling pathway is activated by many cytokines and growth factors and has been participated in a diversity of cellular functions in neuronal, hematopoietic and hepatic system. Additionally, it plays a critical role in mediating pro-inflammatory cytokines like interleukin-6 (IL-6) in mammals and it is responsible to regulate immune responses to bacterial and viral infections (Schindler, 1999; Levine and Wernig, 2006; Darnell et al., 1994). In the liver cells, jak-stat pathway is activated in response to cytokines, hormones and growth factors, mediating a variety of cellular functions including defense against pathogens, proliferation, differentiation, cell metabolism and apoptosis (Kisseleva et al., 2002). Over the last decades, jak-stat components and its negative regulators have been under intense investigation (Naka et al., 1999). JAK-STAT pathway in the liver is activated by phosphorylation of the Janus protein tyrosine kinases, upon ligand binding to cognate receptors. Followed by phosphorylation and activation of STAT protein. Subsequently, activated STAT translocate to the nucleus where they modulate expression of target genes (Kisseleva et al., 2002). Animal models have highly contributed to our understanding of JAK-STAT components which regulate the physiological functions of liver cells. However, this pathway is less complex in *Drosophila* (Brown et al., 2003). There are three related cytokines encoded in the *Drosophila* genome, called unpaired (upd), unpaired 2 (upd2) and unpaired 3 (upd3), which are released due to injury of infection. To regulate the activity of JAK-STAT signaling, e.g. IL-6-like cytokines (upd3) bind a gp130-like cytokine receptor named domeless (Dome) (Brown et al., 2001; Arbouzova and Zeidler 2006; Wright et al. 2011). The Dome receptor then in turn activates the Janus Kinase (JAK), Hopscotch (Hop) (Binari and Perrimon, 1994). Subsequently, in order to activate transcription of genes that are critical to the inflammatory response, activated Hop induces phosphorylation of the signal transducer and activator of transcription STAT92E. Hopscotch shows greatest similarities to the human JAK2 (27% identity) and STAT92E is most similar to human STAT5 (37% identity) [Fig. 7] (Zeidler et al., 2000; Arbouzova and Zeidler, 2006).

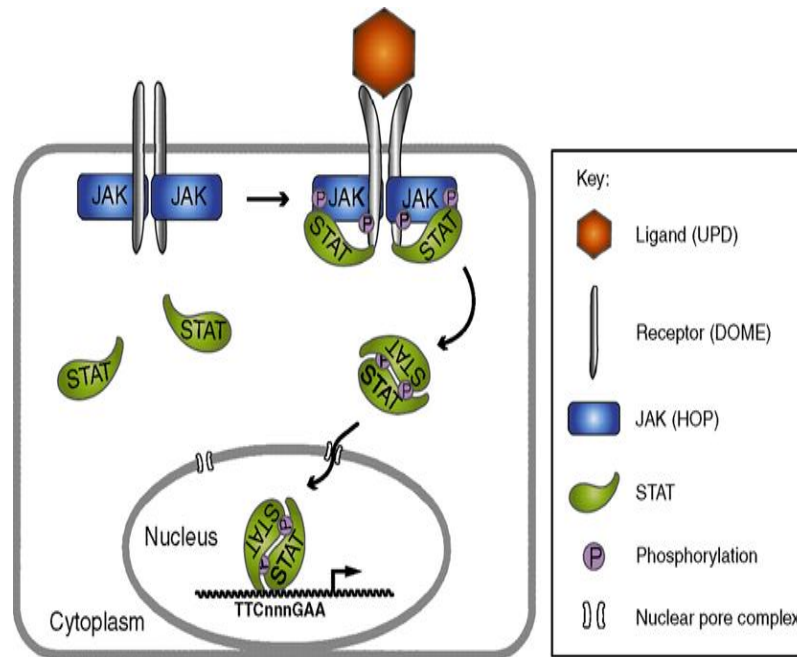


Figure 7: JAK-STAT pathway in *D. melanogaster*.

The main components of the JAK/STAT pathway in *Drosophila* consist of: three ligands (UPD, UPD2 and UPD3); a transmembrane receptor named Domeless (DOME); a JAK kinase known as Hopscotch (HOP); and a transcription factor of the STAT family (Arbouzova and Zeidler, 2006).

1.9 Host–microbiota interaction

Recently, and due to the gut anatomical, structural and functional similarities between *Drosophila* and vertebrates, researchers used *Drosophila* as a model organism to study human gut related disorders (Vodovar et al., 2004; Apidianakis and Rahme, 2011). Besides these, published scientific reports suggested the utilization of fruit flies as models to detect the role of gut microbes in improving some physiological parameters such as life span, gut stem cell activity, metabolic and developmental homeostasis, dietary habits, growth promoting activity, behavior, activation of the immune system and mid-gut regeneration (Ryu et al., 2008; Ohlstein and Spradling, 2006; Blum et al., 2013). Natural populations of *D. melanogaster* live in constant association with microorganisms inhabiting rotting fruits, including a variety of yeasts and bacteria of the *Acetobacteraceae* and *Lactobacillales* taxa. In the laboratory, members from the same families of microbes are also found to be extensively associated with their fly partners and in the food. Therefore, *Drosophila* serves as an excellent model to study host–microbiota interaction, as well as liver–microbiota interactions (Téfit et al., 2017).

1.10 Role of Gut Microbiota in Liver Diseases

For a long time, the microbial products were considered one of the major contributors in the onset and progression of liver disease. However, a more detailed picture of the gut microbiota contributing to liver disease started to develop. The intestine is colonized by a large numbers of microbes that help in digestion, immune response, and generate a variety of microbial products that result from its metabolic activities. These products together with host-microbiota interactions affect both normal physiology and disease susceptibility (Llorente & Schnabl, 2015). A disorder of the symbiosis between microbiota and host is known as dysbiosis and can have profound impact on health. Qualitative changes such as bacterial overgrowth, reduced levels of beneficial bacteria and increased proportions of harmful bacteria have been associated with liver disease such as fatty liver disease and cirrhosis (Schnabl, 2014; Yan et al., 2011). Understanding the interaction between the pathophysiology of liver diseases and functional and compositional changes of the gut microbiota will help in the design of effective therapies. In this study, I focus on the role of gut microbiota in the accumulation of lipids in the liver like cell, oenocyte and its effect on some physiological parameters of adult *Drosophila*.

1.11 Aims of the study

The major aim of this project was to study the role of *Drosophila* oenocytes, as hepatocyte-like cells, during periods of starvation and to unravel their key role in the control of lipid homeostasis. To study the hormonal regulation of this complex process, I manipulated the cAMP and JAK-STAT pathways in oenocytes in order to evaluate if different hormonal signals converging onto these two pathways modify their physiology. My study comprises three major aspects. The first part focused on the role of cyclic AMP as a second messenger on the function of fly's oenocyte in the fat accumulation during starvation conditions and its impact on physiological parameters such as life span, metabolic rate and lipid amount in the adult flies. For the same reasons, I tested the effect of JAK/STAT activation and knockdown in the

oenocyte, in the second part of my study. Furthermore, I measured the expression of some interesting genes which are related with lipid metabolism and immunity in starved flies. Lastly, this work described the gut microbiota-oenocyte interaction in adult flies and its effect on the fat accumulation after starvation as well as on some physiological aspects. Using of fruit fly, *Drosophila* for such studies could provide rapid insights into genetic mechanisms of liver diseases and allow screening of new compounds for alternative therapeutic interventions.

2. Materials and Methods

2.1 Materials

2.1.1 Drosophila strains and husbandry

The fly stocks used in this project were raised on standard medium and kept at 25°C with 50%-60% relative humidity under a 12 h light/dark cycle. Moreover, all assays utilized virgin females and unmated males at a density of 25-30 adults in each vial.

Table 1: Fly stock, genotype and donator

Stocks name	genotype	donator
W¹¹¹⁸	W¹¹¹⁸	Bloomington No: 5905
Oenozyten-Gal4	w+; PromE(800)-Gal4 (Line 2M); +	Joel Levine, University of Toronto
STAT-GFP 2nd	w: 10x STAT-GFP 2nd	Bach EA et al. Gene Expr Patterns. 2007,7:323-331.
STAT-GFP 3rd	w: 10x STAT-GFP 3rd	Bach EA et al. Gene Expr Patterns. 2007,7:323-331.
UAS-bPAC	w[*];P{UAS-bPac.S}/Cyo	a gift of Martin Schwärzel; Free University Berlin
UAS-STAT92E	P{w[+mC]=UAS-STAT92E) synonym UAS-mr	Dustin W. Perry, Univ. Kentucky, USA
UAS-STAT92E-RNAi	y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HMS00035}attP2	Bloomington No: 33637

UAS-Upd3-RNAi TRiP	y[1] sc[*] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HMS00646}attP2	Bloomington No: 32859
upd3-Gal4;UAS-GFP	Expresses Gal4 in the pattern of upd3	BestGene 5070-3-2F*)
UAS-GFP	w ¹¹¹⁸ ; P[UASCameleon.2.1]82	Bloomington No: 6901

2.1.2 Fly media

2.1.2.1 Standard medium

The ingredients of the fly standard food were composed of 6.25% yeast, 6.25% cornmeal, 1% agar powder, 2% glucose and 3% molasses and syrup. All components were dissolved in sterile water and boiled for 15 minutes. After cooling, 3% nipagin and 1% propionic acid were added as preservatives to protect media from being infected.

2.1.2.2 High sugar medium

For well-fed condition, high sugar medium was used. This medium was composed of the same ingredients of standard media with additional 10% sucrose.

2.1.2.3 Axenic medium

Axenic medium is a microbe free media required for generation of germ-free flies since food is a main source of germ contamination.

This medium carries the normal ingredients of standard fly food, all components were dissolved in sterile water and were boiled for 15 minutes. Subsequently, the mixture was autoclaved for 20 min. Later propionic acid and nipagin were added inside laminar condition.

2.1.3 Solutions

Phosphate Buffered Saline Tween-20 (0.3% PBST):

The following was dissolved in 800 ml of distilled water, 8 g NaCl, 0.2 g KCl, 1.44 g Na₂PO₄, 0.24 g KH₂PO₄, 3 ml Tween-20. Subsequently, the pH was adjusted to 7.2 and additional distilled water was added up to 1 liter. The solution was autoclaved.

Paraformaldehyde in PBS (4% PFA):

2 g paraformaldehyde powder was dissolved in 50 ml PBS solution under continuous stirring at 45°C. The fixing solution was aliquoted and stored at 4°C after it became clear.

2.1.4 Antibodies

Mouse α -coracle	Development Studies Hybridoma Bank, Iowa, IA, USA
Goat α -mouse Daylights 549	Jackson ImmunoResearch Laboratories, Dianova, Germany

2.1.5 Molecular biology enzymes

Table 2: List of molecular biology enzymes

DNase	Thermo fisher scientific, Waltham, MA, USA
RNase inhibitor (40U/ μ l)	Invitrogen, Darmstadt, Germany
SuperScript™IV Reverse transcriptase	Invitrogen, Darmstadt, Germany
Taq DNA Polymerase (5 U/ μ l)	Invitrogen, Darmstadt, Germany

2.1.6 Oligonucleotides

Table 3. Oligonucleotides used for PCRs

primers	Sequence(5` to 3`)
<i>5-HT1A-F</i>	GCTGCAATTAAGCGAAGCGA
<i>5-HT1A-R</i>	CTACATTCGGCTGTTCGGGA
<i>5-HT2A-F</i>	TACGCATTGACGATTCCCCT
<i>5-HT2A-R</i>	CCGTCGCCCATGATAGGAA
<i>5-HT2B-F</i>	AGTTCTGGGTCTGTCCACCA
<i>5-HT2B-R</i>	TAGAGTGC GTTCTTGGGCAG
<i>Bmm-F</i>	CTGTGCAAGATCACCCCAAT
<i>Bmm-R</i>	CTAGCATCCGACTCGGTCTC
<i>D2R-F</i>	CGATGAATGCGCCTTCTACA
<i>D2R-R</i>	GTC TGG GCA ATG TTC TCG
<i>Dilp6-F</i>	ACGGTGGCCCTTGGCGATGTA
<i>Dilp6-R</i>	CCTGTAGGTGCAGCCACCCGA
<i>DopR2-F</i>	CTTCTCGCTGTCCAGGAAAC
<i>DopR2-R</i>	GCAGGCGTAAATCACAGGAT
<i>DopR-F</i>	CCCAGCGAGTTTCTCAGTTC
<i>DopR-R</i>	TGTCATTGGAACGAGGATGA
<i>OA2-F</i>	GGCAACGAGTAACGGTTTGG
<i>OA2-R</i>	TCATGGTAATGGTCACGGGC
<i>OAMB-F</i>	CGGTTAACGCCAGCAAGTG
<i>OAMB-R</i>	AAGCTGCACGAAATAGCTGC
<i>Octb2R-F</i>	TCCTGTGGTACACACTCTCCA
<i>Octb2R-R</i>	CCACCAATTGCAGAACAGGC
<i>Octb3R-F</i>	TGTGGTCAACAAGGCCTACG
<i>Octb3R-R</i>	GTGTTCCGGCGCTGTTAAGGA

<i>Rpl32-F</i>	CCGCTTCAAGGGACAGTATC
<i>Rpl32-R</i>	GACAATCTCCTTGCGCTTCT
<i>Socs36E-F</i>	GAGATCCTCACAGAGGCCACT
<i>Socs36E-R</i>	GCGAAACTTTCCACCTGACC
<i>Thor-F</i>	AGCGACTTGGTCTGCTTGAT
<i>Thor-R</i>	TCCTGGAGGCACCAAACCTTA
<i>TyrR I-F</i>	AGACGAGGTGCAAGGTGTTG
<i>TyrR II-F</i>	TGCAGTCTTTGCCACCTTCA
<i>TyrR III-F</i>	GAAGTTGGCCATCACCGACT
<i>TyrR II-R</i>	GTTGCCACGAGCCTATGAGA
<i>TyrR I-R</i>	TTCCCCGACTTCTTTGACTGC
<i>TyrR-III-R</i>	GTGACGGCGAGATACCTGTC
<i>Upd2-F</i>	AGCAGAAGAGCCTCAACGAG
<i>Upd2-R</i>	CTGGCGTGTGAAAGTTGAGA
<i>Upd3-F</i>	GAGAACACCTGCAATCTGAA
<i>Upd3-R</i>	AGAGTCTTGGTGCTCACTGT

2.1.7 Chemicals

Table 4: Chemicals

Agar-Agar	Carl Roth, Karlsruhe, Germany
Agarose	Biozym, Oldendorf, Germany
Bacto TM yeast extract	Becton, Dickinson and
BODIPY(493/503)	Molecular Probes life technologies, Darmstadt, Germany
Chloroform	Carl Roth, Karlsruhe, Germany
DAPI	Invitrogen, Darmstadt, Germany

Ethanol	Carl Roth, Karlsruhe, Germany
dNTPs	Promega, Mannheim, Germany
Glucose	Carl Roth, Karlsruhe, Germany
Goat serum	Sigma-Aldrich, Steinheim, Germany
Isopropanol	Carl Roth, Karlsruhe, Germany
Molasses	Kanne Brottrunk GmbH & Co. KG, Germany
Nipagin	Carl Roth, Karlsruhe, Germany
Paraformaldehyde	Sigma-Aldrich, Steinheim, Germany
Propionic acid	Carl Roth, Karlsruhe, Germany
RNAmagic	Bio-Budget, Kredeld, Germany
Sucrose	Carl Roth, Karlsruhe, Germany
Triglyceride	Invitrogen, Darmstadt, Germany
TritonX-100	Sigma-Aldrich, Steinheim, Germany
Tween-20	Carl Roth, Karlsruhe, Germany

2.1.8 Equipments

Table 5: Equipments

Agarose Gel electrophoresis unit	Biometra GmbH, Göttingen, Germany
Autoclave Steam Sterilizer Varioklav	H+P Medizintechnik GmbH, Oberschleisheim, Germany
Bead Ruptor 24	Denver Instruments, New York, USA
Centrifuge 5417 R, rotor FA-45-30-11	Eppendorf, Hamburg, Germany
Centrifuge, 5415 D; rotor: F45-24-11	Eppendorf, Hamburg, Germany
Electrophoresis power supply, EV245	Consort, Parklaan, Belgium

Flourescence microscope Axio Imager.Z1	Zeiss, Oberkochen, Germany
Ice machine Manitowoc®	Herborn, Germany
Incubation shaker, Heidolph Unimax 1010	Heidolph Instruments GmbH & Co. KG, Schwabach, Germany
Incubator 37°C	Memmert GmbH & Co.KG, Heilbronn, Germany
Leica TCS SP1 confocal Microsystems	Leica, Heidelberg, Germany
NanoDrop (ND-1000UV)	Peqlab, Erlangen, Germany
Olympus SZX12 microscope	Olympus GmbH, Hamburg, Germany
Omni Bead Ruptor 24	Omni International Inc, Kennesaw, USA
PCR amplification	Bio-RadLaboratories GmbH, München
pH meter, model 330	Wissenschaftlich-Technische Werkstätten GmbH Weilheim, Germany
Spectrophotometer	Multiscan MCC/340P version 2.32
StepOnePlus™ System	Applied Biosystems, Life Technologies
Thermocycler (Labcyler)	SensQuest GmbH, Göttingen, Germany
Vortex-2 Genie	Scientific Industries, INC., Bohemia, N.Y.,USA

2.2 Methods

2.2.1 GAL4/UAS crossing

Without otherwise statement, the crossings were raised at 25°C, 10 -15 virgin females of the UAS strains and corresponding GAL4 males were used and the F1-generation was collected for the analysis.

For cyclic AMP activation, F1-generations of crosses with blue-light inducible strains were kept either in the dark or the fly vials were covered with aluminum foil to protect them from UV-light.

2.2.2 Starvation resistance

Starvation experiments were performed by placing 25 flies in each vial (100 flies per replica) with water-soaked filter paper. Mortality rates were determined by counting the number of dead flies at fixed time intervals.

2.2.3 Measuring number and size of fat droplets

Fat droplets from the digital images were analyzed on a computer by drawing a line over the scale that was introduced into the image field. Using the line tool, numbers and diameters of 100 fat droplets were manually measured. The output of measurements was loaded into GraphPad Prism 6 and evaluated for average number and size of lipid droplets.

2.2.4 Measurement of metabolic rate

This method was described in the paper from (Yatsenko et al., 2014) and used with some modifications. A scheme showing the construction of the respirometer for CO₂ measurement is shown [Fig. 8]. In detail, a long capillary micropipette was settled and sealed in the head of a 1 ml pipette tip. Soda lime, which was used as a CO₂ absorbent, was placed in the tip and insulated by two pieces of foam in front and back. 3 adult flies of the genotype of interest were placed in the pipette tip. Using plasticine, the respirometer was sealed completely at the top. Two respirometers without flies were used as the atmospheric control. At the bottom of the chamber, a blue dye solution was added to make the liquid visible. The respirometer was placed in straight with the micropipette tips submerged into the coloured solution. The chamber was covered with glass to prevent it from temperature and pressure fluctuations. The assay was performed for 2 h; subsequently a photograph of the chamber was taken. The amount of CO₂ production (μl/h/fly) was calculated with the formula:

$$\frac{(\pi \times R^2) \times (\Delta d - \Delta c) \times 1000}{n \times h}$$

R: radius of capillary micropipette (cm).

Δd: height of the liquid in experimental samples far from the colored solution (cm).

Δc: height of the liquid in negative controls far from the colored solution (cm).

n: number of flies used.

h: hours of assay performed.

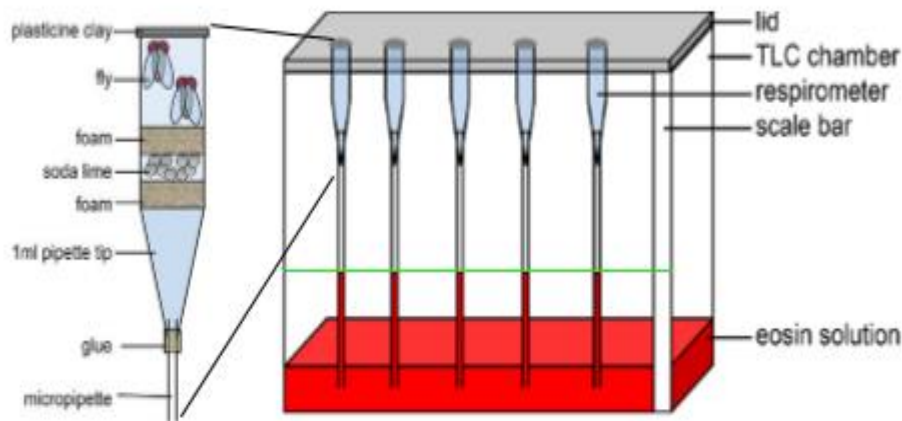


Figure 8: Scheme of setups used for measurement of CO₂ production.

The construction of the individual respirometer and the chamber. Eosin solution was alternatively replaced by blue dye (FD&C Blue No.1). Source adoption from Yatsenko et al., 2014.

2.2.5 Body fat quantification

For total triacylglycerol (TAG) quantification in flies, the *Coupled Colorimetric Assay (CCA)* method was used as described previously (Hildebrandt et al., 2011). Glycerol trioleate was used as TAG standard for standard curve establishment, which was diluted in a series of 2-fold dilution with starting quantity of 100 µg. For experiments, 5 females or 8 males per group as one biological replicate were weighted. Then the flies were transferred to a 2 ml screw-cap tube containing 1 ml 0.05% Tween-20 and 4 ceramic beads, and homogenized by using a Bead Ruptor 24 at a speed of 3.25 m/s for 2 min. Homogenates were heat-inactivated at 70°C for 5 min followed centrifugation for 3 min at 3000 rpm. Sample supernatant of 50 µl was pipetted to a 96 well microliter plate. The various concentrations of TAG standard were treated in an identical way. The absorbance of samples was measured at 562 nm using a microplate reader as blank values. 200 µl of pre-warmed triglyceride reagent was added into each well, then incubated 30 min at 37°C with mild shaking. The absorbance was measured at 562 nm again and the standard curve was set up by subtraction of blank values.

The final quantity of samples was calculated per mg of fly wet weight base on the standard curve. Each genotype was measured in triplicate.

2.2.6 Optogenetic manipulation of oenocytes with blue light

In order to investigate the physiological impact of cAMP-dependent signaling in the oenocyte of *D. melanogaster*, transgenic animals expressing the photoactivated adenylyl cyclase (bPAC) were subjected to blue light. The blue light lamp was arranged approximately 25 cm above the vials and a lux meter was used for measuring illumination (approximately 740 lux (Fig. 9)). In order to ensure light-induction was only performed by the blue-light, vials were packed in aluminum foil until blue-light was applied.

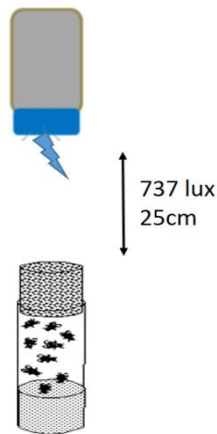


Figure 9: Schematic view of blue light stimulation

Animals were exposed to blue light to observe changes within the oenocyte of the fly.

2.2.7 Bodipy staining

The neutral lipids in the oenocyte were stained with the amine-reactive 4,4-difluoro-1,3,5,7,8-pentamethyl-4-bora-3a,4a-diaza-s-indacene (BODIPY® 493/503) dye, which could be used to create green-fluorescent bioconjugates. The dye was dissolved in dimethyl sulfoxide (DMSO, 1mg/ml), and diluted 1:1000 (v/v) in PBS for working solution.

For each genotype, abdominal fillet dissection for 5 flies were performed, and 500 μ l 4% (w/v) paraformaldehyde (PFA) was added for fixation for 15 min at RT. After washing three times with PBS 1x, 200 μ l of BODIPY working solution was given over the flies and incubated

for 30 min. in the darkness at RT. It was followed by two more washes in PBS and the preparations were observed under a fluorescent microscope.

2.2.8 Immunohistochemistry and imaging

Abdominal fillet dissections of 3rd instar larvae and 7-day-old adult flies were performed in hemolymph-like saline (HL-3) buffer under a microscope, fixed it in 4% PFA for 30 min at room temperature, and rinsed three times for 5 min each in PBST (0.3% Triton X-100), blocked in blocking-buffer (1×PBS + 1% Triton X-100 + 5% goat serum) for 30 min at RT. Subsequently, the primary antibody was added to incubate overnight at 4°C, after three times of 5 minutes washing, the diluted secondary antibody was added and incubated for 3 h. at RT in a constant darkness. The material was washed three times for 10 min each, finally the tissue was mounted on slide by immersing in DAPI. Each image was photographed by Carl Zeiss fluorescent microscope and Zeiss AxioVision 4.8 software.

The following antibodies and dilutions were used in this study:

- Mouse α -coracle 1:200, as a primary antibody.
- Goat α -mouse Dylight 549 1:250, as a secondary antibody.

For fluorescent intensity measurement, Zeiss Axio Imager Z1 with Apotome was applied and the quantification of cell fluorescent intensity was measured using Image J and calculate the corrected total cell fluorescence (CTCF). $CTCF = \text{Integrated Density} - \text{Area of selected cell} \times \text{Mean fluorescence of background readings}$ (Gavet and Pines, 2010).

2.2.9 Germ-free fly assays

Eggs which have been laid overnight on normal medium were collected using a brush. Dechoriation of embryos was done by treating with 6% Sodium Hypochlorite for 2-3 min. Next, embryos were washed twice with 70% Ethanol followed by three washes with sterile 1x PBS. Dechorionated eggs were transferred to axenic media. Subsequently, flies were checked for the absence of gut microbiome using RT-PCR (Sabat et al., 2015).

2.2.10 Molecular biological methods

2.2.10.1 Agarose gel electrophoresis

Agarose gel electrophoresis was used for analytical and preparative purposes. For a casting gel, 1-2 % (w/v) agarose was dissolved by heating it in a microwave oven in 1 x TBE buffer. To make DNA bands visible, 1 μ l ethidium bromide was added to agarose gels. The DNA samples were mixed with DNA loading buffer and loaded onto the wells, DNA marker run in parallel. Electrophoresis was performed at a constant voltage of 100 V for 45–60 min, depending on the desired resolution. For the purposes of documentation, DNA bands were visualized under UV light (312 nm) and gels were photographed using the gel imaging system.

2.2.10.2 Total RNA extraction

Manual isolation of oenocyte ribbons was performed in HL-3 buffer on ice. After that, the ribbons were homogenized in 1 ml RNA magic for 1 min at 3.25 m/s speed, with the help of ceramic beads using Bead Rupture 24 implement. Following addition of 200 μ l of chloroform with shaking by hands 15 times, the mixture was centrifuged for 5 min at 15000 g. The upper phase was transferred carefully into a clean tube, mixed with an equal volume of isopropanol. The mixture was centrifuged for 30 min at maximum speed at 4°C there after incubated at -20°C overnight. The RNA pellet was washed twice with 500 μ l cold 70% ethanol. Finally, all aqueous phase materials were discarded and air dried, the RNA resolved in RNase-free water and was stored at -80°C.

2.2.10.3 cDNA synthesis

The procedure was designated to convert mRNA into first strand of complementary DNA. The following components were used: 500 ng of total RNA, 0.5 μ l Oligo-dT7 I (10 pmol), 0.5 μ l CapFinder SP6rG1 (10 pmol), 1 μ l dNTPs mix (10 mM), water added up to 6.75 μ l, the mixture

was incubated at 65 °C for 5 min, then placed on ice for at least 1 min. The following components were mixed in the indicated order: 2 µl 5 × FSB buffer, 0.25 µl RNase Inhibitor (10 U), 0.5 DTT (0.1 M) and 0.5 µl Superscript III Reverse Transcriptase (100 U), gently mixed and incubated at 50 °C for 1 h, and the reaction was terminated at 70°C for 15 min to inactivate the transcriptase. Then it was chilled on ice for immediate usage or stored at -20°C.

The second-strand cDNA synthesis was performed by PCR. The following reagents were added: 5 µl 10 × LA buffer, 8 µl dNTPs mix (10 mM), 1 µl cDNA template, 1 µl AdaptorSp6-PCR (10 pmol), 1 µl OdT T7 II (10 pmol), 0.5 µl LA-Taq and 33.5 µl ddH₂O was added up to a total volume of 50 µl. The mixture was vortexed and centrifuged for a short time.

The reaction was carried out in a thermocycler with the following program:

95 °C	1 min	
95 °C	20 sec	} 30 cycles
58 °C	20 sec	
72 °C	2 min 30 sec + 5 sec/cycle	
72 °C	5 min	
4 °C	∞	

A small volume of PCR products was estimated by gel electrophoresis on 1% w/v agarose. The samples were loaded in gel wells with staining by loading buffer. The voltage at 100 V was applied for 30 min, and 1 kb DNA ladder served as a reference. DNA fragments were visualized underlying Molecular Imager Gel Documentation camera.

2.2.10.4 Purification of PCR Products

The reagent SureClean™ was used according to the manufacturer's instruction for purification of PCR products. An equal volume of PCR products and SureClean solution was mixed thoroughly, incubated at RT for 10 min, and then centrifuged at maximum speed (21000 g) for 10 min and the pellet retained. The pellet was washed twice in cold 70% ethanol at 2 × original volume, and resuspended it in 15 µl water. The final concentration and purity of cDNA was measured using a NanoDrop 1000.

2.2.10.5 Reverse transcriptase PCR analysis

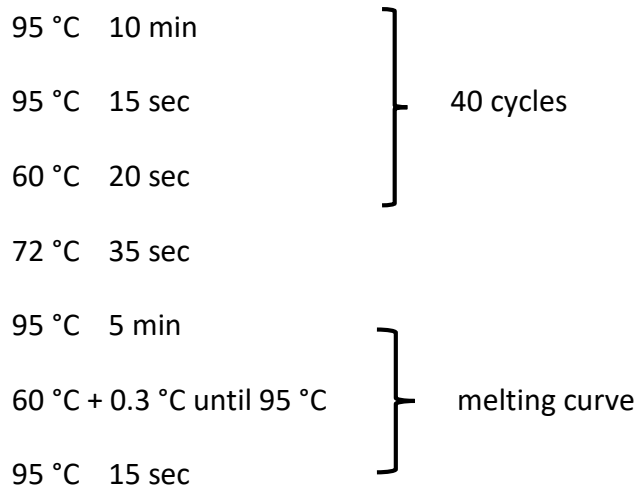
The reverse transcriptase polymerase chain reaction (RT-PCR) was used to check the expression of different genes in the oenocyte of adult flies. cDNA-synthesis was performed at 50°C for 60 min using a conventional oligo (dT) primer and SuperScript III reverse transcriptase. PCR amplification was carried out using Taq DNA polymerase in a total volume of 25 µl and the gene specific primers were used for PCR. The program was processed according to table 3. Subsequently, the PCR products were checked by 1 % agarose gel electrophoresis.

Table 6: RT-PCR program

window	Denaturation 95°C	Primer-Annealing 55°C	Elongation 72°C	Cycles
1	1 min	---	---	1
2	30 s	30 s	1 min	30
3	---	---	5 min	1
4		Cool down 4°C ∞		

2.2.10.6 Relative quantifications of qRT-PCR

For relative quantitative PCR, the reaction was performed using StepOnePlus™ Real-Time PCR System. The total volume of 10 µl each reaction was loaded into 48 wells plate which consists of 5 µl SYBR Green MasterMix, 0.25 µl ROX, 0.5 µl forward primer (5 µM), 0.5 µl reverse primer (5 µM), 1 µl cDNA (20 ng) and 2.75 µl water. The amplification was run as below:



In a preliminary test, the primer efficiencies for both genes of interest and reference were analyzed basing on the standard curve and the slope, which was derived from serial dilutions of cDNA from 50 ng to 3.12 ng. The primer efficiencies were calculated according to $E=10^{-1/\text{slope}}$ formula (Pfaffl, 2001). Thus, the relative expression ratio (fold change) of a target gene was determined based on E and CT values in comparison to a reference gene. In triplicates, the fold changes for each group were represented as mean \pm S.D. for statistical analysis. In this case, RPL32 was served as a reference gene for all assays.

$$\text{Ratio} = \frac{(E_{\text{target}})^{-\Delta CT_{\text{target}}(\text{sample-control})}}{(E_{\text{ref}})^{-\Delta CT_{\text{ref}}(\text{sample-control})}}$$

2.2.11 Statistical analysis

In all experiments, GraphPad Prism version 6.00 was mainly used for statistical analyses. The comparison between two groups was done by the unpaired two-tailed student's t-test, if multiple comparisons were done, one-way ANOVA with Tukey Post-hoc test were used. All

histograms were represented as mean \pm S.D. The survival curves of lifespan were analyzed with the log-rank (Mantel-Cox) test. Statistical analysis of significant difference was defined as *P<0.05, **P<0.01 and ***P<0.0001 and ns not significant.

3. Results

3.1 Targeted manipulation of oenocytes

To manipulate the oenocytes in *Drosophila*, specific gal4 lines are available. In order to evaluate its specificity, the oenocyte-driver line (Oen-GAL4) was crossed with the UAS-GFP reporter line and the signal was analysed in F1 animals. This analysis was performed in larvae and adult flies using fluorescent microscopy [Fig. 10].

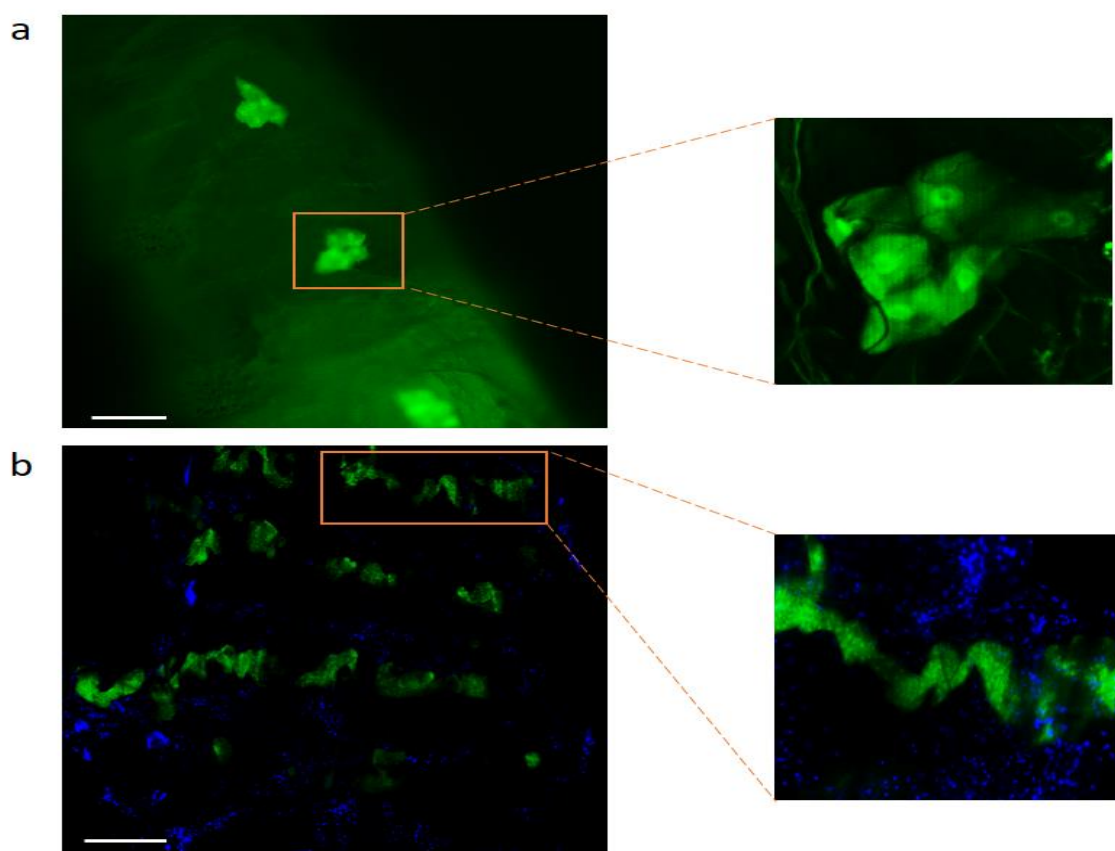


Figure 10: Testing available driver lines specifically targeting oenocytes.

Abdominal fillet dissection of the 3rd instar larva (a) and adult (b) flies showed GFP signaling (green) in the targeted oenocytes which are arranged as a cell clusters in larva and as ribbons in adult. Crosses were performed using UAS-GFP. Scale bar is: 50 μm .

3.2 Short-term starvation causes fat accumulation in the adult oenocyte

I first examined how fat is redistributed throughout the fly body after food deprivation. Using Bodipy staining (Qiu and Simon, 2016), two cell types in the adult flies were found to contain numerous lipid droplets under fed and/or starved conditions: the fat body cells and the oenocytes [Fig. 11]. Despite lipid loss over a 24 hours' period of fasting, the fat body still had a larger number of lipids stored in form of lipid droplets, which are strongly stained with Bodipy (493/503) under fed conditions. As previously reported, lipid released from the fat body during starvation correlates with lipid droplet aggregation, which is seen as the occurrence of larger droplets (Colombani et al., 2003). In contrast to the fat body, the second cell type of interest, the oenocytes showed not staining with Bodipy (493/503) under fed conditions but they do contain numerous lipid droplets after 24 hours of fasting. Thus, similarly as the mammalian liver, oenocytes are highly atypical cells that accumulate fat droplets specifically during phases of food deprivation.

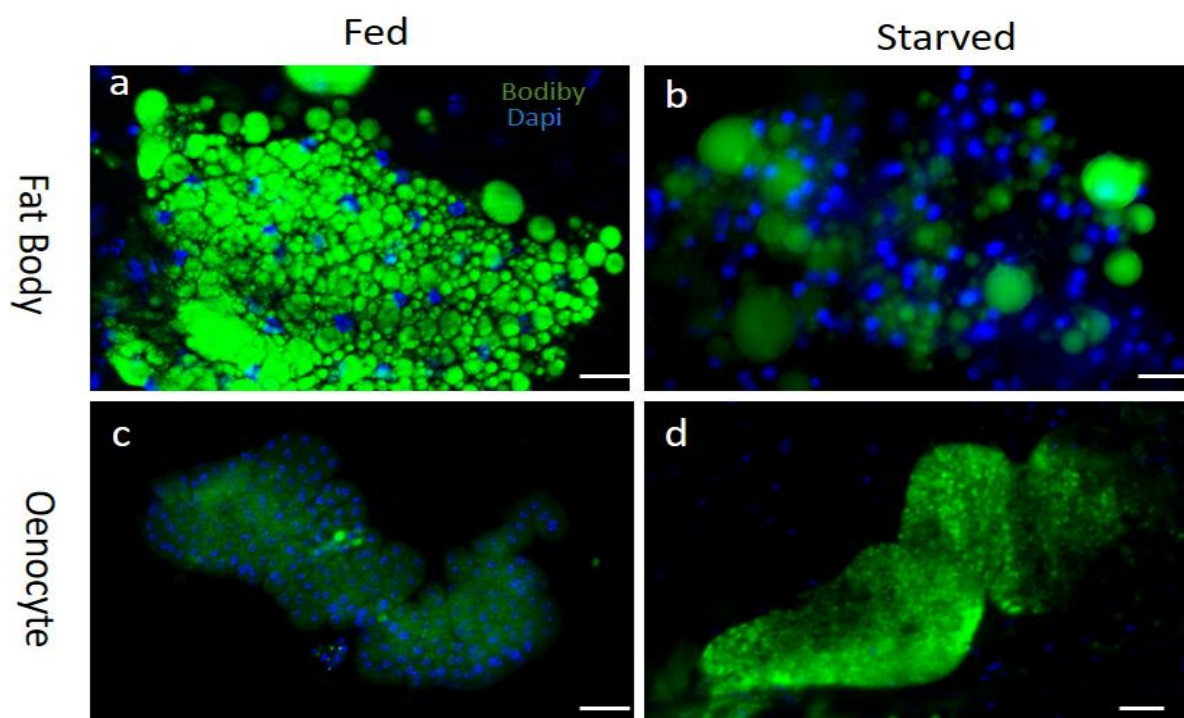


Figure 11: a–d, Bodipy staining of fat bodies and oenocytes in the adult flies under fed and starved conditions.

After 24 h of fasting, lipids in the fat body are significantly depleted (b) compared with fed conditions (a). Individual oenocyte ribbons (c, d) showing that lipid droplets accumulate specifically and consistently in oenocytes after fasting. Bodipy (green), DAPI (blue), Scale bar is: 50 μ m.

3.3 Effect of starvation and cAMP activation in oenocytes on lipid accumulation.

To provide energy, oenocytes are highly relevant cells that accumulate lipids during food deprivation (Gutierrez et al., 2007). Since cyclic AMP is a second-messenger that plays an important role in lipid homeostasis and energy expenditure (Zingg et al., 2017), the first part of my thesis was devoted to evaluate the role of cAMP in controlling the physiology of oenocytes. Thus, the levels of lipid accumulation in the oenocyte and some physiological parameters of flies during fed and starved conditions were analysed related to different cAMP levels. The recently found adenylyl cyclase of *Beggiatoa* (bPAC) is a powerful tool for optogenetic manipulation in *D. melanogaster*, which is achieved via illumination with blue light. I used the Gal4/UAS-system to specifically express bPAC in oenocytes and activated it by irradiation with blue light.

The Oen-GAL4 was used to drive a bPAC under transcriptional control of an UAS promoter. Thus, the progeny of Oen-GAL4>UAS-bPAC carry this photoactivatable enzyme specifically in oenocytes. To minimize the genetic background aberration, the parental control lines Oen-GAL4 and UAS-bPAC were crossed to w¹¹¹⁸ respectively.

3.3.1 Specific activation of bPAC in oenocytes of *D. melanogaster* affects lipid accumulation

Simple expression of bPAC in the adult oenocytes in those flies that were exposed to dark light had no effect on the occurrence and distribution of fat droplets in the oenocytes. Using Bodipy staining showed no obvious differences in the fat accumulation between treatment and control of fed and starved groups [Fig. 12]. On the other hand, Activation of bPAC, thus increasing cAMP concentration in the oenocyte in those flies expressing this gene exclusively in oenocytes, had remarkable impact on the lipid accumulation during starvation as well as during fed conditions. Compared with control, Oen>bPAC animals showed a marked increase of oenocyte lipids after 24 and 48 hours' starvation. Even in well fed condition some fat droplets were observed in oenocytes of the treatment group [Fig. 13].

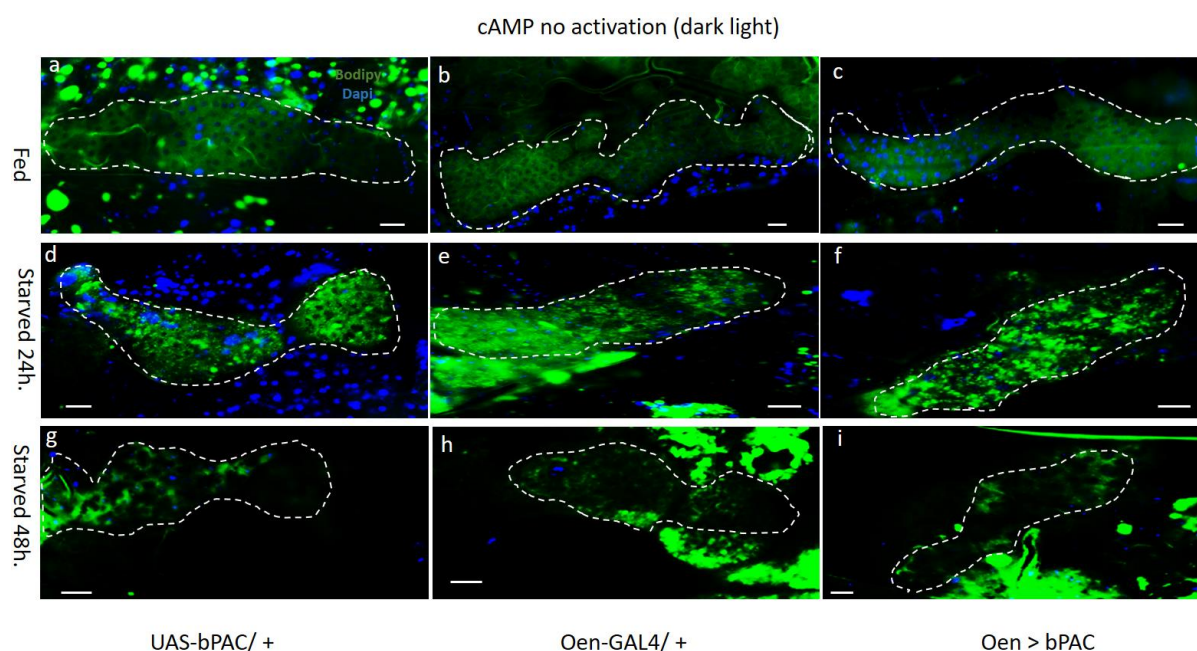


Figure 12: No ectopic cAMP activation in the adult oenocyte during fed and starved conditions.

Compared with fed flies (a-c), oenocyte ribbons from adult female flies accumulate lipid droplets after 24 h of starvation as seen by Bodipy staining (d-f). The oenocytes of Oen>bPAC and its controls contain more Bodipy positive lipid droplets than fed flies after 24 h of starvation and they lost most of the accumulated lipids after 48h of fasting (g-i) without a marked difference between treatment and control of all fly groups. Bodipy (green), DAPI (blue), Oenocyte ribbons are circled. Scale bar is: 50 μ m.

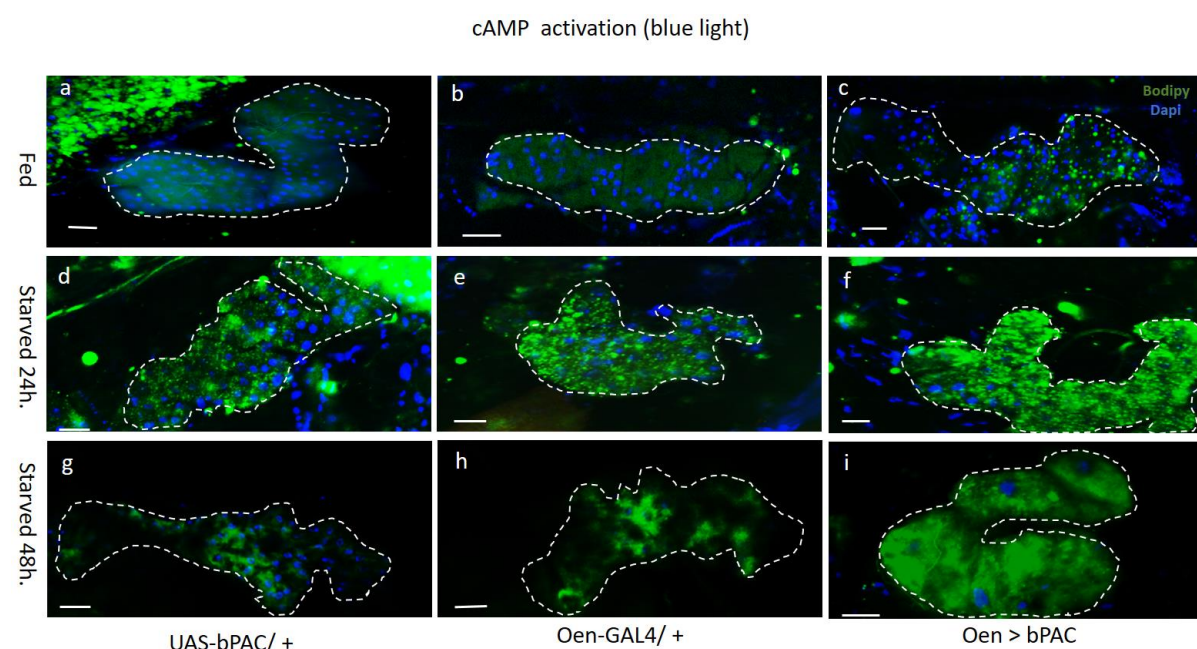


Figure 13: Ectopic cAMP activation increase lipid droplets accumulation in the adult oenocyte after starvation.

bPAC activation by blue light in the Oen>bPAC group caused a marked increase in the fat accumulation after 24 h (f) and 48 h (i) of starvation compared to the control groups at 24 h (d, e) and 48 h (g, h) respectively. Even

in well fed conditions, ectopic cAMP activation of Oen>bPAC exhibit some accumulated fat droplets (c), while no lipids in the ribbons of fed control groups (a, b). Bodipy (green), DAPI (blue), Oenocyte ribbons are circled. Scale bar is: 50 μ m.

3.3.2 Activation of bPAC in oenocytes of *D. melanogaster* affects number and size of fat droplets

Short-term starvation results in a significant increase in both, number and size of lipid droplets. Simple expression of bPAC in oenocytes had no significant effect on the number and size of fat droplets in the oenocytes in the both, fed and starved groups [Fig. 14]. Light induced activation of a bPAC that is exclusively expressed in oenocytes enhanced this effect significantly. This was also seen under well-fed conditions [Fig. 15].

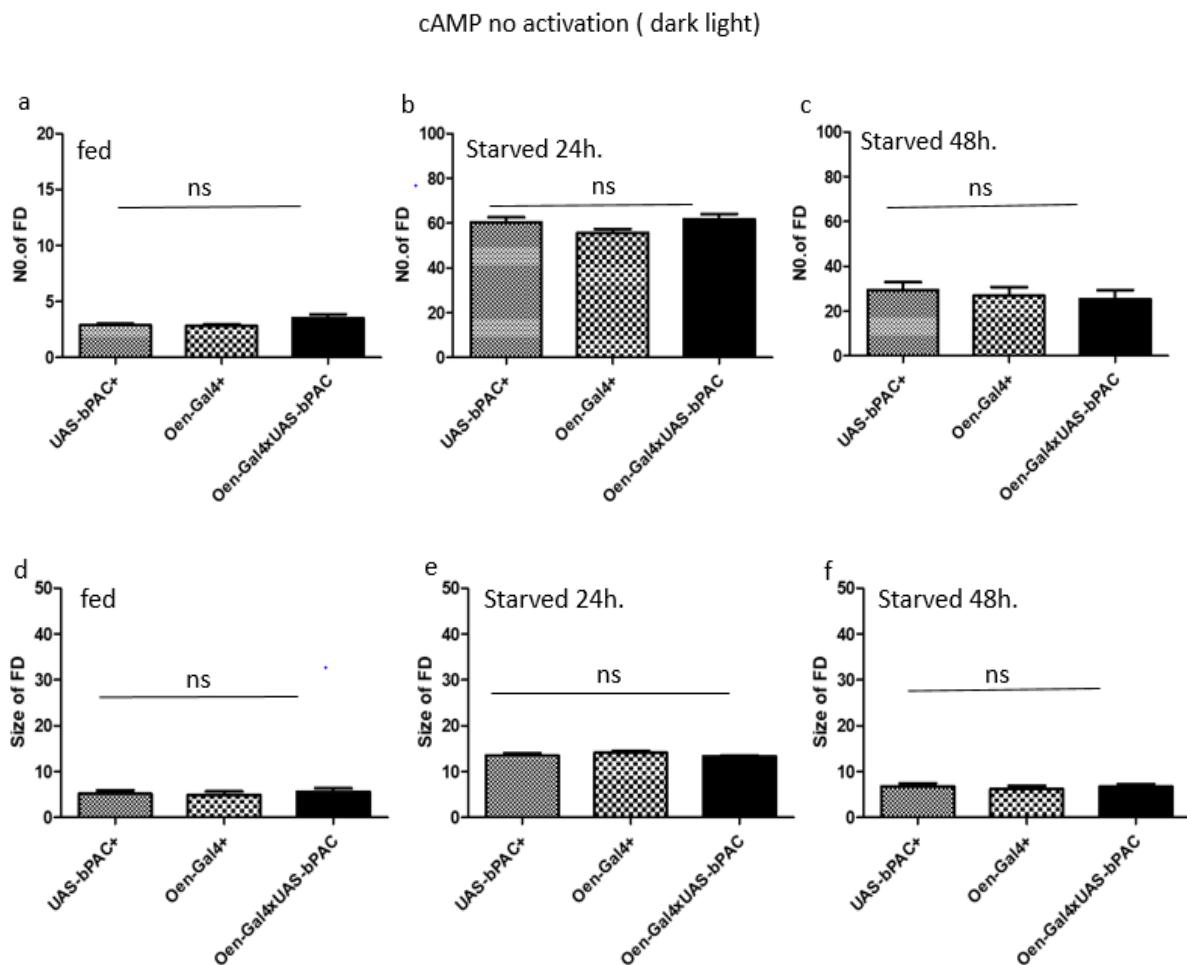


Figure 14: Number and size of fat droplets without oenocyte's cAMP activation.

Without cAMP activation (a-f) no significant differences in the number and size of fat droplets between Oen>bPAC and control groups during fed and starved condition were observed. Data are presented as mean \pm SEM; one-way ANOVA was used for analysis. ns means not significant.

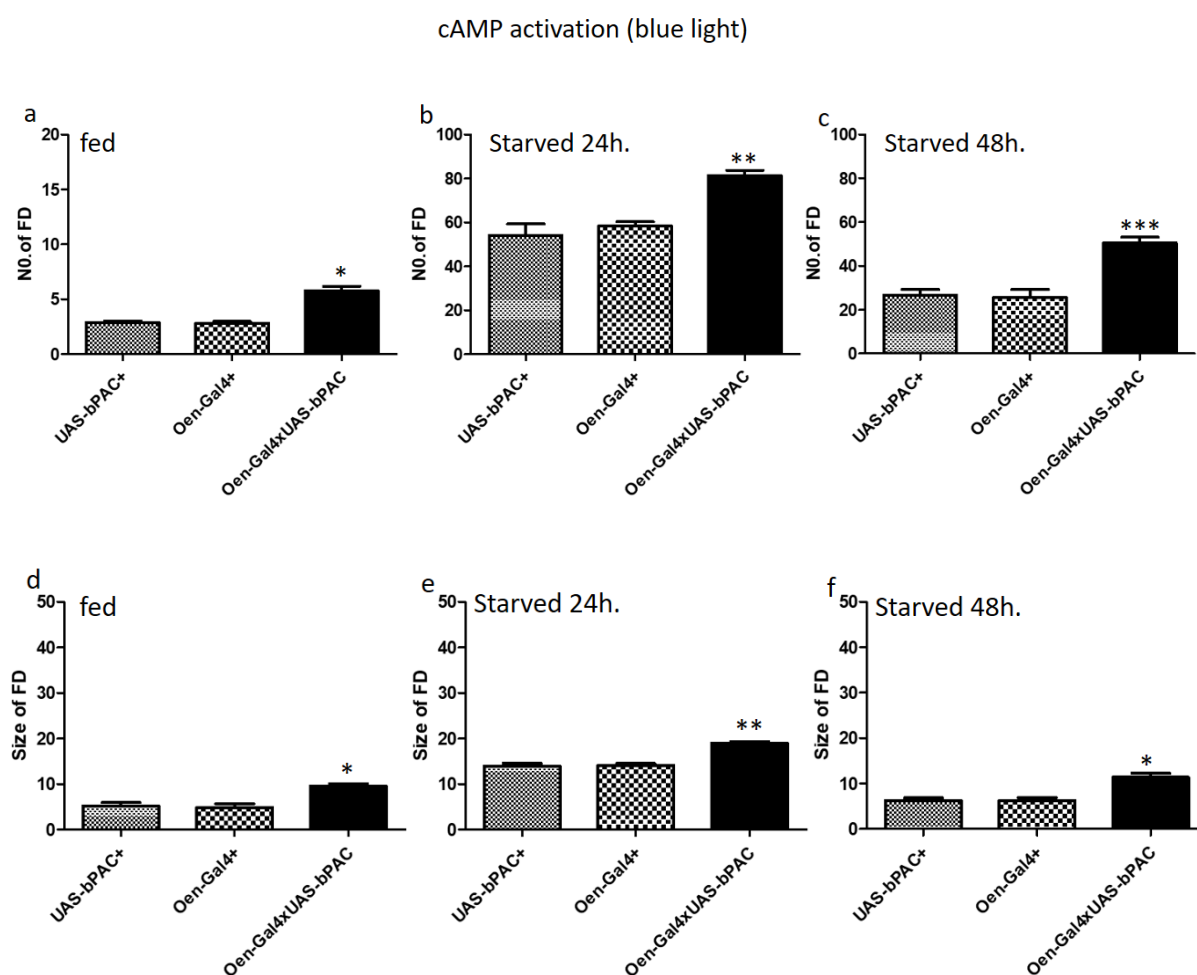


Figure 15: Dual effect of starvation and oenocyte's cAMP activation increase number and size of fat droplets.

The activation of cAMP (a-f) leads to a significant increase in the number (b and c) and size (e and f) of the oenocytes' fat droplets in the Oen>bPAC group after 24 and 48-hour starvation. Furthermore, even in well fed flies, cAMP activation in Oen>bPAC results in a significant increase in the number (a) and size (d) of accumulated fat droplets in the adult oenocytes compared to controls. Data are presented as mean \pm SEM; one-way ANOVA was used for analysis. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns not significant.

3.3.3 Activation of cAMP in the oenocyte affects the metabolic rate in adult *Drosophila*

To further elucidate the role of cAMP in regulation of metabolism, a modified respirometric analysis was performed to measure CO₂ production from flies, which is an indicator of the metabolic rates. Flies of the experimental genotype as well as those of the two control crossings that were not irradiated with light and that therefore had no increased cAMP levels, showed no significant differences between genotypes, either in starved or fed animals [Fig. 16]. It was found that, when compared to the control flies, Oen>bPAC flies that experienced cAMP activation, had a significantly decreased CO₂ production (measured as per hour × fly), associated with high levels of accumulated lipids in the adult oenocytes after 24 and 48 hour starvation.

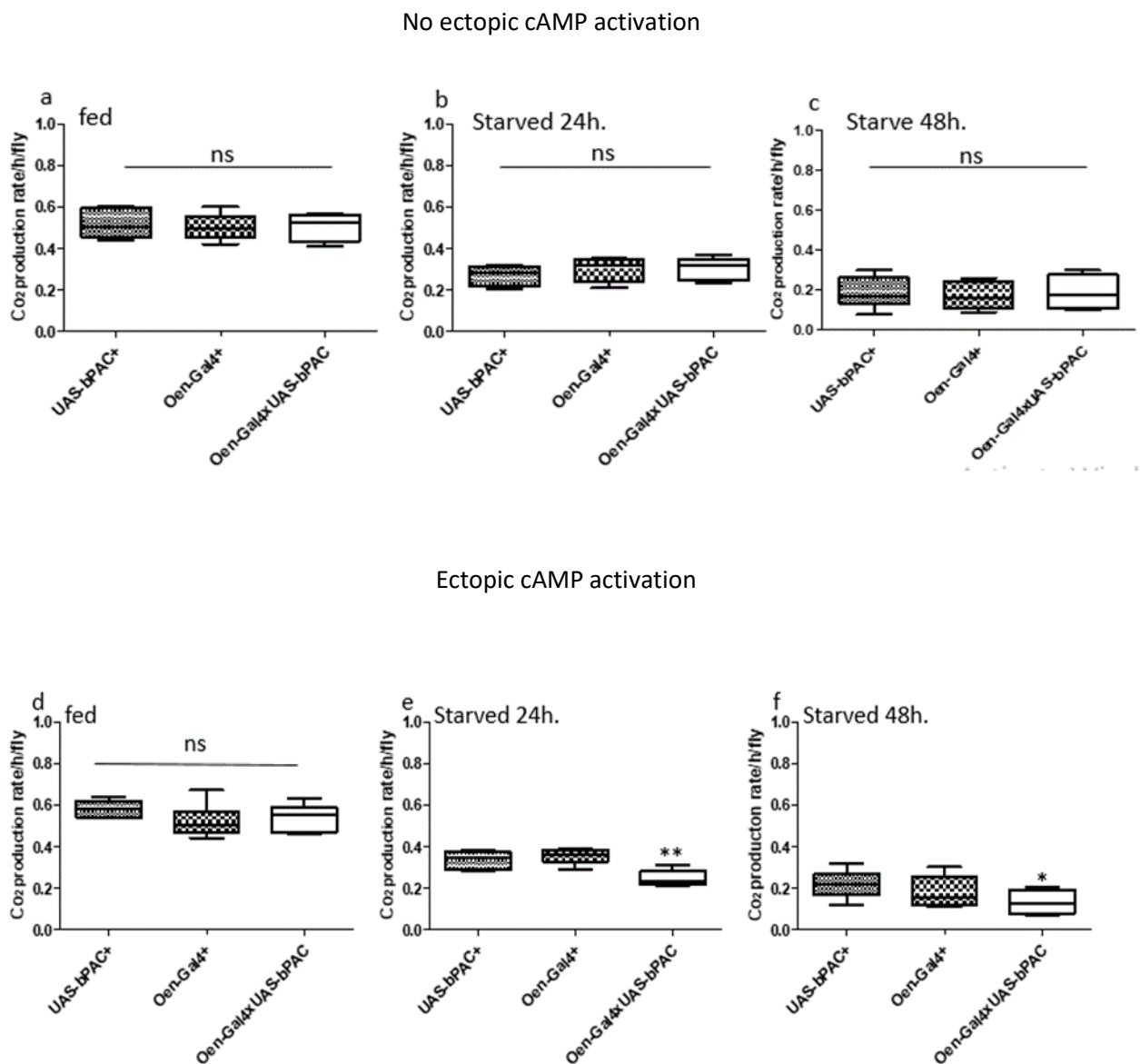
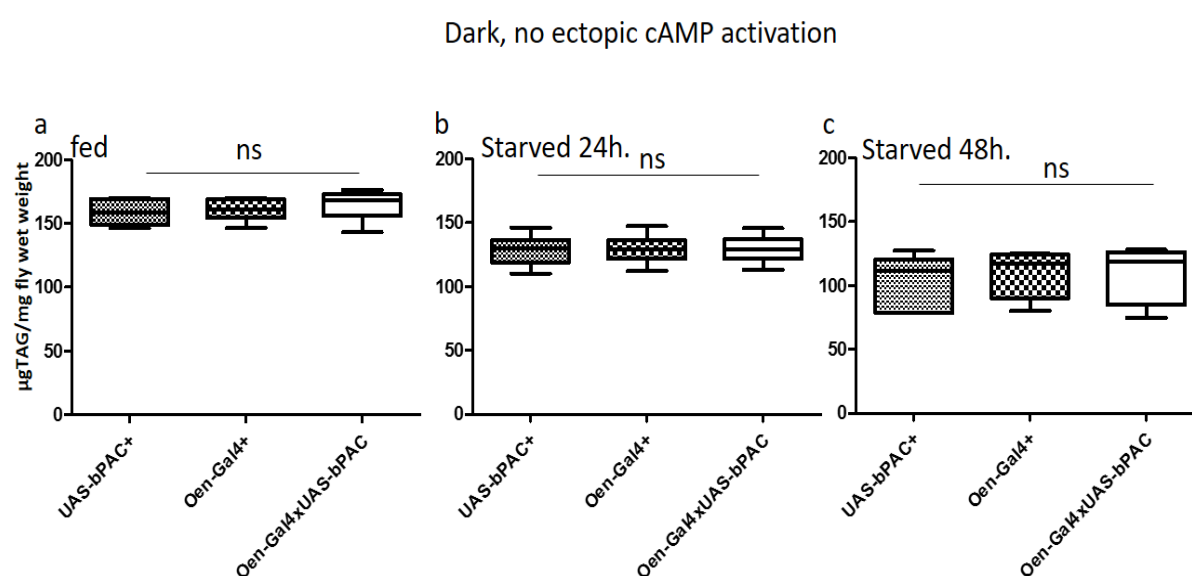


Figure 16: CO₂ production is affected by the combined activity of starvation and cAMP activation in oenocytes.

CO₂ production was measured in fed and starved flies either without ectopic cAMP activation (a-c) or with cAMP activation (d-f) using respirometry. Flies without cAMP activation showed no significant differences in the CO₂ production between Oen>bPAC and control groups both, under fed and starved conditions. Measurement of CO₂ production in cAMP activated flies showed significant decrease in the Oen>bPAC group compared to control groups in starved flies, but not under fed conditions. Data are presented as mean \pm SEM; one-way ANOVA was used for analysis. * $p < 0.05$, ** $p < 0.01$, ns not significant.

3.3.4 Activation of cAMP in oenocytes leads to mild increases in body fat during starvation

To test whether cAMP plays a role in modulating energy metabolism, I measured the levels of triacylglycerol, TAG (also called triglycerides), the major form of fat droplet storage in insects (Meyers et al., 2017). Whole body TAG levels were measured in adult flies raised on control medium for the entire time (fed) or that had been starved for 24h or 48h. Moreover, they were subjected to blue light or raised in the dark. Compared to the controls, a significant increase of lipid content was observed in Oen>bPAC group after 24 and 48 hour of starvation associated with ectopic bPAC activation [Fig. 17].



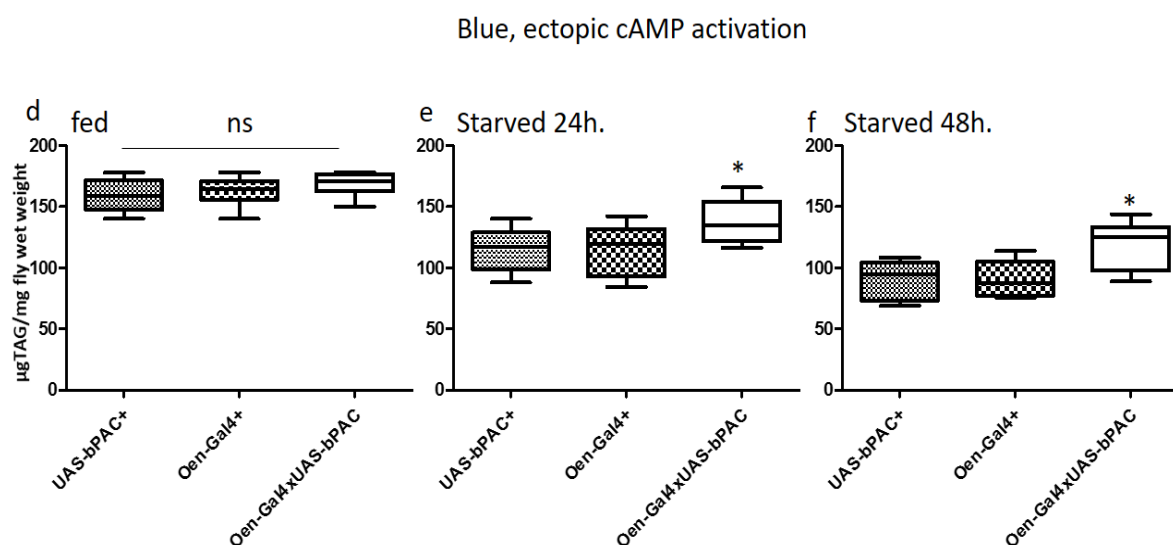


Figure 17: Fat content of flies which experienced cAMP manipulation in adult oenocytes.

Total TAG levels were measured in Oen>bPAC and control flies under fed and starved conditions using the Coupled Colorimetric Assay (CCA). Flies housed in the dark (without ectopic cAMP activation (a-c)) did not show significant differences between experimental and control groups in fed and starved conditions. Compared with the matching controls Oen>bPAC subjected to blue light have significantly higher TAG levels in response to starvation (e, f) but not in the fed (d) state. Data are presented as mean \pm SEM; one-way ANOVA was used for analysis. * $p < 0.05$, ns not significant.

3.3.5 Activation of cAMP in the oenocyte has a significant effect on starvation resistance of adult flies

Most animals face periods of food deficiency and are thus expected to improve adaptations enhancing starvation resistance. *Drosophila* offers a unique occasion for an integrated study of the various aspects of adaptation to nutritional stress which are strongly associated with energy accumulation and hormonal signaling pathways (Rion and Kawecki, 2007; Djawdan et al., 1998; Jeong et al., 2012). To examine whether manipulation of cAMP levels in oenocytes has an effect on starvation resistance of *Drosophila*, I performed a longevity test of transgenic flies with altered cAMP level.

Flies which experienced a chronically enhanced cAMP synthesis in oenocytes had a decreased starvation resistance compared to the genetic-matched controls in both males and

females. Adult males were more sensitive to starvation in control groups as well in response to ectopic cAMP manipulation [Fig. 18].

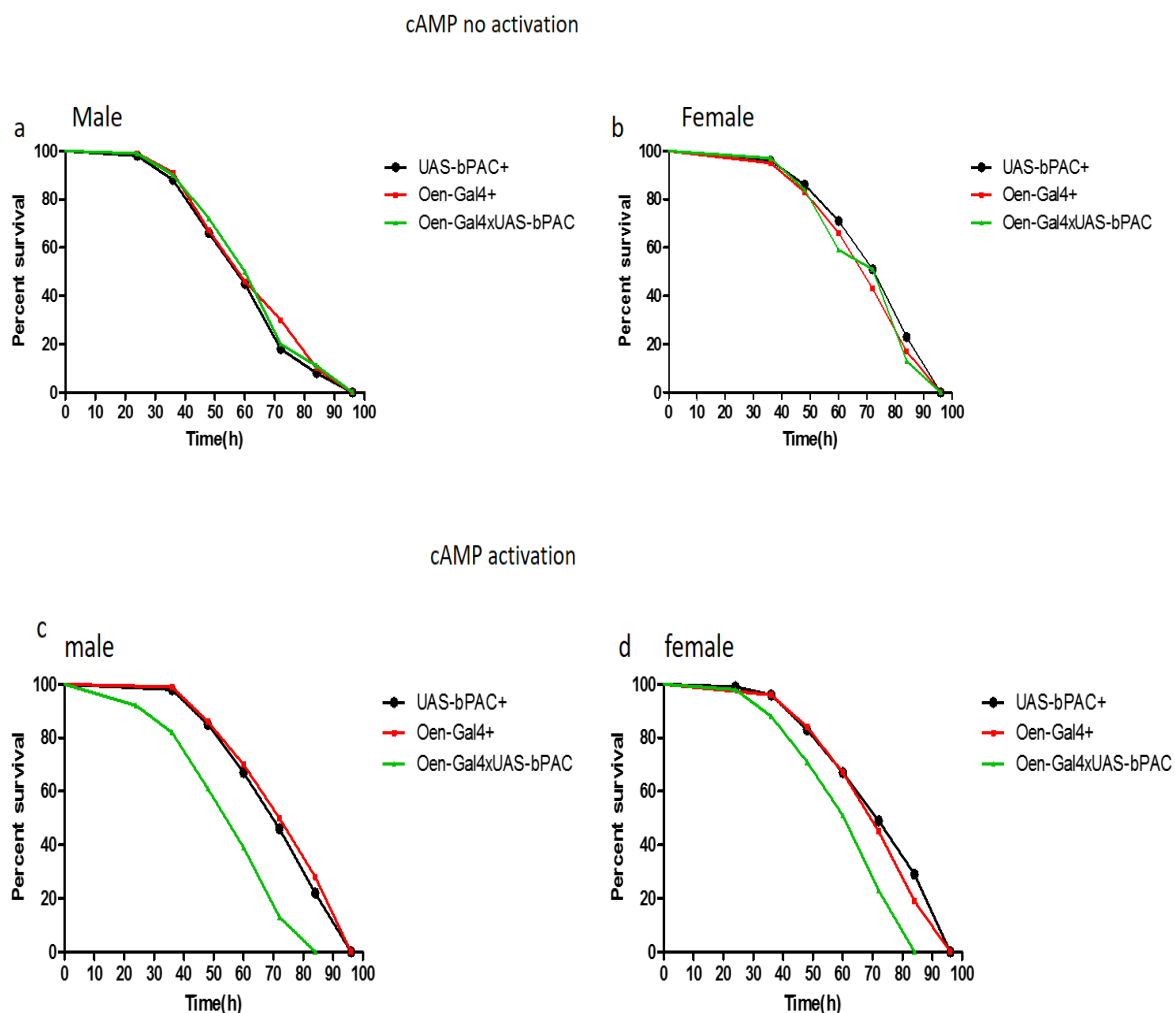


Figure 18: Flies releasing more cAMP in the oenocyte are sensitive to starvation stress.

Starvation resistance was assayed using starved flies. Without cAMP activation (a, b) male and female did not show differences in the survival curve both in mutant and its parental controls. Flies with cAMP activation (c, d) lived shorter than its controls both in males and females. Log-rank test for survival analysis showed significant differences, $p < 0.001$.

3.4 Analysis for hormone receptors expression in the adult coenocytes using qRT-PCR of manually isolated cells

Changes of the cAMP levels are usually mediated through the activity of G-protein coupled receptors, similarly as it is known for other second-messengers. Thus, G-protein-coupled receptors have the potential to modulate cAMP levels under normal conditions in oenocytes. In this part of my thesis, the expression of G-protein coupled receptors from the group of bioamine receptors including octopamine, tyramine, dopamine and 5-hydroxytryptamine receptors in oenocytes was evaluated by RT-PCR. Octopamine (OA) and tyramine (TA) share similarities regarding their structure, and their pharmacological characteristics as well as their sets of G-protein coupled receptors (Roeder, 2005). OA exerts its effects through specific OA receptors (OAMB, OA2/Oct β 1R, Oct β 2R, Oct β 3R) and TA through a different class of specific TA receptors (TyrR I, TyrR II, TyrR III). Dopamine (DA) receptor has been shown to play a substantial role in regulating basal locomotion as well as learning and memory, addiction and courtship in *Drosophila*. Recently, the participation of DA in more complex behaviors such as decision making, attention, and appetite have also been discussed (Yamamoto and Seto, 2014). DA acts via three different receptors named DopR, DopR1 and D2R. 5-hydroxytryptamine (5HT) named also serotonin, acts as a messenger substance in most animal phyla. It modulates a great diversity of important behavioral and physiological processes including aggression, sleep, circadian rhythms, feeding, responses to visual stimuli, and learning (Blenau and Thamm, 2011). The function of serotonin is mediated by binding to G-protein-coupled receptors through 5HT1A, 5HT2A and 5HT2B receptors.

To analyze expression of the corresponding receptor genes, extraction of RNA from the manually isolated oenocytes was performed. RT-PCR analysis was used and is shown in [Fig. 19]. It is noted that only TyrRII is strongly expressed, and minor expression to DopR, DopR1 and 5HT1A was observed.

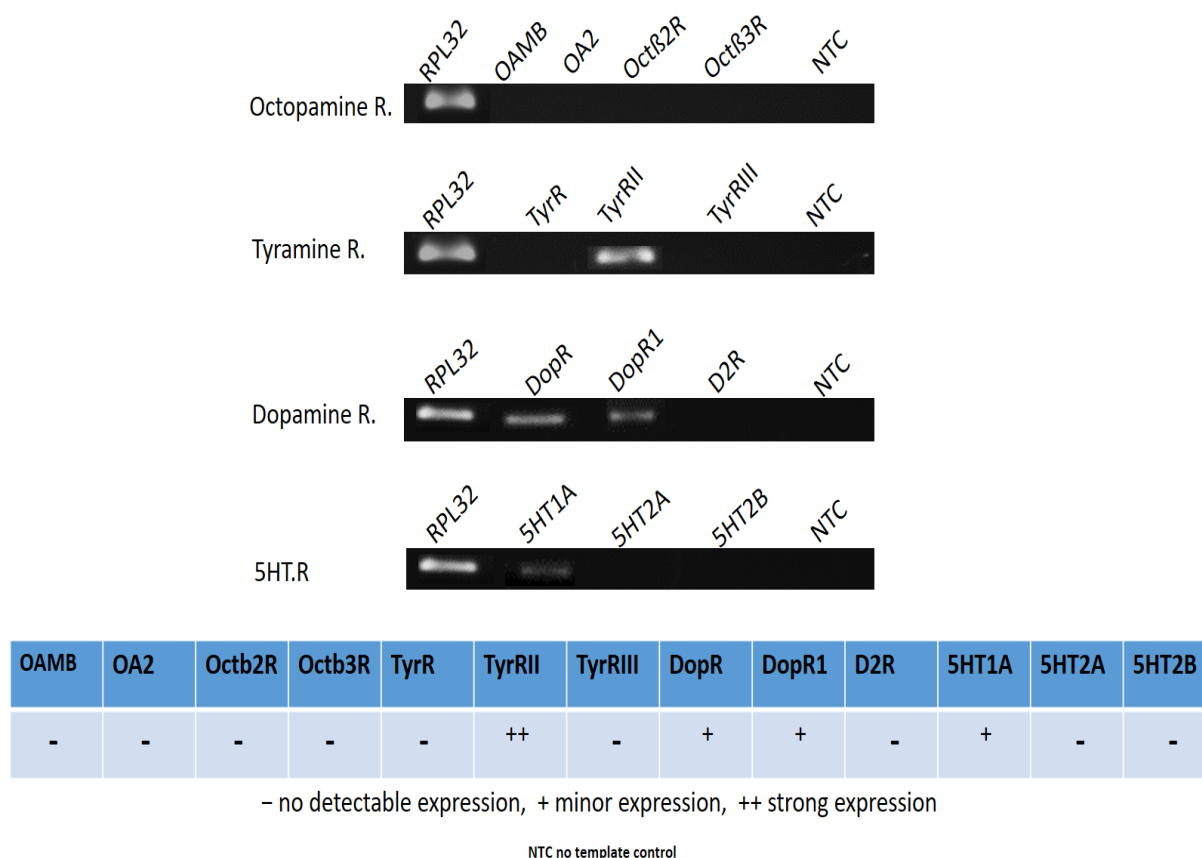


Figure 19: Receptors gene expression in adult oenocytes.

RT-PCR analysis with RNA isolated from adult oenocytes showed a strong expression of TyrRII and minor expression of DopR, DopR1 and 5HT1A. RPL32 serves as a positive control, NTC is no template control.

3.5 Effect of JAK-STAT signaling on oenocyte physiology

3.5.1 Activation of JAK-STAT signaling in oenocytes in response to starvation

To identify pathways, which are induced after food deprivation in oenocytes of flies, I used GFP reporter lines and exposed them to starvation. In this experiment I exposed early third instar larvae and seven days old adult of the 10XSTAT92E-GFP reporter line to 12h and 24h of starvation, respectively. JAK-STAT signaling was activated in the oenocytes in response to starvation and may play a physiological role as a key signaling pathway for food deprivation related responses [Fig. 20].

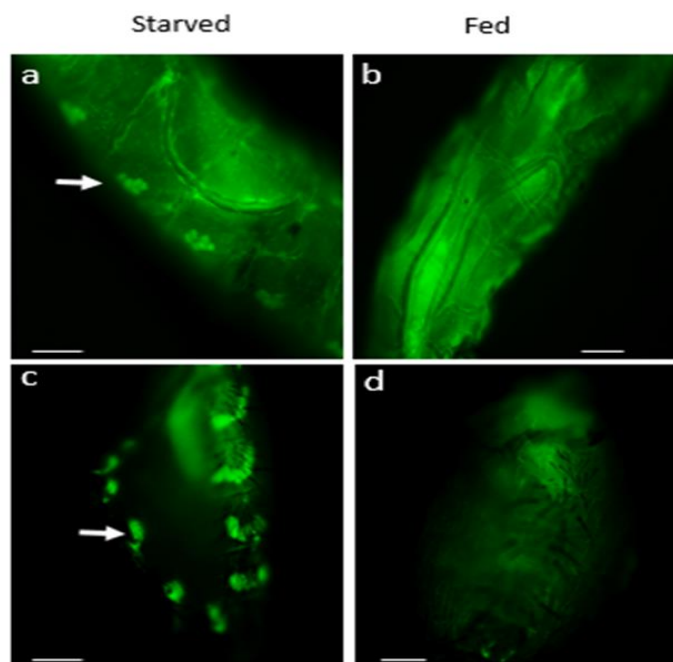


Figure 20: Starvation effects on third instar larva and adult in the JAK-STAT reporter line.

The 10XSTAT92-GFP reporter (green) detects JAK-STAT pathway activation after starvation at the oenocyte clusters of third instar larva (a) and at the oenocyte ribbons of adult flies (c). Strong GFP expression (green) occurred in starved animals (a, c) when compared to fed animals (b, d). The scale bar is 100 μ m.

3.5.2 Activation of the JAK-STAT pathway in the adult oenocyte affects lipid accumulation during starvation

To investigate the role of JAK-STAT signaling in the adult oenocyte, I tested in the second part of my thesis two important components of the JAK-STAT pathway, Upd3 and STAT92E (Amoyel and Bach, 2012), in oenocytes of *Drosophila*. I examined the structural and functional consequences of their ectopic overexpression exclusively in oenocytes using UAS-Upd3- and STAT92E-UAS lines respectively with help of the Oen-GAL4 line. Overexpression of the IL6-related cytokine Upd3 as well as of the STAT transcription factor itself was used to evaluate if this manipulation can alter the oenocyte's lipid status and the physiology of adult flies. Interestingly, I observed a remarkable increase of lipid accumulation in adult oenocytes during starvation in those animals that experienced Upd3 or STAT overexpression. As well as, even in

well fed state some lipid droplets were observed in the oenocyte of those flies with STAT overexpression. Moreover, a downregulation of this signaling system was achieved by ectopic expression of the corresponding RNAi constructs [Fig. 21, 22].

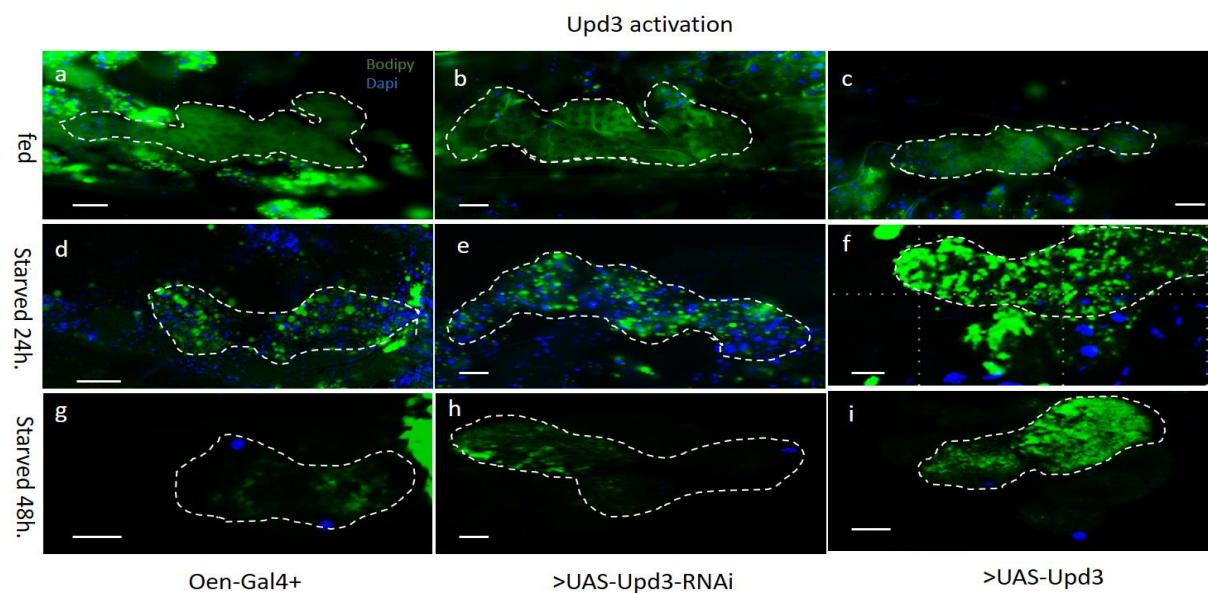


Figure 21: Upd3 activation increases lipid droplet accumulation in the adult oenocyte after starvation.

Oenocyte ribbons from adult female flies accumulate lipid droplets after 24 h of starvation as seen by Bodipy staining. The oenocytes of oen-Gal4>UAS-Upd3 and its controls of starved adult flies contain more Bodipy positive lipid droplets than well fed flies (a-f). Scale bar is: 50 μ m.

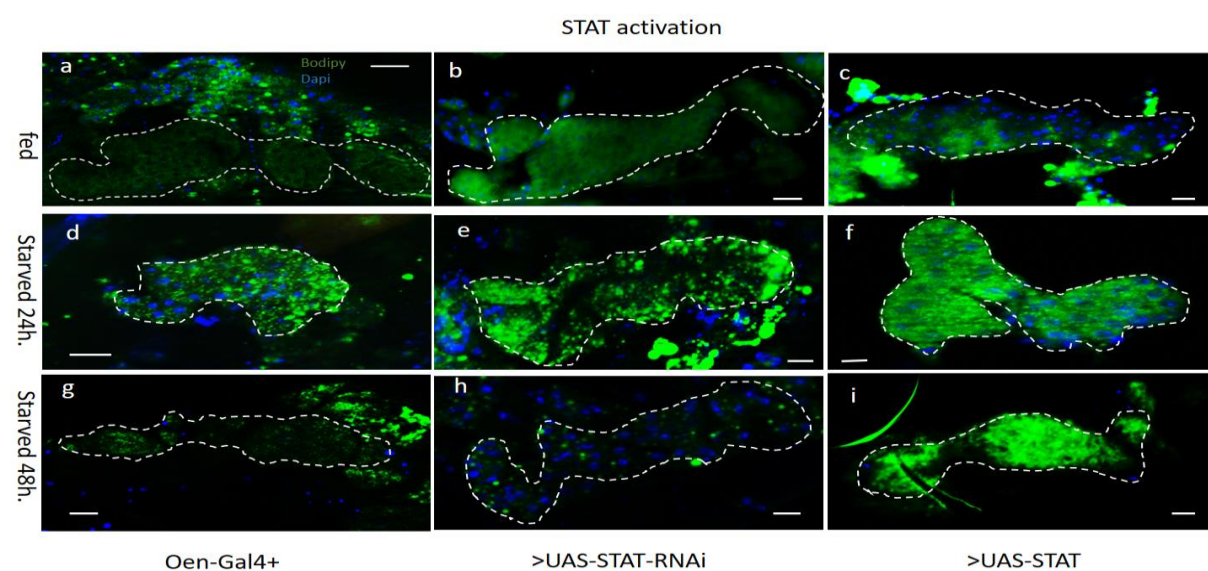
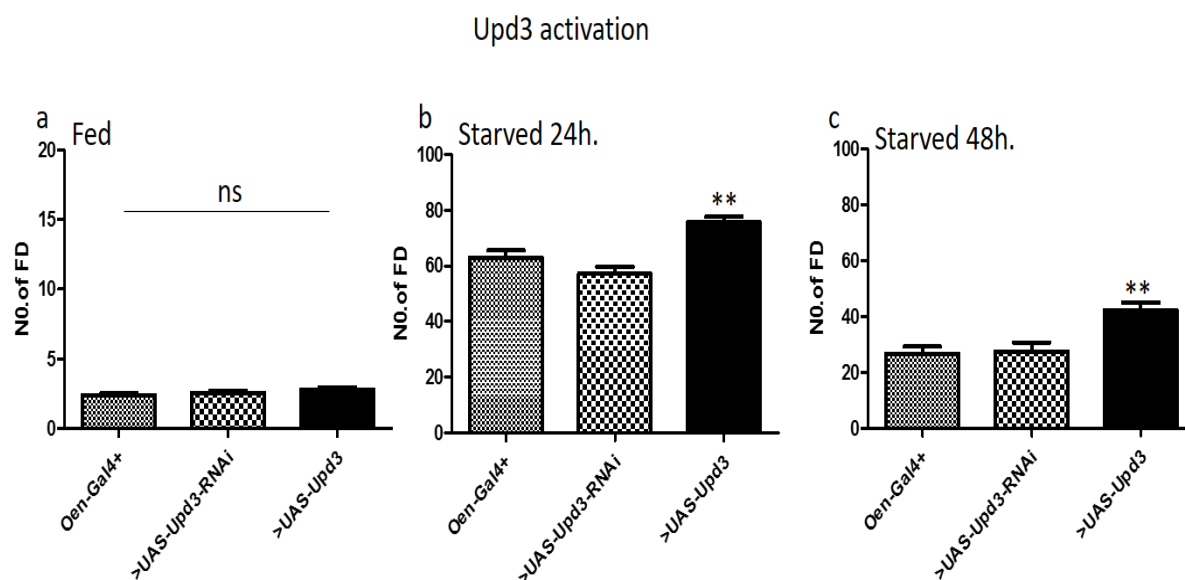


Figure 22: STAT activation increases lipid droplet accumulation in the adult oenocyte after starvation.

STAT overexpression in starved *oen-gal4>UAS-STAT* (l) caused a marked increase in the fat accumulation compared to the control groups (j, k). Even in well fed conditions *>UAS-STAT* exhibit some accumulated fat droplets (i), while no lipids in the ribbons of fed control groups (g, h). Bodipy (green), DAPI (blue), Oenocyte ribbons are circled. Scale bar is: 50 μ m.

3.5.3 Activation of the JAK-STAT pathway in the adult oenocyte affects number and size of lipid droplets during starvation

Overexpression of both components of the JAK-STAT pathway (*upd3* and *STAT*) induced an obvious increase in the number and size of fat droplets in oenocytes compared with controls and those animals subjected to JAK-STAT inhibition in their oenocytes. Furthermore, even in fed condition flies with *STAT* activation showed a significant increase in the number of fat droplets [Fig. 23, 24]. This result indicates that the JAK-STAT-signaling pathway plays an important role controlling the oenocyte's physiology during starvation.



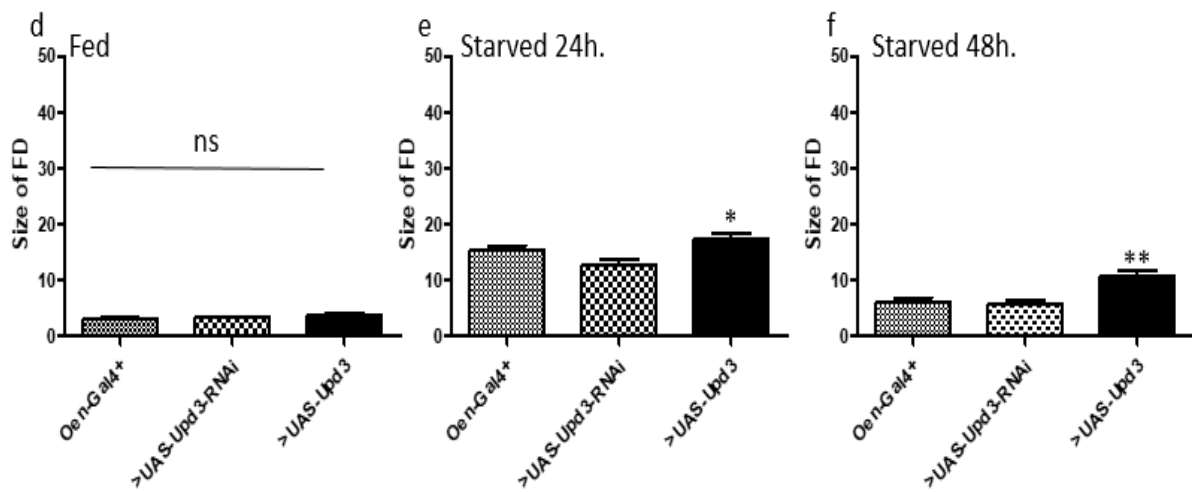
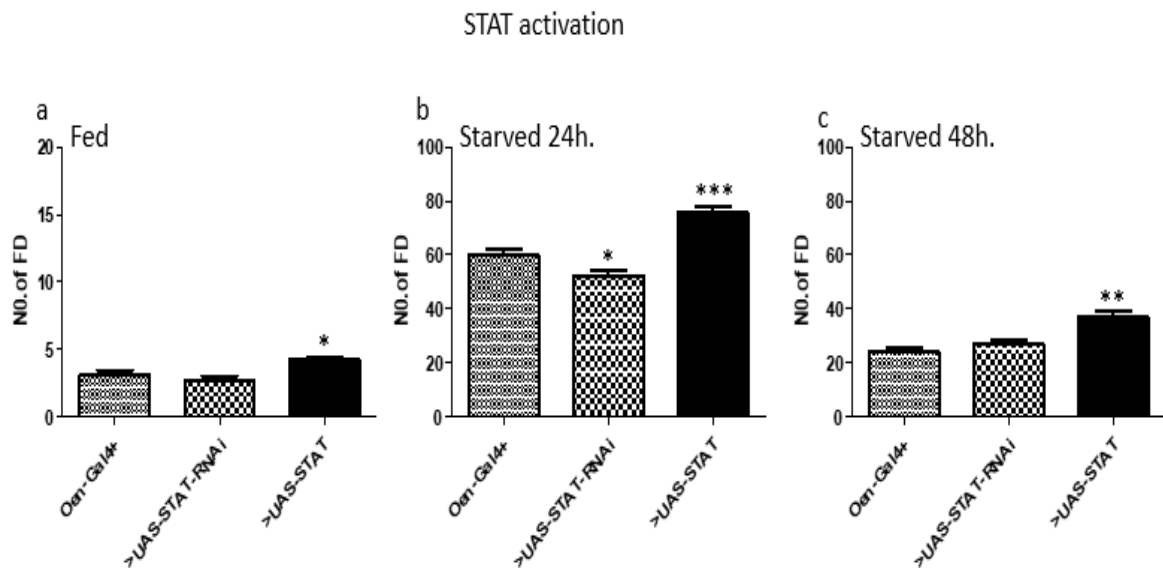


Figure 23: Starvation and oenocyte's upd3 overexpression affect number and size of fat droplets in the adult oenocytes.

Flies with JAK-STAT activation via Upd3 overexpression, >UAS-upd3 (a-f) showed a significant increase in the number and size of fat droplets after 24 and 48-hour starvation compared to control and flies with JAK-STAT inhibition, UPD3-RNAi. Data are presented as mean \pm SEM; one-way ANOVA was used for analysis. * $p < 0.05$, ** $p < 0.01$, ns not significant.



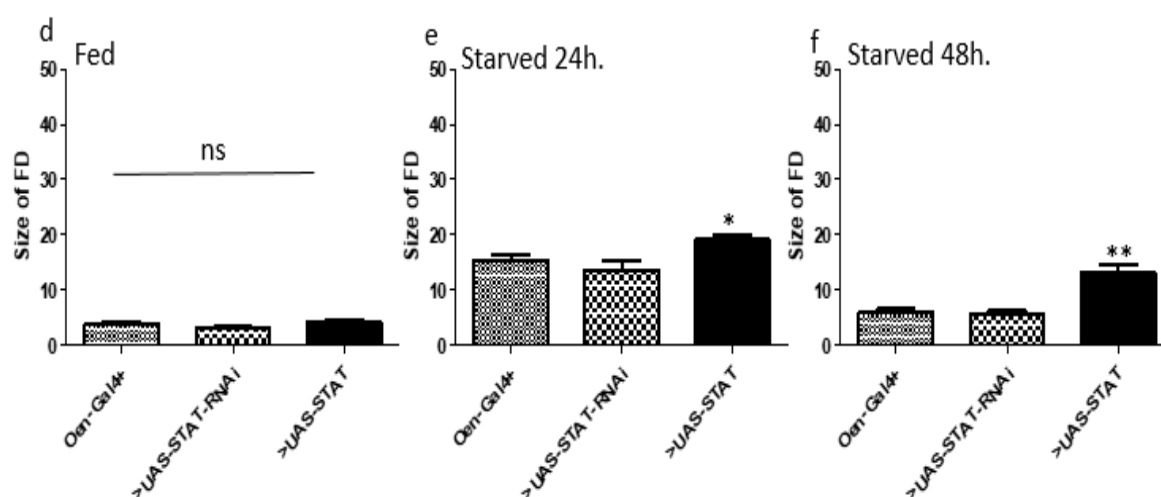


Figure 24: Starvation and oenocyte's STAT overexpression increase number and size of fat droplets in the adult oenocytes.

Flies with JAK-STAT activation via STAT overexpression, >UAS-STAT (a-f), showed a significant increase in the number and size of fat droplets after 24 and 48 hour starvation compared to control and flies with JAK-STAT inhibition, STAT-RNAi. Furthermore, even in fed condition flies with STAT activation showed a significant increase in the number (g) but not in the size (j) of fat droplets. Data are presented as mean \pm SEM; one-way ANOVA was used for analysis. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns not significant.

3.5.4 Effect of JAK-STAT activation in adult oenocyte on the metabolic rate of adult *Drosophila*

Furthermore, it was evaluated if ectopic overexpression of components of the JAK-STAT signaling pathway in adult oenocytes also lead to alterations of the metabolic rate. For this purpose, overexpression of Upd3 and STAT in adult oenocytes was done during starvation and fed conditions. I observed a significant decrease in the metabolic rate of the flies associated with high fat aggregation in the oenocyte after starvation in comparison to control groups. Flies with downregulated Upd3 and STAT levels respectively, induced by Upd3-RNAi and STAT-RNAi, showed no significant differences in their metabolic rates compared with the matching control groups [Fig.25]. Based on these results, I hypothesize that JAK-STAT activation in oenocytes affects systemic metabolic adaptation to fasting conditions.

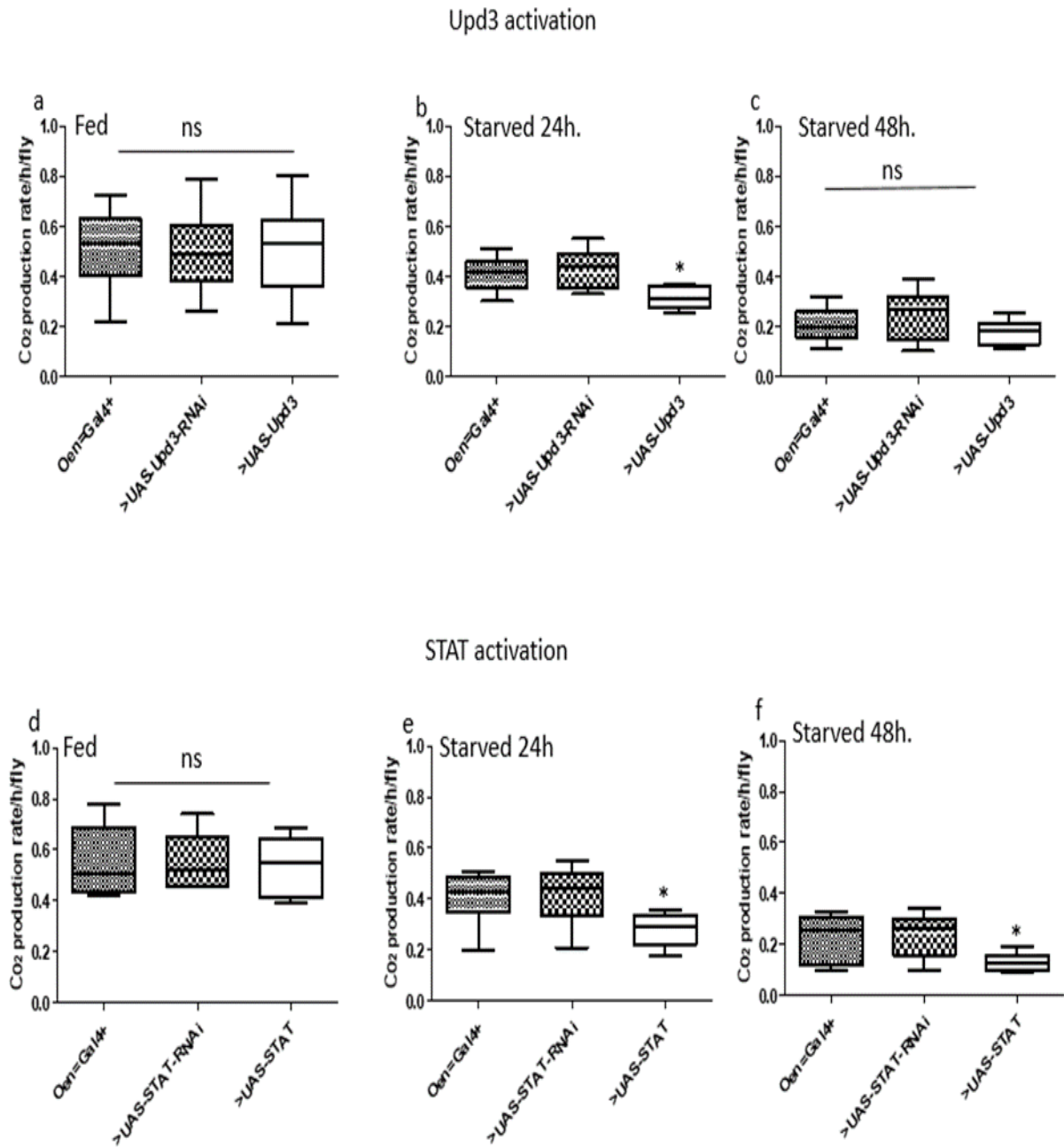


Figure 25: Oenocyte's JAK-STAT activation affect the metabolic rate of adult flies during starvation.

Flies with Upd3 activation, oen-Gal4>UAS-Upd3 (a-c) in the adult oenocyte showed a significant decrease in the metabolic rate after 24h. starvation compared to Upd3 knockdown, >UAS-Upd3-RNAi, and control. Additionally, the same results were recorded in the flies with STAT activation oen-Gal4>UAS-STAT (d-f) after 24 hour starvation (e) as well as 48 hour (f). Data are presented as mean \pm SEM; one-way ANOVA was used for analysis. * $p < 0.05$, ns not significant.

3.5.5 JAK-STAT activation in adult oenocyte affect the amount of fat in *Drosophila*

To look further if overexpression of Upd3 and STAT in adult oenocytes could also affect the amount of body fat, whole body TAG levels were measured in adult flies that were raised on control medium or subjected to starvation. Compared to the controls, a significant increase of lipid amount was observed in Upd3 and STAT overexpressing flies after starvation, but not in Upd3 and STAT knockdown flies [Fig. 26].

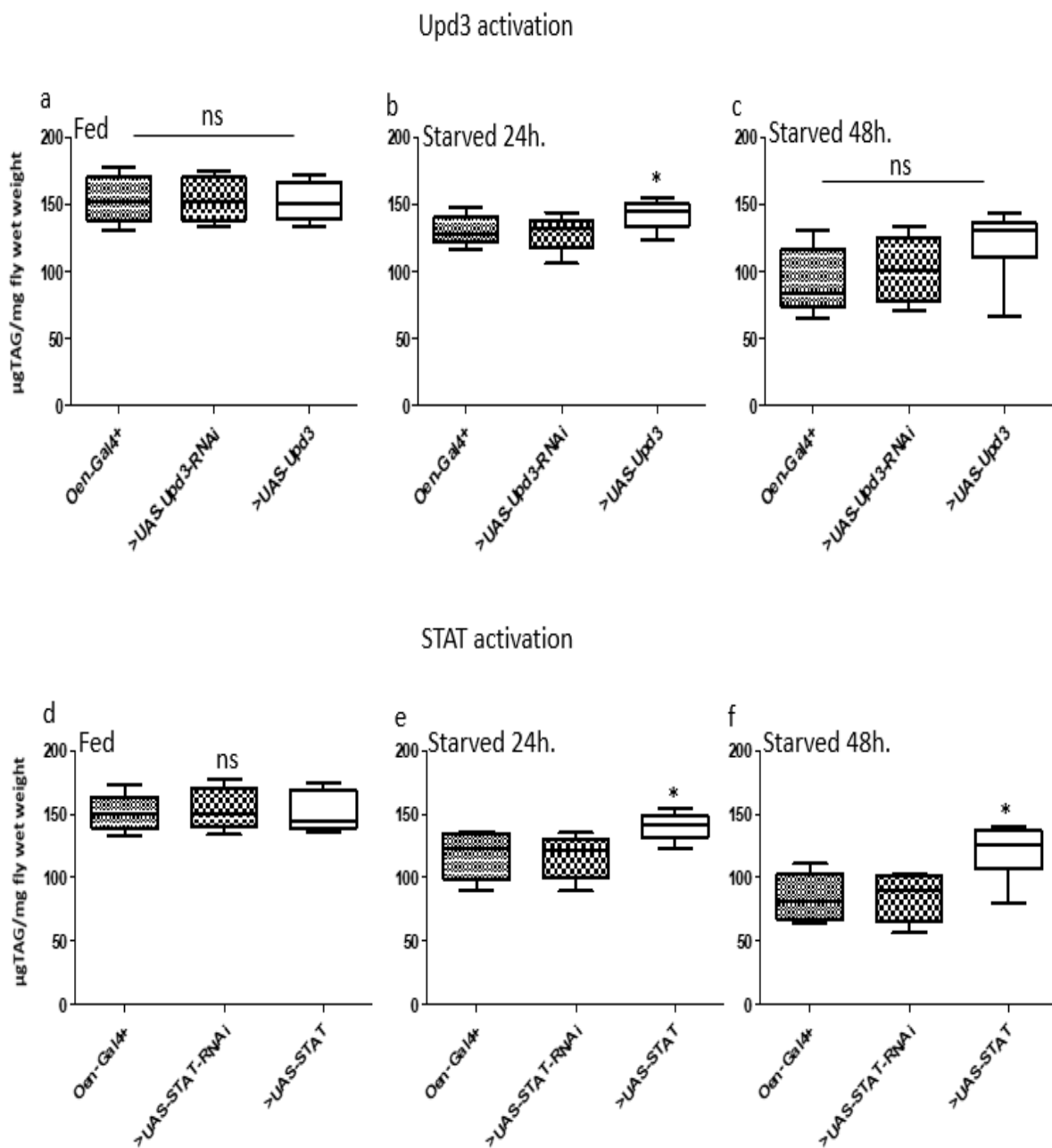


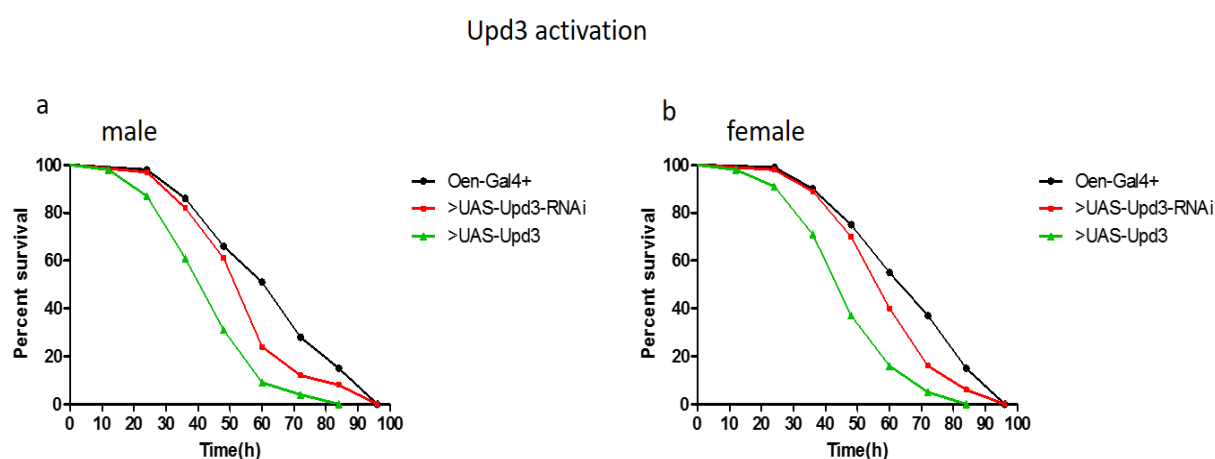
Figure 26: Overexpression of Upd3 and STAT in the adult oenocyte affects the total TAG level after starvation.

(a-c) The graphs show the triglyceride levels of flies with Upd3 overexpression in the adult oenocyte under fed and starved conditions. A significant increase was seen in the total TAG level associated with Upd3 overexpression in oenocytes compared with Upd3 knockdown and control animals after 24 hour of starvation. No differences were observed in well fed conditions. Flies with oenocyte's STAT overexpression (d-f) showed a significant increase in the body fat after 24h and 48h starvation. Data are presented as mean \pm SEM; one-way ANOVA was used for analysis. * $p < 0.05$, ns not significant.

3.5.6 JAK-STAT activation in adult oenocyte affects starvation resistance

Lifespan of many organisms can be extended by both, environmental conditions and genetic factors such as reducing calorie intake and increasing stress-protective responses (Kenyon, 2005).

To examine whether JAK-STAT-signaling in oenocytes acts on starvation resistance in *Drosophila*, I performed a longevity test of transgenic flies with altered Upd3 and STAT level exclusively in their oenocytes. Flies releasing more Upd3 and STAT in the oenocyte had a significantly decreased lifespan compared to the controls for all in both, adult males and females. Adult males were more sensitive to starvation than females [Fig. 27].



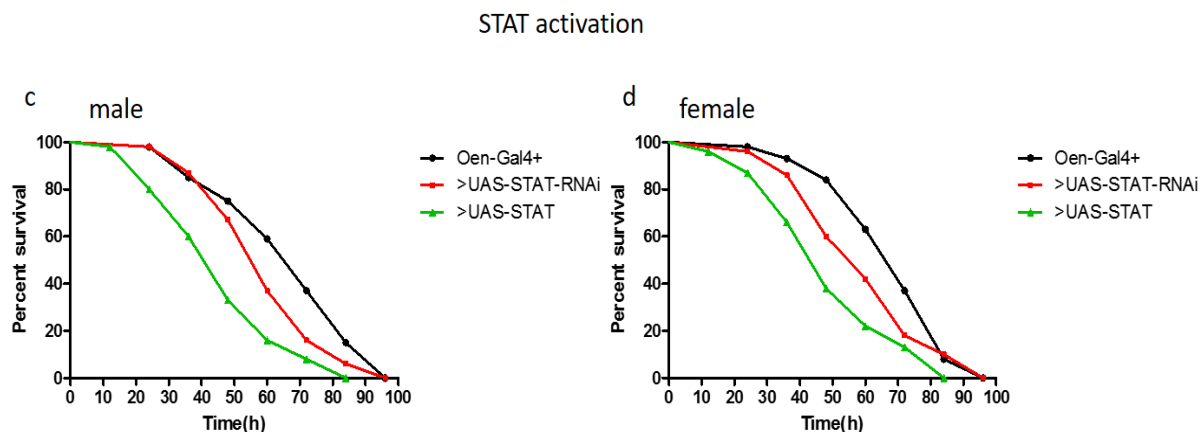


Figure 27: JAK-STAT activation in the adult oenocyte decreased starvation resistance of male and female.

Upd3 overexpression flies lived shorter than the Upd3 knockdown and control flies both in male and female (a, b). As well as, flies with oenocyte's STAT overexpression (c, d) were showed the same result. Log-rank test for survival analysis showed that $p < 0.001$ for both sexes.

3.6 Quantitative real-time PCR validation of differential gene expression

To show the differences in transcript levels in adult oenocyte after starvation, six genes associated with fat metabolism and immunity were selected for further validation, including brummer lipase (*bmm*), *Drosophila* insulin like peptid-6 (*dilp6*), *Thor*, *Upd2*, *Upd3* and *Socs36E*.

Brummer lipase play a key role in the regulation of lipid storage (Grönke et al., 2005). *dILP6*, which is induced in the fat body in response to starvation, mediates the accumulation of fat droplets in the oenocyte (Chatterjee et al., 2014). *Drosophila* 4E-BP, *Thor* is a member of the 4E-binding protein family has an effector role in cell growth, dFOXO activation and immune induction (Bernal and Kimbrell, 2000). Unpaired 2 (*Upd2*) and Unpaired 3 (*Upd3*) play a role as a binding ligand to activate the JAK-STAT signaling pathway (Yang et al., 2015). *Drosophila* *Socs36E* acts as a negative regulator of activated JAK/STAT pathway signaling (Stec et al., 2013).

Confirming the role of oenocytes in lipid metabolism, genes that are involved in lipid processes in starved oenocytes were significantly more highly expressed compared with material derived from flies of the fed state. A prominent example is *bmm*. It is induced to a higher extent in the oenocyte. Furthermore, similar differential induction and expression

levels were observed for the *dilp6* and *Thor*. The specific high expression of these genes support the notion that adult oenocytes have a key role in coordinating metabolic adaptation to fasting. As well as, the transcript levels of all selective genes that related with immunity shows significant upregulation except the transcript level of *Upd2*. The data calculated from five biological replicates are shown in [Fig. 28]

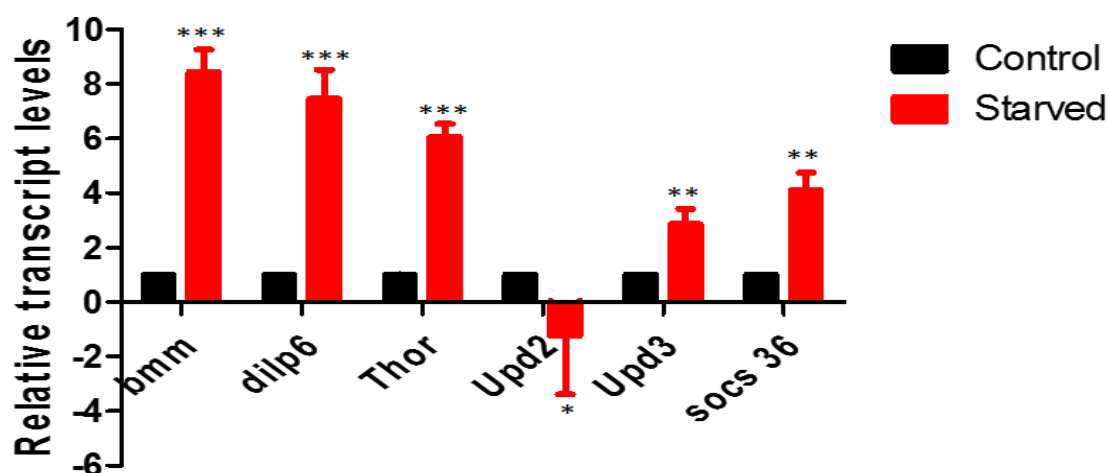


Figure 28: Validation of differential expression levels using relative qRT-PCR.

The relative fold change of six selected genes was normalized by an endogenous reference gene of RPL32. The red bars indicate gene expression levels in starved adult oenocytes compared with control, black bars. All values represent as mean \pm SEM. from five independent replicates. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

3.7 Oenocyte-Gut microbiota interaction

In the past few years, there has been an increasing interest in the effects of the microbiota on the host. This comprises understanding the importance of the gut microbiota in modulating host physiology, behavior, ecology and finally evolution (Heys et al., 2018). In this part, the interaction between the oenocyte and gut microbiota and its effect on the lipid homeostasis as well as some physiological parameters in the adult flies during starvation and well fed conditions was analysed.

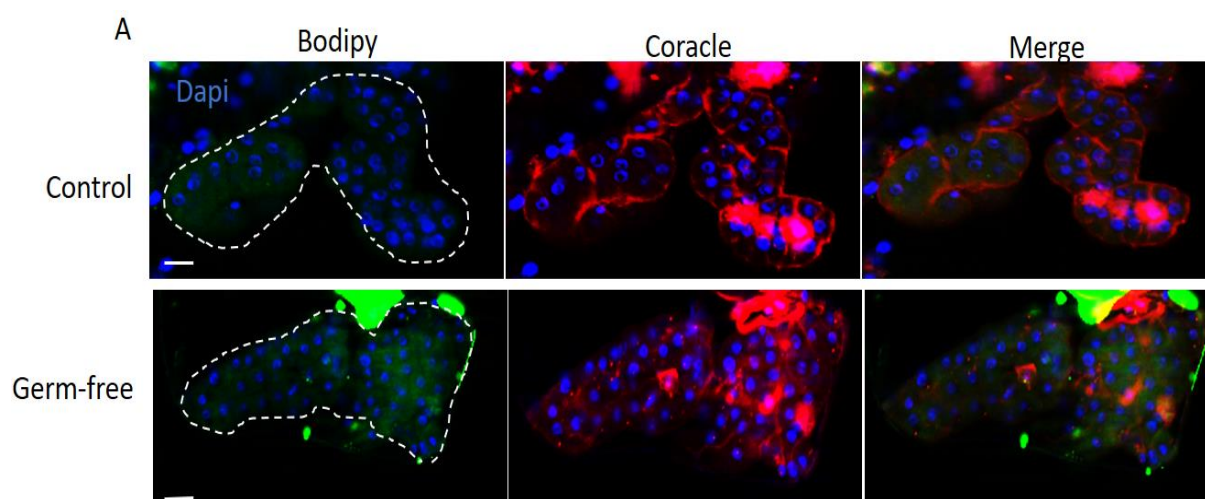
For this purpose, sterile or axenic wild type flies were made using egg dechoriation. Eggs were divided into two groups, the first group was placed on the axenic media for maintaining

them germ-free flies, and the other group was recolonized on the same media with a mixture of naturally occurring bacteria of the *Drosophila* microbiota, such as *Lactobacillus*, *Acetobacter*, *Enterobacter* and *E. coli* as a control group. All these steps were performed under otherwise sterile conditions.

3.7.1 Effect of gut microbiota on lipid accumulation in oenocytes of starved adults

As part of their function in lipid turnover, oenocytes accumulate fat droplets during starvation (Tian et al., 2011). This phenotype was assessed to unravel the effects the gut microbiota in lipid turnover during starvation. For this issue, germ-free flies were compared with recolonized ones. Interestingly, after 24h of starvation, oenocytes of germ-free flies showed a significant decrease in lipid accumulation [Fig. 29] as well as in the number and size of fat droplets [Fig. 30]. However, no significant differences were observed between the two groups under well fed conditions.

Taken together, these results show the role of gut microbiota in the lipid mobilization and accumulation in the starved adult oenocytes.



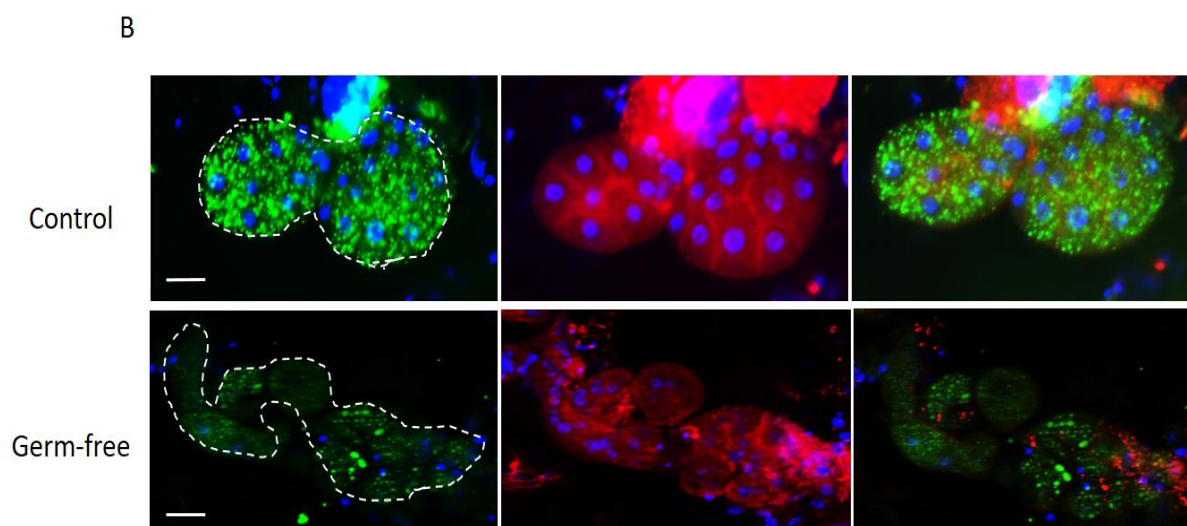


Figure 29: Role of gut-microbiota in the oenocyte's fat accumulation during starvation.

IHC staining of adult oenocyte showed no fat accumulation both in control and germ-free flies during well fed condition (A). After 24h starvation, lipid accumulation of germ-free was less than control group (B). This result indicates that gut-microbiota might play a significant role in the oenocyte physiology during starvation. Bodipy (green), DAPI (blue), coracle (red), Oenocyte ribbons are circled. Scale bar is: 50 μ m.

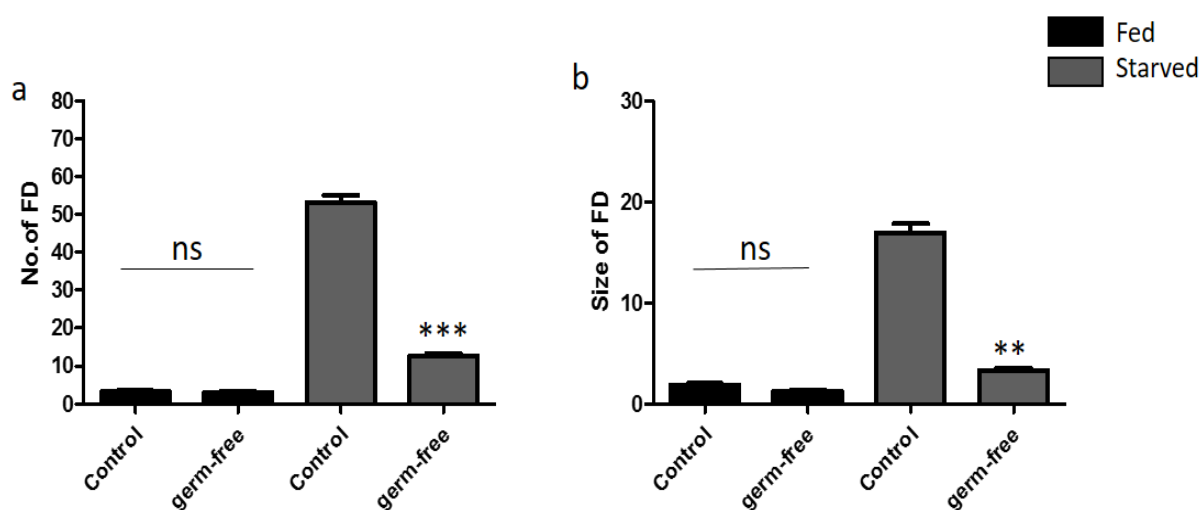


Figure 30: Gut microbiota affects the number and size of accumulated fat droplets in adult oenocytes.

Germ-free flies showed a significant decrease in the number (a) and size (b) of oenocyte's lipid droplets after 24h. starvation. No significant difference was seen between germ-free flies and control groups in fed conditions. Data are presented as mean \pm SEM; the unpaired two-tailed Student's t-test was used for analysis. ** $p < 0.01$, *** $p < 0.001$, ns not significant.

3.7.2 Relation of gut microbiota and metabolic rate of adult *Drosophila* during starvation

In mammals, metabolites that are secreted by the intestinal microbiota modulate adult metabolism and juvenile development (Ichimura et al., 2009; Hara et al., 2014). To investigate the role of gut microbiota in the regulation of metabolic rate in the adult flies, I tested the germ-free flies under fed and starved conditions. Compared to controls, germ-free flies displayed a significantly decreased metabolic rate after 24h of starvation, but not if they were well-fed [Fig. 31].

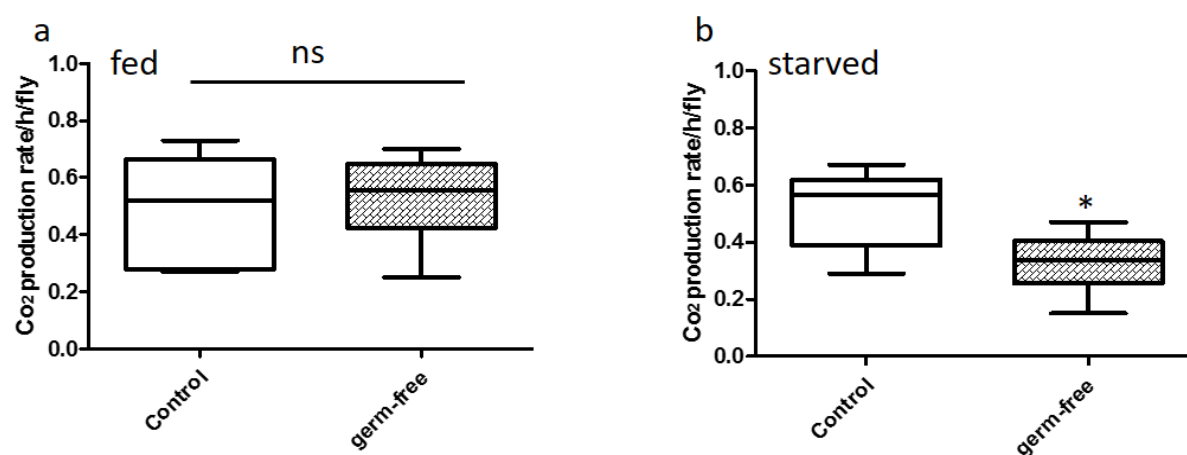


Figure 31: Metabolic rate of germ-free and recolonized adult flies.

The metabolic rates were measured in germ-free and recolonized flies in fed and starved conditions. No significant difference was observed in the fed state (a). Germ-free flies showed a significant decrease in the metabolic rate after 24h of starvation compared to recolonized animals (b). Data are presented as mean \pm SEM; the unpaired two-tailed Student's t-test was used for analysis. * $p < 0.05$, ns not significant.

3.7.3 Effect of gut microbiota on the body fat amount of adult *Drosophila*

To address the role of microbiota in the level of body fat amount during starvation and under well-fed conditions, CCA tests were employed. Starved, germ-free flies showed a mild but not significant decrease in body fat compared to recolonized animals. No significant differences between these two groups were seen in the well fed state [Fig. 32].

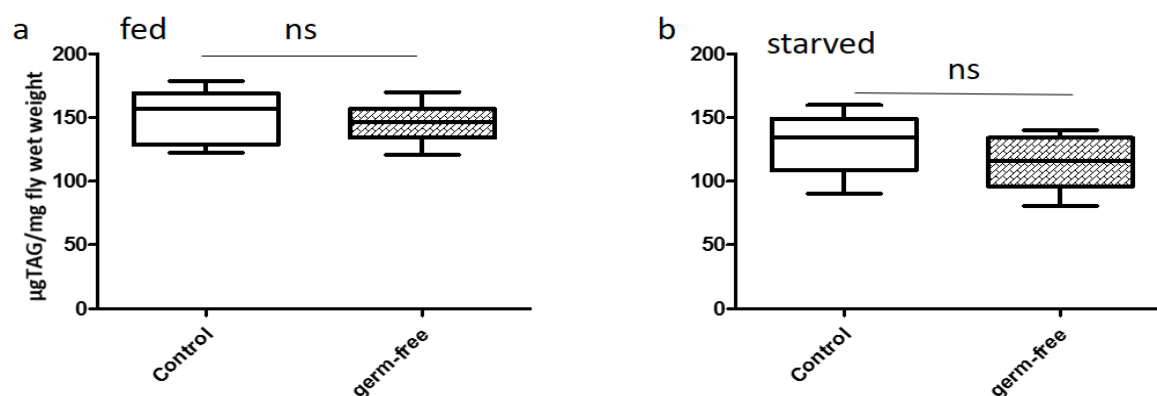


Figure 32: TAG level in the germ-free and reconstituted flies during fed and starved conditions.

Total TAG levels were measured in germ-free and reconstituted flies under fed (a) and starved (b) conditions. No significant differences between the two groups were observed. Data are presented as mean \pm SEM; the unpaired two-tailed Student's t-test was used for analysis. ns, not significant.

3.7.4 Role of gut microbiota in starvation resistance

I tested whether gut microbiota affects survival during starvation. Experimental and control flies were exposed to food deprivation by only feeding water. However, germ-free flies displayed a significantly lower survival with respect to the controls in both male and female [Fig. 33].

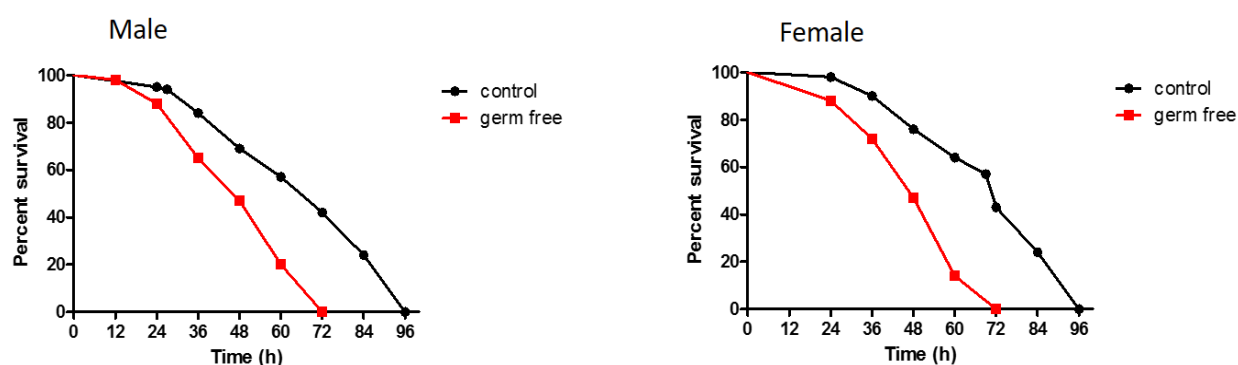


Figure 33: Germ-free flies are sensitive to starvation stress.

Germ-free flies lived shorter than its controls with normal microbiota, both in male and female. Log-rank test for survival analysis showed that $p < 0.001$ for both sexes.

4. Discussion

4.1 Modeling a fatty liver in *D. melanogaster*?

The goal of this study was to unravel if *Drosophila* could be used as a model for the development of fatty liver diseases. In this work, I have presented for the first time a *D. melanogaster* model to study the direct effects of starvation and manipulation of intracellular signaling systems as major factors that control lipid accumulation in the hepatocyte like cells of flies, the oenocytes (Cinnamon et al., 2016).

Liver disease is an important international ailment with mortality rates reaching almost 2 million deaths per year. Non-alcoholic fatty liver disease (NAFLD) and alcoholic liver disease (ALD) are the primary disease phenotypes seen in chronic liver disorders. Pathologically, NAFLD and ALD share an identical patterns of hepatic disorders ranging from simple steatosis to steatohepatitis, fibrosis and cirrhosis. It is now recognized that enhancing the identification of new pharmacological strategies is required, because no FDA-accredited therapy for both NAFLD or ALD is yet available (Wahlang et al., 2018).

Most recently, genetically tractable models of fatty liver disease comprise mice (Monteillet et al., 2018), rat (Cao et al., 2018) and guinea pigs (Ipsen et al., 2018). Invertebrate models, especially fruit flies as the most important invertebrate disease models have not been used in this research field. Therefore, simple model such as *Drosophila melanogaster* can be considered as a new way to study the role of fatty liver-genes and their effects on the physiological function of the organism.

In the current study, I have highlighted the advantages of *Drosophila* to model fatty liver-like phenotypes and elucidated to what extent the fly can be used to study chronic diseases of this organ. Moreover, when compared to vertebrates, the oenocytes of the fruit fly share striking similarities with respect to its physiology and reaction towards pathogens and other biotic stimuli with the vertebrate liver (Ugur et al., 2016). Beside the remarkably decreased lifespan

of *D. melanogaster* in response to excessive lipid accumulation in adult oenocytes, the corresponding animals demonstrated several fatty liver-related phenotypical features.

4.2 Fat body-Oenocyte interaction during starvation

The fruit fly and mammals share a surprisingly similar organ composition, for example, the fat body and oenocyte are functional analogues of the mammalian adipose tissue and liver, respectively (Chatterjee et al., 2014). In this project I highlighted the main role of fat droplets, which are accumulated in the oenocyte during a starvation period.

Lipids are important energy carriers and play key roles in redistribution of resources throughout the body during periods of fasting (Roberts et al., 2014). In mammals, complex coordinating systems act to regulate lipid homeostasis and metabolism with energy request, including physiological interactions between the intestine, adipose tissue, liver and central nervous system (Finn and Dice, 2006). Dietary lipids are digested in the small intestine with a help of pancreatic lipase into fatty acids, which are absorbed from intestinal cells and broken down into free fatty acids (FFAs). These products are moved into the circulatory system and taken up by either peripheral tissues for direct energy production or adipose tissues for storage in the form of triacylglycerol (TAG). During starvation, endocrine signals activate lipases (adipose triacylglycerol lipase [ATGL] and hormone-sensitive lipase [HSL]) in the adipose tissues to convert the storage lipids into FFAs. Surprisingly, food deprivation stimulates fat accumulation in liver cells. However, to provide an additional source of energy, liver cells oxidize at least some of these accumulated lipids to form ketone bodies (Chatterjee et al., 2014). The interaction between fat cells and liver during starvation is therefore critical for maintaining appropriate energy homeostasis, and dysregulation of these processes can lead to hepatic steatosis and is a major cause of metabolic diseases (Van den Berghe, 1991; Saltiel and Kahn 2001).

Drosophila and other insects store lipids in the form of intracellular droplets in the fat body (Ad et al., 1985). Diet-derived lipids, exported from the midgut to the haemolymph as lipoproteins, are taken up by the fat body via a mechanism including lipophorin receptors (Canavoso et al., 2001; Lee et al., 2003). In response to food deprivation, a sensor mechanism

in fat body cells that is controlled by the insulin/IGF1 signaling (IIS) pathway and TOR signaling, triggers the accumulation of fat droplets into larger structures, the upregulation of lipases such as the ATGL-like enzyme Brummer, as well as the release of lipids into the hemolymph (Grönke et al., 2005). Whereas the fat body is an important lipid and glycogen storage organ in *Drosophila*, it also plays a key role as an endocrine organ to regulate metabolic homeostasis (Musselman and Kühnlein, 2018).

Similar to mammalian hepatocytes, oenocytes express genes with homology to liver-specific enzymes. Furthermore, oenocytes take, similar as liver cells, a critical role in lipid aggregation during starvation (Billeter et al., 2009). This process is required for functional mobilization of lipids from the fat body and it represents a metabolic change resembling hepatic steatosis, suggesting a close interaction between fat body and oenocytes in regulation of fat metabolism.

Apparently, oenocytes play a critical role in the metabolic homeostasis via the conserved insulin/IGF1 signaling (IIS) pathway, which acts as a central regulator of metabolic adaptations. IIS coordinates responses to environmental stressors and nutritional changes in order to regulate metabolism, growth, reproduction and longevity (Karpac and Jasper, 2009; Xu et al., 2012). In the mammalian liver, IIS stimulation activates glucose uptake, glycogenesis, and fatty acid synthesis (Saltiel and Kahn, 2001), whereas reduced IIS activity under starvation leads to activation of the transcription factor Foxo, which promotes glycogenolysis and fatty acid beta oxidation (Nandi et al., 2004). In flies, IIS activity is activated by a number of different insulin-like peptides (ILPs) (Brogiolo et al., 2001; Grönke et al., 2010). Whereas secretion of most types of *Drosophila* ILPs is significantly decreased in response to nutrient deprivation (Ikeya et al., 2002), fat body-derived *Drosophila* insulin-like peptide 6 (dILP6) has recently been found to induce lipid uptake in adult oenocytes in nonfeeding states thus increasing starvation tolerance of the animal (Bai et al., 2012; Chatterjee et al., 2014). Furthermore, tissue-specific manipulations of the Slimfast amino-acid channel, the Brummer lipase and the Lsd2 fat-storage regulator showed that oenocytes act downstream of the fat body. In turn, oenocytes play a role in depleting stored lipid from the fat body during starvation (Gutierrez et al., 2007).

Thus, the functions of glycogen storage and lipid processing seem to be divided in *Drosophila* between the fat body and oenocytes. Lipid mobilization from the fat body during fasting produces lipid droplet accumulation in oenocytes, a metabolic change resembling hepatic

steatosis, and the near-distance between fat body and oenocyte helps to orchestrate this strong relationship between them.

4.3 cAMP signaling pattern - Utilization of photoactivatable adenylcyclases to study cAMP signaling in *D. melanogaster*

Usually, cyclic AMP, an intracellular second messenger is activated by binding of neurotransmitters and hormones to G protein-coupled receptors (GPCRs). Cyclic AMP performs many of its physiological functions by stimulation cAMP-dependent protein kinase (PKA), which in turn phosphorylates and regulates the functions of downstream protein targets including enzymes, ion channels and transcription factors (Yang and Yang, 2016), many of these effects appear through the activation of the cyclic AMP response element binding protein (CREB), which acts as a transcription factor that regulates the expression of genes involved in a variety of different processes (Ravnskjaer et al., 2015).

Since the cAMP pathway plays an important role in lipid homeostasis and energy production by increasing lipolysis and fatty acid β -oxidation (Zingg et al., 2017), an increase in cAMP levels induced by food deprivation (La vine et al., 1975) may contribute to starvation resistance. cAMP secretion in the liver leads to increased glucose production through stimulation of glucose producing pathways by increasing the transcription of gluconeogenic enzymes, such as phosphoenolpyruvate carboxykinase (PEPCK), glucose 6-phosphatase (G6Pase), and pyruvate carboxylase (PC) (Jitrapakdee, 2012). Additionally, phosphorylation of key enzymes involved in glycolysis (phosphofructokinase-2 and pyruvate kinase) inactivates them. Furthermore, cAMP inhibits glycogen synthase and activates glycogen phosphorylase through phosphorylation, leading to suppressed glycogenesis and activated glycogenolysis, respectively. On the other hand, hepatic cAMP also leads to inhibition of lipogenesis through phosphorylation of key players in fatty acid synthesis, such as pyruvate dehydrogenase and Acetyl-CoA carboxylase, thereby inhibiting their action (Lent and Kim 1983).

In this study, I have demonstrated that high levels of cAMP in oenocytes of adult flies in the fasting state enhanced fat fluxes and its accumulation in this cell type similar to what happens in the case of the fatty liver. Both, the number and size of fat droplets is increased in response

to high cAMP levels, which is even observed under well fed condition, where this is usually never seen. Furthermore, it seems that this massive amount of accumulated lipids in these cells has a negative effect on their functions, especially those functions related to fatty acid metabolism and very long chain fatty acid (VLCFA) synthesis, a process that is necessary to maintain the water-tightness of the respiratory system and to protect the adult flies from desiccation (Parvy et al., 2012; Neven and Mitcham, 2008). Disruption of this function could cause the obvious decrease in the rate of metabolism and in the starvation resistance of adult flies as observed in the current experiment, which is associated with high lipid levels in the oenocytes. Therefore, strategies to control cAMP efflux in the hepatocyte could serve as a potential new tool to prevent metabolic diseases-associated hepatic steatosis.

Similar as the mammalian glucagon, the adipokinetic hormones (AKHs) play a major role in insects during starvation by activation the cAMP pathway in the fat body through the stimulation of G protein-coupled receptors (GPCRs) (called AKH receptors, AKHRs) expressed on the fat body cells. This process leads to elevated rates of breakdown of stored triglyceride and glycogen, to produce and release circulating glucose and free fatty acid (Gáliková et al., 2015). Additionally, glucagon plays a key role to maintain glucose and lipid homeostasis in the mice hepatocyte during the periods of starvation through induction of intracellular cAMP production (Lv et al., 2017). Glucagon also regulates fatty acid oxidation in the liver and reduces plasma triacylglycerol levels. Indeed, glucagon receptor knockout mice show increased triacylglycerol levels during the fasted state (Longuet et al., 2008)

In connection with these results, I checked the expression of selective hormone receptors in the adult oenocyte, such as octopamine (OA), tyramine(TA), dopamine(DA) and 5-hydroxytryptamine(5HT) receptors according to its role in the metabolic homeostasis and physiological behavior. It is highly probable that these receptors might be those that regulate cAMP levels under natural conditions in oenocytes thus serving as the molecular substrate for this type of manipulation of these highly interesting cells. OA and TA are the invertebrate homologs of the vertebrate adrenergic transmitters, epinephrine and norepinephrine. They are decarboxylation products of the amino acid tyrosine, with TA as the biological precursor of OA. both monoamines, OA and TA, are independent neurotransmitters that mediate their action through sets of G protein coupled receptors (Roeder, 2005). Four different octopamine receptors (OAMB, Oct β 1R, Oct β 2R, Oct β 3R) and 3 different tyramine receptors are known

(TyrR, TyrRII, TyrRIII) and I have checked their presence in the adult oenocyte of *Drosophila melanogaster*. Additionally, DA is a neurotransmitter which modulates a wide array of behaviors, ranging from sleep and locomotion to courtship and learning, acts via three different receptors (DopR, DopR1 and D2R) (Van Swinderen and Andretic, 2011; Beaulieu et al., 2015), which I have also tested with respect to expression in adult oenocytes. Furthermore, 5-HT which is also named serotonin, acts as a messenger substance to modulate a great diversity of important behavioral and physiological processes including aggression, sleep, circadian rhythms, feeding, responses to visual stimuli, and learning (Blenau and Thamm, 2011). The function of serotonin is mediated by binding to G-protein-coupled receptors through 5HT1A, 5HT2A and 5HT2B receptors (Saudou et al., 1992) which I checked out in this study.

By using RT-PCR I have tested the expression of all these receptors in the adult oenocyte, and I have shown that only TyrRII is strongly expressed, while DopR, DopR1 and 5-HT1A showed minor expression. The study by El-Kholy et al. (2015) performed in parallel showed that from the all the different types of TA receptors, only TyrRII was expressed in the larva oenocyte of fruit flies without any expression of the OA receptors (El-Kholy et al., 2015). OA and TA appear to play a key role in the regulatory network responsible for inter-organ communication (Rajan and Perrimon, 2011). Release of OA and TA modulate several behaviors and metabolic processes in order to shift the animal's physiology to a high performance and high energy-expenditure state. Moreover, it was shown recently that OA regulates the rate of resting metabolism and affects directly the rate of fat storage (Li et al., 2016). Consequently, manipulation of both monoamines signaling properties could lead to metabolic alterations. such as those associated with body fat storage are most conspicuous. Despite the structural similarities between OA and TA, there effect on the major metabolic trait, fat storage is opposed. Whereas animals without of OA are obese, those devoid of OA and TA are not (Li et al., 2017).

Dopamine (DA) is a monoamine neurotransmitter synthesized from the amino acid tyrosine and plays a role in social and physiological relations (Draper et al., 2007). Physiological actions of Dopamine are mediated by the three closely related G-protein coupled receptors (DopR, DopR1and D2R), they have ability to modulate adenylyl cyclase (AC) and can stimulate the

formation of cyclic adenosine 3',5'-Monophosphate (cAMP) (Jaber et al., 1996), but their role in modulation lipid metabolism is until now completely unknown.

Serotonin is a neuromodulator that is present in most animal phyla and is involved in a variety of function by activation of G protein-coupled receptor via three serotonin receptors (5HT1A, 5HT2A and 5HT2B). The 5HT1A receptor stimulates adenylate cyclase while the 5HT2A and the 5HT-2B receptors are negatively coupled to adenylate cyclase and activate phospholipase C (Saudou et al., 1992). Again, also for serotonergic neurotransmission, a direct involvement in fat metabolism has not been shown yet in fruit flies.

4.4 Role of JAK-STAT signaling in the oenocyte lipid homeostasis

The Janus kinase (JAK)/signal transducer and activator of transcription (STAT) intracellular signaling pathway plays a key role in transducing signals from a diversity of metabolically related cytokines and hormones including leptin, erythropoietin, growth hormone, interleukin 4, interleukin 6 and Interferon- γ . This signaling pathway is activated by the interaction of ligands with their receptors, causing receptor stimulation and transphosphorylation of receptor-associated JAK molecules. This leads to the phosphorylation of tyrosine residues on the receptors and phosphorylation of downstream signaling molecules called STATs, which translocate to the nucleus where they modulate expression of target genes (Ladyman and Grattan, 2013). JAK-STAT pathways are dysregulated in the context of metabolic diseases and obesity (Gurzov et al., 2016; Dodington et al., 2017).

Until now very little is known about the role of JAK/STAT signaling in the regulation of lipid metabolism. To unravel these mechanisms, I mimicked the situation of JAK-STAT manipulation in the oenocyte of the adult fruit fly *D. melanogaster* during starvation. Firstly, I have shown that activation of JAK-STAT signaling occurred in the oenocyte clusters of 3rd instar larvae and ribbons of adult flies in response to high fat accumulation during starvation by utilizing the 10xSTAT-GFP reporter. The signal in oenocytes indicate that lipid accumulation has caused an inflammation-like state and JAK-STAT signaling is mediating this reaction at least in part.

Liver disease progression is controlled by a large number of cellular mediators, including growth factors, cytokines, hormones, and among others. Of the various downstream signaling

pathways, the JAK-STAT pathway has been shown to play a critical role in the pathogenesis of liver diseases. Accumulated research data from the last decade suggest that the JAK-STAT signaling helps in controlling liver injury, inflammation, fibrosis and regeneration (Gao et al., 2012).

To date, four mammalian JAKs family members (JAK1-3 and Tyk2) and seven STAT proteins (STAT1-4, 5a, 5b, and 6) have been identified (Aaronson and Horvath, 2002). Interestingly, STAT1,2 and STAT3,5 stimulation play opposing roles in the progression of liver pathophysiology, including liver injury and repair. Whereas STAT1 and STAT2 activation in hepatocytes leads to hepatocytes death and increased liver damage, STAT3 and STAT5 activation protects against cells death and is critical for liver regeneration (Sun et al., 2006). On the other hand, the roles of STAT4 and STAT6 in liver injury, inflammation, and regeneration is still unknown (Gao et al., 2012). JAK-STAT pathway stimulation affects the fat metabolism directly by influencing the expression of enzymes such as Acyl-CoA oxidase and fatty acid synthase. Dysregulation of the JAK-STAT pathway would lead to a multiple metabolism disorders and compounds specifically interfering with this signaling pathway may become highly important for the treatment of various diseases including the metabolic syndrome (Xu et al., 2013). During starvation, cAMP-dependent processes regulate the classical pathways of lipolysis activation in adipose tissue. JAK-STAT signaling has been identified in recent years as a pathway involved in this lipolysis response (Chaves et al., 2011). The elevated rates of triglyceride lipolysis lead to enhanced FFA flux to the hepatocyte. Moreover, overexpression of STAT3 in hepatocytes, decreases a multitude of mRNAs for Acetyl-CoA carboxylase and fatty acid synthases. (Kinoshita et al., 2008).

In the fruit fly *Drosophila melanogaster*, the core components of the JAK-STAT pathway include three related ligands named Unpaired (Upd), Unpaired 2 (Upd2) and Unpaired 3 (Upd3). Binding of the ligand to the Domeless (Dome) receptor stimulates signaling and urges the receptor associated JAK tyrosine kinase Hopscotch (Hop) to phosphorylate both, itself and the cytoplasmic tail of the Dome receptor to create docking sites for the latent STAT92E protein, which is most similar to mammalian STAT3 and STAT5. STAT92E is in turn phosphorylated, dimerises and translocates into the nucleus where it is capable of binding to a palindromic response element to induce target gene expression (Hou et al., 1969; Chen et al., 2002).

Woodcock et al. also showed that shorter lifespan, increased fat storage, reduced insulin sensitivity, and hyperglycemia were associated with systemic activation of the Janus kinase and signal transducer and activator of transcription (JAK-STAT) pathway (Woodcock et al., 2015). In fact, I have found that the dual exposure to starvation and activation of JAK-STAT signaling by overexpression of *upd3* or *STAT* in the adult oenocytes leads to high fat accumulation as well as significant increase in the number and size of fat droplets. Furthermore, these changes negatively affected metabolic rate and survival of the adult flies. Thus, it is clear that appropriate regulation of JAK-STAT signaling is required for the normal physiology of oenocytes.

4.5 Profile analysis for genes expression in the adult oenocytes

In this part of the thesis, I compared the changes of the oenocyte's mRNA profiles involved in lipid metabolism and immunity after 24 h of starvation. Transcription levels were validated by qRT-PCR for 6 selected genes including brummer lipase (*bmm*), *Drosophila* insulin like peptide-6 (*dilp6*), *Drosophila* 4E-BP (*Thor*), Unpaired 2 (*Upd2*), Unpaired 3 (*Upd3*), and *Drosophila Socs36E*.

The *bmm* gene encodes the fat storage droplet-associated TAG lipase Brummer, a homolog of human adipocyte triglyceride lipase (ATGL). Starvation or chronic *bmm* overexpression leads to decrease in the body fat stores *in vivo*, whereas loss of *bmm* action causes obesity in flies (Grimke et al., 2005). Additionally, *bmm* plays a key role in both basal and stimulated lipolysis as well as in storage fat mobilization (Haemmerle et al., 2006; Grönke et al., 2007) suggesting a general role of *bmm* in body lipid balance control. Fat break-down in the oenocyte lipid droplets helps in fat beta oxidation, energy production and starvation resistance, which might explain the upregulated of oenocyte's *bmm* observed in this study after starvation. In parallel, nonfeeding states increase *dILP6* expression through dFoxO-mediated feedback regulation of the IIS pathway. Therefore, *dILP6* plays a central role in promoting growth during environmentally or developmentally induced starvation (Slaidina et al., 2009; Chatterjee et al., 2014). On the other hand, *Thor* strongly affects fat metabolism in *Drosophila* via works as a metabolic brake that is activated under the environmental stress conditions to control lipid metabolism. *Thor* mutants lack this regulation, reducing their ability to survive under

unsuitable conditions (Teleman et al., 2005). Together, the upregulation of *dILP6* and *Thor* in this study suggests their role in lipid metabolism of adult oenocyte during starvation.

JAK/STAT Signaling regulates inflammation that is associated with metabolic related diseases including insulin resistance and obesity (Dodington et al., 2017). In *Drosophila*, the Unpaired (*Upd*) family is associated with JAK-STAT stimulation. While *upd2* regulates body size through insulin signaling and plays a role in growth and energy metabolism. *Upd3* expression in some tissues is essential for a subset of JAK-STAT mediated developmental functions such as cell proliferation, differentiation, cell migration, and apoptosis as well as *upd3* has also been implicated in immune function and tissue homeostasis in larvae and adult fruit flies (Wang et al., 2014). In this study, upregulation of *upd3* and downregulation of *upd2* in the starved oenocyte suggest that only *upd3* is expressed in the oenocyte in the nonfeeding state. Interestingly, my work suggests that the lipid accumulation in the starved oenocyte may cause inflammation and stimulate the JAK-STAT pathway. Additionally, The Suppressor of Cytokine Signaling (*SOCS*) family has been found as an important physiological negative regulators of JAK/STAT pathway (Stec and Zeidler, 2011). Thus, activation of JAK, STAT and *SOCS* protein hint for their role during normal oenocyte physiology.

4.6 The interplay between gut microbiota and oenocyte

The gut microbiota has recently been identified as an environmental factor that may play a role in metabolic diseases. The interplay between gut microbiota and oenocytes in the adult *Drosophila* remains still unclear. In this part, I focused on the role of the gut microbiota in the lipid metabolism and its effect on the phenotype and physiology of adult oenocytes. To investigate the effect of gut microbiota on the oenocyte's lipid metabolism and host physiology, I compared the number and size of accumulated fat droplets in the adult oenocyte, host metabolic rate, host fat amount as well as survival curve between conventional and germ-free flies during starvation and fed conditions.

Firstly, I have shown that starvation of adult flies leads to decrease in the oenocyte's lipid accumulation and decrease in the number and size of fat droplets in germ-free flies during the starved state. Secondly, I have found significant decrease in the host metabolic rate of germ-

free flies and decreases in the host fat amount but it was not significant after starvation. Furthermore, germ-free flies show significantly decreased starvation resistance for both males and females.

These results are strongly correlated with previous findings in mammals. Martinez-Guryn et al, demonstrated that the microbiota is essential for host adaptation to dietary lipid changes by regulating gut epithelial processes involved in their digestion and absorption. Additionally, in mice, gut microbiota exhibit increased lipid absorption even when fed a low-fat diet (Martinez-Guryn et al., 2018). On the other hand, the study of Bäckhed et al., on the conventionalized and germ-free mice shows decrease in body fat content and host metabolic rate. Furthermore, germ-free mice have decreased hepatic triglyceride levels compared with conventionally raised mice and are resistant to diet-induced obesity (Bäckhed et al., 2004; Bäckhed et al., 2007). These findings suggested the role of the gut microbiota in the intestinal lipid absorption, whereas in response to consumption of high-fat foods, several studies have shown that gut microbiota can multiply within few hours in the intestine to produce and release a digestive enzyme into the bowel, these enzymes break down dietary fat, enabling the rapid absorption of calorie-rich foods. At the same time, the microbiota releases bioactive compounds which activate the intestinal absorptive cells to package and transport lipids for absorption, a case that could cause obesity (Velagapudi et al., 2010; Ramakrishna, 2013; Cox et al., 2014; Chang and Martinez-Guryn, 2018). Sequentially, the gut microbiota of obese mice was found to be responsible for increased production of endogenous ethanol, which reaches the liver via portal blood, leads to hepatocyte lipid accumulation (Cope et al., 2000).

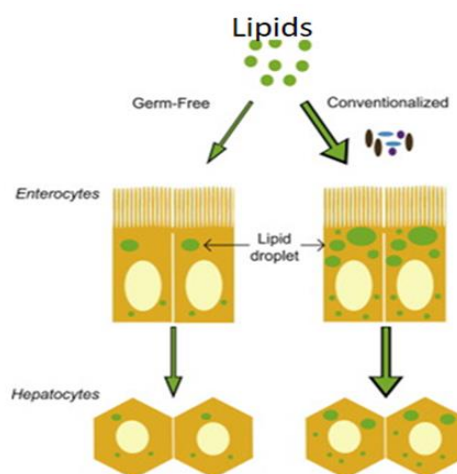


Figure 34: Role of gut microbiota in lipid intestinal absorption and hepatocyte accumulation.

Gut microbiota play a role in the intestinal lipid digestion and absorption by enterocytes, a case which leads to in the end an increases of fat accumulation in the hepatocytes

Similar to the results of Brummel et al. (2004), I have shown and due to the role of microbiota in the metabolism homeostasis and vitamins production, microbiota play an important role in the *Drosophila* longevity and starvation resistance. Thus, manipulation of gut microbiota may serve as a novel therapeutic strategy for treatment or prevention of such metabolic related diseases like obesity, diabetes and fatty liver.

Finally, the current study paves paths for the establishments of *D. melanogaster* as a model for human fatty liver metabolic related diseases using hepatocyte like cells, oenocytes in the adult flies.

Summary

Fatty liver is among the most common liver diseases with continuously growing incidences worldwide. It describes the pathological buildup of lipids in hepatocytes. For a more detailed analysis of the underlying molecular mechanisms, a simple and reliable model of fatty liver is desired and *Drosophila melanogaster* is considered as a candidate to develop these models. In *Drosophila*, as in other insects, the functional role of the liver is taken by so called oenocytes that accumulate fat in times of starvation, because they act as a relay tissue for the usage of fatty acids.

The major goal of this project was to unravel the regulation of this complex process of lipid transport and processing. For that, firstly, I manipulated the cAMP level in oenocytes in order to evaluate if hormonal signals converging onto the second messenger cAMP modify their physiology. It is evident that short-term starvation results in a significant increase in both, number and size of accumulated lipid droplets. Light induced activation of a photo-activatable bacterial adenylate cyclase that was exclusively expressed in oenocytes enhanced this effect significantly. Moreover, even under well-fed conditions, cAMP activation induced the occurrence of lipid droplets in these cells. Furthermore, I could show expression of some hormone receptors and genes in the adult oenocyte that might be responsible for the in vivo regulation of cAMP in these cells. Only the tyramine receptor TyrRII is strongly expressed, whereas minor expression was obvious for the dopamine receptors DopR, DopR1 and the serotonin receptor 5HT1A. Moreover, I could show upregulation of *bmm*, *dilp6*, *Thor*, *upd3* and *SOCS*, genes which are play a role in the lipid metabolism and immunity, in response to starvation. Secondly, I manipulated the JAK-STAT signaling pathway in adult oenocytes via the overexpression of either the ligand upd3 or the transcription factor STAT proteins. I showed a significant increase in the accumulated lipid droplets associated with the combined effects of starvation and JAK-STAT pathway activation. Excessive amounts of accumulated lipids in oenocytes had a negative effect on the basic metabolic rate and starvation resistance of the adult flies. The observed phenotype mirrors the situation found in humans with hepatosteatosis, meaning fatty liver. In the last part of this study, I highlighted the gut

microbiota-oenocyte axis that plays an important role in lipid metabolism and host physiology. However, this relationship still poorly understood in *Drosophila*. To investigate the effect of gut microbiota on the oenocyte's lipid metabolism and host physiology, I compared the number and size of accumulated fat droplets in the adult oenocyte, host metabolic rate, host fat amount as well as survival between conventional and germ-free flies during starvation and and fed conditions. In germ-free flies, I have shown that short-term starvation leads to a significant decrease in the number and size of accumulated fat droplets in the oenocyte without any effect on the total body fat. Moreover, germ-free flies showed a significant decrease in the metabolic rate as well as in their lifespans.

Taken together, the present work could pave the way for better understanding of the complex regulatory network that controls the activity of the hepatocyte-like cells and to understand molecular events underlying the pathogenesis of metabolic inflammation of the liver.

Zusammenfassung

Die Fettleber gehört zu den weltweit häufigsten Lebererkrankungen, die sich außerdem durch eine ständig wachsende Inzidenz auszeichnet. Die Erkrankung wird durch das pathologische Speicherverhalten von Lipiden in der Leber ausgelöst. Für eine detailliertere Analyse der zugrundeliegenden molekularen Mechanismen ist ein einfaches und zuverlässiges Modell der Fettleber wünschenswert und die Taufliege *Drosophila melanogaster* hat alle Voraussetzungen, um die entsprechenden Anforderungen zu erfüllen. In *Drosophila*, wie auch in anderen Insekten, wird die Funktion der Leber von so genannten Oenozyten übernommen, die in Zeiten des Hungers Fett ansammeln, da sie, ebenso wie die Leber, als Relaisstation für die Verwendung von Fettsäuren dienen.

Das Hauptziel dieser Arbeit bestand darin, die Regulierung dieses komplexen Prozesses des Lipidtransports und der Lipidverarbeitung zu entschlüsseln. Dazu habe ich einerseits den cAMP-Spiegel in Oenozyten experimentell manipuliert, da eine Vielzahl unterschiedlicher Hormone einen Einfluss auf den cAMP-Spiegel von Zielgeweben haben. Es ist offensichtlich, dass kurzfristiges Hungern zu einem signifikanten Anstieg sowohl der Anzahl als auch der Größe der Lipidtröpfchen in den Oenozyten führt. Die Aktivierung einer photo-sensitiven bakteriellen Adenylatcyclase, deren Expression ausschließlich auf Oenozyten beschränkt wurde, verstärkte diesen Effekt deutlich. Darüber hinaus führte die cAMP-Aktivierung auch unter guten Ernährungsbedingungen zum Auftreten von Lipidtröpfchen in diesen Zellen. Darüber hinaus konnte ich die Expression einiger Hormonrezeptoren in adulten Oenozyten zeigen, die für die *in vivo*-Regulation des cAMPs in diesen Zellen verantwortlich sein könnten. Nur der Tyraminrezeptor TyrR11 wird stark ausgedrückt, während für die Dopaminrezeptoren DopR, DopR1 und den Serotoninrezeptor 5HT1A eine geringere Expression nachweisbar war. Außerdem konnte ich die Hochregulation nach Steigerung des cAMP-Spiegels von *bmm*, *dilp6*, *Thor*, *upd3* und *SOCS* zeigen, Gene, die eine Rolle im Fettstoffwechsel und in der Immunität spielen. Des Weiteren habe ich den JAK-STAT-Signalweg in adulten Oenozyten über die Überexpression des Liganden *upd3* oder des Transkriptionsfaktors STAT- manipuliert. Ich zeigte einen signifikanten Anstieg der akkumulierten Lipidtröpfchen, die mit den kombinierten Effekten von Hunger und Aktivierung des JAK-STAT-Signalwegs einhergehen. Größere Mengen angesammelter Lipide in Oenozyten hatten einen negativen Einfluss auf den Grundumsatz und die Hungerwiderstandsfähigkeit der erwachsenen Fliegen. Der beobachtete Phänotyp spiegelt die Situation beim Menschen mit Heptosteatose, der Fettleber, wider. Im letzten Teil dieser Studie habe ich die Darm-Mikrobiota-Oenozyten-Achse eingehender untersucht, die eine wichtige Rolle im Fettstoffwechsel und in der Wirtsphysiologie spielt. Allerdings ist diese Beziehung in *Drosophila* noch wenig verstanden. Um die Wirkung von Darm-

Mikrobiota auf den Lipidstoffwechsel und die Wirtsphysiologie der Oenozyten zu untersuchen, habe ich die Anzahl und Größe der angesammelten Fetttröpfchen in der adulten Oenozyten, den Wirtsstoffwechsel, die Wirtsfettmenge sowie die Lebensspannen keimfreier Fliegen und solcher mit normaler Microbiota miteinander verglichen. Bei keimfreien Fliegen habe ich gezeigt, dass kurzfristiges Hungern zu einer signifikanten Abnahme der Anzahl und Größe der angesammelten Fetttropfen in den Oenozyten führt, ohne Auswirkungen auf den Körperfettgehalt zu zeigen. Darüber hinaus zeigten keimfreie Fliegen einen signifikanten Rückgang des Stoffwechsels und der Lebenserwartung.

Zusammengenommen könnte die vorliegende Arbeit das Verständnis des komplexen regulatorischen Netzwerks verbessern, das die Aktivität der Hepatozyten-ähnlichen Zellen der Insekten steuert, und dabei helfen, molekulare Ereignisse zu verstehen, die der Pathogenese der metabolisch induzierten Entzündung der Leber zugrunde liegen.

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Declaration

I, Ali Al-lami, here declare that my doctoral thesis entitled (Using of fruit fly, *Drosophila melanogaster* for study some physiological and molecular aspects of human fatty liver disease) and the work presented in it are my own work apart from supervisor's guidance and design of the paper. This dissertation has not been submitted for the award of doctoral degree to another examining body and was prepared according to the Rules of Good Scientific Practice of the German Research Foundation.

Erklärung

Hiermit erkläre ich, Ali Al-lami, dass diese Dissertation mit dem Titel (Using of fruit fly, *Drosophila melanogaster* for study some physiological and molecular aspects of human fatty liver disease) sowie die darin beschriebenen Arbeiten, abgesehen von der Beratung durch meinen akademischen Lehrer, nach Inhalt und Form meine eigene Arbeit ist. Diese Arbeit wurde weder im Ganzen noch zum Teil an anderer Stelle im Rahmen eines Promotionsvorhabens eingereicht. Sie wurde nach den Regeln guter wissenschaftlicher Praxis der Deutschen Forschungsgemeinschaft angefertigt.

Kiel,

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