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GUT AND FAT BODY SPECIFIC MITO-CHONDRIAL DYSFUNCTION IN DRO-SOPHILA INFECTION MODEL

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ABSTRACT

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Mitochondria are gaining recognition on their pronounced role in the organism beyond the classical energy metabolism organelle, instead panning across immunity, signalling and cellular stress responses. One yet to be understood facet of mitochondrial function is how mitochondrial metabolism affects immune response and tissues associated with immune response. To address this need, *Drosophila melanogaster* (fruit fly) is an excellent model organism in investigating innate immunity with a high conservation across immune response associated tissues and genes. Recent work in our Mitochondrial Immunometabolism-research group at Tampere University with *D. melanogaster* has shown that manipulating the oxidative phosphorylation (OXPHOS) complex genes in certain tissues can alter the organism's fitness to combat infection, depending on the nature of mitochondrial interference and target tissues.

In my thesis, the role of mitochondrial OXPHOS metabolism in infection was investigated in the fruit fly model using RNA interference (RNAi) to perturb OXPHOS complex III (cIII) genes' *oxen* and *UQCR-C1* function in the fat body and gut tissues using the GAL4-UAS system. Furthermore, this perturbation was only introduced after the flies had eclosed and matured using the temporal tubulin-GAL80 temperature-sensitive system (GAL80^{TS}) as a modulator. Previous work and preliminary experiments showed that simply knocking down the OXPHOS cIII genes in the fat body and gut specifically since embryogenesis proved disastrous for the fly, regardless of other tissues being able to normally express genes of interest, highlighting the role of the fat body and gut in organism fitness.

To verify function of the fat body and gut targeted RNAi, RNA was extracted from whole flies, and analysed with RT-qPCR to quantify RNA levels of the gene of interest. This proved problematic due to the primer design and length and overlap of target genes and RNAi sequence. However, a strong phenotype was observed, and correct tissue specific localization was observed with RedStinger label when imaged. Additionally, two new transgenic fly lines were created with the temporal activation Gal80^{TS} component for further dissection of the role of the fat body and midgut separately in infection outcomes, and their function was verified by crossing these newly created fat body-specific and gut-specific driver lines with UAS-RedStinger. The localization of RedStinger expression was correct for both created lines when investigated under red fluorescent light.

Following mitochondrial perturbation in fat body and gut tissues, adult flies were infected with gram-negative bacteria *Providencia rettgeri*, a natural fly pathogen. Female flies of both genes survived better under the septic injury infection than controls, whereas knockdown males were similar to controls in terms of survival. Sexual dimorphisms in immunity have been observed before, although more often females are reported to be more susceptible to infection than males.

Further endeavours to elucidate the role of mitochondrial metabolism in response to infection and outcomes is needed, such as investigating the various metabolic readouts, mode of infection and distinguishing between the role of the fat body and gut in adulthood infection resistance.

Keywords: mitochondrial metabolism, oxidate phosphorylation, *Providencia rettgeri*, *Drosophila melanogaster*, *UQCR-C1*, *oxen,* infection

PREFACE

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LIST OF SYMBOLS AND ABBREVIATIONS

☿	Symbol denoting virgin female fly
C564-GAL4	GAL4-driver specific to the fat body and digestive tract
CFU	colony-forming unit
DSB	double-strand break, a type of DNA modification
fb-GAL4	GAL4-driver specific to the fat body
GAL4-UAS	GAL4-UAS expression system enabling RNAi
GFP	green fluorescent protein
h.p.i	hours post infection
IMS	intra-membrane space
NP1-GAL4	GAL4-driver specific to the midgut and digestive tract
OXPHOS	oxidative phosphorylation
RedStinger	red fluorescent protein dye
RFP	red fluorescent protein
RNAi	RNA interference
ROS	reactive oxygen species
tub-Gal80^{TS}	temperature-sensitive repressor of GAL4-UAS system
w;x;y;z	denoting contents and order of Drosophila chromosomes

1 INTRODUCTION

Mitochondria are ubiquitous across eukaryotic life forms, but highly heterogenous. The endosymbiont theory on the origin of mitochondria consists of two distinct versions that diverge in the suggested benefit of endosymbiosis, and the stage at which this has happened with regards to host evolution. The first theory posits that an early anaerobic eukaryote phagocytosed an obligate anaerobe, and that this symbiosis could confer benefit thorough the means of processing toxic oxygen to the anaerobe (Roger et al., 2017). However, this poses two challenges: if the host organism was already a eukaryote, then all other eukaryotic ancestors would be assumed extinct, and mitochondria produce reactive oxygen species (ROS) so this would pose a problem rather than a solution to an anaerobe host. The second theory states that mitochondria originate from an event where a prokaryotic archaebacterium phagocytosed a bacterium. The archaebacterium provides an enclosed environment for the endosymbiont, which in turn provided the prokaryote with means to energy via H_2 – a phenomenon which is seen in bacterial colonies even today (Martin & Mentel, 2010). In further support for the latter theory of their evolutionary origin, mitochondria share similarity with bacteria: in their structure, such as the shape and size of their circular DNA, size of the organelle/organism itself and transport systems. Additionally, mitochondria self-replicate in a similar fashion to bacteria, and share similar patterns in inflammatory response and recognition to bacteria (Calfee & Matthay, 2010) Thus, in addition to the acquired benefits discussed earlier, this endosymbiosis event could have had additional benefit of providing the cell with the precursor of host defense signalling molecules and points towards mitochondrial activity in innate immunity. (Mills et al., 2017; Murphy & O'Neill, 2024)

Generally, mitochondrial perturbation is devastating to the host, and many mitochondrial diseases of the OXPHOS assembly have a poor prognosis and manifest as various syndromes. It has been recently shown in Mitochondrial Immunometabolism research group as well as in other research groups that mild mitochondrial perturbations can in turn offer situational benefit, depending on target tissue and gene of interest. (Pacheu-Grau et al., 2018a) This was shown in the fruit fly model by knocking down mitochondrial OXPHOS complex genes from hemocytes, the fly blood cells (Vesala et al, 2024). The OXPHOS knockdown larvae were noted to have immune activated blood cells prior to infection, and upon parasitoid wasp infection they had improved killing rate of the wasp eggs or larvae when compared to control larvae. The adverse effect was observed when the perturbation was done in the fat body, analogous to liver and adipose tissue in mammals (Vesala et al, 2024).

In my master's thesis I strove to find out if a tissue-specific mitochondrial dysfunction has harmful, neutral, or beneficial effects on immune response in the context of bacterial infection due to mitochondrial perturbation. Building on the previous research conducted in the group where OXPHOS genes were silenced in the fat body (*fb*) and hemocytes, my master's thesis work expanded the target tissues to the entire gut and fat body together and combined this perturbation with a temporal control element so that the perturbation could be introduced at a certain developmental stage. The mitochondrial function was thus disturbed in the experimental setup by genetically inducing tissue-specific mitochondrial dysfunction in the adult fly gut and fat body tissues both sequestered and simultaneously. The flies were then infected with a known fly pathogen, *P. rettgeri* by septic injury, and monitored for survival.

As part of the master's thesis work, I also learned how to create new transgenic fly lines that have combined tissue-specific driver (GAL4) (Brand & Perrimon, 1993) with temperature-sensitive repressor (tubulin-Gal80^{TS}). My thesis work will produce useful transgenic fly lines for my site of work for future research endeavors, as well as one model of gut tissue-specific mitochondrial dysfunction under infection stress from bacteria.

2 LITERATURE REVIEW

2.1 Mitochondria in immune response

The molecular intricacies of the immune response have been under investigation for decades, and the underlying signalling pathways are often intertwined with cellular metabolism of the organism. This line of study – termed immunometabolism – explores the role of metabolic pathways in immune response (Arnoult et al., 2011; Weinberg et al., 2015) Notoriously called the "powerhouses of the cell", mitochondria have garnered research interest in immune systems as they are essential to cellular metabolism. While the classical school of thought considers mitochondria and their role in immunity as actors in sustaining the metabolic environment for different immune cell phenotypes, growing evidence suggests that mitochondria actively signal and/or feedback to drive certain immune phenotypes (Mahsa et al., 2022; Mohanty et al., 2019) Both the innate and adaptive immune pathways are implicated in mitochondrial metabolism, and most recent findings rather attribute mitochondria to be "the powerhouse of immunity" (Bahat et al., 2021; Mills et al., 2017)

Mitohormesis could be described as the mitochondrial version of the phrase "What doesn't kill you makes you stronger", where persistent, non-lethal stress results in better adaptation of the mitochondria and the host cell to future stressors (Ristow & Zarse, 2010) Mitohormesis has been indicated in better outcomes under duress in especially *Caenorhabditis elegans* and *Drosophila* models, but also in mammalian mouse models of viral infections under antibiotic dosage (Mottis et al., 2022; Obata et al., 2018; Zarse & Ristow, 2021) Most of the work done on mitohormesis has focused on longevity and fitness against infections, but not all mitohormesis events confer an advantage. Recent insights on mitohormesis especially in cancer cell progression indicate that some mitochondrial stress signals are priming certain cancer cell types to metastasize more effectively. As such, mitohormesis appears beneficial in non-pathological settings, but persisting mitochondrial stress might benefit tumor growth and metastasis (Kenny et al., 2019)

2.2 Mitochondrial OXPHOS and disease

Oxidative phosphorylation (OXPHOS) is a multi-unit complex system in the mitochondria and the major supplier of adenosine triphosphate (ATP) as the culminating step after the tricarboxylic acid (TCA) cycle. The OXPHOS system is in proximity of the TCA cycle on the mitochondrial inner membrane and comprised of five complexes with genetic origins both from the mitochondrial and the nuclear genomes, as illustrated in Figure 1 (Koopman et al., 2013). The first four of these complexes form the electron transport chain (ETC) whereas only the last complex, complex V or ATP synthase phosphorylates ADP to ATP.

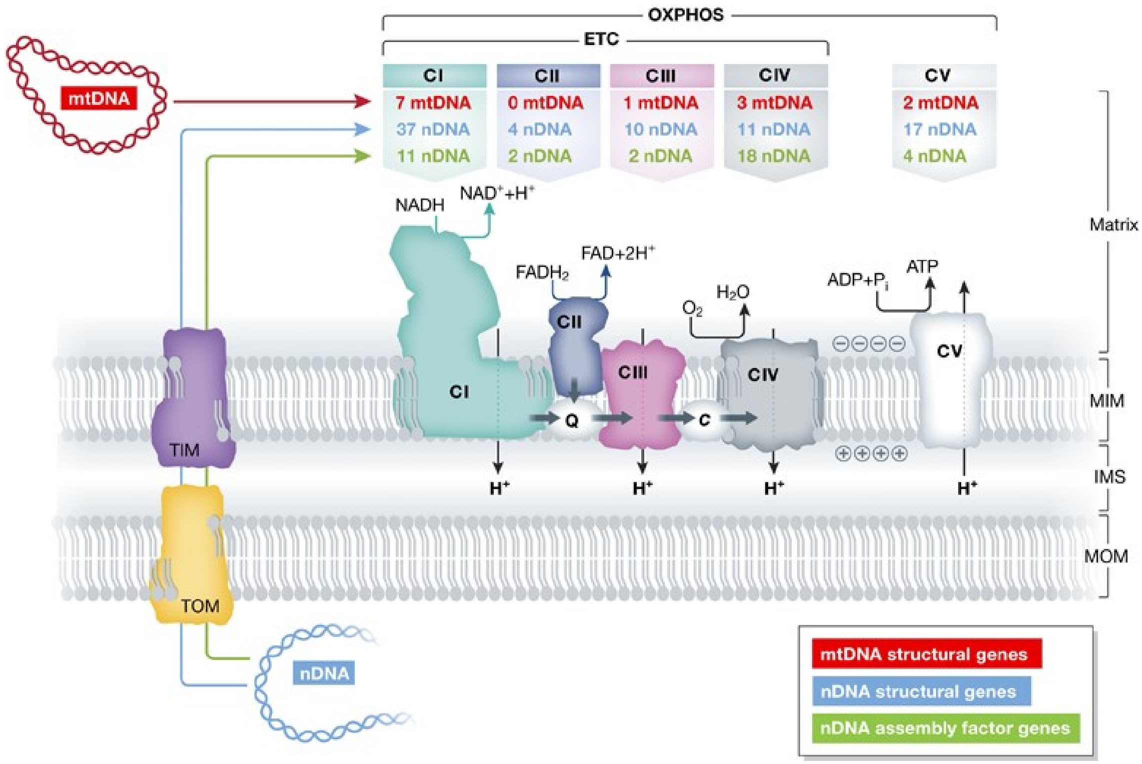

Figure 1 – Mitochondrial OXPHOS complexes and their mitochondrial or germline genetic origins. Nuclear DNA (nDNA) is divided into genes that contribute to either the structure (blue) or assemblage (green) of the OXPHOS machinery. nDNA is transported into the mitochondria via TOM and TIM complexes. Q = ubiquinone, c = cytochrome c, CI = Complex I (NADH dehydrogenase), C II = complex II (succinate dehydrogenase), C III = complex III (ubiquinol cytochrome C oxidoreductase), CIV = complex IV (cytochrome C oxidase) and CV = complex V (ATP synthase). Koopman et al, 2013.

Complex I (NADH dehydrogenase) is the largest in size and produces reactive oxygen species (ROS) as a normal byproduct of its function in electron transfer and proton translocation across the mitochondrial inner membrane. Complex II (succinate dehydrogenase) does not extend through the inner membrane and does not contribute to hydrogen proton transfer. Electrons acquired via complexes I and II are then transported to ubiquinone (Q) that is further reduced to ubiquinol. Complex III (ubiquinol cytochrome c oxidoreductase) then oxidizes the produced ubiquinol, which in turn enables electron transfer through cytochrome C (c), making complex III another ROS producer both into the matrix and the intermembrane space (Formosa & Ryan, 2018a; Tang et al., 2020).

As electrons go through cytochrome C, they are transported to complex IV (cytochrome C oxidase) and oxygen is reduced to water. This movement of hydrogen protons into the intermembrane space (IMS) creates a proton concentration in the IMS, which is resolved by complex V that re-introduces protons into the matrix. The movement of hydrogen protons through the complex powers the production of ATP from ADP (Zhao et al., 2019).

Because the respiratory system of mitochondria is governed by both the nuclear and mitochondrial genomes, it is vulnerable to mutations in both genomes. Mutations in OXPHOS genes manifest in changes in the structural integrity, further complicating the delicate assembly of its parts and proper function of energy homeostasis and communication between the two genomes (Formosa & Ryan, 2018b; Tang et al., 2020b) Thus the proper function of the OXPHOS system is crucial to any respirating organism, as both the production of ATP and maintaining normal physiological levels of ROS rely on the system. This importance is also illustrated by the fact that many mitochondrial OXPHOS diseases present as multi-symptomatic syndromes albeit single-symptom diseases subsist (Fernandez-Vizarra et al., 2020).

Variation in the mitochondrial genome – the OXPHOS system among them – is more common than that in the nuclear DNA (Allio et al., 2017). mtDNA copy number also varies between species and tissues, mtDNA is not packed in histones and is inherited maternally. Besides normal mutation in the germline repair and assembly mutations, mtDNA is subject to spontaneous somatic mutations. Due to the multicopy nature of mtDNA, these mutations can present in heteroplasmic (both mutated and wild type mtDNA in the same mitochondrion or cell) or homoplasmic (either-or). Especially in heteroplasmic mutations, the proportion of mutated mtDNA needs to surpass a threshold to that of wild type to display aberrant function (threshold effect). Higher mutation rates in mtDNA than nDNA are attributed in part due to the immediate proximity of ROS due to normal function of mitochondria. This could pose a “vicious cycle” if ROS triggers DNA damage, which in turn promotes dysregulated respiration and ultimately more ROS and damage (Salminen & Vale, 2020).

The severity of mitochondrial OXPHOS diseases depends heavily on whether the underlying defect(s) is in the germline or mitochondrial DNA and how and where these defects appear in the host. Additionally, the duration and nature of the mitochondrial perturbance is of importance, such as whether the defect is permanent in the germline or in response to a temporary stressor that can be bypassed by adapting short-term, such as exposure to mitochondrial function inhibiting antibiotics like ciprofloxacin, nutritional shortage, or a temporary alteration in oxygen availability (hypoxia). For example, it has been shown that some antibiotics can cause mitochondrial toxicity (Suárez-Rivero et al., 2021). However, this mitochondrial toxicity does not always confer a negative effect and with mild dosage appears to benefit the host by increasing tolerance to infection (Colaço et al., 2021).

Thus, mitochondrial perturbations in certain tissues or genes can confer tissue-specific responses. The pathologies of mitochondrial stress are highly heterogenous and are typically classified into either multi-symptom syndromes or single tissue affecting disorders. However, the endpoint phenotype is highly similar across mutations as most often mitochondrial stress affects the OXPHOS system or mitochondrial translation. Affected respiratory chains or translation can affect target tissue or cell size and function, such as hearing loss after antibiotic treatment. Mitochondrial perturbation in altered expression of genes can also alter the size or shape of mitochondria themselves so much so that their function is impaired, such as *OPA1* mutations affecting mitochondrial fusion. Defects in the OXPHOS system often result in a misbalance of ROS in the cells, and increased ROS concentration in cells can trigger cellular death cascades, further crippling the operating environment of the target tissue. (Pacheu-Grau et al., 2018b)

2.3 *Drosophila melanogaster* as a model organism

The fruit fly *Drosophila melanogaster* has a short life cycle (Figure 2), produce numerous offspring, and require little space to maintain. Regardless of having only four chromosomes, the fly harbors many (60%) orthologs to human genes, especially in the scope disease-associated genes (75%), making it an excellent model organism for clinical application studies (Yamaguchi & Yamamoto, 2022). The appeal to use the fruit fly as a model organism in studies is its economic size and rearing requirements, fast and extensive academic network of using the model and previously validated toolkits available.

An example of such a toolkit is the comprehensive online database FlyBase (https://flybase.org). FlyBase has genetic and molecular information readily available on *D. melanogaster* among other *Drosophila* species and is specifically targeted for researchers. While genetic manipulation of the fruit fly is possible in-house, many stock centers exist for different genotype flies, omitting the need to create transgenic flies. Examples of such widely used stock centers are the Vienna Drosophila Resource Center (VDRC, Vienna, Austria), Bloomington Drosophila Stock Center (Illinois, USA) and Kyoto Drosophila Stock Center (Kyoto, Japan).

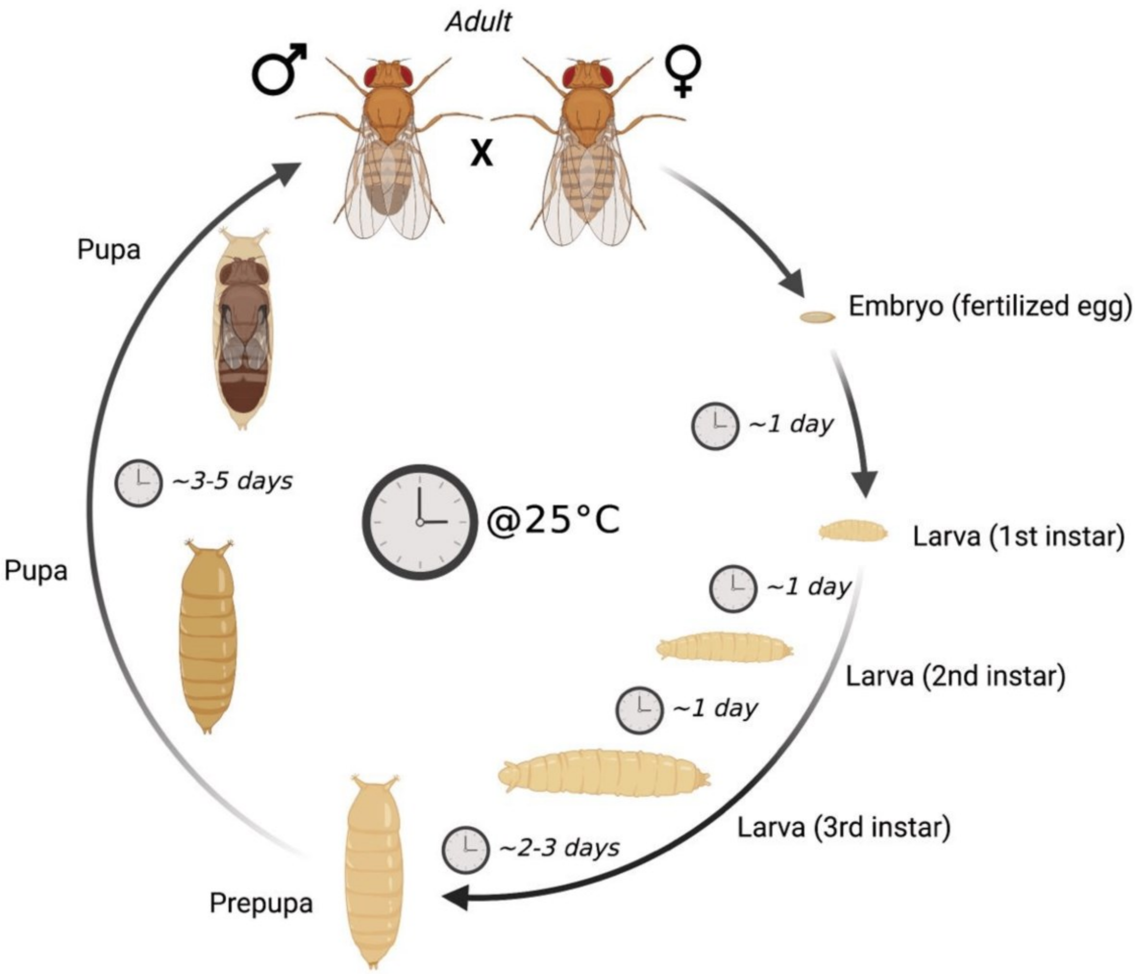

Figure 2 – Lifecycle of *Drosophila melanogaster* reared at 25 °C. The fruit fly developmental cycle from fertilized egg to eclosed adult takes approximately 8-11 days in standard laboratory conditions, depending on factors such as nutrition or temperature. Each female lays numerous eggs, and the egg's embryogenesis lasts 24 hours. The larval stage follows the embryonic stage, and the larva passes through two molts, emerging from its food at the 3rd instar stage to pupariate within 2-3 days. The metamorphosis from larva to adult fly happens in the pupal stage over a period of 3-5 days, after which an adult fly emerges from the pupa casing. The eclosed adult fly is fertile after ~24 hours of eclosion. Newly eclosed i.e., virgin flies can be identified by the yet unpassed meconium visible through their still translucent abdomen. Depending on laboratory conditions such as nutrition composition and availability, rearing temperature and population density, an adult fly will live typically 2 to maximum 3 months. Illustration by Brischigliaro et al., 2023.

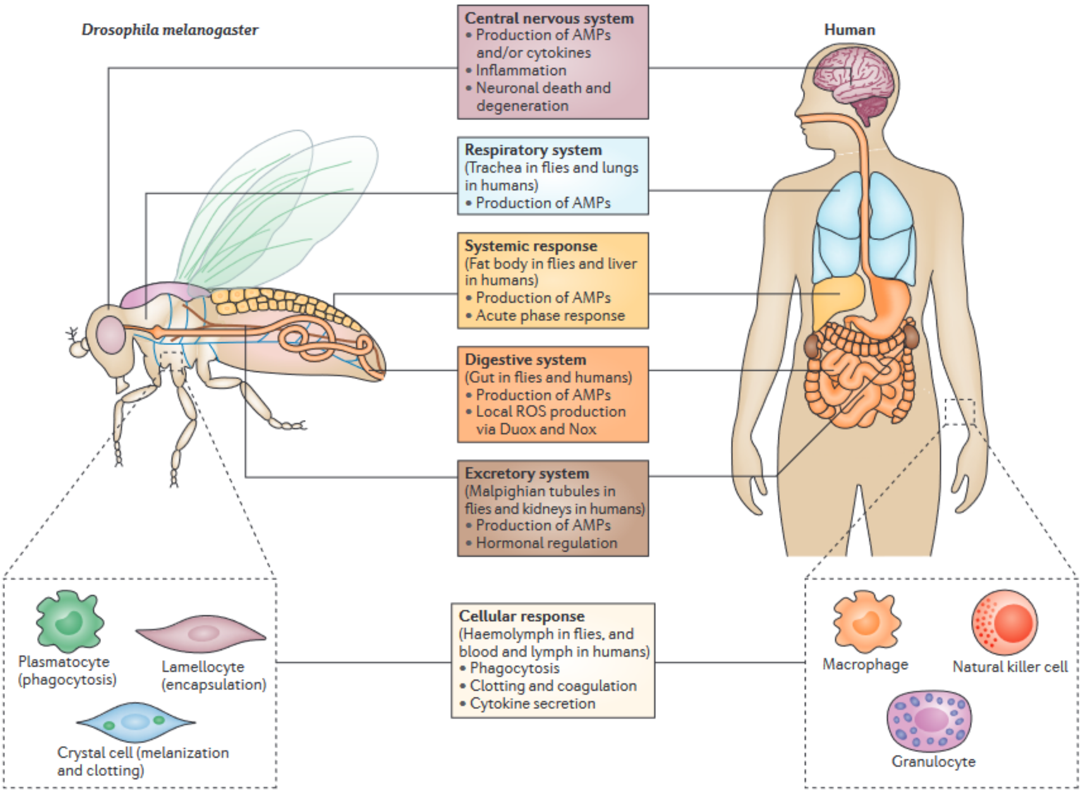

Figure 3 – The fruit fly shares multiple facets of immunity with the human immune system, although the fly anatomy and organs diverge from their human counterparts. Illustration by Buchon et al, 2014.

D. melanogaster lacks adaptive-like immunity but has been extensively used as a model organism for innate immunity, as the organism shares similar structures in immune response related tissues and cells. The immune response counterparts have been summarized in Figure 3. (Buchon et al., 2014) Due to its low cost and burden of use as a model organism in research and high homology with human genetics and disease, it comes as no surprise that several Nobel Prizes in Physiology or Medicine laureates across more than a hundred years have used *Drosophila* as a model, since Thomas Hunt Morgan first began refining the earlier Mendelian principles of inheritance with the fruit fly in 1900. (Morgan, 1910)

2.3.1 The fat body and the gut of *Drosophila*

As stated previously, many tissues and functions of *Drosophila* and mammals are similar, including the tissues and organs responsible for energy metabolism.(Buchon et al., 2014) However, the most striking difference regardless of organ-level similarities is that the fly does not have a closed circulatory system, and as such the transport of nutrients and oxygen are separate. The nutrients from food are transported to the organs via an open circulatory system, called the hemolymph. Oxygen is supplied to tissues via a separate organ, the trachea. The skeletal muscle and gut are key tissues in energy consumption like in mammals, but the fly's fat body deviates from general adipose tissue in mammals. The fly fat body has an auxiliary role as a triglyceride storage in addition to being the key organ in metabolism, nutrient sensing and storing glycogen, suggesting that the fat body performs the roles of what in mammals would be white adipose tissue and liver. (Chatterjee & Perrimon, 2021)

In addition to serving as a triglyceride storage and metabolic center, the fat body is also a key organ site for the Toll and Imd (immune deficiency) pathways that are key to danger signalling in response to infection. The end products of both pathways are antimicrobial peptides (AMPs) which affect pathogen function and prevent pathogen proliferation. While the gut epithelium is also capable of producing AMPs, the fat body is more prolific in their expression. AMPs can be expressed specifically in response to certain pathogens or danger signals, and some AMPs function in synergy. The 20 AMPs identified in *Drosophila* so far have been categorized into seven groups (Bland, 2023; Hanson & Lemaitre, 2020)

Although the mammalian and fly gut differ physiologically, they share many similarities in their function and composition as well as conservation across their genetic-regulatory axis. Proper homeostasis and barrier function of the gut is orchestrated by a complex signalling network, paired with intestinal stem cell proliferation. The gut is a key organ in immune response, especially in the case of persisting infections and maintaining beneficial microbiota. Dysregulation of the gut and microbiome interaction of the host has indeed been implicated in many diseases outside of the intestine, including but not limited to tissue damage and chronic inflammation that can progress to cancer. In the *Drosophila* model, two immune signalling systems are key to the gut: the Imd pathway in AMP production and dual oxidase (DUOX) in ROS production. The DUOX system has been implicated especially in gut microbiota maintenance as key discriminator between pathogenic and symbiont microbes. (Colombani & Andersen, 2020; Kim et al., 2014)

2.4 Ethical considerations of *Drosophila* as a model organism

Another benefit of using *D. melanogaster* as a model organism is that its use for research carries less ethical concern than vertebrate model organisms. While prior authorization from the Project Authorization Board (known as the Animal Experiment Board) is not required for working with invertebrates in Finland, this does not exclude the need for ethical considerations when working with live animals (Laboratory Animals - Animals - Licences, Notices and Applications - Individuals - Services - Aluehallintovirasto, n.d.).

The 3R principle – a term coined first in the 1959 book "The Principles of Humane Experimental Technique" by Russell and Burch – is an acronym for the terms 'replace', 'reduce' and 'refine'. The principle has since then evolved to reflect the changed scientific landscape and advent of new techniques. This has also invited different definitions of the 3R principle in modern-day setting (Tannenbaum & Bennett, 2015). In Finland, the 3R principles are based on the EU directive 2010/63/EU and supervised by the Council on the Protection of Animals Used for Scientific or Educational Purposes (TOKES). In short, the 3R principle invites to replace animal experiments with alternative methods such as *in vitro* or *in silico* methods, reduce the discrete number of animals used in experiments to a minimum by either reducing replicates or combining several projects into one animal, and refine the handling and experimental setup so that the animal enjoys as good as possible quality of life from its birth to its death. (https://fin3r.fi/en/the-3rs, accessed 18.11.2023)

Important consideration when working with invertebrates is the concept of nociception versus what is defined as pain by humans. Most animals encounter risk of tissue damage throughout their lifespan and protect against said damage with a nociceptive reflex. The reaction itself is not as insightful as the behavior of the animal *following* the noxious stimuli. For example, *Drosophila* larvae have aversion to touch in a reflex-like manner, but when exposed to higher degree of mechanical stimuli, the larva begins to "barrel roll" away from the stimuli. The plasticity of this response invites discussion into what is considered nociception and pain in invertebrates. (Elwood, 2011; Putz & Heisenberg, 2002)

Regardless of whether a treatment to an animal can be unambiguously determined as painless and simply a reflex to noxious stimuli, the animals are treated in the *Drosophila* facility in such a way that it inflicts as little discomfort, stress, or pain as possible in a laboratory setting. The fruit flies are provided with natural light-cycle, ample food, and space in their vials to avoid known stressors such as starvation and overpopulation. The adult flies are anesthetized on CO_2 pads that are cleaned with rubbing alcohol to prevent pathogen spread, and all handling is done with soft-bristled brushes to prevent mechanical burden to the fly. Proper care must be taken when handling the larvae so as not to puncture their soft epidermis.

2.5 *D. melanogaster* – the genetic toolbox

2.5.1 GAL4-UAS

The GAL4-UAS-system origins are in the GAL4 gene encoding for a transcriptional activator in the yeast *Saccharomyces cerevisiae.* It was first described as a transcriptional activator outside its yeast origins in *D. melanogaster* by (Brand & Perrimon, 1993; Fischer et al., 1988) and is a versatile tool in genetic expression manipulation, where modified target constructs can be expressed in select tissues of the fly by crossing transgenic GAL4 fly lines with another transgenic fly line containing the target UAS-gene.

The GAL4 fly line – also termed as “driver” – has in its genome a gene encoding for GAL4 and a tissue-specific promoter upstream of it. The GAL4 line expresses GAL4 in select tissues, but without a binding site does not do much for the animal. The second fly line, the UAS-line – termed “reporter” – contains a UAS sequence and the target sequence downstream of it. The UAS sequence acts as a GAL4 binding site, and without GAL4 bound to the UAS-site, the expression of the target sequence is repressed (Duffy, 2002).

The offspring of GAL4 driver and UAS reporter lines will thus have both components of the GAL4/UAS system in its genome functioning, with GAL4 expressed in target tissue binding to the UAS site, driving the expression of the target sequence only in tissues where GAL4 is present.

The GAL4-lines used in this work are expressed in specific tissues of the fruit fly to illustrate the effects of tissue-specific mitochondrial perturbation and how the animal might compensate or succumb due to these perturbations. C564-GAL4 driver is expressed in the larval and adult fly fat body and digestive system and has been shown to be also expressed in the adult midgut. (Valanne et al., 2019) NP1-GAL4 – also termed Myo31DF – is expressed in adult fly enterocytes and the midgut (Gramates et al., 2022).

The GAL4-UAS-system can be further refined with complementary elements that enable the system to be modified in multiple ways, such as increasing temperature to enhance the efficiency of the system or introducing complementary proteins to modify the system. An example of such a complementary tool is the temperature-sensitive repressor of the GAL4-UAS-system, $GAL80^{TS}$. At 18C, $GAL80^{TS}$ binds to GAL4, preventing the binding of transcription machinery and subsequently represses expression of the UAS target gene. In higher temperatures, $GAL80^{TS}$ is digested, allowing for the transcription machinery to bind to GAL4 and begin transcription from the UAS-site and begin expression of the target sequence. (Caygill & Brand, 2016; Suster et al., 2004)

2.5.2 RNA interference

RNA interference (RNAi) is a naturally occurring gene silencing mechanism brought about by double-stranded RNA (dsRNA) molecules that initiate a pathway eventually degrading mRNA or repressing its translation. It was first discovered in the nematode

Caenorhabditis elegans, although similar gene silencing due to RNA had been described in other kingdoms such as plants as well. (Fire et al., 1998; Hammond, 2005) The RNA trigger can be introduced to the organism exogenously through synthetic small interfering RNA (siRNA) or due to microRNA (miRNA) produced by the organism itself to regulate its gene expression. Commercial libraries exist for different model organisms, where RNA constructs are tailored to silence specific genes of interest, such as the VDRC in Vienna, Austria (Dietzl et al., 2007).

The trigger of RNAi pathway is either long dsRNA or mature miRNA that are introduced to the nucleus. The pathway begins with an enzyme called Dicer and Drosha which cleave the triggering RNAs into siRNAs that act as the trigger for the pathway. The generated siRNA fragments are loaded on to the RNA-induced silencing complex (RISC) where the double-stranded siRNA is unwound. The resulting single strand siRNA or miRNA binds to its complement mRNA strand with the RISC complex in tow. The mRNA bound by the RISC complex can then be cleaved by one of the complex's components, Argonaute protein family, effectively halting the expression of the gene of interest. In the case of non-complementary binding of the mRNA to the RISC complex, the silencing is achieved by translational inhibition rather than cleaving the mRNA. (Dalmay, 2013)

An important note in the RNA-interference silencing is that it is a knockdown, intentionally differentiating from knockout as RNAi does not guarantee total elimination of the target gene expression.

2.5.3 Classical fly genetics: Balancer chromosomes

The versatility of *D. melanogaster* as a model organism has been discussed in previous chapters, but it is especially true for classical genetics and when creating new fly genotypes. Balancer chromosomes are rearranged chromosomes with multiple inversions that are either sterile or lethal as homozygotes, and the balancer chromosome's inversions hinder DSB repairs when paired with a normal chromosome and thus, suppressing crossovers. This mechanism enforces the heterozygosity in fly stocks with balancer chromosomes and prevents recombinant products. Balancer chromosomes often have visible mutations – either dominant or recessive – that makes it convenient to follow which progeny has the gene of interest. (Hentges & Justice, 2004; Miller et al., 2019)

The fruit fly only has four chromosomes out of which the male sex chromosome y and autosomal chromosome 4 do not contain many genes, meaning that most of the work with balancer chromosomes is done with autosomal chromosomes 2, 3 as well as the female sex chromosome X. However, proper care must be taken in choosing the right balancer, considering the location of the balancer to the desired mutant gene of choice. Ideally, the chosen balancer should have breakpoints situated in a way that suppresses recombination to prevent the fly lines from "breaking." (Ashburner, M., Golic, K.G., Hawley, 2005a; Greenspan, 2004) Additionally, as the genetic positions of the balancer breakpoints have been elucidated, some balancers have been shown to alter the function of other genes by either directly interfering with the gene region or changing the chromatin structure. (Miller et al., 2019b)

2.6 Immune response in *D. melanogaster*

Because the immune response signal transduction pathways and regulators are highly conserved, the fruit fly is a highly valid tool in studying pathogen-host interactions. However, the study of immune response in *D. melanogaster* goes well beyond the classical mechanism of microbial recognition and defense into the study of regulation and coordination of said response. (Buchon et al., 2014; Neyen et al., 2014; Younes et al., 2020) Particularly adult flies have been used in these studies on immune response, although larvae have been proposed as a model in later years (Kenmoku et al., 2017).

The fruit fly's immune system has been believed to rely entirely on innate immune responses, but a growing number of experimental works suggest the possibility of adaptive-like immunity although this type of immunity is better described as innate immune response with memory properties. The fruit fly does not produce antibodies or have specific immune memory cells such as mammals do, hence the preference of term for innate immunity with memory rather than adaptive immunity. (Arch et al., 2022). Many studies into the innate immune memory in *Drosophila* revolve around priming, a concept in which the immune system is challenged by non-lethal pathogen load confers protection against secondary immune challenge, which does not carry over generations. Recent studies have shown that this priming protection does not appear to be universal and suggests that the outcome is reliant on the pathways induced and not the infectious agent itself (Acuña Hidalgo & Armitage, 2022; Christofi & Apidianakis, 2013). Thus, it is important to note that the innate immune response is still the hallmark mode of response in many insects, including *Drosophila*. (Mondotte et al., 2020; Pham et al., 2007; Tassetto et al., 2017)

The innate immune response is achieved by the humoral response and the cell-mediated response, like in vertebrates. The key organs in the fruit fly's humoral immune system are the fat body, gut and the circulatory system, hemolymph. The humoral response functions mainly by the secretion of AMPs from the fat body or the gut into the hemolymph, which is the fruit fly's open circulation system that delivers the AMPs to their destination. The humoral response also entails increased reactive oxygen species (ROS) secretion and clotting factors through the prophenoloxidase (PO) cascade. While majority of the work on this thesis is focused on inducing the humoral immune response mechanisms of the fly, it is worthwhile to mention the cell-mediated aspect of the *Drosophila* defense mechanism. The *Drosophila* hemolymph contains distinct types of blood cell types that form the cell-mediated layer of the immune response. These blood cells can be roughly categorized based on their morphology and marker genes, although this classification is likely too narrow to catalogue the full spectrum of blood cell types in *Drosophila.* (Tattikota et al., 2020) The three distinct categories of mature blood cells in the fly are lamellocytes, crystal cells and plasmatocytes. When compared to vertebrate blood cells, lamellocytes and crystal cells are analogous in their function with clotting and granuloma formation, although the *Drosophila* achieve these by melanization and encapsulation. The plasmatocytes perform similar functions with vertebrate macrophages, as they can phagocytose bacteria. (Parsons & Foley, 2016; Vlisidou & Wood, 2015; Wood & Martin, 2017)

The fruit fly's humoral immune response can be induced in multiple ways, including pattern recognition such as recognition of bacterial and fungal cell wall component peptidoglycan via peptidoglycan receptor proteins (PGRPs) that are specific to the type of peptidoglycan encountered, and bacterial metabolites such as uracil via uracil-specific receptor proteases that yet remain unidentified (Bier & Nizet, 2015) or microbial proteases. The mechanism of RNAi used widely in the GAL4-UAS system even in this work acts as a mechanism of defense against viral infections. (Dionne & Schneider, 2008)

With the recognition of microbial molecular patterns, the immune effector pathways Toll and Imd are induced towards AMP production, specific to the microbe encountered. The Toll pathway is induced in response to recognition of gram-positive bacteria cell wall component Lys-peptidoglycan and beta-glucan in the case of fungi by PGRPs, which then signal transcription factors further down in the cascade which upregulate genes producing AMPs. Similarly, Imd pathway is activated by the recognition of DAP-type peptidoglycan cell wall component in gram negative bacteria as well as some members of the gram-positive bacilli in the *Clostridium* and *Bacillus* families, albeit in the case of gram-negative bacteria the wall is much thinner and closer to a component of the outer cellular membrane. Although these pathways are presented here as two separate pathways, there are several exceptions to these inducer "rules," and both pathways work in parallel as well as have upstream effectors in common in danger sensing and signalling, as pictured in Figure 4 below. (Buchon et al., 2014a; Cytryńska et al., 2016; Dionne & Schneider, 2008; Gordon et al., 2005; Lemaitre & Hoffmann, 2007; Myllymäki et al., 2014; Valanne et al., 2011)

Many danger sensing signals in *Drosophila* rely on serine protein cascades, with multiple serine proteases involved in the immune response activation cascade on both regulatory and propagator ends. Some of the same serine proteases involved in Toll response described earlier in this chapter are also part of the phenoloxidase pathway, essential for the melanization response in response to wound site healing and battling parasite infections. (Dudzic et al., 2019) Melanotic nodules, also called melanotic tumors or pseudo tumors, are dark, encapsulated masses that form in the fly tissues in response to aberrant structures which activate the specific fly blood cells known as lamellocytes due to e.g., parasite invaders or self-tissue or impaired regulation of lamellocyte differentiation. As the formation of melanotic nodules follows lamellocyte activation, the presence of melanotic nodules in the fruit fly tissues can be considered an indicator of immune system activation. (Avet-Rochex et al., 2010; Minakhina & Steward, 2006) Even sterile injury induces the immune response pathway, and pre-existing injury has even been shown to affect survival outcome depending on the site of injury. (Chambers et al., 2014)

Serine protease pathways rely on the detection of microbial metabolites and are not necessarily specific the mode by which abnormal proteases are introduced to the cell, as these proteolytic pathways are also used in maintenance of host cell fitness. One such protease sensor is the Persephone serine protease, which contains a “bait” region for exogenous proteases but also detects divergent host cells, and cleavage of Persephone enables the downstream signalling to induce the Toll-pathway. (Buchon et al., 2014; Gong et al., 2019; Issa et al., 2018; Lemaitre & Hoffmann, 2007)

Another layer introducing variance in the *Drosophila* immune response is sex, as the fly tissues, behavior and responses to immunity challenges are different across the sexes. However, these differences in their immunity do not necessarily predict outcome of the response. Although this dichotomy is well appreciated in *Drosophila*, only in recent years has more interest been cast on the mechanisms and scope of these dimorphisms in immune responses, apart from *Spiroplasma* and *Wolbachia* infections where host sex bias has been well described.

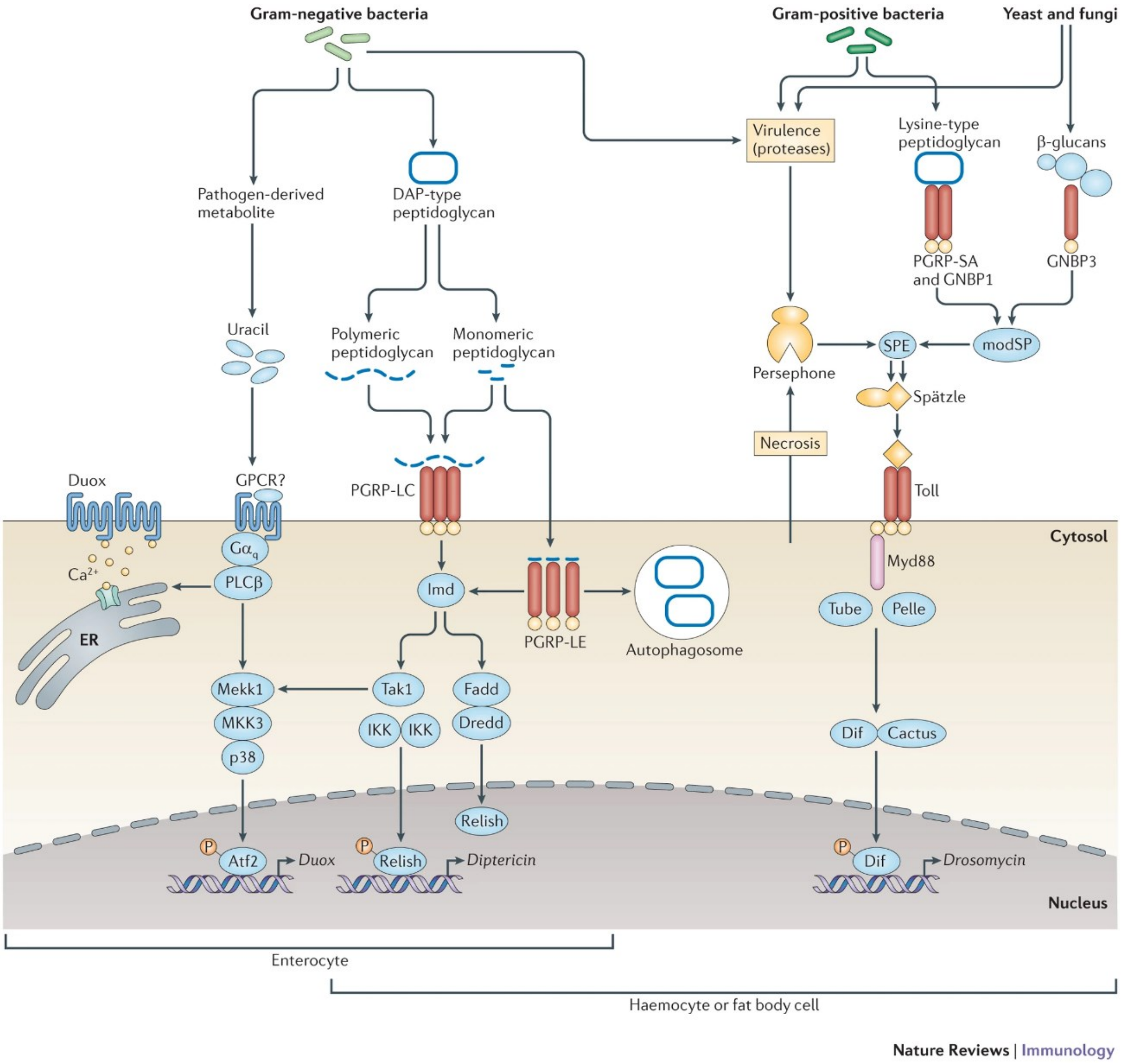

Figure 4 – Overview of the *Drosophila* immune response pathways in response to microbes depending on their recognition mechanisms. Majority of bacterial infection signalling pathways involve peptidoglycan receptors proteins (PGRPs) which detect bacterial or fungal cell wall components, and activate either the Toll or Imd pathways, which then induce expression antimicrobial peptides such as Diptericin and Drosomycin or in the case of gut epithelial cells can induce the release of ROS via dual oxidase (Duox) induction. As displayed in the illustration, gram-negative bacteria can trigger both Toll and Imd pathways, and many pathways shown here are truly interlinked, providing the organism the benefit of multi-level immune signalling and response pathways. Some pathways described above do not rely on microbial cell wall components, but rather their metabolites such as proteases or uracil. Uracil sensors work transcriptionally both in the gut nucleus and cytosol. Uracil detection in the cytosol encourages the endoplasmic reticulum to release calcium ions, which induces Duox to release ROS outside the cytosol. In the nuclei, the same pathway which encourages Ca2+ release from the ER also induces transcription of Duox, further advancing the ROS production in the gut epithelial cells. The recognition and response to uracil is suggested to be one of the main mechanisms by which the immune system senses and maintains a healthy gut microbiota. (Kim et al., 2014) In addition to microbial proteases, the Persephone protease plays a danger-sensing signalling role in the immune cascade, activating the Toll pathway to produce AMPs even in sterile conditions, such as abnormal proteolysis following necrosis. Illustration by Buchon et al, 2014.

A clear physiological dimorphism is that females have a larger fat body – the key organ in immunity - in relation to their size than males, and functional dimorphisms are evident both at baseline transcriptional response as well as in response to immune challenge and over lifespan. In the case of microbial infection survival bias, the outcome is highly heterogenous between pathogen species and no single immune response pathway can be identified as having a ubiquitous sex-based bias in survival, and such complexity invites caution into making any statements about general outcome expectations for infection survival based on sex. (Belmonte et al., 2020)

P. rettgeri infections tend to kill females more efficiently in wild-type setting, but another gram-negative bacilli *Serratia marcescens* does the opposite. In the case of *P. rettgeri*, the Toll pathway has been identified as the "culprit" in the survival bias between sexes although the established pathway in gram-negative bacteria such as *Providencia* would have been the Imd pathway. Toll-signalling has also been shown to be dimorphic in both genome-wide expression levels as well as under induction after infection. (Duneau et al., 2017) This further enforces the notion that the immune response is multi-layered, and infection clearance is achieved not only by secreting AMPs through one signalling pathway, but also other immune response events such as induction of ROS, the physical epithelial barriers, cell-mediated defense mechanisms of clotting, phagocytosis and melanization of divergent components.

2.7 Bacterial infection models in *D. melanogaster*

Bacteria, among other microbes, continue to pose a threat to the global population and the study of their pathogenesis is necessary in the face of antibiotic resistance and global warming changing the virulence of previously non-pathogenic microbes. As described in the chapters above, *Drosophila* continues to be a great tool in investigation of innate immune response to acute or chronic infections, such as *Pseudomonas* infections in cystic fibrosis (Galac & Lazzaro, 2011). Several models of infection techniques exist for bacterial infections, and these can model various infection types. Examples are a systematic infection via septic injury to the fly thorax, or a chronic infection in the gastrointestinal tract by feeding the fly with a predetermined load of bacteria in its food. In addition to the choice of infection model, the infectious injury site can impact mortality depending on pathogen of choice. (Siva-Jothy et al., 2018; Chambers et al., 2014; Khalil et al., 2015)

While many bacterial species of clinical interest have similar pathologies in *Drosophila* such as *Staphylococcus aureus* and many members of the *Enterobacterales* order, some virulence factors and immunopathologic mechanisms require further work in mammalian models to elucidate their clinical significance. (Panayidou et al., 2014) The bacterium used in this study – *P. rettgeri* – is a known opportunistic pathogen in a variety of infections in many different organisms (including humans) but has been recovered from wild-type *Drosophila* hemolymph that should be sterile. Thus, the presence of *P. rettgeri* in a sterile fluid in the fly can be considered an infection and does not suggest colonization such as bacterial species found in the fly gut. What makes *P. rettgeri* specifically interesting to study is that while it appears in some wild-type *Drosophila* hemolymph and is clearly an infectious agent, it only achieves about a 40-50% kill rate at high bacterial burden, whereas other flies clear the infection at low bacterial load. Other *Providencia* species are also found in the fly hemolymph, but these other species rarely infect humans. However, these *Providencia* species such as *P. alcalifaciens* and *P. sneebia* achieve a kill rate of 90% in the fly. (Galac & Lazzaro, 2011)

Bacterial infections assays can measure a multitude of aspects in pathogenesis, from the ultimatum of survival against time to quantifying bacterial loads, ROS, mRNA, and associated protein content at set points in time. Imaging-based assays such as melanization, ROS imaging and phagocytosis assays can also provide additional insights to the survival assays. (Troha & Buchon, 2019)

3 MATERIALS AND METHODS

3.1 Fly husbandry and stocks

There are several readily available commercial stock centers for *Drosophila* genetic lines, and the Tampere Drosophila facility keeps selected key *Drosophila* fly lines accessible by maintaining them. The fly stocks used in my experiments were *C564-GAL4;tubulin-Gal80*^{TS} made in the Drosophila core facility by combining *C564-GAL4* (a gift from professor Bruno Lemaitre) and *tubulin-Gal80*^{TS} (a gift from professor Dan Hultmark), *NP1-GAL4* (a gift from prof. Lemaitre), *FB-GAL4* (a gift from prof. Hultmark), RNAi (UAS) constructs against OXPHOS cIII genes *Ubiquinol-cytochrome c reductase core protein 1* (*UQCR-C1*; Vienna Drosophila resource center, VDRC #40466) and *oxen* (*ox*; VDRC #35828), and a control line *w*^{1118} also called *w*^{iso} (Bloomington Drosophila stock center, #5906). RedStinger fluorescent reporter stock was a gift from Professor Robert Schulz (stock id #118C201).

The fly stocks used in the experiments were reared on fly food vials at 25^{o}C (except for the temperature-sensitive tubulin-GAL80^{TS} fly stock at 18 ^{o}C). The flies were reared on a 12:12 light/dark cycle. Adult flies were transferred to fresh food vials minimum two times per week. Fly food medium consisted of mashed potato powder, agar powder, corn syrup, dry baker's yeast, nipagin and ascorbic acid. All females in the experimental crosses were unmated females, i.e., females collected into separate vials <8h after eclosion or a visible meconium is seen in the abdomen to ensure they were virgin, and all offspring was of the desired genotype.

C564GAL4; tub-Gal80^{TS} unmated females and males from the UAS-RNAi construct fly lines were allowed to mate in 25 ^{o}C for 24h and transferred to fresh food vials the following day. This process was repeated twice, and the parental flies were then euthanized by discarding the vial contents into ethanol. Each cross was made in technical replicates of three, with each vial containing minimum 10 females and 5 males to ensure sufficient egg yield. After each mating, the food vials with eggs were moved into 18 ^{o}C. The crosses were done on five biological and three technical replicates.

To temporally control the activation of the *C564*-GAL4-UAS-system and allow the experimental flies to develop normally into maturity, the experimental crosses' eggs and larvae were kept at 18 ^{o}C to suppress the driver activity by keeping tub-Gal80^{TS} intact before mature flies had eclosed. After eclosion, adult flies were introduced to a temperature of 29 ^{o}C which will digest tub-Gal80^{TS} and unblock the activation of the RNAi system in the target tissue. All further experiments performed on these flies were done after 48 hours in permissive temperature.

3.2 Creation of transgenic fly lines

As an alternative to commercial fly strains and as an exercise in classical fly genetics, I combined two other GAL4-drivers, fat body-specific fb-GAL4 and midgut and digestive tract specific NP1-GAL4 with the temperature-sensitive tubulin-Gal80^{TS}-construct. This was achieved using balancer chromosome flies. Balancer chromosomes are fly genotypes which have a visible phenotypic marker, and thus choosing right genotype offspring can be done by choosing a specific phenotype by observing specific characteristics of the fly offspring, such as body color, size, eye shape and wing shape. This system makes it possible to “genotype by the naked eye,” although there are limitations to the use of this system and created crosses need to be tested for correct expression.(Ashburner, M., Golic, K.G., Hawley, 2005b; Bloomington Drosophila Stock Center: Indiana University Bloomington)

The tissue-specific GAL4-drivers are both on chromosome 2, making it easier to plan the crossing scheme and use the same balancer fly lines for both combinations. The GAL4-regulator tubulin-GAL80^{TS} is located on the third chromosome. (Gramates et al., 2022) The crossing schemes for creating GAL4-driver lines combined with Gal80^{TS}-regulator are below in Figures 5 and 6.

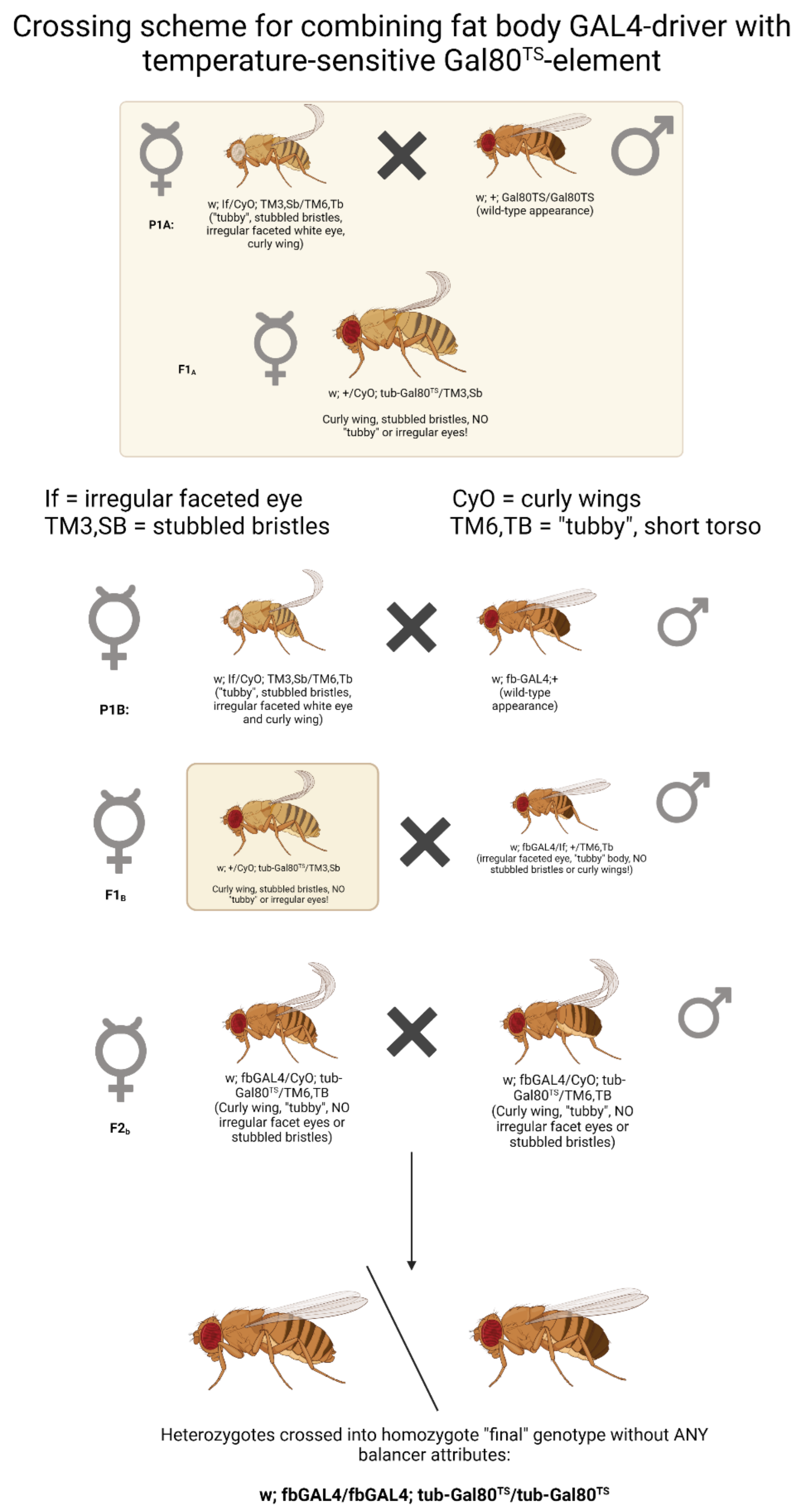

Figure 5 – Crossing scheme for combining fat body-specific GAL4-driver with a temperature-sensitive tubulin-Gal80^{TS} element to allow for tissue-specific expression controlled by temperature. F1$_B$ female is the same offspring from F1$_A$. All females in the crossing schematic are unmated i.e., virgin females. Created with Biorender.com.

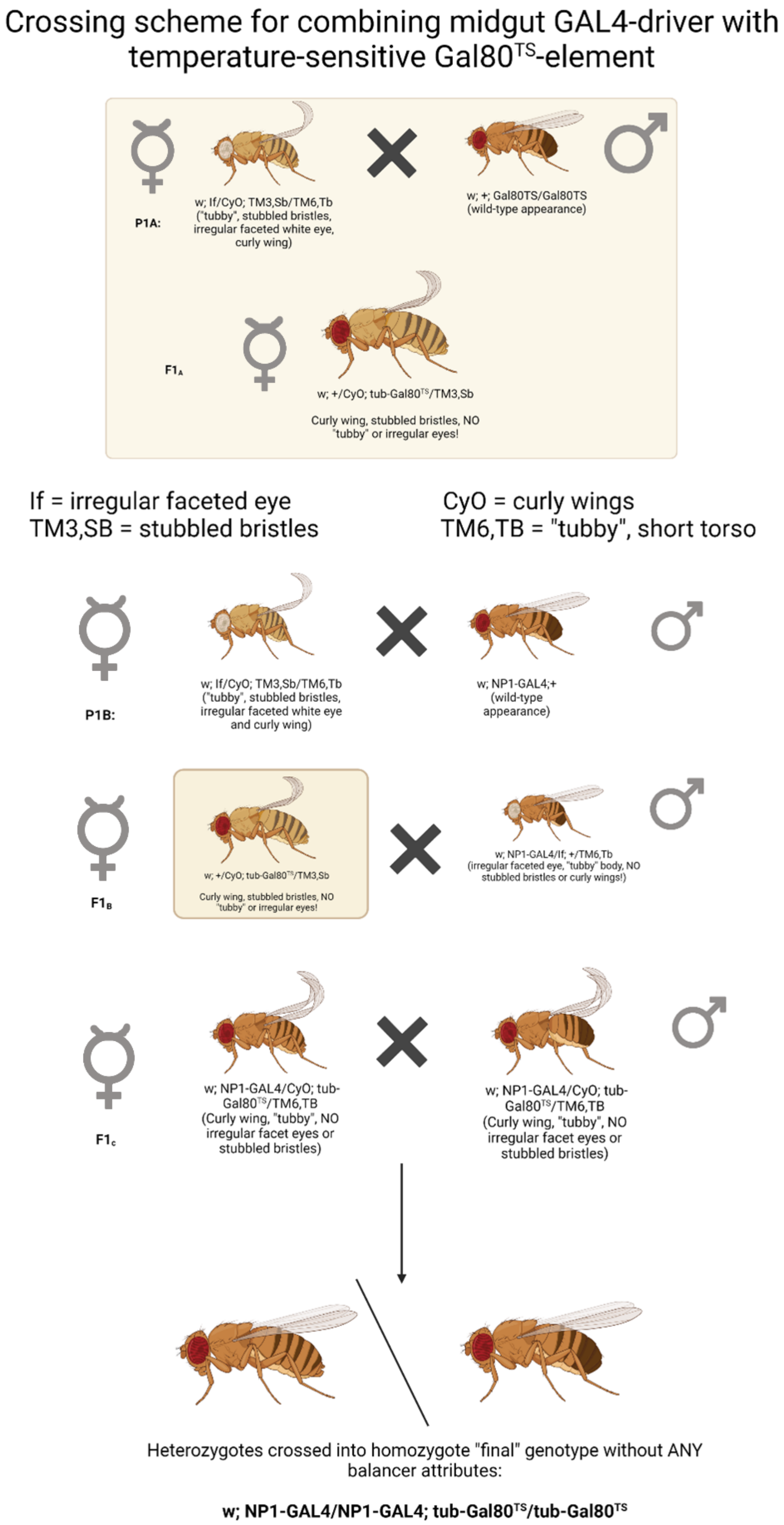

Figure 6 – Crossing scheme to combine midgut-specific NP1-GAL4-driver with a temperature-sensitive tubulin-Gal80^{TS} element to allow for tissue-specific expression controlled by temperature. F1$_{B}$ female is the same offspring from F1$_{A}$. All females in the crossing schematic are unmated i.e., virgin females. Created with Biorender.com.

3.3 Verification of transgenic fly lines

To verify correct localization and function of the newly combined drivers, lethality assays were performed to confirm the function of the driver. In addition, the created lines were crossed with RedStinger red fluorescent protein-expressing fly lines to be able to image the expression of the driver in desired tissues. *NP1-GAL4; tubulin-Gal80^{TS}* and *fb-GAL4;tub-Gal80^{TS}* driver lines were both crossed with UAS-RNAi lines *oxen* and *UQCR-C1* to disturb normal mitochondrial function in the target tissues, and vials containing the eggs from these crosses were put into 29 °C immediately. The vials in which the RNAi silencing activity was introduced from conception were followed for any larvae or fly viability for 10 days.

To image the driver function in select tissues, the same driver lines were crossed with a red fluorescent protein reporter line RedStinger, which returns a red, nuclear-localized fluorescence. Third instar larvae were collected, sexed, and rinsed with filtered water to remove any fly food residue, dried and placed on a chilled 76 x26 mm microscope slide (Thermo Scientific) in a drop of ice-cold 65% glycerol with a 24 x 50 mm cover slip (Menzel-Gläser). The larvae were imagined both intact while immobilized by 15 minutes in -18 °C and dissected, after being euthanized in freezing temperature on the slide. The imaging was done using microscope Olympus SZX16 with SDF PLAPO 1xPF objective, combined with Olympus Ush-1030L Hg-lamp with a filter set for RFP. Images were captured using CellSens Standard v1.7 software.

3.4 RNA sample extraction and RT-qPCR

RNA extraction was done in 3 biological and 2 technical replicates, each sample consisting of 5 whole flies grouped by sex. Two timepoints were chosen after RNA*i* induction*;* 48 and 96 hours after introduction to permissive temperature. RNA was extracted with Invitrogen™ TRIzol™ (Thermo Fisher Scientific, USA) from the whole animal after euthanizing the flies by snap-freezing and proceeding with the extraction protocol as per manufacturer's specifications. Obtained RNA concentrations were measured with NanoDrop™ 2000 (Thermo Fisher Scientific, USA) and diluted to a final concentration of 10 ng/µl.

RT-qPCR assay was run with iTaq Universal SYBR Green One-Step Kit (Bio-Rad) in a 10 µl reaction following manufacturer's RT-qPCR-protocol. Expression levels were analyzed by expression level fold-change against a housekeeping gene *histone H3.3B* (*His3.3b*).

3.5 Systemic bacterial infection assay

To prepare the flies for infection, newly eclosed flies at 18°C were introduced separated by sex and batches of 10 flies into fresh vials in 29°C and subjected to permissive temperature for 48 hours. The bacterium used for the infections was a gram-negative bacillus, *Providencia rettgeri*. The strain used in the septic injury experiments (DSM4542/ATCC 29944) was originally recovered from a fruit fly and contains DAP-type peptidoglycan.

A fresh bacterial solution was prepared the day before by inoculating a frozen bacterial stock into sterile LB broth and leaving the suspension overnight in an incubator at 35 °C with an oscillating speed of 200 rpm. The following morning, 200 µl of the original suspension was transferred to a fresh sterile LB broth tube and monitored for optical density at 600 nm (OD600) to ascertain the fresh suspension has reached its exponential growth phase at OD600 = 0.4 – 0.6. The suspension was subsequently diluted with sterile 1 x PBOS to OD600 = 0.1 and was placed in smaller Eppendorf vials to decelerate the growth of the bacteria while infections were performed.

The infection model for bacterial infection assays was septic injury technique, where the microbe is entered into the fly by pricking with a bacteria-laden tungsten needle. The tungsten needle (Austerlitz Ø0.2mm) was prepared and dipped into the bacterial solution before each needle prick. The flies were anesthetized on a CO_2 pad vial by vial, and the needle was then inserted into the fly thorax from its side, the mesopleuron pictured in Figure 7. The flies were then placed in fresh food vials on the side to prevent the flies to succumb to the food while anesthetized and monitored for survival for 96 hours at 25 °C, recording the quantity of succumbed flies per vial. The bacterial assay was done in replicated of five vials per sex, each containing 10 females or males.

Figure 7 - The mesopleuron region of a D. melanogaster male. Adapted from https://www.ento.csiro.au/biology/drosophila/melanogaster.html

To control for needle-pricking technique and ascertain the injury itself is not lethal, a separate batch of flies were pricked with 1 x sterile PBS. Ensuring that the bacterial solution of *P. rettgeri* is sufficiently lethal, a batch of Relish^{E20} knockdown flies were also injured with the same solution as the experimental flies. These control lines were monitored for survival identical to the experimental setup.

A majority of Relish^{E20} flies should succumb to gram-negative bacterial infection if the bacterial solution and the needle prick technique are correct. Injury with 1x sterile PBS should kill little to no control flies. (Khalil et al., 2015)

0-hour bacterial load assay was done immediately after infecting the flies. A population of 3 female and male flies were chosen after pricked with the bacterial solution, and the site of injury was allowed to heal for 2 minutes before surface sterilizing each fly by submerging them in 70% EtOH. After 30 seconds, the EtOH was removed by pipetting and each specimen was allowed to air-dry completely. Once the specimen was dry, the entire fly was homogenized with 100 µl of 1x sterile PBS and plated on LB agar medium. The LB agar with the fly homogenate was incubated overnight at 37 C and recorded for colony forming units (CFU) per fly to ensure initial bacterial loads were sufficient.

3.6 Statistical analysis

Bacterial survival experiments were plotted and analyzed using the GraphPad Prism v 8.0 (GraphPad Software) software. Excel v 16.0 (Microsoft) was used for plotting qPCR data and statistical analysis, including plotting standard deviation and t-test.

4 RESULTS

4.1 Viability of knockdown flies without temporal control

To check for lethality and developmental delay resulting from the induced mitochondrial perturbation in selected tissues, the chosen driver lines C564-GAL4, NP1-GAL4 and fb-GAL4 were crossed with RNAi UAS constructs without any regulator to their activity. Because these drivers do not have the tub-Gal80^{TS} regulator incorporated, the RNAi-silencing is functional from the earliest stages of development. The crosses were put into two different temperatures (25 °C and 29 °C) and monitored for development time, stage, and presence of melanotic nodules (Figure 8) as stressor signs, as described in Table 1 and 2, respectively. Additionally, any eclosed flies were monitored for eclosion. This was to ensure that the flies could be brought into eclosion and adulthood to be able to perform the bacterial assay, and that the flies would not succumb to the mutation alone without septic injury.

Figure 8 – Melanotic nodules (in blue rings) on anesthetized females that have *ox*-silencing in their gut and fat body (C564GAL4 driver) at 25 °C. As described below in Table 1, this genotype induced from the embryonic stage onward had limited potential to eclose from pupa and presented melanotic nodules, indicating an unfavorable outcome from silencing the gene of concern in the gut and fat body. Melanotic nodules are often a result of overstimulated hemocytes, such as in response to an induced immune system response in the fly.

With the silencing of the OXPHOS genes in the gut and fat body, the flies were not able to eclose fully, and this effect was more pronounced with a higher temperature, as the RNAi is stronger with higher temperatures. While some of the flies were able to eclose and reach adulthood at a slightly more permissive temperature at 25 °C, all the surviving flies had melanotic nodules, indicating that the flies were under immune duress. When introduced to a higher temperature and degree of RNAi, the effect was further increased as most silenced genotypes could not eclose a single living adult fly. In the case of silencing *oxen* in the fat body only, none of the flies developed further from the pupal stage, regardless of the temperature. *UQCR-C1* had a similar viability effect only in the gut tissues and at a higher temperature.

Table 1 – Viability of selected genotypes when reared at 25 °C. Controls are coded in blue color, adverse or non-wild type outcome in red background and wild-type or non-adverse event in green background. Each genotype was tested in three biological and three technical replicates. The flies were considered as eclosed fully if most larvae were able to pupate and eclose into adult flies. In some experimental genotypes, a limited number of adult flies were able to eclose, but majority of their siblings were not able to eclose. These genotypes are denoted by a positive outcome in eclosion by 14 days, but an adverse outcome recorded in the eclosed fully-bracket.

Viability of genotype at 25 °C					
Target tissue (GAL4)	**RNAi target gene**	**Eclosion by 14d**	**Eclosed fully**	**Developmental stage prior to death**	**Melanotic nodules**
gut	*UQCR-C1*	Yes	**No**	Pupal	**Yes**
fat body + gut	*UQCR-C1*	Yes	**No**	Pupal	**Yes**
fat body	*UQCR-C1*	Yes	**No**	Pupal	**Yes**
gut	*oxen*	Yes	**No**	Pupal	**Yes**
fat body + gut	*oxen*	Yes	**No**	Pupal	**Yes**
fat body	***oxen***	**No**	**No**	**Pupal**	**Yes**
gut	w^{ISO} (control)	Yes	Yes	N/A	No
fat body + gut	w^{ISO}(control)	Yes	Yes	N/A	No
fat body	w^{ISO} (control)	Yes	Yes	N/A	No
N/A	*UQCR-C1*	Yes	Yes	N/A	No
N/A	*oxen*	Yes	Yes	N/A	No

Table 2 – Viability of selected genotypes when reared at 29 °C. Controls are coded in blue color, adverse or non-wild type outcomes in red and wild-type or non-adverse outcomes in green. Similar to Table 1, if only a minority of knockdown genotype flies were able to eclose, it is denoted as favorable outcome in eclosion by 14 days, but a negative outcome in arrested developmental stage. Compared to the viability of the flies reared at a lower temperature, these flies were under much duress and as such could not be used for the bacterial infection work, both because underlying strong immune stress as well as a survival bias against the RNA interference. Under 29 oC, silencing *UQCR-C1* from the gut at all appears more drastic whereas silencing the *oxen* gene in the fat body is equally harmful but silencing the same gene in the gut allows some flies to eclose and survive.

Viability of genotype at 29 °C					
Target tissue (GAL4)	**RNAi target gene**	**Eclosion by 14d**	**Eclosed fully**	**Developmental stage prior to death**	**Melanotic nodules**
gut	*UQCR-C1*	No	No	Larvae	Yes
fat body + gut	*UQCR-C1*	No	No	Larvae	Yes
fat body	*UQCR-C1*	Yes	No	Pupal	Yes
gut	*oxen*	Yes	No	Pupal	Yes
fat body + gut	*oxen*	No	No	Pupal	Yes
fat body	*oxen*	No	No	Pupal	Yes
gut	w^{ISO} (control)	Yes	Yes	N/A	No
fat body + gut	w^{ISO}(control)	Yes	Yes	N/A	No
fat body	w^{ISO} (control)	Yes	Yes	N/A	No
N/A	*UQCR-C1*	Yes	Yes	N/A	No
N/A	*oxen*	Yes	Yes	N/A	No

The results from these initial viability schemes illustrated the need to have an element of temporal control in the silencing, as silencing either of the genes before eclosion had a dramatic effect on the survival of the knockdown flies and would introduce a significant baseline difference to the viability of the knockdowns even without introducing any infectious dose.

4.2 RT-qPCR assay

To confirm proper function of the silencing in the tissues of interest, both control and knockdown flies were collected and homogenized for RNA extraction, each replicate containing five whole flies of the same sex and in three biological and two technical replicates. The sample was chosen to be whole flies as this has been shown to confer expression level differences on whole fly-level. The experiment was repeated in two timepoints after introducing the flies to permissive temperature, 48 and 96 hours after the adult flies had been moved to the permissive temperature of 29 °C.

The silencing of *UQCR-C1* in the gut and fat body could not be unanimously shown in the qPCR results (Figure 9). The expression levels of *UQCR-C1* were higher in males than in females, although there was no significant difference between genotypes.

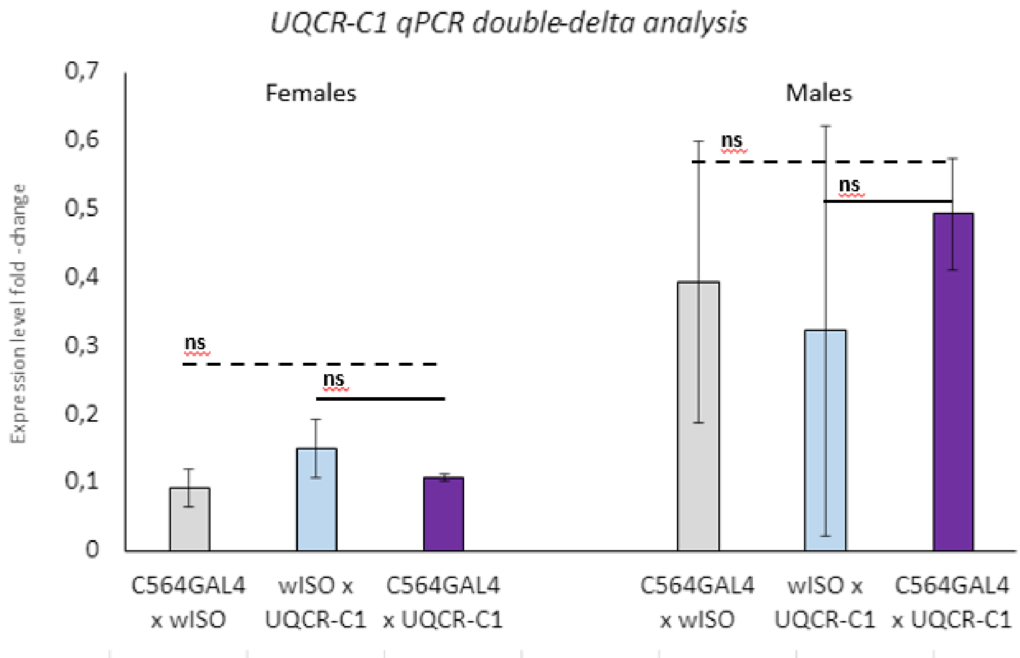

Figure 9 – qPCR expression levels of *UQCR-C1* fold-change against a steady expression gene (*His3.3b*). Results are grouped by sex (F/M) and genotype from *C564GAL4;tub-Gal80*^{TS} x *UQCR-C1* cross with both the driver and the reporter controls ($w^{1118} = w^{ISO}$). No fat body or gut tissue specific silencing of *UQCR-C1* can be seen in the qPCR analysis from whole fly samples. Standard deviation numerical values portray an extremely high deviation in the qPCR expression levels, especially in males. p-values of knockdown crosses (purple column) indicated above relative to each control (gray and blue columns.) Each sample consisted of five whole homogenized flies; each sample done in three replicates.

Similarly to *UQCR-C1* expression, no significant fold change in expression was observed in the *oxen* RNAi flies with respect to either genetic background control, but the dimorphism between sexes is reversed with females suggested to have a higher overall expression level to males (Figure 10). However, the deviation in the female expression levels is so extreme with both genotypes that this dimorphism is likely less profound than observed in the results. The possible reasons as to why the qPCR assay did not portray statistically significant differences such as the survival assay are discussed further in the discussion, but some caveats were identified in the design of the *oxen* primers overlapping with the hairpin structure of the *oxen* gene.

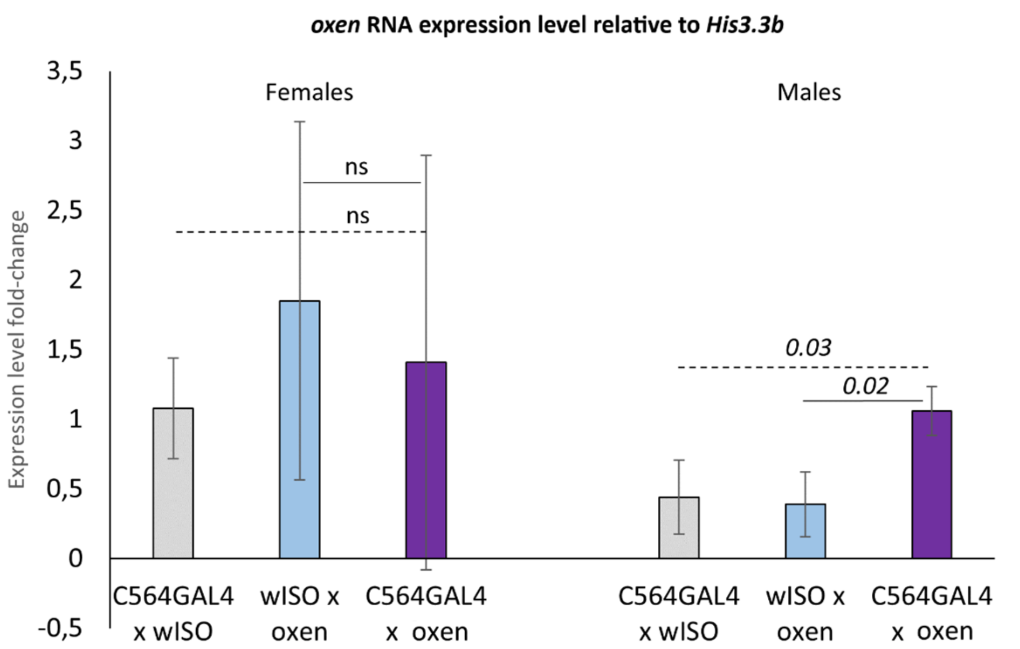

Figure 10 – qPCR expression levels by fold-change against a control gene (*His3.3b*) using the double-delta analysis method for the *C564GAL4;tub-Gal80^{TS}* x *oxen* knockdown fly lines and controls for both the driver and reporter lines. No silencing activity of *oxen* in target tissues can be seen on either sex based in the qPCR assay using whole fly as sample material. The poor standard deviation could be due to the overlapping of the RNAi construct with the short *oxen*-gene, which introduces variability into the qPCR results. p-values of knockdown crosses (purple column) indicated above relative to each control (gray for construct control and blue for driver control.) Each sample consisted of five whole homogenized flies; each sample done in three replicates.

4.3 Phenotypical confirmation of knockdown fly lines

Following the results of qPCR analysis that indicated no silencing of neither *UQCR-C1* nor *oxen*, a follow up experiment was conducted with a viability assay much like the ones described earlier in this work. The flies were either introduced to and reared in the higher, permissive temperature of 29 °C at conception or only as adults eclosed within 12 hours. All adult flies introduced to the permissive temperature after eclosion were able to survive for the observatory period of 14 days after eclosion and able to produce progeny, although these vials did not have live or roaming larvae in them, consistent with the results from the knockdown flies reared in 29 °C that would not develop into adulthood (Table 3).

Table 3 – Summary of the results from the viability evaluation of the gut and fat body-specific driver *C564GAL4; tubulin-Gal80^{TS}* crossed with the two genes of interest (*oxen* and *UQCR-C1*) following the inconclusive results from qPCR analysis. Each genotype was done in three biological replicates and two technical replicates. All genotypes were monitored until eclosion, or up to a period of 14 days. The genotypes that could not eclose were verified to be lethal, and the larvae had expired in their pupa. Control lines and rearing temperatures are highlighted in brown colour, ideal outcomes in green and detrimental outcomes in red colour background.

Target tissue	**RNAi target gene**	**°C**	**Eclosed Y/N**	**°C**	**Eclosed (Y/N)**
Gut and fat body	*UQCR-C1*	29	N	18	Y
Gut and fat body	*w^{1118}*	29	Y	18	Y
N/A (w^{1118})	*UQCR-C1*	29	Y	18	Y
Gut and fat body	*oxen*	29	N	18	Y
N/A (w^{1118})	*oxen*	29	Y	18	Y

The results from the viability assay portray a clear lethal phenotype that supports the hypothesis that the driver line and RNAi construct are working as intended in conjunction with the imaging experiments performed, which are described later in chapter 4.4 of this work.

4.4 Transgenic fly lines combined with temporal control

To enable further investigations into tissue-specific mitochondrial perturbations and following the observation that certain knockdown fly lines could not eclose properly and face significant developmental delay and stress without temporal element to their RNAi activation, other knockdown driver lines specific to only fat body or gut were necessary to establish with the GAL4-UAS modulator GAL80^{TS}. As the first knockdown line used has combined both the midgut and fat body (*C564*) with the temporal control element of Gal80^{TS}, follow-up studies would benefit from creating a fly line specific to each tissue, the gut (NP1-driver) and fat body (fb-driver). Following the crossing scheme described earlier in chapter 3.2, both fly lines were established and tested at the laboratory. The correct function and localization of the GAL4-UAS system was verified by crossing the newly created driver lines (NP1-GAL4;Gal80^{TS} and fb-GAL4;Gal80^{TS}) with a fluorescent nuclear-signal RedStinger reporter line. Both lines show correct localization at 29 °C in Figures 11 and 12, with no nuclear fluorescence in the target tissues at 18 °C where Gal80^{TS} is not yet digested and is blocking the function of GAL4-UAS system as intended.

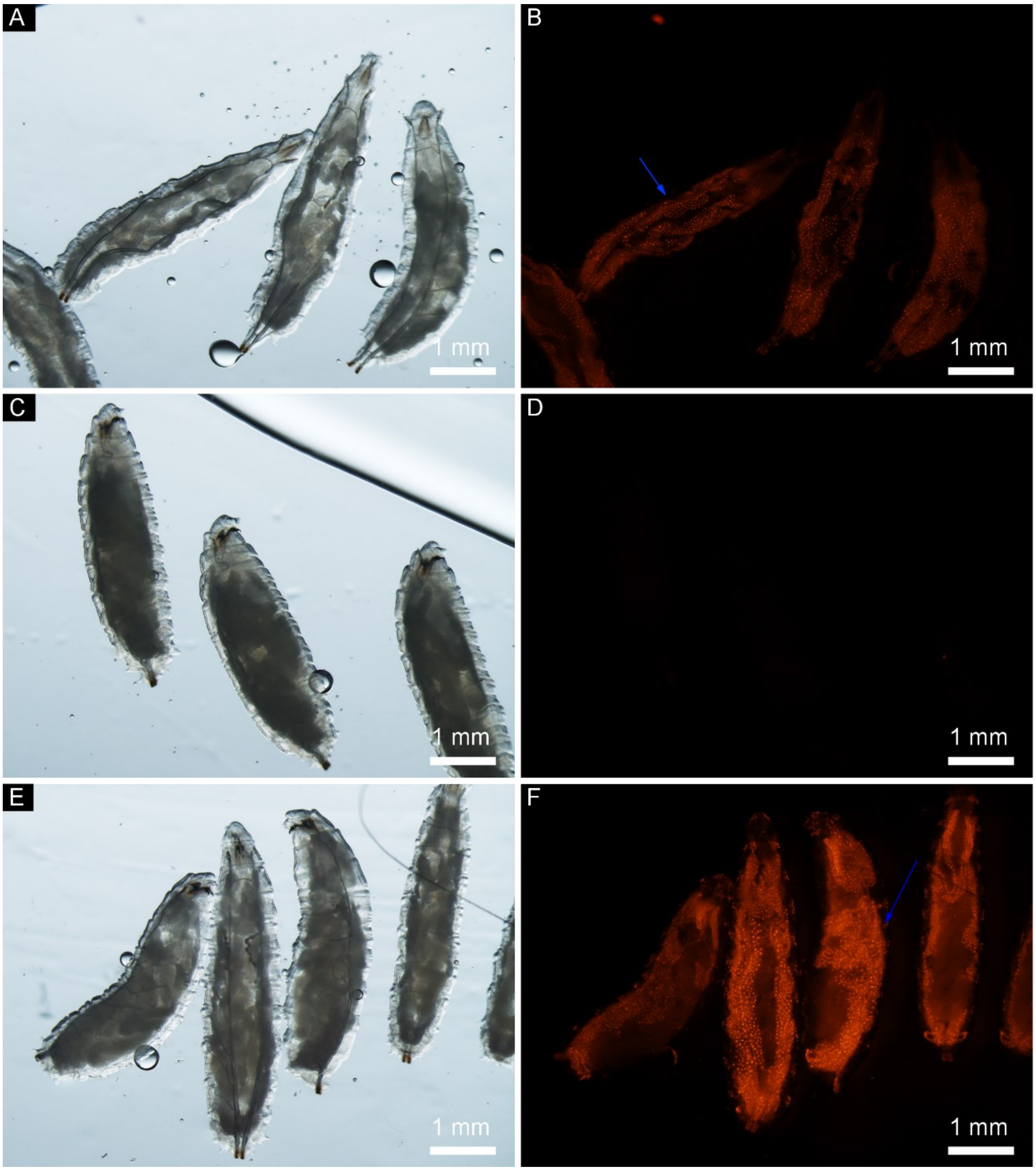

Figure 11 – Overview of the fat body specific GAL4-driver line under 20x magnification. A & B) 3rd instar *fb-GAL4* x UAS-RedStinger larvae with fb-GAL4-construct only reared at 18 °C. As these larvae do not contain the *Gal80^{TS}-GAL4*-modulator, RedStinger RFP is visible even when reared at 18 °C. The signal is weaker, but nuclear localization can be seen in the fat body (blue arrow). C & D) *fb-GAL4;tub-Gal80^{TS}* x UAS-RedStinger larvae, reared at non-permissible temperature of 18 °C. Under RFP filter, the larvae do not fluoresce at all. The picture has been adjusted for high exposure to ensure absence of low intensity fluorescence. E & F) Same genotype as in C/D but reared at permissible temperature of 29 °C. Nuclear-localized, strong red fluorescence in the fat body that envelops the larvae (denoted by blue arrow).

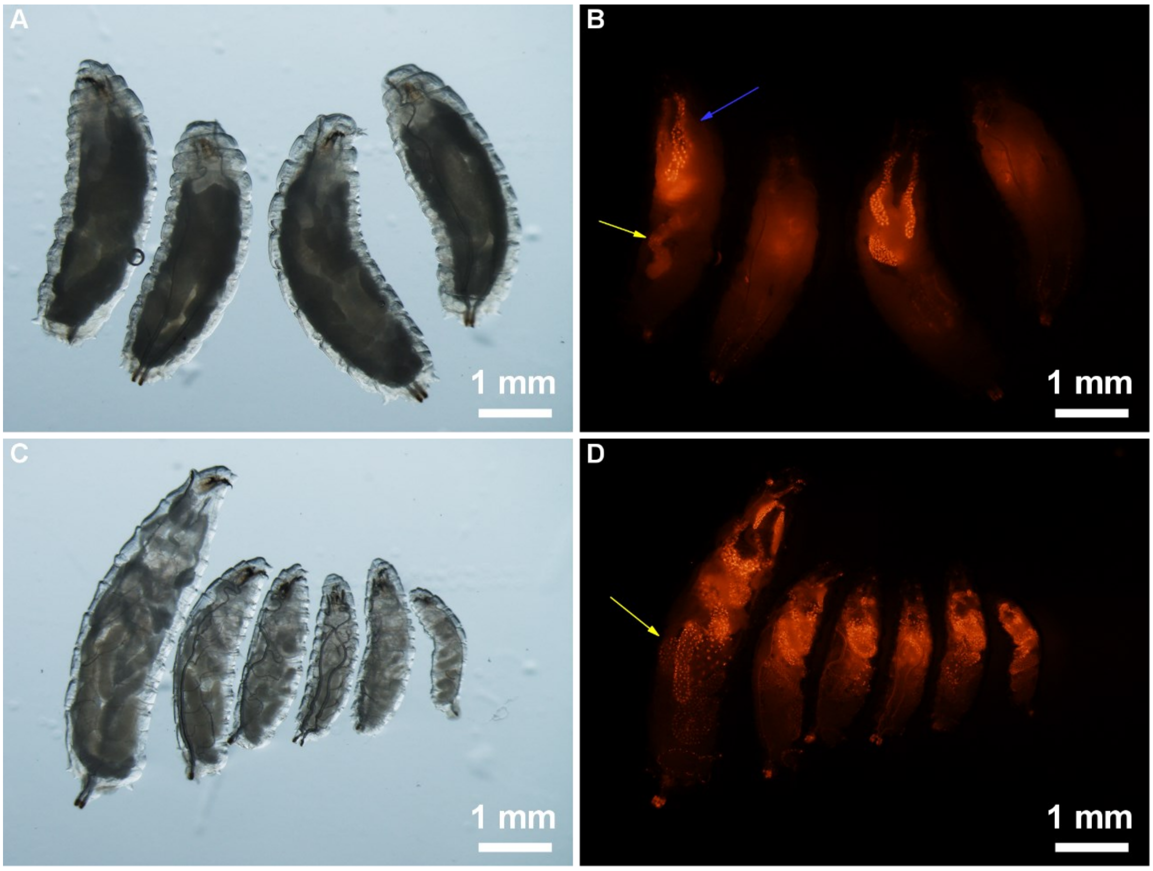

Figure 12 – Overview of the gut-specific transgenic fly line reared in 18 °C and 29 °C at 20x magnification. **A)** *NP1-GAL4;tub-Gal80^{TS}* x UAS-RedStinger 3rd instar, close to pupating larvae reared at non-permissible temperature (18 °C) captured at 20x under bright-field microscopy. **B)** Same larvae and magnification as in A, now captured under red fluorescent protein (RFP) filter. There is particularly strong nonspecific autofluorescence in the salivary glands (blue arrow) and the gut region (yellow arrow.) RedStinger-fluorescence signal is nuclear-localized, so general autofluorescence without nuclear signal can be ignored. **C)** Same genotype and magnification of the larvae, this time reared in permissible temperature at 29 °C under bright-field microscope. These larvae are further from pupating, hence more translucent body structure. **D)** Same genotype and magnification of the larvae under RFP filter. Regardless of autofluorescence, there is a strong nuclear signal from the gut of the larvae, best seen in the larger individual (yellow arrow.)

4.5 Bacterial infection survival assay

The pricking and bacterial solution controls performed up to expectations, where 30-40% of the flies clear the infection, with a slight adverse outcome for females. (Fig. 13C). Both female fly lines with OXPHOS cIII RNAi in the fat body and gut tissues against *oxen* and *UQCR-C1* consistently resisted infection better than controls, with a survival rate above 75% for both experimental fly lines while controls were below 50%. Highest kill rate was achieved within the 25-50 hours post infection window, after which it is likely the remaining flies have cleared the infection. (Fig. 13A) A similar, significant effect is not seen in males, where both the knockdown males and controls succumb to the infection at a similar rate and survival endpoint (Fig. 13B).

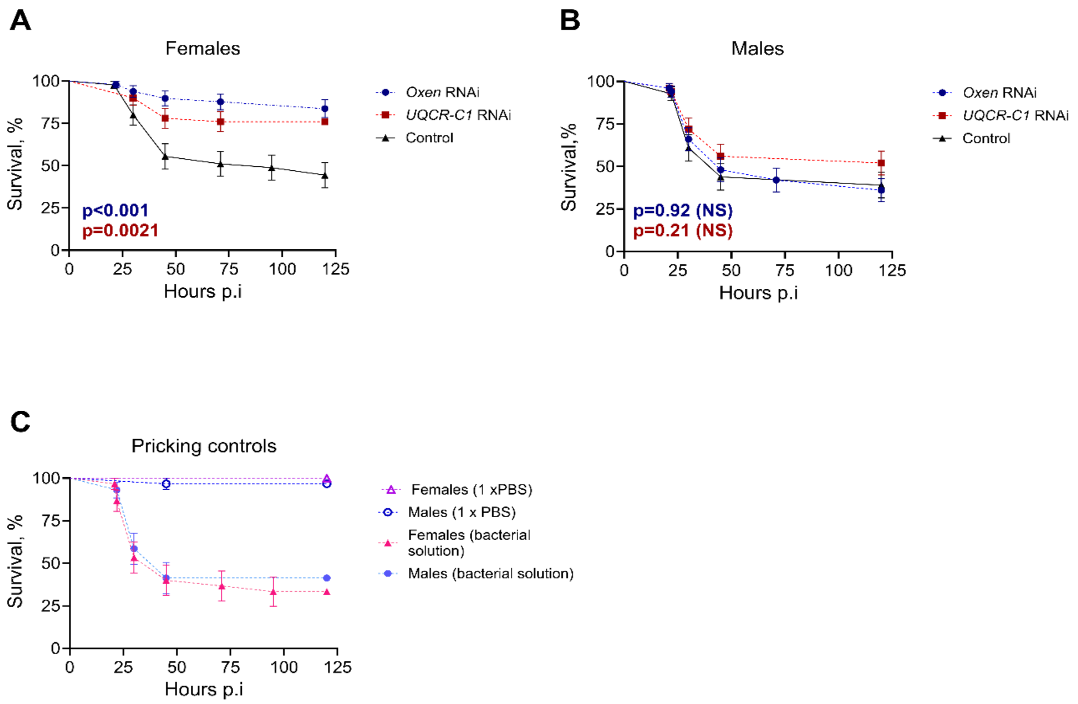

Figure 13 – *P. rettgeri* infection survival against hours post infection from flies that have mitochondrial perturbation specifically in their gut and fat body tissues. **A.** Survival rates of female *Drosophila* RNAi lines (*oxen* and *UQCR-C1*) crossed with fat body and gut-specific driver (*C564GAL4;tub-Gal80*^{TS}) against a wISO control. *oxen* p<0.0001, *UQCR-C1* p = 0.0021. n = 5 replicates x 10 flies per sex. **B.** Survival rates of male *Drosophila* same RNAi lines crossed with the fat body and gut-specific driver (*C564GAL4;tub-Gal80*^{TS}) against a *w*^{ISO} control. *oxen* p =0.9621 (ns) *UQCR-C1* p= 0.2142 (ns) N = 5 replicates x 10 flies. **C.** Control *Drosophila* flies were pricked with sterile 1 x PBS to account for flies succumbing due to the technique of the person performing the needle prick. As none succumbed to the injury alone, there is no baseline killing rate. Similarly, the bacterial solution is tested to ensure a killing concentration of *P. rettgeri* is achieved in wild-type flies. The bacterial solution is an aliquot from the original infectious solution at OD600nm = 0.1. n = 3 x 10 for both PBS and bacterial solution experiments.

5 DISCUSSION

In line with previous findings from the effect of mitochondria on innate immunity, perturbing mitochondrial metabolism – be it induced in the germline or temporally - does not necessitate a universally adverse outcome in response to infectious disease. Whether the outcome is beneficial, neutral, or harmful for host survival is reliant on the site and length of the perturbation as well as on the pathogen the host is challenged with, and the exact mechanisms by which this is achieved are not yet well understood. Colaço et al. (2021) have shown in murine models that ubiquitous perturbation of cI enhanced the model organism's resistance to sepsis, and Vesala et al. (2024) showed that disturbing any of the complexes in the hemocytes, but not in the fat body, significantly enhance the killing rate of parasitoid wasps. Perturbing the mitochondrial OXPHOS cIII genes *UQCR-C1* and *oxen* in fat body and gut in the *D. melanogaster* model appears to provide only the female flies with a benefit of surviving the infection better than controls, if the perturbation is introduced at adulthood. The effect is curious as when wild-type flies are subjected to *P. rettgeri*-infection, males typically clear the infection better than females. Dimorphisms between the sexes in terms of immunity and survival are well-recognized, although only a small portion of articles on *Drosophila* immunity report results by sexes. The consequence of this shortage on papers stratifying by host sex is that we do not yet know whether dimorphisms in immunity stem from plastic functional mechanisms or transcriptionally from gene expression, and what regulatory pathways could be at play. (Belmonte et al., 2020; Millington & Rideout, 2018)

There appears to be a threshold to the beneficial effect of perturbing the OXPHOS system, as ubiquitous knockdown of OXPHOS genes is devastating for host development, fertility, and infection tolerance but a milder perturbation of the same assembly can offer the host situational benefit. However, also in the case of milder or temporal perturbation, the affected tissue introduces heterogeneity into the outcome: disturbing OXPHOS in the fat body alone lowered resistance to wasp infection, versus silencing the same assembly in the hemocytes conferred better killing efficiency of the wasp eggs and larvae as shown by Vesala et al, 2024. In this experiment, the modification was taken even further by introducing mitochondrial perturbation to these tissues only after the flies reach adulthood. When the OXPHOS system was perturbed in the fat body and gut only at a later stage when all flies had eclosed, improved survival against infection was observed in female flies. Interestingly, the survival against infection was in line with controls with males, and no genotype displayed increased susceptibility to infection either – a phenomenon which has been reported previously when silencing of the OXPHOS assembly was introduced at embryogenesis. Considering only the outcome of surviving septic infection with *P. rettgeri,* disturbing the OXPHOS genes in these tissues in adult flies does not seem to compromise immunocompetence, and instead enhance it.

Priming the immune system by pre-existing mild stress appears to prevent the females from succumbing to the infection, but this work did not further assay other readouts such as longevity or bacterial load after survival, AMP levels upon infection, transcriptomes, ROS, or mitochondrial membrane potential. Delving into these assays could provide additional clues towards what events take place on a molecular level, and to potential reasons why females are able to clear the infection better than males. Quantifying the bacterial load from surviving flies would be slightly challenging as the flies likely shed some of the bacteria into their food, perpetuating the bacterial load in their gut by ingesting the food. Whether the surviving flies clear the bacteria entirely or maintain a different bacterial load than controls could also offer insight into any tradeoffs from surviving the infection. Previous work with chronic infections with *P. rettgeri* has found that surviving the acute infection garners protection against a secondary infection at the cost of lowering tolerance to starvation (Chambers et al., 2019). Campos et al., (2021) showed that mitochondrial perturbation to other cIII genes (isp-1 and nuo-6) in a nematode model resulted in increased lifespan, and improved resistance to pathogens that are mediated by p38 signalling, and that ATFS-1, a mitochondrial unfolded protein response regulator, was at the core of this resistance.

Perturbing the mitochondrial OXPHOS system will likely also affect the fat body function and metabolism as the major immunocompetent organ in the fly, resulting from ROS accumulation and danger signaling due to a partly malfunctioning OXPHOS system following disruption of cIII function. CIII is a major ROS producer, and ROS constitutes the major signalling molecule in mitochondria, prompting immune response both by production of AMPs as well as priming hemocytes for cell-mediated level immune response. It is worthwhile to note that AMP upregulation is not only done in response to microbes, but also in cellular maintenance tasks. Thus, sterile but otherwise aberrant host cells and tissues can trigger an immune response that resembles a transcriptional state under microbial infection. (Hanson & Lemaitre, 2020; Myers et al., 2018; Owusu-Ansah & Banerjee, 2009; Yu et al., 2022; Zhao et al., 2019) Quantifying ROS in the knockdown flies before infection could provide insight into whether the infection resistant phenotype arises from ROS priming the immune system before infection, hastening the response to infection when encountered compared to non-challenged controls. As females have a larger fat body relative to their body mass, it could be part of the resolution as to why they are able to survive infection better but remains a subject for speculation.

The sex-specific differences between survival against several different bacteria were recorded by (Duneau et al., 2017) and the underlying cause for dimorphism was identified as the Toll pathway but not through cell-mediated immunity. The Imd pathway was identified as key repressor of *P. rettgeri* proliferation as mutants of this pathway showed increased susceptibility to infection, but in mutants of any stage of the pathway the females still succumb to the infection faster than males. Thus, Imd pathway is necessary for *P. rettgeri* infection survival, but does not appear to be the defining factor in the sexual dimorphism. Mutant Toll ligand *spaetzle* flies retained susceptibility to infection, but both sexes succumbed at a similar rate. Further knockdowns revealed the serine protease Persephone as responsible for danger-sensing in Toll pathway to be the key differentiator in sexual dimorphism upon *P. rettgeri* infection. However, other bacteria do not rely on the same concept, fortifying the statement that while Toll pathway seems to be at the heart of male and female dimorphisms in immunity, the root cause for this is yet unelucidated. Many theories suggest hormonal baseline signalling as the differentiator, at least in the case of mated flies. In mated females, the susceptibility to infection has been shown to be due to hormonal signalling by juvenile hormone (JH) (Schwenke & Lazzaro, 2017) and females having a higher variation in mated immunosuppression expression (Short & Lazzaro, 2010) in response to male seminal fluids (Short et al., 2012). However, in the case of this work, the females were all unmated virgins and the sexual dimorphism in survivorship was in their favor.

A higher degree or multiple layers of mitochondrial disruption will trigger the mitochondrial unfolded protein response (UPR^{mt}), including inhibitors of cIII. UPR^{mt} is a transcriptional response ensemble that is highly conserved, and its primary function is to maintain cellular function by upregulating genes that aim to repair and maintain mitochondria and its components such the OXPHOS complex, mito-nuclear protein balance and ROS neutralization. UPR^{mt} is a normal event in development, and its activation is milder in adulthood. As UPR^{mt} activity declines in senescence, maintaining UPR^{mt} transcriptional profile appears an attractive alternative to slow aging. Conversely, a sustained UPR^{mt} has an opposite effect on longevity as well as organism fecundity. Instead, it has been shown to accumulate damage in mitochondrial DNA, and some of these mutated mitochondria are able to escape the normal maintenance by mitophagy. These plastic properties of UPR^{mt} highlight the threshold effect on innate immunity. (Shpilka & Haynes, 2017) Vesala et al.

2024 also showed similarly that UPR^{mt} is likely part of the driving force behind the activated immune cells prior to infection in OXPHOS knockdown hemocytes, and the improved infection outcome against wasp infection.

Results from this work, including the transgenic fly lines with temporal control elements to overcome developmental stress and delay in larvae, build on the growing number of research work supporting the role of mitochondria in innate immunity and its regulation, and the possibility of situational benefits instead of the canonical notion that mitochondrial dysfunction is always detrimental to the host organism. The qPCR quantification proved difficult due to the short length of the *oxen* gene and the impossibility to design qPCR primers for *oxen* gene outside of the region that overlaps the hairpin RNAi. However, with the strong phenotype on the viability assays suggests strongly that the knockdown of *oxen* and *UQCR-C1* is achieved, albeit not irrefutably. While the mechanism by which the susceptibility to P. rettgeri infection has been reversed in female flies that have OXPHOS perturbation in their gut and fat body will not be elucidated by this work, future research endeavors provide interesting avenues to identify possible effectors, such as measuring ROS, mitochondrial membrane potential and the possible AMP level differences between the sexes and controls. While not recorded in these experiments, it would have been interesting to record if there was any sexual dimorphism in the flies that were able to eclose at 25 ^{o}C from the knockdown lineages without the $Gal80^{TS}$ repressor element.

6 CONCLUSIONS

Building on the previous findings done by the Mitochondrial Immunometabolism research group, this work shows that mitochondrial perturbance has an impact on the immune response and could have sexual dimorphisms to it yet poorly understood. Perturbation in the mitochondrial OXPHOS cIII genes *oxen* and *UQCR-C1* in the fly midgut and fat body appears to confer a favorable survival outcome in response to septic injury infection by *P. rettgeri* only in females in the *D. melanogaster* model, when the perturbation is introduced after eclosion, in adults. Previously we have shown that early-onset whole-animal or fat body knockdowns of OXPHOS genes has devastating impact on the viability or infection resistance of the organism (Vesala et al, 2024). However, when this silencing was introduced at a later life stage, the effect was reversed. A similar effect on survival was not observed with male *Drosophila* where gene knockdown animals and controls survived the infection at similar rates.

These results add to the growing number of evidence that mitochondrial function impacts the immune response, and the tissue and strength of any perturbance introduces heterogeneity in the outcome. Investigating the mechanisms and effectors behind mild mitochondrial perturbation that give rise to situational benefits when confronted with an immune challenge will be key in understanding the plasticity of the immune response.

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