AMERICAN UNIVERSITY OF BEIRUT

ANALYSIS OF THE *WOLBACHIA* ENDOSYMBIONT EFFECTS ON THEIR *Drosophila melanogaster* HOST

by
HASSAN ALI MUSTAPHA

A thesis
submitted in partial fulfillment of the requirements
for the degree of Master of Science
to the Department of Biology
of the Faculty of Arts and Sciences
at the American University of Beirut

Beirut, Lebanon
April 2018

AMERICAN UNIVERSITY OF BEIRUT

ANALYSIS OF THE *WOLBACHIA* ENDOSYMBIONT EFFECTS ON THEIR *Drosophila melanogaster* Host

by

HASSAN ALI MUSTAPHA

Approved by:

Zakaria Kambris

Dr. Zakaria Kambris, Associate Professor — Advisor
AUB, Department of Biology

Heinrich Burggraf zu Dohna-Schlobitten

Dr. Heinrich Burggraf Zu-Dhona Schlobi, Assistant Professor — Committee Member
AUB, Department of Biology

Khouzama Knio

Dr. Khouzama Knio, Professor — Committee Member
AUB, Department of Biology

Date of thesis/dissertation defense: April 23, 2018

AMERICAN UNIVERSITY OF BEIRUT

THESIS, DISSERTATION, PROJECT RELEASE FORM

Student Name: Ali Mustapha Hassan
Last First Middle

☑ Master's Thesis ○ Master's Project ○ Doctoral Dissertation

☑ I authorize the American University of Beirut to: (a) reproduce hard or electronic copies of my thesis, dissertation, or project; (b) include such copies in the archives and digital repositories of the University; and (c) make freely available such copies to third parties for research or educational purposes.

☐ I authorize the American University of Beirut, to: (a) reproduce hard or electronic copies of it; (b) include such copies in the archives and digital repositories of the University; and (c) make freely available such copies to third parties for research or educational purposes

after : **One ---- year from the date of submission of my thesis, dissertation, or project.**

Two ---- years from the date of submission of my thesis, dissertation, or project.

Three ---- years from the date of submission of my thesis, dissertation, or project.

May 7-2018

Signature Date

ACKNOWLEDGMENTS

I would like to thank my supervisor Dr Zakaria Kambris the most. He was my mentor throughout those two years, helping me grow both on the personal and scientific levels.

Thank you for Dr. Heinrich Burgraff Zu-Dhona Schlobi and Dr. Khouzama Knio for serving on my committee and guiding me with their rich knowledge and experience.

I would like to that my lab colleges as well: Farah Ayash, Maria Janeh, and Sana Jaber for the support and collaboration.

A special thank you goes for my family and friends who have been my support and motivation so I develop into an ambitious young scientist.

AN ABSTRACT OF THE THESIS OF

Hassan Ali Mustapha for Master of Science
Major: Biology

Title: Analysis of the *Wolbachia* Endosymbiont Effects on its *Drosophila melanogaster* host

Wolbachia is a known endosymbiont of many insects including the fruit fly *Drosophila melanogaster* and the disease vectors *Culex pipiens* and *Aedes albopictus*. It is present in the gonads and induces cytoplasmic incompatibility; a partial one in *Drosophila* and a complete one in mosquitoes. Therefore, understanding how this microbe manipulates its host to induce this reproductive alteration would be of great importance. It would indeed fill in a gap in knowledge present in the field pertaining to the nature of this interaction: is *Wolbachia* perceived as a parasite by the host? Does its presence impose some sort selective advantage or disadvantage on its host's fitness? Moreover, deciphering the sequence of events leading to cytoplasmic incompatibility would indeed open new possibilities for biological control of some disease vectors, of which an example of would be the introduction of infected males to an environment of *Wolbachia* free females, leading to cytoplasmic incompatibility and thus population control. In this study we aim to understand some of the mechanisms employed by *Wolbachia* to manipulate its natural host *Drosophila melanogaster,* and how in turn does this fly counteract and respond to the bacterium studying the immune responses activated by *Drosophila* specifically. In this study, we use the genetic tools available for *Drosophila* in order to tackle the interactions taking place at the molecular levels between the endosymbiont and its host. We try to address how the bacterium is manipulating its host at the molecular and gene levels, and in turn check how the host is responding to control *Wolbachia*.

CONTENTS

ILLUSTRATIONS

Figure Page

TABLES

Page

ABBREVIATIONS

ul: micro-liters

g: gravitational force

DNA: Deoxy-ribonucleic acid

RNA: Ribo-nucelic acid

PCR: polymerase chain reaction

mV: milli Volts

V: Volume

cDNA: Complementary Deoxy-ribonucelic Acid

OD: optical Densisty

nl: nano lites

IR: inverted Repeats

RNAi: Ribonucleic Acid Interference

gDNA: genomic DNA

ml: milli liters

n: number of samples

ATG: autophagy-linked protein

%CG: percentage of cytosine and guanine in sequence

bp: base pairs

WftsZ: for *Wolbachia* quantification

DRS: Drosomycin

Dpt: Diptericin

Rpl32: ribosomal protein 49

His: histone

F: Forward Primer

R: Reverse Primer

CHAPTER I

INTRODUCTION

A. *Wolbachia* and its hosts

Wolbachia is a natural endosymbiont present intracellularly mostly in the gonads of many insects and inherited vertically from female to offspring, yet exact numbers revealing *Wolbachia*s' infection incidence across different species are not present or show much discrepancy in literature (Carrington *et al* 2011). One of the impacts of this symbiotic existence is the induction in the host of a phenomenon known as cytoplasmic incompatibility (CI) , a biological observation that has been under study since a long time (Poinsot *et al* ., 1998). CI is mainly characterized by a decrease in the hatching number of eggs during certain mating events through which *Wolbachia* is able to impose reproductive restrictions over its host (Carrington *et al* 2011). This reduction in surviving eggs is due to zygotic modifications whenever uninfected females mate with infected males, or with males infected with another incompatible *Wolbachia* strain (Duron 2008). Despite the fact that much research has been invested in this topic, many gaps in knowledge are still present, and a lot of questions are constantly addressed such as: how does the host perceive this endosymbiont? Is it a disadvantageous parasite against which host defenses are activated? Or does it confer some selective advantage through a mutualistic relation with

the host? Or even is it a neutral existence now resulting from a long history of coevolution? And indeed the question of how *Wolbachia* manipulates its host remains a debatable topic

B. Gaps in knowledge in *Wolbachia* research

Despite the abundance of research data concerning this topic, a clear picture of *Drosophila-Wolbachia* interactions has not been formulated yet. Many questions remain unanswered. Further research is needed to better fathom this biological relation that could be mutualistic, parasitic, or neutral, which could have basic and practical implications on the field of science.

In this study, we aimed to fill in some of the gaps in knowledge that are present in the literature of *Wolbachia-Drosophila* interactions. We used the genetic tools available for *Drosophila melanogaster* to try understanding some of the mechanisms employed by *Wolbachia* for CI induction, and then try to point out some immune response pathways activated by *Drosophila* against *Wolbachia*'s infection. Possible fitness improvement through enhancing survival against bacterial infections by *Wolbachia* has also been addressed.

C. *Wolbachia* research in *Drosophila*

Most of the *Wolbachia*- related questions have been tackled in *Drosophila melanogaster*, and other *Drosophila* species. This is not surprising as this model is famous for being a robust genetic model with many tools available to aid in biological research.

This focus is mainly emphasized by the presence of an easily accessible targeted gene expression: UAS-gal4 system (Brand and Perrimon 1993). As Brand and Perrimon describe, a sequence encoding Gal4, the yeast transcriptional activator is inserted in any location in the genome. Then other strains are built including the Upstream Activating Sequence (UAS) to which Gal4 bind, being inserted in the promoter of any desired gene. Neither of the two strains will have altered gene expression on of the desired gene, rather whenever they are crossed together, the offspring will now have both Gal4 and UAS, which will lead to the binding of Gal4 to the UAS, thus driving the expression of any desired gene (Brand and Perrimon 1993).

The first major question raise is concerning the nature of *Wolbachia* itself: is it a facultative parasite similar to what *Wolbachia* is considered in *Drosophila simulans* and *Aedis aegypti?* Or does it fall under a mutualistic relation which is the case in *Asobara tabida*? (Kremer *et al* 2009). To investigate this issue, scientists have looked into both possibilities focusing on how *Wolbachia* enhances the fitness of *Drosophila* on one hand, and how it imposes restrictions on this fitness on the other hand.

D. *Wolbachia* enhances *Drosophila*'s fitness

Drosophila fitness and *Wolbachia* has been addressed from two major points in the literature: *Wolbachia*'s effect on *Drosophila's* survival in the case of viral infections, and in normal conditions when fitness of infected flies are compared to uninfected ones. In 2010, Glaser *et al* have showed that *Wolbachia* enhances *Drosophila's* resistance to the

West Nile Virus upon infection. This was demonstrated through a 500- fold higher infectious dose 50 and 100000 fold lower virus titer production in flies harboring *Wolbachia* compared to those which are *Wolbachia* free. This was further emphasized in 2014 when Martinez *et al* showed that viral resistance is a observable in *Wolbachia* infected flies upon the infection of two other viruses: *Drosophila* C virus (DCV) and the Flock House Virus (FHV). This observation has been previously reported along with an enhanced resistance upon Nora viral infections in the fruit flies (Texeira *et al* 2008). When it comes to *Wolbachia*'s effect on *Drosophila'*s bacterial resistance, very little data is available. Recently in 2017, Gupta *et al* have touched on this issue, but only studied one bacterium which is *Pseudomonas aeruginosa.* They showed that flies infected with the *Wolbachia* strain present in *Drosophila melanogaster* (wMel) show enhanced survival rates upon this bacterial infection, but depending on the infection route: systemic infections of *Pseudomonas aeruginosa* did not portray any fitness differences between infected and uninfected flies, but enteric ones did. Even in the absence of any infections, Fry and colleagues have given evidence for the presence of baseline enhanced longevity in *Wolbachia* positive flies. This was the result of different genetic crosses between infected and uninfected *Drosophila* flies, showing a strong correlation between inheriting the enhanced survival phenotype of prolonged life time and *Wolbachia* positive status (Fry *et al* 2002). This could be the result of what later has been proposed by this research group that the fecundity, defined as number of eggs laid per female, was always significantly higher in infected females (Fry *et al* 2004). This observation was reemphasized in 2011 when Sullivan *et al* gave evidence to an increased egg laying in flies infected with the *Wolbachia* strain of *Drosophila mauritiana* (wMau).

E. *Wolbachia* has a neutral effect on its host fitness

Other researchers show supporting evidence that *Wolbachia* either has no effect on certain aspects of *Drosophila*'s fitness, or provide data linked to a disadvantageous effect imposed by *Wolbachia* on the survival of infected flies, and thus a defense mechanism is activated to fight back what is possibly perceived as a pathogen. In 2004, Harcombe provided some support for the neutral effect hypothesis. Flies infected with wMel showed no change in survival fitness concerning heat resistance when compared to uninfected ones, although some differences between flies of either both infection statuses were present when comparing heat shock protein levels in them. This could either mean that *Wolbachia* has no major effects, or that other processes are manipulated so that *Wolbachia* ensures its continuous existence in *Drosophila melanogaster* (Harcombe *et al* 2004). In 2012, Rottschaefer *et al* tested the effect *Wolbachia* could have on the fitness of *Drosophila melanogaster* infection in light of intracellular pathogenic infection *Listeria monocytogene* and *Salmonella typhimurium*, or the extracellular one *Providercia rettegi*, in both cases a negative result was shown hinting again to a neutral effect by *Wolbachia* on its host fitness.

F. *Wolbachia* infection as a drawback for host fitness

On the other hand, other scientists hint to a possible drawback for *Wolbachia*'s infection portrayed through this cytoplasmic incompatibility that is the major effect of the endosymbiont on its host. This restriction on mating choice and the decrease in egg hatch

could be perceived as a control over the machinery of the host in order to drive the evolution of the host in a specific direction which could only be beneficial for *Wolbachia* but not for the host (Duron 2008). In that case, defenses against *Wolbachia* would be an intuitive thing to think of. A study published in 2012 by Voronin *et al* reveal that autophagy is a mechanism recruited by filarial nematodes against *Wolbachia*, specifically finding ATG8a to be significantly associated with anti-*Wolbachia* autophagy immune response.

G. *Drosophila* immunity

If *Wolbachia* could be considered as a pathogen and thus immune responses could be activated against, then it is worth studying those immune pathways to check this host-pathogen interaction. The innate immune responses could be divided into humoral and cellular. Cellular immune responses, as Discussed by Govind, are coagulation, autophagy and melanization which the latter depends on the phenol-oxidase gene forming black clot-like structures (Govind 2008). In addition to the autophagy discussed previously which is considered a cellular response, the humoral innate immune responses in *Drosophila* are also of great importance (Govind 2008). *Wolbachia* is characterized by two major immune pathways: the ImD pathway activated against gram-negative bacteria leading to the production of the Anti-microbial peptide Diptericin under the activity of the transcription factor Relish, and the Toll pathway activated against gram-positive and fungal infections leading to anti-microbial peptide Drosomycin production under the effect of transcription

factor Dif (Govind 2008). The nitric oxide synthase enzyme has been shown to have significant impact on the activation of *Drosophila* immunity (Foley *et al* 2003).

H. *Wolbachia* manipulates its host

Regardless of what *Wolbachia* would be labelled as, understanding the impact it does on the host, and how it manipulates its system *in vivo* remain indispensable questions to understand host-endosymbiont interactions. Extensive research is continuously conducted tackling this issue from different perspectives. Sullivan *et al* . showed that wMau enhances the mitotic activity of stem cells in the gonads of infected *Drosophila Mauritania* flies. Through immunofluorescence, an increased division in the germline stem cells (GSCs) and a downregulation of programmed cell death has been linked to *Wolbachia*'s infection. This indicates an impact of this bacterium at the genetic level manipulating cell division events. Studies have been conducted as well to check the metabolic and biochemical alterations induced by *Wolbachia*. In 2009, comparative transcriptome analysis of infected and uninfected insects showed that there is a difference in iron metabolism between both groups. Overexpression of ferritin has been observed in uninfected females, to which *Wolbachia* would also be sensitive and in turn respond by changing its bacterioferritin expression levels (Kremer *et al* 2009). A more recent study revealed a correlation between host's diet and *Wolbachia*. Ponton *et al* gave evidence to that through altering yeast and sugar components of *Drosophila* food and then recording observable outcomes. They report that diet-induced modifications to the guts of flies resulted in changes in *Wolbachia* population levels (Ponton *et al* 2015).

I. *Wolbachia* and Cytoplasmic incompatibility

When it comes to CI induction, different factors have been studied in order to try to understand this mechanism. The male age appears to be an important factor whereby the strongest CI levels happen to show when the male is young, and then those levels tend to get weaker as the male age increases (Reynold *et al* 2002). Poinsont hypothesizes as well that CI promotion is controlled by different factors in males and females. This came up when males infected with the *Wolbachia* strain present in *Drosophila melanogaster* did not induce CI when mated with females infected with that strain of *Drosophila sim*ul*ans*, wRi (Poinsot *et al* 1998). Other researchers have been interested with studying molecular interactions, and showed evidence of correlation between the transcription profile of host and *Wolbachia*'s localization. Some data reveal that the localization of *Wolbachia* in the female oocyte of *Drosophila* overlaps with the localization and distribution of two host mRNAs, *oskar* and *bicoid*, along the posterior-anterior axis (Veneti *et al* 2004).

J. *Wolbachia* manipulates host transcription profile

In 2010, Kambris *et al* have given evidence that *Wolbachia* manipulates the expression of some of its host genes. Through somatic infection, the research group introduced the over proliferating *Wolbachia* strain: Wmelpop into the malaria vector *Anopheles gambiae.* Through comparative analysis of transcription profile of both infected and control flies, six immunity genes were shown to be uprefulated in infected flies

including two that are related to responses against the malaria vector (LRIM and TEP1). In agreement with this, the titer of malaria *Plasmodium* was decreased significantly more in the stable cell line infection than what was evident in the transiently somatically infected flies. Taking into consideration tha cytoplasmic incompatibility is most likely a result of gamete development manipulation by *Wolbachia* (LePage *et al* 2014), LePage and his research group hypothesized that DNA methylation could be differentially regulated in the infected flies compared to control ones. As expected, they show that *Wolbachia* infection in the testes of *Drosophila melanogaster* lead to an upregulation of DNA methylation in infected flies by 55%. This manipulation in the transcription profile of the host by its endosymbiont *Wolbachia* aligns with the results Pinto *et al* got in 2013. They show that *Cul*e*x pipiens* mosquitoes infected with *Wolbachia* exhibit an upregulation in a Zinc finger protein, homologue to grauzone, a protein that keeps female meiosis in *Drosophila melanogaster* under control.

K. *Wolbachia* induces CI by altering protein levels in host

Very recently, Beckmann and his colleagues have given insight to a new potential mechanism by which *Wolbachia* is introducing cytoplasmic incompatibility. They show that *Wolbachia* secretes a deubiquitylating complex which they named DUB (Beckmann *et al* 2017). This complex consists of two Cytoplasmic Incompatibility inducing DUB, named as CidA and CidB. They also give evidence to the complexity and functionality of this complex: CidB removes ubiquitin from proteins in the *Drosophila* system they worked on, and CidA binds to the other DUB. All the male flies having cida-cidb complex

witnessed cytoplasmic incompatibility in their descendants when crossed with the females. This CI was confirmed through the absence of normal condensation in male pronucleus (Beckmann *et al* 2017). Taking into consideration tha this is an important part of spermatogenesis (Blattner *et al* 2016), this observation indicates that *Wolbachia* manipulates the normal meiosis which could be the reason to induce CI

L. Overview on *Drosophila* meiosis and link to *Wolbachia*

Meiotic events in *Drosophila* vary based on sex. Separation of homologous chromosomes in anaphase I for example is regulated by the formation of bivalents in a canonical manner in females, while it is conveyed through an Alternative System of Conjugation involving several proteins such as MNM, SNM and the protease Separase in males (Blattner *et al* 2016). Nevertheless, the major events such as chromatin remodeling, condensation of chromosomes and the role of centromeric proteins in meiosis, are overall conserved in both sexes (Raychaudhuri *et al* 2012). Raychaudhuri discusses the role of several centromeric proteins in *Drosophila* meiosis such as Cid, Cenp and Cal-1, explaining that Cid out of all is the only one that remains throughout the whole meiosis even after chromatin remodeling. This remodeling comes in the later stages in meiosis before the maturation of the parental gametes, something discussed by Fabian *et al* in 2012. The meiotic events are governed by several molecular interactions such as the action of Grauzone in oogenesis that lead to a mature oocyte (Pinto *et al* 2013), or those that lead to a mature sperm after spermiogenesis. *Drosophila* spermiogenesis is the morphological change of the spermatocyte into a motile sperm and the last step in male meiosis (Fabian *et al* 2012). Spermiogenesis initiates by mitochondrial aggregation and polarization of the

nuclear envelope the latter which is regulated by Cyclin Dependent Kinases, CDKs (Onischenko *et al* 2005). This ends with chromatin remodeling in which histones are removed and replaced by sperm specific proteins such as Mst77F, Protamine A, Protamic B. This is necessary because those proteins are small enough to fit into the functional needle-shaped sperm, resultant of the nuclear envelope polarization events (Fabian *et al* 2012). In 2002, Tram and his team have pointed out to a link between those meiotic events and the cytoplasmic incompatibility induced by *Wolbachia*. Based on experimental research on *Nassonia,* they show that cytoplasmic incompatibility is caused by a failure in chromosome condensation. They claim that *Wolbachia* leads to a delay in the condensation of chromosomes of the paternal side leading to delay in the sperm maturity, and thus an incompatibility with the earlier matured oocyte. This asynchrony is "rescued" whenever the female also harbors *Wolbachia* leading to a delay of maturation in both gametes, and as a result normal fertilization. This could be the result of manipulations by *Wolbachia* which could possibly affect a Cenp protein, Cenp-A leading to abnormal chromatin condensation. *Wolbachia* could also be delaying chromatin remodeling through affecting histone 3 phosophorylation by CDKs (Tram *et al* 2002).

M. *Wolbachia* and host phylogenetics

The phylogenetic research tackling the potential evolutionary link between the *Wolbachia* and its host has become an interesting research field recently. In 2013, Ilinsky constructed a phylogeny of the mitochondrial DNA (mtDNA) of different *Drosophila melanogaster* flies from different geographic locations. This resulted in a correlation

between two clades of flies diverging in Africa and their respectively associated *Wolbachia* group. This indicated a potential role of *Wolbachia* in inducing mtDNA variation of its host leading to diversification. Very recently in 2018, Zu Dhona *et al* built a phylogenetic tree for the different *Wolbachia* strains. The phylogeny gave a phylogenetic signal when it comes to the host phenotype on the tree which means that this can be considered as a *Wolbachia* trait. Upon further investigation, no evidence where deduced to prove that cytoplasmic incompatibility is ancestral to *Wolbachia* in arthropods.

N. Unnatural *Wolbachia*-Host Association

The interaction between the endosymbiont and its natural host has to be compared with artificial symbiosis. Pan and colleagues have forced an artificial endosymbiosis between *Wolbachia* and a naturally *Wolbachia* free insect, *Aedis aegypti* (Pan *et al* 2017). They have noticed that *Wolbachia* takes advantage of the host by boosting its immune system in order to be able to compete with other bacteria coexisting in the host so it could form its own niche with minimum competition over resources. Whether or not this mechanism is found in naturally occurring symbiotic relation has to be looked into. Another artificial association occurred when introducing the over-replicating *Wolbachia* strain Wmelpop of *Drososphila* into *Aedes aegypti* mosquitoes. This lead to a shortening of the life span of those mosquitoes, and hindered the thriving of filarial nematodes in them (Kambris *et al* 2009).

O. Significance and aim of our study

As indicated before, there still exist a lot of gaps in knowledge in the literature when it comes to this specific field of study. There has been a lot of separate studies trying to address the effect *Wolbachia* has on the fitness of the host in light of pathogenic infections. Here we test this effect on a wider range of pathogens: two gram negative bacteria, two gram positive and two fungal species.

The interactions between *Wolbachia* and *Drosophila* on the molecular level are still unclear. This is why we will test how the endosymbiont alters the host's immunity through checking the levels of anti-microbial peptide secretion of infected versus control flies, at the basal as well as upon pathogenic infection. In turn, we checked how the host keeps the pathogen under control through altering the immunity of the host and then studying the fitness of *Wolbachia* in the different altered immunity strain, trying to deduce a certain regulatory mechanism for homeostatic control of *Wolbachia* levels.

How *Wolbachia* induces cytoplasmic incompatibility at the molecular level is a recently emerging topic. In this study, we screened thirteen different genes linked to *Drosophila* meiosis to check a potential manipulation of their expression levels by *Wolbachia*.

The main impact this research would have is tackling a debatable topic that has many gaps in knowledge. Going more in-depth into how are the interactions between *Wolbachia* and *Drosophila* regulated and maintained would indeed pave the way for better understanding of other endosymbiotic relationships. Moreover, since *Wolbachia* is present

in many disease vectors, such as *Aedes* species (Kremer *et al* 2009), and bearing in mind that CI is also present in those species, then understanding this biological phenomenon would allow potential biological control mechanisms to replace insecticide usage. An example of that would be controlling the population levels of disease vectors through increasing the number of *Wolbachia* infected males into an ecology of infected females resulting in CI induction. A major aspect of this project would be its evolutionary impact. Understanding some immune responses activated by the host against *Wolbachia*, and in turn trying to dig into the mechanisms employed by *Wolbachia* to manipulate the host system will help improve our knowledge about driving forces for evolution and possible coevolution mechanisms that ensured *Wolbachia* population maintenance in its host for years.

Specific Aim 1

Survey of the incidence of *Wolbachia* in different insect species of Lebanon and phylogenetic analysis.

To do that**:** different insects sampled from random geographic areas in Lebanon will be tested for harboring *Wolbachia* through PCR analysis following DNA extraction. This will be followed by a Phylogenetic analysis across Lebanese insect species of the rate shifts between the two states: harboring *Wolbachia* or not.

Specific Aim 2

Analyzing the effect *Wolbachia* has on its host's immunity

A. Generate a *Wolbachia*-free strain for comparative analysis

   To do that: The wild-type *Drosophila* strain Oregon (Or), that naturally harbors *Wolbachia*, will be treated with antibiotics for several generations to clear the bacteria. After a recovery phase of at least two generations, this treatment will generate the *Wolbachia*-free strain Or-Tet. Absence of *Wolbachia* will be confirmed through PCR analysis.

B. Compare the effect of *Wolbachia*'s presence on host survival after different microbial infections.

   To do that: Org and Or-Tet flies will be micro-injected with different microorganisms (two gram-negative bacteria, two gram-positive bacteria, and two fungal strains). This will be followed by monitoring the survival of the infected flies over time.

C. Compare expression levels of anti-microbial peptides Diptericin and Drosomycin in Or and Org-Tet strains.

i. Local expression levels in the flies' gonads.

To do that: RNA will be extracted from dissected ovaries of both strains, followed by reverse transcription and qRT-PCR.

ii. Systemic levels in whole flies.

To do that: RNA will be extracted from whole flies of both strains, followed by reverse transcription and qRT-PCR. This will be analyzed in absence or after secondary infection.

Specific Aim 3

Check *Wolbachia* population levels in the context of different host immunity backgrounds.

A. Elevated host immunity

To do that: *Drosophila* strains overexpressing both antimicrobial peptides will be generated through genetic crosses. After introducing *Wolbachia* into this system, the bacterial populations will be quantified through qRT-PCR.

B. Suppressed host immunity

To do that: *Drosophila* strains expressing low levels of both antimicrobial peptides will be generated through genetic crosses. After

introducing *Wolbachia* into this system, the bacterial populations will be quantified through qRT-PCR.

C. Suppressed autophagy responses in the host

To do that**:** *Wolbachia* will be introduced by genetic crosses into *Drosophila* strains with RNAi knock-downs of specific autophagy genes. This will be followed by bacterial population quantifications through qRT-PCR.

Specific Aim 4

Compare the effect of *Wolbachia*'s presence on the host reproductive physiology.

A. Test the effect of *Wolbachia* on different host candidate genes involved in *Drosophila* oocyte meiosis such as *grauzon* and *cortex*.

i. Quantify the levels of expression of the candidate genes in Or and Or-Tet flies.

To do that: RNA will be extracted from whole flies, followed by reverse transcription and qRT-PCR.

CHAPTER II

MATERIALS AND METHODS

A. *Drosophila* stocks, rearing and stock maintenance

Or strain is the wild type strain present in the lab. Or-tet was obtained through tetracycline treatment as indicated below. All inverted repeat lines were obtained from Vienna *Drosophila* RNAi Center (VDRC). To rear stocks, 50 ml vials were utilized. The food medium was a mixture of soy flour, polenta (cornmeal), agar, molasses and propionic acid. Flies were kept at two different temperatures: either 18 degrees or 25 degrees. To ensure stocks are in close to natural habitat conditions, a 12 hour light: 12 hour dark cycle was maintained in the lab.

B. *Drosophila* Crosses

Virgin females were collected from the required strain were collected at 18 degrees. 10 of those were crossed to the desired male strain and the cross was kept at 25 degrees to collect the progeny. In cases of using IR strains, the cross was removed from 25 degrees at the second larval stage and kept at 29 degrees until progeny hatching in order maximize gene knock down.

C. *Wolbachia* free strain

In order to develop a *Drosophila* strain that is genetically similar to the wild type, but for comparative purposes is *Wolbachia* free, wild type Or flies have been raised on a medium containing tetracycline for three generation. Equal volumes (5ml each) of instant *Drosophila* medium and tetracycline solution (a 1/15 distilled water diluted solution of 25mg/ml tetracycline stock solution). 4 samples (1 fly) of the Or-tet strain compared to 1 fly of the Or strain have been tested to check they are *Wolbachia* free through DNA extraction and PCR analysis as discussed below. After the third generation, flies were raised on regular cornmeal medium for three generation before starting experiments to rule out any effect of tetracycline in the results.

D. DNA extraction and PCR amplification

For DNA extraction, one fly was grinded in livak-100 ul then cooked at 65degrees for 20 minutes. 40ul of sodium acetate was added to the tube and left on ice for 20 minutes. The mixture was spun at 15000g for 10 minutes at 4 degrees. 100 ul of the supernatant was transferred to a new tube. 20ul of 100% ethanol was added to it and then centrifuged for 15 minutes at 4 degrees. After this step, the supernatant was discarded and the pellet was washed with 500ul of 70% ethanol to be followed by a quick 5 minutes spin at 20000g. Ethanol was discarded and the pellet was air dried at room temperature for 5 minutes. Finally, it was re-suspended in 40ul nano-pure double distilled water.

DNA was amplified using PCR amplification. 2ul of DNA was added to a mixture of 13ul nano-pure double distilled water, 2ul 10x buffer, 1ul MgCl2, 0.5ul dNTPs, 0.5 forward and 0.5 reverse primers of a *Wolbachia* specific primers (WSP primers), and 0.5ul of the Taq polymerase. Samples were kept at 95 degrees for 5 minutes then another 30 seconds, proceeded with 30 seconds at 53 degrees then 72 degrees for 2 minutes. Steps 2-4 were repeated 30 times. Step six was to keep them at 72 degrees for 5 minutes, and finally leave at 4 degrees for collection.

To visualize the *Wolbachia* infection status, samples then were run on a 1% agar-TBE1x gel containing 3.2ul eithidium bromide at 90mV for 40 minutes. The gel was then imaged using the BioRad chemiDoc.

E. RNA Extraction, Reverse Transcription and quantitative Real-Time PCR

RNA extraction was performed as follows: on average 15 flies were homogenized in 500 ul TRizol reagent (Invitrogen). Extracted RNA was separated from cellular components through centrifugation at a speed of 20000g for 20 minutes at 4 degrees. The pellet was discarded and 100 ul was added to the supernatant. The mixture was vortexed for a minute then a second step of centrifugation was performed under the same previous conditions. The aqueous phase was transferred to a new eppendorf to precipitate the RNA by 0.7V isopropanol followed by a third identical centrifugation step. The supernatant was discarded, and the RNA pellet was washed with 70% ethanol and re-suspended in 50ul

nanopure double distilled water. RNA was extracted to a concentration of 500ng/ul and 2ul were used for cDNA synthesis.

In the case of RNA extraction from ovaries, same protocol was followed with minor changes in the volume of reagents added: 250ul trizol initially, and 70ul chloroform was added, and finally 20ul nano-pure double distilled water was used to re-suspend the pellet. The concentration of RNA was diluted to 200ng/ul and then 5ul were used for cDNA synthesis.

cDNA was synthesized using a Qiagen cDNA synthesis kit. First genomic DNA was degraded out of the diluted RNA using the gDNA wipe-out mix by incubation at 42 degrees for 2 minutes. Synthesis then was followed by the addition of 1ul the Reverse transcriptase enzyme, 4ul of the enzyme mix and 1ul of the primer mix to the previous RNA mix. Synthesis took place for 25 minutes at 42 degrees, and then stopped by a two minutes incubation at 95 degrees.

5ul of the cDNA was added to a mixture of 0.5ul reverse and 05 ul of forward primers of the gene of interes, and QuantiFAST SYBR green PCR min (10ul, Qiagen). Placed in BioRad low profile strips and capped with ultraclear flat caps, they were subjected to 40 cycles of denaturation at 95 degrees, annealing of the primers at 60 degrees. Elongation of the transcript was performed at 72 degrees. Resultant construct levels were quantified at every cycle.

For the quantification of the anti-microbial peptide levels forward and reverse primers of Drosomycin and Diptericin were used. *Wolbachia* level in the different altered

immunity strains was quantified using forward and reverse primers of the *Wolbachia* FtsZ protein. Candidate genes hypothesized to have differential expression levels due to manipulations by *Wolbachia* have been quantified using forward and reverse primers of the candidate genes. In all cases levels were normalized to the *Drosophila* ribosomal protein rp49 using the delta Ct method. Student t-test was then performed for statistical analysis. Unless otherwise stated, each real-time analysis has been done with three replicates (n=3).

F. Microbe preparation

The bacterial cultures, in LB, were grown at 37 degrees overnight in a shaker. Then, they were exposed to spinning at 4000g for 10 min, after which the cell pellet was re-suspended in LB, optical density measured by a spectrophotometer at a wavelength of 595nm, then diluted to the specific OD needed for infection. Fungal species were grown on PDA plated for a three weeks' time. This was proceeded by spore collection, filtration and appropriate adjustment to the needed spore concentration (spores/nl).

G. Infection of *Drosophila* and survival analysis

Fifteen female flies of both strains Or and Or-tet were collected at the age of 3-5 days and injected with 32.2nl of the following microbes: S.aurea OD… This was done through a nano-injector supported by a capillary needle. After injection flies were returned to vials with normal cornmeal *Drosophila* food to an incubator at 25 degrees. To perform survival analysis, dead flies were counted twice per day. Percent survival versus time were

then plotted using GraphPad Prism Software, and statistical significance was through Cox Regression Analysis. In case RNA extraction was intended after infection, flies were frozen at -80 degrees one or two days post infection in the case of bacterial or fungal nano-injection respectively.

H. Insect Survey

Insects were collected from different areas of Lebanon, labelled and photographed. The pictures were sent to Dr. Knio for taxonomic identification. The samples were stored at -20 degrees until DNA extraction was performed through the Livak method. *Wolbachia* infection status (positive or negative) was determined through PCR amplification with WSP primers as discussed later. All positive strains were prepared for sequencing to determine exact *Wolbachia* strain for future phylogenetic analysis. This was done by PCR amplification of the positive template and DNA purification.

I. Altered immunity strains development and *Wolbachia* fitness testing

To check the regulatory mechanisms employed by the host to regulate the levels of *Wolbachia*, the bacterium has been introduced through genetic crosses into different host immune backgrounds. This was possible though the UAS-gal4 system in which we have used a fat body specific driver:C565. The following strains have been developed: strain of elevated immunity: UAS Relish-C654 gal4 and UAS toll-C564 gal4. Strains of suppressed immunity: Relish mutant, Dif mutants, autophagy suppressed strains: UAS atg8a IR-C564

gal4 and UAS atg13 IR-C564 gal4, and UAS Nitric Oxide Synthase (NOS) IR- C564 gal4 and Black Cell mutants with a mutation in the phenol oxidase gene (Neyen *et al* 2014).

The autophagy 8a and 13 IR lines have been chosen after a selection from different lines present in our lab. All available strains were previously crossed with C564 driver followed by survival analysis after pathogenic infection with *Erwinia carotovora carotovora.* Both strains were tested as positive compared to the control mutants indicating functional knock down of those genes.

In the case of the other strains, functional activity was tested through RNA extraction and real time PCR using drosomycin or diptericin levels compared to control flies to check upregulation in the case of UAS Relish- C564 gal4 strain or downregulation in the other strains. This real-time has been performed once. Levels of AMPs were compared to the W strain (W1118) control strain having a genetically similar background to the above strain but with an intact immune background.

The BC mutant phenotype flies were confirmed by the presence of naturally occurring melanization spots even in the absence of infection (Govind 2008).

DNA extraction followed by PCR analysis to ensure the colonization of *Wolbachia* into the ovaries was performed.

After that, altered immunity strains containing *Wolbachia* were exposed to RNA extraction reverse transcription and Real-Time PCR using *Wolbachia* FtsZ primers to quantify the levels of the bacterium. The levels of *Wolbachia* in all strains were compared to the control which is the wild type Org strain, naturally harboring *Wolbachia*.

J. Candidate genes differential regulation

A set of 13 different genes involved in *Drosophila* meiosis have been chosen to check their expression levels in infected and control flies. Those genes are: the chromatin linked proteins histone H3, histone H4, Protamine A, Protamine B and protamine like 99c. The chromatin remodeling proteins: histone deacytelase, histone acetyl- transferase, ISWI and ACF1 (ito *et al* 1999). Female meiosis regulator proteins Cortex and Grauzone. As well as the two centromere linked proteins Cid and Hira.

RNA was extracted as described from whole flies of both control and infected groups followed by reverse transcription and real time analysis using primers of the respective genes.

K. Primers Sequence

Targeted genes were search on Fly Base in order to get their coding sequence. Then using Primer3 software, primers were designed. For Real-time PCR, primers were intended to have %CG of about 50%, size of around 20bp and amplifying an amplicon of 200 nucelotides maximum.

Primer Used for PCR amplification of *Wolbachia* band

wsp81F	5' TGGTCCAATAAGTGATGAAGAAAC 3'
wsp691R	3' AAAAATTAAACGCTACTCCA 5'

Table 1: Table showing the respective sequences of the primers used in PCR experiment

Primers used in Real time PCR

WftsZ-F	5’ TGATGCTGCAGCCAATAGAG 3’
WftsZ-R	5’ TCAATGCCAGTTGCAAGAAC 3’
Dpt-F	5’ GCTGCGCAATCGCTTCTACT 3’
Dpt-R	5’ TGGTGGAGTGGGCTTCATG 3’
Drs-F	5’ CGTGAGAACCTTTTCCAATATGATG 3’
Drs-R	5’ TCCCAGGACCACCAGCAT 3’
rpl32-F	5’ GACGCTTCAAGGGACAGTATCTG 3’
rpl32-R	5’ AAACGCGGTTCTGCATGAG 3’
ACF1-F	5’ GAGCTATCCGTTCTGCGTTC 3’
ACF1-R	5’ CAAGCGCATCACTTCGTTTA 3’
Cid-F	5’ AATCGCTATCCCACAACCAG 3’
Cid-R	5’ CGGATCTCACGATCCATTCT 3’
CortexF	5’ TCCTACCGCAAGCTGTTCTT 3’
CortexR	5’ TATGGGTGGGAGCAAACTTC 3’
GrauzoneF	5’ ACCATGTTCCAGACGAGGAG 3’
GrauzoneR	5’ GTACTCCGTGGCAAAAGCAT 3’
Hira-F	5’ AATGGTCAGAACCTGGCATC 3’
Hira-R	5’ TAGACATCGTTGGGTGACCA 3’
his4-F1	5’ AAACTCGCGGTGTGCTAAAG 3’
his4-R1	5’ TAACCGCCAAATCCGTAAAG 3’
histone acetyltransferaseF	5’ CTGAAACTCAATCCCCTCCA 3’
histone acetyltransferaseR	5’ AAGCGCTCCATTTTCTTCAA 3’

histone deacytelase-F	5’ TGGGCTCTATCGAAAAATGG 3’
histone deacytelase-R	5’ ATCTTCGCCGACATTGAAAC 3’
ISWI-F	5’ CACCCGCCTACATCAAAAGT 3’
ISWI-R	5’ GATGACGATGTGTGGTCCAG 3’
ProtamineB-F	5’ AGTCAGAAGTGCAGCAAGCA 3’
ProtamineB-R	5’ ATCCGGCGGTATCTATCCTT 3’
Protamine like 99c-F	5’ TGCAGATCAGCAAAGAATCG 3’
Protamine like 99c-R	5’ AGTTGCCGTCTTCAGCAGAT 3’
Protamine A-F	5’ AAGCCAATGAAGTCCTGTGC 3’
Protamine A-R	5’ CGCGGTTTCAAGTTACAGTG 3’

Table 2: Table showing the respective sequences of the different primers used in Real-time PCR

CHAPTER III

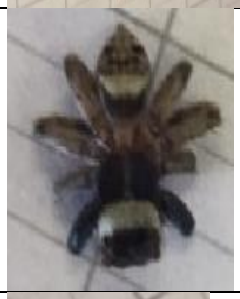
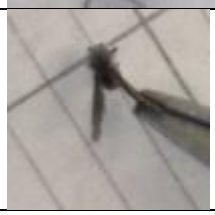
RESULTS

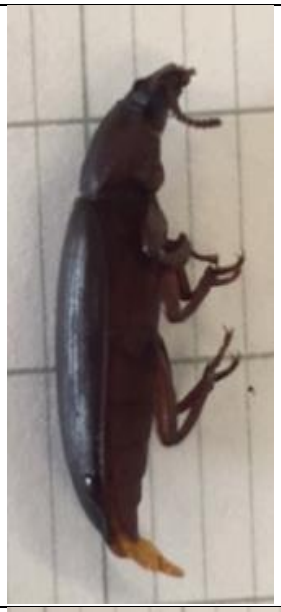
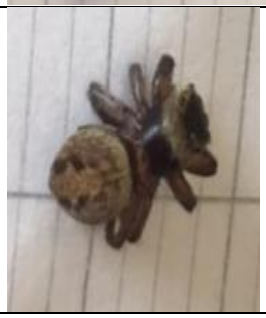
A. Insect Survey

Following are the results of the *Wolbachia* infection status analysis across different Lebanese insect species.

	Subphylum: Uniramia Class: Insecta Order: Diptera Family: Syrphidae *Scaeva selenitica* (Meigen, 1822)	*Wolbachia* Negative
	Subphylum: Uniramia Class: Insecta Order: Diptera Family: Bibionidae *Bibio* sp (Geoffroy, 1762)	*Wolbachia* Positive
	Subphylum: Uniramia Class: Insecta Order: Diptera Family: Tephritidae *Tephritis* sp (Latreille, 1804)	*Wolbachia* Negative
	Subphylum: Uniramia Class: Insecta Order: Hemiptera Family Pyrrhocoridae	*Wolbachia* Negative

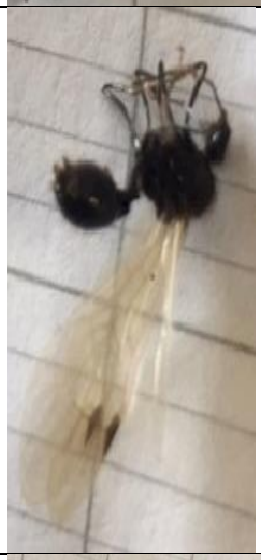
	Subphylum Crustacea class Malacostraca Order Isopoda suborder Oniscidea wood louse	*Wolbachia* Negative
	Subphylum: Uniramia Class: Insecta Order: Coleoptera Family Chrysomelidae *Cassida* sp (Linnaeus, 1758)	*Wolbachia* Negative
	Subphylum: Uniramia Class: Insecta Order: Diptera Family: Phoridae	*Wolbachia* Negative
	Subphylum: Uniramia Class: Insecta Order: Lepidoptera Family Arctiidae *Ocnogyna* sp. (Lederer, 1853)	*Wolbachia* Positive
	Subphylum: Uniramia Class: Insecta Order: Hemiptera Family Lygaeidae *Spilostethus pandurus* (Scopoli, 1763)	*Wolbachia* Negative

		Subphylum: Uniramia Class: Insecta Order: Coleoptera Family: Carabidae	*Wolbachia* Negative
		Subphylum: Uniramia Class: Insecta Order: Hymenoptera Family: Apidae	*Wolbachia* Negative
		Subphylum: Uniramia Class: Insecta Oder: Diptera Family: Tipulidae	*Wolbachia* Negative
		Subphylum: Chelicerata Class: Arachnida Order: Aranea Family: Thomisidae	*Wolbachia* Negative
		Subphylum: Uniramia Class: Insecta Order: Diptera Family: Psychodidae	*Wolbachia* Negative

	Subphylum: Uniramia Class: Insecta Order: Coleoptera Family: Tenebrionidae	*Wolbachia* Negative
	Subphylum Chelicerata Class Arachnida; Order Aranea	*Wolbachia* Positive
	Subphylum Chelicerata Class Arachnida; Order Aranea	*Wolbachia* Negative

	Subphylum: Uniramia Class: Insecta Order Neuroptera: Family Chrysopidae lacewing	*Wolbachia* Positive
	Subphylum: Uniramia Class: Insecta Order Lepidoptera Family Geometridae	*Wolbachia* Positive
	Subphylum: Uniramia Class: Insecta Order Lepidoptera Family Pyralidae *Pyralis* sp. (Linnaeus, 1758)	*Wolbachia* Positive
	Subphylum: Uniramia Class: Insecta Order Diptera Family Muscidae *Musca* sp. (Linnaeus, 1758)	*Wolbachia* Positive

	Subphylum: Uniramia Class: Insecta Order Hemiptera Family Pentatomidae	*Wolbachia* Negative
	Subphylum: Uniramia Class: Insecta Order Hemiptera Family Pentatomidae Nymph (immature)	*Wolbachia* Negative
	Subphylum Uniramia Class Diplopoda (millipede)	*Wolbachia* Negative
	Subphylum: Uniramia Class: Insecta Order: Coleoptera Family: Scarabeidae	*Wolbachia* Negative

	Subphylum: Uniramia Class: Insecta Order: Diptera	*Wolbachia* Positive
	Subphylum: Uniramia Class: Insecta Order: Hymenoptera	*Wolbachia* Positive
	Subphylum: Uniramia Class: Insecta Order: Lepidoptera Family: Noctuidae	*Wolbachia* Negative

	Subphylum: Uniramia Class: Insecta Order: Diptera Family: Drosophilidae *Drosophila melanogaster* (Meigen, 1830)	*Wolbachia* Positive
	Subphylum: Uniramia Class: Insecta Order: Diptera Family: Culicidae *Culex pipiens* (Linnaeus, 1758)	*Wolbachia* positive
	Subphylum: Uniramia Class: Insecta Order: Diptera Family: Culicidae *Aedis albopictus* (Skuse, 1894)	*Wolbachia* positive

Table 3: Table showing the different insects collected with their respective pictures, taxonomic identifications and *Wolbachia* infection status.

A total of 31 different insect species have been screened for their *Wolbachia* infection status out of which 12 have been tested as positive (38.7%) and 19 tested as negative (61.3%).Those were divided between three subphyla: Uniramia, Crustacea and Chelicerata.

Under the subphylum Uniramia, n=27 insect species where tested, of which 12 (44.4%) tested as positive and 15 (55.6%) tested as negative. Under this subphylum, two classes were screened, Class Insecta, n=26, 11 positive and 15 negative, and Class Diplopoda n=1 negative. Six orders fell under the screened class Insecta: Order Diptera

n=11 belonging to 10 different families out of which 6 tested positives and 5 tested negative, Order Hemiptera n=3 negatives belonging to 3 different families, Order Lepidoptera with n=4, 3 positives and 1 negative, belonging to 4 different families, Order Coleoptera with n=4 belonging to 4 different families all of which tested negative, Order Hymenoptera with n=2 one positive and one negative each belonging to a different family, and the Order Neuroptera with 1 positive insect under the family Chrysopidae.

Under subphylum Crustacea one specimen tested as negative belonging to the Class Malacostracea, Order Isopoda of the collected insects.

Under Subphylum Chelicerata, 3 specimens where collected belonging to the class Arachnida, Order Aranea, 2 of which testes negative, and one was positive harboring *Wolbachia*.

Taxonomically, they were divided according to the following scheme:

Subphylum	Class	Order	Family
Uniramia	Insecta	Diptera	Syrphidae *Scarea selentica* Negative n=1
			Bibionidae *Bibio* sp Positive n=1
			Tephritidae *Tephritis* sp Negative n=1
			Phoridae Negative n=1
			Tipulidae Negative n=1
			Psychodidae Negative n=1
			Muscidae *Musca* sp.

			Positive n=1
			TO BE IDENTIFIED Positive n=1
			Drosophilidae *Drosophila melanogaster* Positive n=1
			Culicidae Positive n=2 *Culex pipiens* *Aedes albopictus*
		Hemiptera	Pyrrhocoridae Negative n=1
			Lygaeridae *Spilostethus pandurus* Negative n=1
			Pentatonidae Negative n=1
		Lepidoptera	Arctiidae *Ocnogyna* sp Positive n=1
			Geometridae Positive n=1
			Pyralidae *Pyralis* sp Positive n=1
			Nocturidae,negative, n=1
		Coleoptera	Chrysomelidae *Cassida* sp. Negative n=1
			Carabidae Negative n=1
			Tenebrionidae Negative n=1
			Scarabidae Negative n=1
		Hymenoptera	Apidae Negative n=1
			TO BE IDENTIFIED Positive n=1
		Neuroptera	Chrysopidae Lacewing Positive n=1

	Diplopoda (Millipede) Negative n=1		
Crustacea	Malacostracea	Isopoda Suborder: Orniscidea Wood louse Negative n=1	
Chelicerata	Arachnida	Aranea	Thomisidae Negative n=1
			TO BE IDENTIFIED Negative n=1
			TO BE IDENTIFIED Positive n=1

Table 4: Table of the taxonomic distribution of the different screened insects

B. *Wolbachia* free strain

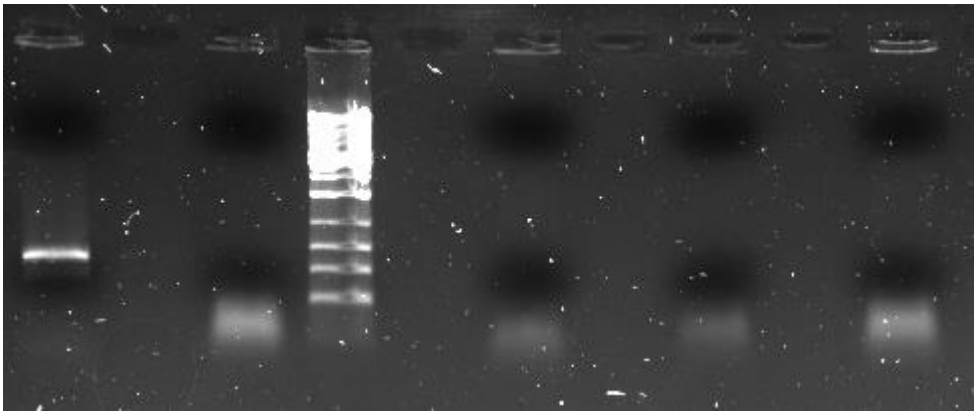


Figure 1: Image showing results of Gel electrophoresis done for Org-tet *Wolbachia* free status confirmation

The first sample to the left is the Org sample showing the *Wolbachia* band indicating it being positive for *Wolbachia*. The four other samples, one before the ladder and three after, lack this band indicating their *Wolbachia*-negative status.

C. Survival Analysis

Following are the survival curves resulting from infection of both strains org and org-tet compared to the mutant controls.

BB= *Beauvaria bassiana;* AN: *Aspergillus nomius*; ECC: *Erwinia carotovora carotovora*; EF*: Enterococcus feacalis*; SA: *Staphylococcus aureas*; Serratia: *Serratia marscecens*

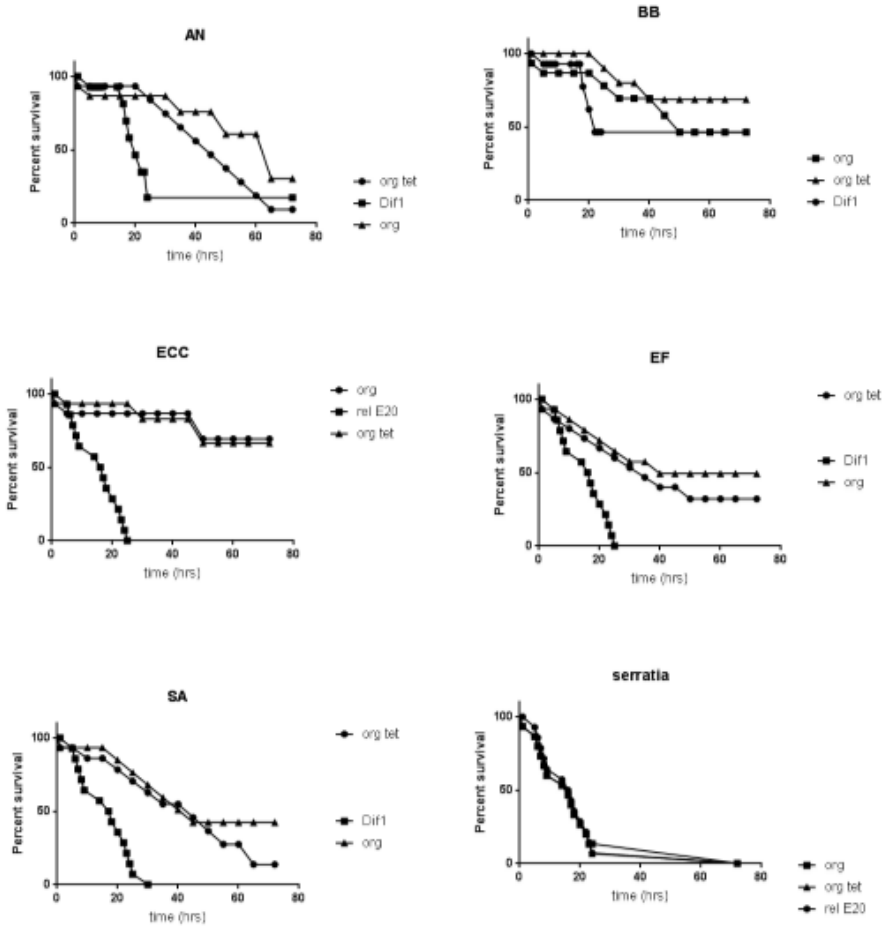


Figure 2: Graph showing survival curves of both strains org and org t*et al* ong with the positive controls of the six different injected microorganisms.

Overall, no microorganism showed a difference in the survival between the two strains Org and Org-tet upon systemic infection.

Two gram-negative bacteria were injected: ECC and Serratia. For ECC, their was no significant difference in the survival analysis between the two strains org and org-tet upon Cox regression (p-value 0.99 greater than 0.05 with the 95% confidence interval chosen for analysis). Nevertheless, both strains tested positive and survived better than the mutant Rel E20 (p-values <0.0001 for both strains). In serratia, a similar result was shown whereby both strains survived similarly upon infection (p-value 0.841), but they died as severely as the positive control (p-values 0.99 and 0.83 for the org and org-tet strains respectively compared to the control survival).

The gram positive bacterium EF did not show a difference in killing any of the two strains upon infection (p-value 0.45), but killed the positive control significantly more than both strains org and org-tet (respective p-values: 0.0003 and 0.0011). A similar scheme was shown upon the infection of the other gram-positive bacterium SA: p-value comparing survival of org and org-tet (0.457), p-value comparing org and org-tet survival to the control (<0.0001 and 0.0002 respectively).

Upon BB fungal infection, both strains survived similarly (p-value 0.3112). The Org strain survived similar to the negative control (p-value 0.436), yet the Org-tet survival showed a slight statistically significant enhanced survival compared to the Dif1 mutant with a p-value of 0.039. In AN fungal infection, just like BB infection, both strains had similar lethality (p-value 0.18). Nevertheless, both strains died similar to the control with p-values of org and org-tet being 0.059 and 0.139 respectively.

D. AMP quantification

The levels of both Anti-microbial peptides, Drosomycin and Diptericin did not show any significant difference at the level of the whole flies when comparing Org and Org-Tet females. The insignificance was similar at basal levels without immune induction, and upon infection of both a gram-negative ECC bacterium as well as a gram-positive EF bacterium.

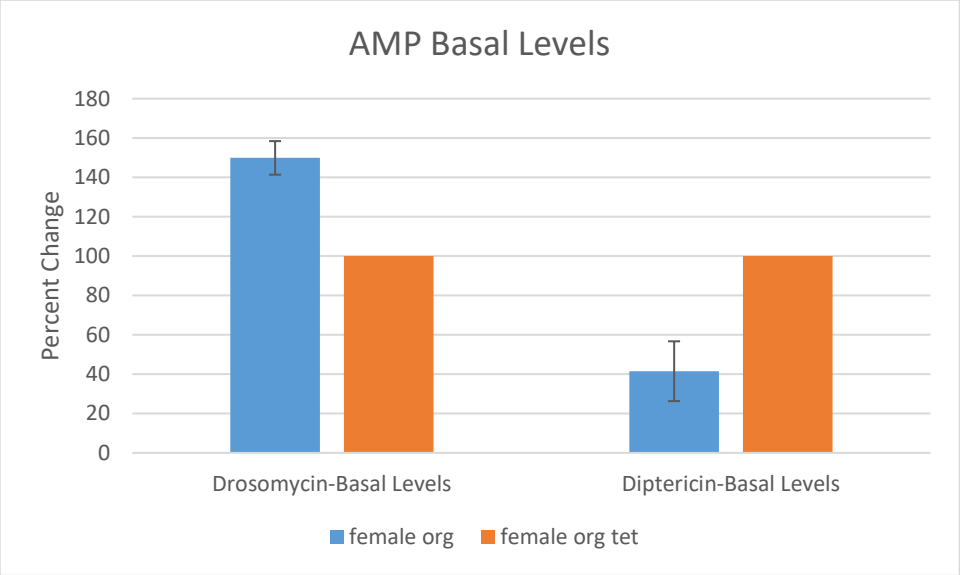


Figure 3: Histogram comparing the levels of Drosomycin and Diptericin between Org and Org-tet flies at basal levels. P-value Drosomycin analysis: 0.08, P-value Diptericin analysis: 0.41

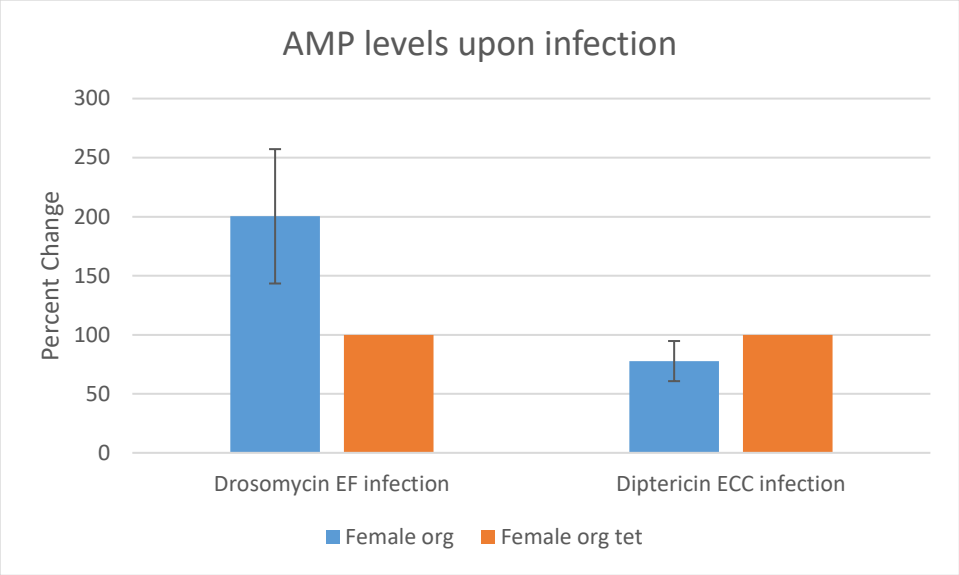


Figure 4: Histogram showing the levels of anti-microbial peptides Drosomycin and Diptericin upon infection in both Org and Org-Tet strains. P-value Drosomycin analysis: 0.27, P-value Diptericin analysis: 0.50

When comparing the levels of Anti-microbial peptides Drosomycin and Diptericin locally at the level of ovaries, where *Wolbachia* mainly resides, the results where different. The levels of both AMPs where significantly much higher in Org flies, harboring *Wolbachia*, than the Org-tet females, free of *Wolbachia*.

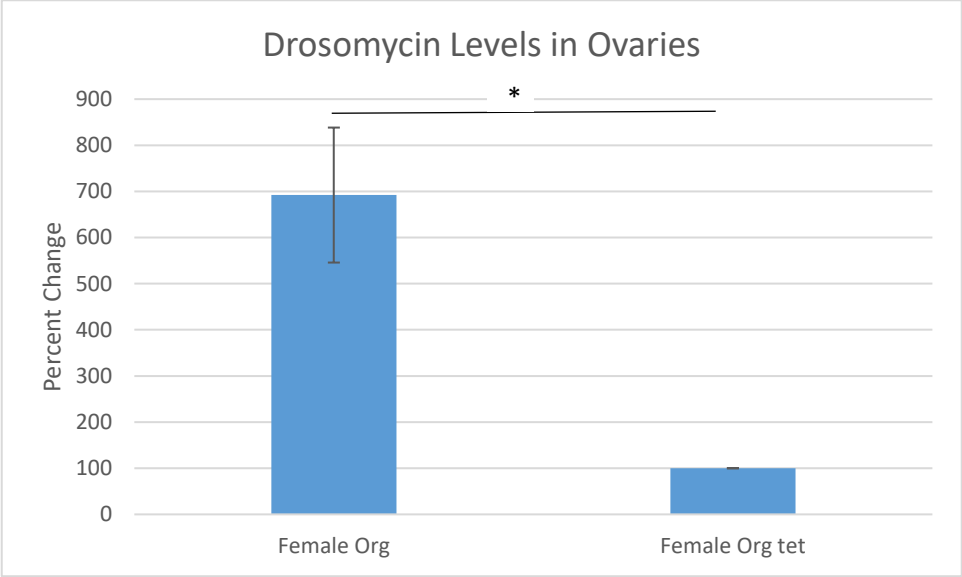


Figure 5: Histogram showing the levels of Drosomycin in the ovaries of both Org and Org-tet females. * indicates significant p-value (<0.05). P-value 0.03

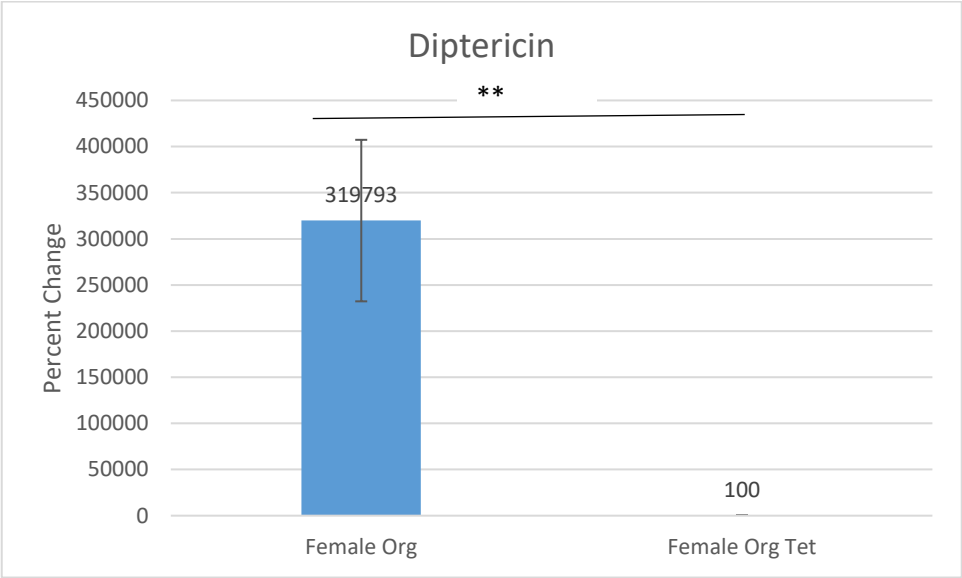


Figure 6: Histogram showing the levels of Diptericin in the ovaries of both Org and Org-tet females. ** indicates highly significant p-value (<0.01). p-value: 0.008

E. Building the altered immunity strains harboring *Wolbachia*

Following are the results of the survival curves of the different IR RNAi strains of different autophagy-related proteins crossed to the C564 gal4 driver.

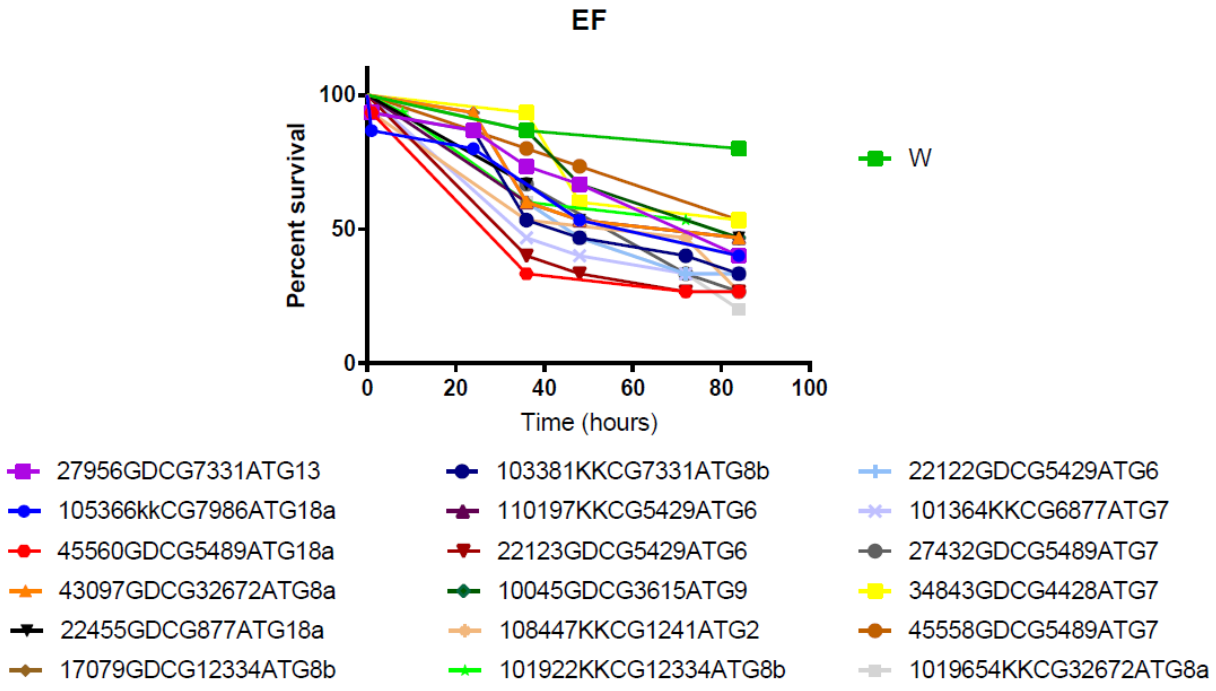


Figure 7: Survival curve showing the change of the percentage of surviving *Enterococcus feacalis* infected flies of the respective genetic background: ATG IR-RNAi- C564 gal4 over time.

Out of those, the two autophagy-linked proteins ATG13 and ATG8a have been chosen and checked for being positive compared to the control. Both survival curves have tested positive compared to the control with a p-value of 0.027 and 0.0009 respectively.

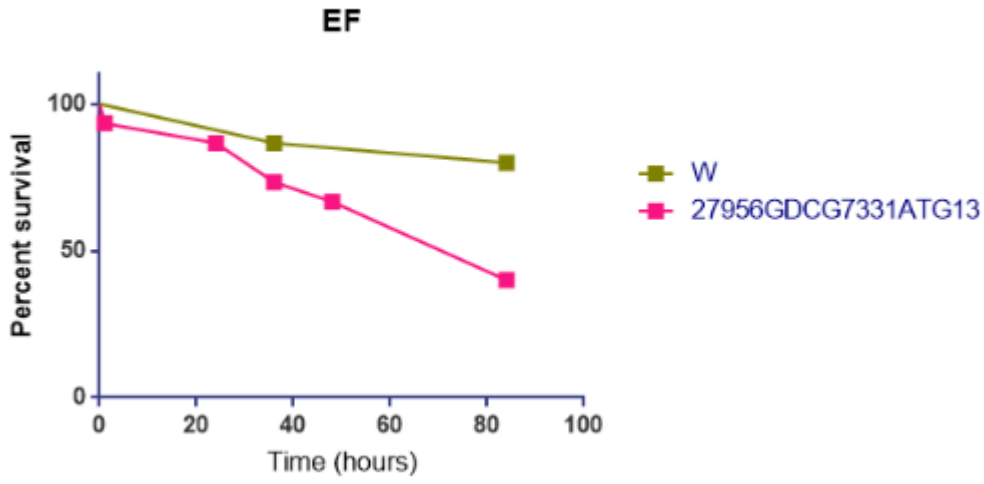


Figure 8: Survival curve showing the change of the percentage of surviving *Enterococcus feacalis* infected flies of the respective genetic background: ATG13 IR-RNAi- C564 gal4 over time. P-value: 0.027

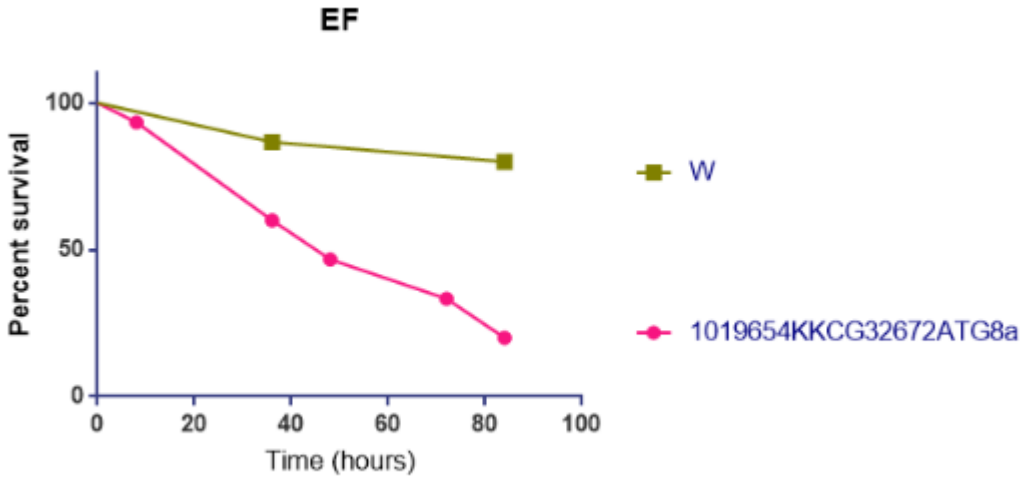


Figure 9: Survival curve showing the change of the percentage of surviving *Enterococcus feacalis* infected flies of the respective genetic background: ATG8a IR-RNAi- C564 gal4 over time. P-value: 0.00009

Upon generating the strain over activating the toll pathway, lethality has occurred in most of the flies of the genetic background UAS toll-C564 gal4, and only very

few melanized flies have escaped this lethality, weak and not sufficient to proceed with the genetic crosses.

Figure 10: An image showing flies of the genetic background UAS toll-C564 gal4 with the melanization spot pointed at with a black arrow.

A similar result as figure 2 has been shown in the BC mutant phenotype confirming the genetic background.

Flies mutant for Dif (Dif1 flies), showed low levels of Drosomycin upon quantification by real-time PCR compared to the control, W flies.

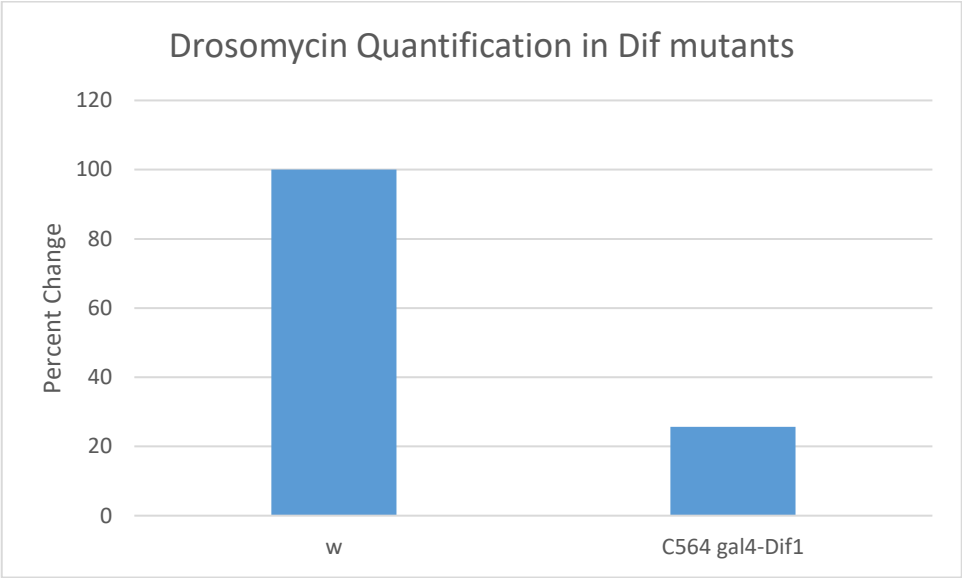


Figure 11: Histogram showing Drosomycin quantification in Dif mutant flies compared to the control.

For the flies of altered ImD pathway activity, both strains overexpressing and suppressing the ImD pathway have been tested for functionality through Real-time PCR, quantifiying the levels of anti-microbial peptide Drosomycin produced. Successful functionality of both strains have been confirmed from the upregulated Drosomycin production in the UAS Relish-C564 gal4 (approximately more than doubling) and a decrease to less than half in the Rel E20 mutant, both compared to the control W flies.

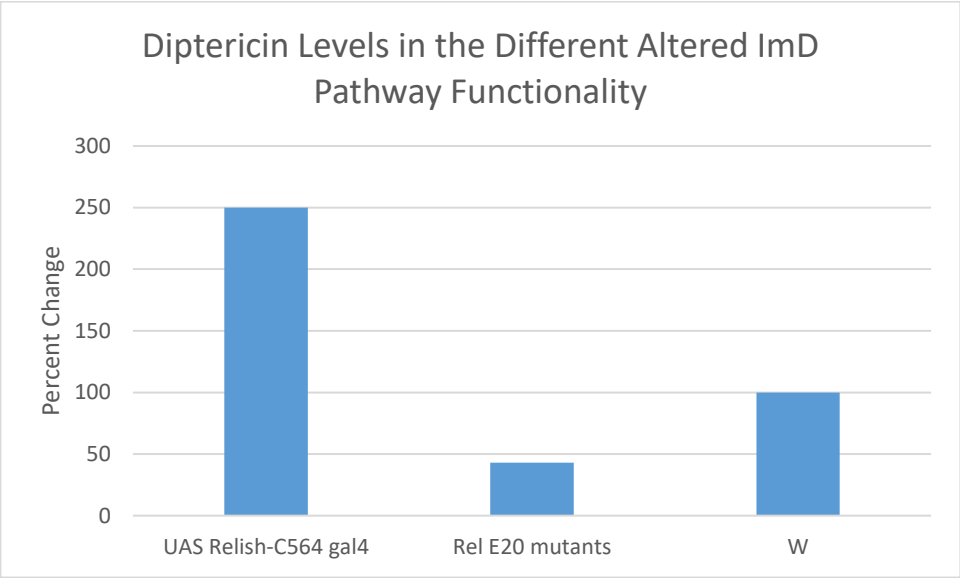


Figure 12: Histogram showing the Diptericin production of the two altered ImD pathway activity compared to that of the control through Real-time PCR quantification results.

The IR-RNAi strain of the Nitric Oxide Synthase enzyme has been used as a positive control when crossed with C564 gal4 driver in several infection experiments in our lab, therefore, its functionality did not have to be further validated.

After confirming functional activity, colonization of *Wolbachia* to the ovaries was confirmed in all strains through the presence of the *Wolbachia* band in all six strains used, shown in the image below.

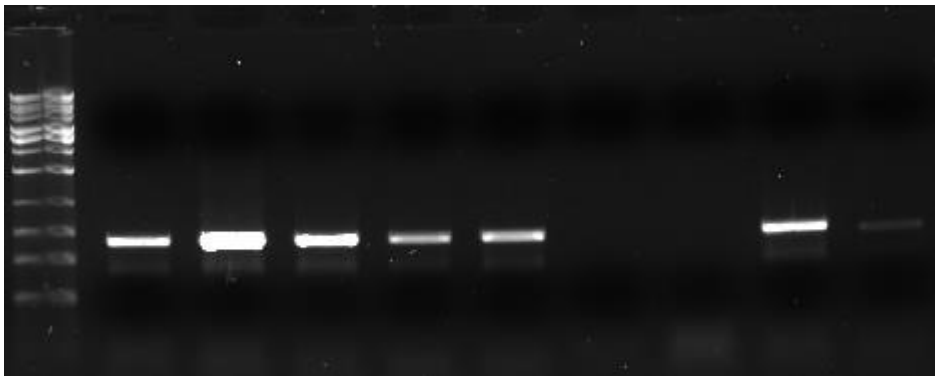


Figure 13: A gel image showing positive samples of the strains: from the ladder, the strains are: C564 gal4 UAS Relish; C564 gal4 UAS NOS IR RNAi; C564 gal4 BC; C564 gal4 ATG 13 IR RNAi; C564 gal4 ATG8a IR RNAi; Negative Control Water; Negative Control Org-tet; Positive Control Org; C564 gal4 RelE20.

F. *Wolbachia* Quantification in the Different altered immunity strains

Following are the results of the *Wolbachia* quantification in different host immunity background:

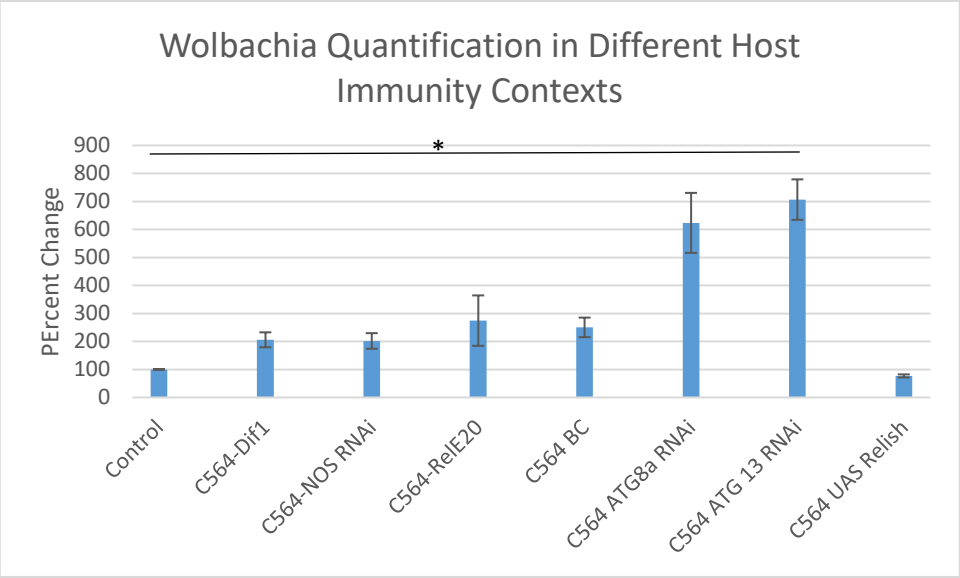


Figure 14: Histogram showing *Wolbachia* quantification in the different host immune backgrounds. P values of respective strains compared to control starting from C564-Dif1 to C564-UAS Relish: 0.52; 0.78; 0.91; 0.51; 0.54; 0.04; 0.43

Although there has been a trend in which levels of *Wolbachia* showed to be elevated in immune-suppressed strains (C564-Dif1; C564-NOS RNAi; C564 RelE20; C564 BC) and decreased levels in the strain that has increased immunity (C564 UAS Relish), those changes did not differ much from the control (Org wild type strain) and remained below statistical significance threshold.

Both strains with suppressed autophagy showed strikingly high levels of *Wolbachia* compared to the control, but only the ATG13 knock down strain had levels that reached statistical significance with p-value< 0.05 (p-value=0.048).

G. Candidate genes upregulation

The expression of 10 different genes probably involved in CI has been quantified in male Org flies and compared to that in Org-tet ones. The results are as shown in the below histogram.

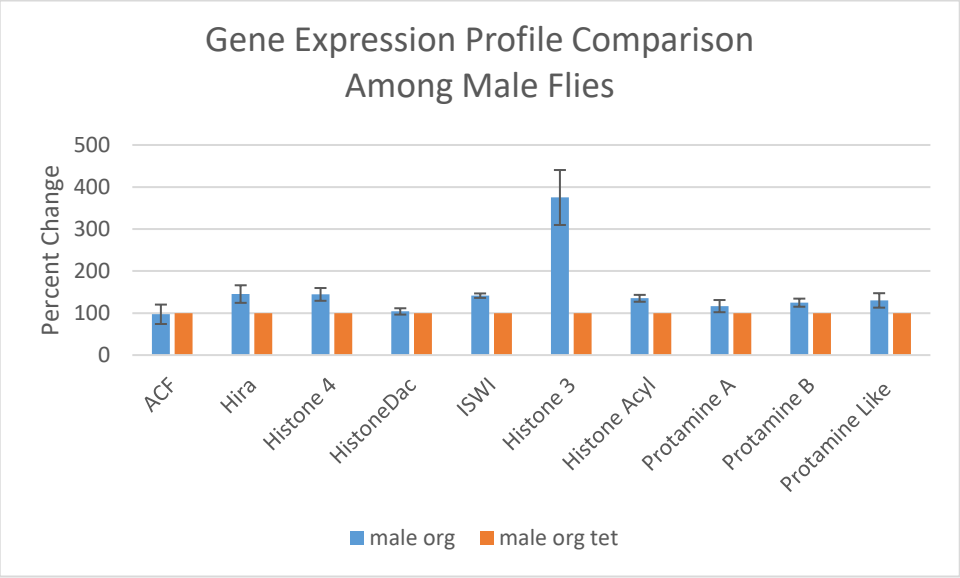


Figure 15: Histogram showing the expression levels of different genes in male Org flies compared to male Org-Tet Flies.
P-values starting ACF analysis to Protamine like 99c: 0.77; 0.8; 0.48; 0.91; 0.35; 0.13; 0.43; 0.97; 0.79; 0.68

Nine out of the ten genes did not show any difference in their expression levels and remained almost unchanged. Histone 3 only stood different and showed an increased level of expression in Org males harboring *Wolbachia*. Nevertheless, this increase did not reach statistical significance.

Ten genes have also been checked for expression in female flies, and a very similar result was shown.

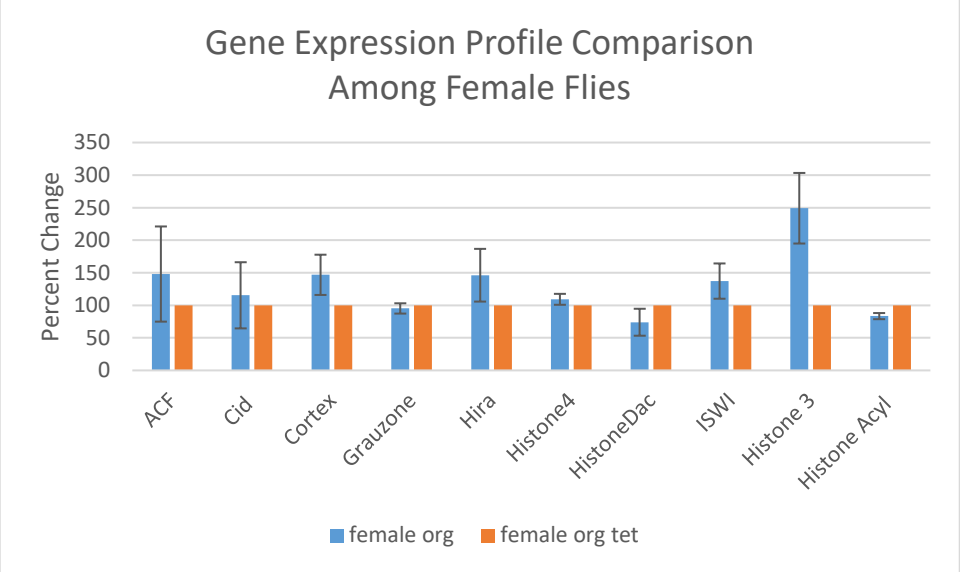


Figure 16: Histogram showing levels of ten different genes comparing their expression between female Org flies and female Org-tet flies.
P- values of analysis starting ACF to Histone Acetyl Transferase: 0.86; 0.47; 0.91; 0.68; 0.95; 0.68; 0.81; 0.89; 0.38; 0.39

As in the case of males, most of the genes did not show any significant differential expression between the two strains. As in males as well, Histone 3 levels portrayed elevated expression in Org females, yet not reaching the threshold of statistical significance either.

CHAPTER IV

DISCUSSION

In this study, we screened 31 different insect species and tested them for *Wolbachia* infection. We reported 14 out of those to be harboring *Wolbachia*. After this general screen, infection status was confirmed through PCR analysis followed by gel elelctrophoresis. We moved to study the fruit fly *Drosophila melanogaster* as a model organism for the endosymbiotic interaction with its natural endosmbiont *Wolbachia*. Before proceeding with any study on *Wolbachia,* we first generated a genetically similar model to the wild type (Org strain) but free of *Wolbachia* through tetracycline treatment, which we refer to as the Org-tet strain. We then moved on to start our comparative analysis dividing it into a scheme tackling the effect *Wolbachia* has on its host and how the host affects *Wolbachia* titers in its system and thus keeps it under control. To check the first part, survival analysis of both strains Org and Org-tet were compared upon six different microbial infections, of which all revealed no difference in survival. This was paralleled with a real-time PCR quantification for the levels of AMPs in the host at the level of the whole organism (both at basal levels and upon infection) as well as locally in the ovaries. Both AMPs had no significantly different levels the level of the whole organism neither at basal levels nor upon infection. This was different from the case at the level of the ovaries which revealed significantly higher levels of both AMPs in the Org strain. To check a potential manipulation of *Wolbachia* to its host transcriptome for cytoplasmic incompatibility induction, a set of ten different genes have been assessed for any

differential expression levels between both strains, among males and females, but all gave negative results with no much altered expression. To check how the host alters *Wolbachia*'s levels, different host immune backgrounds have been generated. The results came out to suggesting the involvement of the autophagy cell-mediated immune response in the control of *Wolbachia* titers in the host system.

The insect survey results revealed approximately 45% *Wolbachia* infection among the sample of insects collected from Lebanon. Nevertheless, the sample size of 31 is relatively small for a generalization of a *Wolbachia* infection status among Lebanese insects and has to be increased to at least a 100 to get a better idea. Still this result will have a great impact for paving the way for future research. Phylogenetic analysis could be conducted on those insects to check a possible bias between the transition rates of *Wolbachia* infection (gain of *Wolbachia*) and its absence (Loss of *Wolbachia*) which will give an idea about a potential preference in the overall pool of insects to harbor *Wolbachia* or get rid of it. Moreover, positive samples will be sent for sequencing to get the exact sequence of the *Wolbachia* strain present in each insect, to be followed by overlapping the host and the endosymbiont phylogenies to check potential correlated evolution. Whether *Wolbachia*'s cytoplasmic incompatibility effect could participate in speciation, by which mating preference for example could occur when non-infected females could prefer non-infected females only, can be addressed by generating a bioinformatics model for speciation with trying to plug in this effect and check if it could have an effect. Population genetics could also be done through collecting more samples of each positive insect and check what percentage of the members of each species is infected with *Wolbachia* and study it along

the geographical location. An important aspect of this part was to find potential insect candidates that tested positive for *Wolbachia* and could work as a model for *Wolbachia* research of which some could be better than the fruit fly in having more severe CI and thus more obvious effects can be evident.

We showed that *Wolbachia*'s infection is not affecting the survival of the *Drosophila* flies, neither negatively nor positively. We have extended evidence given in the literature for this effect by checking a broader range of microbes at the same time: two gram-positive bactera, two gram-negative bacteria and two fungi. Those results could mean one of two things either *Wolbachia* has coevolved with its fruit fly over millions of years and the relation changed into a mutualistic neutral effect, or that *Wolbachia* induces minor effects on its host and thus can't be observed at the broad organismal level. The AMP quantification levels came out to support the latter possibility. No difference in both AMP levels showed different levels among both strains Org and Org-tet at the organismal level. This result means that the endosymbiont is not altering its host system much at the basal levels, and that it does not help its host upon infection since no difference in the AMP levels was evident upon bacterial injections, thus, supporting the observations of the survival analysis. On the other hand, this significantly elevated levels of Drosomycin and Diptericin in the ovaries, where *Wolbachia* resides, gives evidence for minor alteration of the host system. *Wolbachia* could potentially be doing that to compete with other bacterial species so it maintains its existence in the ovaries, or it could be that the host is activating both of the immune pathways to keep *Wolbachia* levels under homeostatic control. The fact that overall no immune stimulation has occurred could be a selective advantage for the host

that could have been selected for in the course of coevolution of *Wolbachia-Drosophila* in order to minimize energy loss by the host for AMP production ubiquitously when it could be concentrated only in the ovaries. This could be looked into further by fluorescent microscopy through staining *Wolbachia* bacterial cells and check their counts. Then this could be correlated with possible differences that could be observed at the level of the ovaries' size or oocyte count.

The involvement of cell-mediated Autophagy in regulating *Wolbachia* is highly plausible from the elevated levels of *Wolbachia* obtained upon ATG8a and ATG13 knock downs. The involvement of ATG13 has more evidence because of the statistical significance observed in its case that was absent upon ATG8a knock down. This result has to be further looked into by checking *Wolbachia* counts through fluorescent microscopy which could also be linked to the elevated immunity present in the ovaries to check if this autophagy activated against *Wolbachia* as well as the elevated immunity there is affecting the ovaries' physiology. The results we got from the other strains are not decisive because the driver used was C564 gal4 which is a fat body specific driver. For further analysis, an ovary specific gal 4driver has to be used to build the same strains and then check *Wolbachia*'s levels.

The absence of any major difference in the transcription levels of the selected genes in the presence or absence of *Wolbachia* could be a supporting evidence for the lack of major CI in *Drosophila,* yet it cannot be conclusive. Proteomic modifications have to be always kept in mind especially after the recent results showing the presence of the DUB *Wolbachia* protein (cited in introduction). Nevertheless, if the same genes were

tested in a species that has more severe CI such as *Culex pipiens*, and the results showed to be differentially regulated, then the idea of transcriptomic modification could be correlated with severity of CI. It should be noted that the levels of mRNA of those genes have been quantified at the overall organism level, so it makes sense to repeat this real-time experiment and quantify the expression of those genes at the levels of ovaries and testis of the flies. The unique result of elevated histone 3 mRNA levels could be just a statistically and biologically insignificant result, or it could be confirmed if significant upregulation of this protein was shown at the levels of the gonads.

The major limitation that we have faced was the high standard error in real-time PCR experiments which was mainly due to the fact that we are using female flies for quantification which could have a lot of variability among them due to differences in the eggs each has and its fertility. The usage of females was necessary because *Wolbachia* is maternally transmitted, so checking the interaction at the maternal level would give more insight into the *Wolbachia*-host interaction. This could be the reason why statistical insignificance was not attained in the ATG8a knock out strain, therefore, future microscopic cell count of *Wolbachia* between control and the ATG8a knock out strain could help get over this issue. The difference in the efficiency of the RNAi system is something that cannot be controlled much and will normally occur and thus increase the variability among different flies as well.

References

Beckmann, J. F., Ronau, J. A., & Hochstrasser, M. (2017, September 1). A *Wolbachia* Deubiquitylating Enzyme Induces Cytoplasmic Incompatibility. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/28248294

Blattner, A. C., McKee, B. D., & Lehner, C. F. (2016). Separase Is Required for Homolog and Sister Disjunction during *Drosophila* melanogaster Male Meiosis, but Not for Biorientation of Sister Centromeres. Retrieved April 01, 2018, from http://journals.plos.org/plosgenetics/article?id=10.1371/journal.pgen.1005996

Brand, A. H., & Perrimon, N. (1993, June). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Retrieved April 15, 2018, from https://www.ncbi.nlm.nih.gov/pubmed/8223268

Carrington, L. B., Lipkowitz, J. R., Hoffmann, A. A., & Turelli, M. (2011). A Re-Examination of *Wolbachia*-Induced Cytoplasmic Incompatibility in California *Drosophila* simulans. *PLoS ONE*,*6*(7). doi:10.1371/journal.pone.0022565

Duron, O. (2008). Insights beyond *Wolbachia–Drosophila* interactions: Never completely trust a model: insights from cytoplasmic incompatibility beyond *Wolbachia–Drosophila* interactions. *Heredity*,*101*(6), 473-474. doi:10.1038/hdy.2008.113

Fabian, L,. & Brill, A. J. *Drosophila* spermiogenesis. (2012). Retrieved April 15, 2018, from http://www.tandfonline.com/doi/abs/10.4161/spmg.21798

Foley, E., & O'Farrell, P. H. (1970, January 01). Nitric oxide contributes to induction of innate immune responses to gram-negative bacteria in *Drosophila*. Retrieved April 15, 2018, from http://genesdev.cshlp.org/content/17/1/115.full

Fry, A. J., & Rand, D. M. (2002). *Wolbachia* Interactions That Determine *Drosophila* Melanogaster Survival. *Evolution*,*56*(10), 1976. doi:10.1554/0014-820(2002)056[1976:witddm]2.0.co;2

Fry, A. J., Palmer, M. R., & Rand, D. M. (2004). Variable fitness effects of *Wolbachia* infection in *Drosophila* melanogaster. *Heredity*,*93*(4), 379-389. doi:10.1038/sj.hdy.6800514

Glaser, R. L., & Meola, M. A. (2010). The Native *Wolbachia* Endosymbionts of *Drosophila* melanogaster and Culex quinquefasciatus Increase Host Resistance to West Nile Virus Infection. *PLoS ONE*,*5*(8). doi:10.1371/journal.pone.0011977

Govind (2008). Innate immunity in *Drosophila*: Pathogens and pathways. Retrieved April 15, 2018, from http://europepmc.org/articles/PMC2871703

Gupta V, Vasanthakrishnan RB, Siva-Jothy J, Monteith KM, Brown SP, Vale PF. 2017 The route of infection determines *Wolbachia* antibacterial protection in

Drosophila. Proc. R. Soc. B 284: 20170809. http://dx.doi.org/10.1098/rspb.2017.0809
Harcombe, W., & Hoffmann, A. (2004). *Wolbachia* effects in *Drosophila* melanogaster: in search of fitness benefits. *Journal of Invertebrate Pathology,87*(1), 45-50. doi:10.1016/j.jip.2004.07.003

Ilinsky, Y. (2013). Coevolution of *Drosophila* melanogaster mtDNA and *Wolbachia* Genotypes. Retrieved April 15, 2018, from http://journals.plos.org/plosone/article?id=10.1371/journal.pone.0054373

Ito, T., Levenstein, M. E., Fyodorov, D. V., Kutach, A. K., Kobayashi, R., & Kadonaga, J. T. (1999, January 01). ACF consists of two subunits, Acf1 and ISWI, that function cooperatively in the ATP-dependent catalysis of chromatin assembly. Retrieved April 15, 2018, from http://genesdev.cshlp.org/content/13/12/1529.full

Kambris, Z., Cook, P. E., Phuc, H. K., & Sinkins, S. P. (2009, October 2). Immune activation by life-shortening Wolbachia and reduced filarial competence in mosquitoes. *Science,326*(5949). Retrieved from http://science.sciencemag.org/content/326/5949/134.long

Kambris, Z., Blagborough, A. M., Pinto, B., Blagrove, M. S., Godfray, H. C., Sinden, R. E., & Sinkins, S. P. (2010). *Wolbachia* Stimulates Immune Gene Expression and Inhibits Plasmodium Development in Anopheles gambiae. Retrieved April 15, 2018, from http://journals.plos.org/plospathogens/article?id=10.1371/journal.ppat.1001143

Kremer, N., Voronin, D., Charif, D., Mavingui, P., Mollereau, B., & Vavre, F. (2009). *Wolbachia* Interferes with Ferritin Expression and Iron Metabolism in Insects. *PLoS Pathogens,5*(10). doi:10.1371/journal.ppat.1000630

LePage, D. P., Jernigan1, K. K., Bordenstein, S. R., G, B., L., B., . . . Neill SLO. (2014, December 09). The relative importance of DNA methylation and Dnmt2-mediated epigenetic regulation on *Wolbachia* densities and cytoplasmic incompatibility. Retrieved April 15, 2018, from https://peerj.com/articles/678/

Martinez, J., Longdon, B., Bauer, S., Chan, Y., Miller, W. J., Bourtzis, K., . . . Jiggins, F. M. (2014). Symbionts Commonly Provide Broad Spectrum Resistance to Viruses in Insects: A Comparative Analysis of *Wolbachia* Strains. *PLoS Pathogens,10*(9). doi:10.1371/journal.ppat.1004369

Neyen, C., Binggeli, O., Roversi, P., Bertin, L., Bou Sleiman, M., & Lemaitre, B. (2014, December 24). The Black cells phenotype is caused by a point mutation in the *Drosophila* pro-phenoloxidase 1 gene that triggers melanization and hematopoietic defects. Retrieved April 16, 2018, from https://www.sciencedirect.com/science/article/pii/S0145305X14003206

Onischenko, E. A., Gubanova, N. V., Kiseleva, E. V., & Hallberg, E. (2005). Cdk1 and Okadaic Acid-sensitive Phosphatases Control ... Retrieved April 15, 2018, from

http://www.bing.com/cr?IG=6C7686E0375B4A15909B14EAE4DDDBA5&CID=05C6CA52B82B661D37AEC180B9846768&rd=1&h=8EBPzufmmPmHNMgrb6EBDMMNg08zj3ZCMsh2lF30ASY&v=1&r=http://www.molbiolcell.org/content/16/11/5152.full.pdf&p=DevEx,5067.1

Pan, X., Pike, A., Joshi, D., Bian, G., McFadden, M. J., Lu, P., . . . Xi, Z. (2017). The bacterium *Wolbachia* exploits host innate immunity to ... Retrieved April 15, 2018, from https://www.bing.com/cr?IG=37D6CC109FF9497DA9EE97A37AAFC91A&CID=1B5FDEBBF20F6F3D1BADD568F3A06E7C&rd=1&h=W1BhPupMrttbSzhpXn5dkNDXDU_ijlpHJO8_ulBRaio&v=1&r=https://www.researchgate.net/publication/320837123_The_bacterium_*Wolbachia*_exploits_host_innate_immunity_to_establish_a_symbiotic_relationship_with_the_dengue_vector_mosquito_Aedes_aegypti&p=DevEx,5066.1

Pinto, B., Stainton, K., Harris, S., Kambris, Z., Sutton, E. R., Bonsall, M. B., . . . Sinkins, S. P. (2013). Transcriptional Regulation of Culex pipiens Mosquitoes by *Wolbachia* Influences Cytoplasmic Incompatibility. Retrieved April 16, 2018, from http://journals.plos.org/plospathogens/article?id=10.1371/journal.ppat.1003647

Poinsot, D., Bourtsiz, K., Markakis, G., Savakis, C., & Merc¸ot, H. (1998). *Wolbachia* Transfer from *Drosophila* melanogaster into D. simulans: Host Effect and Cytoplasmic Incompatibility Relationships Denis Poinsot,*,1 Kostas Bourtzis,†,‡,1 George Markakis,§ Charalambos Savakis†,**. *Genetics*. Retrieved from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1460311/.

Ponton F, Wilson K, Holmes A, Raubenheimer D, Robinson KL, Simpson SJ. 2015 Macronutrients mediate the functional relationship between *Drosophila* and *Wolbachia*. Proc. R. Soc. B 282: 20142029. http://dx.doi.org/10.1098/rspb.2014.2029

Raychaudhur, N., Dubruille, R., Orsi, G. A., Bagheri, H. C., Loppin, B., & Lehner, C. F. (2012). Transgenerational Propagation and Quantitative Maintenance ... Retrieved April 15, 2018, from https://www.bing.com/cr?IG=3A10A0A9B4AD48B4819BBE09A0DD69F1&CID=0E68ADEED9D968C018C6A63DD87669FE&rd=1&h=0YZi49vOMvZPp-3nCiZ8iczzZ-CkAjdZs2jBCzYu3D4&v=1&r=https://www.researchgate.net/profile/Nitika_Taneja2/publication/234089974_Transgenerational_Propagation_and_Quantitative_Maintenance_of_Paternal_Centromeres_Depends_on_CidCenp-A_Presence_in_*Drosophila*_Sperm/links/54c6eeec0cf289f0cecc9e20.pdf?inViewer=true&pdfJsDownload=true&disableCoverPage=true&origin=publication_detail&p=DevEx,5062.1

Reynolds, K. T., & Hoffmann, A. A. (2002). Male age, host effects and the weak expression or non-expression of cytoplasmic incompatibility in *Drosophila* strains

infected by maternally transmitted *Wolbachia*. *Genetics Research,80*(02). doi:10.1017/s0016672302005827

Rottschaefer, S. M., & Lazzaro, B. P. (2012). No Effect of *Wolbachia* on Resistance to Intracellular Infection by Pathogenic Bacteria in *Drosophila* melanogaster. Retrieved April 16, 2018, from http://journals.plos.org/plosone/article?id=10.1371/journal.pone.0040500

Sullivan, W. (2011). Faculty of 1000 evaluation for *Wolbachia* enhance *Drosophila* stem cell proliferation and target the germline stem cell niche. *F1000 - Post-publication peer review of the biomedical literature*. doi:10.3410/f.13357229.14775140

Teixeira, L., Ferreira, Á, & Ashburner, M. (2008). The Bacterial Symbiont Wolbachia Induces Resistance to RNA Viral Infections in *Drosophila* melanogaster. *PLoS Biology,6*(12). doi:10.1371/journal.pbio.1000002

Tram, U., & Sullivan, W. (2002). Role of Delayed Nuclear Envelope Breakdown and Mitosis in ... Retrieved April 15, 2018, from http://www.bing.com/cr?IG=1BD32C018586481C95D593691E5A7558&CID=24053CAA033968B6204E3779029669F1&rd=1&h=xprB8VzKyl9fp7K1T7A67Er2-f8Vg0AB2lybj6SlKN0&v=1&r=http://science.sciencemag.org/content/296/5570/1124&p=DevEx,5068.1

Veneti, Z., Clark, M. E., Karr, T. L., Savakis, C., & Bourtzis, K. (2004). Heads or Tails: Host-Parasite Interactions in the *Drosophila-Wolbachia* System. *Applied and Environmental Microbiology,70*(9), 5366-5372. doi:10.1128/aem.70.9.5366-5372.2004

Voronin, D., Cook, D. A., Steven, A., & Taylor, M. J. (2012). Autophagy regulates *Wolbachia* populations across diverse symbiotic associations. *Proceedings of the National Academy of Sciences,109*(25). doi:10.1073/pnas.1203519109

Zu Dhona, H., Houry, C., & Kambris, Z. (2018). Heinrich zu Dohna | American University of Beirut, Beirut ... Retrieved April 15, 2018, from https://www.bing.com/cr?IG=C59045AC0D68492CA8C1D3556521616D&CID=1B6FD31A6311674338CBD8C962BE66A8&rd=1&h=sUk_alwN7B2BvdhTYbF4NyHMn_sXdDjmu_rG0oNPwQ4&v=1&r=https://www.researchgate.net/profile/Heinrich_Dohna&p=DevEx,5067.1