AMERICAN UNIVERSITY OF BEIRUT

INVESTIGATION OF CANDIDATE GENES FOR A POTENTIAL ROLE IN *DROSOPHILA'*S RESPONSE TO THE SYMBIONT *WOLBACHIA*

by

FARAH SAMI EL-AYACHE

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AN ABSTRACT OF THE THESIS OF

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Title: Investigation of candidate genes for a potential role in *Drosophila'*s response to the symbiont *Wolbachia*

Drosophila melanogaster lacks adaptive immunity and only possesses innate immune responses to fight pathogens, which makes it a good model to study innate immunity independently of adaptive immunity responses. Interestingly, innate immunity reactions in *Drosophila* share similar evolutionary roots with those of humans, and studies of the symbiotic flora both in insects and in mammals have shown that these symbionts affect host immunity. *Wolbachia* is a maternally transmitted obligate intracellular symbiont, estimated to be present in approximately 50% of all insect species. It is naturally found in *Drosophila melanogaster* and although it does not induce an immune response, it has been found to confer its host an increased resistance against certain pathogens. However, when introduced into a novel host, *Aedes aegypti*, a strain of *Wolbachia* (*wMelPop*) led to an upregulation of the expression of genes related to the immunity and protected the mosquitoes against other infections. The relationship between insect hosts and the *Wolbachia* they harbor remains poorly characterized. We propose to study *Wolbachia* /*Drosophila* interactions in an attempt to determine which signaling pathways are involved in controlling *Wolbachia*. We will analyze the effects of *Wolbachia* on host immunity and reproduction by examining local immune responses, ovary size and egg production. In parallel, we will follow an *in vivo* RNAi approach to analyze the effects of selected candidate genes of unknown function in the control of *Wolbachia* and in flies' immunity to pathogens. The results of this project are expected to provide information on *Wolbachia* natural host interactions. A long-term application of this study would be to use *Wolbachia* as an alternative pest control method.

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ABBREVIATIONS

*:	Crossed with
UAS:	Upstream Activating Sequence
AMP:	Antimicrobial peptide
PGRP:	Peptidoglycan recognition proteins
NF-Kb:	Nuclear factor kappa-light-chain-enhancer of activated B cells
CI:	Cytoplasmic incompatibility
RNA:	Ribonucleic acid
qRT-PCR:	Quantitative real-time Polymerase chain reaction
ATG:	Autophagy-linked protein
IR:	Inverted Repeats
ML:	*Micrococcus luteus*
EF:	*Enterococcus faecalis*
ECC15:	*Erwinia carotovora carotovora*
BB:	*Beauveria bassiana*
LB:	Luria-Bertani
g:	Gravitational force
OD:	Optical Density
nl:	Nano-liters
ul:	Micro-liters
DNA:	Deoxy-ribonucleic acid
PCR:	Polymerase chain reaction
mV:	milli-Volts
cDNA:	Complementary Deoxy-ribonucleic Acid
RNAi:	Ribonucleic Acid Interference
Ct:	Cycle threshold
Rp49:	Ribosomal protein 49
V:	Volume
bp:	Base pairs
wsp:	*Wolbachia* surface protein
WftsZ:	Filamentation temperature-sensitive protein Z (*Wolbachia)*
F:	Forward Primer
R:	Reverse Primer
ACF:	ATP-utilizing chromatin assembly and remodeling factor
ISWI:	Imitation SWI (SWitch)
mm:	millimeter
n:	Number of samples
GFP:	Green Fluorescent Protein

CHAPTER I

INTRODUCTION

A. The Model

Drosophila melanogaster, commonly known as the fruit fly, belongs to the order Diptera and family Drosophilidae. *Drosophila melanogaster* is an intensively used model organism in the study and identification of important molecular mechanisms that relate to vertebrate organisms, especially humans. Studies have shown highly conserved molecular pathways between humans and this fruit fly (Reiter, Potocki et al. 2001). Other characteristics, such as its short development time, its convenient size and simple diet, its known genomic sequence, and its simple genetics, have also contributed in making *Drosophila melanogaster* a powerful and well-studied organism (reviewed in Hughes, Allen et al. 2012). This led to an expanding range of genetic methods that could be applied to this model, one of which is the UAS:GAL4 system that is able to modify gene expression in specific cells or tissues (Figure 1). First described by Brand and Perrimon, this system could be used to overexpress a gene of interest in a target tissue using a tissue-specific enhancer. Similarly, it can be used to knock-down a given gene if a hairpin RNA is produced instead of the normal mRNA (Brand and Perrimon 1993).

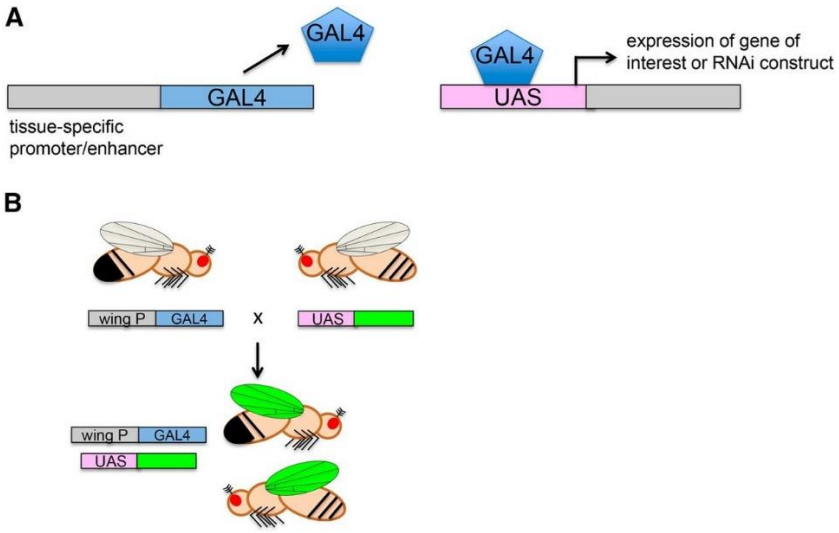


Figure 1: GAL4/UAS system used to modify gene expression in *Drosophila* (Hales, Korey et al. 2015). A fly expressing the GAL4 transcription factor under the control of a specific promoter is crossed to another transgenic fly that carries the cDNA of the gene of interest under the control of the Gal4 upstream activating sequence (UAS). In the progeny, this will lead to the over-expression of the target gene in a specific tissue-manner. A variant of this system, in which inverted repeat sequences (IR) of a target gene are placed under the control of UAS is used to knock-down genes expression.

B. *Drosophila* Immunity

Drosophila species have mechanisms that provide immune responses against different pathogens that could impose a threat. The first line of defense is the epithelial barrier that is directly in contact with the bacteria such as in the trachea and the gut. Immune responses could be classified as cellular or humoral responses. The cellular response includes the activation of haemocytes that are responsible for phagocytosis of pathogens, whereas the humoral response mainly involves a systemic response that leads to the production of Antimicrobial peptides (AMPs) by the fat body via the Toll and IMD pathways (reviewed by Buchon, Silverman et al. 2014).

Insects lack an adaptive immune system and rely on innate immune responses to detect and combat infection. In *Drosophila*, after microbial infection and recognition of

microbial determinants, signaling pathways are activated and antimicrobial peptides (AMP) are produced. These AMPs are regulated via two distinct signaling pathways: Imd (Immune deficiency) and Toll. These are respectively the homologues of TNF-R and TLR cascades found in humans (reviewed by Lemaitre and Hoffmann, 2007). In *Drosophila*, the majority of immunity genes is either under Toll or Imd control, or both (De Gregorio, Spellman et al. 2002). Several studies have identified and investigated *Drosophila* immune-regulated genes (DIRGs), some of which are of unknown function, but suggested to be associated with immunity due to their differential expression upon immune challenge (De Gregorio, Spellman et al. 2001, Irving, Troxler et al. 2001) .

C. Immune pathways

The systemic induction of AMP in the fat body is mainly done via the activation of the Toll and Imd pathways, each of which depends on the type of microbe that infects the fly. Gram-positive bacteria and fungal pathogens induce the Toll pathway after the recognition of microbial determinants by secreted PGRPs. This recognition initiates an extracellular proteolytic cascade leading to the maturation of Pro-Spatzle to Spatzle, a cytokine and the Toll receptor ligand. Activating Toll results in a DEATH-domain protein complex of MyD88, Tube and Pelle kinase. This results in the dissociation of the transcription factor Dif from the inhibitory protein Cactus and its nuclear translocation to drive the expression of AMPs such a *Drosomycin* (reviewed in Buchon, Silverman et al. 2014).

Unlike Toll, the Imd pathway is induced against gram-negative bacteria mainly by PGRP-LE and PGRP-LC that induce an NF-kB signaling pathway, eventually leading to the cleavage of the inhibitory tail of Ankyrin repeats on the NF-kB protein Relish. Relish, in

turn, translocates into the nucleus and activates AMP expression, such as *Diptericin* (Govind 2008) (Figure 2). Mutations in these components for both signaling pathways are expected to increase the fly's susceptibility to their respective microbial infections.

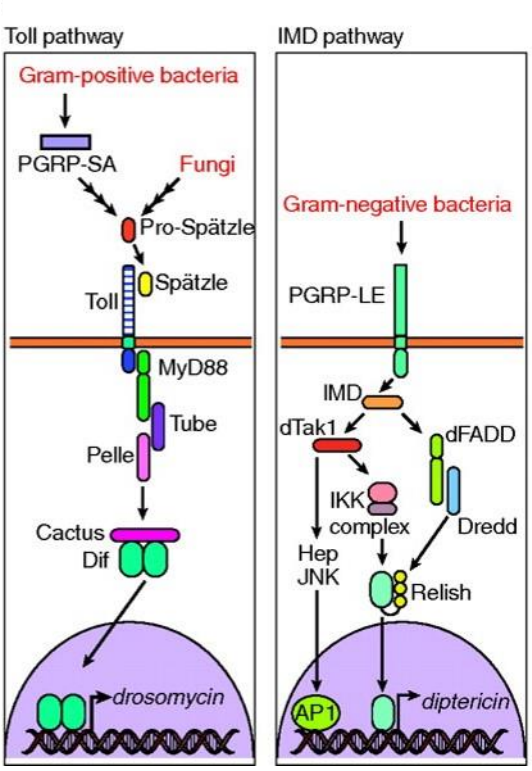


Figure 2: Diagram representing the Toll and Imd immune pathways. (modified from Bier and Guichard 2012). Gram-positive bacteria or fungi are responsible for the induction of the Toll pathway through a series of events that start with their recognition by PGRPs and eventually lead to the expression of AMPs, mainly *Drosomycin*. On the other hand, the Imd pathway is activated by gram-negative bacteria after the recognition of bacterial determinants by distinct PGRPs, leading to the activation of a cascade that ends in the production of AMPs including *Diptericin*.

D. *Wolbachia*

Wolbachia is a maternally inherited bacterial endosymbiont that belongs to the order Rickettsiales. It is mostly present in reproductive tissues in a wide range of insect hosts, estimated to range between 20 and 60% of all insect species. *Wolbachia* induces a set of variable effects, depending on its strain and the host, which could range from parasitism to mutualism.

In order to ensure its transmission in the invertebrate host, *Wolbachia* has the ability to manipulate the reproductive system. Indeed, the success of the persistence of this endosymbiont can be mainly attributed to its wide range of reproductive manipulation on the invertebrate host. These manipulations include: feminization which causes a change in an individual's sex and a genetic male starts to develop as a female (Rousset, Bouchon et al. 1992) , parthenogenesis, where female virgins infected with *Wolbachia* produce unfertilized eggs (Stouthamer, Breeuwert et al. 1993), male- killing which is the death of infected male embryos but the survival of females, and cytoplasmic incompatibility (Sinkins 2004).

E. Cytoplasmic Incompatibility

Cytoplasmic incompatibility is one of the common reproductive manipulations imposed by *Wolbachia* on its hosts and it aims to allow *Wolbachia* to better spread and persist within a population, by providing females with a reproductive advantage. Unidirectional CI is a phenomenon that produces a viable progeny if the female is infected with *Wolbachia*, regardless of the male's infection status. However, *Wolbachia*-infected males mating with uninfected females cause embryo death (Sinkins 2004) (Figure 3). The molecular basis of this phenomenon remains unknown, as different hypotheses are being

proposed and studied to explain CI. One of the main models suggested is a modification-rescue model, such that this incompatibility occurs due to a modification that happens in the sperm, however, it could be rescued when the same *Wolbachia* strain is found in the egg, which is where the rescue mechanism must be found (Werren 1997).

Cytoplasmic incompatibility has been suggested to result from disruption in post fertilization behavior between the egg and sperm pronuclei, however, the mechanism of this disruption remains to be unknown. Studies in *Nasonia* show that this could result from a delay between the male and female pronuclei, as the paternal nuclear envelop breakdown is slower in infected males and thus delaying paternal chromosome condensation (Tram, Fredrick et al. 2006). Similarly, studies on *Drosophila simulans* explain that this starts as a delay in the deposition of histones after protamine removal and thus affects nucleosome assembly (Landmann, Orsi et al. 2009). Also, Zheng *et al*. proposed the involvement of histone H3 chaperone, HIRA, in the CI mechanism as its expression was proved to be down regulated in infected male *Drosophila simulans* (Zheng, Ren et al. 2011). Therefore, it seems essential to study the factors contributing to CI at the level of embryogenesis, specifically the first mitosis.

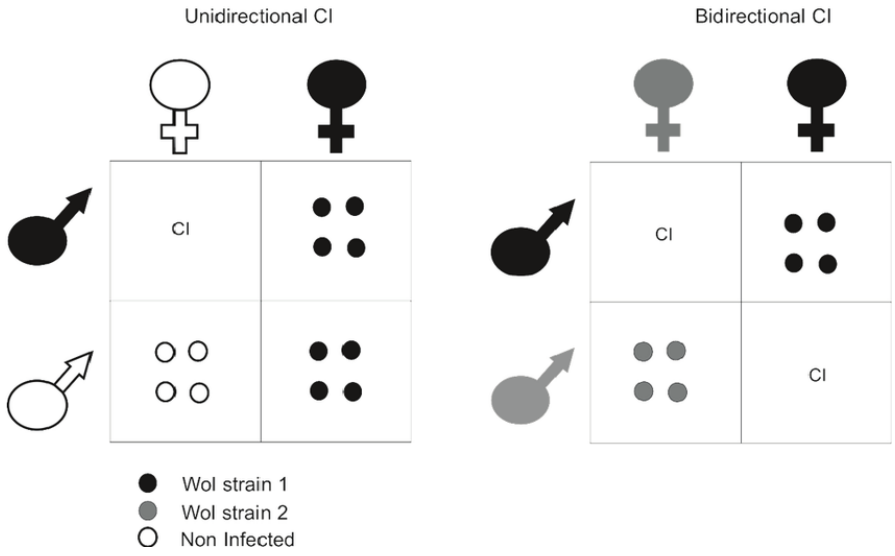


Figure 3: The basis of cytoplasmic incompatibility induced by *Wolbachia* (Kean, Rainey et al. 2015). In unidirectional CI, a *Wolbachia*-infected female crossed with an infected or uninfected male is able to produce a viable progeny, however, a *Wolbachia*-infected male can only give rise to viable progeny if the female is infected. In bidirectional CI, production of a viable progeny requires both male and female to be carriers of the same or compatible *Wolbachia* strains otherwise CI occurs.

F. Other effects on reproductive physiology

Comparing the fitness between *Wolbachia*-infected and uninfected strains has shown other possible effects of *Wolbachia* on its host reproductive physiology. Since *Wolbachia* resides in the gonads of their host, it is possible to assume that it may impose various effects on reproduction that could, in turn, aid in its persistence, like CI for example. Early studies in *Tribolium confusum* show decreased fecundity in *Wolbachia pipientis* infected females compared to uninfected ones (Wade and Chang 1995). Similarly, a *Drosophila simulans* population showed approximately 10% decreased fecundity of infected females compared to uninfected females (Hoffmann, Turelli et al. 1990). This analysis, however, done twenty years later on the same population changed the advantage towards infected *Drosophila* (Weeks, Turelli et al. 2007). *Wolbachia's* effect on fecundity is still under study but has

shown variable results between different hosts, possibly depending on the *Wolbachia* and host species and the different methods used by *Wolbachia* to ensure its persistence.

G. Effect of *Wolbachia* on Immunity

Aside from reproductive manipulation, *Wolbachia* has been shown to have other effects, one of which is on immunity. Understanding *Wolbachia*-host interactions concerning immunity remains deeply studied and very interesting, especially since *Wolbachia* supposedly affects immune pathways, yet is able to evade them. Early studies on hosts naturally harboring *Wolbachia*, such as *Drosophila simulans* and *Aedes albopictus*, show that AMP expression is not induced by the gram-negative bacterium *Wolbachia*, but remains inducible upon other bacterial infections (Bourtzis, Pettigrew et al. 2000). Similarly, the classical immune pathways are not induced by the presence of the symbiont in *Drosophila melanogaster*, however, it did cause an increased resistance to viral infection (Hedges, Brownlie et al. 2008, Teixeira, Ferreira et al. 2008).

Wolbachia has also been introduced into insect species that do not naturally harbor it in order to take advantage of its ability to manipulate host reproduction for a potential utilization in the control of pest-insects. Upon the embryonic microinjection of *w*MelPop, an over proliferative *Wolbachia* strain, into *A. aegypti* mosquitoes (McMeniman, Lane et al. 2009),the expression of several immunity genes was upregulated. This resulted in an increased resistance to *Erwinia* bacteria and in an inhibition of filarial nematodes development in *A. aegypti* (Kambris, Cook et al. 2009). Also, *Drosophila simulans Wolbachia* strain caused an increase in AMP expression in *Drosophila melanogaster*, whereas its naturally harbored strain did not (Xi, Gavotte et al. 2008). An upregulation of

immune genes expressions are evident in novel hosts, however, they do not show the same effect on their native host.

In order to gain more insight about the *Wolbachia*-host interactions, it is interesting not only to study the effect of the endosymbiont *Wolbachia* on the different immune pathways, but also to determine the effect the immune pathways have on the harboring of this bacterium. Recent studies in mosquitos have shown that the suppression or boosting of the immune pathways have an effect on *Wolbachia* load, especially the Imd pathway since *Wolbachia* is a gram-negative bacterium. Pan *et al.* have shown that suppressing the Imd pathway in *A. aegypti* mosquitos lead to a decrease in *Wolbachia* density, whereas, an increase in mosquito immunity caused an increase in *Wolbachia* load. It is, therefore, suggested that *Wolbachia* density and the immune pathways could be part of a positive feedback loop that allow for this successful symbiosis (Pan, Pike et al. 2018).

H. Significance

This study focuses on further investigations of the *Wolbachia*-host interaction, specifically in *Drosophila melanogaster*. Establishing a clearer view on how *Wolbachia* managed to evolve and successfully maintain its transmission would provide a better understanding of the mechanism that was established to achieve this symbiosis and an insight on how to utilize this in procedures aimed to induce *Wolbachia* infection. This not only provides knowledge on this particular topic, but may also help understand the basis of host-symbiont interaction, as well as the possible use of *Wolbachia* as a pest and vector control method.

I. Specific aims

1. *Specific aim 1*

Testing candidate genes of unknown function for the involvement in *Drosophila* immunity. For this we will:

- Achieve the knock-down of genes of unknown functions and inject them with gram negative bacteria, gram positive bacteria or fungi.
- Select genes that show compromised immunity
- Determine the effect of these genes on the immune pathways by injecting different bacteria, extracting RNA, followed by reverse transcription and RT-PCR.

2. *Specific aim 2*

Compare the effect of *Wolbachia*'s presence on *Drosophila* reproductive physiology. For this we will:

- Test for cytoplasmic incompatibility between Or^{E20} and Or^{E20}-*Tet* flies using egg hatching experiments.
- Determine the effect of *Wolbachia* on Or^{E20} and Or^{E20}-*Tet* ovaries size and physiology using fluorescent microscopy.
- Test the effect of *Wolbachia* on different host candidate genes involved in *Drosophila* local immunity and reproduction. To do that: RNA will be extracted from ovaries and testes, followed by reverse transcription and qRT-PCR to quantify the levels of expression of the candidate genes in Or^{E20} and Or^{E20}-*Tet* gonads.

3. Specific aim 3

Investigating *in vivo* the potential role of a selection of *Drosophila* genes in *Wolbachia* control/tolerance. For this we will:

- Achieve the knock-down of representative genes from different immune pathways (IMD, Toll and ATG) with transcriptional profiles shown to be up-regulated after immune challenge (based on previous microarray analysis and previous preliminary results)
- Determine the effect of genes knock-down on *Wolbachia* densities by qRT-PCR.

CHAPTER II

MATERIALS AND METHODS

A. *Drosophila* Strains and maintenance

The *Drosophila* strains are maintained in the lab at two different temperatures 18°C or 25°C, depending on the purpose of use, with a 12 hour light: 12 hour dark cycle. The medium used for their maintenance consists of polenta, yeast, soy flour, agar, molasses and propionic acid. The *Drosophila* strains that are used as controls are the wild-type flies: W^{1118} and *Oregon*, as well as the mutants of the Toll and Imd pathways that are Dif^{1} and Rel^{E20}, respectively. Also, a *Wolbachia*-free strain will be used, *Oregon-Tet*, which is a strain previously obtained in the lab by tetracycline treatments.

B. Crosses

In order to perform a cross, approximately 15 virgin females are collected and mated with 7 to 10 males and placed at a temperature of 25°C. The virgin females are collected from vials maintained at 18°C at a maximum of 16 hours after emergence to ensure virginity. If the cross contains IR constructs, the cross is transferred to a temperature of 29°C upon reaching the second instar larval stage in order to favor gene knock down.

C. Microbes and microbial preparation

The microbes used have already been utilized as elicitor of *Drosophila's* immune response in laboratory conditions. These are *Micrococcus luteus*, *Enterococcus faecalis* as Gram-positive bacteria and *Erwinia carotovora* (Ecc15) as gram-negative. The fungus used is *Beauveria bassiana*.

Bacterial cultures were incubated in LB in a shaker, overnight, at a temperature of 37°C. The culture then undergoes spinning for 10 minutes at 4000g. After re-suspending the obtained pellet in LB, a spectrophotometer is used to measure the optical density (OD) at a wavelength of 595nm. This OD is then diluted to obtain the specified OD necessary for injection. As for the fungus *Beauveria bassiana*, its spores are filtered after collection from the PDA plates they were growing on, then counted on a slide under the microscope to quantify the number of spores/ml. The fungus suspension is then diluted into the desired number of spores/nl for injection purposes.

D. Infection and survival assays

Batches of fifteen wild-type (or mutant) flies are used in survival experiments. In all experiments, male and female flies are used separately, and are collected at an age range two to five days. Flies are anesthetized using a CO_2 flow bed and microinjected with 32nl of a microbial suspension of a specific concentration (*M. luteus* OD: 0.01, *E. faecalis* OD: 0.05, *E. carotovora* OD: 0.1, *B.*

bassiana concentration of approximately 4 spores/nl) into the fly thorax by a capillary needle. The vials containing the injected flies are then put in an incubator at the desired temperature (preferably 29°C if using a IR constructs) and the surviving flies counted at different time points. Each experiment is repeated at least three independent times, survival curves are plotted and statistical tests performed. For the purpose of RNA extraction and Real-Time PCR, flies are frozen after injection at -80°C, after a duration depending on the injected microbe (24 hours for *M. luteus,* 12 hours for *E. carotovora*, and 48 hours for *B. bassiana*).

E. DNA Extraction

A sample of one fly is ground in 100µl of Livak grind buffer (80mM NaCl, 0.16M sucrose, 130mM Tris Base, 50.8mM EDTA Ph= 8.0 and 5mM SDS) and incubated at 65°C for 20 min. Then, 40µl of sodium acetate are added and this solution is incubated on ice for 20 minutes, followed by centrifugation at 4°C at 15000g for 10 min. 100µl of the supernatant was added to 20µl of 100% ice cold ethanol and centrifuged for 15 min at 4°C at 20000g to obtain a DNA pellet. The supernatant is discarded and the pellet is rinsed with 500µl of cold 70% ethanol, followed by a quick spin at 20000g for 5 min. After discarding the ethanol, the pellet is air dried for 5 min at room temperature, then re-suspended in 40µl of double distilled water.

F. PCR

PCR is used to amplify the obtained DNA by adding 2ul of it to a mix (13ul nanopure water, 2ul 10x buffer, 1ul MgCl2, 0.5ul dNTPs, and 0.5ul Taq polymerase) and to 0.5ul of each of the *wsp* primers. Samples were then subjected to 5 minutes of 95°C, followed by 30 cycles of 30 seconds at 95°C, 30 seconds at 53°C, and 2 minutes at 72°C, ending with 5 minutes at 72°C. Gel electrophoresis is then performed to view the bands obtained, by running the samples on a gel (1% agar-TBE 1x, 3.2 ul ethidium bromide) for 30 minutes at 90mV.

G. qRT-PCR

To monitor the expression levels of a certain gene, qRT-PCR can be performed using specific primers that detect the transcript of the gene under study. To do that, first RNA is to be extracted using TRIzol™ reagent from *Drosophila melanogaster* whole flies (or gonads). In the case of whole flies, a sample of 15 flies is used, whereas in the case of gonads, the sample is made up of 15 pairs of ovaries or 30 pairs of testes. RNA is then used to synthesize cDNA using SuperScript II enzyme (Invitrogen) and following the respective protocol. The cDNA obtained is then diluted (1:20) in nuclease-free water in order to be used for qRT-PCR. Using dsDNA dye SYBR Green I and the resulting cDNA, qRT-PCR is performed. The cycle threshold (Ct) values are determined and background fluorescence is subtracted. To calculate the gene expression levels of target genes, they are first normalized relative to the ribosomal protein gene Rp49 (ribosomal protein).

H. Egg production Assay

To test for egg production, 10 virgin females and 10 males are sorted separately from vials placed at 18°C and left for 24 hours. These one-day old flies are then mated together (with the same strain) and left for another 24 hours at 25°C. The flies are then transferred to apple agar plates and left for approximately 24 hours at 25°C. The number of eggs on the plate are counted and compared.

I. CI Assay

Egg hatching experiments are used to determine the percentage of hatched versus unhatched eggs to assess cytoplasmic incompatibility. Each vial consists of 20 1-day-old virgin females mated with 20 1-day-old males and placed at 25°C for 24 hours. The vial is then turned and placed on an apple agar plate for approximately 4 to 5 hours at 25°C, then transferred to a different plate. The eggs on the plate are aligned and counted. 24 hours after the original placement of the vial on the plate, the eggs are scored for the percentage of hatched and unhatched eggs.

J. Fluorescence microscopy

Two to five-day old female ovaries are dissected and placed in PBS, then fixed in 100 µl 4% PAF for 30 minutes. Washes in PBS-Triton 0.1% are then administered for 15 minutes and repeated three times. This is followed by blocking using PBS-Triton 0.1%-BSA 1% for 30 minutes, after which the dye is applied, which is in this case DAPI, followed by another three 15 minute PBS-Triton 0.1% washes. The ovaries are then mounted on slides using a mounting medium of glycerol:PBS (1:5), and viewed under an

upright fluorescence microscope. The respective length (l) and width (w) of the ovaries were measured and used to calculate the volume of the ovaries. The volume formula used was that of an ellipsoid: $V = \frac{4}{3}$ πabc, where a is half the length, and b and c are half the width.

K. Developing altered immunity strains

In order to be able to obtain altered immunity strains that harbor *Wolbachia*, we first started by introducing *Wolbachia* into the drive that will be used in the upcoming crosses. Using standard genetic crosses and balancers, we obtained the C564:GAL4 fat body driver that now carries *Wolbachia*. Virgin females were then collected and crossed to males of flies of the necessary IR RNAi strain. Since *Wolbachia* is maternally inherited, this ensures the progeny now carries *Wolbachia* and the targeted gene is knocked down by the UAS:GAL4 system.

L. Primers coding sequences used in PCR

Table 1: Coding Sequences of the primer used to amplify *Wolbachia* in PCR.

wsp81F	5’ TGGTCCAATAAGTGATGAAGAAAC 3’
wsp691R	3’ AAAAATTAAACGCTACTCCA 5’

M. Primers coding sequences used in Real-Time PCR

Table 2: List of primers used in Real-Time PCR and their coding sequences.

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’
Rp49-F	5’ GACGCTTCAAGGGACAGTATCTG 3’
Rp49-R	5’ AAACGCGGTTCTGCATGAG 3’
ACF1-F	5’ GAGCTATCCGTTCTGCGTTC 3’
ACF1-R	5’ CAAGCGCATCACTTCGTTTA 3’
Cid-F	5’ AATCGCTATCCCACAACCAG 3’
Cid-R	5’ CGGATCTCACGATCCATTCT 3’
Cortex-F	5’ TCCTACCGCAAGCTGTTCTT 3’
Cortex-R	5’ TATGGGTGGGAGCAAACTTC 3’
Grauzone-F	5’ ACCATGTTCCAGACGAGGAG 3’
Grauzone-R	5’ GTACTCCGTGGCAAAAGCAT 3’
Hira-F	5’ AATGGTCAGAACCTGGCATC 3’
Hira-R	5’ TAGACATCGTTGGGTGACCA 3’
Histone 4-F	5’ AAACTCGCGGTGTGCTAAAG 3’
Histone 4-R	5’ TAACCGCCAAATCCGTAAAG 3’
Histone acetyl transferase- F	5’ CTGAAACTCAATCCCCTCCA 3’
Histone acetyl transferase- R	5’ AAGCGCTCCATTTTCTTCAA 3’
Histone deacetylase – F	5’ TGGGCTCTATCGAAAAATGG 3’
Histone deacetylase – R	5’ ATCTTCGCCGACATTGAAAC 3’
ISWI – F	5’ CACCCGCCTACATCAAAAGT 3’
ISWI – R	5’ GATGACGATGTGTGGTCCAG 3’
Protamine B – F	5’ AGTCAGAAGTGCAGCAAGCA 3’
Protamine B – 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’

CHAPTER III

RESULTS

A. Survival analysis of flies with knock-down in genes with unknown function

For this study, we selected a list of genes with no attributed function that have been shown to have increased transcriptional profiles upon immune challenge in previous microarray analysis (De Gregorio, Spellman et al. 2001, Irving, Troxler et al. 2001). Because of the high lethality we observed when we knocked-down these genes using the ubiquitous Act-Gal4 driver, we used a spatially restricted GAL4 driver: C564:GAL4. This fat-body specific driver was crossed to IR RNAi fly strains in order to achieve the gene knock-down in the fat body (the main site of AMP production). Upon this cross, the knock-down of CG 10882 and CG 14938, still lead to lethality and these two genes were not further analyzed.

For all others (13 genes), fifteen flies of the progeny of C564:Gal4 x Target gene-IR crosses were collected at an age ranging between two to five days and infected. Survival curves were plotted after infection with three different microbes. The microbes used include a gram-positive bacterium *Enterococcus faecalis* (EF), a gram-negative bacterium *Erwinia carotovora carotovora* (Ecc15), and a fungus *Beauveria bassiana* (BB). A representative curve for each microbe is shown below (each experiment was performed at least three times).

Depending on the type of microbe used, appropriate control flies were included. With the bacterium EF, the wild-type flies were used as controls, as

well as their crosses with the fat body specific driver. As for the positive controls, a mutant of a Toll pathway transcription factor, Dif, was infected. After infection of the flies with EF, *Drosophila* with a knock-down in genes CG 5150, CG 2217, CG 9186, CG 5729 showed compromised survival in a consistent manner compared to the wild-type flies. In addition, one gene CG 3829 showed inconsistent susceptibility towards this type of infection (Figures 4-5).

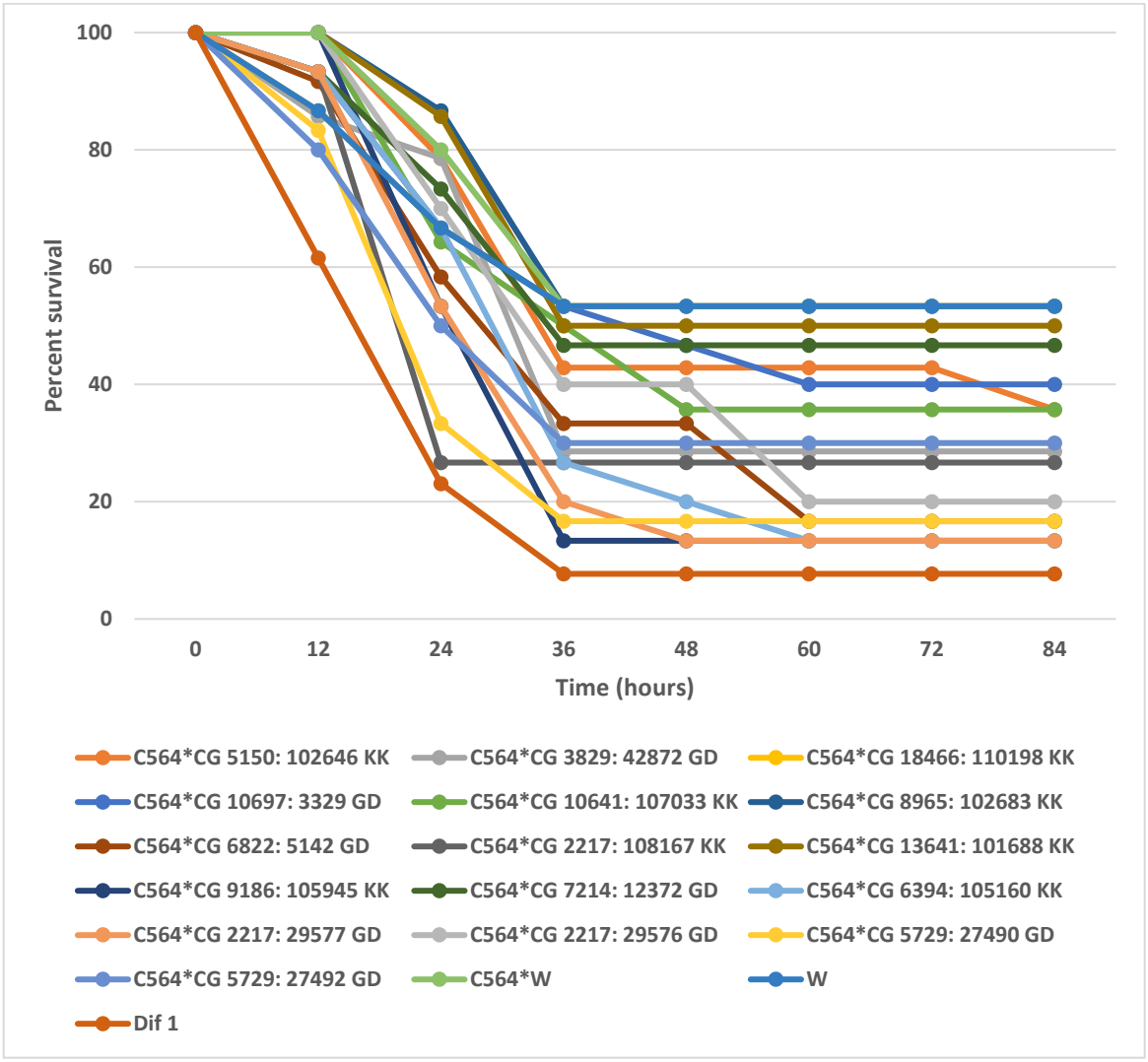
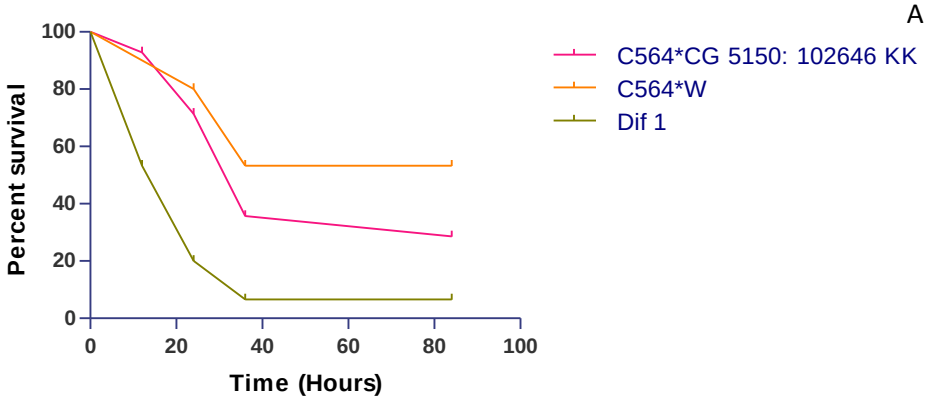


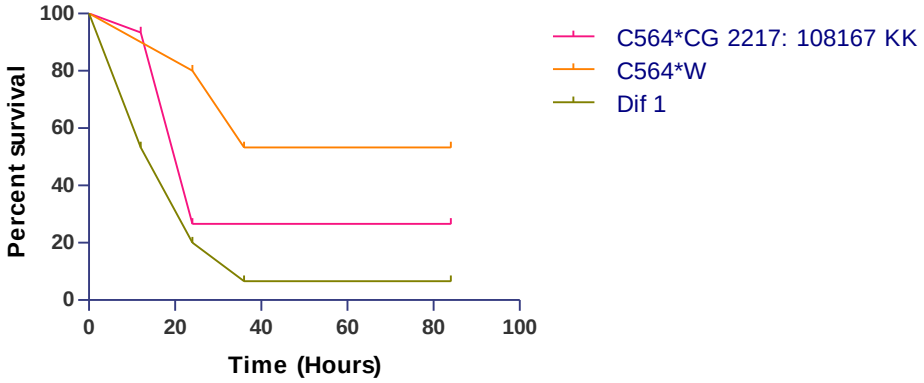
Figure 4: Survival curves for the progeny of C564:Gal4 x UAS:IR crosses after injection of EF (OD 0.05). The percent survival of each progeny after infection is shown as a function of time. The curves were

compared to those of wild-type flies W^{1118} and Or^{E20}. *Dif*^{1} is a Toll pathway mutant that was included as a control.

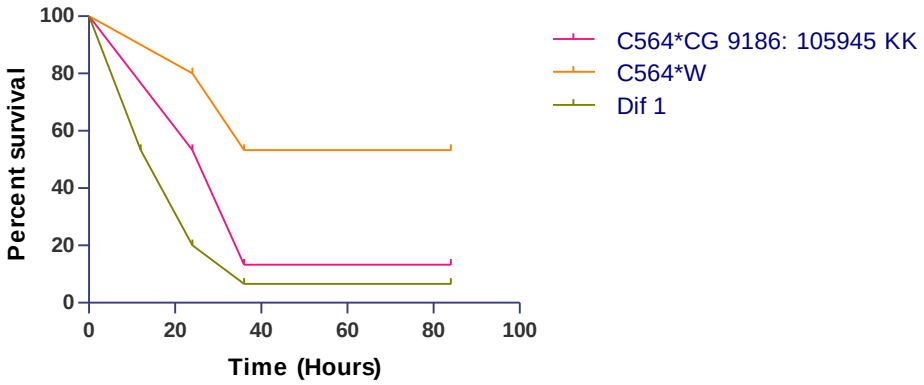
A



B



C



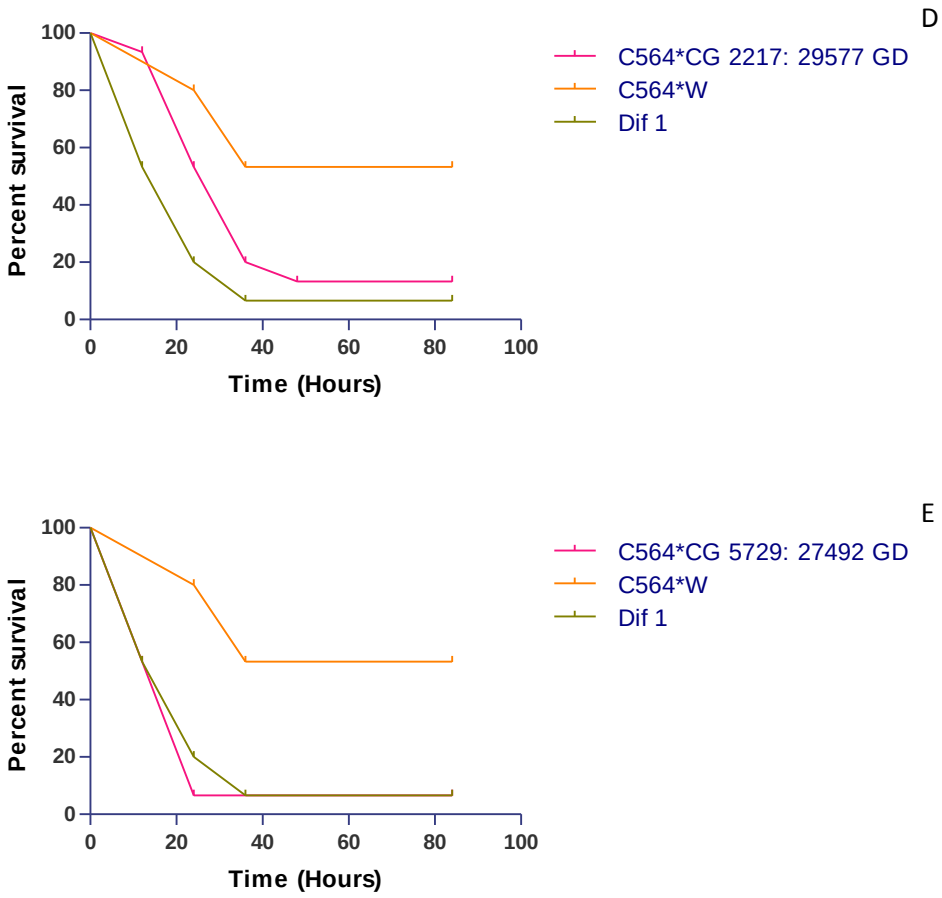


Figure 5: Survival curves for the progeny of selected C564:Gal4 x UAS:IR crosses after infection with EF (OD 0.05). The percent survival of each progeny after infection is shown as a function of time and compared to wild-type flies W^{1118} and Dif^{1}control flies. The respective p-values of the curves are: 0.19, 0.05, 0.02, 0.01, and 0.0001 (A-E).

The Toll pathway also controls the fly's antifungal response. We therefore assayed the survival of flies with gene knock-downs after BB infections. Among the five genes that led to increased susceptibility to EF infection when knocked-down, two (CG 9186 and CG 5729) seems to be required to fight BB infection since their knock-down led to increased susceptibility to infections with this fungus. However, knock-down in the other three genes (CG 5150, CG 2217 and CG 3829) did not show compromised survival to BB infections. In

addition, the knock-down of another gene (CG 18466) showed inconsistent susceptibility to BB infections (Figure 6-7).

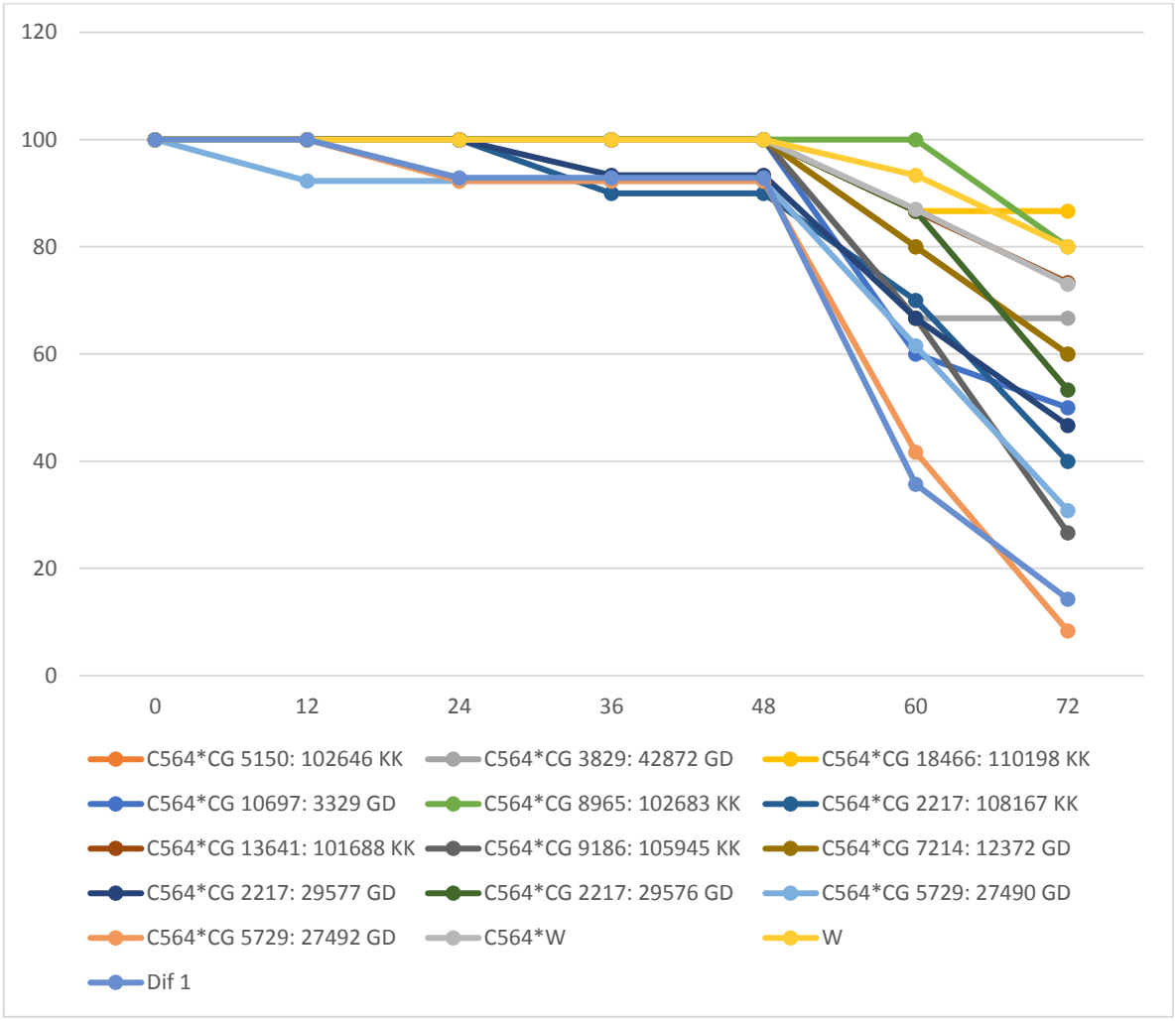
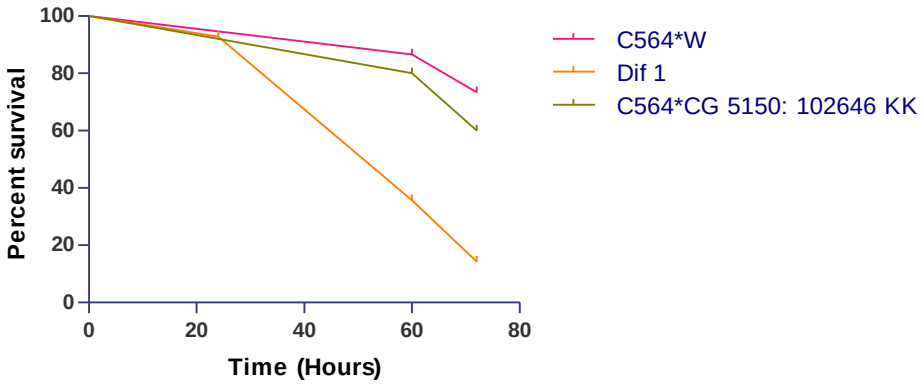
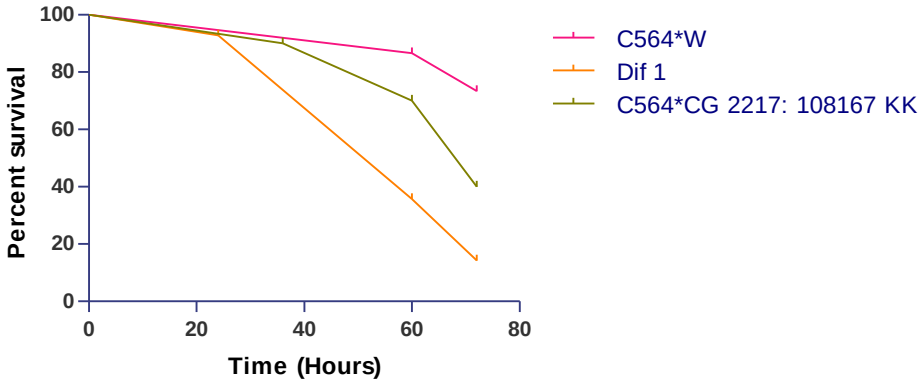


Figure 6: Survival curves for the progeny of C564:Gal4 x UAS:IR crosses after injection of BB. The percent survival of each progeny after infection is shown as a function of time. The curves are compared to those of the controls: wild-type flies W^{1118} and Or^{E20}, and *Dif* mutant which is a mutant in the Toll pathway and the positive control.

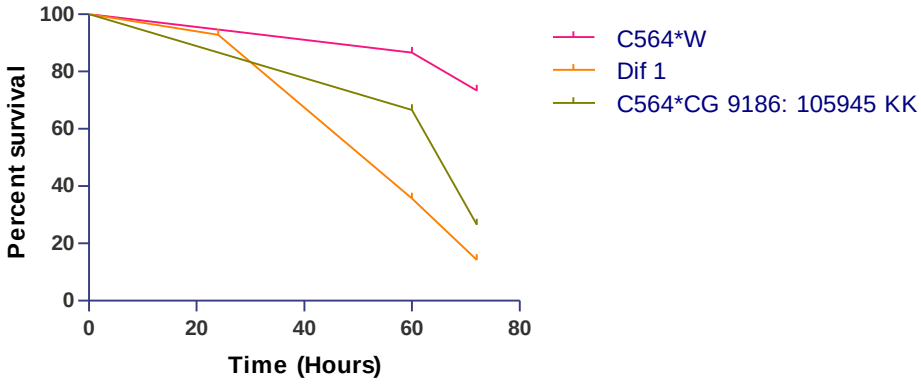
A


Percent survival
100
80
60
40
20
0
0
20
40
60
80
Time (Hours)
C564*W
Dif 1
C564*CG 5150: 102646 KK

B


Percent survival
100
80
60
40
20
0
0
20
40
60
80
Time (Hours)
C564*W
Dif 1
C564*CG 2217: 108167 KK

C


Percent survival
100
80
60
40
20
0
0
20
40
60
80
Time (Hours)
C564*W
Dif 1
C564*CG 9186: 105945 KK

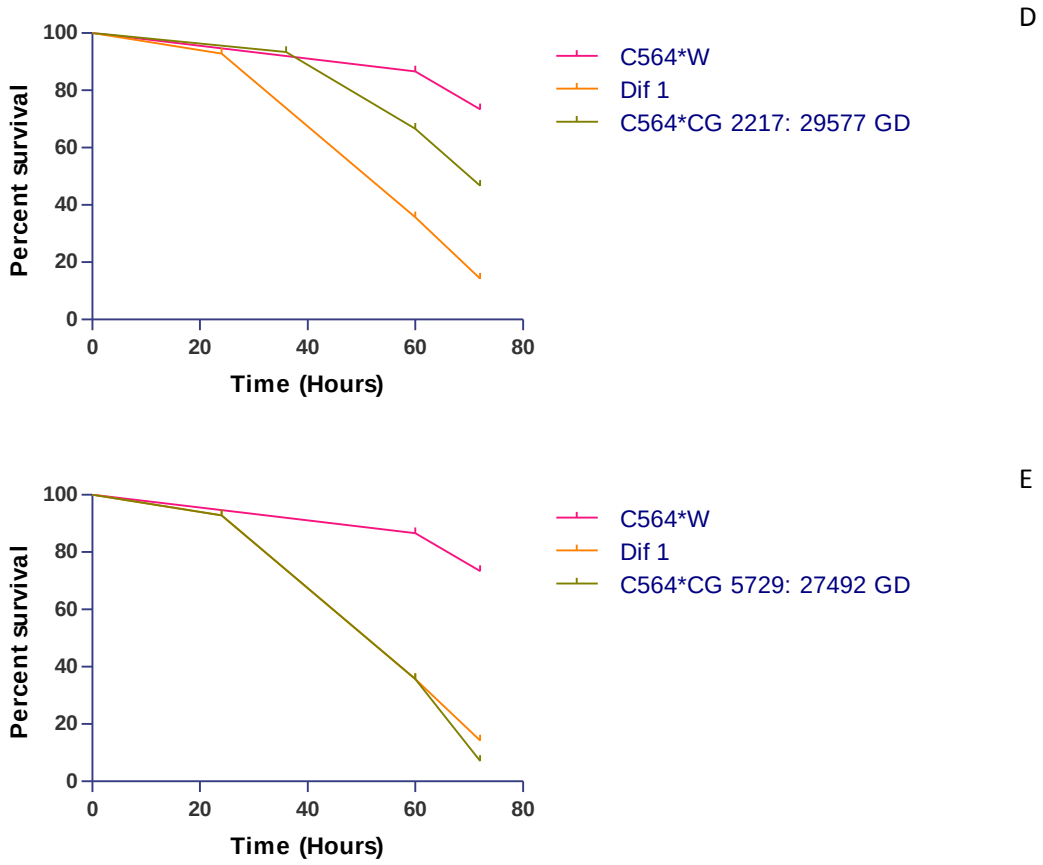


Figure 7: Survival curves for the progeny of selected C564:Gal4 x UAS:IR crosses after infection with BB. The percent survival of each progeny after infection is shown as a function of time and compared to wild-type flies W^{1118} and the positive control Dif^{1}. The respective p-values of the curves are: 0.44, 0.09, 0.01, 0.11, and 0.0002 (A-E).

To determine if some candidate genes are required for the activation of the IMD pathway, flies silenced for the different genes were tested for their ability to survive Gram-negative bacterial infections. Two genes knock-downs led to increased susceptibility to infection with the ECC15: CG 10697 and CG 5729. Another gene CG 18466 showed inconsistent susceptibility when silenced (Figures 8-9).

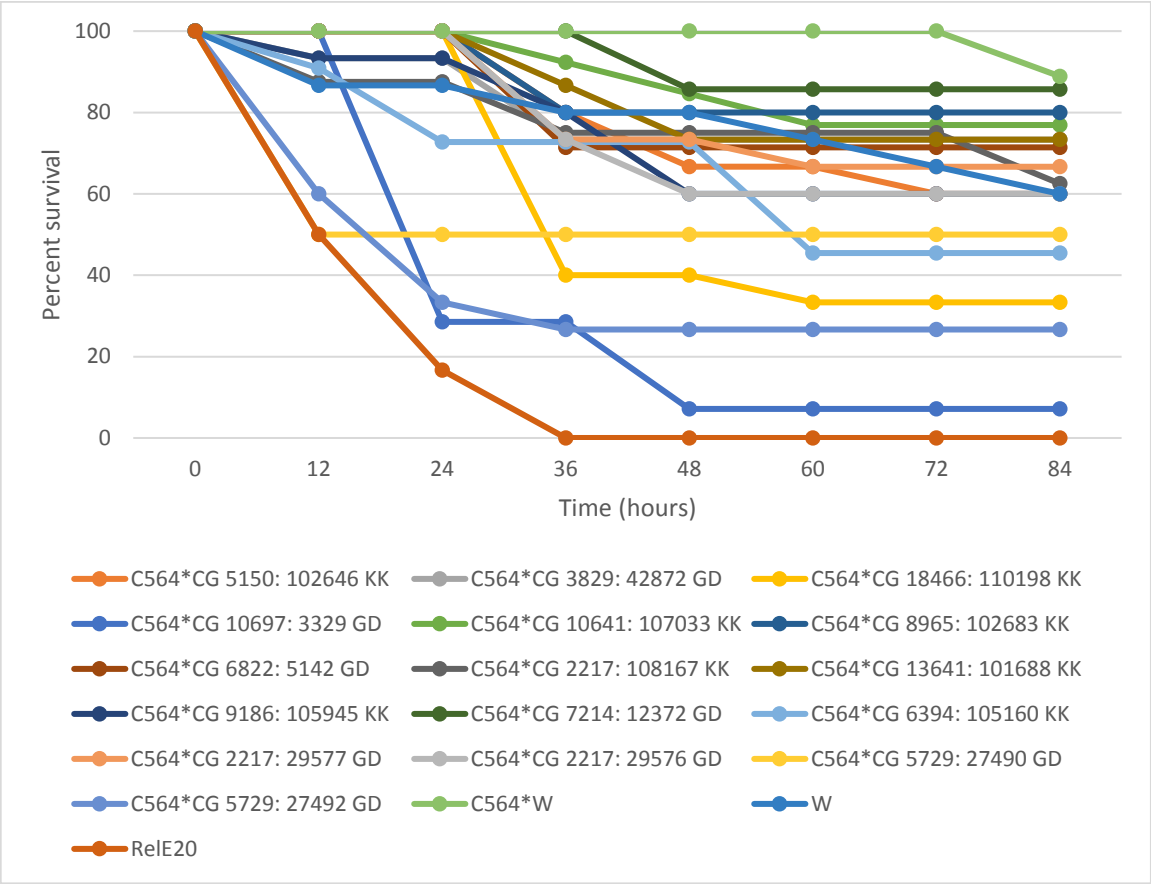


Figure 8: Survival curves for the progeny of C564:Gal4 x UAS:IR crosses after injection of ECC15 (OD 0.1). The percent survival of each progeny after infection is shown as a function of time. The curves are compared to those of the controls: wild-type flies W^{1118} and Or^{E20}, and to that of Rel^{E20} flies which are mutant in the IMD pathway.

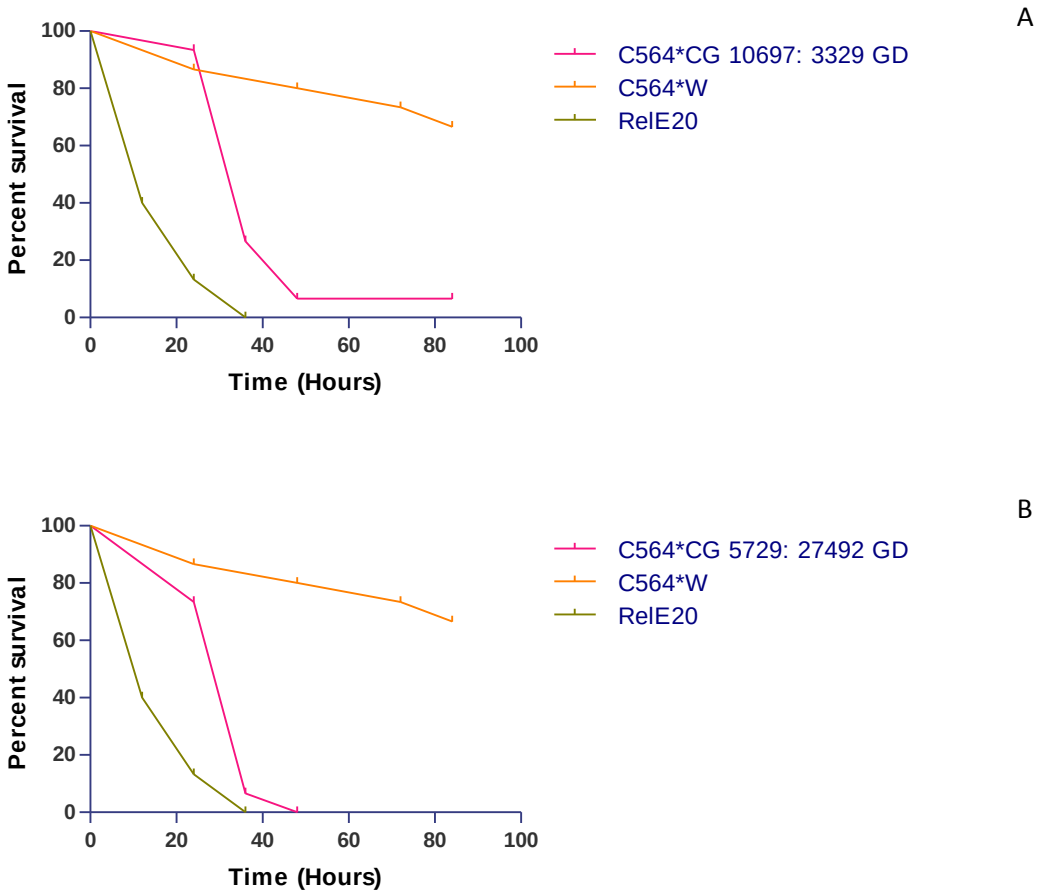


Figure 9: Survival curves for the progeny of selected C564:Gal4 x UAS:IR crosses after infection with ECC15 (OD 0.1). The percent survival of each progeny after infection is shown as a function of time and compared to wild-type flies W^{1118} and the positive control Rel^{E20}. The respective p-values of the curves are: 0.0004 and 0.0001.

B. Quantification of AMP for selected genes

The genes for which knock-down showed compromised immunity were then assayed for AMP levels. For this, knock-down flies were subjected to infection with microbes, followed by RNA extraction, reverse transcription and Real-Time PCR with AMP-specific primers. The microbes used are a gram-positive bacterium *Micrococcus luteus* (ML), a gram-negative bacterium *Erwinia carotovora carotovora* (Ecc15), and a fungus *Beauveria*

bassiana (BB). ML was chosen in this experiment instead of EF as a gram-positive representative since it is less virulent and the aim here is eliciting an immune response that enables us to quantify AMP levels rather than assaying survival. The flies infected with gram-positive bacteria or fungi were tested for *Drosomycin,* whereas, those infected with gram-negative bacteria were tested for *Diptericin,* as read outs of the Toll and Imd pathways respectively.

The negative controls used were the W^{1118} wild-type strain with similar genetic background to the crosses performed, as well as progeny of the cross C564:GAL4 x W^{1118} (or the wild-type Or^{E20} strain), to ensure that there is no effect of the driver on the obtained results. As positive controls, mutants from the respective immune pathways are used depending on the microbe injected, as well as progeny of the cross between C564:GAL4 x UAS:IR of a gene in the respective immune pathway in order to confirm proper knock-down of the gene (Dif for Toll and Relish for Imd). The quantified levels are shown in the following graphs.

Flies with the knock-down of genes CG 5150, CG 3829, and CG 9186 showed lower induction of *Drosomycin* after infection with ML. These results were consistent with those of the survival assays: all these flies also showed compromised survival upon injection with a gram-positive bacterium. On the other hand, CG 2217 and CG 5729 genes knock-down flies did not show a decrease in *Drosomycin* expression levels (Figure 10).

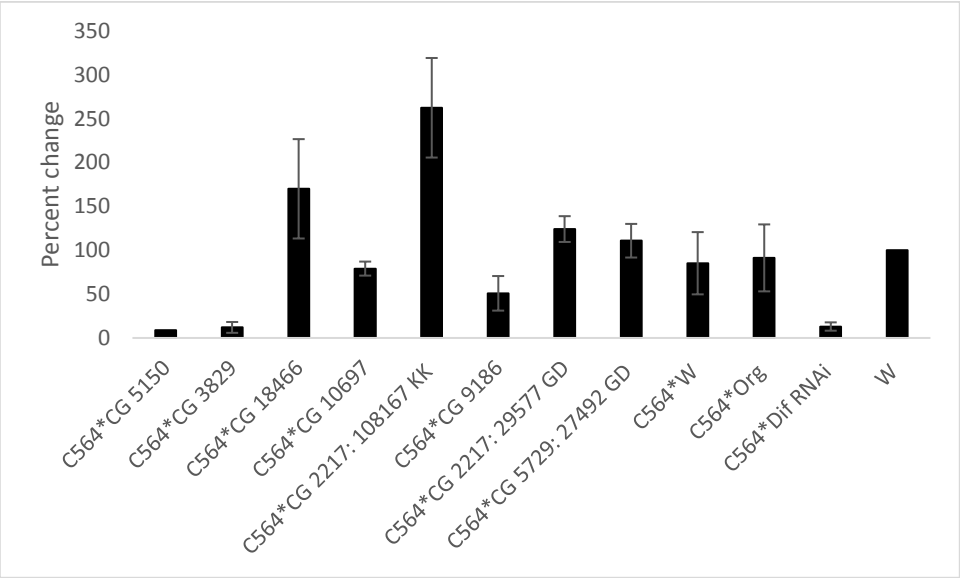


Figure 10: Quantification of *Drosomycin* levels in flies after infection with ML. The histogram represents the change in the expression levels of *Drosomycin* in flies after the knock-down of the selected genes and infection with the gram-positive bacterium ML. The negative controls used are the W^{1118} wild-type strain, *C564:Gal4** W^{1118} and *C564:Gal4*Or*^{E20}. *C564:Gal4*Dif-IR* flies are included as a positive control for Toll pathway impairment. The bars are the respective standard errors.

After BB infection, CG 9186 and CG 5729 knock-down flies showed decreased survival when compared to the control in survival assays (Figure 6). However, only CG 5729 knock-down flies displayed a decreased expression level of *Drosomycin*, while CG 9186 knock-down flies displayed normal levels (Figure 11). In addition, a low level of *Drosomycin* was observed for flies silenced for the gene CG 3829.

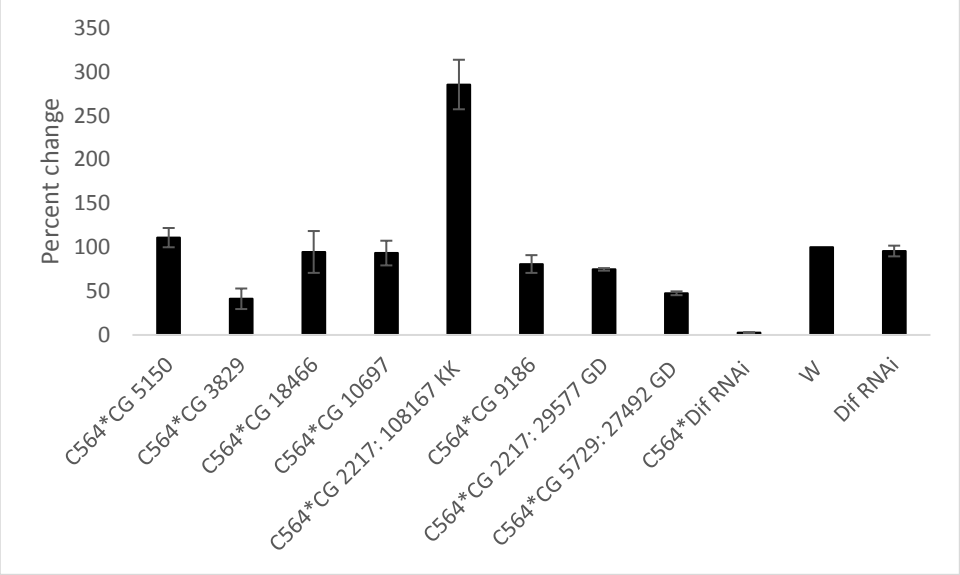


Figure 11: Quantification of *Drosomycin* levels in flies after infection with BB. The histogram represents the change in the expression levels of *Drosomycin* after gene knock-down and infection with the fungus BB. Wild-type controls used are the W^{1118} wild-type strain and the flies with a *Dif*-IR not crossed to any GAL4 driver. The positive control is *C564:Gal4*Dif-IR*. The bars are the respective standard errors.

For the genes that led to increased sensitivity to ECC15 infections when silenced, *Diptericin* levels were assayed after this type of infection. Flies with knock-down in gene CG 5729 showed a decrease in the *Diptericin* expression quantified relative to the wild-type flies (W^{1118}). CG9186 knock-down also led to lower expression of *Diptericin* although this gene's knock-down did not lead to compromised survival after ECC15 (Figure 12).

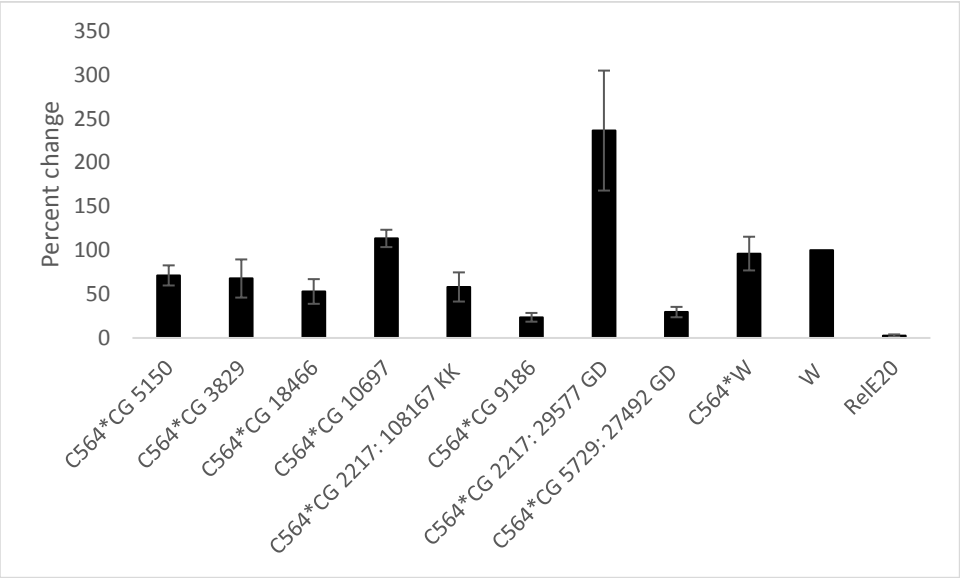


Figure 12: Quantification of *Diptericin* levels in flies after infection with ECC15. The histogram represents the change in the expression levels of *Diptericin* in flies after gene knock-down and infection with the gram-negative bacterium ECC15. The negative controls used are the W^{1118} wild-type strain and *C564:Gal4*Or^{E20}*. The positive control used as an Imd pathway mutant is Rel^{E20}. The bars are the respective standard errors.

C. Detection of *Wolbachia* in different *Drosophila* strains

Tetracycline treatment of the wild-type *Wolbachia*-harboring fly Oregon resulted in an Or^{E20}*-Tet* strain that is free of *Wolbachia*. To verify that the established Or^{E20}*-Tet* strain is does not harbor *Wolbachia* 10 generations after its treatment, DNA was extracted from Or^{E20}, Or^{E20}*-Tet* and W^{1118} flies, amplified by PCR, and followed by gel electrophoresis. The primers used were the general *wsp* primers that detect the presence of different *Wolbachia* strains. The positive control being the Or^{E20} strain as it carries *Wolbachia*, and the negative control W^{1118} as the *Wolbachia*-free wild-type strain. The results did not show a band for Or^{E20}*-Tet* flies (Figure 13), confirming that *Wolbachia* has been eliminated from this line.

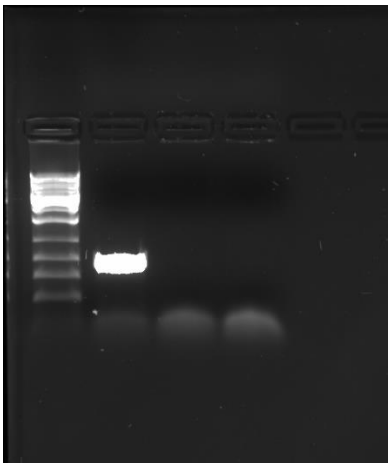


Figure 13: Gel image showing the detection of *Wolbachia* in different *Drosophila melanogaster* strains. *Wolbachia* specific primers (*wsp)* are used to amplify bacterial DNA from extracted fly gDNA and amplicons were analyzed by agarose gel electrophoresis. A band of approximately 600 bps reveals the presence of *Wolbachia*. The above lanes correspond to (from left to right): The DNA ladder, Or^{E20} **(**positive control), Or^{E20}*-Tet*, and W^{1118} (negative control).

D. Quantification of CI genes in *Drosophila* gonads

Thirteen genes were selected as potential candidates in the induction of CI. These genes are the homologues of genes identified in a microarray investigation that compared *Wolbachia*-infected to uninfected *Culex* embryos and testes (Kambris, unpublished results). In order to test for the possible involvement of the 13 different *Drosophila* genes in the CI mechanisms, we wanted to compare their expression profile between gonads of *Wolbachia*-containing and *Wolbachia*-free flies. Three genes (Cid, ISWI, and histone deacetylase) were excluded from the results due to the unsuccessful amplification with the primers. The results obtained with the ten remaining genes are shown in the following histograms comparing, separately, expression levels in the ovaries (Figure 14) and testes (Figure 15).

The results showed no significant change in the expression levels of these genes between Or^{E20} and Or^{E20}-*Tet* ovaries, with a maximum increase of two-folds in Grauzone, ACF and Histone 4 transcripts levels. This increase, however, was statistically insignificant (p-value > 0.05).

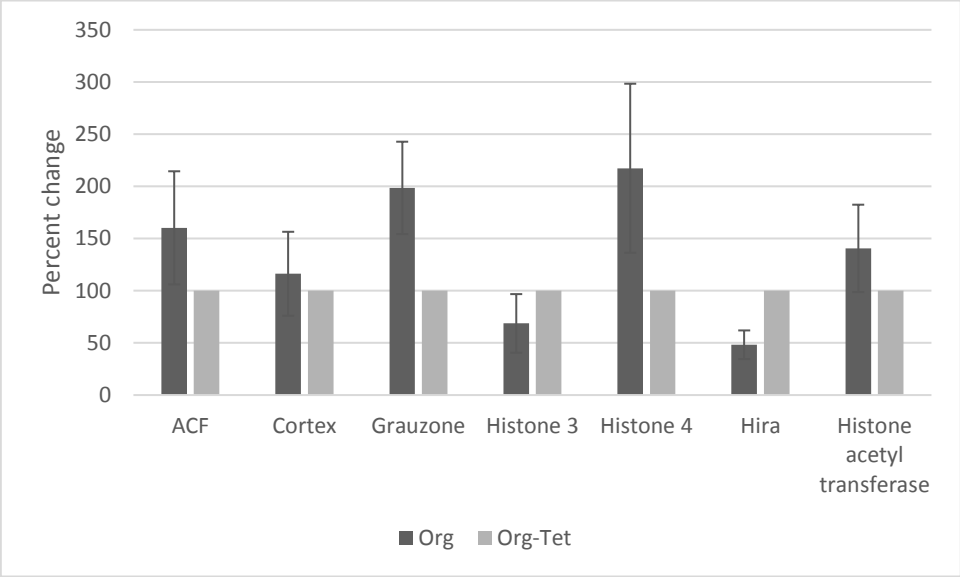


Figure 14: Quantification of CI candidate genes expression in the ovaries of Or^{E20} and Or^{E20}-*Tet* flies. The histogram represents the change in the ovary expression levels of different genes between a *Wolbachia*-infected (Or^{E20}) and a *Wolbachia*-uninfected (Or^{E20}-*Tet*) strain. The bars are the respective standard errors.

Similar to what has been observed in the ovaries, the expression levels of these genes remained almost the same when we compared Or^{E20} and Or^{E20}-*Tet* testes, with a small increase in the Protamine levels, and a two-fold increase in ACF. These increases, as well, were not statistically significant (p-value > 0.05).

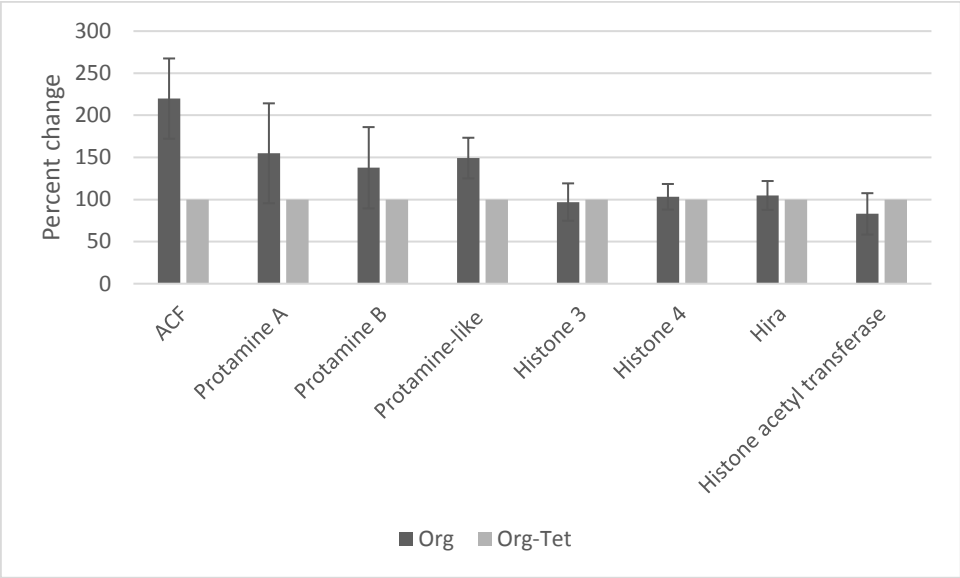


Figure 15: Quantification of CI candidate gene levels in the testes of Or^{E20} and Or^{E20}-*Tet* flies. The histogram represents the change in levels of different genes between *Wolbachia*-infected (Or^{E20}) and *Wolbachia*-uninfected (Or^{E20}-*Tet*) testes. The bars are the respective standard errors.

E. Quantification of AMPs in *Drosophila* gonads

To further investigate the effect of *Wolbachia* on *Drosophila*, specifically on the gonads where it resides, and in order to test whether harboring *Wolbachia* affects the immune response in the gonads, AMP levels of *Drosomycin* and *Diptericin* were quantified and represented in the following histograms.

Results on the ovaries show a higher expression level of both AMPs in Or^{E20} ovaries compared to Or^{E20}-*Tet* ovaries. The expression level of *Drosomycin* in Or^{E20} ovaries reached approximately a ten-fold increase, but remained a statistically insignificant increase with respect to Or^{E20}-*Tet* (p-value > 0.05) due to high variability (Figure 16).

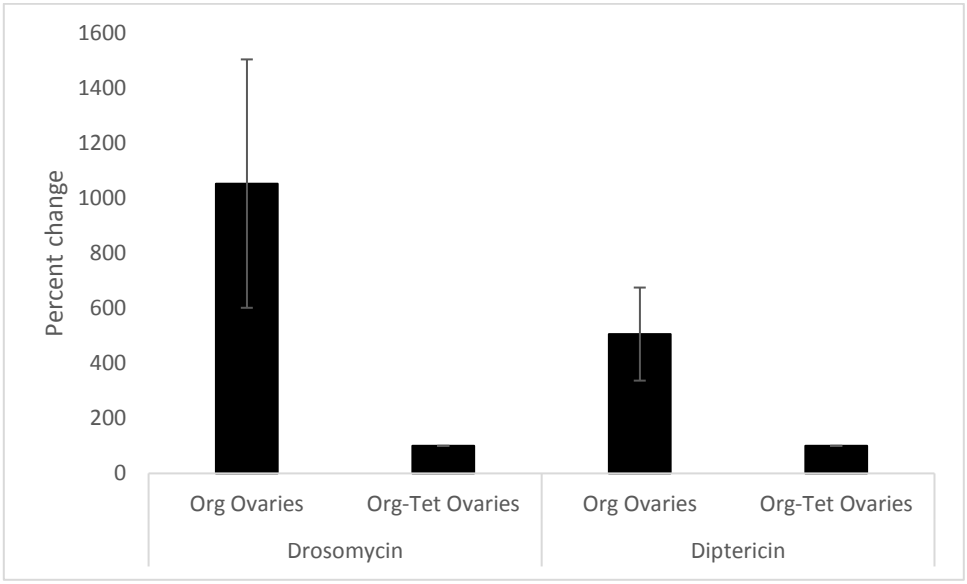


Figure 16: Quantification of AMPs levels in the ovaries of Or^{E20} and Or^{E20}-*Tet* flies. The histogram represents the change in the levels of the AMPs *Drosomycin* and *Diptericin* in the ovaries of a *Wolbachia*-infected (Or^{E20}) compared to *Wolbachia*-uninfected (Or^{E20}-*Tet*) strain. The bars are the respective standard errors.

AMP levels in Or^{E20} testes seem to also show an increase when compared to Or^{E20}-*Tet* testes (Figure 17). The percent increase is somehow lower in the testes as compared to the ovaries and having a statistically insignificant value as well.

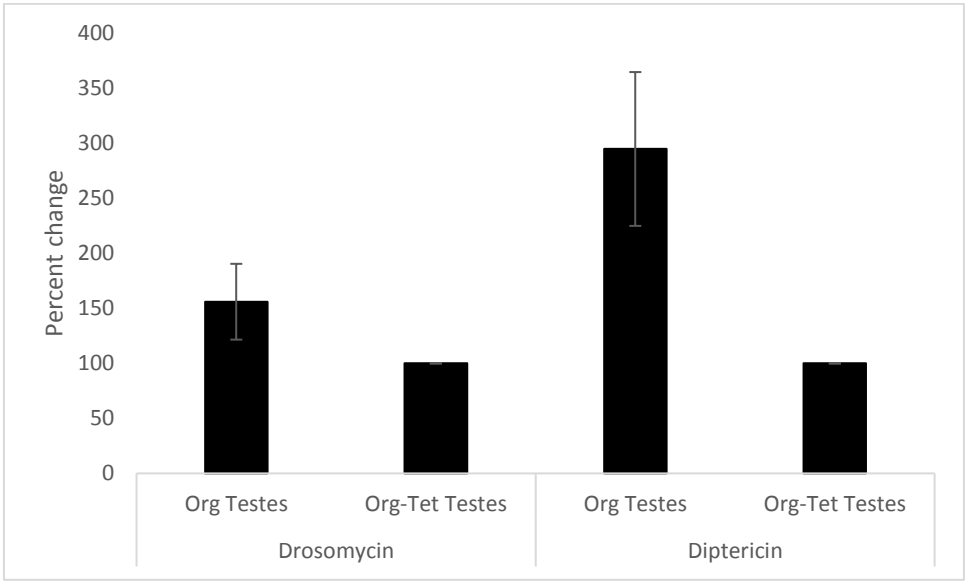


Figure 17: **Quantification of AMPs levels in the testes of Or^{E20} and Or^{E20}-*Tet* flies.** The histogram represents the change in the testis expression levels of the AMPs *Drosomycin* and *Diptericin*. The comparison was made between a *Wolbachia*-infected (Or^{E20}) and a *Wolbachia*-uninfected (Or^{E20}-*Tet*) strain. The bars are the respective standard errors.

F. CI Analysis

CI tests were done by comparing the percentage of eggs hatched in crosses that involve *Wolbachia*-infected (Or^{E20}) and *Wolbachia*-free strains (Or^{E20}-*Tet*). The crosses included a control of female and male Or^{E20}-*Tet* cross, a female Or^{E20} crossed to male Or^{E20}-*Tet*, and vice versa. The results did not show any difference between these three crosses, as all the laid eggs in the crosses hatched after a certain time. Thus, no CI was shown between these two strains obtained in our laboratory.

G. Egg production analysis

For the purpose of testing whether *Wolbachia* increases egg production in the flies it harbors, the overall number of laid eggs was counted in the *Wolbachia*-

infected and *Wolbachia*-free strain under the same conditions during 24 hours. The following graph shows the average number of eggs laid per female between Or^{E20} and Or^{E20} *Tet* flies. On average, a slightly higher, but statistically insignificant, number of eggs was obtained in Or^{E20}-*Tet* females (9.06 eggs/female) compared to Or^{E20} females (7.6 eggs/female) (Figure 18).

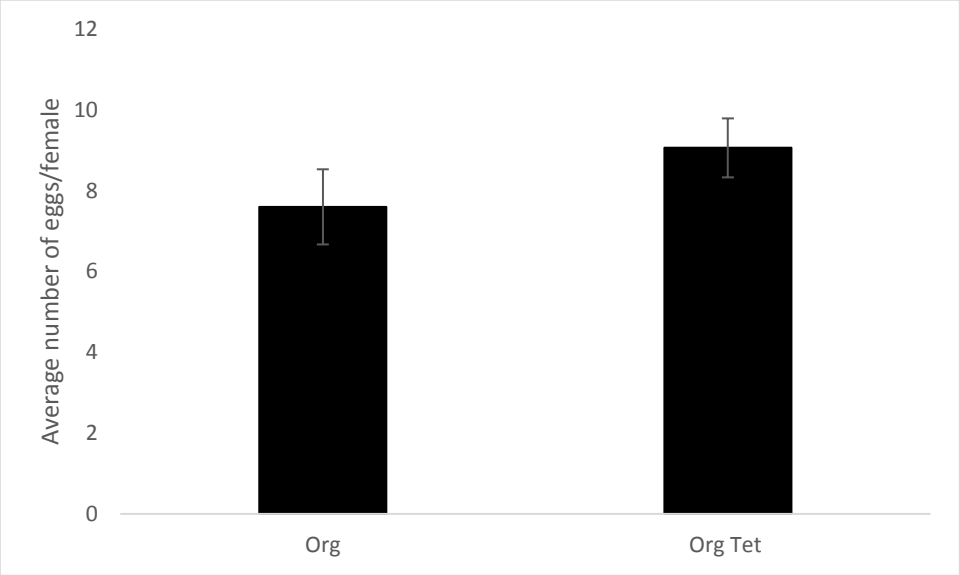


Figure 18: Egg production in different *Drosophila melanogaster* strains. The histogram represents a comparison in the average of number of eggs laid by females of Or^{E20} and Or^{E20}-*Tet* strains. The bars are the respective standard errors.

H. Ovary size Analysis

Another physiological effect of *Wolbachia* on its host could be a change in the size of ovaries, where *Wolbachia* is mainly present. Using fluorescent microscopy to visualize and measure ovary dimensions, the approximate size of each ovary was calculated as the volume of an ellipsoid. More than 90 ovaries were

dissected and their lengths and widths measured (Figure 19) for each of the strains Or^{E20} and Or^{E20}-*Tet*.

The results obtained show that Or^{E20} ovaries, carrying *Wolbachia*, had a slightly smaller volume on average (0.511 mm^3) when compared to ovaries of the uninfected strain Or^{E20}-*Tet* (0.618 mm^3) (Figure 19). This difference was statistically significant ($p < 0.05$).

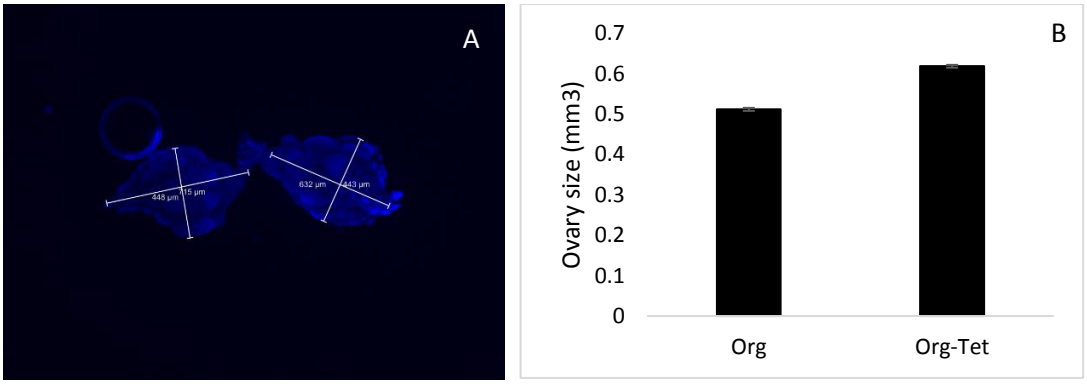


Figure 19: Average volume of ovaries in Or^{E20} and Or^{E20}-*Tet* *D. melanogaster* strains. A fluorescent image showing how the measurement of the length and width were taken (A). The histogram represents a comparison in the average volume of ovaries of Or^{E20} and Or^{E20}-*Tet* strains (n=90, p-value 0.031). The bars are the respective standard errors (B).

I. Quantification of *Wolbachia* in altered immunity strains of *Drosophila melanogaster*

As opposed to the previous experiments that investigated the effect of *Wolbachia* on the host immunity and physiology, in this experiment, the aim was to test whether immune pathways of the host affect *Wolbachia* density. To do this, genes from different immune pathways in the fly were either knocked-down or over-expressed using the UAS:GAL4 system.

For the Toll and Imd pathway altered immunity strains, the genes targeted were those corresponding to Toll, and to IMD and Relish, respectively. In addition to those genes, two different genes from the autophagy pathway were used: ATG8a and ATG13. These two genes were chosen on the basis of previous results in our lab that have shown compromised survival of flies with a genetic knock-down of either of those genes (Mustafa et Kambris, unpublished). The control used in this experiment was UAS:GFP crossed with the same driver C564:GAL4. This was done in order to ensure that the C564:GAL4 driver had no direct effect on *Wolbachia* titers.

The obtained results show that upon the knock-down of ATG8a and ATG13 from the *Drosophila* flies, a two-fold increase of *Wolbachia* density was shown compared to the control. Similarly, the over-expression of both IMD and Relish from the Imd pathway show a trend of increased *Wolbachia* density in those flies (Figure 20). However, these increases were not statistically significant due to high variability.

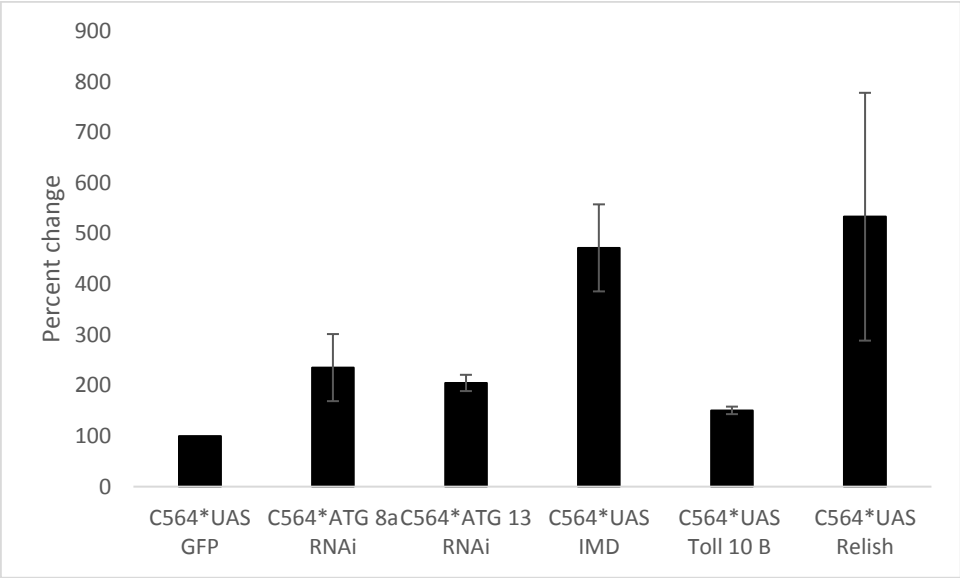


Figure 20: Quantification of *Wolbachia* density in flies with altered immune context. The histogram represents the change in the levels of *Wolbachia* in flies after the knock-down (RNAi) or over-expression of selected genes. The control used was C564:Gal4*UAS:GFP. The bars are the respective standard errors.

CHAPTER IV

DISCUSSION

In this study, the aim was to investigate the possible involvement of candidate genes in *Drosophila* innate immune responses and to look into the interaction between *Drosophila melanogaster* and *Wolbachia* by studying the effect of this endosymbiont on the immune and reproductive system of its host. The mechanisms underlying *Wolbachia*-host interactions remain unclear and seem to vary depending both on *Wolbachia* strain and on the host species carrying it. Despite this variation and the remaining gaps in knowledge on this topic, the significance of studying this interaction lies in the possible exploitation of *Wolbachia* as a biological method for pest control, as well as in providing further information on host responses to endosymbionts. Here, we analyzed the possible effects of *Wolbachia* on its natural hosts *Drosophila melanogaster* by comparisons done between a *Wolbachia*-harboring (Or^{E20}) and a *Wolbachia*-free strain (Or^{E20}-*Tet*). Also, the analysis of the immune pathways that may have a role in controlling *Wolbachia* in the host was done through *Wolbachia* quantification in flies with altered immunity.

The effect of the knock-down of thirteen candidate genes in the fat bodies of the flies was first assessed using survival assays after infection with three different types of microbes, followed by AMP quantification of the flies for which the gene knock-down showed compromised survival. For each type of microbe (gram-positive bacteria, gram-negative bacteria, fungi), the results of both the survival assay and the level of quantified

AMP were compared. For gram-positive bacteria, five different gene knock-downs in flies displayed compromised survival, but only three of which: CG 5150, CG 3829, and CG 9186 also showed a decrease in the induction of the AMP *Drosomycin*. Thus, it seems that these flies' survival could have been affected by their inability to activate the Toll pathway, reducing *Drosomycin* production, and thus incapable of targeting the invading bacteria. Also responsible for activating the Toll pathway is the fungus BB, of which its infection into flies showed compromised survival for two gene knock-down flies, only one of which (CG 5729) displayed a decrease in *Drosomycin* induction.

As for ECC15, survival assays displayed compromised survival for two gene knock-down flies, but only one: CG 5729 was shown to decrease *Diptericin* induction by Real-Time PCR, which may suggest that its low survival could have been due to its inability to activate the Imd pathway. This gene showed positive results in both gram-negative bacteria and fungal infection, making it interesting candidate gene. Further experiments using different microbes from each type could be a start for confirmation, followed by experiments allowing us to find where they might be acting.

Concerning *Wolbachia-Drosophila* interaction, we first tested ten genes for their possibility of playing a role in CI mechanisms. The comparison was made between the gonads of the two strains Or^{E20} and Or^{E20}-*Tet*, and the results obtained by Real-Time PCR show no change in the transcriptional profiles of most of these genes. A few genes showed a maximum increase of two-folds (ACF, Grazuone and Histone 4 in the ovaries and ACF and Protamines in the testes) but were not statistically significant. Having no change in the expression levels of these genes may be explained with the egg hatching results obtained

that showed no CI between these two strains. These results are consistent with previous studies showing that *D. melanogaster* displays weak CI which could range from partial CI to being inexistent (reviewed in Werren, 1997). As for the genes with slightly upregulated transcriptional profiles, this increase could be biologically insignificant as it is statistically. To verify whether it is significant or not, perhaps the same study could be done on a species with a more evident CI, if the increase persists, then it could be interesting to look further into.

The levels of AMPs (*Drosomycin* and *Diptericin*) were slightly increased in the *Wolbachia*-harboring strain, Or^{E20}, compared to Or^{E20}-*Tet*, in both ovaries and testes, but also statistically insignificant. This could mean it is also biologically insignificant or it could be because of variability due to technical errors. If we take the latter into consideration, we could speculate that this increase might be the effect of *Wolbachia* on the gonads, where it mostly resides, in the aim of protection against other bacteria, or maybe a host protection mechanism to control *Wolbachia* levels.

Other effects of *Wolbachia* on host fitness were studied, mainly egg production and ovary size. Concerning egg production, Or^{E20} females displayed a slightly, statistically insignificant, reduced number of eggs per female when compared to Or^{E20}-*Tet* egg production. As for ovary size, a significant decrease of ovary size in Or^{E20} is observed in comparison to Or^{E20}-*Tet*. Although the former result is statistically insignificant, it is, however, consistent with the latter. The strain with a bigger ovary size on average, is producing more eggs, and vice versa. A recent study suggested that *Wolbachia*-induced apoptosis in the *Culex* ovaries may be causing egg degeneration and hence a lower egg

production (Almeida and Suesdek 2017).Thus, it is interesting to monitor apoptosis in these ovaries in order to test if it could be the reason behind this result.

We then wanted to investigate the effect of the *Drosophila melanogaster* immune system on *Wolbachia* control. For this, we altered the immunity of flies by either knock-down or over-expression of representative genes from the Toll, Imd and autophagy pathways. Results show that the knock-down of two autophagy genes (ATG8a and ATG13) lead to an increase in *Wolbachia* density. Although statistically insignificant, this increase is consistent with previous results in our lab on these genes (Mustafa and Kambris, unpublished), and suggests a possible involvement of autophagy in the control of *Wolbachia*.

Also, the over-expression of IMD and Relish from the Imd pathway showed an increase in *Wolbachia* density, as if this pathway favors *Wolbachia*. This is consistent with a recent study that suggests an activation of the Imd pathway in the mosquito *Aedes aegypti* could be enhancing *Wolbachia* survival suggesting these two factors could be involved in a positive feedback loop (Pan, Pike et al. 2018). Both these results should be investigated further by fluorescent microscopy to better confirm the results obtained. Also, this experiment was done using a fat body specific driver, so perhaps using an ovary-specific driver and investigating *Wolbachia* density in the ovaries could better verify these suggestions.

In summary, the results obtained from this study, along with further confirmation, would add more information on *Wolbachia* interaction with its hosts. Understanding these

interactions should contribute towards the possibility of using this symbiont as a biological control method.

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