AMERICAN UNIVERSITY OF BEIRUT

INVESTIGATION OF CANDIDATE GENES' ROLES IN *DROSOPHILA MELANOGASTER*'S INNATE IMMUNITY

by
SARAH BASSAM HALABI

A thesis
submitted in partial fulfillment of the requirements
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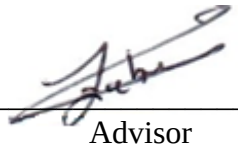

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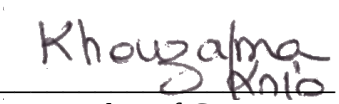

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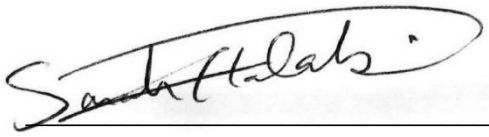

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ABSTRACT
OF THE THESIS OF

Sarah Bassam Halabi for Master of Science
Major: Biology

Title: Investigation of Candidate Genes' Roles in *Drosophila melanogaster*'s Innate Immunity

Drosophila melanogaster is an efficient genetic model to study immunity, and by understanding the fly's innate immunity we can draw similarities and figure out the homologies in humans, which will help us understand our own innate immune system. There are candidate genes with unknown functions suspected to be involved with *D. melanogaster*'s innate immune responses based on previously conducted microarray studies. RNA interference (RNAi) was utilized to knockdown the candidate genes' expression and test for the effects of their silencing on the flies' innate immunity. For this, the survival is assayed after different microbial infections and bacterial proliferation assays are performed. The expression levels of antimicrobial peptides (AMPs) are determined by real-time polymerase chain reaction (RT-PCR). The results allow us to determine whether a candidate gene is a component the Toll or the IMD pathway or if it acts independently of these pathways. Thirty-one different genes were selected based on previous microarray studies and were screened by conducting survival assays using Gram-positive bacteria, Gram-negative bacteria and fungi as the immune challenges. Based on the survival analysis results, 14 genes were selected and subjected to bacterial proliferation assays to study the bacterial clearance. Interestingly enough, the Gram-negative bacteria did not affect the survival rate nor the bacterial clearance rate of the candidate genes. For the Gram-positive bacteria, however, some genes were shown to overlap in the positive selection for the survival assay and bacterial clearance. Statistical analysis method proved that there is a significant correlation between survival rate and CFU count using. Although the RT-PCR runs on control flies were successful, the runs for testing the candidate genes were not completely finalized due to the malfunctioning of the RT-PCR machine. Regardless, the samples were saved so they can be tested in the future.

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ABBREVIATIONS

RNAi	RNA interference
%	Percent
°C	Degrees Celsius
g	Grams
LB	Luria-Bertani
nm	Nanometers
OD	Optical density
PDA	Potato dextrose agar
nL	Nanoliters
SA	Staphylococcus aureus
ECC	Erwinia carotovora
EF	Enterococcus faecalis
BB	Beauveria bassiana
RT-PCR	Real-time Polymerase Chain Reaction
h	Hours
µl	Microliters
RT	Reverse transcription
Ct	Cycle threshold
mg	Milligrams
mL	Milliliters
WT	Wild-type
F1	First filial generation
AMP	Antimicrobial peptide
IMD	Immunodeficiency pathway
SPE	Spatzel processing enzyme
ModSP	Modular serine protease
PGRP	Peptidoglycan recognition protein
GNBP	Gram-negative binding protein
DAP	Diaminopimelic acid type
dsRNA	Double-stranded RNA
siRNA	Small interfering RNA
RISC	RNA-induced silencing complex
UAS	Upstream activating sequence
cDNA	Complementary DNA
hpRNA	Hairpin RNA
NFAT	Nuclear factor of activated T cells
OD	Optical density

CHAPTER I

DROSOPHILA MELANOGASTER AND INNATE IMMUNITY

Drosophila melanogaster is an efficient genetic model to study immunology seeing as there are many parallels between human innate immunity and *D. melanogaster* innate immunity due to evolutionary conservation. The DNA of *D. melanogaster* has been fully sequenced and there is a variety of genomic and bioinformatic tools available to aid in conducting experiments that can define the roles of certain genes.

A. *Drosophila melanogaster* Immune Pathways

There are two main signaling pathways involved with regards to gram positive and gram negative bacterial infections; the Toll pathway and the immune deficiency pathway (IMD)(Buchon, Silverman, & Cherry, 2014). The Toll pathway is involved to fight Gram-positive bacteria or fungi while the IMD pathway controls the response to Gram-negative bacterial infections. In both pathways, a series of signaling cascades is activated whereby the final product includes the production of important antimicrobial peptides (AMPs) that the body uses as an innate immunological defense mechanism against the infections.

1. The Toll Pathway Heading

a. Gram-positive bacteria

When gram positive bacteria infect the D. melanogaster, bacterial determinants (like lysine-type peptidoglycan and microbial proteases)(Silverman, Paquette, &

Aggarwal, 2009), initiate a cascade of proteases which leads to the activation of Spatzle (the Toll receptor ligand) turning proSpatzle into Spatzle via Spatzle processing enzyme activation (SPE) (Valanne, Wang, & Ramet, 2011). These serine proteases includes Grass, spirit, spheroide, and sphinx1 and sphinx 2 (which are also respond to fungal responses) (Kambris et al., 2006). Modular serine protease (ModSP) recognizes the peptidoglycan recognition protein (PGRP)-SA and integrates the signals to the Grass-SPE-Spatazle signaling cascade (Valanne et al., 2011). Persephone is a protease that recognizes Gram positive bacterial virulence factors (proteolytic activities secreted by the bacteria) and subsequently activates SPE (Chtarbanova & Imler, 2011). Gram-negative binding protein1 (GNBP1), PGRP-SA and PGN-SD recognize the Lys-type peptidoglycan of Gram-positive bacteria. After that, PGRP-SA and GNBP1 will form a complex which activates the GNBP1. This subsequently leads to the GNBP1 hydrolyzing the Lys-type peptidoglycan and producing a glycan with reducing ends that interacts with PGRP-SA and subsequently triggers the serine protease cascade (Kurata, 2014).

Cleaved Spatzle binds to the Toll receptor and activates it. This, in turn, causes the Toll receptor to bind to MyD88, an adaptor protein, through the Toll's TIR domains. This will lead to the subsequent recruitment and formation of MyD88-Tube-Pelle complex through their death domains. After this complex formation, Cactus (the Drosophila IκB factor), becomes phosphorylated by Pelle, and becomes tagged for degradation which causes it to be released from the NF-κB transcription factor Dorsal/Dif (Dorsal-related immunity factor). It is important to note, Cactus acts as an inhibitor of Dorsal/Dif when bound. After its release, Dorsal/Dif

translocates into the nucleus where it activates the transcription of the target genes including those encoding AMPs.

The difference between Dorsal and Dif is that Dorsal is involved in dorsal-ventral polarization while Dif is not. Also, Dif induces Drosomycin (antifungal peptide) in adults, meanwhile, Dorsal and Dif can both act in the larvae stages (Valanne et al., 2011).

b. Fungi

When an infection is caused by a fungal agent, there are slight differences in how the Toll pathway becomes activated; the majority of the differences occurs upstream of Toll receptor activation. For starters, the ModSP-Grass-SPE becomes activated when the β-glucan component of the cell wall of fungi becomes recognized by GNBP3. Persephone can also recognize foreign proteases secreted by the fungi (namely PR1) and directly activate SPE (Silverman et al., 2009). The remainder of the signaling cascade is the same as the Gram-positive bacterial activation of the Toll pathway.

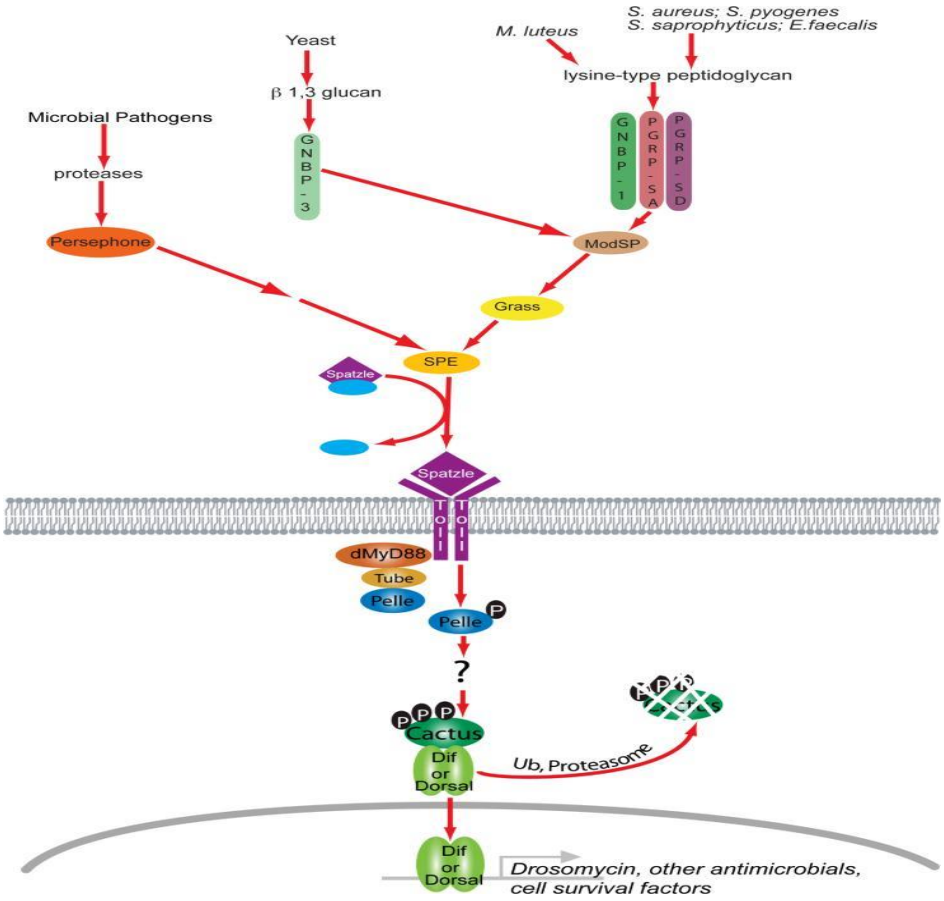


Figure 1. The activation of the Toll pathway (Silverman et. al 2009). A series of proteases will activate SPE which turns pro-Spatzel into Spatzel, the ligand for the Toll receptor. After binding, Toll becomes activated which initiates the signaling cascade that leads to the production of antimicrobial cell survival factors via Dif activation.

2. The IMD Pathway

The IMD pathway is activated when Gram-negative bacteria infects *D. melanogaster*. It starts off when PGRP-LC recognizes the peptidoglycan that is found on Gram-negative bacteria, diaminopimelic acid type (DAP), or when intracellular full length PGRP-LE binds to monomeric peptidoglycan fragments (Myllymaki, Valanne, & Ramet, 2014).

After PGRP-LC becomes activated, an Imd-dFadd-Dredd complex will become recruited. Dredd becomes ubiquitinated by lap2 (which is associated with

UEV1a, Ubc13, and Ubc5). Dredd becomes activated and will cleave Imd creating a binding site for lap2 where it ubiquitinates Imd. This recruits the Tab2/Tak1 complex which will phosphorylate and activate the Drosophila IKK complex which is responsible for phosphorylating and subsequently activating the transcription factor Relish. Relish will activate the transcription for genes that code for AMPs (Kurata, 2014).

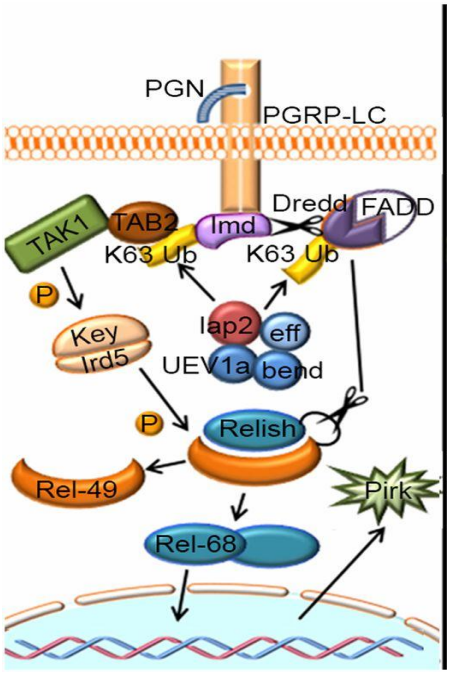


Figure 2. The activation of the IMD pathway (Myllymaki et al., 2014). The peptidoglycan of the Gram-negative bacterial cell wall becomes recognized by PGRP-LC and leads to a signaling cascade that ends with the activation of Relish. Relish will then transcribe for AMPs.

3. Cross Talk

Interestingly, the Toll and IMD pathways are not completely independent of one another. Although not much is known about the signaling pathway of this cross talk, there are reports that show that the injection of Gram-negative bacteria and Gram-positive bacteria upregulated effector genes of both pathways which means cross talk between the two pathways exists. It was also shown that when knocking down IMD pathway genes, the upregulation of effector molecules for both the Toll and IMD pathways were suppressed with the IMD effector molecules being more affected. The same results were shown for the knocking down of Toll pathway genes except the Toll effector molecules were more affected (Nishide et al., 2019).

B. *Drosophila melanogaster* UAS/Gal4 RNAi system

In order to knock down genes for experiments without compromising the stock's health, the UAS/Gal4 RNA interference (RNAi) system is utilized.

1. RNAi

RNAi is a post transcriptional gene silencing mechanism that occurs as a way to protect the host from foreign nucleic acids, viruses and accumulation of transposons and repetitive sequences (Zamore, Tuschl, Sharp, & Bartel, 2000). In order for the RNAi pathway to be triggered, the presence of double stranded RNA (dsRNA) or hairpin RNA (hpRNA) needs to be recognized by RNAse III (Dicer) whereby it cleaves it into small interfering RNA (siRNA). The siRNA has an overhang involving 2 nucleotides at the 3' end. The antisense strand is the guide strand that gets incorporated into the RNA-induced silencing complex (RISC), however, the sense strand gets degraded. The guide strand complexed with the RISC then create base pairs with the complementary mRNA whereby it gets degraded via the endonuclease Argonaute protein found within the RISC complex (Majumdar, Rajasekaran, & Cary, 2017).

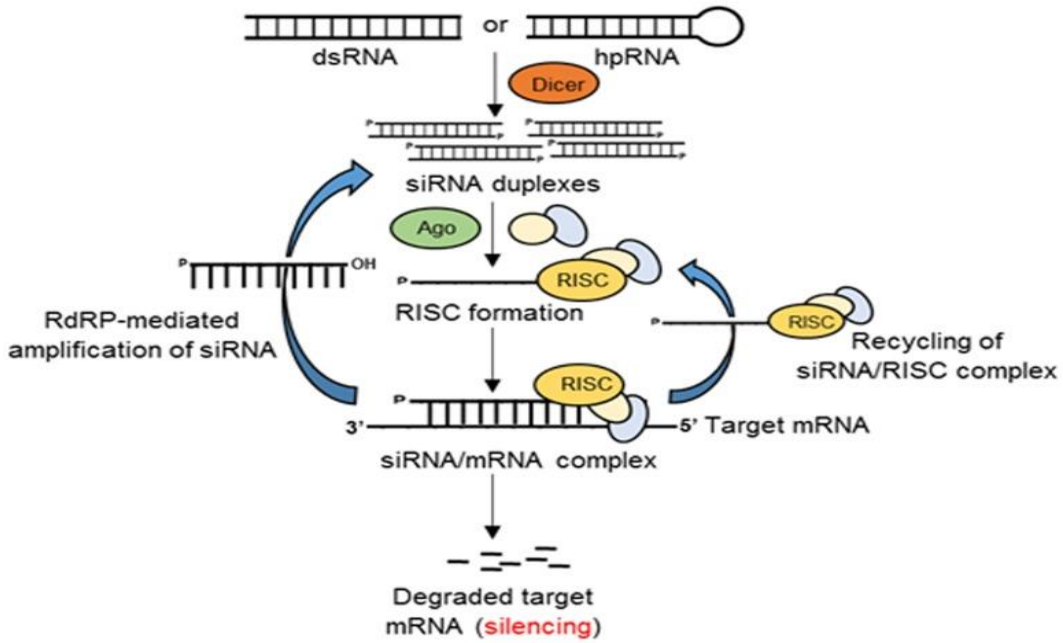


Figure 3. Gene silencing using RNAi (Blake, Finger, Hardy, & Ables, 2017). Whenever cells sense dsRNA or hpRNA, Dicer will cleave them into siRNA where the antisense siRNA gets incorporated into the RISC complex and will guide the complex to the mRNA that needs silencing. Upon complementary binding to the mRNA, Argonaute will degrade the mRNA.

2. UAS/Gal4 system

Having an RNAi system that is controlled by the experimenter is vital as certain genes can weaken flies or even inhibit their development into mature adult flies, hence, putting the fly stock at risk of extinction from the lab. Thus, the UAS/Gal4 RNAi system is an incredibly useful tool for controlled gene knockdown. Gal4 is a transcription factor that is endogenous to yeast and binds to the DNA response element, the UAS. The UAS will precede an inverted repeat DNA fragment that is complementary to the gene that is supposed to be knocked down. Due to the inverted repeats, expression of this gene will give a dsRNA hairpin which will trigger the RNAi response. Typically speaking, there are two lines of flies involved, one containing the UAS-inverted repeats gene fragment and another line of flies that contains and

expresses the Gal4 transcription factor. When these two lines are crossed, the progeny will contain the knocked down gene of interest when put under 29°C (Blake et al., 2017).

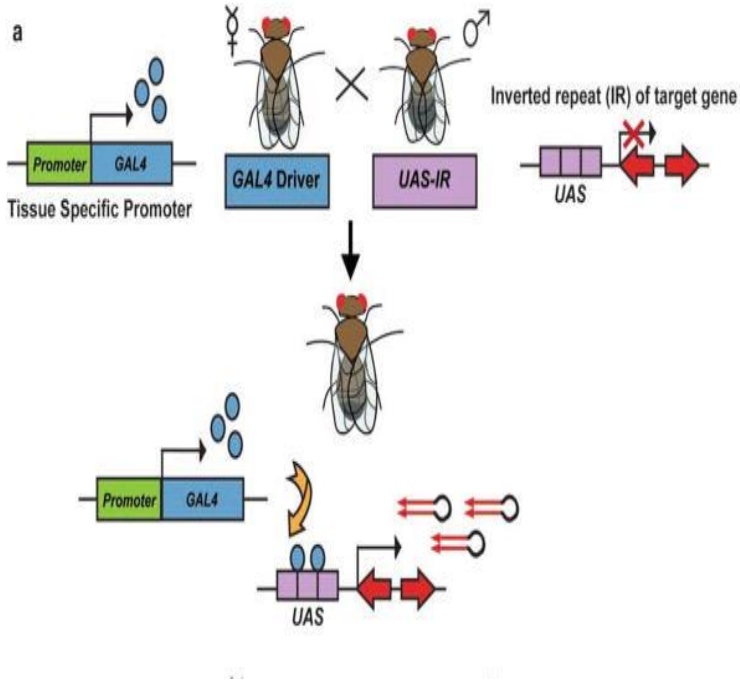


Figure 4. UAS/Gal4 RNAi knockdown *D. melanogaster* system (Blake et al., 2017). Female flies containing the Gal4 driver are crossed with male flies containing the UAS followed by the inverted repeats gene fragment that is complimentary to the mRNA that is needed to be silenced under conditions that keep the temperature at 29°C. The progeny will contain the silenced gene.

C. *Drosophila melanogaster* life cycle

D. melanogaster is incredibly efficient model to work with. The adult progeny takes approximately 10 days to emerge under 25°C (it takes twice as long under 18°C) from fertilization into adulthood. Its lifespan can be anywhere from 60 to 80 days and its life cycle can be broken down into four stages: the embryo, larvae, pupae and adult stages. The embryo stage takes about 24 hours before it goes into the first instar larvae stage. Afterwards, it will enter the second instar larvae stage where the larvae starts to burrow deeper into the culture. Finally, once the fly reaches the third instar larvae stage, it will start to search for a place to begin its pupariation by wandering about the walls of the vials. The larvae stage takes about 3 days. Once the fly undergoes pupariation, it will take around four days for the adult fly to finally emerge (Fernandez-Moreno, Farr, Kaguni, & Garesse, 2007).

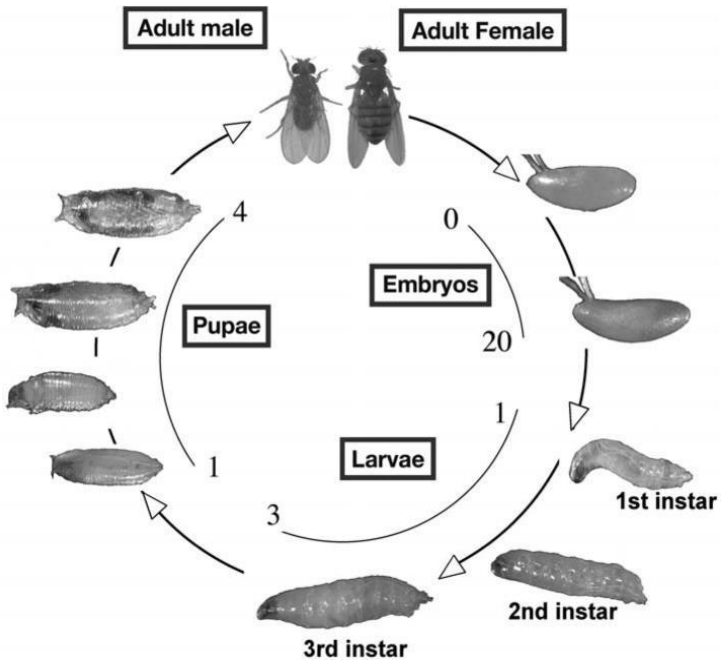


Figure 5. *D. melanogaster* life cycle (Fernandez-Moreno et al., 2007). The embryo stage is represented in hours, the larvae stage is represented in days and the pupae stage is also represented in days.

D. NFAT

1. In humans

Nuclear factor of activated T cells has been found to be involved in human immune responses. It is a calcium dependent AP-1 transcription factor first described in T cells. Calcineurin is a calcium dependent phosphatase that dephosphorylates NFAT which leads to their translocation into the nucleus. They have an important role in humoral immunity as they regulate cytokines, chemokines and growth factors in immune cells. They are also crucial for the differentiation of T helper cells. The activation, antigen presentation, proliferation and apoptosis of B cells have also been shown to be affected by NFAT (Vaeth & Feske, 2018).

2. In D. melanogaster

NFAT in *D. melanogaster* has been found to be involved in the regulation of the pre-synaptic development of the fly (Freeman, Franciscovich, Bowers, Sandstrom, & Sanyal, 2011). NFAT is also involved in the regulation of homeostatic synaptic plasticity in cholinergic neurons (Eadaim, Hahm, Justice, & Tsunoda, 2020). It's also been shown to regulate osmotic balance similar to its mammalian counterpart (Keyser, Borge-Renberg, & Hultmark, 2007). Unfortunately, little is known about NFAT in *D. melanogaster* in the context of immunity.

D. Significance and Aims of the Project

Although the entire genome of the fruit fly has been sequenced, there is still a gap in knowledge about the functions of many genes. Since we are interested in innate immunity, it would be of great importance if we can attribute an immune function to

such genes. Doing so will open the door to more questions that can be explored and can further describe the fly's innate immunity especially when it comes to signaling pathways.

We first inactivated the candidate genes using the UAS/Gal4 system with the C564 driver which expresses Gal4 in the fat body of the fly. The system was validated and the positive controls showed susceptibility to the microbe infection. Then, we conducted survival assays and test to see which genes become compromised after knockdown following a microbe challenge. We also performed bacterial proliferation assays in order to measure the bacterial clearance of selected candidate genes. Finally, we tested to see whether or not these genes are directly involved in the Toll and IMD pathways by measuring their effector molecules (AMPs).

1. Specific Aim 1

We hypothesize that the inactivation of candidate genes will elicit the desired effect. To test our hypothesis, we performed the following experiments:

- Knock down the candidate genes by crossing the available inverted repeats lines to the fat body driver C564.
- Check survival assay to see if the positive controls tested positive.
- Check to see if the cross was lethal and the progeny did not emerge.

2. Specific Aim 2

We hypothesized that a number of candidate genes will be lethally affected by the microbial challenges and the survival assays will reveal the positive hits. This would

be due to their supposed role in the flies' immunity. To test our hypothesis, we performed the following experiments:

- Knock down the candidate genes by crossing the available inverted repeats lines to the fat body driver C564.
- Inject the candidate genes' progeny with microbes that will challenge the flies' immunity.
- Record the death rate of the candidate genes and compare it to the positive and wild type controls.

3. Specific Aim 3

We hypothesized that of the positively selected candidate genes from the survival assays will have a low bacterial clearance rate compared to the positive controls. To test our hypothesis, we performed the following experiments:

- Knock down the candidate genes by crossing the available inverted repeats lines to the fat body driver C564.
- Inject the candidate genes' progeny with microbes that will challenge the flies' immunity.
- Plate the flies and count the CFUs.

4. Specific Aim 4

We hypothesized that the positively selected candidate genes are involved in the Toll or IMD pathways due to their susceptibility towards a microbial challenge. To test our hypothesis, we performed the following experiments:

- Knock down the candidate genes by crossing the available inverted repeats lines to the fat body driver C564.
- Inject the candidate genes' progeny with microbes that will challenge the flies' immunity.
- Measure the AMP production using RT-PCR

CHAPTER II

MATERIALS AND METHODS

A. *Drosophila melanogaster* stocks, rearing and stock maintenance

All of the *D. melanogaster* lines were obtained from the Vienna *Drosophila* RNAi Center. 50 mL vials are used to rear stocks and the medium within these vials contains a mixture of soy flour, polenta (cornmeal), molasses, agar, and propionic acid. Flies are either stored at 18°C or at 25°C depending on how quickly it is needed for the flies to emerge with the former taking a longer time. A 12 hour light:dark cycle is used to maintain the stocks.

B. *Drosophila* crosses

Virgin females containing the driver were collected from vials stored at 18°C from 0-16 hours post-eclosion from pupae. Approximately speaking, ten virgin females were crossed to 4-8 males carrying the UAS-inverted repeats gene fragments in vials containing fresh medium and the crosses were maintained at 25°C until the progeny reached the third instar larval stage. After that, crosses were transferred to an incubator that is kept at 29°C since this temperature is needed for maximum efficiency for the UAS/Gal4 RNAi knockdown system.

C. Microbe preparation

Bacterial cultures were incubated overnight at 37°C with shaking. The cultures were then spun at 4000G for 10 minutes, cells were re-suspended in LB. OD is measured with the spectrophotometer at 595nm and adjusted to the desired

concentration. BB fungus was grown on PDA plates for three weeks and the spores were later collected via filtration and adjusted to the desired number of spores/nL.

D. Infection of *D. melanogaster*

Fifteen to twenty male or female flies of the progeny of each *Drosophila* cross were collected at an age ranging from three to six days. Flies were injected with 32.2 nL of the microbe (SA OD 0.002, ECC OD 0.05, EF OD 0.05 or 5 spores/nL of BB) using a nano-injector armed with a capillary needle. They were returned to their vials and maintained at 25°C . For survival assays, dead flies were counted at regular intervals. Survival graphs were then plotted as percent survivals as a function of time. Flies that were infected for RNA extraction and RT-PCR were frozen at -20°C for 24h after bacterial infection and 48h after fungal infection.

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

For RNA extraction, approximately 15 flies were homogenized in 500 µL of Trizol reagent (Invitrogen) and RNA was then separated from its cellular constituents by spinning at 15,000G for 10 minutes at 4°C. 100 µL of Chloroform were added to the supernatant, and the mix was vigorously vortexed for 2 minutes and later spun at 20,000G to separate the phases. The aqueous phase was transferred to a clean Eppendorf and the RNA was precipitated by the addition of 0.7 volumes of isopropanol and was later spun at 20,000G for 20 minutes at 4°C. The pellets were then washed in 70% ethanol and resuspended in 50 µL of nanopure double distilled water.

The extracted RNA was then diluted to a concentration of 200 or 100 ng/µL and then 5 µL were reverse transcribed into cDNA using the iScript cDNA Synthesis

Kit (Biorad). The master mix was first created by putting 2 µL of the mix, 2.5 µL of nanodrop water, and 0.5 µL of the reverse transcriptase enzyme. After which, 5 µL of the of the master mix was distributed to the eppendorfs followed by the addition of 5 µL of the extracted RNA sample. The first step is to put the mix at 42°C for 50-60 minutes, and then the reaction was stopped by a five minute incubation period at 85°C followed by a 2 minute incubation period at room temperature. Then using RT-PCR, the gene transcription level was quantified using a 1:15 dilution of the RT product.

A mixture of *Drosophila* cDNA (4 µL), reverse and forward primers specific to the gene of interest (0.5 µL each) and QuantiFAST SYBR green PCR mix (5 µL of the 10 µL Qiagen kit) were subjected to 40 cycles of denaturation at 95°C, annealing of the primers at 57°C, and elongation at 72°C followed by the quantification at the end of each cycle. The expression levels of *Drosomycin* and *Diptericin* were used as read-outs for the Toll and IMD pathways respectively. The gene that encodes for the ribosomal protein Rp49 was considered as a reference gene for normalization and the Delta Ct method was used for calculations.

F. Bacterial Proliferation Assay

Two flies of the same sex were injected with bacterial suspensions. The incubation period depends on the microbe, after the time has passed, the two flies were grinded in 200 µL of LB. The lysates were then serially diluted and plated on LB agar plates that contain the antibiotic that is specific to the resistance of the injected microbe. The plates were then incubated at 34 °C overnight to score the CFUs.

CHAPTER III

RESULTS

A. Screening of Candidate Genes Using Survival Assay

Candidate genes were selected from previously conducted microarray studies that showed differential expression levels after an immune challenge (De Gregorio, Spellman, Rubin, & Lemaitre, 2001; Irving et al., 2001). The male flies were first crossed with C564 female flies and the F1 progeny (which has the activated RNAi for the target gene) was collected and used for the experiment. Flies were injected with 32nL of EF (OD of 0.05), ECC (OD of 0.05), BB (5 spores/nL) and SA (OD of 0.002). The flies were then monitored and the death rates were recorded for approximately 72 hours for every microbe except BB; BB was monitored for a full week. Survival plots were then plotted as percent survival as a function of time. Thirty-one different genes were screened (Figures 6,7, and 8). Dif-1 null mutant and DifKK RNAi mutant were used as positive controls for the Toll pathway and Relish null mutant and dFadd RNAi mutant were used as positive controls for the IMD pathway. Oregon (crossed and not crossed) and White (crossed and not crossed) were used as wild type controls. For the sake of simplicity, the genes will be referred to by their corresponding serial numbers (see Table 1). It's interesting to note that genes CG14936 7779/GD, CG10882 37543/GD, CG14938 7779/GD, CG1725 41134/GD, CG4257 43866/GD, CG6713 27725/GD were lethal when knocked down. Genes 1, 8, 10, 14, 15, 16, 21, 22, 25, 30, 35, 36, 37, and 38 were picked for survival assay confirmation and further experimentation (Figure 9). Genes 21, 25, 36, 37, and 38 were the most vulnerable to

EF infection (Figure 9). All of the candidate genes were primarily affected by EF and not ECC infection.

Table 1 Candidate genes and their serial numbers

Serial Number	Gene			Serial Number	Gene	
1	CG2217	29576/GD		**22**	CG7780	100014/KK
2	CG2217	29577/GD		**24**	CG6426	102243/GD
3	CG2217	108167/KK		**25**	CG5118	34937/GD
4	CG6394	105160/KK		**26**	CG5118	106292/KK
5	CG7214	12372/GD		**27**	CG3759	15602/GD
6	CG9186	105945/KK		**28**	CG4257	43866/GD
8	CG13641	101688/KK		**29**	CG3019	104716/KK
10	CG13641	14717/GD		**30**	CG3019	25597/GD
11	CG5150	102646/KK		**31**	CG3759	108677/KK
12	CG3829	42872/GD		**33**	CG3131	2593/GD
13	CG18466	110198/KK		**35**	CG11172	107032/KK
14	CG10697	3329/GD		**36**	CG12172	30782/GD
15	CG10641	107033/KK		**37**	CG12172	30783/GD
16	CG8965	102683/KK		**38**	CG9460	24036/GD
18	CG6822	5142/GD		**39**	CG7219	106228/KK
19	CG7294	27492/GD		**40**	CG11331	107404/KK
20	CG5729	27490/GD		**42**	CG6877	101364/KK
21	CG10592	104767/KK		**43**	CG7331	103381/KK

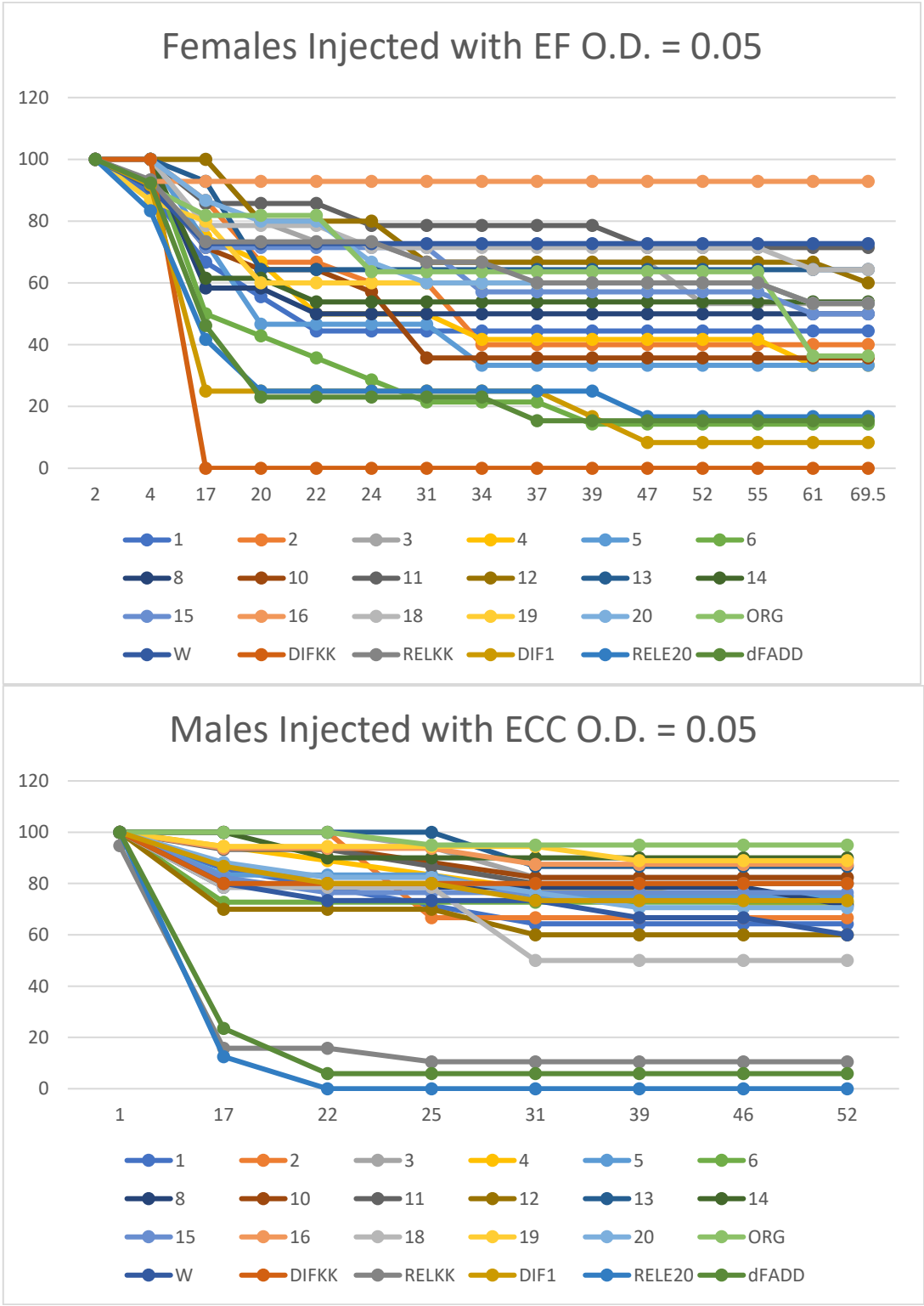


Figure 6 Survival assays displaying males and females injected with ECC and EF with an OD of 0.05 respectively (1). Oregon and White are the wild type controls and DifKK, dif-1 are the Toll pathway positive controls and RelKK, RelE20 and dFadd are the IMD pathway positive controls. Genes 1, 8, 10, 14, 15, and 16 were selected since they were vulnerable to EF infection.

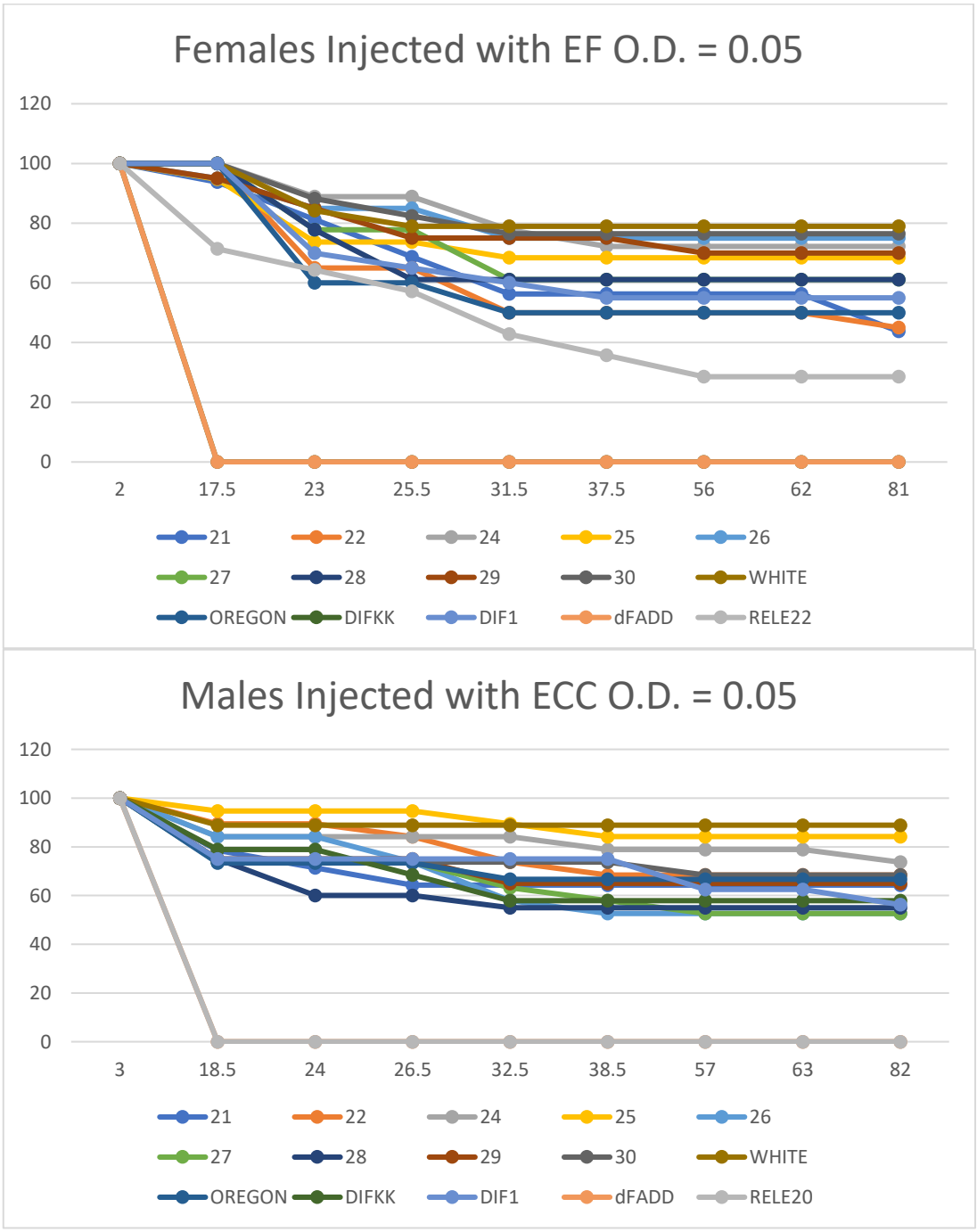


Figure 7 Survival assays displaying males and females injected with ECC and EF with an OD of 0.05 respectively (2). Oregon and White are the wild type controls and DifKK, dif-1 are the Toll pathway positive controls and RelE20 and dFadd are the IMD pathway positive controls. Genes 21, 22, 25, and 30 were selected since they were vulnerable to EF infection.

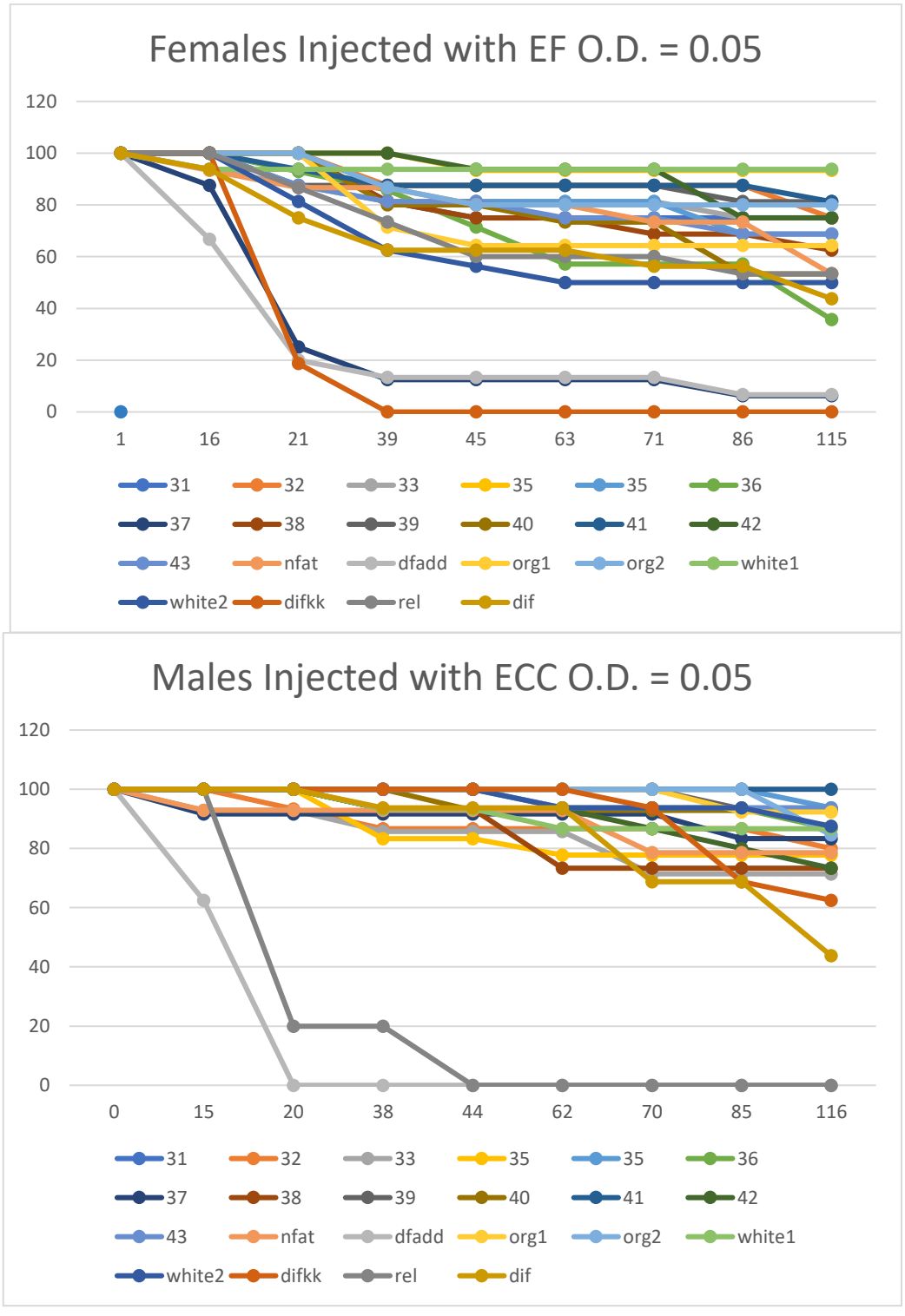
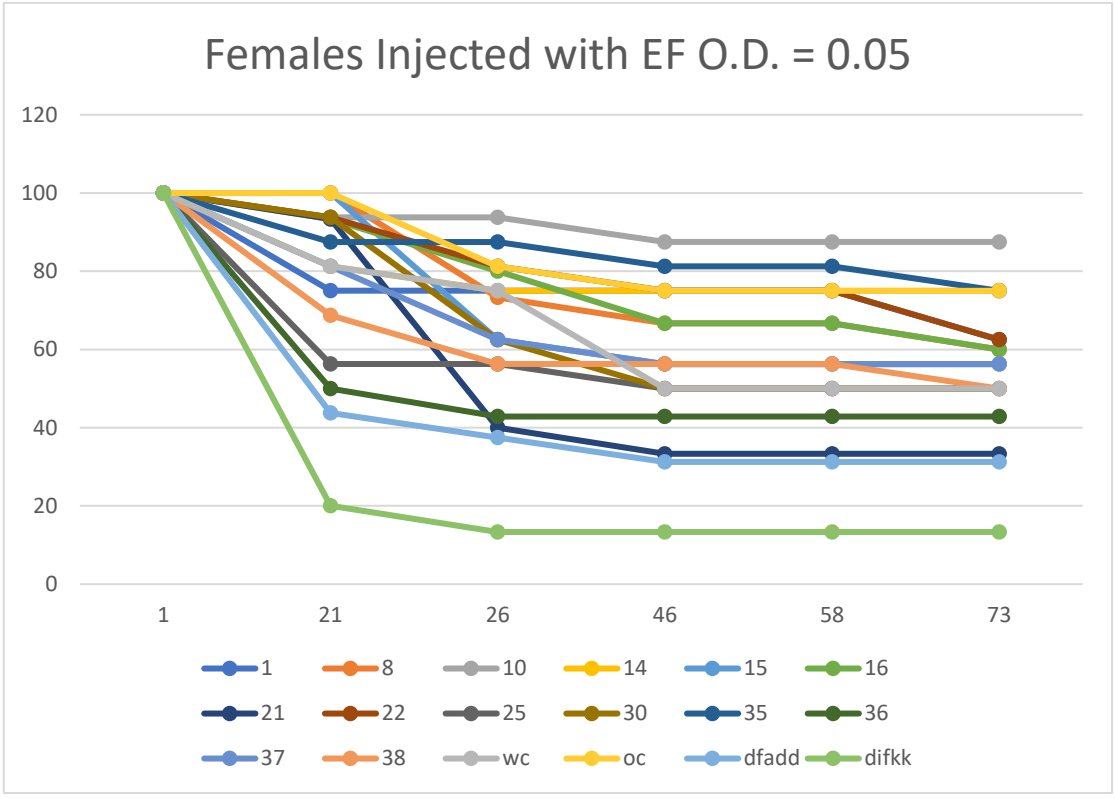


Figure 8 Survival assays displaying males and females injected with ECC and EF with an OD of 0.05 respectively (3). Oregon and White are the wild type controls and DifKK, dif-1 are the Toll pathway positive controls and RelE20 and dFadd are the IMD pathway positive controls. Genes 36, 37, and 38 were selected since they were vulnerable to EF infection.



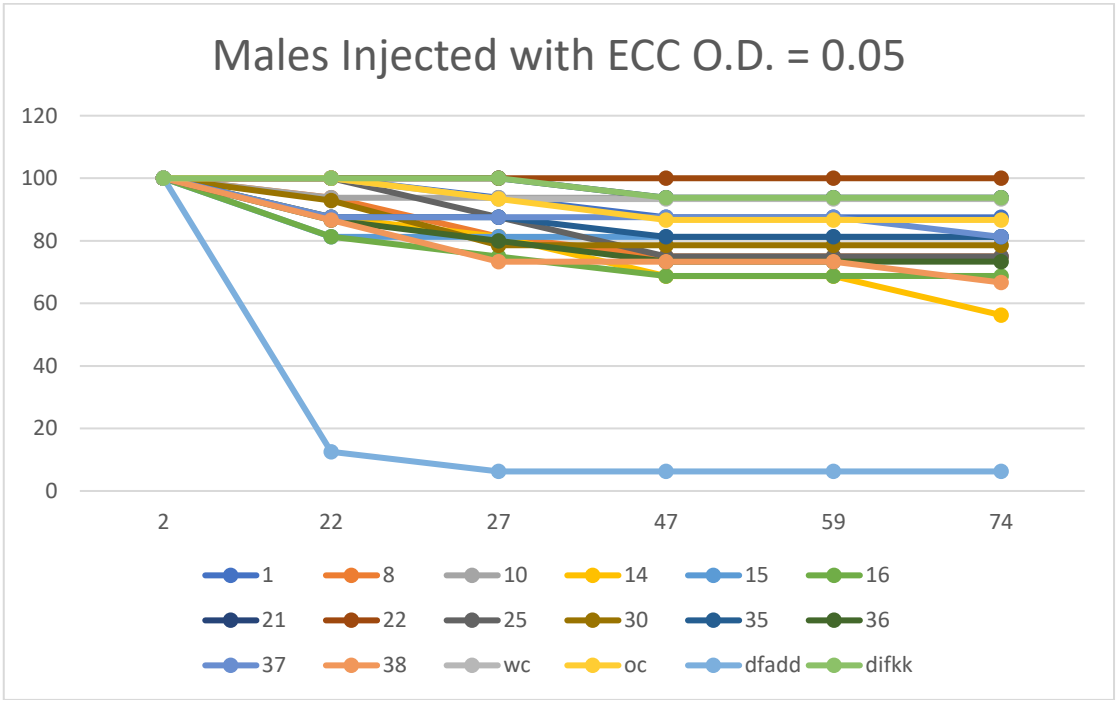


Figure 9 Survival assays displaying males and females injected with ECC and EF with an OD of 0.05 respectively (4). Crossed Oregon and White (oc and wc respectively) are the wild type controls and DifKK is the Toll pathway positive control and dFadd is the IMD pathway positive control. Genes 21, 25, 36, 37, and 38 were the most affected by EF infection.

B. Bacterial Clearance

After the survival assay, bacterial clearance was analyzed for the 14 selected genes. The progeny was injected with tetracycline resistant SA (OD 0.002) and ampicillin resistant *E. coli* (OD 0.05) with an incubation period of 24 hours for the SA infected flies and 0, 6 and 12 hours for the *E. coli* infected flies. Afterwards, the flies injected with SA were diluted (1:10) and plated on tetracycline plates. The flies injected with *E. coli* were diluted (1:10) and plated on ampicillin plates. The colonies were counted after incubating the plates at 37°C. The bacterial clearance rate was poor for SA; the CFUs were much higher at the 24 hour mark than at the 0 hour mark. Unfortunately, the dilution was too concentrated genes 1, 10, 15, 22, 25, 30, 35, 37 at 24 hours formed carpets which cannot be represented by the graph (Figure 10).

The bacterial clearance for *E. coli* was quite high. The majority of the bacterial count tended to be high at zero hours, at 6 hours it stayed the same or declined slightly and at 12 hours, the bacterial count declined (Figure 11).

The same experiment was conducted to confirm these results where the dilution for the SA injected flies was increased to a 1:100 ratio to obtain less carpets and a better resolution of the bacterial colonies. The flies were plated at 12 hours for *E. coli* and 18 hours for SA. Similar to Figure 11, Figure 12 also shows the bacterial clearance rate was high for the *E. coli* injected flies and none were comparable to the dFadd control which was a carpet (not shown in the graph). However, with the SA infection, genes 14, 22, 25, 37, and 38 were found to have very low clearance rates in comparison with the Oregon and White crossed controls (Figure 13) and were comparable to the DifKK control which isn't shown in the graphs because it was a carpet (plates shown in figure 14). Figure 14 shows a sample of what the plates looked like. The plates

represent the SA injected flies at 24 hours; Gene 37 and DifKK are carpets while the White cross (WC) has 167 CFUs.

Figure 15 displays a scatter plot of the survival rate as a function of the log of the CFU count and it is clear that there is an inverse relationship between the two. Using Spearman's correlation statistical testing on the pooled Gram-positive and Gram-negative, it was proven that there is a significant negative correlation between survival rate and CFU count (p-value = 5.335e-06 << 0.05). However, without pooling the data, the Gram-positive data alone and the Gram-negative data alone were not enough to prove a significant correlation. In addition, there is no significant correlation between the two CFU counts and between the two survival rates of the Gram-positive and Gram-negative data.

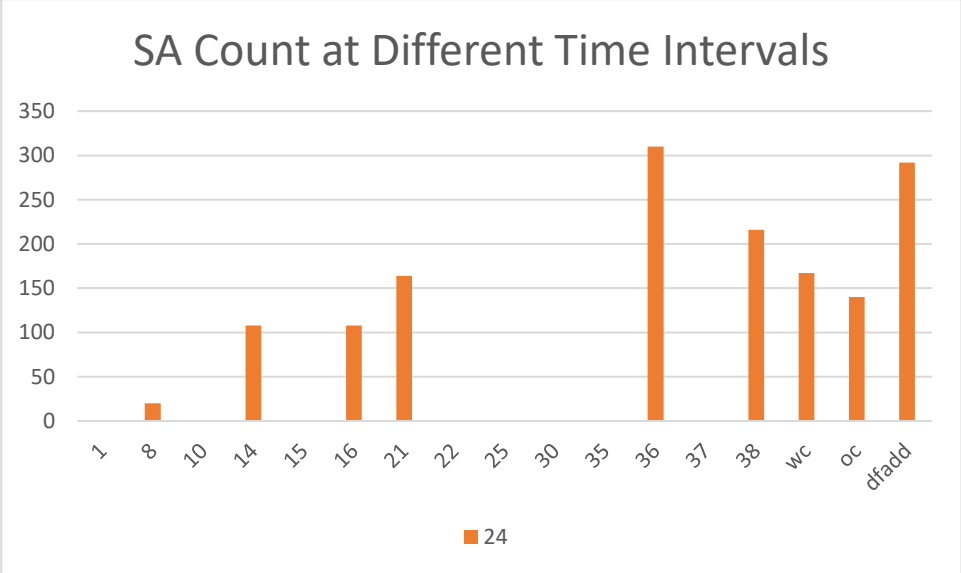


Figure 10 CFU count of SA injected females with an O.D. of 0.002 24 hours post-infection. DifKK controls not numerically represented as they formed a carpet. X-axis represents the gene number or symbol and the y-axis represents the number of CFUs counted. wc and oc represent White crossed and Oregon crossed flies. The genes at the certain time intervals that were not represented due to them forming a carpet are genes 1, 10, 15, 22, 25, 30, 35 and 37.

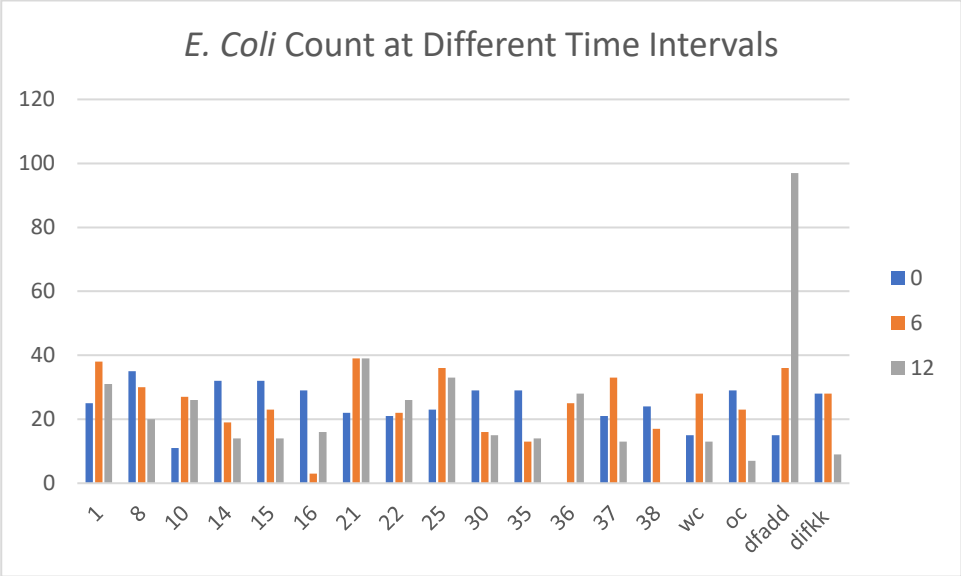


Figure 11 CFU count of *E. Coli* injected females with an O.D. of 0.05 at different time intervals. dFadd controls not numerically represented as they formed a carpet. X-axis represents the gene number or symbol and the y-axis represents the number of CFUs counted. Blue bars represent the count after 0 hours, the green bars represent the count after 12 hours, and the red bars represent the count after 24 hours. wc and oc represent White crossed and Oregon crossed flies. The clearance rate was high for all candidate genes.

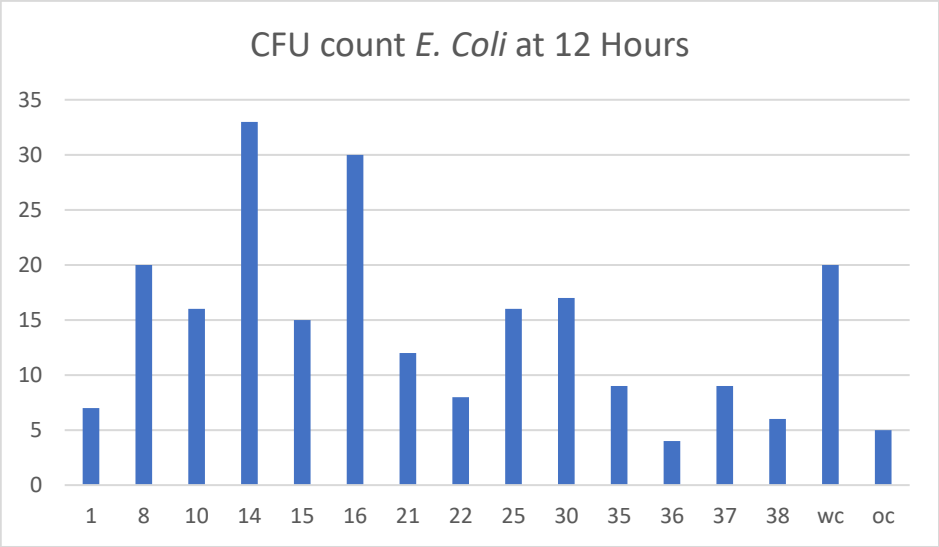


Figure 12 CFU count of *E. Coli* injected females with an O.D. of 0.05 at 12 hours. dFadd controls not numerically represented as they formed a carpet. X-axis represents the gene number or symbol and the y-axis represents the number of CFUs counted. wc and oc represent White crossed and Oregon crossed flies. The clearance rate was high for all candidate genes.

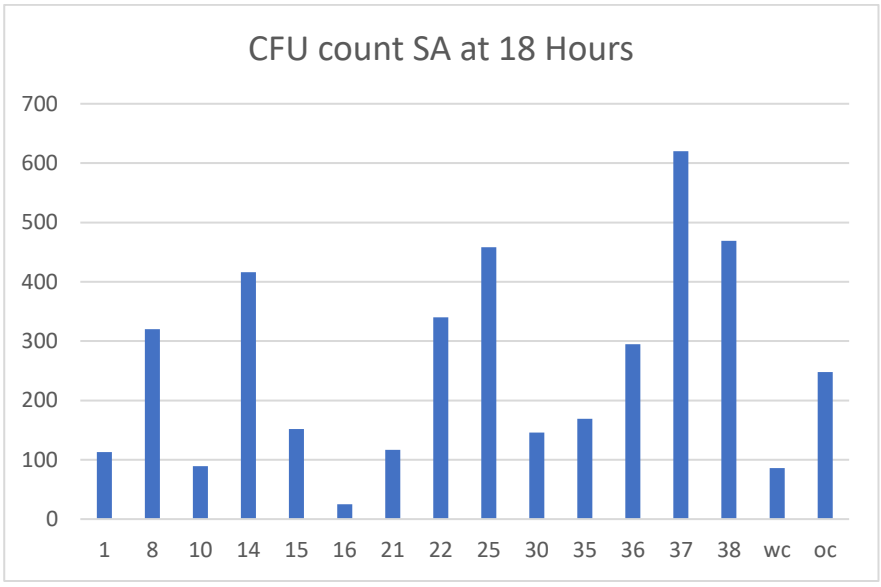


Figure 13 CFU count of SA injected females with an O.D. of 0.002 at 18 hours. DifKK controls not numerically represented as they formed a carpet. X-axis represents the gene number or symbol and the y-axis represents the number of CFUs counted. wc and oc represent White crossed and Oregon crossed flies. Genes 14, 22, 25, 37, and 38 were found to have very low clearance rates.

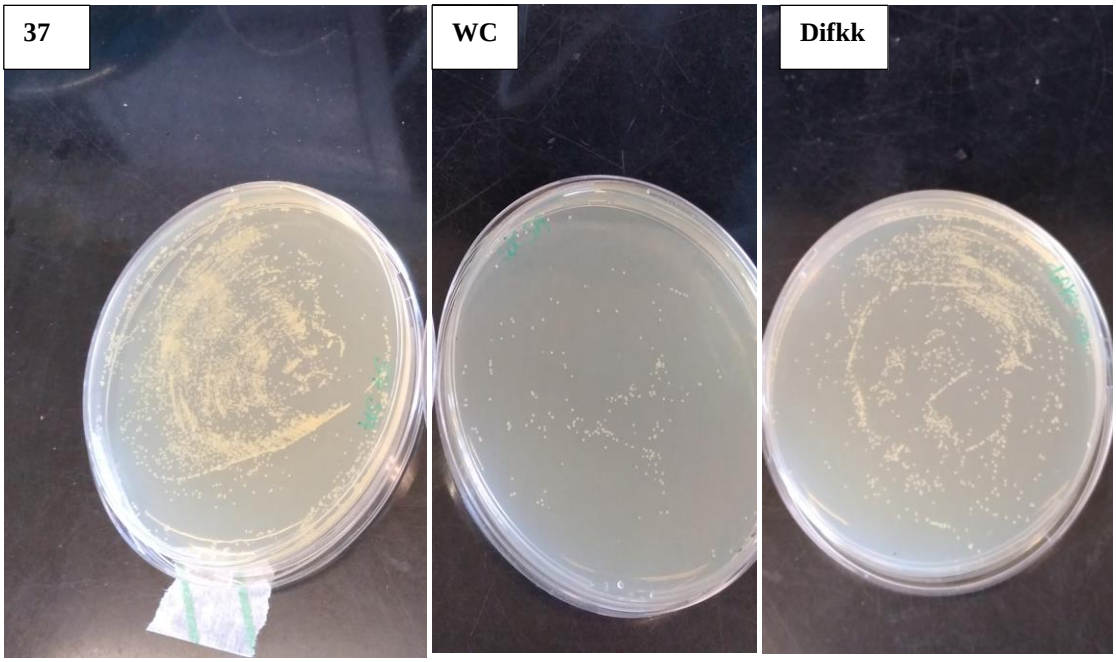


Figure 14 Sample plates of flies injected with SA (O.D. 0.002) at 24 hours. Gene 37 and Difkk are carpets while the White cross (WC) has 167 CFUs.

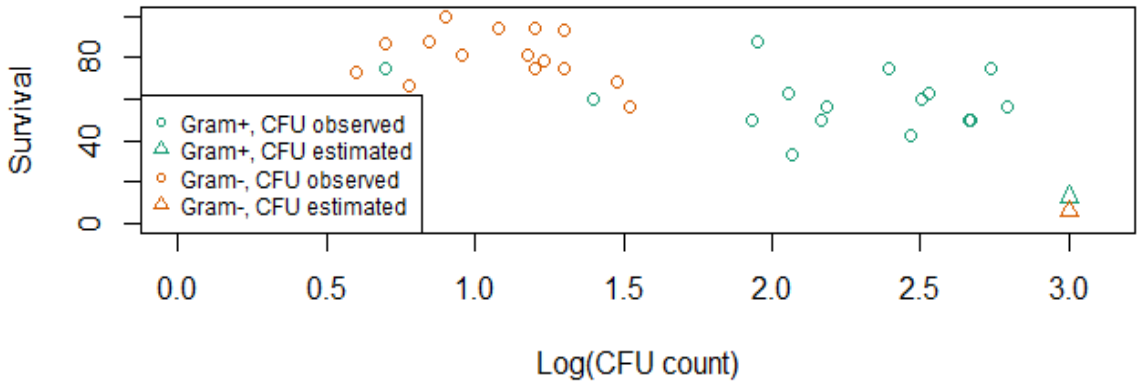


Figure 15 Survival rate as a function of log(CFU count) scatter plot. Green represents Gram-positive infection while orange represents Gram-negative infection. Circles represent observed values while the triangles represent estimated values (Difkk and dFadd). Pooled data shows a significant correlation between survival and CFU count (p-value = 5.335e-06 << 0.05).

C. RT-PCR

The RT-PCR model was first tested using a control system. We injected ECC into White and dFadd flies and froze them after 6 hours. After RNA extraction and reverse transcription, the Diptericin levels were measured using RT-PCR for the uninfected White flies (control unchallenged) and ECC infected dFadd and White flies (control challenged). The dFadd flies only had 2.24 times more Diptericin levels than the unchallenged control and the challenged control remarkably had 41 times more Diptericin levels than the unchallenged control (Figure 16). This validated the experimental model and conditions. Next, genes 21, 25, 36, 37, and 38 were selected for RT-PCR to study the production of AMPs in the Toll pathway. Flies were injected with ML (OD 0.1) and were frozen after an incubation of 18 hours. RNA extraction and reverse transcription were conducted, and the cDNA product was used for the RT-PCR where Drosomycin levels would be measured. These genes were selected based on the survival assays and the bacterial clearance rate. Unfortunately, due to machine malfunctioning, the results were could not be interpreted.

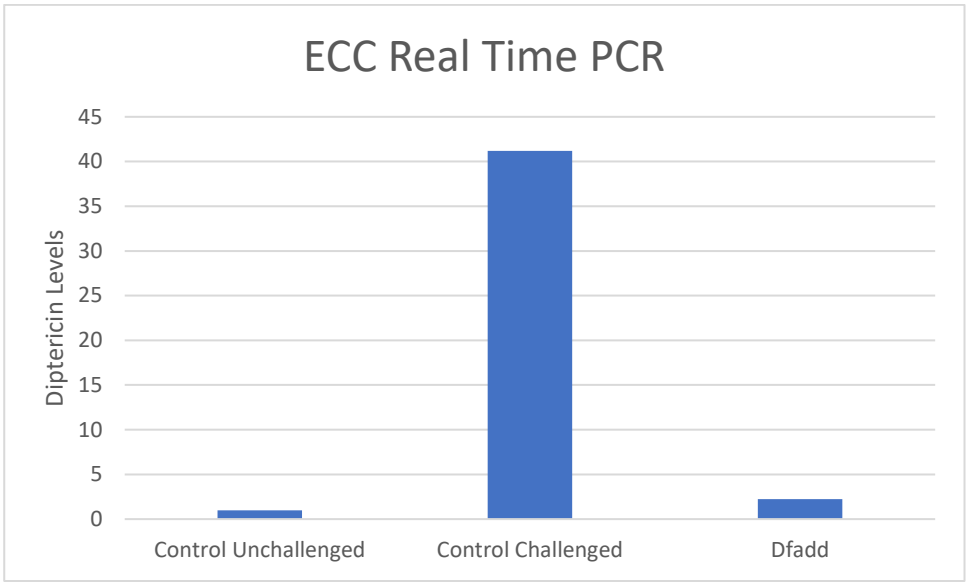


Figure 16 RT-PCR controls test. Uninfected White flies (control unchallenged) Diptericin levels compared to ECC injected dFadd and White flies (control challenged). dFadd levels were only 2.24 times more of Diptericin levels than the unchallenged control and the challenged control remarkably had 41 times more Diptericin than the unchallenged control. This proves the validity of this experimental model.

CHAPTER IV

DISCUSSION

In this work, the main focus was to uncover the candidate genes' roles in the *D. melanogaster's* innate immunity. We used the *in vivo* RNAi system to knock down the target candidate genes followed by introducing a microbial challenge. Resilience would mean that these genes don't play an essential role in the fly's innate immune system.

The survival assays proved to be very helpful and powerful guides as to narrow down which candidate genes would be used for further experimentation since it would be a waste of time and money to do irrelevant genes. The six genes that were lethal when knocked down must have a function in the flies' development since their knockdown did not produce a progeny. It's worth mentioning that with the EF infection, the dFadd and RelE20 was also affected but not as much as the DifKK and Dif-1 (Figures 6,7,8, and 9). This supports the theory that there might be cross talk between the Toll and IMD pathways. DifKK and Dif-1 are not as affected by the ECC infection, however, it is more affected than the wild type controls Oregon and White (Figures 7 and 8).

Figure 8 shows that NFAT mutant is vulnerable to EF infection but not so vulnerable to ECC infection. This supports previous unpublished findings (Battina Habib) where a mutant NFAT gene also showed vulnerability towards Gram-positive bacterial infection. This may mean that there is a link between NFAT and the fly's innate immune system similar to how the human NFAT is linked to the human's immune system.

Although we did introduce BB as a microbial challenge, the spores were unfortunately too old to illicit an effect on neither the candidate genes nor the positive controls, however, it is predicted that the same genes that were affected by the EF infection will also be susceptible to the BB infection since both Gram-positive and fungal infections induce the Toll pathway. Although the screening detected more than 14 possible candidate genes, we were limited in our time and resources, so we focused on only 14.

Among the 14 candidate genes, the survival chart for the ECC challenged group had a high resilience and this was further confirmed by the bacterial proliferation assay where the bacterial clearance rate was high compared to the dFadd and wild type controls in *E. coli* infected flies (Figures 9 and 11). This means that the candidate genes are not involved in the IMD pathway so they are not affected by Gram-negative bacterial infections.

With regards to the EF survival assay, genes 1, 8, 16, 21, 22, 25, 30, 36, 37, 38 all seemed to be remarkably affected. However, only genes 22, 25, 37, and 38 had a low bacterial clearance rate compared to the DifKK and wild type controls in SA infected flies (Figure 13). The high bacterial clearance rate in the genes that were vulnerable in the survival assay could be explained; there are protective mechanisms other than AMP production such as phagocytosis, coagulation, ROS production, encapsulation and melanization (Govind, 2008). Since the flies' innate immune pathways have not been completely uncovered, it's not surprising that the genes with a low bacterial clearance rate may be involved in more than one innate immune pathway, hence, having a much higher bacterial CFU count since more than one protective pathway is being affected.

With regards to the RT-PCR experiment, the Diptericin levels were the lowest in the unchallenged flies, they were slightly increased in the dFadd mutant but not significantly because a mutation in dFadd negatively affects the induction of AMPs coming from the IMD pathway. Diptericin levels were remarkably increased in the challenged control flies as expected. This RT-PCR control model proved that the experimental model was valid for the aims for this project even though the malfunctioning of the RT-PCR machine did not allow us to interpret the candidate genes' results in the end. The RNA extraction can also be verified to be successful because the NanoDrop spectrophotometer displayed a high RNA yield, as well as an acceptable A260/A280 ratio of a minimum of 1.8 (which proves RNA purity from DNA and proteins).

CHAPTER V

CONCLUSION AND PERSPECTIVES

This work suggests that the function of the selected genes is related to *D. melanogaster*'s innate immunity, however, there is much more work to be done. There were 6 genes that seem to be involved with the flies' development since the progeny did not emerge when these genes were knocked down. Also, since there is cross talk between the Toll and IMD pathway, it wouldn't be surprising to think that the selected genes are also involved in other pathways. Due to time constraints and the limitations that Covid-19 had put on campus access, we unfortunately could not repeat the RT-PCR experiment in order to measure the Drosomycin levels. These results are imperative to pinpointing the functions of these candidate genes. If Drosomycin levels were affected, it would mean that the candidate gene is directly involved in the Toll pathway. Finally, due to BB spores being too weak to illicit an immune response in the flies, it would be interesting to see if there is an overlap with the Gram-positive infected flies and the flies infected with the spores.

Prospective work should test the candidate genes with BB spores and see if there is overlap with the EF infection. Future work should also further investigate the 6 genes that are suspected to be involved with the flies' development in their developmental and immune roles. The RNA samples of candidate genes 21, 25, 36, 37, and 38, along with the controls, were frozen and preserved, so future work would include to test the Drosomycin levels of these candidate genes and validate whether or not they are involved in the Toll pathway. Finally, future work should investigate if the selected genes are also involved in cross talk with other signaling pathways.

REFERENCES

Blake, A. J., Finger, D. S., Hardy, V. L., & Ables, E. T. (2017). RNAi-Based Techniques for the Analysis of Gene Function in Drosophila Germline Stem Cells. *Methods Mol Biol, 1622*, 161-184. doi:10.1007/978-1-4939-7108-4_13

Buchon, N., Silverman, N., & Cherry, S. (2014). Immunity in Drosophila melanogaster--from microbial recognition to whole-organism physiology. *Nat Rev Immunol, 14*(12), 796-810. doi:10.1038/nri3763

Chtarbanova, S., & Imler, J. L. (2011). Microbial sensing by Toll receptors: a historical perspective. *Arterioscler Thromb Vasc Biol, 31*(8), 1734-1738. doi:10.1161/ATVBAHA.108.179523

De Gregorio, E., Spellman, P. T., Rubin, G. M., & Lemaitre, B. (2001). Genome-wide analysis of the Drosophila immune response by using oligonucleotide microarrays. *Proc Natl Acad Sci U S A, 98*(22), 12590-12595. doi:10.1073/pnas.221458698

Eadaim, A., Hahm, E. T., Justice, E. D., & Tsunoda, S. (2020). Cholinergic Synaptic Homeostasis Is Tuned by an NFAT-Mediated alpha7 nAChR-Kv4/Shal Coupled Regulatory System. *Cell Rep, 32*(10), 108119. doi:10.1016/j.celrep.2020.108119

Fernandez-Moreno, M. A., Farr, C. L., Kaguni, L. S., & Garesse, R. (2007). Drosophila melanogaster as a model system to study mitochondrial biology. *Methods Mol Biol, 372*, 33-49. doi:10.1007/978-1-59745-365-3_3

Freeman, A., Franciscovich, A., Bowers, M., Sandstrom, D. J., & Sanyal, S. (2011). NFAT regulates pre-synaptic development and activity-dependent plasticity in Drosophila. *Mol Cell Neurosci, 46*(2), 535-547. doi:10.1016/j.mcn.2010.12.010

Govind, S. (2008). Innate immunity in Drosophila: Pathogens and pathways. *Insect Sci, 15*(1), 29-43. doi:10.1111/j.1744-7917.2008.00185.x

Irving, P., Troxler, L., Heuer, T. S., Belvin, M., Kopczynski, C., Reichhart, J. M., . . . Hetru, C. (2001). A genome-wide analysis of immune responses in Drosophila. *Proc Natl Acad Sci U S A, 98*(26), 15119-15124. doi:10.1073/pnas.261573998

Kambris, Z., Brun, S., Jang, I. H., Nam, H. J., Romeo, Y., Takahashi, K., . . . Lemaitre, B. (2006). Drosophila immunity: a large-scale in vivo RNAi screen identifies five serine proteases required for Toll activation. *Curr Biol, 16*(8), 808-813. doi:10.1016/j.cub.2006.03.020

Keyser, P., Borge-Renberg, K., & Hultmark, D. (2007). The Drosophila NFAT homolog is involved in salt stress tolerance. *Insect Biochem Mol Biol, 37*(4), 356-362. doi:10.1016/j.ibmb.2006.12.009

Kurata, S. (2014). Peptidoglycan recognition proteins in Drosophila immunity. *Dev Comp Immunol, 42*(1), 36-41. doi:10.1016/j.dci.2013.06.006

Majumdar, R., Rajasekaran, K., & Cary, J. W. (2017). RNA Interference (RNAi) as a Potential Tool for Control of Mycotoxin Contamination in Crop Plants: Concepts and Considerations. *Front Plant Sci, 8*, 200. doi:10.3389/fpls.2017.00200

Myllymaki, H., Valanne, S., & Ramet, M. (2014). The Drosophila imd signaling pathway. *J Immunol, 192*(8), 3455-3462. doi:10.4049/jimmunol.1303309

Nishide, Y., Kageyama, D., Yokoi, K., Jouraku, A., Tanaka, H., Futahashi, R., & Fukatsu, T. (2019). Functional crosstalk across IMD and Toll pathways: insight into the evolution of incomplete immune cascades. *Proc Biol Sci, 286*(1897), 20182207. doi:10.1098/rspb.2018.2207

Silverman, N., Paquette, N., & Aggarwal, K. (2009). Specificity and signaling in the Drosophila immune response. *Invertebrate Surviv J, 6*(2), 163-174.

Vaeth, M., & Feske, S. (2018). NFAT control of immune function: New Frontiers for an Abiding Trooper. *F1000Res, 7*, 260. doi:10.12688/f1000research.13426.1

Valanne, S., Wang, J. H., & Ramet, M. (2011). The Drosophila Toll signaling pathway. *J Immunol, 186*(2), 649-656. doi:10.4049/jimmunol.1002302

Zamore, P. D., Tuschl, T., Sharp, P. A., & Bartel, D. P. J. C. (2000). RNAi: double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals. *101*(1), 25-33.