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PREVALENCE OF WOLBACHIA IN WILD INSECTS AND ITS EFFECT ON MOSQUITO HOST

by
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AN ABSTRACT OF THE THESIS OF

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Mosquito-borne diseases are a considerable threat to human and animal health. In order to limit their spread, several methods have been developed. However, the control of these diseases is still difficult and novel means of vector control are required as the threat becomes more eminent. The use of *Wolbachia*, an endosymbiotic alpha-proteobacterium, is one of the most exciting approaches used to limit virus transmission from mosquito to vertebrate. *Wolbachia* has been extensively studied in vector-control research because of its ability to induce cytoplasmic incompatibility. *Wolbachia* is thought to infect 20–70% of all arthropod species and several nematodes by being vertically transmitted through the maternal lineage. However, there is a debate concerning the percentage of insects infected with *Wolbachia*. Using PCR followed by DNA sequencing, our study shows that 20% of insects sampled from random geographic areas in Lebanon harbor *Wolbachia*. Previous studies showed that *Wolbachia*'s presence is responsible for pathogen blocking, while other studies showed that in its native host the protective effects of the endosymbiont are not detected. In this context, we aimed to analyze the effect of *Wolbachia* on *Culex* mosquito host immunity. We showed that *Wolbachia* enhances the survival of old mosquitoes in response to injection with gram-negative bacteria without affecting the survival of young mosquitoes. In summary, *Wolbachia* is present in 20% of our sampled insect population and its presence leads to an age-dependent immune modulatory role in *Culex* mosquito.

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ABBREVIATIONS

A. albopictus: Aedes Albopictus

AMP: Antimicrobial peptide

Bp: Base pairs

C. pipens: Culex pipiens

cDNA: Complementary Deoxy-ribonucleic Acid

CI: Cytoplasmic incompatibility

D. *melanogaster*: *Drosophila Melanogaster*

DNA: Deoxy-ribonucleic acid

Dome: Domeless

Ecc15: *Erwinia carotovora carotovora 15*

Ef: *Enterococcus faecalis*

F: Forward Primer

Hop: Hopscotch

JAK/STAT: Janus Kinase/Signal Transducer and Activator of Transcription

LB: Luria-Bertani

NF-Kb: nuclear factor kappa-light-chain-enhancer of activated B cells

OD: Optical Density

PCR: Polymerase chain reaction

PGRP: Peptidoglycan recognition proteins

qRT-PCR: Quantitative real-time Polymerase chain reaction

R: Reverse Primer

RNA: Ribonucleic acid

RNAi: Ribonucleic Acid Interference

Rp49: Ribosomal protein 49

RVFV: Rift Valley fever virus

SLEV: St. Louis encephalitis virus

Upd: Unpaired

WftsZ: Filamentation temperature-sensitive protein Z (*Wolbachia)*

WNV: West Nile virus

Wsp: *Wolbachia* surface protein

CHAPTER 1

INTRODUCTION

A. Mosquitoes are Vectors of Diseases

Mosquitoes (Diptera Culicidae) are a public health concern due to their ability to transmit diseases. They are a diverse group of insects with more than 3000 species spread all over the world. The life cycle of mosquitoes is comprised of four distinct stages egg, larvae, pupae and adult. It requires approximately two weeks depending on temperature and availability of nutrients. Throughout the life span of female mosquitoes, it can lay up to five batches of eggs. The female mosquito lays her eggs on stagnant water surfaces after taking a blood meal and within two to three days, the eggs will hatch into larvae. It takes around one week for the four larval stages to occur, during which they molt and increase in size after which they transition to a non-feeding pupal stage. When the development is complete, the pupae will metamorphose into an adult. The juvenile stages of mosquitoes are aquatic unlike the terrestrial adult stages that are ready to mate few days after metamorphosis. Male mosquitoes feed mainly on sugar sources, whereas most female mosquitoes are anautogenous, which means that the adult females must ingest a blood meal from a vertebrate host to obtain nutrients necessary for the production of eggs. Blood feeding exposes mosquitoes to pathogens that they might transmit to human or animal host during subsequent blood meals [1].

The majority of mosquito-borne viruses are RNA viruses primarily associated with global morbidity and mortality. They belong to three families Flaviviridae, Togaviridae and Bunyaviridae. Yellow fever virus (YFV), dengue virus (DENV), Japanese encephalitis virus (JEV), West Nile virus (WNV) and Zika virus (ZIKV) belong to the Flaviviridae family. However, DENV is considered the most important among the previously mentioned knowing that it causes more than 300 million infections annually and it is transmitted primarily by *Aedes aegypti* (WHO. Dengue control strategies (2017)). In addition to that, ZIKV was also declared in February 2016 as a public health emergency by the World Health Organization (WHO). Culicine and anopheline mosquitoes are considered most effective vectors of human and animal pathogens.

In Lebanon, several species occur naturally such as *Aedes Albopictus* (*A. albopictus)* and *Culex Pipiens* (C. Pipiens). *Aedes Albopictus*, the Asian tiger mosquito is a vector of several viruses including Dengue, West Nile, Chikungunya and Zika virus. Similarly, *Culex* Pipiens are efficient vectors of Rift Valley Fever virus (RVFV), St. Louis encephalitis virus (SLEV), West Nile virus (WNV) and Sindbis virus (SINV).

B. Tissue Barriers to Infection in the Mosquito Vector

After the mosquito takes a blood meal from an infectious individual, it can transmit the virus to a vertebrate host if it succeeds to replicate in the mosquito midgut epithelium and salivary glands [2] [3]. The virus from the blood meal can penetrate into the midgut epithelium if it can cross the first cellular barrier, which is the midgut infection barrier

(MIB). The midgut contains the blood meal post-feeding through its layer of epithelial cells, which makes it one of the first obstacles the virus has to overcome for successful viral replication and transmission. During the blood digestion process a peritrophic matrix will be secreted by the midgut epithelial cells into the lumen to envelope the blood meal and prevent virions from gaining access to the epithelial cells [4] [5]. After the virus replicates in the midgut epithelium, it should cross the midgut escape barrier (MEB) into the basal lamina. After crossing MEB, the virus enters the haemocoel, an open body cavity in which the hemolymph circulates. Then specific immune cells present in the mosquito's open circulatory system called hemocytes together with the hemolymph will allow the dissemination of the virus to reach the salivary glands, which are the final physical barrier for effective transmission of arboviruses. After this, the mosquito is ready to transmit the virus by injecting the infected saliva into the vertebrate host.

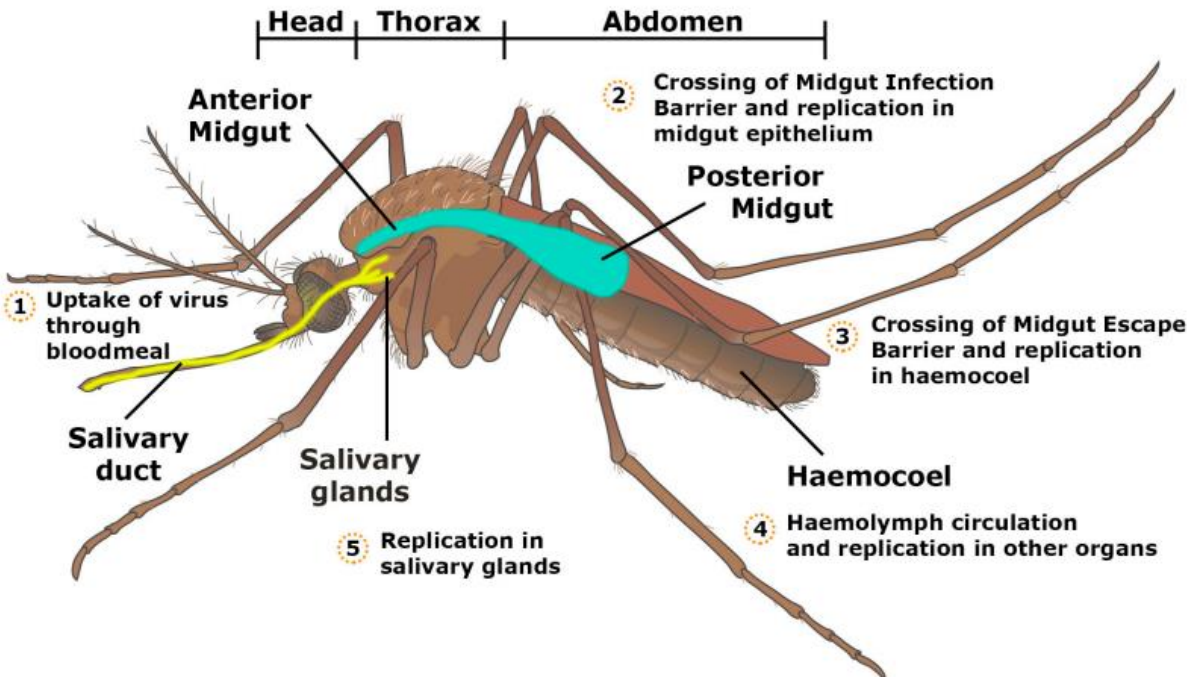


Figure 1: Schematic representation of the virus replication in mosquitoes.[1].

C. Mosquito Innate Immunity

The key determinant for a successful transmission of viruses is their ability to survive the innate immune response [6]. Mosquitoes lack an adaptive immune response, which makes them depend on their innate immunity to fight the pathogens. The activation of innate immune pathways in response to viral infection leads to transcription of genes having an antiviral response. The innate immune system of mosquitoes is divided into cellular and humoral responses which act together to protect mosquitoes against a wide variety of pathogens including bacteria, yeast and viruses. *Drosophila melanogaster* is used as a genetic model to widen the knowledge about insect antiviral immunity. There are orthologs of the core components of the *Drosophila* immune pathways present in the

genomes of major vector mosquitoes. Therefore, it is hypothesized that the immune signaling pathways are highly conserved between *Drosophila* and mosquitoes [7] [8] [9]. The cellular immune responses are mediated by hemocytes and include phagocytosis, nodulation and encapsulation of the pathogens [10] [11]. However, the signaling pathways that lead to the synthesis of effector molecules such as antimicrobial peptides (AMPs), reactive oxygen species (ROS) and components of the phenoloxidase cascade are referred to as humoral responses [12] [13] [14]. AMPs and ROS are secreted by epithelial cells in the mosquito midgut as a first line of defense against many pathogens acquired from blood feeding. In addition to that, the fat body of mosquitoes plays an important role in production and secretion of AMPs, which makes it the primary site of humoral responses. Signaling cascade pathways, including the Janus kinase-signal transducer and activator of transcription (JAK-STAT), Toll and immune defciency (Imd) pathways are responsible for the transcription of innate immune genes encoding for AMPs [15] [16].

1. Immune Pathways in Mosquitoes

a. Toll Pathway

The Toll pathway was first characterized in *Drosophila melanogaster* for to its role in embryonic development and immunity. It acts against fungi, gram-positive bacteria and viruses [17, 18]. The Toll pathway is activated upon the recognition of pathogen-associated molecular patterns (PAMP) by host derived pattern recognition receptors PRRs. PGRP-SA and GNBP1 act upstream Toll pathway. The binding of PAMP to a PGRP leads to the

activation of SPE that cleaves the cytokine Späetzle [19] [20]. After the cleavage of Späetzle, it binds to and activates the Toll receptor. This will initiate several intracellular events involving the adaptor proteins MyD88, Tube and the kinase Pelle [21] [22]. Signaling through these proteins will result in the phosphorylation and proteasomal degradation of Cactus; a negative regulator that binds to and sequesters the NF-kB-like transcription factor Dorsal (Rel1 in mosquitoes) in the cytoplasm. The degradation of Cactus allows the translocation of Dorsal/Rel1 to the nucleus leading to the transcription of AMPs such as Diptericin and Cecropin that are active against fungi and Gram-positive bacteria [23, 24].

b. Imd Pathway

The binding of DAP-type PGNs to PGRP-LC triggers the activation of Imd pathway. Although PGRP-LC is the main receptor linked to the activation of Imd pathway, its activity is enhanced by the cytosolic PGRP-LE that binds to DAP-PGNs thus promoting the activity of PGRP-LC. Activation of Imd pathway leads to intracellular signaling through the adaptor Imd protein and various caspase-like proteins and kinases which results in the functional split in the pathway into two downstream branches [25-27]. One branch activates the transcription factor AP-1 via JNK signaling like the mammalian c-Jun/JNK pathway [28] [29]. However, the second branch allows the activation of the NF-kB transcription factor Relish (Rel2 in mosquitoes) via caspase-mediated cleavage of its carboxy-terminal

end [29] [30]. Five proteins are involved in the cascade of Imd signaling pathway (IMD, FADD, Dredd, Tak1 and IKK) which results in the expression of the NF-κB transcription factor Rel2. Gram negative bacteria and viruses activate the Imd pathway leading to the degradation of the negative regulator Caspar and translocation of Relish 2 (Rel2) to the nucleus resulting in the transcription of AMPs [12] [31].

Despite the role of Toll and Imd pathways in antifungal and antibacterial responses in mosquitoes, their role in antiviral responses is not well characterized [32]. The key components of Toll and Imd pathways are conserved between *Drosophila* and mosquitoes. *A. aegypti, C. quinquefasciatus* and *A. gambiae* have homologous genes from Toll and Imd pathways. The Toll pathway genes (GNBP, Toll5A and MYD88 genes) were upregulated in the salivary glands during DENV infection of *A.Aegypti*. A slight increase of DENV viral titre in the midgut was observed due to MYD88 silencing [33]. Rel1 and its downstream antimicrobial peptides were found to be upregulated to control infection against DENV [34] and SINV [35]. However, in *Culex* mosquitoes, the transcription factor Rel2 of the Imd pathway activates the secretion of an antiviral peptide against WNV.

c. JAK-STAT Pathway

The Janus kinase/signal transducers and activators of transcription (JAK-STAT) pathway was first identified in vertebrates as an interferon (IFN)-induced signaling pathway in addition to its role in antiviral immunity in mammals. [36] [37] [38]. This pathway is conserved in invertebrates and it was originally identified in *Drosophila* through

its role in embryonic segmentation. It was shown to have an antiviral role against *Drosophila* C virus (DCV) and Flock House virus (FHV). In addition to that, mosquitoes also express cytokine receptor, Domeless (Dome) and the tyrosine kinase Hopscotch (Hop) which are the main components of JAK-STAT pathway. The binding of the Unpaired (Upd) peptide ligand to the extracellular region of the transmembrane receptor Dome leads to conformational modification and dimerization of Dome which results in self-phosphorylation of the receptor-associated Janus kinase Hop. The self-phosphorylation of Hop results in its activation, then it phosphorylates the cytoplasmic tail of the Dome receptor, generating docking sites for the recruitment of STAT proteins. The Dome/Hop activated complex will phosphorylate the recruited STAT resulting in STAT activation and dimerization. Following the dimerization of STAT, it translocates to the nucleus to induce the expression of effector genes. [39] [40]. When *A. aegypti* mosquitoes were genetically modified to overexpress Dome and Hop, the mosquitoes were more resistant to DENV infection, but not to CHIKV and ZIKV infection. This proves that the molecular response possessed by *A. Aegypti* mosquito varies from one virus to another [41]. Despite the fact that Dome is the most well characterized cytokine receptor in mosquitoes, studies proved the presence of other cytokine receptors that activate the JAK-STAT pathway. Vago is a secreted peptide present in *Culex* mosquito and it was shown to be upregulated in response to WNV infection. It reduces the viral load by subsequently activating the JAK-STAT pathway through another receptor since knockdown of Dome did not inhibit signaling of the JAK-STAT pathway, indicating that Vago activated JAK-STAT via another unknown receptor [15].

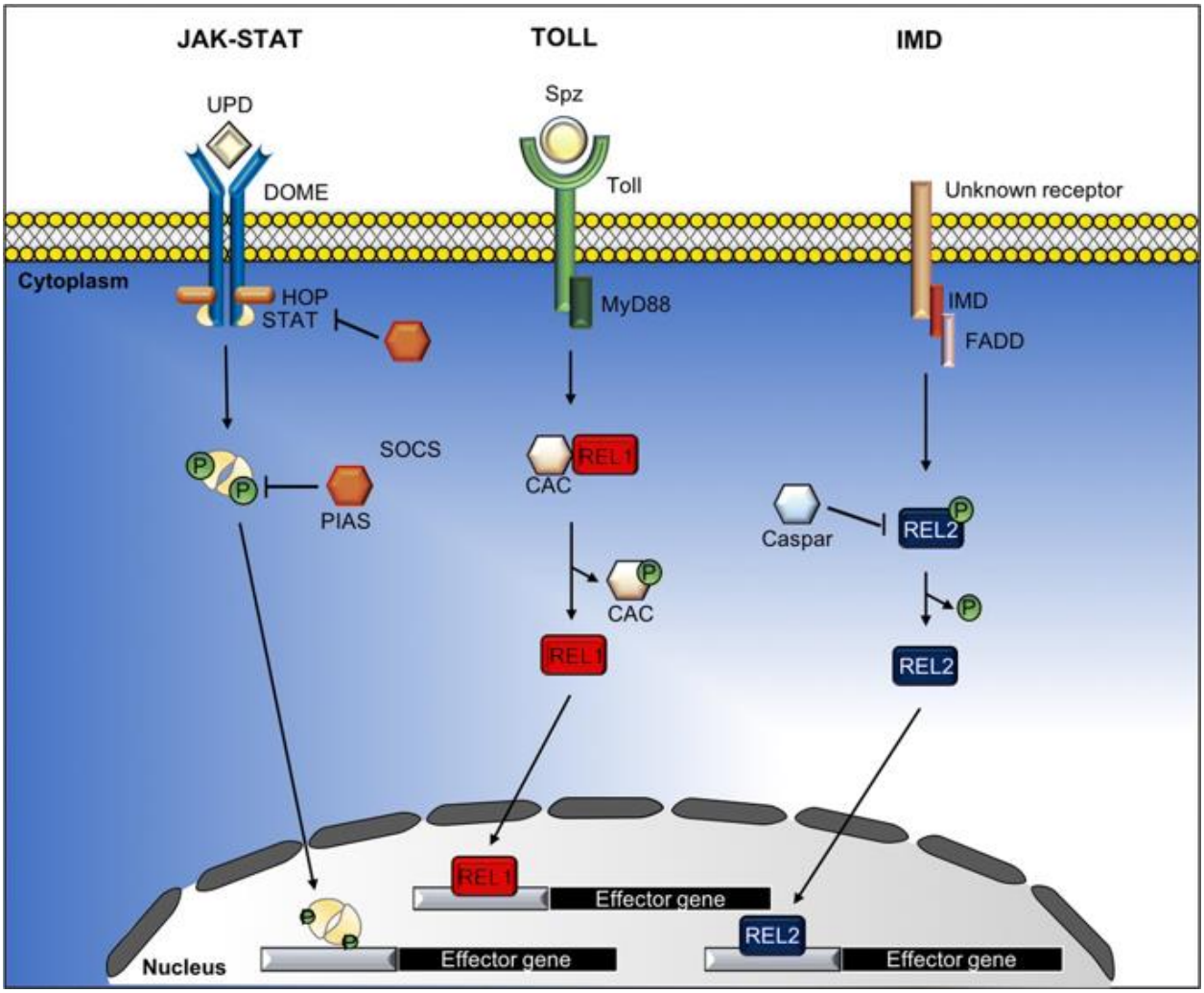


Figure 2: Schematic representation of the main mosquito innate pathways [6].

d. RNA Interference (RNAi) Pathways

Although the activation of Toll, Imd and JAK-STAT limit viral replication, RNA interference (RNAi) pathway has been shown to be the most robust antiviral defense. It

controls virus infection through degradation of RNA, also known as RNA silencing. The production of small RNAs from long viral double-stranded RNA (dsRNA) is the key event in this pathway. Small RNAs can be divided into three major groups small interfering RNAs (siRNAs), microRNAs (miRNAs) and PIWI-interacting RNAs (piRNAs) knowing that siRNAs is the main antiviral responses in mosquito. The genome of mosquito –borne viruses is mainly a single stranded RNA that is either positive-sense (+) or negative-sense (−) [42] [43]. These viruses generate dsRNA as an intermediate product during genome replication. Dicer-2-R2D2 is a complex that consists of RNase III enzyme, called Dicer-2 (Dcr-2) and an associated protein, called R2D2. After binding of Dicer-2-R2D2 complex to the viral dsRNA, RNase III domain of Dcr-2 cleaves the dsRNA to produce siRNA of 21–23 nucleotides (nt) in length [44]. Then the siRNA will activate the RNAi machinery by binding to another multiprotein known as the RNA-induced silencing complex (RISC) which unwinds the double stranded RNA and degrades one of the siRNA strands keeping the other for targeted degradation of single-stranded viral RNA with sequence complementary to the siRNA by the host endonuclease, Argonaute-2 (Ago2).

The second type of small RNAs is the miRNAs, which is a class of endogenous small-non-coding RNAs (20-25 nt). They play an essential role in the posttranscriptional regulation of target genes in multiple metabolic processes either by cleavage of target mRNAs or repression of mRNA translation. The miRNA genes are transcribed into primary miRNA (pri-miRNA) by host polymerase II. Then they are processed by the nuclear protein, Drosha, into pre-miRNAs, which are subsequently exported from the nucleus into the cytoplasm. In the cytoplasm, Dcr-1 will further process the pre-miRNAs into 22-nt

duplex miRNAs and then it will be loaded into Ago-1 of the RISC complex, which guides the binding of the complex to complementary mRNA for degradation. The antiviral role of miRNAs has not been reported in mosquitoes since replication of RNA viruses occurs in the cytoplasm preventing its access to Drosha in the nucleus. However, this pathway plays a critical role in the modulation of host genes that control viral replication and dissemination [45] [46] [47].

The third antiviral RNAi pathway is piRNA pathway. The primary role of this pathway is silencing transposons and maintaining the integrity of the animal's germline. These small RNAs originate from distinct genomic loci, termed piRNA clusters that give rise to long single stranded RNA transcripts that are processed into piRNA. This pathway does not require Dicer, however it involves piRNA-induced silencing complex (piRISC) which is made up of P-element induced wimpy testis (Piwi), Aubergine (Aub) and Argonaute 3 (Ago3). This pathway is initiated in *Drosophila* by the synthesis of primary piRNA pool from single stranded precursors. Then, the primary piRNAs can be associated with Aub and Piwi proteins which help them undergo an amplifying process known as the 'pingpong' cycle to further amplify the Aub-bound piRNAs and to refine the piRNA pool. The aim of this cycle is to ensure an efficient piRNA-mediated silencing of the target RNA [48]. There are few differences between this pathway between drosophila melanogaster and mosquitoes. The main difference is the composition of the of the protein players like Aub and Piwi which have no direct orthologs in mosquitoes. Instead, *Culex, Aedes* and *Anopheles* have an expansion of Piwi proteins [49]. In addition to that, no clear role for this pathway has been established outside the germ line in *Drosophila* since Ago-3 and Aub do

not appear to be expressed in the *Drosophila* soma. However, the Piwi-clade Ago proteins of *A. aegypti* include Piwi 1-7 and Ago-3 which are broadly expressed in the soma unlike *Drosophila*.

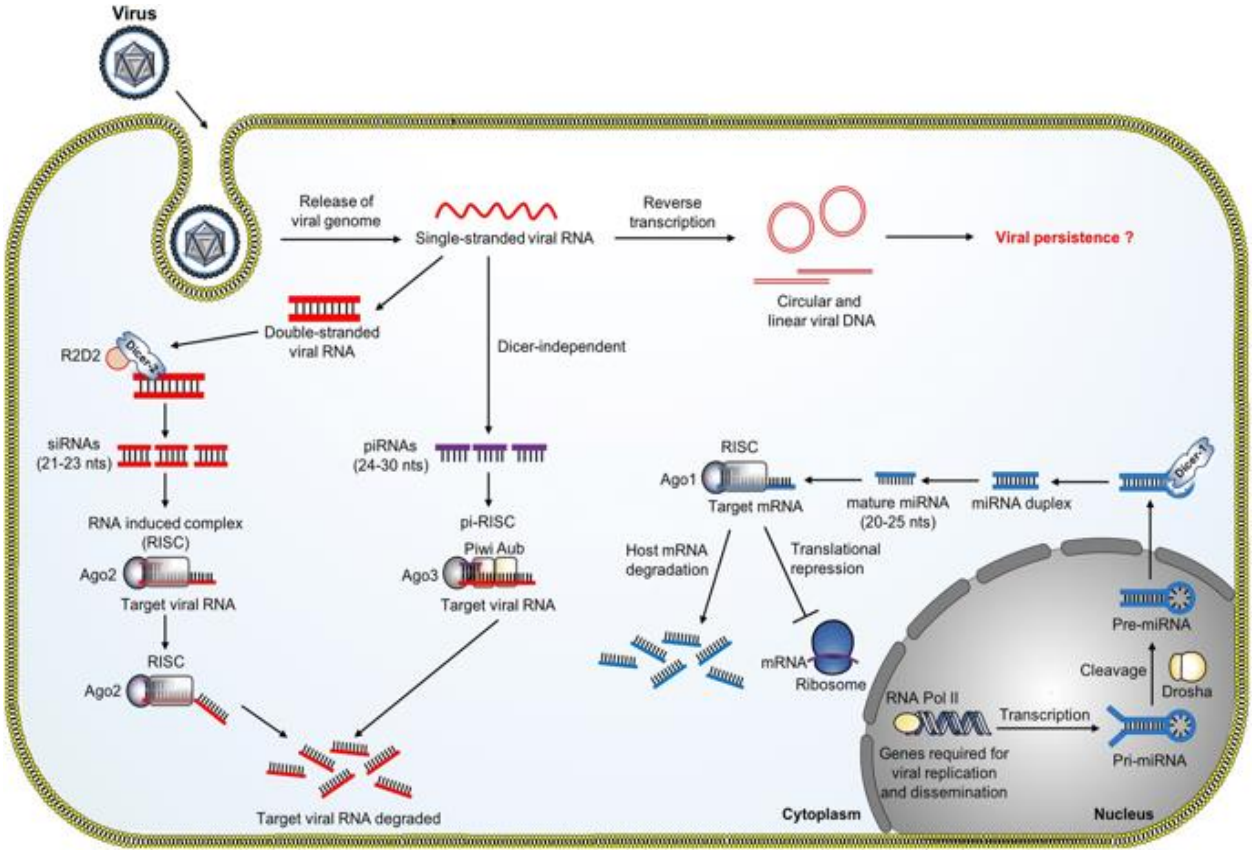


Figure 3: Schematic representation of the RNAi pathways in mosquitoes.

The three major types of small RNAs present in mosquitoes are small interfering RNAs (siRNAs), microRNAs (miRNAs) and Piwi-interacting RNAs (piRNAs), with siRNAs being the main antiviral response in mosquitoes. Modified from [6].

2. Humoral Responses

Hemocytes elicit humoral responses by activating the previously mentioned signaling pathways. These responses control infection against invading pathogens by the synthesis and secretion of soluble effector molecules such as AMPs, ROS and components of phenoloxidase cascade into the hemolymph.

a. AMP Production

The activation of Toll, Imd and JAKSTAT in response to viral infection initiates the formation of a multiprotein complex that consists of protein kinases, transcription factors and other regulatory molecules which control the expression of downstream innate immunity genes like genes encoding AMPs [12, 13]. AMPs are potent immune-inducible peptides that have an antimicrobial activity and act rapidly in response to pathogens. During an infection with bacteria (gram positive or gram negative), filamentous fungi and yeast, a wide spectrum of AMPs are produced by hemocytes, fat bodies and epithelial cells which are produced in response to PRRs and other recognition machinery present in the insect. AMPs are transported to the hemolymph after their synthesis to be then transported to their site of action. These AMPs either act by direct killing of the pathogen or by recruiting and activating immune cells [50] [51]. *Drosophila* was used as a model in most studies on the regulation of AMPs. However, the regulation of AMPs in mosquitoes is poorly understood since they vary among different mosquito species and the induction of AMPs is regulated by multiple immune signaling pathways that are highly dependent on

the type of pathogen that elicited the response. 17 AMPs have been identified in *A. aegypti* and they are divided into five different families: Defensins (cysteine rich peptides), Cecropins (α-helical peptides), Diptericin (glycine-rich peptides), Attacin (glycine-rich peptides), and Gambicin (cysteine-rich peptides [52]. The mode of killing of AMPs differs depending on the invading pathogens. Defensins are active mainly against gram-positive bacteria and parasites. Their ability to disrupt the membrane permeability barrier makes them highly toxic since they cause loss of motility. On the other hand, Cecropins are positively charged peptides that bind to negatively charged lipids in the membrane causing a change in the biological structure of the membrane. Another possible mode of killing used by Cecropins is the inhibition of nucleic acid and protein synthesis. Defensins and Cecropins have been found to be expressed in *A. gambiae* mosquitoes in the midgut, thoraxand abdominal tissues during parasite infection. Despite the fact that Gambicin has been found to be induced in the midgut, fat body and hemocytes of *A. gambiae* mosquitoes, its role in antiviral immunity is not understood. Cecropins were shown to be upregulated in DENV-2 infected *A. aegypti* mosquitoes; they were also shown to exhibit an antiviral activity DENV and CHIKV [33].

b. Reactive Oxygen Species/Reactive Nitrogen Species

Reactive nitrogen species (RNS) and reactive oxygen species (ROS) are two major components of the mosquito immune response [53]. They have been shown to play a major role in the case of bacterial and plasmodial infections. ROS are a group of oxygen derived radical species that are mainly formed during cell respiration at the level of the

mitochondria. However, another source of ROS are the membrane-bound enzymes NADPH oxidases (NOX1, NOX2, NOX3, NOX4, NOX5) and dual oxidases (DUOX1 and DUOX2) which catalyze the reduction of molecular oxygen to generate superoxide and/or hydrogen peroxide using NADPH as an electron donor [54]. The production of ROS serves as the first line of defense in the gut immunity of *Drosophila melanogaster*. In addition to that, they are required for the development of an effective immune response against Plasmodium parasites in *A. gambiae* [55].

c. Melaniztion

In mosquitoes, melanization is important immune response that is involved in the killing of Plasmodium, filarial nematodes and bacteria. It is described as the deposition of a darkened proteinaceous capsule that surrounds invading pathogens. This process involves a series of reactions that lead to conversion of tyrosine into melanin precursors, which is followed by the cross-linking of proteins to form a layer of melanin that surrounds and sequesters invading pathogens. It often leads to the death of the pathogen through oxidative damage brought on by unstable intermediates created during melanogenesis or by starvation. It is initiated by the proteolytic cleavage of a pro-phenoloxidase zymogen into its active form phenoloxidase, which then hydroxylates tyrosine to form dopa. The latter is then oxidized by phenoloxidase to form dopaquinone, which is then converted to dopachrome and then 5,6-dihydroxyindole which is oxidized into indole-5,6-quinone by phenoloxidase and then cross-linked with hemolymph proteins to form melanotic capsules. In an alternative pathway, Dopa will be formed by the hydroxylation of tyrosine and then it

will be decarboxylated by dopa decarboxylase to form dopamine, which is then converted into melanin by phenoloxidase and other enzymes. [56]. Now concerning the cellular immune responses, they take place by immune cells called hemocytes that undergo phagocytosis, nodulationand encapsulation of the pathogen. In addition to that, hemocytes also evoke mosquito humoral immunity by activating the previously mentioned signaling pathways which lead to the synthesis and secretion of soluble effectors molecules such as AMPs and components of the phenoloxidase cascade into the hemolymph to fight invading pathogens [57].

3. Cellular Responses

Hemocytes comprise a key component of the mosquito immune system due to their ability to kill pathogens via phagocytic, lytic and melanization pathways. Around 500 to 4000 hemocytes are present within an individual mosquito and they can be divided into three populations: granulocytes, oenocytoids and prohemocytes. Granulocytes comprise 85% of the hemocyte population and their main function is to kill pathogens by phagocytosis or lysis. Oenocytoids, on the other hand, are responsible for the production of the enzymes required for melanization while prohemocytes participate in phagocytosis. In addition to that, these immune cells could be divided based on their anatomical location into circulating hemocytes that circulate within the hemolymph and sessile hemocytes that are tissue resident. Circulating hemocytes comprise 75%, however the sessile ones comprise a lower proportion 25%. Both circulating and sessile hemocytes participate to defend the host against pathogens [58].

a. Phagocytosis

The hemocyte-mediated immune response in insects includes phagocytosis, encapsulationand nodulation of the pathogen. Phagocytosis is a cellular process used by mosquitoes to neutralize and remove microorganisms such as bacteria, yeast and plasmodium. It is initiated when the foreign body is recognized by the humoral PRR that acts as an opsonin followed by the internalization of the foreign body into a membrane-delimited phagosome. The phagosome will fuse with a lysosome present in the cytosol allowing the neutralization of the microorganisms that will be hydrolytically digested by the enzymes present in the lysosome. The mosquito pattern recognition receptors (PRRs) are involved in the regulation of phagocytosis [58]. The complement-like protein, thioester containing protein 1 (TEP1) is the most studied among these factors. It opsonizes bacteria and targets them for phagocytosis. Other members of the TEP protein family (TEP3 and TEP4) are leucine rich repeat containing proteins (LRRs; LRIM1), fibrinogen-related proteins (FBN8) and DSCAM, which is a hypervariable immunoglobulin that can be transpliced into over 31 000 variants. All play a role in the phagocytic response [59] [60]. Despite the fact that some of these proteins opsonize pathogens, other proteins like LRIM1 act upstream of the opsonization response. Some pathogens require the deposition of melanin onto the surface of the foreign entity before the initiation of phagocytosis. In addition to that, β integrin (BINT2), a peptidoglycan recognition protein (PGRPLC), a low-density lipoprotein re, whereas ceptor-related protein (LRP1) and a protein containing both zinc finger and LITAF domains are all examples of transmembrane receptors that are exposed on the surface of hemocytes and are implicated as PRRs [61]. They might

recognize the pathogens directly or after they have been opsonized by humoral factors. Finally, there are certain proteins responsible for the internalization of the pathogen like CED2, CED5and CED6. CED2/CED5 pathway controls TEP4- and BINT2-mediated phagocytosis, whereas CED2/ CED5 pathway controls TEP4- and BINT2-mediated phagocytosis.

b. Encapsulation

The binding of hemocytes to larger targets such as parasites, protozoa and nematodes is referred to as encapsulation. This process is observed for example when parasitoid wasps lay their eggs in the hemocoel of *Drosophila* larvae. It involves the formation of a multilayer capsule around the invader and this will be ultimately accompanied by melanization. The invader will be killed within the capsules by the local production of cytotoxic free radicals ROS and RNS, or by asphyxiation [10].

D. Mosquito Control Strategies

Mosquitoes are a key threat for millions of people worldwide, since they represent the major arthropod vectors of human diseases transmitting malaria, lymphatic filariasis and arboviruses such as dengue virus and Zika virus. Unfortunately, no vaccine or drug is available for the treatment of these diseases. In this scenario, vector control is the main form of prevention.

1. Chemical Control Methods

All the traditional vector control strategies have focused on killing mosquitoes using insecticides. Different methods have been used for personal protection against mosquito-borne diseases which involve the use of mosquito repellents such as N,N-diethyl-meta-toluamide (DEET), dimethyl phthalate (DMP), N,N-diethyl mendelic acid amide (DEM), as well as plant-borne molecules and mosquito bed nets. Removing breeding sites of mosquitoes has often been used alongside chemical or microbiological ovicides, larvicidesand pupicides especially in areas where pandemic mosquito-borne diseases occur [62]. However, the great reproductive capacity of mosquitoes and their genomic flexibility have led to the failure of mosquito control strategies. The two previously mentioned characteristics are exemplified by two observations. First of all, mosquitoes and other insects have developed resistance to insecticides which has been reported worldwide in most vectors and against every class of chemical insecticide [63]. In addition to that, the use of insecticides including organophosphates, carbamates and pyrethroids, can also have a harmful effect on the environment and on human.

2. Genetic Control Methods

a. Sterile Insect Technique

The Sterile Insect Technique (SIT) is a genetic control method that has been shown to be species-specific, effective and environmentally friendly technique of insect control. It has been widely used since it succeeded in controlling agricultural pests and, in certain cases,

mosquito vectors [64] [65]. The concept of introducing sterile insects into the population as a form of controlling pests with agricultural importance was introduced in 1995. The concept of SIT is based on mass rearing of mosquitoes which is followed by the sterilizing the mosquitoes by radiation. Then a large number of sterilized males will be released into a given target area. Thus, mating with a sterile insect will result in no offspring and eventually the population will decline with time if enough sterile insects are released [66]. The advantage of this technique is that it requires the release of only males and not females. The fact that sterile males are required to mate with wild type males represents a major difficulty with SIT since sterilizing the insects by irradiation causes a dramatic loss of their mating ability compared to wild type. Another disadvantage of SIT is that it requires the release of a large number of males over a long period knowing that mosquitoes need to be sorted by sex before the release. Hence, there is always a risk of releasing females by mistake which could have hazardous effects. Taking this into account, the use of SIT against mosquitoes is problematic.

b. The RIDL System

The RIDL (Release of Insects Carrying a Dominant Lethal Gene) system was proposed by Thomas et al. (2000) consists of the introduction of a dominant lethal gene that is under the control of a female-specific promoter, such as the vitellogenin gene. Then, the genetically modified males carrying female acting transgenes will be released into the wild. Treatment with tetracycline can inactivate the expression of the lethal gene that allows the colony to be maintained. When the separation of males and females is required, tetracycline

is removed from the system, causing death of all females [67]. In this case, male will carry and deliver female acting transgenes into the population. One approach uses a construct that decreases the expression of a gene active in flight muscles in female pupae. As a result, the daughters of the released males will lose their ability to fly to find mates or human hosts [68]. Another approach relies on transgenes that induce mortality either in pupae or in adults. This could be ;accomplished in the lab by placing the transgenes under the control of a repressor that inhibits expression in the presence of tetracycline [69]. Although RIDL seems to be more effective than SIT, it still faces some of the previous disadvantages of SIT such as the ability of transgenic males to mate with wild type females and the need to release a large number of males.

3. Biocontrol Strategies

In addition to the previously mentioned genetic methods, naturally occurring organisms that are pathogenic to mosquitoes can be used as biocontrol strategies.

a. Fungi

Entomopathogenic fungi are known to produce infective spores (conidia) that attach to the cuticle of mosquitoes then penetrate it releasing toxins that result in mosquito death. Previous studies have shown the pathogenic effect of fungi on malaria mosquito vectors and on *A. Aegypti* [70] [71] [72]. Fungi like *Beauveria bassiana* are known to induce lethality in insects by germinating on host cuticle then penetrating it. After 4-14 days, the blastospores will circulate through the body and kill the insect. Then, they will transform

into mycelium and produce more conidia on the exterior of the insect. The evolution of fungus resistance is predicted to be much slower than the evolution of insecticide resistance, which is considered an advantage of entomopathogenic fungi. However, the slow killing rate (up to 2 weeks) and high cost of production and their high specificity make them unsuitable for commercial use, which requires broad range killers.

b. Bacteria

Bacillus Thuringiensis (Bti) is the most common larvicide used currently in European countries. It is a gram-positive, spore-forming bacterium that releases insecticidal toxins and virulence factors that target the larval stages of insects precisely [73] [74]. Although Bti has been used to reduce *A. Aegypti* mosquitoes and their larvae, the long term use might lead to the development of resistance to Bti toxins[75]. Bti toxins are produced during the sporulation phase as parasporal crystals comprised of one or more proteins like Crystal (Cry) and Cytolitic (Cyt) toxins, also called d-endotoxins, which are very specific to the target insect while being harmless to humans, vertebrates and plants.

4. Wolbachia as a Promising Alternative

Several studied showed that the microbiota of mosquito vectors has a strong impact their ability to transmit diseases. *Wolbachia* is a genus of intracellular alpha-proteobacteria belonging to the order Rickettsiales. It is thought to infect 40–75 % of all arthropod species and several nematodes [76, 77]. Its genome ranges from 1.2 to 1.6 Mb and contains WO

prophages. *Wolbachia*, which was first discovered in the mosquito *Culex* pipiens, has now been found in a number of mosquito vector species [78]. Eighteen supergroups of *Wolbachia* have been identified and the majority is present in arthropods. The success of *Wolbachia* resides in their unique capacity to infect and manipulate the host germ line in order to facilitate their vertical transmission through maternal lineage. *Wolbachia* has been extensively studied because of its ability to alter the reproductive physiology of the host in order to facilitate its own transmission. Its effects include male killing, parthenogenesis, feminization and cytoplasmic incompatibility (CI) [79]. Cytoplasmic incompatibility is the most common *Wolbachia*- induced reproductive phenotype. It refers to the failure of *Wolbachia*-infected males to produce viable offspring when mating with either uninfected females or females infected with a different strain of *Wolbachia* [80]. The inability of *Wolbachia*-infected males to successfully mate with uninfected females is said to be unidirectional CI, because it will promote the expansion of only one subpopulation composed of *Wolbachia*-infected mosquitoes. However, in the second scenario CI occurs when *Wolbachia* infected males mate with females infected with a different strain of *Wolbachia* which is referred to as bidirectional CI because it can result in the development of divergent subpopulations, each infected with one of two or more opposing *Wolbachia* strains [81].

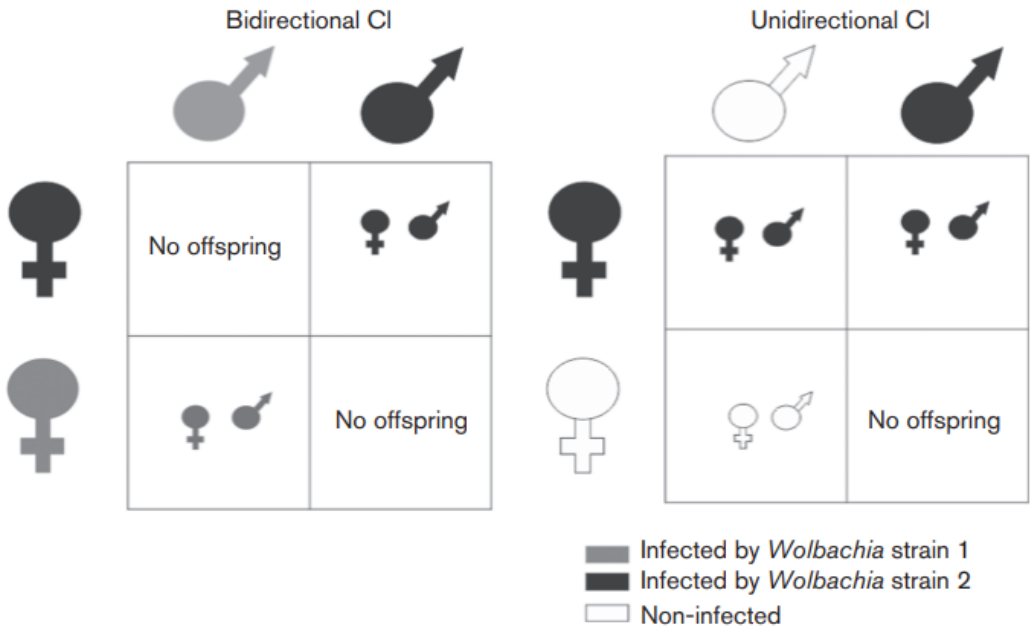


Figure 4: *Wolbachia* can cause a reproductive phenotype called cytoplasmic incompatibility.

It can occur in two forms (a) Bidirectional CI occurs when males and females infected with two different strains of *Wolbachia* are unable to produce viable offspring. (b) Unidirectional CI occurs when infected females are able to successfully mate with both uninfected males and males infected with the same strain of Wolbachia [82].

The ability of *Wolbachia*-infected females to mate successfully with infected or uninfected males provides them with a reproductive advantage over uninfected females. During gametogenesis, *Wolbachia* organisms are abundant, however later they become excluded from the male gametes during the process of sperm maturation, which suggests that the *Wolbachia*-free sperm derived from an infected male contains *Wolbachia*-secreted factors responsible for the CI phenotype. CI induces defects in paternal chromatin condensation that prevent proper chromosome segregation during the first embryonic mitosis. CI was shown to induce defects in paternal chromatin condensation preventing

chromosome segregation during the first embryonic mitosis and leading to lethal aneuploid or haploid development [83, 84]. The observed chromosomal bridges suggested the presence of incompletely replicated DNA that hindered the segregation of sister chromatids. In a normal cross between compatible male and female insects, the zygotes follow a well-characterized developmental pathway. Immediately following fertilization, the specialized nuclear envelope of the sperm-derived pronucleus will breakdown. Then the protamines, which are small and highly basic proteins used to package paternal DNA, are removed and replaced by maternally supplied histones, including the replication-independent histone H3.3 [85] [86]. Subsequently, male and female pronuclei juxtapose, but do not fuse and they undergo DNA replication before their first zygotic mitosis [87]. Maternal and paternal chromosomes synchronously condense, align at metaphase and then separate at anaphase. However, they do not fuse until the end of the first telophase. In the contrary, the earliest detected abnormality in CI zygotes is impaired maternal H3.3 histone deposition onto the paternal DNA following protamine removal. As a result, the paternal pronuclear nuclear envelope breakdown is delayed. In addition, the cell-cycle kinase CDK1, which is normally responsible for the metaphase-to-anaphase transition, is inhibited in the male pronucleus. The delay in the condensation of paternal chromosomes will often lead to chromosome bridging during anaphase, which is fatal in diploid insects [86]. Further analysis on the cytological defects of CI revealed an abnormal presence of the replicating factor PCNA specifically on the paternal chromatin during mitosis preceded by histone deposition defects during the chromatin remodeling, [81]. The presence of *Wolbachia* in the egg prevents the induction of these defects by the *Wolbachia*-modified sperm. Hence, *Wolbachia* has the ability to modify the sperm through the potential

secretion of a Mod factor(s) which lead to CI. This is called the modification event and it occurs inside *Wolbachia*-infected males during spermatogenesis. However, the presence of a compatible *Wolbachia* strain in the egg prevents CI from occurring through the potential action of a Resc factor(s), leading to a modification-rescue model for CI. *Wolbachia* Mod and Resc effectors driving CI and its rescue have been recently identified after being a longstanding goal for decades. CI factors or cifs have been recently identified as genetic determinants of CI from *Wolbachia*. [88, 89]. *Wolbachia* prophage (WO phage) regions termed eukaryotic association modules (EAMs) contain two-gene operons that encode the cif proteins [90]. These pairs of syntenic genes are found in CI-inducing strains only and contained within the *Wolbachia* phage WO. The downstream genes in the cif operons cifA and cifB of wMel from *D. melanogaster* and their orthologs cidA and cidB of wPip from the mosquito *C. pipiens*, encode enzymatic activities essential to their ability to induce CI when expressed in the germlines of transgenic flies. In fact, it has been shown that these two genes can recapitulate CI traits when transgenically expressed in uninfected male flies. The CI inducing deubiquitylating enzyme (DUB) cidB cleaves ubiquitin from substrates and cidA binds to cidB. Therefore, CI was modeled as a toxin-antidote (TA) system with CidB as the toxin and CidA the antidote [88]. In addition to that, Cifs are annotated with superscripts identifying the *Wolbachia* strain of origin. For example, $CidB^{wPip}$ refers to the toxin from the *w*Pip *Wolbachia* endosymbiont of *Culex pipiens.*

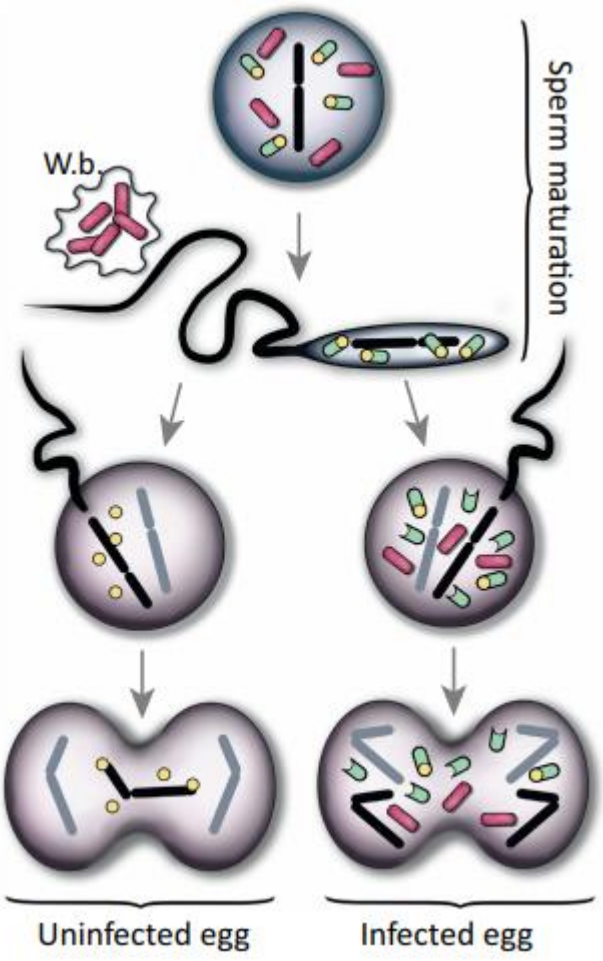


Figure 5: The Toxin-Antidote (TA) model.

A toxin (yellow particles) and its antidote (green particles) are produced by *Wolbachia* bacteria (pink) in the immature sperm. During the sperm maturation process, *Wolbachia* are removed from the maturing sperm into waste bags (W.b). As a result, the antidote will be lost faster than the toxin due to its instability. When the uninfected egg is being fertilized by the sperm, the toxin will hinder the paternal chromosomes leading to embryonic death. However, the antidotes present in the infected egg will bind to the toxin and thus maintain embryo viability. Other CI mechanisms have been studied, but the model depicted here best accounts for all CI features [88].

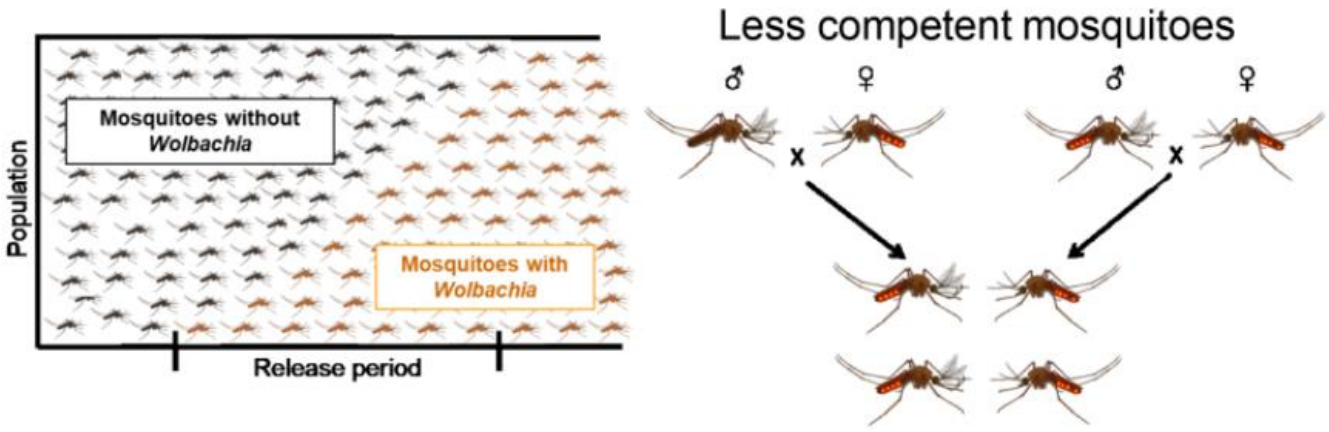
- *Wolbachia* in Limiting Arbovirus Transmission

Wolbachia has been in the spotlight with the discovery of its ability to suppress the replication of vector-borne human arboviruses. The fact that *Wolbachia* is a naturally existing microbe made its use more acceptable than the genetic modification-based approach for the public. *Wolbachia* can be used for population replacement or for population suppression. The former relies on releasing *Wolbachia*-infected female mosquitoes that will mate with males from the wild and produce viable offsprings regardless if the males are *Wolbachia*-infected or not. As a result, *Wolbachia* will spread in the field population making the mosquito vectors less competent individuals. Ultimately, the total number of mosquitoes remains unchanged; however, their ability to transmit diseases will decrease. In contrast, the population suppression method will reduce the total number of mosquito vectors. In this method, *Wolbachia*-infected male mosquitoes are released to mate with uninfected females in the wild. Thus, no viable offsprings are produced due to CI.

Several studies proved that *Wolbachia* could provide fitness advantages to the host fertility or survival. For example, introducing the wMel *Wolbachia* strain artificially to drosophila melanogaster inhibited *Drosophila* C virus (DCV) infection. In addition to that *Wolbachia*-infected flies were much more resistant to DCV than uninfected flies [91]. *Wolbachia* is a promising tool for the control of mosquito-borne diseases due to their ability to invade host populations by inducing CI and to interfere negatively with the transmission of disease pathogens [92]. *A. aegypti* is an urban vector of dengue, Zika, chikungunya and yellow fever virus. It is present in more than 150 countries and is not naturally infected by

Wolbachia. The Eliminate Dengue Programme that emerged in 2008 focused on using the life-shortening wMelPop strain to reduce the number of dengue vectors reaching maturity. This approach took into consideration the fact that mature mosquitoes are more likely to transmit dengue knowing that DENV must incubate in the mosquito for several days before becoming infectious [93]. However, the transinfection of *A. aegypti* with the wMelPop strain induced significant fitness costs like reduction of the longevity of infected adult females and reduction in the viability of eggs. As a result, researchers of the Eliminate Dengue Programme used wMel strain as an alternative because it has a lower fitness cost but still confers sufficient resistance to DENV. A stable transinfection of of A. aegypti with wMel was reported in 2011 [93]. Studies showed that this strain reduced the capacity of *A. aegypti* to transmit dengue. In addition, it successfully invaded wild mosquito populations. As a result, a large-scale release of *Wolbachia*-infected mosquitoes was performed in dengue-endemic areas in Australia. This has led to the suppression of DENV replication in and dissemination by mosquitoes and it was confirmed by vector competence experiments carried out 1 year following the field release [94]. Following the success of the Eliminate Dengue Programme in Australia, trial releases of *Wolbachia*-transinfected *A. aegypti* were performed in other dengue-endemic countries throughout the world such as Colombia, Indonesia, Vietnamand Brazil. The WHO is encouraging countries to use *Wolbachia*-based prevention strategies against other arboviral infections. However, the effectiveness of *Wolbachia* against other arboviral infections is still limited to experimental framework.

a)



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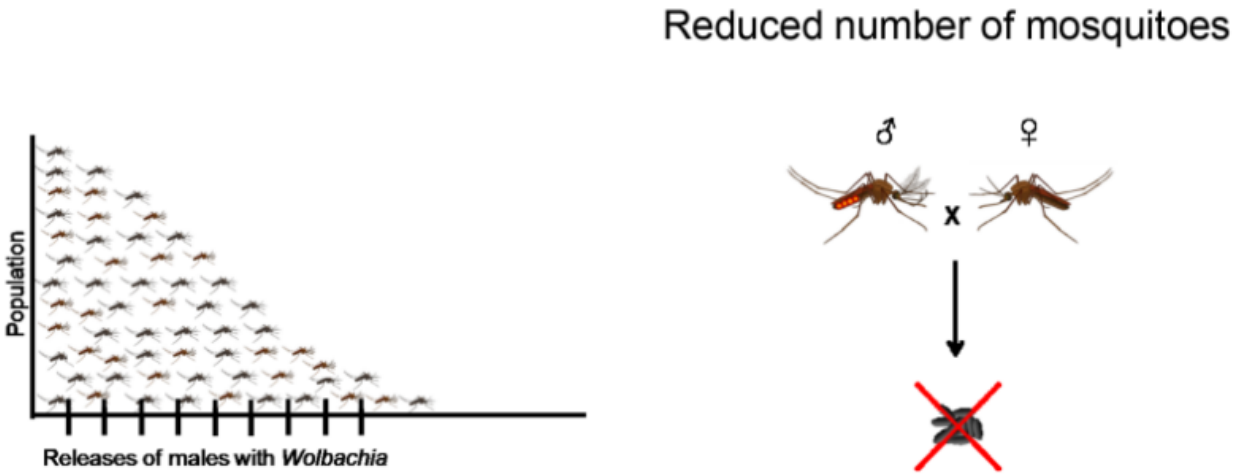

Figure 6: *Wolbachia* is a promising alternative for vector control through population suppression or replacement.

(a) Population replacement method that allows the spread of *Wolbachia* in the population.
(b) Population suppression method that will decrease the total number of mosquitoes due to CI, as modified from [95].

Wolbachia strains that confer the strongest interference with pathogen transmission such as wMelPop are not able to spread easily into local vector populations because of their deleterious fitness effects such as reduced lifespan of larval and/or adult stages, decreased egg viability, or greater susceptibility of *Wolbachia*-infected mosquitoes to some insecticides. Therefore, an adequate *Wolbachia* strain that shows the optimum trade-off between pathogen interference, the strength of cytoplasmic incompatibilityand other potential fitness effects should be selected for *Wolbachia*-based biocontrol strategies. The effects of *Wolbachia* can change dramatically depending on whether *Wolbachia* is present in its native host or transinfected into a novel host. In addition to the *Wolbachia* strain, other factors are crucial for the success of virus blocking such as *Wolbachia* density.

Studies showed that *Wolbachia* could induce resistance to arboviruses through four complementary mechanisms competition for resources, immune priming, induction of the phenoloxidase cascadeand induction of microRNA-dependent immune pathways [96, 97].

- *Wolbachia* Density and Competition for Host Cell Resources

Autophagy is a recycling process by which dysfunctional cellular components are incorporated into lysosomes for digestion. The nutrients resulting from this cellular degradation are then made available for further metabolic processes [98]. Studies showed that *Wolbachia* is not only able to induce autophagy but also to hijack the autophagy system to ensure its survival in the cells of arthropod vectors. In this way, it will reduce the amount of nutrients available for the virus. In addition, *Wolbachia* also competes with

viruses for iron and cholesterol. Studies showed that there is a positive correlation between *Wolbachia* density and the antiviral effect conferred [99]. This is because different *Wolbachia* strains infect vector species at variable densities and they tend to have different tissue distributions [100]. In heterologous host, *Wolbachia* usually grows to higher densities than in native hosts. This may explain why antiviral effects are more often observed in heterologous hosts compared to native host species [101].

- Immune Priming

The transinfection of *Wolbachia* into A. aegypti or any other heterologous arthropod vector that is not naturally infected by *Wolbachia* or that specific *Wolbachia* strain boosts their immune system allowing it to fight microbes more effectively. It activates the three major signaling pathways Toll, Imd and JAK-STAT which results is transcriptional upregulation of immune genes with increased production of antimicrobial proteins (e.g. Cecropin and Defensin). In addition, studies showed that *Wolbachia* is capable of increasing melanization through PO cascade in both homologous and heterologous host [102].

- Induction of MiRNA-Dependent Immune Pathway

Studies showed that *Wolbachia* has the ability to upregulate the microRNA aae-miR-2940 in mosquitoes which leads to the upregulation of the metalloprotease m41ftsh and the downregulation of the DNA cytosine-5-methyltransferase gene, AaDnmt2 [103]. This will

favor the DNA cytosine methylation which indispensable for host immune defense, gene regulation, genome stability, organ differentiation and ageing. In addition, both the metalloprotease m41ftsh and DNA cytosine methylation are essential for maintaining a high density of *Wolbachia* infection in host cells, which could decrease the amount of resources available for the virus.

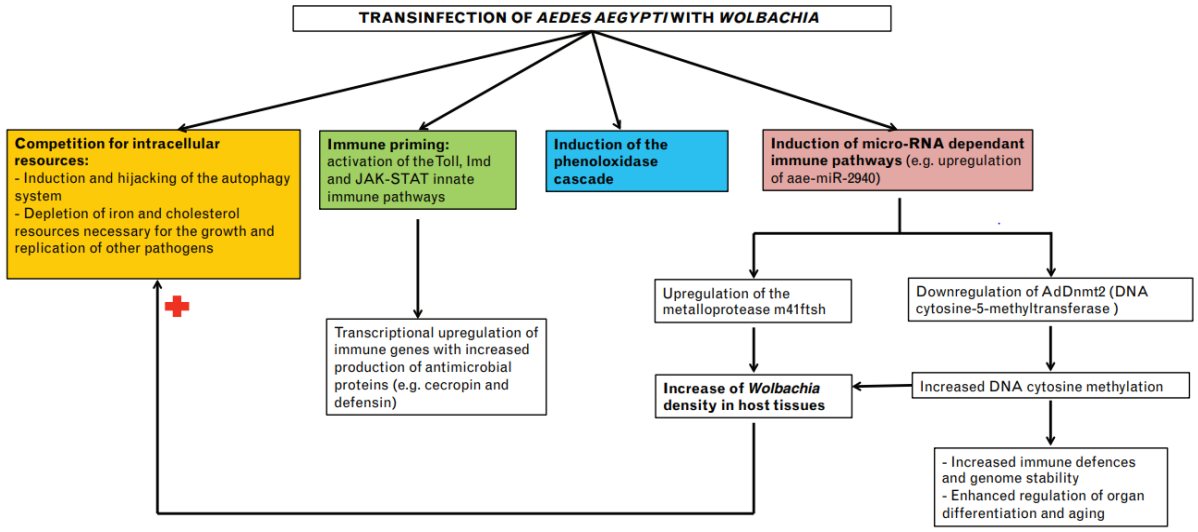

Figure 7: Mechanisms used by *Wolbachia* to induce antiviral resistance in A. aegypti following transinfection [82].

Mosquito host	*Wolbachia* strain	Nature of host-*Wolbachia* association	Virus	Antiviral effect exerted by *Wolbachia*
Aedes aegypti	*w*AlbB	Transinfection	DENV	Reduced virus proliferation
	*w*Mel	Transinfection	DENV	Blockage of viral proliferation
			WNV	No effect
			CHIKV, YFV	Reduced virus proliferation
	*w*MelPop	Transinfection	DENV, CHIKV	Reduced virus proliferation
			YFV	Reduced virus proliferation
			WNV	Reduced virus proliferation
Aedes albopictus	*w*AlbA and *w*AlbB	Natural	CHIKV	No effect
			DENV	No effect on virus replication in midgut; reduced virus dissemination and transmission
			DENV	No effect
	*w*Mel (*w*AlbA and *w*AlbB present)	Transinfection	CHIKV	Reduced virus transmission

Table 1: The antiviral effect of *Wolbachia* in different mosquito/host associations.

CHIKV, Chikungunya virus; DENV, dengue virus; JEV, Japanese encephalitis virus; WNV, West Nile virus; YFV, yellow fever virus [104].

- The Somatic Life of *Wolbachia*

Most studies focused on *Wolbachia* interaction with the host germline because it is the primary route of transmission. However, over the last decade the focus was on prominence of *Wolbachia* in somatic tissues. Previous studies documented the presence of *Wolbachia* not only in reproductive tissues, but also in a variety of somatic tissues.

Organism	Species	Somatic Tissues
Fruit Fly	*D. melanogaster* (adult)	Central brain (intra & extracellular), retina, optic lobe, ganglia, somatic cyst cells, somatic stem cells
	D. simulans (adult)	Head, muscle, midgut, malpighian tubules, wings, hemolymph
	D. melanogaster (larva)	Nerves, malpighian tubules, salivary glands, trachea, fat body, proventriculus
	D. simulans (larva)	Brain, salivary gland, midgut, fat body
Mosquito	*Ae. albopictus*	Salivary glands, some strains no somatic tissue
	An. gambiae (wMelPop)	Brain, sensory organs, mouthparts, hemocytes, fat body, abdomen
	C. pipiens	Head, malpighian tubules, wings, hemolymph
	C. cautella	Head, muscles, midgut, malpighian tubules, wings, hemolymph
	C. tarsalis	Head, muscle, ganglia, fat body, ovary follicles

Table 2: Distribution of *Wolbachia* in somatic tissues of fruit fly and mosquitoes [105].

Wolbachia is prevalent in tissues of the nervous system in *Drosophila* and other flies [106]. *Wolbachia* is also present in digestive and metabolic tissues such as the fat body, gut, salivary glands, hemocytes and malpighian tubules of various arthropod species in addition to being present in the muscle and wings of some species. The fact that *Wolbachia* is present in specific somatic tissues suggests that somatic tissue tropism is not incidental. Several hypotheses may explain the reason behind this somatic tropism. It might be evolutionary maintained because it aids horizontal transmission within and between species, which enhances the genetic diversity of *Wolbachia*. Another explanation might be that somatic *Wolbachia* confers advantageous phenotypes in the host enhancing germline transmission [105].

Like many insects the *Drosophila* egg chamber is made up of a syncytium of 15 nurse cells and an oocyte connected together by cytoplasmic bridges [107]. In addition to the nurse cell cytoplasm being pumped into the oocyte, specific determinants essential for anterior-posterior (AP) axis formation are also transported from nurse cells to specific regions of the maturing oocyte. In order to succeed in vertical transmission from one generation to the next, *Wolbachia* must concentrate at the posterior pole of the maturing oocyte, which is the future site of the germline [108]. Interactions with host microtubules, motor proteins and posterior determinants allow *Wolbachia* to reach the posterior pole of the maturing oocyte. *Wolbachia* that reach the posterior pole are incorporated into the germline. However, the *Wolbachia* that remain dispersed throughout the developing oocyte are incorporated to somatic tissues.

CHAPTER 2

AIMS OF THE PROJECT

Due to the growing expansion of mosquitoes, mosquito-borne diseases have emerged as a global threat to the public health. Several control methods have been developed to limit the spread of mosquito-borne diseases. Unfortunately, these methods have many disadvantages that prevent their use. The lack of licensed vaccines and treatment highlight the urgent need for efficient mosquito vector control. The intracellular bacterium *Wolbachia* is one of the most exciting approaches used to control arbovirus transmission from mosquito to vertebrate. These alpha-proteobacteria propagate through insects by causing manipulations in the host reproductive system such as cytoplasmic incompatibility (CI). CI is described as the inability of *Wolbachia*-infected males to successfully mate with uninfected females. All *Wolbachia*-based control methods rely on the ability of *Wolbachia* to induce CI that results in embryonic death due to incompatible crossings.

Wolbachia infects most arthropods and some filarial nematode species by being vertically transmitted through the maternal lineage. However, there is a debate concerning the percentage of insects infected with *Wolbachia*. Despite the fact that much research has been invested in this topic, many gaps in knowledge are still present. Therefore, the first aim of this project was to answer this question by using different insects sampled from random geographic areas in Lebanon.

C. pipiens mosquitoes are known worldwide to transmit several pathogens to humans such as yellow fever virus and filarial nematodes. In addition, CI was first characterized in this mosquito that harbors a *Wolbachia* endosymbiont correspondingly named wPip. Several studies showed that the infection with *Wolbachia* confers a protection against a plethora bacterial, viral and parasitic pathogens naturally present in arthropods. However, other studies claimed that in its native host, the immune activation by *Wolbachia* might be attenuated and the protective effects of the endosymbiont become abolished. Therefore, our second aim was to investigate the effect of abolishing *Wolbachia* on *C. pipiens* after generating a *Wolbachia*-free strain (referred to as Tet). In addition, we compared the survival of *Culex* and Tet in response to microbial injection. Unraveling those aspects provides additional knowledge that, on the long term, would contribute to the development of alternative control strategies.

<u>Specific Aim1</u> Screen wild caught insects for *Wolbachia* presence

1.1. Test if different insects sampled from random geographic areas in Lebanon harbor *Wolbachia* through PCR analysis following DNA extraction.

1.2. Identify *Wolbachia* strain and the host insect by using the molecular markers WSP and COI respectively.

1.3. Perform phylogenetic analysis across Lebanese insect species**.**

<u>Specific Aim 2</u> Analyze the effect of *Wolbachia* on *Culex* mosquito

1.1. effects on the host immunity and life span

1.2. effects on host physiology (reproduction)

CHAPTER 3

RESULTS

A. Screen Wild Caught Insects for *Wolbachia* Presence

1. Identification of Wolbachia Strain

For this study, different insects were collected from random geographic areas in Lebanon. We started by photographing the collected insects to identify them phenotypically. Then we used Livak method to extract the DNA of 100 insects. Following the DNA extraction, a PCR was performed to identify the *Wolbachia*-positive insects using *Wolbachia* specific primer wsp. 24 samples were found to harbor *Wolbachia* through PCR analysis following the DNA extraction of the collected samples. Actually, 12 species from the 60 species sampled were considered *Wolbachia* positive due to the presence of replicates. Our study showed that 20% of the insects collected harbored *Wolbachia*. A second larger scale PCR was performed on the *Wolbachia* positive insects to amplify the DNA. Then we used phenol-chloroform extraction to purify the DNA before it was sent to AUB-DTS sequencing facility. After sequencing was performed, we used NCBI blast that helped us find 6 *Wolbachia* strains.

2. Molecular Identification of the Host Insect

Our host insects were identified phenotypically only by their photos. However, to be able to perform a phylogenetic analysis comparing the host insects and the *Wolbachia* strain phylogenies, we need to identify the host insect molecularly. To do this, a PCR was performed using cytochrome oxidase 1 (COI) primer. Then, the amplified DNA was purified using phenol-chloroform extraction method and was sent for sequencing. After sequencing was done, we used NCBI blast to find the best match host insect that was double-checked with the photo of the insects. As a result, 5 host insects were identified.

Figure 8: Photo of a selection of the collected insects.

Species	**Sequence**	**Percent identity**
Melanostoma mellinum voucher INV04977 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial	GTTCAGTGCYAAYATTAATTTTACYGKKTTACC YKGGTATRAGTTTTTGATTAYTTCCTCCTTCATT AACTTTATTATTA GTAAGAAGTATAGTAGAAAATGGAGCTGGTAC AGGATGAACTGTTTACCCTCCTCTTTCTTCTAGA ATTGCTCACGGTGG AGCTTCTGTTGATTTAGCTATTTTTTCTCTTCATT TAGCTGGTATATCTTCAATTCTAGGAGCAGTAA ATTTTATTACTA CAGTAATTAATATACGTTCTACAGGTATTTCTTA TGATCGAATACCATTATTTGTTTGATCAGTTGTA ATTACAGCTTTA CTTCTTTTATTATCATTACCAGTTCTTGCTGGAG CAATTACAATATTATTAACAGACCGAAATTTAA ATACTTCTTTTTT TGATCCAGCCGGAGGAGGAGATCCTATTTTATA TCAACATTTATTTTGATTTTTTGGTCATCCAGAA GTTTATATTTTAW TTTTTACCGGGATACATTTTCTTTTCTGA	99.51%
Lasioglossum malachurum cytochrome oxidase I (COI) gene, mitochondrial gene encoding mitochondrial protein, partial cds	GAGCAGTGCATTGTWTKAKWSWTAYCSSTCRG ATWATAATTTAAGTATTCTGASTATTAATTCCA TCAATAATTATATTA TTAACAGGAACAATCTTATCCTCCGGATCAGGA ACAGGATGAACTATTTACCCTCCTTTATCTAGA TCTACATTTCATCC TTCAATTTCAGTTGATTGCACTATTTTTGCTCTT CATATAGCAGGAATTTCTTCCATTATAGGAGCT ATTAATTTTATTG TATCAATTACATTAATAAAAAATTCATCTATTA AATATGATCAACTTCCTCTTTTCCCATGATCAGT AAAAATTACTGCT ATTTTATTATTATTATCCTTACCAGTTTTAGCAG GTGCTATTACAATACTTTTAACAGATCGAAATA TAAATACTTCATT TTTTGATCCTTCAGGAGGAGGAGATCCTATTTT ATATCAACATCTATTTTGATTTTTTGGTCACCCA GAAGTTTATATTT TATTTTTACCGGGAARTWGCTTTTCTTTACC	98.69%

Parisolabis sp. MAN-2016 isolate DM43 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial	TGYCAAAGGATTKTTTGTTTTACYGGGAGTTTA TATTTTWGATTTACCGGGGTGTTACYCCCATCG TTGACCTTTTGCTT TCAGGGAGTATAGKGGACAGAGGGGCTGGAAC GGGGTGGACCGTTTACCCTCCTCTTTCAGGGGC TATTGCTCATGCRGG GGCTGCGGTGGATTTAAGAATCTTTTCTCTTCAC TTAGCTGGRATTTCTTCAATTCTAGGGGCGATT AATTTTATCACTA CAGTAATCAACATGCGTCCAGCCGGCCTTAAAT TAGAACGAATACCTTTGTTTGTGTGGTCTGTAG CTATTACAGCTTTA TTGTTATTACTTTCGTTACCAGTGTTAGCCGGGG CTATTACCATATTATTAACCGATSGGAATTTGA ATACGTCTTTTTT TGACCCAGCRGGAGGGGGGGATCCTATTCTTTA TCAACATTTATTTTGGTTTTTTGGCACCCTGAAG TTTATATTTTAAT TTTACCGGGASGWKTKWTTTATAATTTYACCCC GMGASSGWSTGAWAASACACYCCCCCCACCCA MGAWAACYRTGAGAA CCSCMCCAAMTCACAAGARGKAACCASAGCAS ACKAGTASAWCAGATCACWAGAAGTAAAATCA KCKAWCAGAGTAGAAG CKWGATGATCGYGACTMKTTACATAASACRTM GYASACTCATTMATGCAGCAGYAACGCTARCG TAGMCCGTACCATACG AGCATACTAGTMTGCACA	81.53%
Sympetrum meridionale isolate MR03 cytochrome oxidase subunit I (COI) gene, partial CDS; mitochondrial	GAGMCAATGGATTKTTGKTTTACCGGGAATTTA TTATTTGAATTTTTGACTTCTTCYGMCTTCTTTC TACTCCTTCTAGC TAGAAGAATAGTTGAAAGAGGGGCAGGAACAG GCTGAACTGTCTATCCTCCTTTAGCCGGAGCCA TTGCCCATGCTGGGG CATCCGTAGATTTAACTATTTTTTCATTACATCT TGCAGGAGTGTCCTCAATTCTAGGAGCAATCAA TTTTATTACTACA GTAATTAATATAAAATCTCCTGGGATAAAAATA GATCAAATACCATTATTTGTATGAGCAGTAGTA ATTACTGCAGTTTT ACTTCTCCTATCATTACCTGTACTAGCCGGAGC TATTACAATATTATTAACTGATCGAAATATTAA TACATCGTTCTTTG	98.21%

	ACCCGRCTGGAGGGGGGGACCCCATTCTTTATC AACACCTGTTCTGATTTTTTGGGCATCCTGAAGT TTATATTTTAATT TTACCGGGARKRTTWWATTTTTAWTTTTWMCC GGGRRGGKKTTKTTTTTWTATTTACC	
Syritta pipiens voucher FM18b cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial	ACTCAGTGGATATTTTTSTTTTACCGGGGAGTKA TAATATAAGTTTTTGACTTTTACCTCCTTCTTTA ACTTTATTATTA GTAAGTAGTATAGTAGAAAACGGAGCAGGAAC AGGATGAACAGTATATCCTCCTTTGTCAGCAGG AATTGCTCATAGTGG AGCTTCTGTAGATCTAGCAATCTTTTCTTTACAT TTAGCAGGAATATCATCCATTTTAGGAGCTGTA AATTTTATTACAA CTGTAATTAACATACGAGCAGCAGGAATAACA TATGATCGAATACCTTTATTTGTATGATCAGTA GTTATTACAGCTCTT CTTCTTTTATTATCATTGCCAGTTTTAGCAGGTG CAATTACAATACTTTTAACAGATCGAAATTTAA ATACTTCATTTTT CGACCCTGCTGGAGGAGGAGATCCAATTCTTTA CCAACATTTATTTTGATTTTTTGGACACCCTGAA GTTTATATTTTAA TTTTACCGGGAGGWTWRWTTTTWAWTTTWMC CSGGRGGGGSSGG	99.3
*Onchocerca_ fasciata*_isolate_ Kerman1_cytochrome_c_oxidase_subunit_I_(COX1)_gene_partial_cds_mitochondrial	TGATTGGTGGTTTTGGTAATTGGATGTTGCCTTT AATGTTGGGGGCTCCAGAGATGGCTTTTCCTCG GGTGAATGCGTTGTCTTTTTGGTTTACTTTTGTG GCTTTGTTGATAGTTTATCAATCTTTTTTTATTG GAGGTGGCCCTGGTAGAAGTTGGACTTTTTATC CTCCTTTGAGGGTTGAGGGTCAACCGGAATTGT CTTTGGATACTATGATTTTAGGTTTACATACTGT GGGTGTTGGTTCTTTGTTGGGTGCTATTAATTTT ATGGTTACTACTCAGAATATGCGATCTACTGCT GTAACTTTGGATCAGATTAGTATGTTTGTTTGG	100%

	ACTTCTTATTTGACTTCTTTTTTGTTGGTTTTGTC TGTGCCTGTTTTGGCGGGTTCTTTGTTATTTTTG TTGTTAGATCGTAATTTTAATACTTCTTTTTATG ACACTAAGAAAGGGGGTAATCCTTTGCTGTATC AGCATTTATTTTGATTTTTTGGTCATCCTGAGGT TTATGTTATTATTTTGCCGGTTTTTGGTATTATT AGAGAGGCGGTTTTATTTTTAACTGATAAAGAC CGTTTATTCGGTCAGACTAGGATGACTTTTGCTT CTATTTGGATTGCTGTTTTAGGGACTTCTGTGTG GGGTCATCAT	

Table 3: Identification of insect species using NCBI Blast.

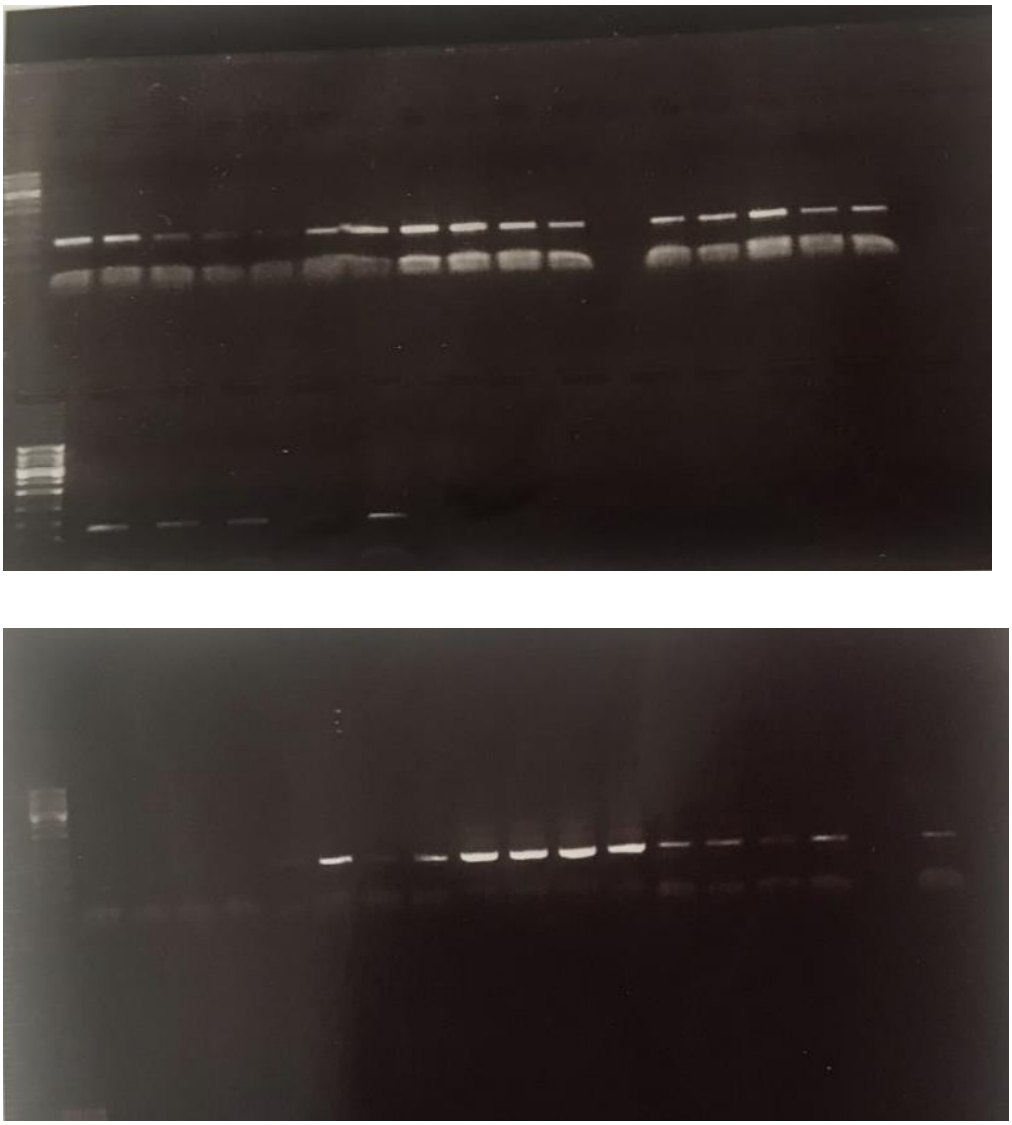

Figure 9: Gel electrophoresis of the PCR products using *Wolbachia* general primers WSP.

Figure 10: Photo of a selection of *Wolbachia* positive insects.

	***Sympetrum meridionale***
	***Melanostoma mellinum***
	***Parisolabis Earwig***
	***Lasioglossum malachurum***
	***Syritta pipiens***

Table 4: Molecular Identification of the Host insect using COI Primer.

3. Phylogenetic Analysis Across Lebanese Insect Species

After the molecular identification of host insect and *Wolbachia* strain, we wanted to perform a phylogenetic analysis to compare phylogeny of the host to the phylogeny of bacteria to determine whether some coevolution has occurred. To do this, phylogenetic trees showing the relationship between host insects and their corresponding *Wolbachia* strains are needed. We used *Onchocerca fasciata* Coi sequence to root the tree of host insects and *Onchocerca cervica* wsp sequence to root the wolbachia tree. Our results showed discordance between *Wolbachia* tree and host tree suggesting that horizontal transmission might have occurred between the species on evolutionary time scale.

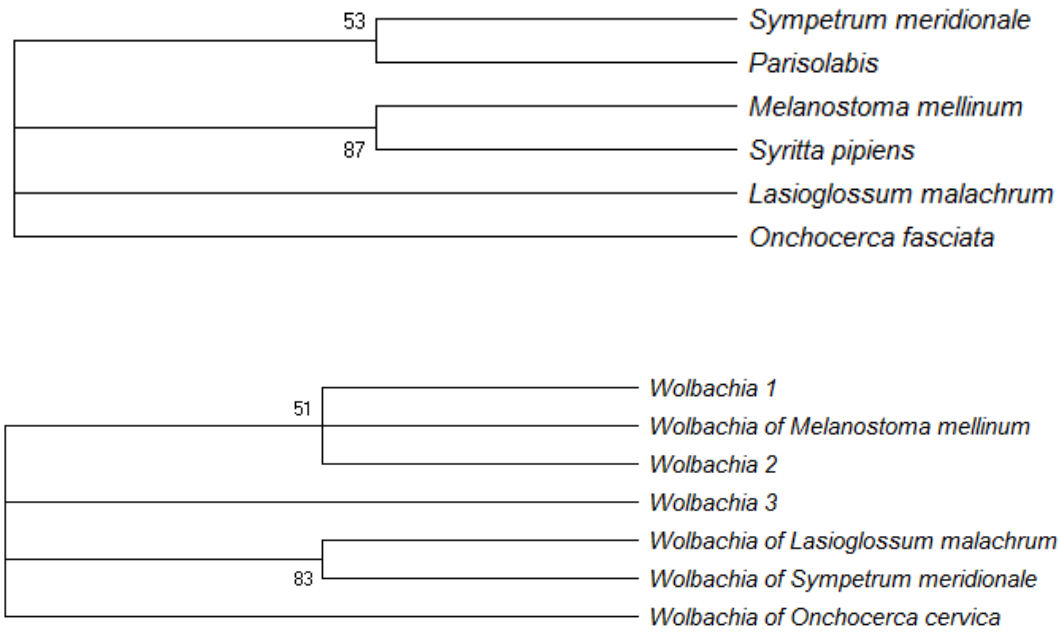

Figure 11: Maximum likelihood was used for tree estimation using mega software [109].

Mega gives the tree with the highest log likelihood value with branch length measured in number of substitutions per site. Bootstrap value, the percentage of trees with the associated taxa clustered together, is shown next to the branches. The bootstrap consensus trees were drawn using majority rule.

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B. Analyze the Effect of *Wolbachia* on *Culex* Mosquito

1. Effect on Host Immunity and Lifespan

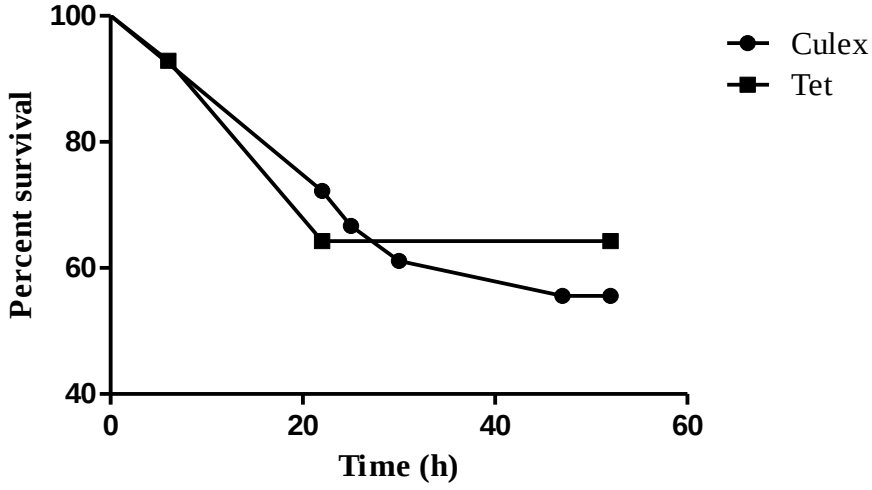
a. Establishment of *Wolbachia*-free Strain

In order to study the effect of *Wolbachia* on *Culex* mosquitoes, we generated a *Culex* strain that is devoid of *Wolbachia* by using tetracycline treatment for two generations. This way, we have two genetically identical *Culex* strains one strain that harbors *Wolbachia* referred to as "*Culex*" and another *Wolbachia*-free "Tet" strain. The DNA was extracted from adult mosquitoes to test the presence of *Wolbachia* using PCR. This method proved to be successful in the clearance of *Wolbachia* from these mosquitoes. We also tested for cytoplasmic incompatibility to prove that our Tet strain is *Wolbachia*-free.
To do this, male *Culex* mosquitoes were crossed with female Tet mosquitoes. As a result, 100% CI was observed.

b. Comparison of the Survival of Young *Culex* and Tet Mosquitoes in Response to Bacterial Infections

We injected both *Culex* and Tet mosquitoes with a selection of microbes by using microinjection method. *Enterococcus faecalis* (*E. faecalis*) was used as a Gram-positive bacteria, Erwinia carotovora carotovora 15 (Ecc15) was used as a gram-negative bacteria. As a result, there was no significant difference in the survival of young *Culex* and Tet in response to *Ecc15* nor to *E.faecalis* bacterial infections.

A



B

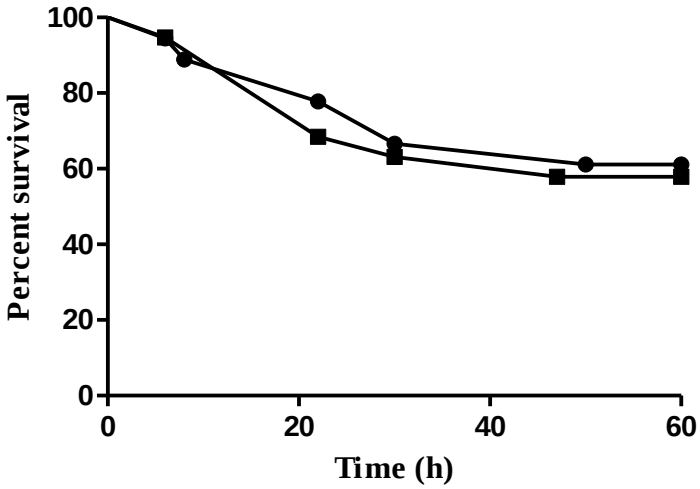
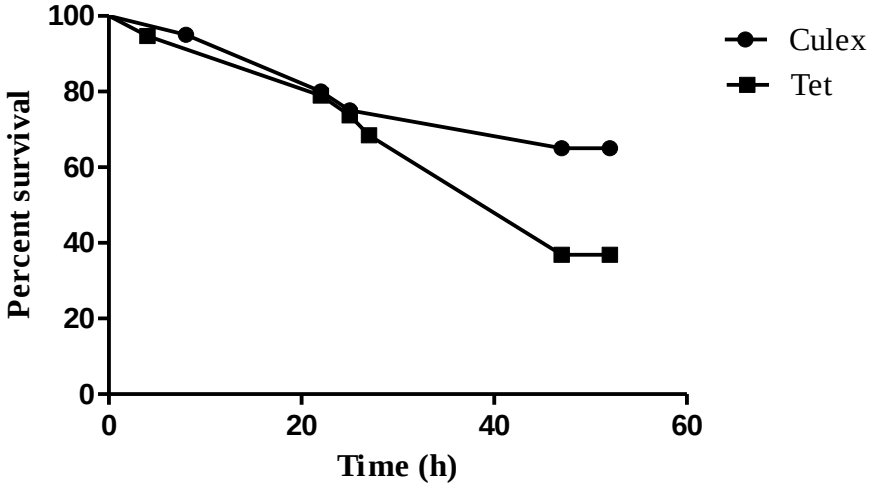


Figure 11: Survival of young *Culex* and Tet mosquitoes in response to bacterial injection with E. faecalis.

Culex and Tet mosquitoes were injected with *E. faecalis* of OD= 0.05. The mosquitoes used were young (4 to 7 days old). The survival curves of *Culex* and Tet were not significantly different with p = 0.75 in **figure 11A** and p = 0.35 in **figure 11B**

A



B

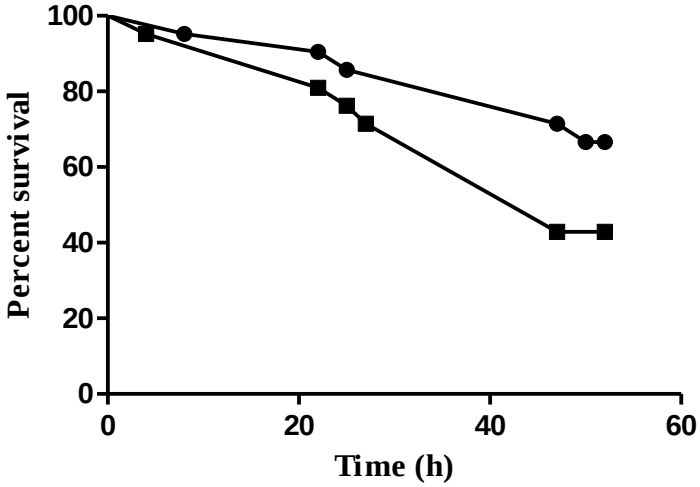


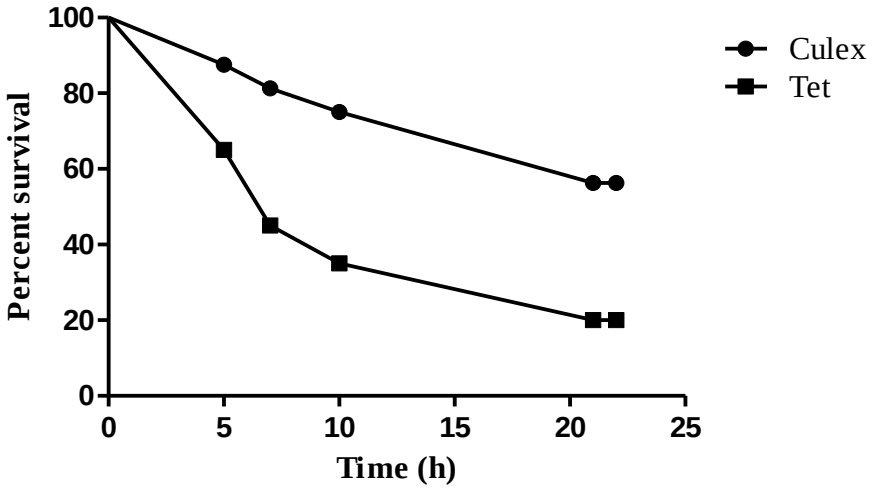
Figure 12: Survival of young *Culex* and Tet mosquitoes in response to bacterial injection with Ecc15.

Culex and Tet mosquitoes were injected with gram-negative bacterium Erwinia carotovora carotovora 15 of OD= 0.05. The mosquitoes used were young (4 to 7 days old). The survival curves of *Culex* and Tet were not significantly different with p = 0.13 in **figure 12A** and p = 0.12 in **figure 12B.**

c. Comparison of the Survival of Old *Culex* and Tet Mosquitoes in Response to Bacterial Injections

This time we injected old *Culex* and Tet mosquitoes, more than 21-days old, with *E. faecalis* and *Ecc15*. As a result, there was no significant difference in the survival of old *Culex* and Tet in response to gram-positive *E. faecalis*. However, injection with gram-negative bacteria *Ecc15* gave a significant difference in the survival rates with a higher survival rate in *Culex* mosquitoes compared to Tet.

A



B

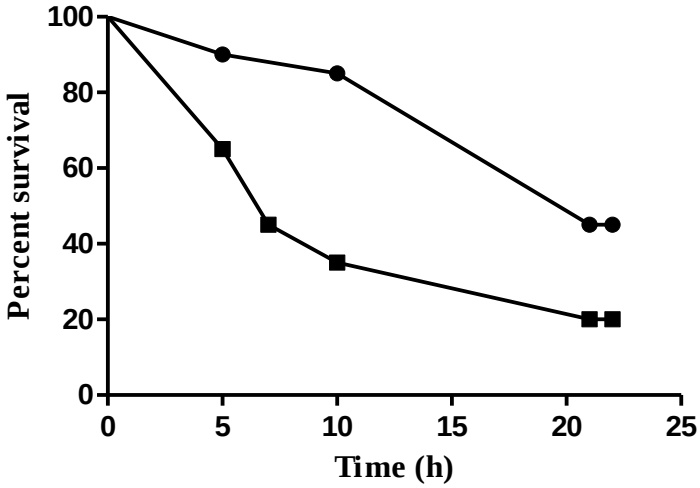
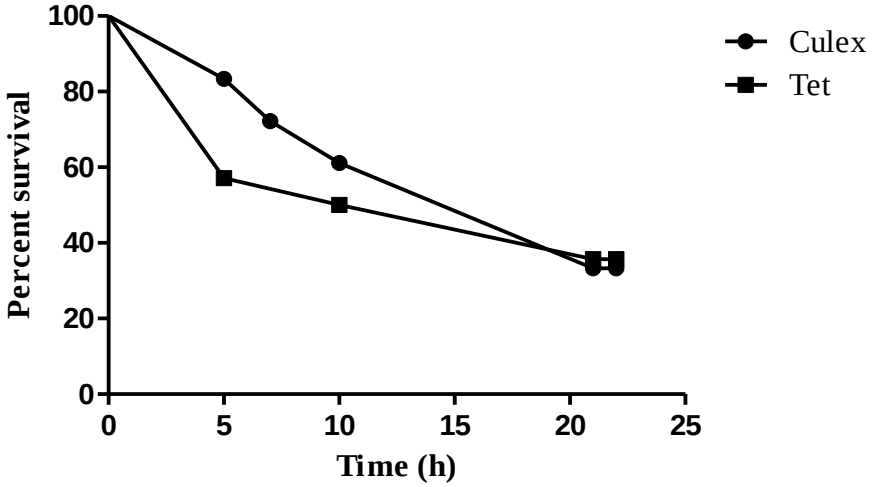


Figure 13: Survival of old *Culex* and Tet mosquitoes in response to bacterial injection with Ecc15.

Culex and Tet mosquitoes were injected with gram-negative bacterium *Ecc15* of OD= 0.05. The mosquitoes used were old (> 21 days old). The survival curves of *Culex* and Tet were significantly different with p = 0.0086 in **figure 13A** and p = 0.0144 in **figure 13B.**

A



B

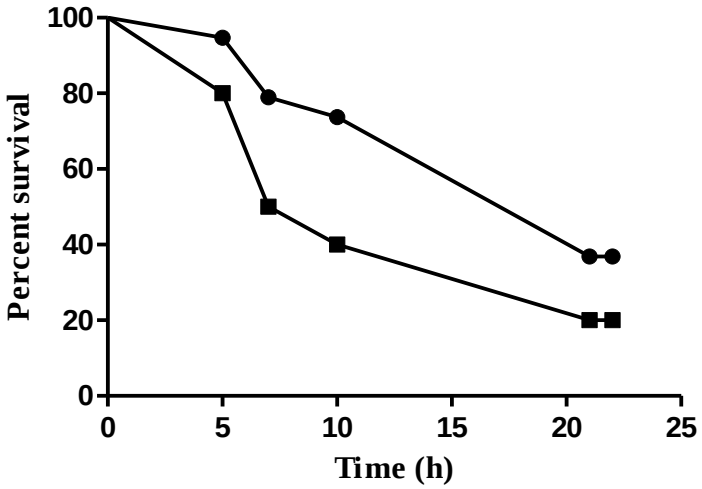


Figure 14: Survival of old *Culex* and Tet mosquitoes in response to bacterial injection with *E.faecalis*.

Culex and Tet mosquitoes were injected with *E. faecalis* of OD= 0.05. The mosquitoes used were old (24 days old). The survival curves of *Culex* and Tet were not significantly different with p = 0.79 in **figure 14A** and p = 0.16 in **figure 14B.**

CHAPTER 4

MATERIALS AND METHODS

A. Ethics Statement

All animal procedures used in this study were carried according to protocols approved by the Institutional Aniamal Care and Use Committee (IACUC) at the American University of Beirut and all methods were carried out in accordance with relevant IACUC guidelines and regulations.

B. Materials

1. Culex Pipiens Mosquito Strains

Culex pipiens mosquitoes were maintained in the insectary at 28ºC and 75% humidity using a 1212 lightdark photocycle. Adults were continuously supplied with cotton pads soaked with 10% sucrose solution and had access to water cups containing clean tap water where they laid egg rafts. Then, the egg rafts were collected from the cages and placed in trays to hatch. Larvae were fed on yeast for the first 24 hours then on fish pellet food till pupation. Pupae were collected with a plastic pipette and placed in water cups inside plastic cages.

2. Bacterial Strains

Escherichia coli, Erwinia carotovora carotovora 15 and *Enterococcus feacalis* were cultured in Luria-Bertani (LB) broth or plated on LB plates

Bacteria were grown overnight at 37ºC in Luria Broth medium containing the appropriate antibiotic. The following day, the bacterial cultures will be pelleted by centrifugation. Then, the pellets were resuspended in LB so that we can measure the O.D.using te spectrophotometer. The O.D. was then adjusted to to the appropriate value depending on the bacteria.

3. Microinjection

Thirty-two nanoliters of the previously mentioned bacterial suspension of optical density (OD) = 0.05 were injected into the thorax of insects using a Nanoject II apparatus. After the injection was done, mosquito survival rates were followed on a daily basis over a span of 10 days. The Kaplan-Meier survival test in GraphPad Prism5 was used to calculate the percent survival.

4. DNA Extraction

Two methods were used for the extracting the DNA from insects: the Livak method and the Sodium-Tris-EDTA (STE) method.

- Livak protocol

Insects were homogenized in 100µL of pre-heated Livak grinding buffer. Then they were incubated at 65ºC for 30 minutes. This was followed by the addition of potassium acetate to obtain a 1 M solution and gentle mixing for few minutes. Then the samples were incubated on ice for 30 minutes before centrifugation at 20000 g for 20 minutes to collect the supernatant. 200µL of ice-cold 100% ethanol was added to the supernatant to be followed by centrifugation at 20000g for 15 min at 4º. Finally, the pellet was washed in 100µL OF 70% ethanol and suspended in 50µL of water.

- STE protocol

Insect samples were homogenized in 100µL STE buffer. This was followed by an incubation at 95ºC for 10 min and centrifugation at 20000g for 5 min. Then, the resulting supernatant was removed and 2 ul were used as a template for PCR.

5. Polymerase Chain Reaction (PCR)

PCR reactions are used to amplify the extracted DNA sample. Specific mixtures of 10x Buffer, MgCl2, dNTPs, forward and reverse primers, Taq polymerase were prepared and added to the DNA template. Then, the samples were placed in a thermal cycler to undergo cycles of denaturation, annealing and elongation.

The primers used were:
Wolbachia surface protein 81 Forward 5' –TGGTCCAATAAGTGATGAAGAAAC-3'
Wolbachia surface protein 691 Reverse 5' –AAAAATTAAACGCTACTCCA-3'
Coi primers sequences:
COI-Forward 5′-GGAGGATTTGGAAATTGATTAGTT-3′
COI-Reverse 5′-CCCGGTAAAATTAAAATATAAACTTC-3′

6. RNA extraction, Reverse Transcription and Real-time PCR

The first step is grinding the mosquitoes in TRIzol. After that, the RNA was extracted using chloroform and precipitated with isopropanol according to the manufacturer's instructions (Invitrogen). The nanodrop spectrophotometer (Thermo) was used to quantify the extracted RNA and 500 ng were retrotranscribed into cDNA (iScript Biorad). Then the cDNA was diluted to a ratio of (1/20). Finally, Real-time PCR was performed on the diluted cDNA using SYBR green (Qiagen) by the BIO RAD thermocycler (CFX 96 Real-time System, C100).

7. Phylogenetic Methods

The program used to draw the phylogenetic trees was mega software [109]. Our data was the alignment and the parameters used were branch length, tree topology and substitution rate. Mega software gave us the tree with the highest log likelihood value. Multiple Sequence Comparison by Log-Expectation (MUSCLE) was used for sequence alignment. Then we used maximum-likelihood method for tree estimation in mega software. Next to the branches, we can see the bootstrap value, which is the percentage of trees in which the associated taxa are clustered together. 200 bootstrap samples were used to draw the bootstrap consensus tree by using majority rule. The trees were drawn to scale, with branch lengths measured in the number of substitutions per site.

8. ***Survivals***

After injecting the mosquitoes with the bacteria, dead insects were counted at different time intervals knowing that each infection was done in triplicates with 15 females per experiment. Then, the rates of survivals were plotted as function of time using GraphPad prism. Gehan-Breslow-Wilcoxon test was performed for the statistical analysis of the survival data.

CHAPTER 5

GENERAL DISCUSSION

Mosquitoes are one of the deadliest insects to humans knowing that they can cause over one million deaths every year. They are responsible for the transmission of several diseases such as yellow fever, dengue, Zika virus and Malaria. Despite the fact that progress is currently being made in combatting some of these diseases, case burdens are still high and the problem is worsening globally. *Culex*, *Aedes* and *Anopheles* are important mosquito species responsible for the transmission of dieseases. In Lebanon, several mosquito species have been identified such as the Asian tiger mosquito *Aedes Albopictus* and the house mosquito *Culex pipiens*. *Culex* mosquitoes are known worldwide to transmit several human pathogens such as yellow fever virus and filarial nematodes. In addition, *A. albopictus* is a known vector of Chikungunya, Dengue and Zika virus. In the past decades, insecticide-based control strategies have been used to target mosquito vectors. However, the prolonged use of the same insecticide has led to the development of insecticide resistance.

In the past 15 years, researchers have been working on alternative vector control strategies that do not take into consideration the use of insecticides or the creation of new vaccine drugs. This has led to the emergence of *Wolbachia* as a promising alternative. *Wolbachia* is an alpha-proteobacterium that was first identified in the ovaries of *Culex* mosquitoes in 1924. It is known as the most-common known endosymbiotic microbe in the biosphere (REF). It is thought to infect 20% up to 70% of the estimated 2 million-5 million

insect species on earth, an unparalleled success stemming in large part from selfish invasive strategies.

Cytoplasmic incompatibility (CI), whereby the symbiont makes itself essential to embryo viability, is the most common *Wolbachia*-induced manipulation of the host reproductive system. Therefore, *Wolbachia* emerged as a promising weapon against vector-borne diseases.

Wolbachia has been in the spotlight with the discovery of its ability to suppress the replication of vector-borne human arboviruses. The fact that *Wolbachia* is a naturally existing microbe made its use more acceptable than the genetic modification-based approach for the public. A stable transinfection of *Wolbachia* wMelPop strain into *A. gambiae*, inhibits plasmodium development in this mosquito. In addition, it inhibits the development of filarial nematodes in *A. aegypti* and halves the lifespan of this mosquito skewing the population toward younger individuals that are no longer infective.

Due to the lack of data throughout literature concerning the percentage of insects infected with *Wolbachia*, our first aim was to target this issue. Actually, there is a debate concerning the percentage of insects infected with *Wolbachia*. It is claimed to infect 20% up to 70% of insect species. Therefore, we started aim1 with screening wild caught insects for *Wolbachia* presence. Our aim was to test if different insects sampled from random geographic areas in Lebanon harbor *Wolbachia* through PCR analysis following DNA extraction. Then, identify *Wolbachia* strain and the host insect by using the molecular markers wsp and COI respectively to be able to perform phylogenetic analysis across Lebanese insect species.

100 insect samples were collected from random geographic areas in Lebanon to be processed and analyzed for *Wolbachia* presence. 24 samples were found to harbor *Wolbachia* through PCR analysis following the DNA extraction of the collected samples. Actually, 12 species from the 60 species sampled were considered *Wolbachia* positive due to the presence of replicates. To be able to identify the *Wolbachia* strain, we amplified the DNA of *Wolbachia*-positive insects by PCR using wsp primer. Then, we used phenol-chloroform to purify the DNA before sending them for sequencing. After sequencing was done we used NCBI blast to identify the *Wolbachia* strains. After the identification of 6 *Wolbachia* strains, our second goal was to identify the host insect. Our host insects were identified phenotypically only by their photos. However, to be able to perform a phylogenetic analysis between host insect and *Wolbachia* strain, we need to identify the host insect molecularly. To do this, a PCR was performed using cytochrome oxidase 1 (COI) primer. Then, the amplified DNA was purified using phenol-chloroform extraction method and was sent for sequencing. After sequencing was done, we used NCBI blast to find the best match host insect. With the help of the photo of the insects, we identified the genus of the 5 hosts. After the molecular identification of host insect and *Wolbachia* strain, we wanted to perform a phylogenetic analysis to compare phylogeny of the host to the phylogeny of bacteria and study coevolution between them. To do this, phylogenetic trees showing the relationship between host insects and their corresponding *Wolbachia* strains are needed. The program used to draw the phylogenetic trees was mega software. However, there was a discordance between the *Wolbachia* and host tree, which suggests that on evolutionary time scales horizontal transmission of *Wolbachia* between species has occurred many times. Our conclusion supports previous studies done in literature that

showed a strong disequilibrium between mitochondrial and *Wolbachia* genomes in a number of species[110] [111]. Direct and indirect interactions allow horizontal transmission to take place within and between species. In intraspecies horizontal transmission happens through direct contact or through the environment such as fruit flies and spiders [112]. Similarly, the interspecies horizontal transfer in intertidal amphipod crustaceans and butterflies sharing the same habitat occurs through the environment [113]. Studies showed that horizontal transfer also occurred plant-feeding pumpkin arthropods that feed on a particular leaf substrate (from arthropod to who?, not the plant!)[114]. The exchange of salivary secretions known as trophallaxys that occurs in colonies of *Cubitermes* termites also facilitates intraspecies transfer of *Wolbachia* between individuals of different castes [115]. In the previous examples, *Wolbachia* transmission relies on somatic tissues and is independent of the germline. The mechanisms of horizontal transmission are still unexplored. It seems that *Wolbachia* can move through host somatic tissues such as the gut and extracellular environment such as the hemolymph, which is a key component in horizontal transmission. However, this area remains unexplored and future studies are needed.

This brings us to the second aim of this project, which is to analyze the effect of *Wolbachia* on *Culex* mosquito-host immunity and life span. In order to study the effect of *Wolbachia* on *Culex* mosquitoes, we generated a *Culex* strain that is devoid of *Wolbachia* by using tetracycline treatment for two generations. In this way, we have two genetically identical *Culex* strains: one strain that harbors *Wolbachia* referred to as "*Culex*" and another *Wolbachia*-free "Tet" strain. The DNA was extracted from adult mosquitoes to test

the presence of *Wolbachia* using PCR. This method proved to be successful in the clearance of *Wolbachia* from these mosquiotes. We also tested for cytoplasmic incompatibility to prove that our Tet strain is *Wolbachia*-free. To do this, male *Culex* mosquitoes were crossed with female Tet mosquitoes. As a result, 100% CI was observed. Then, we injected both *Culex* and Tet mosquitoes with a selection of microbes by using microinjection method. Enterococcus faecalis (E. faecalis) was used as a Gram-positive bacteria, Erwinia carotovora carotovora 15 (Ecc 15) was used as a gram-negative bacteria. As a result, there was no significant difference in the survival of young *Culex* and Tet in response to each bacterial injection separately. Then, we repeated the experiment using mosquitoes that are older than 21 days. As a result, there was no significant difference in the survival of old *Culex* and Tet in response to gram-positive E. faecalis. However, injection with gram-negative bacteria Ecc 15 gave a significant difference in the survival rates with a higher survival rate in *Culex* mosquitoes compared to Tet. It was shown previously in literature that the protective *Wolbachia* host combinations include high densities of *Wolbachia*. For example, in drosophila *Wolbachia* density seems to be positively correlated with the strength of antiviral protection [116]. Similarly in mosquitoes, *Wolbachia* density has also been implicated in antiviral effects [117]. Previous studies showed that there is a link between high *Wolbachia* density in the host and antiviral effects. Therefore, one hypothesis for the observed results is that old *Culex* mosquitoes have a higher *Wolbachia* titer, which is responsible for the higher survival of old *Culex* mosquitoes in response to injection with Ecc15. To test this hypothesis, we should assess and compare *Wolbachia* densities in young and old mosquitoes by using real time PCR or microscopic examination. If old mosquitoes show a higher *Wobachia* density, this will

explain the higher survival rate in old *Culex* mosquitoes upon injection with Ecc15. Previous studies have focused on the ability of *Wolbachia* to activate the immune system of the host enabling it to combat microbes. The wMelPop strain of *Wolbachia* was shown to up-regulate the mosquito's innate immune system and inhibit the development of filarial nematodes in the mosquito [118]. It was also shown to stimulate immune gene expression and inhibits plasmodium development in *A. gambiae* mosquito [119]. Therefore, it would be interesting to compare the level of AMP production in both uninfected *Culex* and Tet mosquitoes and upon bacterial injection knowing that AMP production is one of the most important immune responses in mosquitoes. This will allow us to test the ability of *Wolbachia* to boost the immune system of *Culex* mosquito host.

APPENDIX

Species	**Number of replicates**
Sympetrum meridionale	3 all were *Wolbachia*-positive
Melanostoma mellinum	1 *Wolbachia*-positive
Parisolabis Earwig	2 both were *Wolbachia*- positive
Lasioglossum malachurum	3 all were Wolbachia positive
Syritta pipiens	1 *Wolbachia*-positive

Table 5: This table shows the Wolbachia-positive species with their corresponding number of replicates.

All the other species were Wolbachia-negative knowing that when two or more replicates of the same species were present, they were all *Wolbachia*-negative.

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