AMERICAN UNIVERSITY OF BEIRUT

CHARACTERIZATION OF MIDGUT REGENERATIONCAPACITY AND IMMUNE RESPONSES IN LOCAL AEDES ALBOPICTUS MOSQUITOES

by
MARIA SOUHEIL JANEH

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AN ABSTRACT OF THE DISSERTATION OF

Maria Souheil Janeh   for   Doctor of Philosophy
                                                   Major: Cell and MolecularBiology

Title: Characterization of midgut regeneration capacity and immune responses in local *Aedes albopictus* mosquitoes

Mosquitoes transmit a variety of diseases with important impact on human health. Several mosquito species occur naturally in Lebanon. The presence of these vectors coupled with the environmental changes increase the risk of spread of infectious diseases in the region. Current control strategies comprise several disadvantages, such as insecticide resistance, high cost and the risk of releasing female mosquitoes by mistake. A better characterization of the vectors' immunity and physiology should provide insights for the development of novel control strategies. The gut is an important component of an effective immune response against pathogens. Maintaining gut integrity is therefore very important for successful clearance of invaders. Hence, in the context of this project, we wanted to investigate how mosquito gut integrity is maintained. We established a model for inducing gut damage in *Aedes albopictus* mosquitoes and studied the effect of damage on gut structure and on the proliferation of mitotic cells.

Aedes albopictus is one of the mosquito species constituting a major vector of diseases. Other important examples of mosquito vectors include *Culex pipiens* and *Anopheles gambiae* mosquitoes. Therefore, in the second aim we extended the analysis of gut regeneration to these disease vectors in order to establish a comparative analysis of midgut regeneration capacity and resistance to oral infection

Another aim of the project was to explore the immune responses of the Lebanese *Aedes albopictus* strain after different microbial challenges. We investigated the ability of mosquitoes to tolerate or clear infections with different pathogens. Some previous studies had shown that insects harboring the endosymbiont *Wolbachia* have a better resistance to several pathogens, while other studies have claimed that in its native host, immune activation by *Wolbachia* might get attenuated and the protective effects of the endosymbiont become abolished. Hence, we aimed to establish a model for a comparative analysis by generating *Wolbachia*-free strains in *Aedes*

*albopictus*and *Culex pipiens* mosquitoes. This will allow for the study of the effect *Wolbachia* has on mosquito wellbeing, be it on physiological aspects such as fecundity and egg hatch, or on immunity responses such as tolerance, resistance and melanization.

Unraveling these immune responses and characterizing them adds additional knowledge that, on the long term, would contribute to the development of alternative control strategies.

CONTENTS

FIGURES

ABBREVIATIONS

Aedes albopictus	*A. albopictus*
Culex pipiens	*C. pipiens*
Rift Valley fever virus	RVFV
St. Louis encephalitis virus	SLEV
West Nile virus	WNV
Sindbis virus	SINV
Drosophila melanogaster	*D. melanogaster*
Intestinal stem cells	ISCs
Enteroblasts	EBs
Enterocytes	ECs
Enteroendocrine cells	EEs
Notch Intracellular Domain	NICD
Suppressor of Hairless	Su(H)
Adult midgut progenitors	Aps
Wingless	*wg*
T-cell factor	Tcf
Frizzled2	Fz2
LRP/Arrow	Arr
Dishevelled	Dsh
Janus Kinase/Signal Transducer and Activator of Transcription	JAK/STAT
Unpaired	*Upd*
Epidermal Growth Factor Receptor	EGFR
Spitz	*Spi*
Gurken	*grk*

Keren	*krn*
Vein	*Vn*
Jun-N-terminal Kinase	JNK
Erwinia carotovora carotovora 15	*Ecc 15*
Peritrophic matrix	PM
Reactive Oxygen Species	ROS
Nitric Oxide	NO
Antimicrobial peptides	AMP
Peptidoglycan recognition protein	PGRP
Diaminopimelic acid	DAP
Nitric oxide synthase	NOS
Lipopolysaccharide	LPS
Pathogen-associated molecular patterns	PAMP
Pattern recognition receptors	PRR
Domeless	Dome
Hopscotch	Hop
Phenylalanine hydroxylase	PAH
Phenoloxidase	PO

CHAPTER 1

INTRODUCTION

1. Mosquitoes are vectors of diseases

Mosquitoes are a diverse group of insects, with around 3500 species spread over the world. They belong to the order Diptera, and the family Culicidae. The life cycle of mosquitoes consists of four distinct and separate stages: egg, larva, pupa and adult. The first three stages are mostly aquatic. Eggs can be laid either one at a time, or as egg rafts with up to 200 eggs per raft. Within 48 hours, most eggs hatch into larvae. Larvae go through 4 stages, during which they shed their skins and grow larger: first, second, third and fourth instar. During all stages, larvae live in water and breathe at the surface. After the fourth instar larval stage, larvae change into pupae. Pupae are mobile, and they respond to light changes and movement. At this stage, they do not feed and they are at rest. Pupae go through metamorphosis, and adult mosquitoes emerge from them. Adults rest at the water surface until they are ready to fly. A few days later, adult mosquitoes are ready to mate and blood feed. Male mosquitoes feed only on sugar sources, whereas most female mosquitoes require blood meals in order to develop eggs. It is during the blood meal that female mosquitoes acquire pathogens, that they may transmit to human or animal host upon subsequent blood meals. This makes several species of mosquitoes, such as *Culex*, *Aedes* and *Anopheles*, important vectors of diseases. Vector-borne diseases account for 17% of all infectious diseases, with more than 700000 deaths annually [1].

Many of these diseases may be prevented if protective measures are applied. This calls for the need of developing effective control methods.

2. Mosquito species in Lebanon

In Lebanon, several mosquito species occur naturally, such as *Aedes albopictus (A. Albopictus)* and *Culex pipiens (C. pipiens)*. *A. albopictus*, the Asian tiger mosquito, is a known vector of several viruses, including dengue, chikungunya, West Nile, Zika. Female *A. albopictus* mosquitoes blood feed on humans as well as other mammals such as cats and dogs, and they lay their eggs four to five days after the blood meal just above the surface of the water. Eggs hatch into larvae when covered by rain ensuing the entire aquatic life cycle that occurs within seven to nine days. Adult mosquitoes have a life span of about three weeks [2].

A. albopictus was identified in Lebanon in 2003 [3], and its population size has increased immensely since then. In a study done by *Haddad et al* in 2012 [4], specimens of Lebanese strains of *A. albopictus* were collected from different regions of Lebanon, and vector competence studies were carried out. This study showed that the Lebanese strains of *A. albopictus* were able to transmit chikungunya, dengue and West-Nile viruses, thus demonstrating an important capacity for being vectors of arboviruses. The house mosquito *C. pipiens* is an important vector of several human pathogens, such as the Rift Valley fever virus (RVFV), St. Louis encephalitis virus (SLEV), West Nile virus (WNV), and Sindbis virus (SINV) [5]. In Lebanon, *C. pipiens* mosquitoes are the most widely distributed species, in urban and rural regions. A study carried out in 2018 [6] in which

Culex egg rafts were collected from east of Lebanon showed that the Lebanese strain of *C. pipiens* are efficient vectors of the West Nile Virus, and to some extent Rift Valley fever virus. So far, no cases of mosquito-borne disease outbreak have been reported in Lebanon. However, the presence of endogenous mosquito vectors, coupled with the climatic warming, as well as the increased mobility due to frequent travel, may lead to the spread of mosquito-borne diseases in the area. Hence, a better characterization of mosquito immune responses is important and could provide useful insights for the development of new control strategies.

3. Mosquito Innate Immunity

As vectors of pathogens, mosquitoes are also threatened by the invasion of microorganisms. Normally, mosquitoes are protected from the outside environment by their cuticle. A lesion of that outer layer would lead to the entry of pathogens into the body cavity. Another possible route of entry for pathogens is after ingestion of a bloodmeal [7]. In fact, after the consumption of blood, pathogens enter into the lumen of the midgut, where they face physical and chemical barriers [8-10]. In both cases, mosquitoes respond to the challenge with pathogens by mounting potent innate immune responses in order to clear the infection. These responses can be divided into cellular and humoral responses [11].

1. *Physical Barriers*

The first line of defence against invaders is comprised of the physical barriers, such as the cuticle, and the epithelial barriers (epidermis, intestine, trachea). The cuticle is a chitinous, hydrophobic material forming the exoskeleton of the insect, and lining the foregut, the hindgut, and the tracheal system. This cuticle forms a protective layer hindering direct contact with pathogens in the outside environment [12]. Another possible route of entry of pathogens is through the oral route. The gut therefore is placed at the front line of the battle. At this level, the gut plays a role both a physical barrier inhibiting the dissemination of pathogens, and through activation of local immune responses in an attempt to clear the invading pathogens [13, 14]. As a result of this, maintaining gut integrity is an important factor in an effective immune response against pathogens.

1.1. Gut structure and function

At the larval stage, mosquitoes' alimentary canal is nearly completely autolyzed during pupation, so that the adult digestive system is mostly built anew. While some studies have explored the guts of mosquito larvae [15], the adult mosquito gut remains largely unexplored. *Drosophila melanogaster (D. melanogaster)* has been used as a model to decipher the structure and physiology of the gut, as well as its response after damage. *Drosophila* gut is divided into three distinct domains: the foregut, the midgut and the hindgut [16]. The latter have different developmental origins [17], with the foregut and hindgut having an ectodermal origin, and the midgut an endodermal origin. The foregut is

comprised of the pharynx, the esophagus and the crop which is used for food storage [18]. The cardia, a junction between the foregut and the midgut allows for the regulation of food passage. The midgut is the main site of digestion and nutrient absorption in the gut, and it is surrounded by a peritrophic matrix which forms a barrier between ingested food in the lumen of the midgut and the epithelium. The hindgut, with its associated microtubules allow for excretion and osmoregulation [16]. The midgut itself can be divided into the anterior, middle and posterior midgut, based on the presence of acid-secreting cells in the adult middle midgut [19]. Further morphometric, genetic and histological analysis of the adult midgut suggest the presence of six major regions which can themselves be divided into fourteen subregions with distinct gene expression patterns [20].

The midgut is composed of an epithelium surrounded by visceral muscle, nerves and a tracheal system. This epithelium is renewed every 1-2 weeks. In addition to that, food digestion, ingestion of cytotoxic compounds, enteric infections and molecules produced during the immune response all induce stress and damage to the gut [21]. Pluripotent intestinal stem-cells (ISCs) allow for the renewal of the midgut, both in normal homeostasis and after damage [22, 23]. ISCs divide to produce progenitor enteroblasts (EBs) [24]. EBs then undergo differentiation to either produce enterocytes (ECs), the absorptive cells or enteroendocrine cells (EEs), the secretory cells.
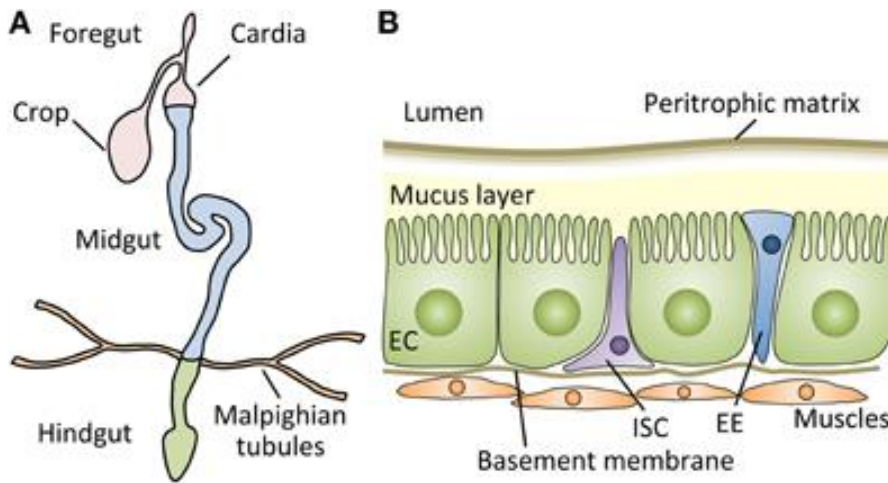
Figure 1: The structure of *Drosophila* gut.
a-A representation of the digestive tract of *Drosophila* flies reveal a tubular structure of the gut divided into foregut, midgut and hindgut with its associated malpighian tubules. **b-**A cross section of the adult *Drosophila* midgut shows that this structure is composed of several types of cells: absorptive enterocytes (ECs), secretory enteroendocrine cells (EEs), and pluripotent intestinal stem cells (ISCs). The midgut is surrounded by a peritrophic matrix on the luminal side, protecting the midgut epithelium from mechanical and bacterial damage. Adapted from [25].

1.1.1. Signaling pathways regulating ISC proliferation and differentiation

The Notch signaling pathway is activated upon interaction of a Notch transmembrane receptor with a ligand, thus initiating proteolytic cleavage of the receptor and release of the Notch Intracellular Domain (NICD) of the receptor. NICD will then translocate into the nucleus where it interacts with Suppressor of Hairless (Su(H)) in flies, and activates the transcription of Notch-regulated genes [26]. In *Drosophila*, Notch signaling determines the fate of differentiation of EBs during different stages of midgut development. At the embryonic stage, high levels of Notch signaling drive the differentiation of EBs into ECs, whereas low levels of Notch signaling lead to the

differentiation of EBs into EEs, or adult midgut progenitors (APs) [27]. In adult

Drosophila midgut epithelium, Notch signaling is important for the development of ECs

from ISCs [22, 28].
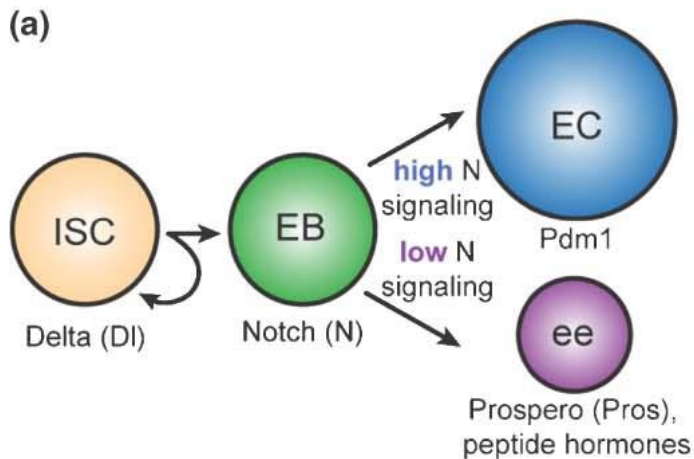
Figure 2: Notch signaling decides the fate of ISCs.
a-Intestinal stem cells (ISCs) divide symmetrically to self-renew, and asymmetrically to produce a progenitor enteroblast (EB) cell. The progenitor cell undergoes differentiation to either produce and enterocyte (EC) or an enteroendocrine cell (EE), depending on the level of Notch (N) signaling in the EB. Modified from [29].

The Wnt signaling pathway is involved in the regulation of cell proliferation, cell

polarity, and cell fate specification [30]. In the absence of the Wnt ligand, *Wingless (wg),*

the transcriptional effector β-catenin/Armadillo (Arm) is kept at low levels through its

constitutive degradation by a protein destruction complex composed of Axin, APC,

GSK3/Zw3, and CK1. The Wnt-regulated genes are therefore kept off by the DNA-

binding transcription factor T-cell factor (Tcf) with the aid of other transcriptional

corepressors. Upon binding of *wg* to its coreceptors Frizzled2 (Fz2) and LRP/Arrow

(Arr), a cascade of cytoplasmic events are initiated leading to the inactivation of the

protein destruction complex through Dishevelled (Dsh), and the subsequent stabilization

and translocation of β-catenin/Arm into the nucleus, where it binds Tcf, and activates the

transcription of the target genes [31]. In the context of the *Drosophila* gut, it has been

shown that the *wg* is expressed at major compartmental boundaries in the adult midgut

[20, 32]. *Wg* also directs pattern formation during *Drosophila* gut formation [33]. Wg

signaling is involved in regulating ISC self-renewal, maintenance and proliferation in the

Drosophila adult gut during homeostasis [34, 35], as well as midgut and hindgut

regeneration after injury [35].
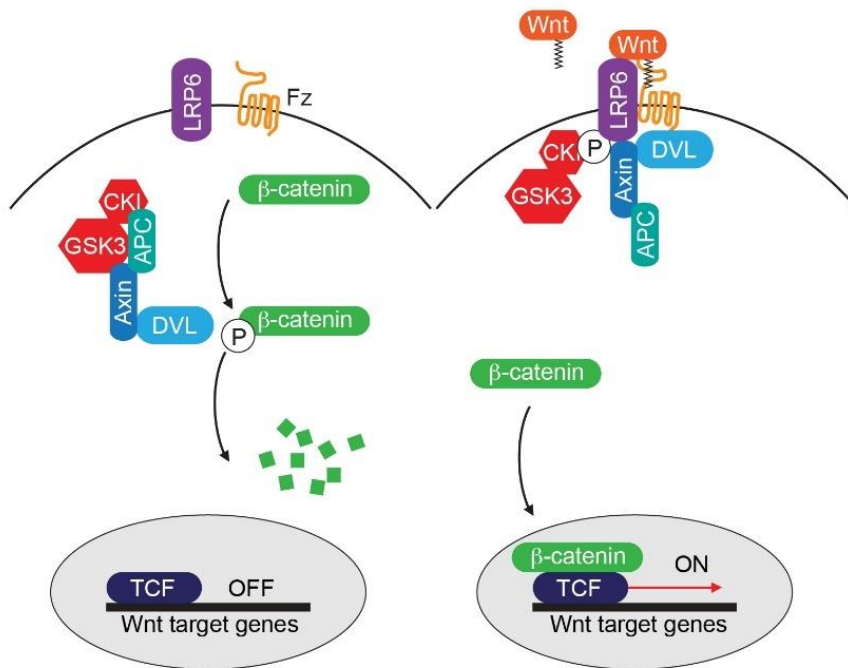
Figure 3: Canonical Wnt signaling pathway.
Binding of the Wnt ligand to its receptors Frizzled and LRP6 inhibits the degradation of
β-catenin, which translocates into the nucleus where it activates the transcription of Wnt
target genes. Adapted from [36]

The Janus Kinase/Signal Transducer and Activator of Transcription (JAK/STAT) pathway is a conserved signaling pathway involved in immunity, development and in several diseases [37]. In *Drosophila*, binding of the *unpaired* ligands (*upd*, *upd2* and *upd3*) to the Dome receptor (encoded by *domeless*) [38] leads to the activation of JAK tyrosine kinase Hop (encoded by *hopscotch*) [39]. Hop will then phosphorylate itself and the cytoplasmic tail of Dome, thus creating docking sites for the transcription factor STAT92E [40]. STAT92E becomes phosphorylated [41], dimerises and translocates into the nucleus where it induces the expression of target genes [42]. SOCS36E is a negative regulator of the JAK/STAT pathway, while also being a direct target gene of the pathway [43, 44]. JAK/STAT has been shown to be involved in the response of ISCs in *Drosophila* during homeostasis and after injury [45-47], as the expression of the cytokines *Upd, Upd2* and *Upd3* is induced after injury [48]. These cytokines activate the JAK/STAT pathway in the midgut progenitors thus promoting the proliferation of ISCs and the differentiation of EBs [49]. This response allows the replacement of damaged enterocytes with newly differentiated enterocytes, and the reestablishment of the midgut. Aside from its role after injury, JAK/STAT is also needed for proper differentiation of the ISC progeny in the adult midgut [45]. Different studies have shown that mutations in*domeless*, *hopscotch*, *Stat92E* in ISCs lead to a reduction in number of mature midgut cells, which become replaced by small midgut progenitors [45, 46, 50].
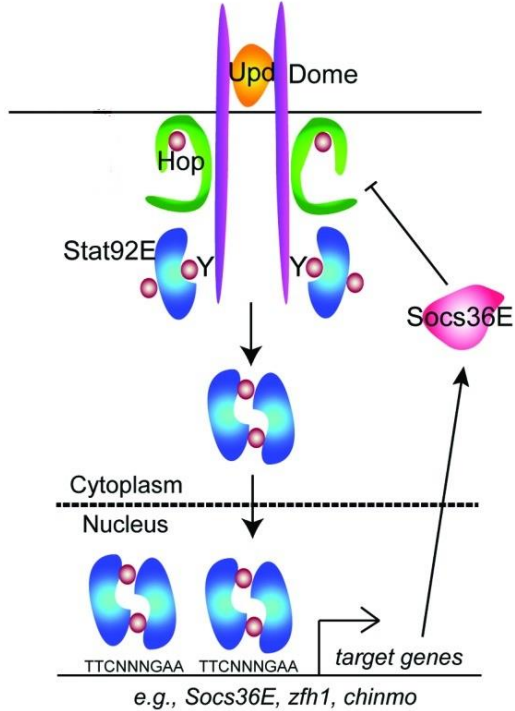
Figure 4: The JAK-STAT pathway in Drosophila.
Upon binding of the Unpaired (Upd) ligands (orange) to the receptor Domeless
(Dome)(magenta), Hopscotch (Hop) is activated (green) leading to the phosphorylation
of Dome (circles). STAT92E (blue) dimerizes and becomes active, this translocating into
the nucleus and activating the expression of target genes. SOCS36E (pink) is a negative
regulator of the JAK-STAT pathway. Modified from [51].

The Epidermal Growth Factor Receptor (EGFR) signaling pathway is active during

development for cell differentiation, proliferation, migration and survival [52]. Activation

of this pathway is initiated through four ligands: *spitz (spi), gurken (grk) and keren (krn)*

belonging to the TGF-α family, and *vein (vn)*, which is a neureglin [53]. EGFR is another

important regulator of ISCs in *Drosophila*. In fact, upon midgut damage, several EGF-

like growth factors are induced, leading to the activation of the EGFR pathway and

subsequent signaling of ISCs divisions [54-57]. The EGFR ligand *vn* is expressed in the

visceral muscle at low levels in normal conditions but will be expressed at much higher

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levels after enteric infections or injury [54-57]. *Spi* and *krn* are expressed in the midgut

epithelial cells and their expression is also induced after injury or infection [54-57]. The

activation of EGFR by these ligands promotes the division of ISCs. In fact, EGFR is

required for ISC proliferation both in normal conditions and after injury [54-57], but it is

not involved in the differentiation of ISCs.
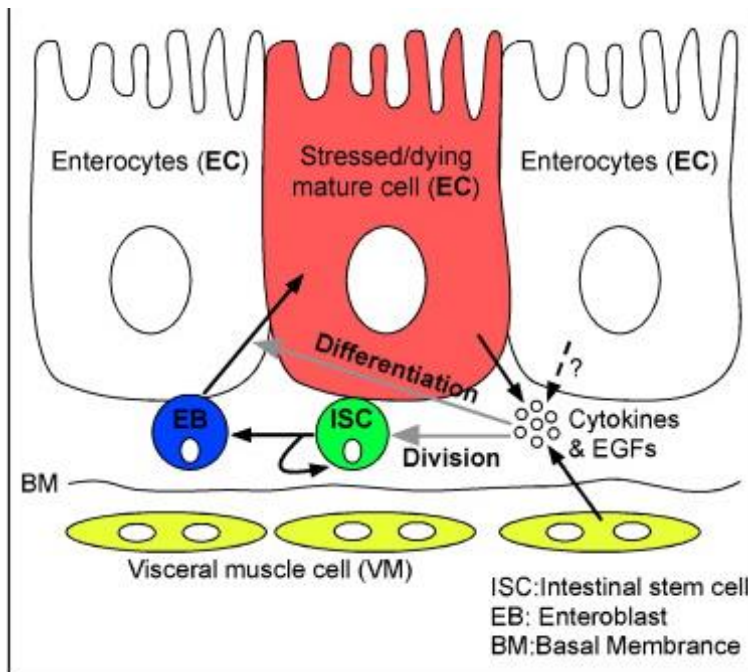
Figure 5: EGFR signaling is involved in the homeostasis and regeneration of
***Drosophila* midgut.**
Different cell types within the *Drosophila* midgut secrete EGFR ligands in response to
stress, thus activation the EGFR pathway and promoting ISC division. Adapted from
[55].

The Jun-N-terminal Kinase (JNK) signaling pathway is evolutionary conserved and

activated by a range of stress signals [58]. Upon activation of a member of the JNK

Kinase Kinase family, a dual-specificity Kinase of the MKK family is phosphorylated

and activated, leading to the phosphorylation of JNK on Serine/Threonine and Tyrosine

residues [59]. Several nuclear and cytoplasmic targets are activated, including AP-1

family members Jun and Fos and the Forkhead Box O transcription factor FoxO [59, 60].

This causes a variety of responses which are context-dependent. In the midgut, JNK is

activated by oxidative damage and pathogenic infection [45, 48, 61]. This leads to the

expression of the Upd cytokines and the EGFR ligands, and consequent ISC proliferation

and regeneration. Studies have claimed that the JNK pathway is required for ISC

mobilization after damage through oxidative stress [61]. The JNK feedback inhibitor,

puckered, is essential for restraining unchecked ISC proliferation in normal conditions,

which could signify that tight control of this pathway is required for normal gut

homeostasis [24]. Following infection, JNK activation depends on the type of pathogen

used and the damage the latter induces. For example, upon infection with pathogens that

directly damage mature midgut cells, such as *Pseudomonas entomophila*, JNK signaling

is activated exclusively at the level of the mature midgut cells [45]. Infection with

bacteria that cause damage through oxidative burst, such as *Erwinia carotovora*

carotovora 15 (Ecc 15), induces activation of JNK in mature ECs and in progenitor cells

[48]. This activation of JNK in mature cells might be facilitating their elimination after

damage and their replacement through ISC proliferation [45, 62]. All in all, studies have

shown that JNK activation likely has three functions in ISCs: first, it functions by

activating stress response genes in ISCs, thus protecting them from oxidative damage

[48]; second, it leads to increased ISC proliferation [56]; and third, it leads to mis-

differentiation of ISCs through induction of high expression of DI in the progenitor cells [61].
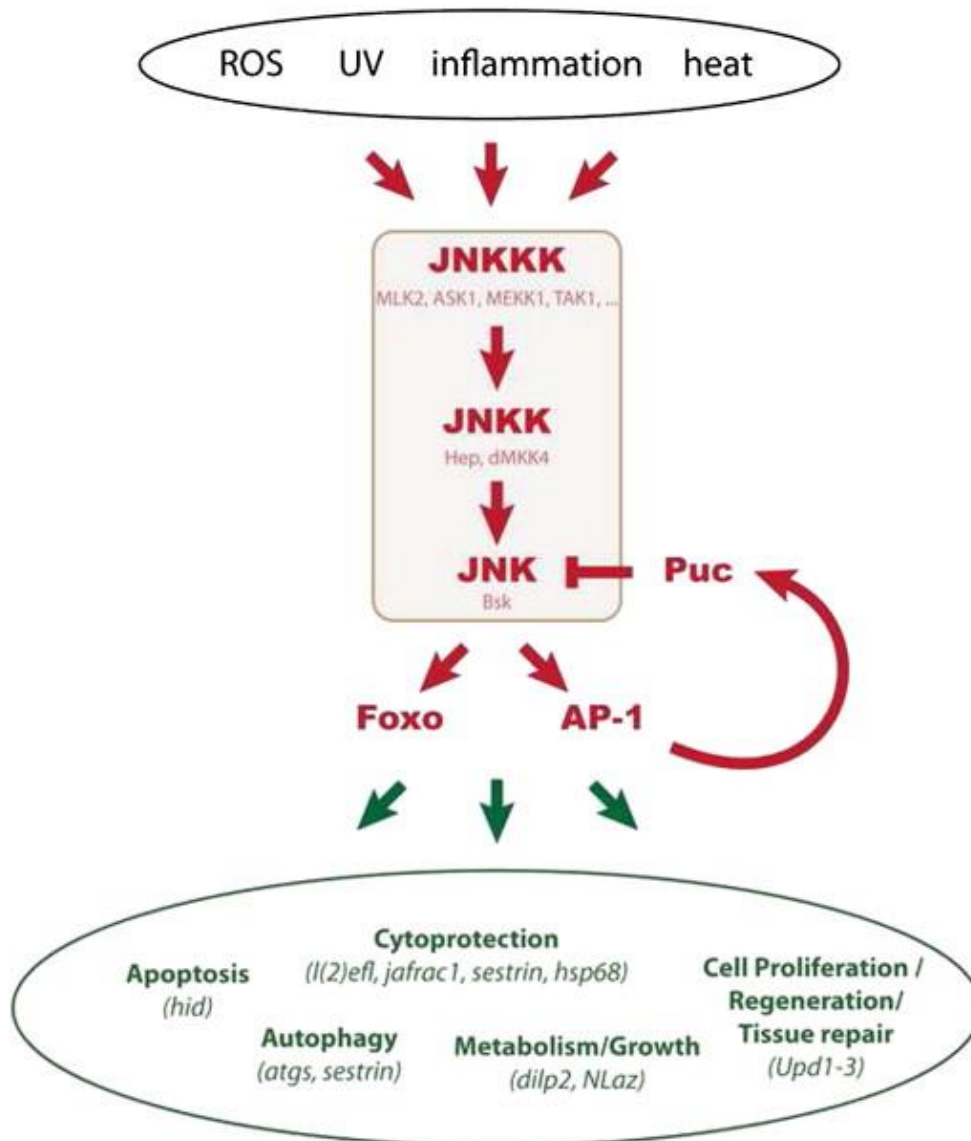
Figure 6: JNK signaling pathway in *Drosophila*.
JNK pathway is activated by a variety of stress signals. This leads to the activation of the transcription factors Foxo and AP-1, thus causing different context-dependent cellular responses. Adapted from [58].

The Hippo/Salvador/Warts pathway is an evolutionary conserved pathway which regulates organ size in *Drosophila*, senses cell adhesion and regulates cell proliferation and survival [63]. This pathway is initiated by a number of stimuli, which cause the activation of Hippo which forms a complex with Salvador, and subsequently phosphorylates Warts. Warts negatively regulates Yorkie, and the unphosphorylated Yorkie translocates into the nucleus where it interacts with its Scalloped transcription factors, thus upregulating the transcription of target genes [64]. In *Drosophila*, Hippo signaling has been shown to be involved in the homeostasis and regeneration of the gut [57, 65-67]. In fact, loss of Hippo signaling in ISCs promotes their proliferation, and loss of this signaling in ECs induces dramatic over-proliferation of ISCs.
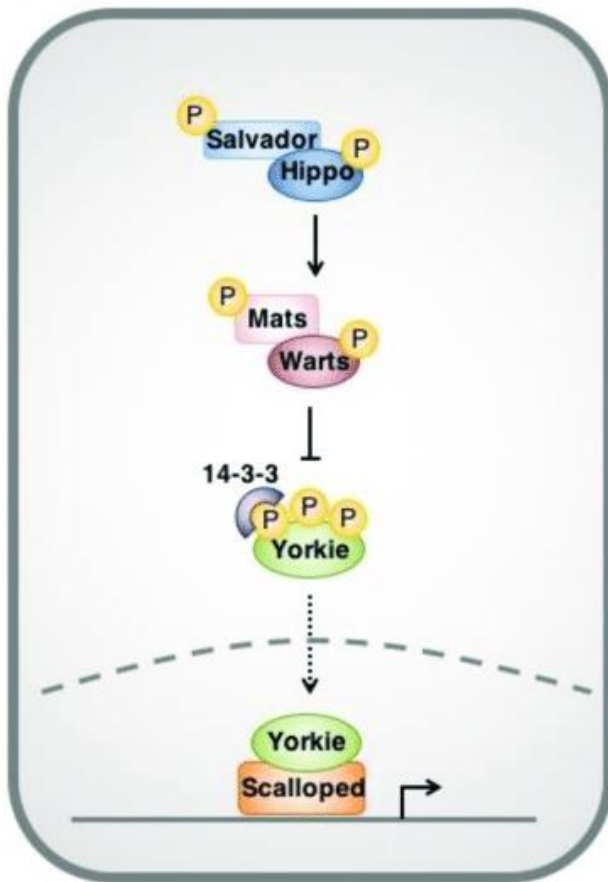
Figure 7: Hippo signaling in *Drosophila*.
A variety of upstream stimuli activate the Hippo pathway, leading to activation of Hippo, subsequent phosphorylation of Warts, and negative regulation of Yorkie. Unphosphorylated Yorkie translocates into the nucleus, where it interacts with Scalloped to upregulate the expression of target genes. Modified from [64].

1.1.2. Insect Gut in Immunity

Insects' guts play a role at the level of immunity through the function of the peritrophic matrix (PM), and the production of Reactive Oxygen Species (ROS), nitric oxide (NO) and Antimicrobial peptides (AMPs) [68] leading to the clearance of pathogens.

1.1.2.1.Peritrophic matrix

The lumen side of insects' guts is lined by a peritrophic matrix (PM), which is a membrane composed of chitin and glycoproteins, with small pores of a maximum size of 10 nm [69]. The PM forms a barrier between the midgut lumen and the epithelium. There are two types of PM: type I PM and type II PM. Type I PM is formed by delamination from the surface of the midgut epithelium and is secreted by the entire midgut. This PM is formed after feeding but may be secreted continuously in some cases. This is in contrast with type II PM, which is secreted constitutively, forming a sleeve-like structure. In type II formation, the PM is secreted by specialized cells of the cardia, present at the anterior midgut [70]. The peritrophic membrane plays several functions in insects. It is a physical and biochemical barrier. It offers mechanical protection and aids in digestion. It also contributes to the immunity of the insects [69].As a physical barrier, the PM protects the midgut from ingested abrasive food particles, digestive enzymes, and some pathogens [10]. As a biochemical barrier, the PM hinders the passage of toxins, and in some cases, it inactivates toxins[69]. In fact, in a study done by *Devenport et al*, it was shown that the *Aedes aegypti* peritrophic matrix protein AeIMUCI is involved in the excretion of toxic reactive oxygen species produced after release of the iron-containing heme during the process of hemoglobin degradation in adult mosquitoes [71].

In the context of immunity, the PM has been shown to provide protective functions against different infections. A study done by *Dessens et al* focused on the rodent malaria parasite *Plasmodium berghei*. Upon knockout of the chitinase *pbCHT1*, the parasite infectivity of *Anopheles stephensi* mosquitoes was reduced by 90 %, indicating an

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important function of the PM in anti-*Plasmodium* defense [72].In another study done on

the malaria parasite *Plasmodium falciparum*, the *PfCHT1* gene (encoding a chitinase)

was disrupted. This caused an impaired ability of the parasites to form oocysts in

Anopheles freeborni midguts [73]. In an *Enterobacteriaceae* infection in *Anopheles*

coluzzii, the PM limits the growth of the bacteria and prevents it from inducing a

systemic immune response [74].

1.1.2.2. AMP production

In the gut, the production of AMPs is under the control of the Imd pathway [75]. Two

members of the pattern recognition receptors of the peptidoglycan recognition protein

(PGRP) family activate this pathway in the gut: PGRP-LC, acting mainly in the anterior

midgut and hindgut; and PGRP-LE, acting in the midgut [76, 77]. The Imd pathway can

be activated through binding of diaminopimelic acid (DAP)-type peptidoglycan (found in

the cell wall of all Gram-negative bacteria and certain Gram-positive bacteria), to these

receptors in different gut compartments [78].

1.1.2.3. ROS and NO production

Complementary to AMP production, the production of ROS and NO was shown to

eliminate invading pathogen in the gut epithelium and trigger downstream immune

responses [79]. In fact, ingestion of *Ecc15* by *Drosophila melanogaster* flies causes an

increase in the levels of ROS produced in the gut [80]. Studies using RNAi suggest that the NADPH oxidase Duox is the main enzyme producing ROS in *Drosophila* [80, 81]. ROS production has also been observed in *Aedes aegypti* midguts as a response to control bacterial growth [82]. The Duox enzyme is thought to act through the direct bactericidal effect of ROS, causing damage to DNA, RNA, proteins and promoting the degradation of lipids in cell membranes [83]. Additional functions of Duox have been suggested. For example, in *Anopheles gambiae*, Duox activity is thought to modulate sclerotization of the PM, thus reducing gut permeability and immune response induction [84]. Another suggested function for ROS is as signaling molecules to induce repair responses [85]. In *Drosophila*, ROS signaling induces intestinal Nitric oxide synthase (NOS) transcription [86]. NOS enzymes generate NO. In *Bombyx mori*, the expression of NOS is induced by Lipopolysaccharide (LPS) stimulation [87]. In *Drosophila*, NO activates the Imd pathway thus inducing AMP expression after bacterial challenge [88]. The gut therefore activates a number of immune responses in an attempt to clear the invading pathogens and protect the insect from infection.
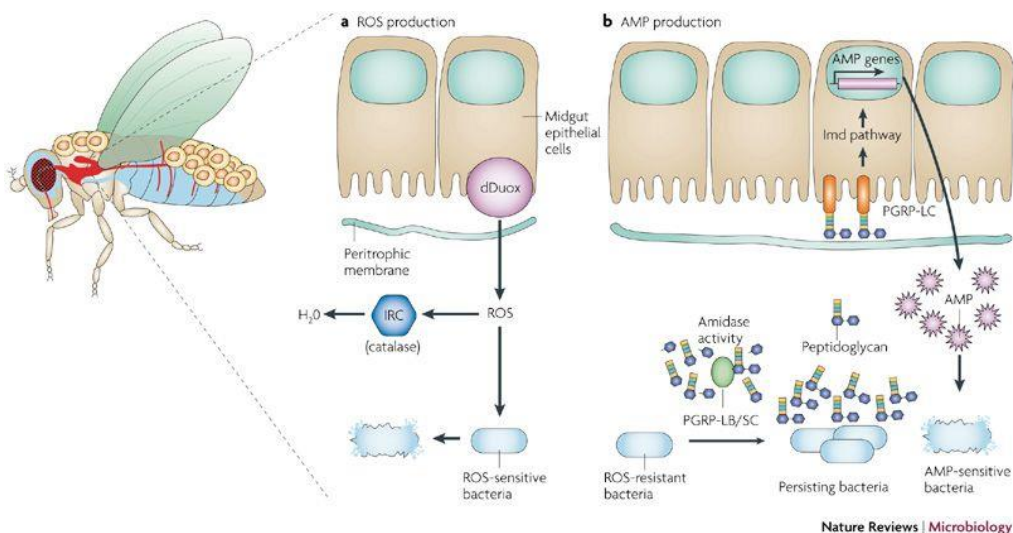
Figure 8: Immune responses at the level of *Drosophila* gut.
Upon bacterial challenge, two immune responses are induced at the level of *Drosophila* gut: **a-**ROS production and **b-**AMP production. Adapted from [89].

2. *Immune Reactions*

2.1. Non-self-recognition

Mosquitoes lack adaptive immunity and depend on innate reactions to fight pathogens [90, 91]. Recognition of pathogen-associated molecular patterns (PAMPs) by host-derived pattern recognition receptors (PRRs) results in signal modulation and transduction that culminates in the activation of several immune effector mechanisms.

2.1.1. Pathogen-associated molecular patterns (PAMPs)

PAMPs exist only in microorganisms, and not in the host. Examples of PAMPs include lipopolysaccharide (LPS), peptidoglycan (PGN), and lipophosphoric acid [78, 92]. PGN is the main component of the bacterial cell wall, classified into either DAP-type PGN or Lys-type PGN depending on the difference in amino-acid residues and cross linking methods [93]. Most gram-negative PGNs are DAP-type, while most gram-positive PGNs are Lys-type [94].

2.1.2. Pattern recognition receptors (PRRs)

One important example of a PRR is the Peptidoglycan recognition protein (PGRP), which can recognize peptidoglycan (PGN), one of the main components of the cell wall of pathogens [95]. PGRPs can be divided into long and short types. In *D. melanogaster*, 13 PGRP genes encoding 19 proteins have been identified [96]. In *A. aegypti*, 7 PGRP genes have been reported [97]. PGRPs are highly conserved during evolution, and they contain a conserved domain consisting of around 160 amino acid residues. PGRPs can be divided into catalytic and non-catalytic PGRPs depending on whether they have amidase activity [98].Catalytic PGRPs are mostly extracellular, usually acting as a bactericide or serving as a regulatory immune pathway preventing the excessive activation of the immune pathway. Non-catalytic PGRPs are intracellular, transmembrane or extracellular. These PGRPs have many functions, such as the activation of hydrolase, signal transduction, or enhancement of activation of the immune pathway. All PGRPs have a conservative L-shaped PGN binding groove that contains 30 to 50 residues of N-terminal fragments.

2.2.Immune signaling pathways

Upon pathogen invasion, three immune pathways are activated: The Toll, Imd and Jak/Stat pathways. The Toll pathway is active against gram-positive bacteria and fungi [94, 99], while the Imd pathway is mainly active against gram-negative bacteria [100, 101]. Some overlap exists between these two pathways in response to gram-positive and gram-negative bacteria. For example, in *Drosophila*, PGRP-SD can recognize gram-

negative bacteria and activate Toll pathway [102]. In some cases, gram-positive bacteria such as *Bacillus* with DAP type peptidoglycan can activate the Imd pathway rather than Toll pathway [101, 103].

2.2.1. Toll pathway

The Toll pathway is active both in immunity and development [104-106]. In immunity, the Toll pathway is activated after infection with Gram-positive bacteria, fungi, viruses, and some plasmodia. This pathway is initiated when a PAMP binds to a PGRP.PGRP-SA, PGRP-SD and GNBP1 act as PRRs upstream of the Toll pathway. In fact, studies in Drosophila have shown that upon mutation of PGRP-SA, Lys-type bacteria could not activate the Toll pathway, while DAP-type bacteria were successful in activating the Imd pathway [107].

Upon binding of the PAMP to the PGRP, Spätzle, an extracellular cytokine, is activated. The latter binds the cellular receptor Toll. This causes the initiation of several intracellular events involving Pelle, Myd88, Tube, leading to translocation of NF- κB transcription factors (Dif/Dorsal, Rel1) to the nucleus, and the activation of the transcription of antimicrobial peptides (AMPs) and other immune effector genes.

2.2.2. Imd pathway

The Imd pathway is mainly active against Gram-negative bacteria, viruses and some plasmodia. Imd is activated after a PAMP binds PGRP-LC or PGRP-LE [108, 109]. This

initiates a signaling cascade involving Imd, Dredd and Fadd [110, 111], thus leading to

the translocation of the NF- κB transcription factors (Relish, Rel2) to the nucleus and the

activation of the transcription of AMPs and other effector molecules. The Jak/Stat

pathway is active against bacteria, viruses and plasmodia. It is involved both in immunity

and development. Binding of the extracellular cytokine Unpaired (Upd) to the cellular

receptor Domeless (Dome) triggers the activation of this pathway. This is followed by the

phosphorylation of Dome by Hopscotch (Hop), and the recruitment of Stat. Stat

dimerizes and is translocated to the nucleus, thus activating the transcription of

antimicrobial genes, such as nitric oxide synthase.
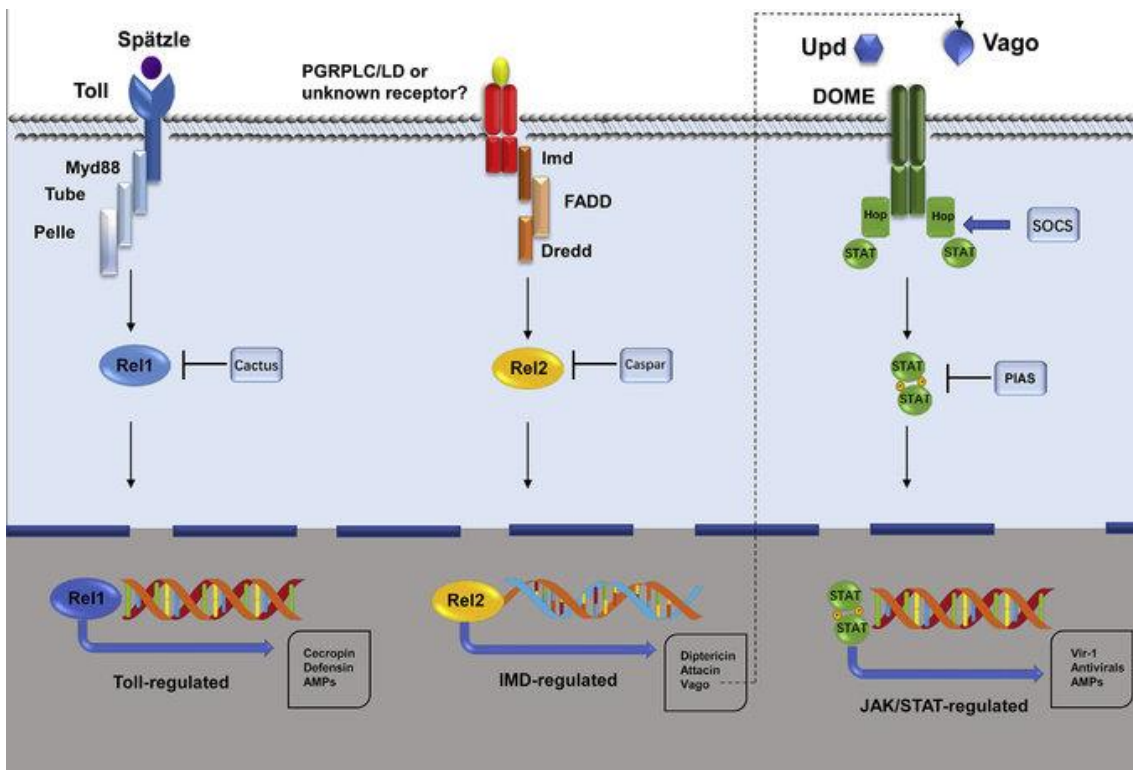
Figure 9: Schematic representation of the main mosquito innate signaling pathways.
Three immune pathways are mainly activated in the mosquito after pathogen invasion:
The Toll pathway, the IMD pathway and the JAK-STAT pathway. Modified from [112].

2.3.Cellular Reactions

The cellular responses are mediated by immune cells called hemocytes [91]. Hemocytes can be divided into three populations: granulocytes, oenocytoids and prohemocytes [113]. Granulocytes are the most abundant type of hemocytes, comprising about 80-95% of circulating hemocytes. These cells are mostly phagocytic [114]. Oenocytoids on the other hand comprise less than 10% of the circulating hemocyte population, and these cells produce phenoloxidase, an enzyme required for the melanization response [115]. Prohemocytes also constitute less than 10% of the circulating hemocyte population. These cells have been found to participate in the phagocytic response [116]. Based on the anatomical location, hemocytes can be divided into sessile (25%) and circulating hemocytes (75%). Responses mediated by hemocytes include phagocytosis, nodulation, melanization and encapsulation [117].

2.3.1. Phagocytosis

Phagocytosis is a rapid response which starts within seconds of pathogen invasion. In mosquitoes, phagocytosis is carried out by sessile and circulating hemocytes [114, 118]. In this case, the invader is recognized by PRRs acting as opsonins. This is followed by internalization of the invader into membrane-delimited phagosome, fusion with a lysosome, and digestion by hydrolytic enzymes [117]. The phagocytic response was shown to be effective against bacteria, yeast, *Plasmodium*[119-121].

2.3.2. Encapsulation

When the invaders are too large to be phagocytosed, they are encapsulated. Encapsulation involves the attachments of granulocytes forming layers around the pathogen. The layer of granulocytes is then surrounded by layers of plasmatocytes, and the binding of additional granulocytes. Encapsulation does not induce the production of antimicrobial peptides [122], but it may be followed by melanization [123].

2.3.3. Nodulation

Nodulation is described as the entrapment of invading microorganisms by aggregates of hemocytes which surround them. This is in most cases followed by melanization [124]. This response involves the adherence of granulocytes to each other and around microorganism aggregates. Granulocytes then release their contents, encasing microorganism in a flocculent material. This is followed by an aggregation of plasmatocytes around the nodule, causing in most cases a melanization of the structure. The molecular mechanisms behind this response remain largely unknown. In the tobacco hornworm *Manduca sexta*, an insect lectin named scolexin was found to be involved in the formation of nodules. Scolexin is produced by epidermal and midgut cells upon wounding or bacterial infection [125].
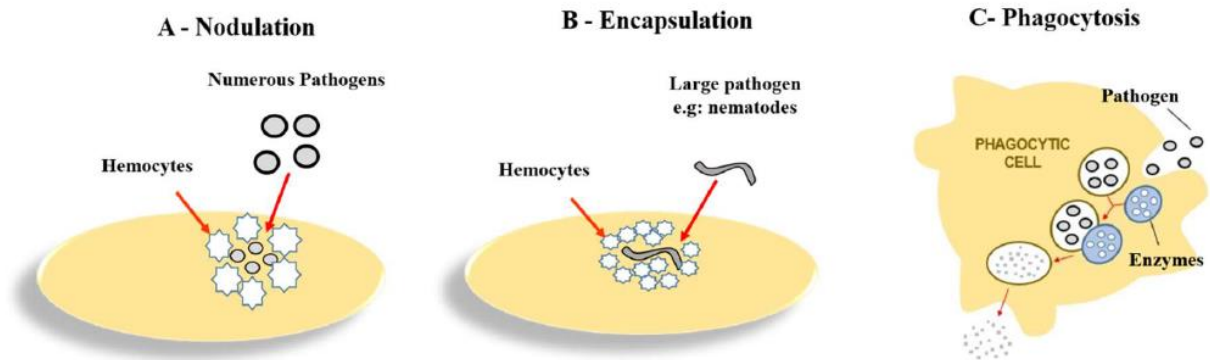
Figure 10: Schematic representation of three cellular immune responses.
This figure illustrates three hemocyte-mediated immune responses: **a-**Nodulation, **b-**encapsulation and **c-**phagocytosis. Adapted from [126].

2.3.4. Melanization

Melanization is a process leading to the death of invaders through the deposition of a

layer of melanin around the invader and its isolation. This leads to the death either

through oxidative damage or starvation [127-129]. This response is an enzymatic process

described as a series of reactions leading to the conversion of tyrosin into melanin

precursors, and the cross-linking of proteins to form melanin. Upon recognition of

PAMPs by PRRs, a serine protease cascade is activated, leading to the cleavage of the

zymogen pro-phenoloxidase to its active form, phenoloxidase [130, 131]. Phenoloxidase

hydroxylates tyrosine to form dopa. Dopa is oxidized by phenoloxidase to form

dopaquinone, which is then converted to dopachrome [127, 128, 132]. The latter is

converted to 5,6-dihydroxyindole by the dopachrome conversion enzyme. 5,6-

dihydroxyindole is oxidized by phenoloxidase into indole-5,6-quinone which is cross-

linked with hemolymph proteins to form melanotic capsules. Melanin is also produced by a complementary pathway, where dopa decarboxylase hydroxylates dopa to form dopamine, which is converted to melanin by phenoloxidase and other enzymes. Tyrosine is the rate-limiting substrate in the melanization pathway. When additional tyrosine is needed, phenylalanine is hydroxylated by phenylalanine hydroxylase. The enzymatic process leading to the production of melanin also produces reactive oxygen species. For this reason, the enzymatic reactions are tightly regulated. Serine protease inhibitors (Serpins), and other factors inhibit the activation of the phenoloxidase cascade [130, 133].

Melanization has been shown to have several different effects at the level of immunity depending on the context. In fact, the active PO may produce highly reactive compounds shown to be cytototoxic to microorganisms and parasites [127]. It has also been implied that PO promotes cellular defense reactions, such as phagocytosis, and increases the efficiency of plasma coagulation. When it comes to pathogen survival, several studies have linked PO activity with decreased pathogen survival, whereas in other cases melanisation was shown not to have an essential function in pathogen clearance. In a study done in *Drosophila*, it was shown that in flies with defective Imd and Toll pathways, melanisation contributed significantly to the clearance of invading bacteria [134]. Melanisation was also shown to increase resistance to fungal infections in *Drosophila*, even in the absence of any mutation of the Toll and Imd pathways [134].
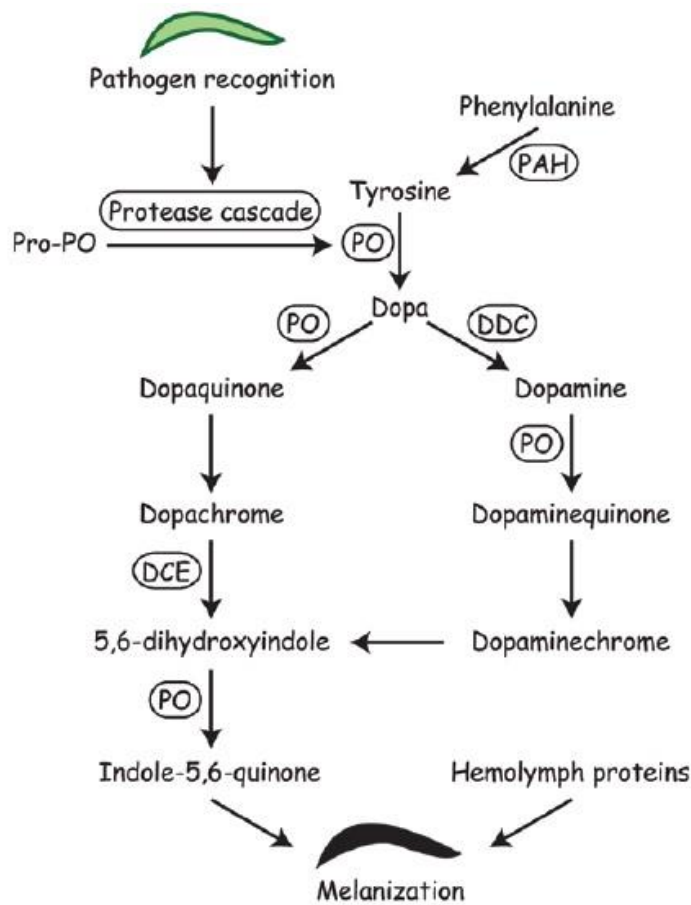
Figure 11: Biochemical pathway leading to the melanization of pathogens.
A representation of the proposed signaling pathway leading to the melanization of
invading pathogens. PAH: phenylalanine hydroxylase; PO: phenoloxidase; DDC: dopa
decarboxylase; DCE: dopachrome conversion enzyme. Adapted from [91].

2.4. Humoral Reactions

The humoral arm of the innate immune system encompasses the production of

Antimicrobial peptides (AMPs) by the fat body, the production of Reactive Oxygen

Species (ROS), and the activation of the prophenoloxidase (proPO).

2.4.1. Production of Antimicrobial Peptides

Antimicrobial peptides are mainly synthesized by the fat body [135], however the gut

[136], the hemocytes [137], the cuticular epithelial cells [138], the reproductive tract

[139] and the salivary gland [140] are also capable of producing AMPs. Over 150 insect

AMPs have been identified since the purification of the first AMP from the pupae of

Hyalophora cecropia in 1980 [141, 142]. Most AMPs are active against bacteria and/or

fungi, and some AMPs have been found to act against some parasites and viruses [143].

Antimicrobial peptides are mostly small and cationic/basic, and they can adopt some

structures, or contain unique sequences allowing their classification into four groups: the

α-helical peptides (such as cecropin and moricin), cysteine-rich peptides (such as insect

defensin and drosomycin), proline-rich peptides (sush as apidaecin, drosocin and

lebocin), and glycine-rich proteins (such as attacin and gloverin) [144, 145]. Most AMPs

are produced as inactive precursor proteins, and they undergo limited proteolysis to

generate the active peptides. Mature peptides act at membranes and kill the target cell by

lysis [146, 147].

2.4.1.1.Insect Defensins

Defensins have been identified in nearly all living organisms. They are small cationic

peptides (34-51 residues) with a size of about 4 kDa, containing six conserved cysteine

residues that form three intramolecular disulfide bridges [143]. Defensins are active against both gram-positive and gram-negative bacteria, however they are highly effective against gram-positive bacteria[148],while being less effective against gram-negative bacteria . Defensins act by formation of membrane channels in the cells, leading to their lysis [149].

2.4.1.2.Cecropins

Cecropins were first isolated from the immunized hemolymph of *H.cecropia* pupae [150], and since then they have been identified in *Lepidopteran*, *Dipteran* and *Coleopteran* insects. Cecropins are cationic AMPs of 31-39 residues, devoid of cysteine with a structure of two α-helices joined by a hinge-region [151]. They have antibacterial activity against gram-positive and gram-negative bacteria, and some of them also act against fungi [152-154]. Cecropin A, for example, has been found to act against the fungus *Beauveria bassiana* in silkworm larvae [155]. Cecropin B has antifungal activity against *Candida albicans*[156]. Cecropins can act though lysis of bacterial cellular membranes, through inhibition of proline uptake, or through inducing leakiness of membranes [157, 158].

2.4.1.3.Attacins

Attacins were first detected in the hemolymph of immunized *H. cecropia* pupae [159]. They have been found also in *Heliothis virescens* [160]*, Bombyx mori* [161], *Trichoplusia*

ni [162], *Manduca sexta* [163], *D. melanogaster* [164] and *Glossina morsitans* [165] among others. Attacins are mainly active against gram-negative bacteria [159]. In fact, they act by blocking the synthesis of the major outer membrane proteins in dividing gram-negative bacteria, causing a disruption of the integrity of the cell wall leading to the growth of the bacteria in long chains [166].

2.4.1.4. Proline-rich peptides

Proline-rich peptides have a molecular size of 2-4 kDa, containing no cystein and at least 25% proline. These peptides include drosocin [167] and metchnikowin [168] from *D. melanogaster*, pyrrhocoricin from the sap-sucking bug *Pyrrhocoris apterus* [169], formaecin [170] from the ant *Myrmecia gulosa*, apidaecin [171] and abaecin [172] from honeybees. They are active against gram-negative bacteria, gram-positive bacteria and some fungi [143].

2.4.1.5. Diptericin

The antimicrobial peptide diptericin has so far been only identified in dipteran species [173, 174]. Diptericins are 9 kDa peptides, which act against gram-negative bacteria. They contain an attacin-like G domain, a C-terminal glycine-rich residue and a short N-terminal proline-rich region containing a consensus site for O-glycosylation [175].

2.4.2. Production of Reactive Oxygen Species

Reactive oxygen Species (ROS) are a group of oxygen-derived radical species, which are mainly formed during cell respiration at the level of the mitochondria [176]. Another source of ROS are the membrane-bound enzymes NADPH oxidases (NOX1, NOX2, NOX3, NOX 4, NOX5) and dual oxidases (DUOX1 and DUOX2), referred to as NOX enzymes, which produce ROS by catalysing the reduction of molecular oxygen to generate superoxide and/or hydrogen peroxide, using NADPH as an electron donor [177]. ROS have been found to play a role in the immune responses of different insects. In *Drosophila melanogaster*, ROS production serves as the first line of defense in gut immunity [80, 178]. This reaction might also be acting as a signal to trigger systemic responses and AMP production in *Drosophila*[86]. Since ROS have harmful effects, their production must be tightly regulated. Immune-regulated catalase (IRC) activity reduces ROS levels after pathogen encounter to decrease the oxidative stress [179]. In *Anopheles gambiae*, ROS are required for the development of an effective immune response against *Plasmodium* parasites and bacteria [180, 181]. In fact, it was shown that refractory *A. gambiae* strains are in a chronic state of oxidative stress, which is amplified after blood feeding, thus resulting in increased steady-state levels of ROS. This favors melanization of parasites [180]. In another study, it was shown that strains of *A. gambiae* mosquitoes with higher levels of ROS survive bacterial challenges better. This effect was eliminated upon dietary administration of antioxidants, thus indicating a requirement of ROS in the immune response against bacterial challenges in *A. gambiae* mosquitoes [181]. Upon

challenge with *Plasmodium*, ROS are produced either by the mitochondria in mosquito midgut cells [182] or by the *Enterobacter* bacterium from the gut microbiota [183]. In *Aedes aegypti*, ROS are produced in the midgut lumen of mosquitoes, and their levels decrease upon a blood meal via blood heme-activated protein kinase C, thus allowing an expansion of gut bacteria [82].

4. Mosquito Control Strategies

Some pathogens, such as viruses and parasites, manage to evade the mosquito immune system, and are transmitted by the mosquito vector during a subsequent blood meal to a human or animal host, thus causing a wide range of diseases, some of which are deadly. This led to the development of various control methods, in order to limit the spread of mosquito-borne diseases. Current control methods include chemical, genetic and biological methods.

1. *Chemical control methods*

Chemical control methods rely on the use of insecticides. Six classes of insecticides are mainly used for the control of adult mosquitoes: organochlorines, organophosphates (OP), carbamates, pyrethroids, pyrroles, and phenyl pyrazoles[184]. In the past, insecticides were used heavily and were successful in limiting mosquito-borne diseases, however the prolonged use of the same insecticides lead to the development of insecticide resistance [185]. Unfortunately, resistance is a major problem, viewed by the

32

World Health Organization (WHO) as a threat for the control of mosquito-borne diseases, and as a contributing factor to the reemergence and spread of arboviruses [186].

2. *Genetic control methods*

Genetic control methods rely on the integration of a foreign DNA fragment into the insect's genome [187]. The two major categories of genetic control methods can be divided into either suppression of mosquito population, or replacement of the mosquito population [188]. The first relies on the reduction of the number of competent vectors, whereas the second relies on the reduction of the vectorial capacity of the mosquitoes [189]. Examples of population suppression methods are sterile-male methods. In this case, males are modified to become sterile, then released into the wild where they mate with wild-type females. No offspring are produced, thus suppressing the mosquito population [189]. Examples of population replacement methods are methods relying on the spread of a novel trait into the target population, such as the reduced ability to transmit a pathogen. In most cases, this trait is deleterious to the mosquito [190, 191].

2.1. Sterile Insect Technique

This technique involves mass rearing of mosquitoes, separating male from female mosquitoes, irradiating or chemically treating male mosquitoes to sterilize them, and then releasing the treated mosquitoes into the wild. Modified males will then mate with wild females, not producing any offspring, and leading to a reduction in the population size

[192]. This technique presents many advantages, such as the release of only male and not female mosquitoes, therefore the biting rates in the targeted area do not increase. However, complete elimination of a mosquito population would require the release of large numbers of males, over a long period of time. Releases will also have to be repeated regularly to maintain the effects of SIT. Since mosquitoes need to be sorted by sex before the release, there is always a possibility of releasing females by mistake, which themselves can become disease-vectors. Another disadvantage is the fitness of the modified male mosquitoes, because these need to compete with wild males for mating [193]. SIT technique therefore presents many disadvantages which may hinder its effects on disease control.
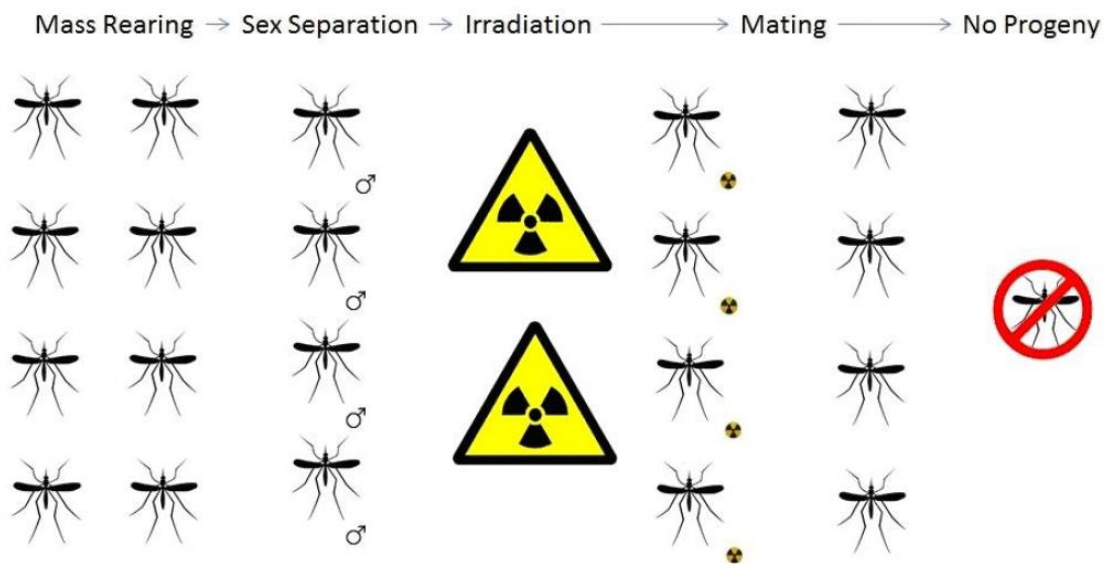
Figure 12: Schematic representation of the Sterile Insect Technique (SIT) method. The SIT method relies on the mass rearing of mosquitoes, followed by manual sex separation. The separated males are then sterilized by irradiation and then released to mate with wild-type females resulting in no progeny. Adapted from [194].

2.2.<u>Release of Insects carrying a Dominant Lethal gene</u>

A modification of the SIT technique would be the Release of Insects carrying a Dominant

Lethal gene (RIDL) technique which results in female killing. RIDL relies on genetically

modified males carrying female-acting transgenes that are released into the wild

population. These transgenes either induce mortality in pupae or adults, or they may

reduce the expression of a gene active in the flight muscle. This would result in the

production of flightless females unable to feed and mate [195]. The fitness of the males in

the RIDL technique is not as compromised as with the SIT because the transgenes are

driven by female-specific promoters, such as the vitellogenin promoter. Although RIDL

has advantages over SIT, it still presents some of the same disadvantages, such as the

ability of the transgenic males to mate with wild females, the need to release large

numbers of males, and the possibility of releasing females by mistake.
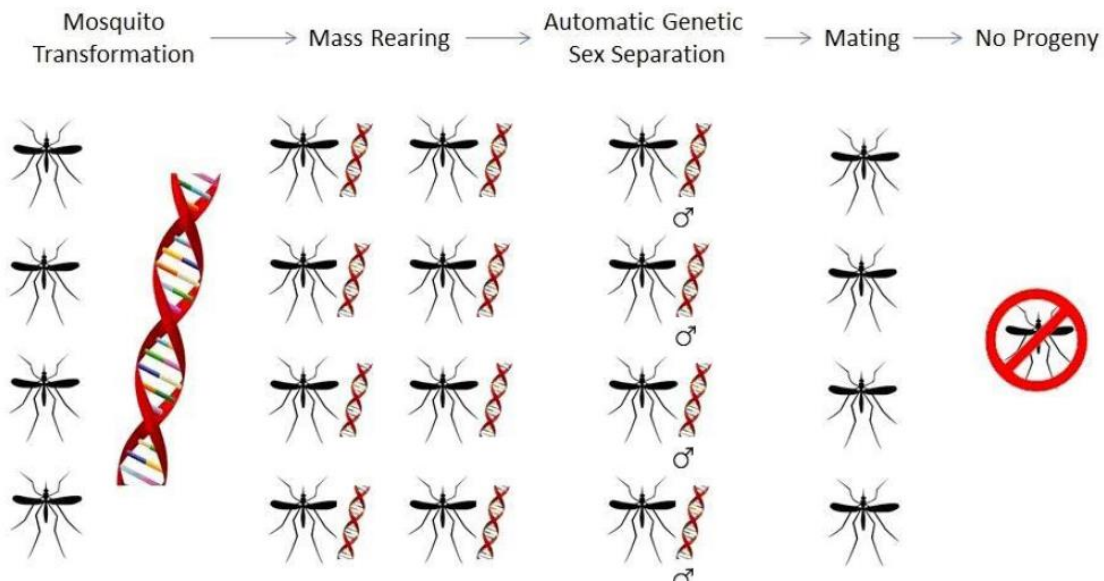
Figure 13: A schematic representation of the RIDL technique.
Genetically modified male mosquitoes carrying a female-acting transgene are released into the wild, where they mate with wild-type females. The resulting offspring do not survive. Adapted from [194]

3. *Biological control methods*

Biological methods include the use of entomopathogenic fungi, bacteria and endosymbionts.

3.1. Entomopathogenic fungi

Entomopathogenic fungi were among the first to be used for biological control of pests.They present many advantages, such as their high specificity to their target. They are harmless to beneficial insects, to the environment and to human health. Insect resistance is not an issue because these fungi have different ways of infection. They secrete toxins which could be exploited for biotechnological research. Some of them play important roles in the activation of the immune system. Their high persistence in the

environment allows for long-term suppression effects on pests [196, 197].Of these fungi, *Beauveria bassiana'* s life cycle has been described in detail [198]. In the absence of a host, *Beauveria* grows through an asexual vegetative life cycle of germination. A lot of mycopesticides have already been commercialized and used in a number of countries, including the United States and United Kindgom [199]. However, these still constitute a small percentage of the insecticide on the market. The pathogenicity of entomopathogenic fungi is different than that of bacteria or virus because they infect bacteria by breaching of the host cuticle. The latter is composed of chitin, proteins, lipids, pigments, and N-acylcatecholamines. In order to successfully infect the insect, the fungi secrete extracellular proteases, chitinases, and lipases [200]. Aside from the secretion of enzymes, the fungi are also able to secrete toxins and metabolites. *Beauveria bassiana*, for example, has been shown to produce low molecular weight cyclic peptides and Cyclosporins A and C with insecticidal properties, Oosporein with antibiotic activity against gram-positive bacteria and cyclic peptides with immunosuppressive activities. Despite the many advantages of the use of entomopathogenic fungi as biopesticides, they also present many disadvantages. These include the slow killing rate (2-3 weeks). Since the pathogenicity process is a biological process, it needs specific conditions such as temperature, humidity and periods of light. Their high specificity makes them unsuitable for commercial use which requires broad range killers. Another disadvantage is the high cost of production, and the necessity of cold storage. And finally, insect-specific application techniques need to be optimized to retain long term effects.
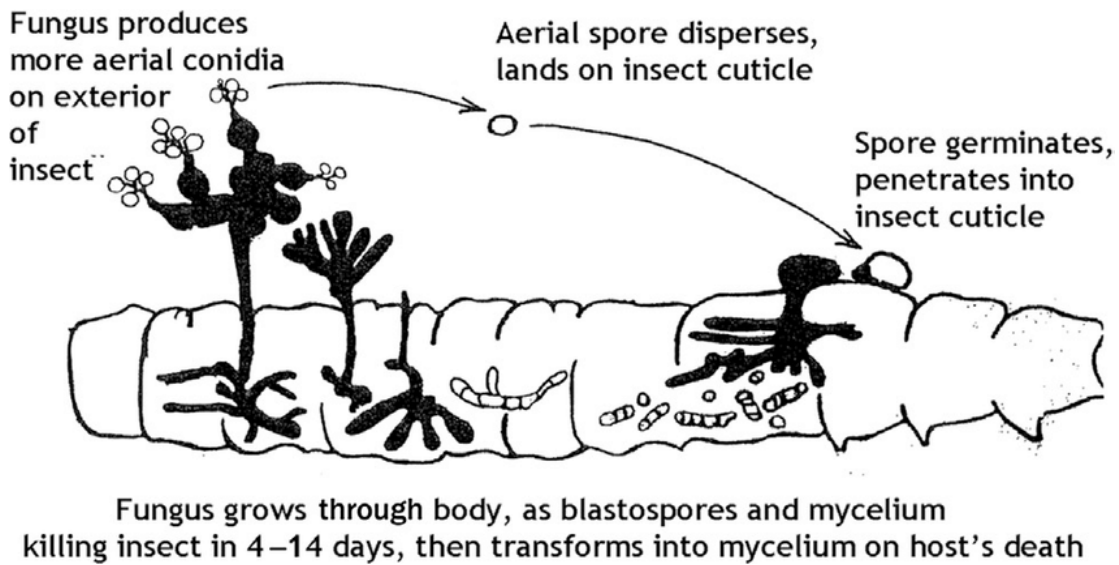
Figure 14: Schematic representation of the life cycle of *Beauveria bassiana*. Entomopathogenic fungi induce lethality in insects by germinating on the host cuticle and penetrating through it. After 4-14 days, the blastospores circulating through the body kill the insect, transform into mycelium and produce more conidia on the exterior of the insect. Adapted from [201].

3.2.Bacteria

Bacteria such as *Bacillus thuringiensis (Bti)* have been used as an effective biocontrol agent against many insects [202]. In fact, *Bti* is highly toxic to different mosquito species, such as *Aedes*, *Culex* and *Anopheles*. *Bti* is a gram-positive, facultative anaerobic sporulating bacterium. It can be found in soils, insects, plants, forests, stored products, aquatic environments, and it can remain latent in the environment until favorable conditions allow for its development [203]. During sporulation, *Bti* forms a crystalline parasporal body composed of protein protoxins (Cry and Cyt proteins). These protoxins are δ-endotoxins, soluble in water, with insecticidal properties. They are very specific to

the target insect, while at the same time being harmless to humans, vertebrates and plants. These toxins are biodegradable. All these properties make *Bti* a sustainable alternative for insect and vector control [204]. Cyt toxins do not bind to receptors, but they interact directly with membrane lipids, and they either insert into the membrane and form pores [205], or they destroy the membrane by a detergent like interaction [196].

Bti toxins have been already used for three major applications. First, for the control of defoliator pests in forests; for the control of mosquito vectors of diseases; and for the development of insect resistant plants. Perhaps the most successful application would be the first mentioned. That does not undermine from the success of the Bti toxins as a vector control method, as they present many advantages, such as the high insecticidal activity, specificity, the lack of resistance, and the lack of toxicity to the non-target organisms [204]. A large number of formulations containing *Bti* exist in the market. For now, the cost is higher than that of chemical insecticides, but that is probably due to its limited use. The development of new technologies will allow for a cost-effective production of this bio-insecticide.

4. *Wolbachia as a promising alternative*

Wolbachia pipientis is a gram-negative α-proteobacterium belonging to the order Rickettsiales. It is a common intracellular endosymbiont of invertebrates, found mostly in the gonads of the host, and it is transmitted vertically, from the infected female to its offspring. *Wolbachia* was first identified in the ovaries of *Culex* mosquitoes in 1924

[206]. This bacterium infects up to 76% of all insect species, making it probably the most common endosymbiont in the biosphere [207, 208]. *Wolbachia* has been shown to alter the physiology of the host, specifically the reproductive physiology in order to facilitate its own transmission [209]. Its effects include male killing, parthenogenesis, feminization and cytoplasmic incompatibility [210].
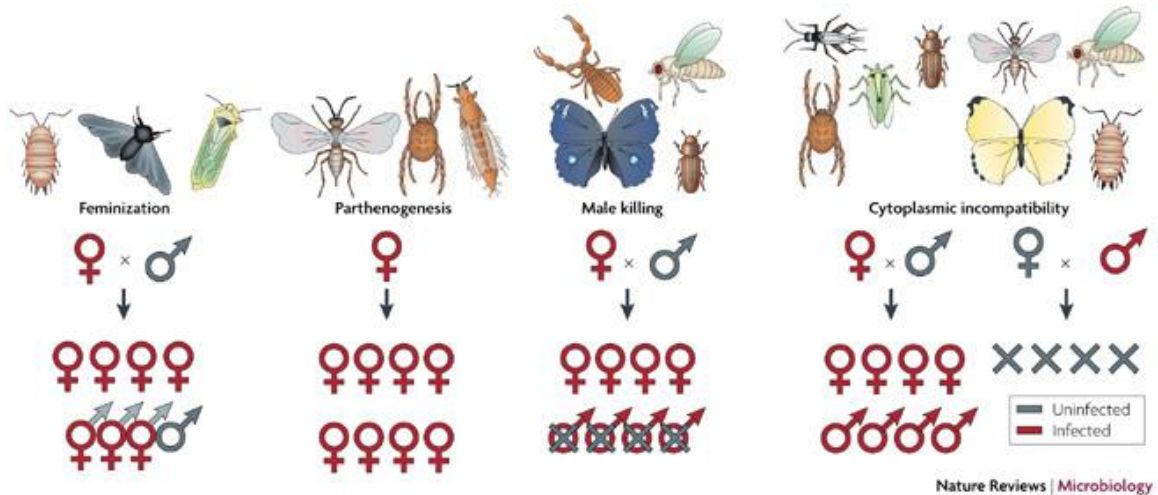
Figure 15: Reproductive phenotypes induced by *Wolbachia*.
Wolbachia induces four reproductive phenotypes in a range of arthropod orders: feminization, parthenogenesis, male killing and cytoplasmic incompatibility. Adapted from [211].

4.1. <u>Male Killing</u>

Male killing has been observed in four different orders: Coleoptera [212], Diptera [213], Lepidoptera [214] and Pseudoscorpiones [215]. This phenomenon occurs during embryogenesis, resulting in an increase of the proportion of infected females [216]. In fact, it was first thought that the cause of the all-female population of infected *Ostrinia scapulalis* was *Wolbachia*-induced feminization, but it was later shown to be the effect of

male killing. This seems to occur through lethal feminization [217]. In some cases, male killing could result in a change of host mating system to accommodate the depletion of males [218]. However, in a study done on the nymphalid butterfly *Hypolimnas bolina*, it has been demonstrated that host suppressor genotypes of male killing can cause complete suppression of a male killer, and that this genotype can spread rapidly within infected populations [219].

4.2. Parthenogenesis

Parthenogenesis is the reproduction of infected females without males. Infected females produce daughters from unfertilized eggs, which in turn are able to transmit the bacteria to their offspring [220-222]. *Wolbachia*-induced parthenogenesis is caused by disruption of the cell cycle during early embryonic development, resulting in diploid development in unfertilized eggs. The infection within some species is fixed, whereas within other species it might be polymorphic, with the chromosomal effects suppressed after a sperm fertilizes an egg [211].

4.3. Feminization

Feminization induces the development of infected males as females or infertile pseudofemales. This occurs through different mechanisms. In some species of the order Oniscidea, *Wolbachia* seems to proliferate within the androgenic gland, leading to androgenic gland hypertrophy and inhibited function [223]. In insects, feminization has

been described in two species, *Eurema hecabe* and *Zyginidia pullula* [224, 225]. The exact mechanism of *Wolbachia*-induced feminization in insects is still unclear, but in *Eurema hecabe*, *Wolbachia* seems to be interfering with the sex-determination pathway and must continuously act throughout development for complete feminization [226].

4.4. Cytoplasmic incompatibility

Cytoplasmic incompatibility (CI) is the most common *Wolbachia*-induced manipulation of the host reproductive system. CI is described as the inability of infected males to successfully mate with uninfected females (unidirectional CI), or with females infected with a different strain of Wolbachia (bidirectional CI) [209]. CI may have different consequences depending on the host; if the host is diploid, CI results in embryonic lethality. If, by contrast, the host is haploid, CI may lead to haploid, and therefore male, offspring [227]. The CI phenomenon was first described in 1938 [228], upon the observation that crosses between some strains of *Culex pipiens* mosquitoes were incompatible in one direction, whereas in the other direction viable progeny were produced. This led to a number of studies focusing on elucidating these observations in the 1940s and 1950s [229-233], and showing that this phenomenon is maternally inherited, suggesting that a extranuclear agent caused this phenomenon, thus giving rise to the term: Cytoplasmic incompatibility [231]. Bidirectional CI was also observed in addition to unidirectional CI in *C. pipiens*[229, 230]. The connection between CI and *Wolbachia* was finally made in the early 1970s [234, 235]. Today, CI is observed in all major insect orders, in mites and in woodlice [236]. CI is also induced by the unrelated

bacterium *Cardinium hertigii* [237, 238]. CI is caused by two different events. The first is called modification, and it occurs inside the *Wolbachia* infected male during spermatogenesis [239]. The second is called rescue, and it happens inside the fertilized egg, where the presence of a compatible *Wolbachia* strain prevents CI from occurring [240]. Normally, upon fertilization the sperm-derived pronucleus undergoes nuclear envelope breakdown and exchanges protamines for maternal histones. Then, male and female pronuclei juxtapose, but do not fuse, and they undergo DNA replication before the first zygotic mitosis. Maternal and paternal chromosome synchronously condense, align at metaphase and separate at anaphase, and they do not fuse until the end of the first telophase [241]. In CI crosses, paternal chromatin does not condense properly for the first mitotic cycle. This causes a lethal mis-segregation and bridging of paternal DNA at anaphase [227, 242]. Delayed histone deposition, improper chromosome condensation and cell division abnormalities all lead to embryonic death and arrest [243]. At the molecular level, it has been shown that a two-gene operon located on the Eukaryotic Association Modules (EAM) in phage WO induces CI. The second gene of this operon is called CI-inducing DUB, Cid B, and this is a deubiquitylating enzyme (DUB), that cleaves ubiquitin from substrates post-translationally. The first gene of the operon is called CidA, and CidA binds CidB [244]. In fact, it has been shown that when these two genes are co-expressed in *Drosophila* males non-infected with *Wolbachia*, CI is induced [245]. It has also been observed that CI strength variation correlates with the number of copies of the pair of genes [245].
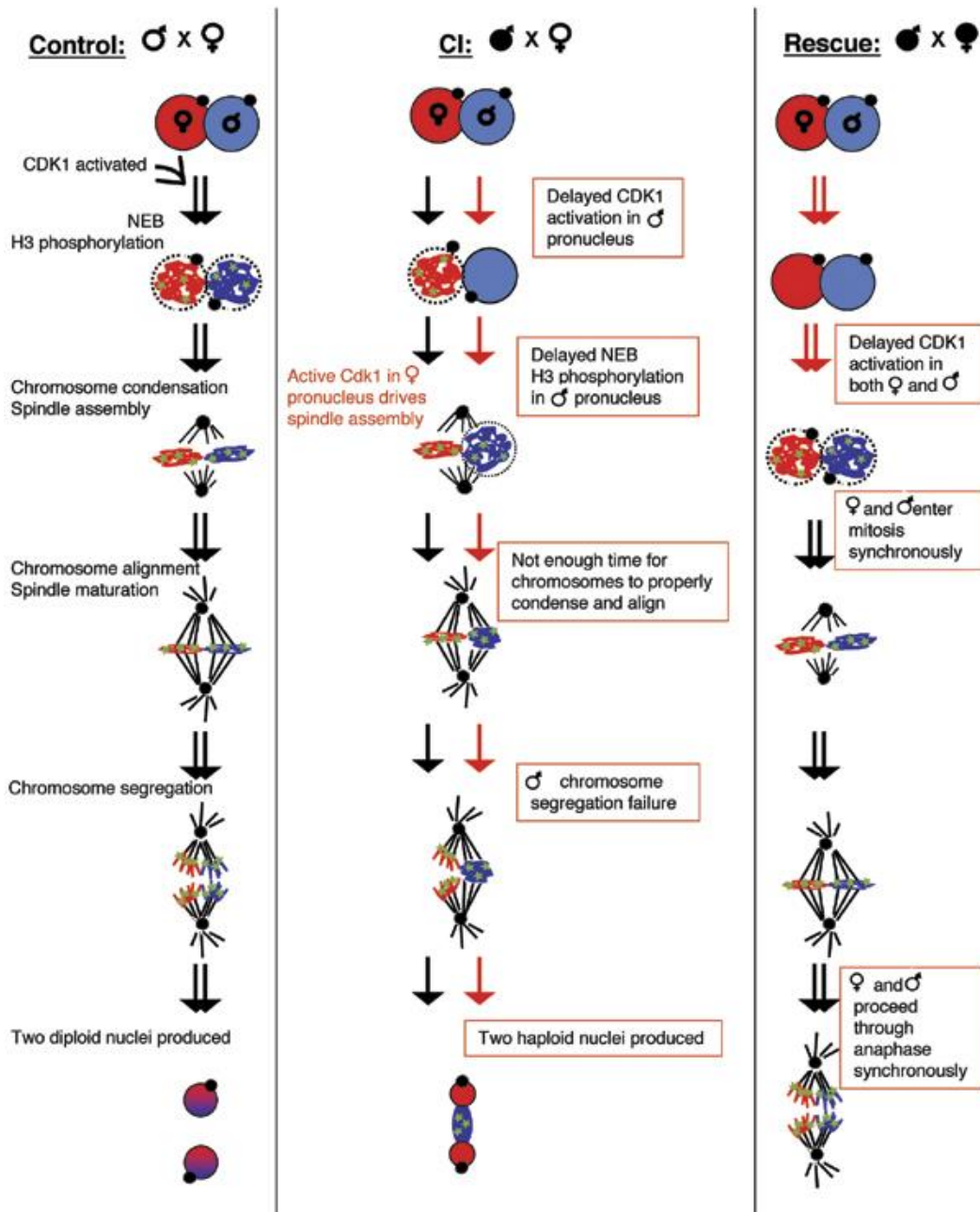
Figure 16: Model of the CI phenotype mechanism.
This schematic representation shows that CI causes a delayed condensation of male chromosomes, leading to the formation of haploid embryos. Upon rescue of this phenotype, both male and female pronuclei display a delayed condensation of the chromosomes, allowing the formation of diploid embryos. Adapted from [246].

Some *Wolbachia* strains can induce more than one of these reproductive phenotypes, depending on the host background they are placed in. For example, the lepidopteran host *Cadra cautella* is normally infected with a strain of *Wolbachia* which causes CI. When this strain is transferred to a new lepidopteran host, *Anagasta kuehniella*, it can cause male killing [247]. In another example, the *Wolbachia* strain naturally found in *Drosophila recens* which induces CI will cause male killing when introgressed into *Drosophila subquinaria* [248].

As for the host, the bacteria's presence can induce positive or negative effects, depending on the strain of *Wolbachia* and the species it infects. In some cases, *Wolbachia* infection has been beneficial to the host, as they may provide fitness benefits. In fact, it has been shown that the infection of *Drosophila melanogaster* flies with *w*Mel *Wolbachia* strain affects the fly lifespan positively [249]. In the parasitoid wasp *Asobara tabida*, *Wolbachia* presence is essential for the development of ovaries, as it downregulates apoptotic processes in the developing ovaries, and therefore removal of the bacteria leads to death of ovarian cells [250]. *Wolbachia* (*w*Mel) may provide *Drosophil*a flies with riboflavin and heme or their intermediates [251, 252], as well as representing an additional source of nucleotides [252].In *A.albopictus*, an infection of two or more strains of *Wolbachia* improves fecundity [253]. On the other hand, In the case of the parasitoid wasp *Leptopilina heterotoma*, *Wolbachia* has been shown to have a negative impact on fecundity and longevity [254].

Wolbachia has also been shown to protect the host against pathogens in some cases. In fact, *Wolbachia* infection can reduce the ability of the host to transmit viruses, and it

protects the host against viral, bacterial, and protozoan parasites. In 2010, *Glaser et al* showed that *Wolbachia* infection of *Drosophila melanogaster* flies increased the host resistance to West Nile Virus infection [255]. In another study, *Wolbachia* was shown to provide protection against oral exposure to *Drosophila C virus* in adult flies [256]. *Hedges et al* showed that *Wolbachia* provided protection against *Drosophila C virus*, *cricket paralysis virus* and *Flock House virus* in *Drosophila melanogaster* flies [257]. *Gupta et al* were able to show that *Wolbachia* provided antibacterial protection against enteric infection with *Pseudomonas aeruginosa* [258]. In terrestrial isopods, *Wolbachia* provided a protective effect against pathogenic intracellular bacteria [259]. In *Anopheles gambiae*, *Wolbachia* infections were found to inhibit the parasite *Plasmodium falciparum* [260, 261]. Similar results were obtained upon infection of *Anopheles stephensi* with the *w*AlbB strain of *Wolbachia*, as infection caused refractoriness to *Plasmodium berghei* [262]. In *Aedes aegypti*, *Wolbachia* inhibits the development of filarial nematodes [263], and limits infection with dengue, Chikungunya, and Plasmodium [264]. In *Aedes albopictus*, *Wolbachia* inhibits dengue transmission [265].

While it is important to characterize the effect of *Wolbachia* on the host immune system, it is equally interesting to observe whether the host itself mounts an immune response against the endosymbiont. In fact, the hosts' response to *Wolbachia* seems to be different in native hosts as opposed to new hosts. In native hosts, *Wolbachia* infection does not seem to cause neither an induction, nor a suppression of AMP gene expression, as demonstrated in *Drosophila melanogaster*, *Drosophila simulans*, *Tetranychus urticae* [266-268]. By contrast, when *Wolbachia* is introduced into a new host, a strong

inductionof AMP gene expression can be observed [263, 264, 269, 270]. *Wolbachia* infection also seems to be affecting the oxidative environment, as it has been shown that *Wolbachia* infection is associated with high levels of ROS in the native host *Aedes albopictus* cell lines [271]. Another study done by *Molloy and Sinkins* however is in contrast with the first study, as it shows no difference in ROS production levels between infected and uninfected insects [272]. In novel hosts, *Wolbachia* causes an induction of ROS production [273-275]. The difference of response between native and novel hosts is probably due to the co-evolution of host and bacteria in native hosts, which would lead to either tolerance of the host to the bacteria, or to the bacteria finding ways to evade the host immune system. In novel hosts by contrast, the endosymbiont would trigger the host immune response aimed at eliminating the invading bacteria [276].

The manipulation of host reproduction offers a selective advantage for the *Wolbachia* endosymbiont and its propagation through the population. This, coupled to its effects on host fitness and immunity, have made *Wolbachia* a promising alternative for vector control.

In fact, releasing male and female mosquitoes infected with *Wolbachia* into a wild population should allow the invasion of *Wolbachia* into that target population. *Wolbachia*-infected females will then have a reproductive advantage over the wild-type females, through the induction of CI, and this would allow *Wolbachia* to spread naturally throughout the population. The *Wolbachia*-infected females will have reduced ability to transmit pathogens, thus allowing a decrease in the proportion of mosquito-borne diseases [277, 278]. Using *Wolbachia* as a vector control method has many advantages.

This method requires the release of fewer number of mosquitoes when compared to other methods such as SIT and RIDL. Also, *Wolbachia* is maintained at high frequency in a population indefinitely, meaning there would be no need for frequent releases every couple of years [279]. In fact, in Australia, initial releases of *Wolbachia*-infected male and female insects were done over a period of 10 weeks, and *Wolbachia* infection has persisted in the target population at frequencies above 90% [280]. *Wolbachia*-based methods are cost effective, and since only one release is needed, continuous funding will not be required. Moreover, since this method involves release of both male and female mosquitoes, there would be no need for sex sorting and the possibility of releasing females by mistake [281].

Wolbachia can be used for either population suppression or for population replacement. In the context of population suppression, *Wolbachia* would need to be stably introduced into a mosquito species, and then only *Wolbachia*-infected males would be released into a target population. The males harboring *Wolbachia* will then mate with wild-type females either not infected with *Wolbachia* or infected with a different strain of *Wolbachia*. This induces CI, thus causing the death of the offspring and overtime a decrease in the size of the population. In the context of population replacement, both males and females infected with the endosymbiont would need to be released. In this case, CI offers a reproductive advantage to *Wolbachia*-infected females, resulting in the spread and establishment of *Wolbachia* in the target population. Newly infected female mosquitoes are resistant to several viral and bacterial infections, thus decreasing their capacity to transmit diseases [281].

a **Suppression of mosquito population**

Cytoplasmic incompatability

Mosquitoes without *Wolbachia*   Mosquitoes infected with *Wolbachia*

b **Modification of mosquito population**

Pathogen blocking

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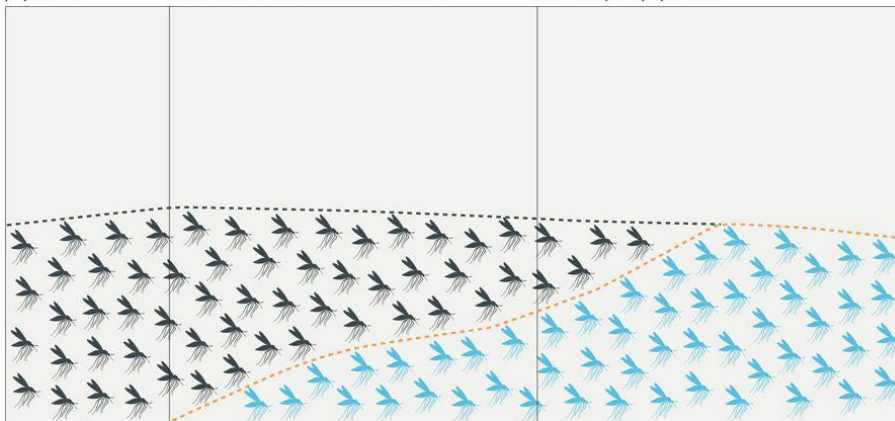
Figure 17: *Wolbachia* **is a promising alternative for vector control through population suppression or replacement.**

a-*Wolbachia* can be used to suppress a mosquito population through the release of Wolbachia-infected males into a population. These males will mate with wild-type females, and because of CI no offspring will be produced. This will lead overtime to a reduction in the size of the population. **b-***Wolbachia* may also be used to replace a mosquito population through the release of male and female mosquitoes infected with the endosymbiont. Owing to CI, *Wolbachia* will spread and establish itself in the population, and the mosquitoes harboring *Wolbachia* will have a reduced ability of transmitting diseases. Adapted from [281].

CHAPTER 2

AIMS OF THE PROJECT

Mosquitoes are known vectors of several diseases, such as yellow fever, dengue virus and Zika virus. In order to limit the spread of mosquito-borne diseases, different control methods have been developed. Unfortunately, these have not proven to be successful as they present many disadvantages, such as insecticide resistance and decreased fitness of mosquitoes. As the threat of mosquito-borne diseases becomes more eminent, the need for the development of alternative effective control methods increases. In our lab, we chose to focus onmosquito immune responses, as this could provide useful insights into the development of novel control strategies. To do so, we chose to work with the local mosquito strains of *Culex pipiens* and *Aedes albopictus*, as these could contribute to the possible spread of mosquito-borne diseases in the region. Most pathogens in mosquitoes are acquired through the oral route.The gut is the first line of defense against these microbes,it functions both as a physical barrier, and through activation of local immune responses [13, 14]. In mosquitoes, no studies so far have shown how gut renewal and homeostasis is maintained, while on the other hand, digestive tract has been extensively studied in *Drosophila melanogaster.*Several studies revealed the presence of a subset of cells called Intestinal Stem Cells (ISCs) responsible for gut renewal and homeostasis[22, 28]. These cells were found to replicate and differentiate in response to damage in order to make up for the loss of damaged enterocytes [282, 283]. In the context of **specific aim**

1 of my project, we were interested in characterizing the gut as an important component of mosquito immunity.We will establish a model using *Aedes albopictus* to study the effect of chemical and bacterial damage on mosquito gut.

C. pipiens and *A. gambia*e.*Culex* mosquitoes are known worldwide to transmit to human several pathogens such as the yellow fever virus and filarial nematodes[284], while *Anopheles gambiae* mosquitoes transmit malaria and are probably the most studied insect vector [285, 286]. A comparative analysis of the gut physiological responses among the three species is important, since it allows us to determine whether differences in these responses exist, and whether this could be related to the vectorial capacity of the mosquitoes. Hence in the context of specific aim 2 we will extend the analysis of adult gut regeneration to these two important disease vector mosquitoes and conduct a comparative analysis of midgut regeneration capacity and resistance to oral infection in three disease-vector mosquitoes.

Certain studies have shown that infection with the endosymbiont *Wolbachia* might confer host resistance to several pathogens, while other studies have claimed that in its native host, immune activation by *Wolbachia* might get attenuated and the protective effects of the endosymbiont become abolished. In the context of **specificaim**3, we will explore the immune responses of the Lebanese mosquito strain of *Aedes albopictus* to different microbial challenges. Additionally, we will investigate the effect of abolishing *Wolbachia* on *A. albopictus*and *C. pipiens*wellbeing aftergenerating *Wolbachia*-free strains in these two mosquito species.

Unraveling these immune responses and characterizing them adds additional knowledge thatwould contribute to the development of alternative control strategies.

Specific Aim 1: Establish a model using *Aedes albopictus* mosquitoes to study the mosquito gut's responses to chemical/bacterial damage

1.1. Characterize the structure of mosquito guts using scanning electron microscopy

1.2. Establish a model for inducing chemical/bacterial damage to the guts. This will be done by feeding the mosquitoes different chemicals/bacteria and observing the effect on gut structure

1.3. Investigate the presence of proliferative cells using immunohistochemistry by probing for markers specific to cellular proliferation

1.4. Investigate which molecular pathways are activated/ suppressed in the gut after damage by real-time PCR

Specific Aim 2: Performa comparative analysis of midgut regeneration capacity and resistance to oral infection in three disease-vector mosquitoes

2.1. Characterize and compare the structure of *Aedes albopictus*, *Culex pipiens* and *Anopheles gambiae*mosquito guts using scanning electron microscopy

2.2. Investigate the presence of proliferative cells using immunohistochemistry by probing for markers specific to cellular proliferation for the three species

2.3. Study the effect of chemical and bacterial feeding on mosquito survival

2.4. Investigate the effect of microflora on gut regeneration in *Culex pipiens* mosquitoes

Specific Aim 3: **Characterize the effect of different microbial infections on** *A.* *albopictus* **mosquitoes**

3.1. Establish microbial infections either by mimicking the natural route of infection (by feeding bacteria through the oral route or spraying a suspension of fungal spores), or by microinjection of bacteria/ fungi intrathoracically

3.2. Characterize *A. albopictus* tolerance and resistance to microbial infections

    3.2.1. Follow the survival of infected mosquitoes over a span of several days

    3.2.2. Determine the rate of clearance of pathogens from the mosquitoes by counting the CFUs (colony forming units) after infection

3.3. Determine the level of AMP activation contributing to the immune responses after microbial infection

3.4. Generate Wolbachia-free strains by treating the larvae with antibiotics

3.5. Compare the physiological characteristics and immune responses of *Wolbachia*-free strains to the original strains

PREFACE TO CHAPTER 3

The following chapter is composed of four parts. The first two parts correspond to published results which have been reformatted to match the desired style of the dissertation, whereas the last two parts correspond to unpublished data. The first part has been published in *Scientific Reports* in 2017 ([287]), whereas the second part is currently under revision in the same journal. These studies describe cell regeneration in the midgut of adult mosquitoes during homeostasis and after damage. The third part presents data corresponding to the role of the microflora in the damage and regeneration of *Culex pipiens* mosquito guts. These results were observed while doing the experiments for the second part, so we decided to expand on them and further characterize whether the microflora is involved in the response observed. The final part corresponds to the characterization of the immune responses of *Aedes albopictus* mosquitoes, and the establishment of *Wolbachia*-free strains allowing for the characterization of the effect of the endosymbiont on mosquito physiology and immunity.

CHAPTER 3

RESULTS

1. Damage-induced cell regeneration in the midgut of *Aedes albopictus* mosquitoes

1. Abstract

Mosquito-transmitted diseases cause over one million deaths every year. A better characterization ofthe vector's physiology and immunity should provide valuable knowledge for the elaboration of controlstrategies. Mosquitoes depend on their innate immunity to defend themselves against pathogens.These pathogens are acquired mainly through the oral route, which places the insects' gut at the frontline of the battle. Indeed, the epithelium of the mosquito gut plays important roles against invadingpathogens acting as a physical barrier, activating local defenses and triggering the systemic immuneresponse. Therefore, the gut is constantly confronted to stress and often suffers cellular damage. In thisstudy, we show that dividing cells exist in the digestive tract of adult *A. albopictus* andthat these cellsproliferate in the midgut after bacterial or chemical damage. An increased transcription ofsignalingmolecules that regulate the EGFR and JAK/STAT pathways was also observed, suggesting a possibleinvolvement of these pathways in the regeneration of damaged guts. This work provides evidence forthe presence of regenerative cells in the mosquito guts, and paves the way towards a molecular andcellular characterization of the processes required to maintain mosquito's midgut homeostasis in bothnormal and infectious conditions.

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2. *Introduction*

Mosquitoes are well known vectors of human and animal diseases. The Asian tiger mosquito *Aedes albopictus*is an important vector for several pathogens, including Chikungunya, Dengue and the recently identified Zikavirus[288]. This mosquito was identified in the Middle East 10 years ago and its population size has increased since then [4]. The presence of endogenous mosquito vectors together with climatic warming may lead to the spread ofmosquito-borne diseases in the near future. It is therefore important to better understand mosquito immuneresponse in order to develop effective control strategies against these vectors of diseases. Like all other insects,mosquitoes depend on innate immunity to fight pathogens[289, 290]. Different immune responses have been describedin mosquitoes including phagocytosis, melanization, complement-like mediated lysis and antimicrobial peptides(AMPs) production[291-293]. AMPs are released when pattern recognition receptors (PRRs) recognize pathogenassociated molecular patterns (PAMPs) and trigger a downstream signal transduction cascade resulting in theactivation of effectors' responses[294, 295].

The gut constitutes an important component of the mosquito's defense against foreign invaders. Besides itsrole in food digestion, the gut forms a physical barrier preventing dissemination of ingested pathogens. An exampleis the Anopheline mosquito species, where the midgut plays a role as a physical barrier against *Plasmodium*parasites due to the presence of the peritrophic matrix lining the midgut epithelium[296]. The mosquito gut epitheliumis also able to clear microbes after the activation of local immune response. It has been shown that theImmune Deficiency (IMD) pathway is activated prior to the

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invasion of the midgut epithelium by the ookinetesin *Anopheles* mosquitoes[297]. Maintaining the integrity of mosquitoes' gut is therefore indispensable for effectivelocal immune defenses against harmful pathogens.

The alimentary canal of larval mosquitoes is nearly completely autolysed and replaced during pupation so thatthe adult digestive apparatus is largely built anew. A few studies have focused on the guts of mosquito larvae[15], while curiously, the adult gut remains poorly explored. Food digestion, ingestion of cytotoxic compounds, entericinfections and molecules produced during the immune response are major gut stress-inducers[21]. In *Drosophila melanogaster*, the presence of such stress-inducers in the gut lumen result in cell damage and loss of the absorptiveand digestive enterocytes (ECs), thepredominant cell type in the gut epithelium[298]. In order to compensate forthe loss of ECs, the gut possesses protective homeostatic mechanisms relying on the activity of intestinal stem cells (ISCs) that are scattered along the midgut epithelium[22, 28, 298]. Upon damage, the *Drosophila* midgut initiates ahomeostatic feedback loop that couples EC loss to ISC division and differentiation. Several signaling pathwaysthat are involved in *Drosophila* gut regeneration have been identified, such as the Janus kinase/signal transducerand activator of transcription (JAK/STAT) and the epidermal growth factor receptor (EGFR) pathways[45, 48, 62, 298, 299]. Gutdamage induces the production of secreted ligands of Unpaired (Upd1, Upd2 and Upd3) and EGF (Spitz, Vein,Keren) families, which activate respectively the JAK/STAT and EGFR pathways in ISCs to promote their rapidproliferation and differentiation, thereby establishing homeostatic regulatory loops[24, 25].

The feeding habits of Drosophila species do not allow these flies to transmit diseases to humans. Mosquitoes bycontrast transmit diseases through pathogen-infected meals. This makes the study of mosquitoes' gut physiologyand immunity highly relevant to human health. Some previous reports suggested the existence of ISCs in mosquitoes'gut based on morphological characteristics[300-303]. Among these, in 1953 Day and Bennetts studied woundhealing in the guts of *Aedes aegypti*; and in 1977, Hook evoked the presence of proliferating cells in *Culex tarsalis*guts after blood meal. In this study, we show that the gut of adult *A. albopictus* mosquitoes contains mitotic cells,which become highly proliferative upon ingestion of damaging chemical compounds or enteric bacterial infections.We also provide insight into the molecular pathways activated in the mosquitoes' gut after damage.

3. Results

a. Structure of the adult *Aedes albopictus* gut

The adult mosquito gut consists of a simple epithelial tubedivided into three discrete structures: the foregut, the midgut, and the hindgut. The foregut allows sugar solutionsintake by the mouth, unidirectional passage of digesta through the pharynx and the esophagus and its storage inthe crop, which is a bag like structure[304]. The midgut serves in food digestion and nutrients absorption and thehindgut with its associated malpighian tubules (functional analogues to mammalian kidney) plays excretory andosmoregulatory roles[305]. The midgut and hindgut are revealed in Fig. 18 using scanning electron microscope (theforegut is not shown). In addition, this figure shows a

clear anatomical difference between male and female guts,male guts being overall smaller. In particular, the midgut compartment is less developed in males as compared tofemales (Fig. 18A and B). This is in agreement with the fact that female mosquitoes require a protein-rich bloodmeal to produce eggs, while males only consume sugars and are smaller than females. Therefore, male guts haveto perform simpler digestive functions. The gut is surrounded by visceral muscles and a dense network of trachealtubes delivering oxygen to and removing carbon dioxide from the gut cells (Fig. 18C and D).
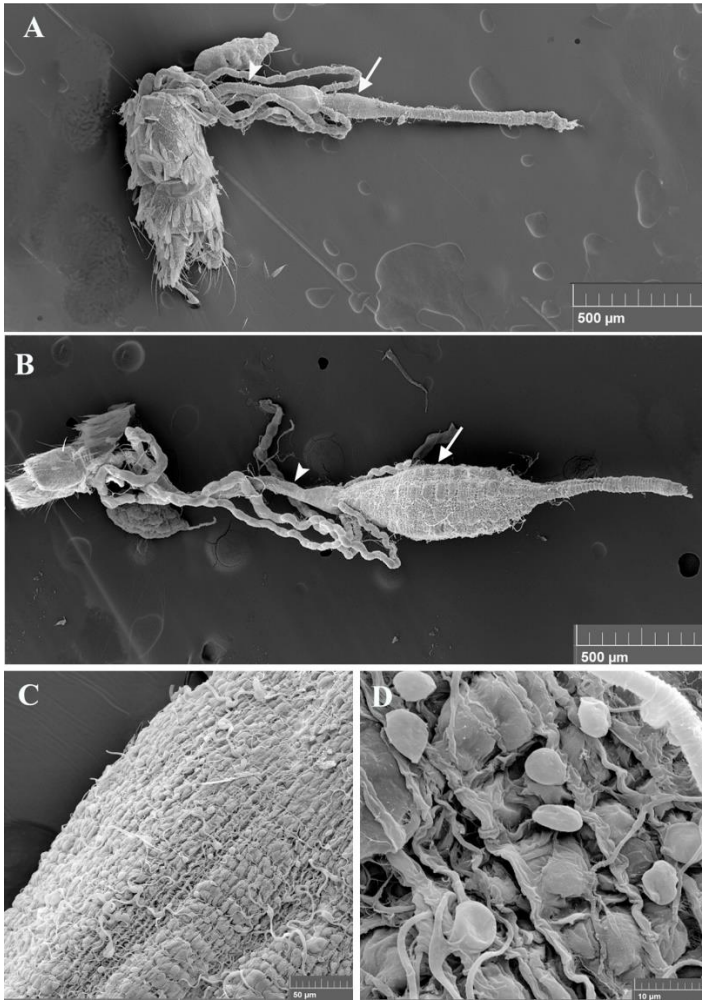
Figure 18: Scanning Electron Microscopy (SEM) images of A. albopictus mosquito guts.
The ultrastructure of the gut of a male (A) and a female (B) as revealed by SEM shows two of the three main compartments: the hindgut (arrowhead) with the associated malpighian tubules and the midgut (arrow). (C and D) are close-up photos of female guts where individual visceral muscle cells as well as tracheal cells and tubing surrounding the gut are visible.

    b. <u>Establishment of a model to induce damage to the gut</u>

To date, it is not known how the mosquitogut integrity is maintained and regulated when

enterocytes suffer damage. To induce damage to mosquito guts,insects were starved for

two hours then fed on a 10% sucrose solution containing 2% Sodium Dodecyl Sulfate(SDS), a chemical used in previous studies to induce damage in Drosophila guts[282]. Mosquito guts were dissected24 hours post treatment and fixed. Staining with fluorescent phalloidin reagent that label the F-actin filamentsallowed us to visualize the global morphology of the gut at different focal planes in order to compare the guts ofSDS treated animals to those of the control group. As shown in Fig. 19, the guts of mosquitoes fed on sucrose supplementedwith SDS (Fig. 19B,F,G and H) were distorted and the F-actin filaments did not show the same homogeneitywhen compared to the gut of control mosquitoes (Fig. 19A,C,D and E).This indicates that SDS treatment isa convenient and reproducible method to inflict damage to mosquito guts and therefore constitute a good modelto analyze the effect of damage on gut structure and physiology.
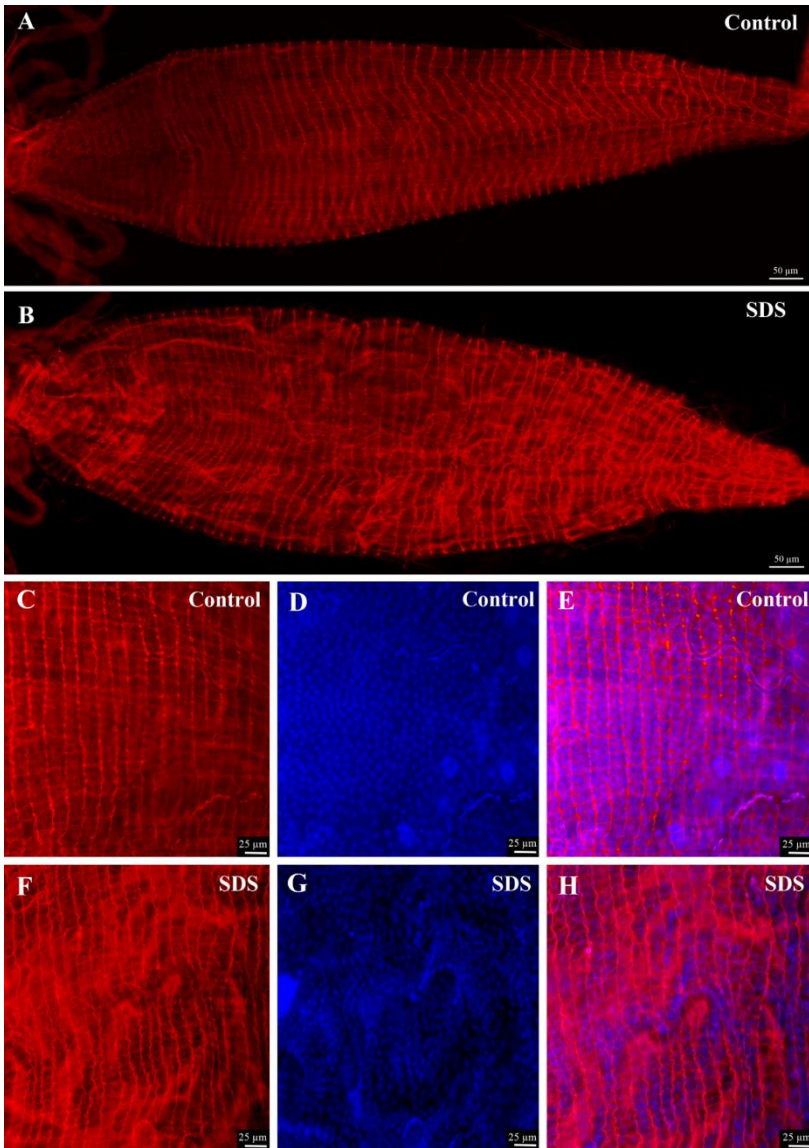
Figure 19: SDS feeding induces damage to the gut.
Phalloidin and DAPI staining of dissected guts shows that the guts of mosquitoes fed for 24 hours on sucrose supplemented with 2% SDS have an altered structure (B) as compared to controls fed on sucrose (A). This can be better appreciated in higher magnification captures showing Phalloidin staining (C and F), DAPI staining (D and G) and the merged pictures (E and H).

c. <u>Proliferating cells are present in the guts of *Aedes albopictus* mosquitoes</u>

To unravel theexistence of proliferating cells in the gut of mosquitoes, we stained the

tissue with antibodies raised againstphospho-histone H3 protein (anti-PH3), a specific

marker of cells undergoing mitosis[306, 307]. Staining was performedon guts of

mosquitoes fed either on sucrose or SDS. Low numbers of small cells with strong PH3

labelingwere observed in the midguts of control mosquitoes (Fig. 20A and B). In many

cases, two PH3 positive nucleiwere observed close to each other, these had a

characteristic coffee-bean shape and very likely correspond totwo sister cells originating

from the recent division of a progenitor cell. A number of PH3 positive cells were

alsoobserved in the tracheal tubes surrounding the midgut, these can be easily

distinguished from small PH3 positivecells located inside the gut epithelium based on

their bigger sized nuclei and on their location and arrangement.A higher magnification

picture focused on midgut cells labeled with anti-PH3 and DAPI staining is shown inFig.

20C and G. When compared to the guts of control mosquitoes, the guts of SDS fed

mosquitoes appeared damagedand distorted confirming the observation shown in Fig. 19.

Interestingly, an increase in the number of PH3positive cells was observed (Fig. 20E and

F) in comparison to control guts. These findings suggest that SDS feedinginduces gut

damage resulting in the activation of local regenerative responses. In addition, we were

able to detectboth in control guts (Fig. 20D) and in damaged guts (Fig. 20H) the presence

of a high numbers of dividing cells in arestricted area of the most anterior part of the
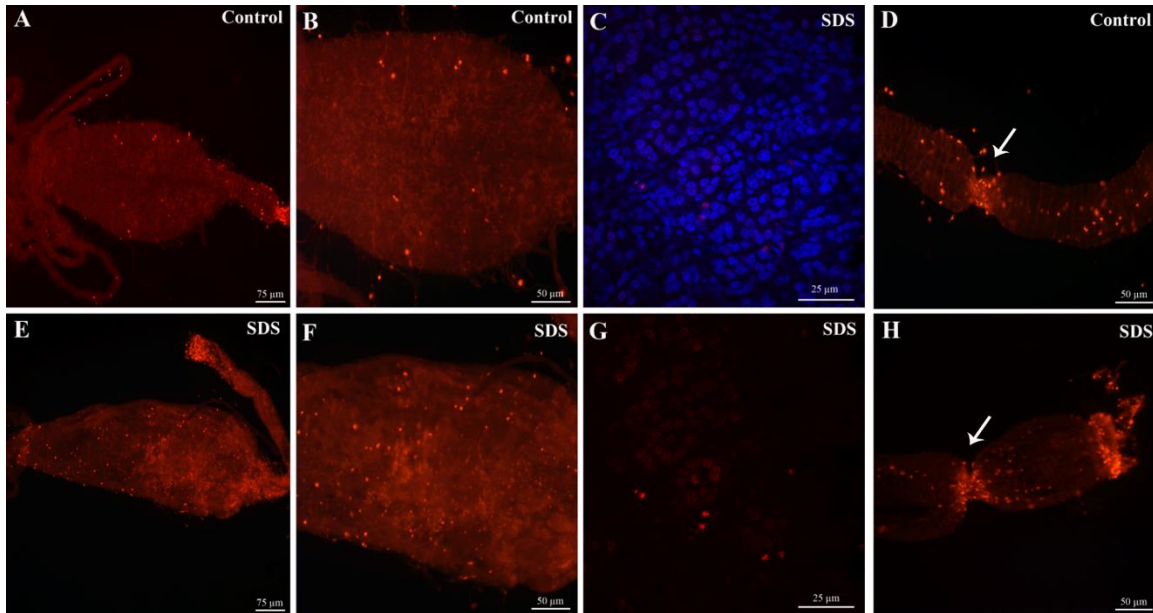
midgut.

Figure 20: Regenerative cells are present in the midgut of adult *A. albopictus*.
Immunofluorescence staining using anti-PH3 antibodies reveals the presence of cells
undergoing division in the gut of adult mosquitoes. An increase in the number of
proliferating cells in the midgut is observed 24 hours after feeding the mosquitoes on
SDS-sucrose (E and F) as compared to the midguts of control mosquitoes (A and B). A
higher magnification picture focused on midgut cells labeled with anti-PH3 and DAPI
staining is shown in (C and G). Two zones of active cell division (arrows) are observed in
the most anterior part of the midgut independently of gut damage (D and H).

d. Chemical and bacterial damage induce a significant increase in the number of
   mitotic cells

First, we performed a quantification of PH3 positive cells residing in the midgut

epithelium of mosquitoesfed on sucrose or SDS solutions. Three independent

experiments were done with 12 guts analyzed foreach condition per experiment. We also

used other chemicals that are classically used to induce gut damagein *Drosophila*

melanogaster, including paraquat, H_2O_2 and Bleomycin[48, 282].Cell counts were

plotted using theGraphPad Prism software and results are shown in Fig. 21A–F. For all

experiments, a statistically significant differencewas found between the numbers of PH3

positive cells in damaged versus control guts. The average numberof dividing cells in

control guts was 2.42 versus 15.56 in SDS treated guts (n = 36). For paraquat (n = 24),

H_2O_2(n = 21) and for Bleomycin (n = 17) feeding, the average number of dividing cells

per gut as compared to controlwas respectively: 12.83 versus 3.16; 10.76 versus 3.47 and

8.70 versus 4.29.To investigate whether enteric infections can also trigger cell

proliferation in the mosquito midgut, we usedthe pathogenic Gram-negative bacteria

Serratia marcescens and *Erwinia carotovora carotovora 15 (Ecc15)*. We fed*Aedes*

mosquitoes on a sucrose solution containing a high concentration of each bacterial

suspension (OD 50).In both cases, we observed a significant increase in the number of

dividing cells permidgut: 12.62 for *S. marcescens*as compared to 3.22 for control guts (n

= 26) and 8.15 for Ecc15 as compared to 3.84 for controls (n = 19)(Fig. 21G,H and

Supplementary Figure 1). These results suggest that damaging the gut of mosquitoes

triggers anintrinsic increase in cell proliferation.
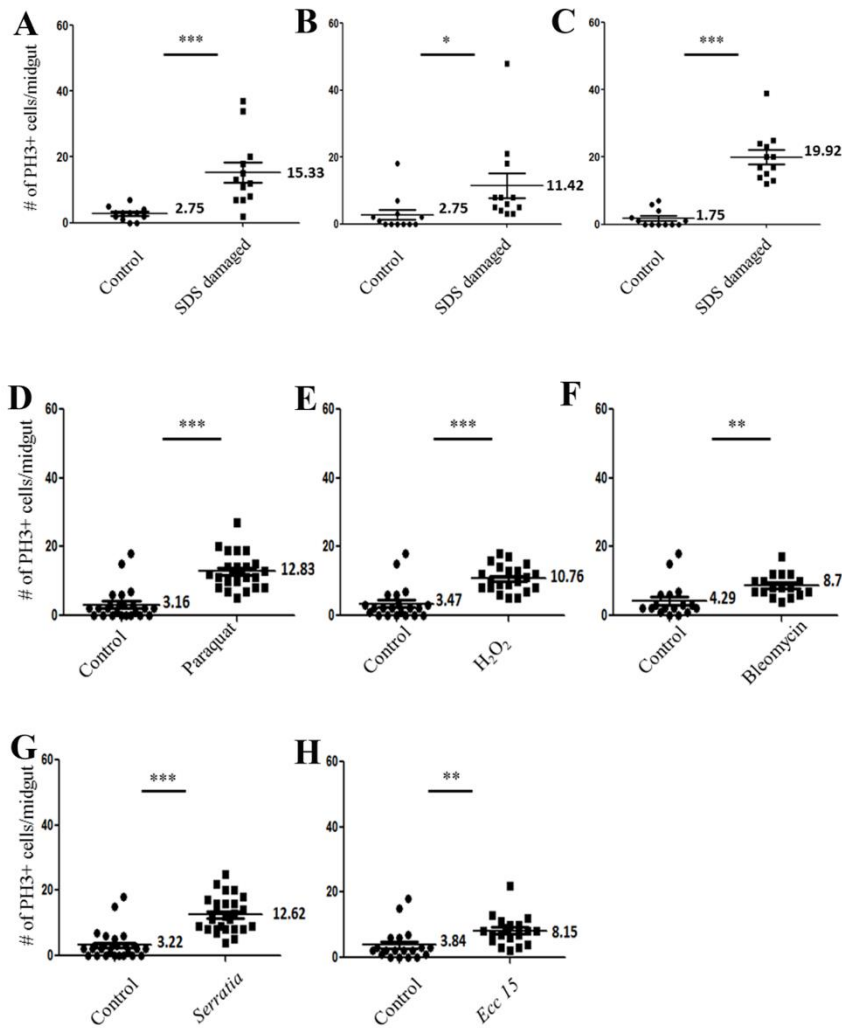
Figure 21: Feeding on stress inducing chemicals or on pathogenic bacteria increases cell division in the midgut.

The number of dividing cells stained by anti-PH3 antibodies per midgut is counted and statistical analysis confirms a significant difference in the number of proliferating cells in damaged guts as compared to control ones. For SDS feeding the experiment was done in triplicates (A–C), and the number of guts analyzed is n = 12 for each replicate. For paraquat feeding (D) n = 24, H2O2 (E) n = 21, Bleomycin (F) n = 17. For *Serratia marcescens* (G) n = 26 and *Erwinia carotovora carotovora 15* (H) n = 19. ***P < 0.001, **P < 0.01, *P < 0.05.

e. <u>Ingestion of pathogenic bacteria induces local AMP production in the gut</u>

We assessed theexpression levels of the antimicrobial peptide CecropinA1 (CecA1) gene in *S. marcescens* fed mosquitoes usingqRT-PCR. This AMP is believed to be active against Gram-negative bacteria based on work realized in *Drosophila melanogaster*[308, 309]. CecA1 transcripts levels were slightly but significantly increased (by approximately 1.4 fold) in the guts of orally infected mosquitoes as compared to controls (Fig. 22A). CecA1 transcriptional upregulation wasnot observed in whole mosquitoes orally infected with *S. marcescens*, indicating that only a local response wastriggered in the gut. This result is in agreement with what has been observed in *Drosophila* after *S. marcescens*feeding[310]; it also gives additional molecular evidence that the mosquito gut acts as a first barrier to protect theorganism from foreign invaders. For comparison, we injected *S. marcescens* and *Escherichia coli* bacteria into <u>the</u>body cavity of mosquitoes to induce the systemic immune response and observed very high expression levels ofCecA1 (respectively 360 and 120 folds as compared to non-injected controls) (Fig. 22A).

f. <u>Analysis of candidate signaling pathways genes expression after gut damage</u>

To gain insightinto the molecular signaling pathways activated in damaged mosquito guts, we looked in the recently releasedgenome of *A. albopictus* for genes with orthologous counterparts that were known to be involved in gut regenerationin

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Drosophila. We were not able to identify an orthologue to any of the three

*Drosophila*unpaired genes(JAK/STAT ligands) in *A. albopitcus* genome. However, we

found an orthologous of the Socs36E, the knowntarget and negative regulator of the

JAK/STAT pathway in *Drosophila*[311, 312]. Alternatively, we looked for the ligandsof

the EGFR pathway that are highly induced in the *Drosophila* midgut following stress-

induced damages[54]. Theligands are usually more up- or down-regulated as compared to

receptors or intracellular components of signalingpathways. We identified an *A.*

albopictus orthologue to the *DrosophilaKeren* gene, one of the four known EGFRligands

in *Drosophila*.

Using specific primers that amplify those genes, we performed qRT-PCR on whole

mosquitoes fed with SDS,with *S. marcescens* or with sucrose. We also did qRT-PCR on

guts isolated from mosquitoes that received thesethree different treatments. SDS feeding

led to a significant increase of both *keren* (2.5 folds) and *Socs36E* (2.9folds) transcripts in

the guts (Fig. 22B and C). This figure shows also a significant increase in the transcripts

levels,both in dissected guts (2.3 folds for *keren* and 8.2 folds for *Socs36E*) and in whole

animals (3.1 folds for *keren* and3.8 folds for *Socs36E*), after *S. marcescens* feeding.

These results suggest that EGFR and JAK/STAT pathways maybe involved in the

regenerative response triggered in the adult *A. albopictus* gut following local cell

damages.
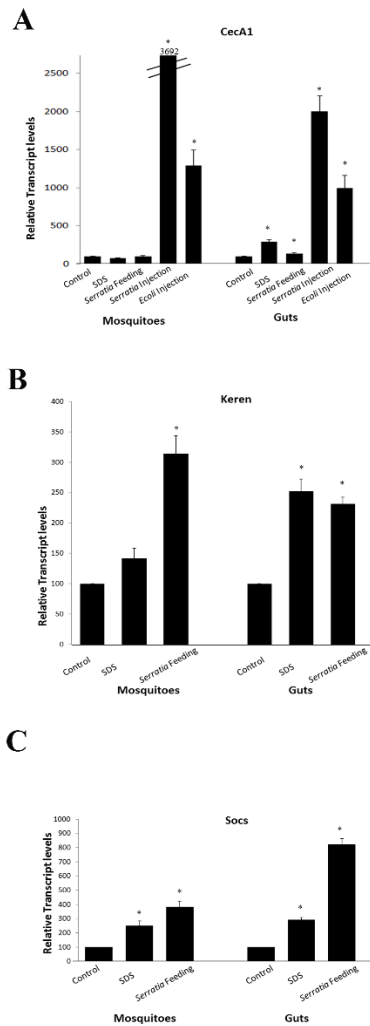
Figure 22: Transcriptional levels of genes encoding CecropinA1 AMP and signaling molecules belonging to the EGFR and JAK/STAT pathways after gut damage.
Feeding *Aedes* mosquitoes on sucrose containing 2% SDS or a high concentration of *Serratia marcescens* (OD = 50) leads to a slight increase in the transcription of the antimicrobial peptide CecA1 encoding gene in the gut. However, this increase was not observed in whole insects, where the levels of AMP transcripts are highly induced only when bacteria (*Serratia* or *E. coli*) is microinjected into the mosquito body cavity causing a systemic immune response (A). Real time qPCR performed on whole mosquitoes or on dissected guts shows that feeding on *Serratia* results in a significantly increased transcription, both at the level of the gut and in whole animals, of the signaling molecules *Keren* (B) and *Socs* (C) that are known to regulate in Drosophila the EGFR and JAK/STAT pathways respectively. SDS feeding also led to a significant increase of Keren and Socs at the level of the gut (B and C). *P < 0.05.

4. *Discussion*

Mosquitoes are one of the deadliest insects responsible of the transmission of diseases that have dramatic impacton human health. In total, vector-borne diseases account for approximately 17% of the estimated burden of allinfectious diseases[313]. Pathogens such as bacteria, viruses and parasites complete part of their life cycle in theinsect midgut, where they are confronted to mosquito-encoded barriers and host effectors that can restrict theirdevelopment. Therefore, the characterization of the cellular and molecular mechanisms required to maintainnormal mosquitoes gut structure and function is highly demanded and could provide novel control strategies ofdiseases vectors.

In this study, we have investigated the ability of the adult *A. albopictus* gut to regenerate in response to chemicalor bacterial challenges. We were able to show the existence of small proliferative cells in the midgut of *A. albopictus*, which are probably intestinal stem cells. These cells showed a regenerative behavior in response tolocal gut damages induced either by chemical compounds or by enteric bacterial infections. At themolecularlevel, the gene expression of known components of the JAK/STAT and EGFR pathways were significantly inducedin response to gut damages. We did also observe a clear difference in gut size and proportions (especially in themidgut) between males and females, but this is not surprising since males and females have different feedinghabits.

SDS feeding triggered a moderate upregulation of the genes encoding the signaling molecules *Keren* (approximately2.5 folds) and *Socs36E* (approximately 2.9 folds) in the gut of *A. albopictus*. These results indicate thatsignaling via EGFR and JAK/STAT

pathways is due to gut damage and the possible entry of bacteria from theintestinal lumen to the body cavity. This signaling is enhanced by the presence of pathogenic bacteria and theimmune response it triggers. Indeed, *Serratia* feeding causes a higher activation of transcription of *Socs36E* andof *Keren*.

Our findings are in agreement with what has been observed in *Drosophila melanogaster*, despite some strikingdifferences between *A. albopictus* and *D. melanogaster* genomes: the DNA content of *Aedes* genome is more than10 times that of *Drosophila* [314].Noteworthy, no orthologous counterpart for several *Drosophila* key immunity genes(such as the *upd* genes) were identified in *A. albopictus*, possibly due to gaps in the first release of the genome.The identification of more orthologues to *Drosophila* genes that participate in pathways controlling intestinalcell division and differentiation is crucial in order to characterize the gut regenerative response in *A. albopictus*.This would help also to describe in an exhaustive manner the intestinal stem lineage in A. albopictus. On anotherhand, a reverse genetics approach should be followed to achieve candidate gene knock-down and determine thecontribution of each pathway to the regulation of intestinal stem cell activity.

Extensive published findings from the *Drosophila* model will certainly help to a better characterization of themechanisms underlying mosquitoes gut homeostasis[315]. Our study provides evidence for the existence of regenerativecells and shows that they proliferate in damaged *Aedes* guts. These results should contribute to a betterunderstanding of how the gut homeostasis is maintained and, together with a more

in-depth characterizationof mosquito's immune responses, should pave the way for the development of alternative control strategies oftheses disease vectors.

2. Comparative Analysis of Midgut Regeneration Capacity and Resistance to Oral Infection in Three Disease-Vector Mosquitoes

The results of this study have been submitted and are currently under revision.

Included here are the results as sent for publication.

1. *Abstract*

Mosquitoes are well known vectors of human and animal diseases. Understanding mosquito biological defenses is an important step towards reducing disease transmission by these vectors. Mosquitoes acquire the pathogens they transmit through ingestion. Therefore, the insects' gut constitutes the first line of defense against invading pathogens. The gut epithelium acts as a physical barrier, activates local antimicrobial peptides production and triggers the systemic immune response. Consequently, gut epithelium is constantly confronted to stress and often suffers cellular damage. We have recently shown that regenerative cells are present in the guts of adult *Aedes albopictus*, and that chemical damage or bacterial infection leads to the proliferation of these regenerative cells in the midgut. In this study we extended the analysis of gut cells response to stress to two other important disease vector mosquitoes: *Culex pipiens* and *Anopheles gambiae*.To investigate the presence of mitotic cells in the midguts, we fed mosquitoes on sucrose solutions or on sucrose supplemented with pathogenic bacteria or with damage-inducing chemicals. We also observedthe survival of mosquitoes following the

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ingestion of pathogenic bacteria. We found that in adult *C. pipiens*, dividing cells exist in the digestive tract and that these cells proliferate in the midgut after bacterial or chemical damage, similarly to what we previously observed in *A. albopictus*. In sharp contrast, we did not detect any mitotic cell in the midguts of *A. gambiae* mosquitoes, neither in normal situation nor after the induction of gut damage. In agreement with this observation, *A. gambiae* mosquitoes were more sensitive to oral bacterial infections compared to *A. albopictus* and *C. pipiens*.This work provides evidence that major differences in gut physiological responses exist between different mosquito species. The presence of regenerative cells in the mosquito guts and their ability to multiply after gut damage affect the mosquito survival to oral infections, and is also likely to affect its vectorial capacity.

2. *Introduction*

Mosquitoes are important vectors of human and animal diseases. In Lebanon, several mosquito species have been identified; some of which are disease vectors.These include the Asian tiger mosquito *Aedes albopictus*[288]and the urban *Culexpipiens*populations – often called the house mosquitoes.*C. pipiens*is a burden all year long and the *A. albopictus* population size has been increasing in the last decade [316]. *Anopheles gambiae* transmits malaria and is probably the most studied insect vector [285, 286]; luckily, it is not present in the Mediterranean area. *Culex* mosquitoes are known worldwide to transmit to human several pathogens such as the yellow fever virus and filarial nematodes[284]; *A. albopictus* is a known vector for several viruses including Chikungunya, Dengue and Zika[288].Cases of mosquito-borne diseases are still

very rare in Lebanon, but the presence of endogenous mosquito vectors together with climatic warming and people increased mobility may dramatically change the *status quo* in the near future. Classical control strategies relying on the use of chemical insecticides often lead to the selection for resistant mosquitoes and have a negative impact on the environment[317, 318]. Therefore the development of effective mosquito control methods is needed [319, 320], and understanding the insect physiology and natural defenses is a valuable element in this perspective.

Mosquitoes and invertebrates depend on their innate immune system to fight pathogens[289, 290]. Several responses have been characterized in mosquitoes including phagocytosis[321], antimicrobial peptide production [322], and melanization [127, 323].The mosquito gut acts as an early immune barrier: it is exposed to both symbiotic microorganisms and pathogens present in ingested food. In addition, gut cells are confronted to the immune effector molecules produced by the insect itself [324].Consequently, the gut faces stress and possibly biological damage, which results in a massive loss of enterocytes[325]. In response, homeostatic repair pathways leading to the preservation of epithelial integrity are activated. This involves the regulation of intestinal stem cells (ISCs) that are necessary for gut regeneration. In *Drosophila melanogaster*, ISCs can proliferate quickly and massively so that enterocytes are completely regenerated in less than 60 hoursin damaged midguts [325-327].

During insects' metamorphosis, gut larval tissue is almost entirely autolysed and replaced by adult new tissue. Some studies have focused on the guts of mosquito larvae [328], but little information is available concerning gut regeneration in adults.Few studies reported the presence of ISCs in adult mosquitoes' guts. The old reports were based on

morphological characteristics and include a study of gut wound healing in *Aedes aegypti* and a study that revealed the presence of proliferating cells in the guts of *Culex tarsalis* guts after blood ingestion [329-332].More recently, we have shown that chemical damage or bacterial infections lead to regenerative cell proliferation in the midgut of adult *A. albopictus*[333]. A similar analysis revealed the presence of adult intestinal stem cells in *A. aegypti*[334].

In the present study, we extended the analysis of adult gut regeneration to two other important disease vector mosquitoes: *C. pipiens* and *A. gambia*e. We found that in adult *C. pipiens,* dividing cells exist in the digestive tract and that these cells proliferate in the midgut after the ingestion of pathogenic bacteria or damaging chemicals, similarly to what we previously observed in *A. albopictus*. In contrast, we did not detect any mitotic cell in the midguts of *A. gambiae* mosquitoes, neither in normal situation nor after the induction of gut damage. We also show that in agreement with this observation, *A. gambiae* mosquitoes were more sensitive to oral bacterial infections compared to *A. albopictus* and *C. pipiens*.

This work provides evidence that major differences in gut physiological responses exist between different mosquito species. The presence of regenerative cells in the guts of adult mosquitoes and their ability to multiply after gut damage affect the mosquito survival to oral infections, and is also likely to affect its vectorial capacity. We expect the results of this study to have implications for vector control methods.

3. Results

a. <u>General structure of the adult mosquito guts</u>

We compared the general gut structure of *C. pipiens* and *A. gambiae* to that of *A. albopictus*. Figure 6 shows the structure of both male and female guts as revealed by scanning electron microscopy for *A. albopictus* (23A, 23B), *C. pipiens* (23D, 23E) and *A. gambiae mosquitoes* (23G, 23H). The gut epithelium is surrounded by visceral muscles and connected to tracheal branches that allow gas exchange. For the three mosquito species, only two of the three main gut compartments are visible: the hindgut with the associated malpighian tubules and the midgut. The foregut and the crop are fragile structures that are lost during the treatment of the samples in preparation for electron microscopy. A clear difference between male and female guts is visible, male guts being overall smaller. In particular, the midgut compartment is less developed in males as compared to females (figure 23A and 23B, 23D and 23E, 23G and 23H). This is in agreement with the fact that male mosquitoes feed on sugars only while female mosquitoes require a protein-rich blood meal to produce eggs, imposing on female guts the burden of performing more complex digestive functions. We can also note a higher degree of similarity between the guts of *C. pipiens* and *A. albopictus* females (figure 23B, 23C and 23E, 23F), while the guts of *A. gambiae* females present an anatomical structure that is more divergent (figure 23H, 23I).
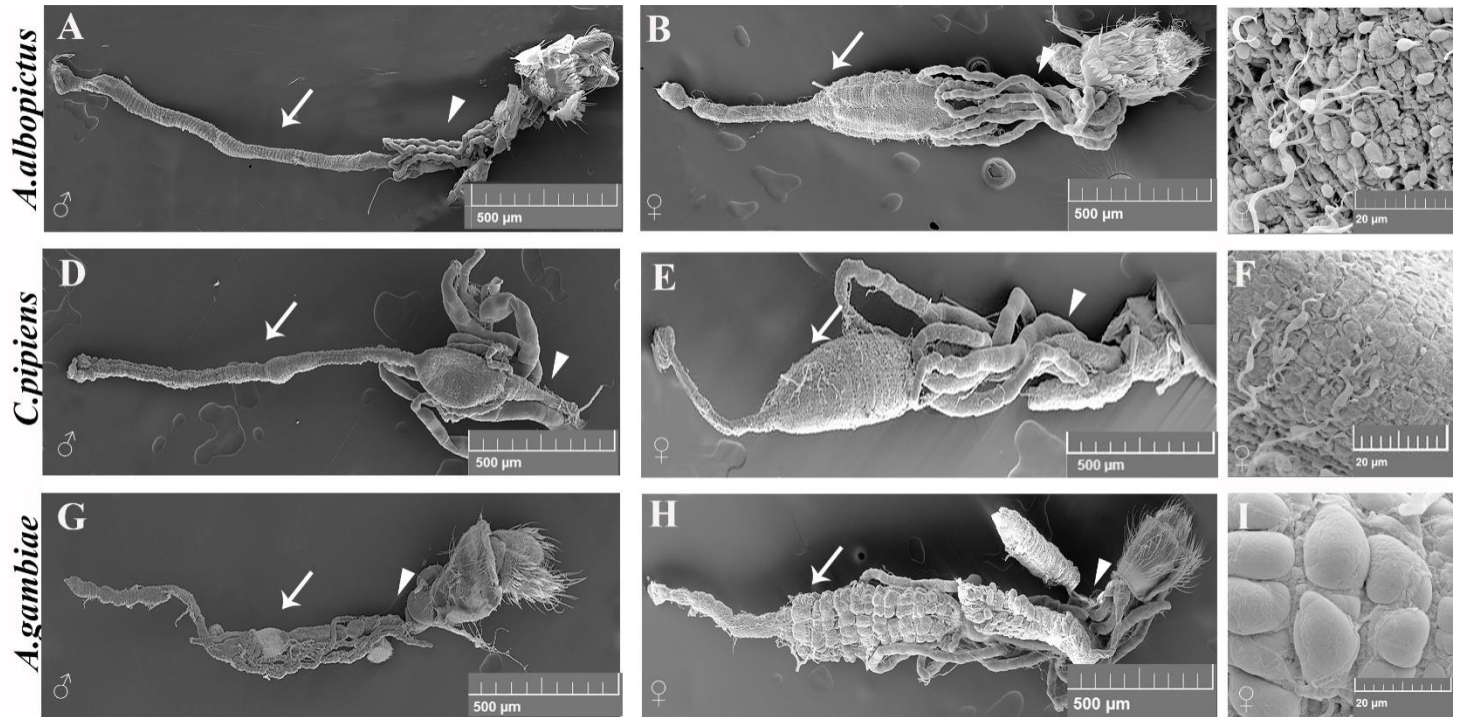
Figure 23: Scanning Electron micrographs (SEM) of *A. albopictus*, *C. pipiens* and *A. gambiae* mosquito guts.
These photos depict the structure of male and female guts respectively as revealed by scanning electron microscopy for *A. albopictus* (23A, 23B), *C. pipiens* (23D, 23E) and *A. gambiae* mosquitoes (23G, 23H). A magnification of the female midgut compartment is shown in 23C for *A. albopictus*, 23F for *C. pipiens* and 23I for *A. gambiae*. Two of the three gut main compartments are visible: the hindgut with the associated malpighian tubules (arrowhead) and the midgut (arrow).

b. <u>Proliferating cells are present in the guts of *A. albopictus* and *C. pipiens* but are not detectable in *A. gambiae* guts</u>

Adult females of the three mosquito species were starved for two hours before being allowed to feed either on sucrose (control), sucrose supplemented with *Serratia marcescens,* or sucrose supplemented with Sodium Dodecyl Sulfate (SDS) to induce gut damage [68, 333-335]. Mosquito guts were dissected 24 hours post-treatment and fixed. Immunohistochemistry was then performed using anti-phospho-histone H3 protein antibodies (anti-PH3), a specific marker of mitotic cells [336, 337].Similarly to what we previously reported for *A. albopictus* [333], we observed a number of small cells with clear PH3 signal in the midguts of control *C. pipiens* midguts (figure 24A and 24D). Some PH3-positive nuclei were observed in pairs, an arrangement characteristic of two sister cells derived from the recent division of a progenitor mother cell. In contrast, the anti-PH3 antibodies were not able to detect any dividing cell in the midguts of *A. gambiae* (figure 24G).

When we compared the guts of mosquitoes fed on sucrose containing either SDS or *S. marcescens* to the control guts, they appeared damaged and distorted (figure 24D and data not shown). Consistent with that, we observed an increase in the number of PH3 positive cells (figure 24B, 24C, 24E and 24F) in the damaged *A. albopictus*and *C. pipiens* guts as compared to control guts (figure 24A and 24D).These findings suggest that SDS or pathogenic bacteria feeding induces gut damage and results in the activation of local regenerative processes. In contrast, we were not able to detect any mitotic cell in the

midguts of *A. gambiae* mosquitoes, neither in normal situation nor after the induction of gut damage (figure 24H and 24I).

To make sure that *A. gambiae* mosquitoes fed on the damaging supplements, we added food colorant to the sucrose solution. We indeed observed blue color in their guts indicating that they did not refrain from feeding when *S. marcescens* or SDS was added to the sucrose solution (figure 25A and 25B). Moreover, the gut of SDS fed *A. gambiae* mosquitoes presented irregular structures as revealed by phalloidin staining confirming that damage to the gut did occur (figure 25D).
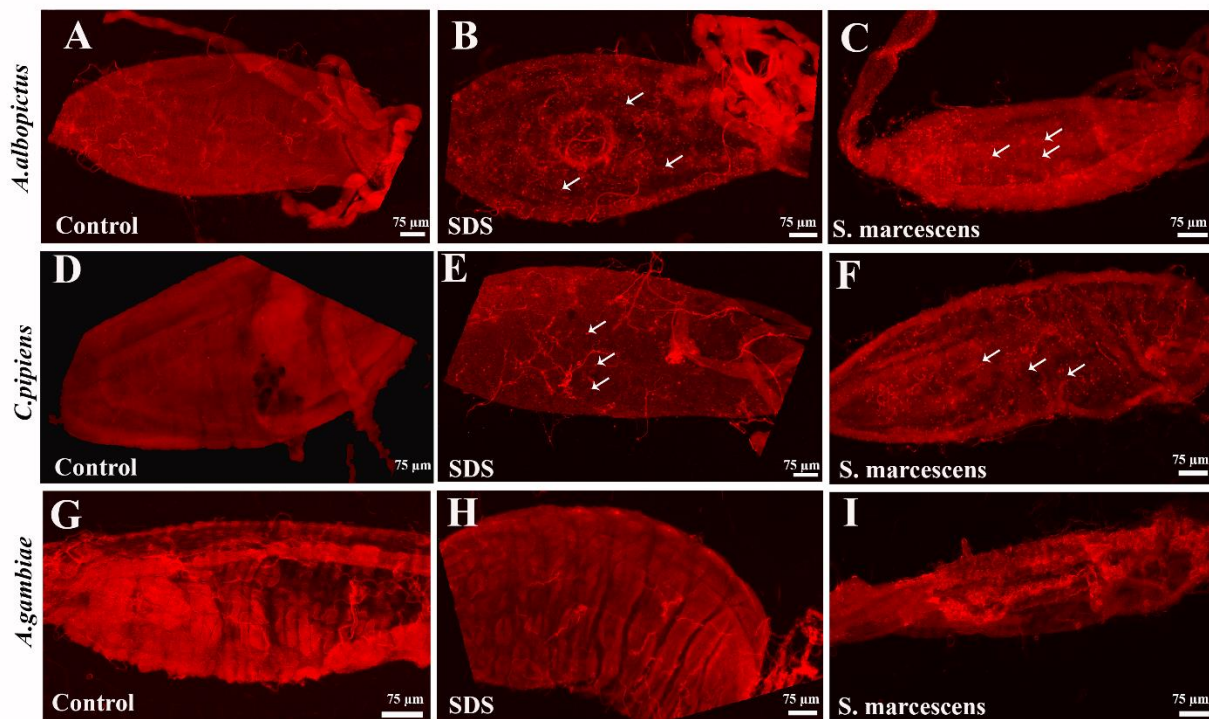
Figure 24: IFAs showing staining for mitotic cells in the guts of *A. albopictus*, *C. pipiens* and *A. gambiae* mosquitoes.
Antibodies raised against phospho-histone H3 protein (anti-PH3) show that replicative cells are present in the midguts of both *A. albopictus* (24A) and *C. pipiens* female mosquitoes (24D). These replicative cells seem more abundant after feeding the mosquitoes on sucrose solutions supplemented with SDS (24B and 24D), or with *S.*

marcescens (24C and 24F). Arrows point to representative PH3-positive cells. In contrast, no PH3-positive cells were detected in the guts of control *A. gambiae* mosquitoes (24G), nor in the midguts of *A. gambiae* fed on sucrose solutions supplemented with SDS (24H) or with *S. marcescens*(24I).
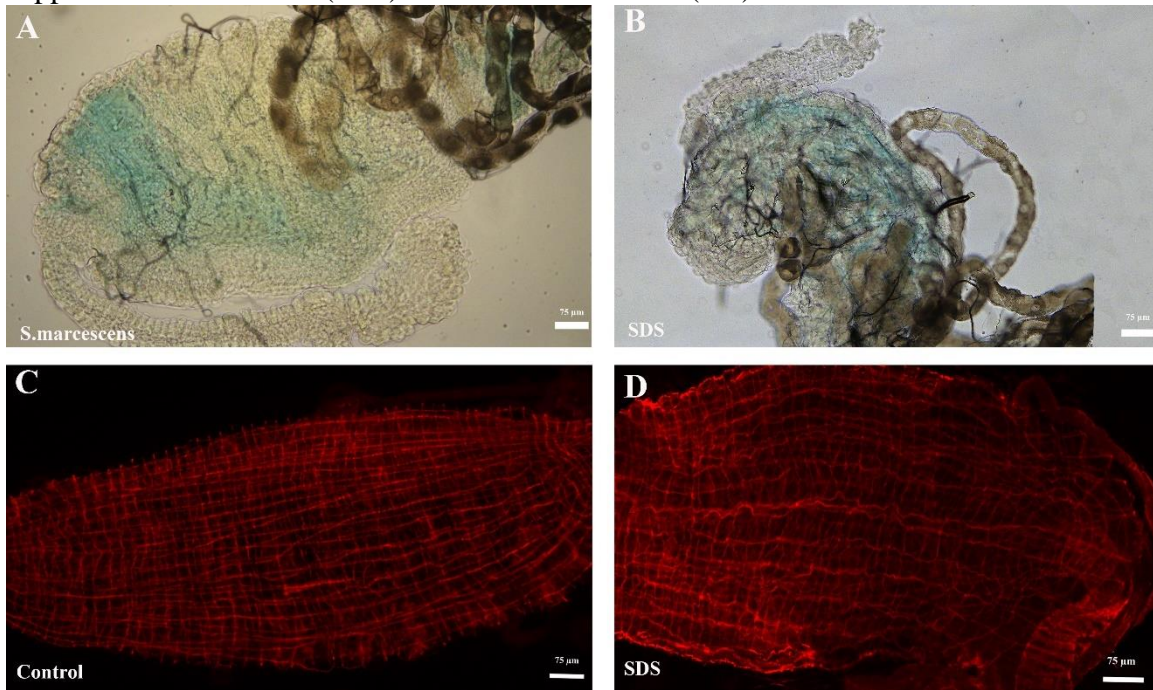
Figure 25: *A. gambiae* **mosquitoes are ingesting the sucrose containing bacteria or SDS and are suffering gut damage.**
Food colorants were added to sucrose solutions supplemented with *S. marcescens* or SDS and fed to *A. gambiae* mosquitoes. 24 hours later, dissected revealed a blue coloration, indicating that the mosquitoes had indeed ingested the *S. marcescens* supplemented sucrose solutions (25A) and not died of starvation. SDS feeding led to the death of most *A. gambiae* mosquitoes after 24 hours, but the dead mosquitoes showed blue coloration at the level of their guts (25B), proving that they had ingested the SDS supplemented solution. Staining with fluorescent phalloidin (a marker that labels the F-actin filaments) reveals that the guts of mosquitoes fed on sucrose supplemented with SDS present a distorted and irregular structure (25D) as compared to the guts of mosquitoes fed on sucrose (25C).

   c.   <u>Ingestion of SDS or pathogenic bacteria increases the numbers of mitotic cells in the midguts of *A. albopictus* and *C. pipiens*</u>

We quantified the numbers of PH3-positive cells per midgut epithelium of mosquitoes fed on sucrose, SDS containing sucrose or *S marcescens* containing sucrose. For each condition, at least 20 guts were analyzed. Cell counts were plotted using the GraphPad Prism software and results are shown in figure 26. Feeding *A. albopictus* mosquitoes sucrose solutions supplemented with SDS induced a significant increase in the number of replicative cells at the level of the guts (27.05 ±1.74; n=20) as compared to sucrose fed mosquito guts (8.06 ±0.51; n=33).This increase was also significant after feeding mosquitoes sucrose solutions supplemented with *S. marcescens* (26.96 ±1.39; n=27) (figure 26). Similarly, *C. pipiens* mosquitoes exhibited a significant increase in the number of replicative cells (19.37 ±1.26; n=30) after being fed sucrose solutions supplemented with SDS, compared to control sucrose fed mosquito guts (5.21 ±0.66; n=38). The guts of *C. pipiens* mosquitoes fed on sucrose solutions supplemented with *S. marcescens* also showed a significant increase in the number of replicative cells (29.67 ±1.65; n=39) when compared to the control guts (figure 26B). This response was not observed with *A. gambiae* mosquitoes fed sucrose solutions supplemented with either SDS or *S. marcescens* (figure 26C).We observed similar proliferative effects when the mosquitoes were fed on sucrose supplemented with paraquat (another chemical used to induce gut stress in *D.* melanogaster)[282, 338] (supplementary figure 1B, 1E and 1H) or sucrose supplemented with *Erwinia carotovora carotovora 15* (*Ecc15*) another strain of bacteria classically used in the lab to study insect immunity (supplementary figure 1C, 1F and 1I). In all cases, no mitotic cells were detected in the midguts of *A. gambiae*, despite the fact that the mosquitoes ingested the stress inducing substance (figure 25A and 8B) and that their guts were damaged (figure 25D). In addition, anti-PH3 antibodies

successfully labeled mitotic cells in *A. gambiae* other tissues, in particular in the ovaries (supplementary figure 2). These results suggest that damaging the gut of mosquitoes triggers an intrinsic increase in cell proliferation in *A. albopictus* and in *C. pipiens* and that there is a major difference in the gut's response to damage between these two mosquito species and *A. gambiae*.
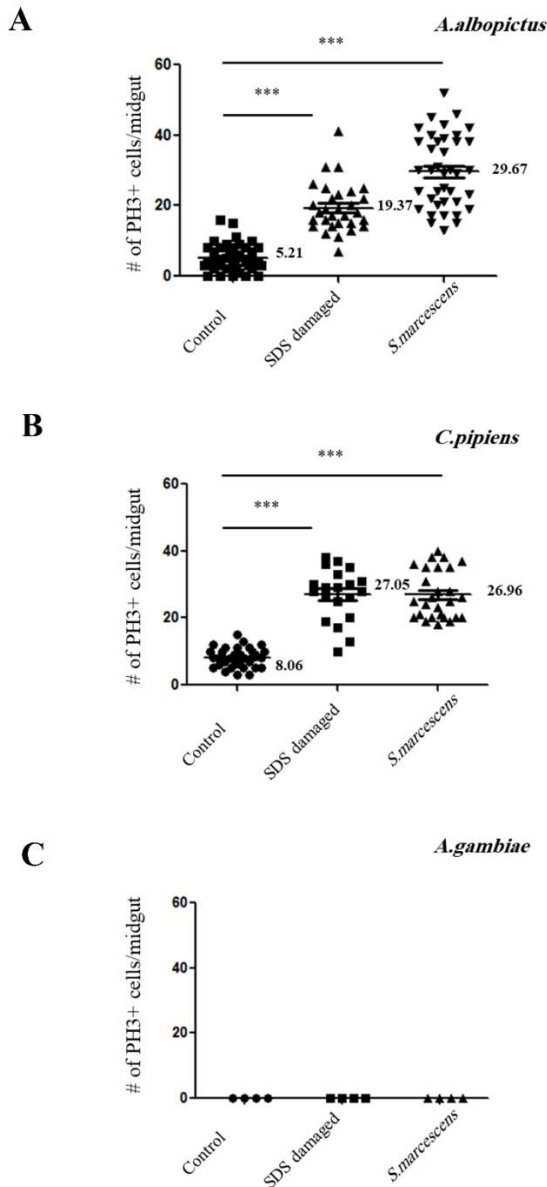
Figure 26: The number of mitotic cells per midgut increases significantly after damage in *A. albopictus* and *C. pipiens* mosquitoes.

Feeding *A. albopictus* mosquitoes sucrose solutions supplemented with SDS induced a significant increase ($p < 0.0001$) in the number of replicative cells at the level of the guts (27.05 ±1.74; n=20) as compared to sucrose fed mosquito guts (8.06 ±0.51; n=33). This increase was also significant ($p < 0.0001$) after feeding sucrose solutions supplemented with *S. marcescens* (26.96 ±1.39; n=27) (26A). *C. pipiens* mosquitoes exhibited a similar response after feeding on sucrose solutions supplemented with SDS (19.37 ±1.26; n=30)

with a significant increase in the number of replicative cells ($p < 0.0001$) when compared to control sucrose fed mosquito guts (5.21 ±0.66; n=38). Guts of *C. pipiens* fed sucrose solutions supplemented with *S. marcescens* (29.67 ±1.65; n=39) also showed a significant increase in the number of mitotic cells ($p < 0.0001$) when compared to the control guts (26B). This response was not observed with *A. gambiae* mosquitoes fed sucrose solutions supplemented with either SDS or *S. marcescens*(26C).

   d.   <u>Differences in mosquito survival after feeding on SDS or pathogenic bacteria</u>

The survival of *A. albopictus*, *C. pipiens* and *A. gambiae* mosquitoes was monitored after feeding on sucrose solutions supplemented with SDS (figure 27A), *S. marcescens* (figure 27B) or *Ecc 15* (figure 27C). Feeding with SDS induced a significant decrease in the survival of mosquitoes when compared to control sucrose fed mosquitoes in all three mosquitoes (figure 27A). When we compared survival amongst the three mosquito species, *A. gambiae* mosquitoes showed the most compromised survival after SDS challenge, and this increased susceptibility was statistically significant when compared to both *A. albopictus* or *C. pipiens*. The difference in survival between *A. albopictus* or *C. pipiens* after SDS challenge was also significant, with *A. albopictus* mosquitoes surviving better. Similarly, *S. marcescens* feeding induced a significant decrease in the survival of mosquitoes when compared to control sucrose fed mosquitoes for all three species (figure 27B).*A. gambiae* showed the highest mortality when compared to *A. albopictus* or *C. pipiens*, whereas the difference in survival between *A. albopictus* and *C. pipiens* was not significant ($p = 0.1739$). Similar results were observed after *Ecc 15* feeding when we compared survivals of the three mosquitoes fed with the bacteria to their control sucrose fed mosquitoes: *A. gambiae* mosquitoes showed strongly compromised survival when compared to the two others, while the difference in survival

between *A. albopictus* and *C. pipiens* was not significant (p = 0.6544). Altogether, the survival assays indicated that, among the three mosquito species studied, *A. gambiae* were the most fragile.

A

B

C
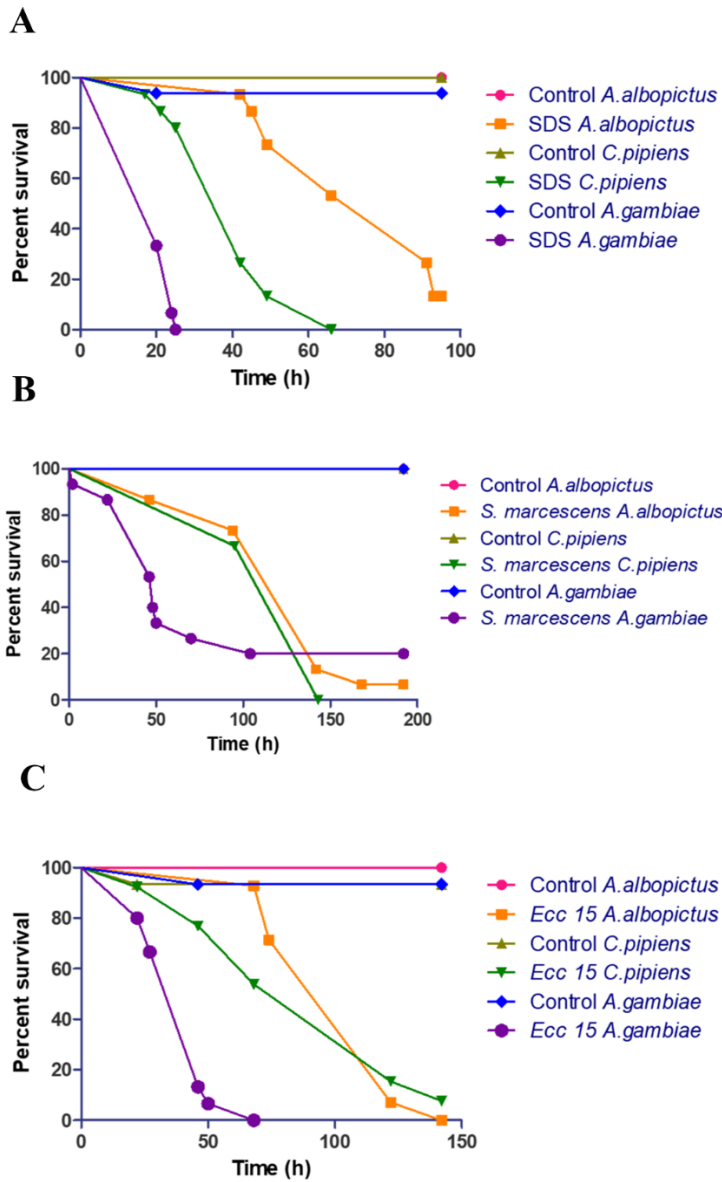
Figure 27: Survival of mosquitoes after feeding on sucrose solutions supplemented with SDS, *S. marcescens* **or** *Ecc 15*.
The survival of *A. albopictus*, *C. pipiens* and *A. gambiae* mosquitoes was monitored after feeding on sucrose solutions supplemented with SDS (27A), *S. marcescens* (27B) or *Ecc 15* (27C). The experiments were done in triplicates with 15 females for each mosquito species per experiment, and the rates of survivals were plotted as function of time. One

representative graph is shown. *A. gambiae* mosquitoes were clearly more sensitive to the three stress-inducer supplements than *A. albopictus* and *C. pipiens*. All statistically significant differences had a p value smaller than 0.001 (p < 0.001).

4. *Discussion*

Due to their ability to transmit various diseases to their vertebrate hosts, mosquitoes represent a serious health threat for humankind. Indeed, it is estimated that approximately one fifth of all people dying of an infectious disease, are dying due to a vector transmitted one [339]. Mosquito-transmitted pathogens and parasites complete part of their life cycle in the insect midgut [340, 341].The midgut is not a passive structure that allows the easy development or passage of pathogens. It constitutes a very hostile environment to the invaders where they are confronted to mosquito-encoded barriers and effector molecules that can restrict their development[13, 14]. For example, *Plasmodium* suffers from the *A. gambiae* response and the number of ookinetes is severely reduced at the level of the mosquito midgut, which creates a bottleneck effect at this stage of the parasite life cycle [342]. The understanding of the cellular and molecular mechanisms that are activated in order to maintain mosquitoes gut homeostasiscan be highly valuablefor the elaboration of novel control strategies of diseases vectors.

The abundant and powerful genetics tools available in *Drosophila* are lacking in mosquitoes. Often scientists rely on findings and extrapolate results in the analysis of other dipteran: for instance, *D. melanogaster* was a landmark model for insect immunity. The mechanisms controlling gut homeostasis have been an active topic of research in the last few years [343] and this can certainly contribute to the understanding of how gut integrity is maintained in mosquitoes. This approach however has its limitation, and it is

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useful to study certain processes directly in organisms of interest, especially that the feeding habits of *Drosophila* are different from those of hematophagous insects such as mosquitoes. We have recently investigated the regeneration of the adult *A. albopictus* guts in response to chemical or bacterial damages. We demonstrated the existence of small mitotic cells in the midgut of*A. albopictus* mosquitoes that have the characteristics of intestinal stem cells[333]. These results were also observed in another closely related mosquito *A. aegypti*[334].

In the present study, we extended the analysis of gut cells response to stress to two other important disease vector mosquitoes. For the three mosquito species, we observed a clear difference in gut size and proportions (especially in the midgut) between males and females, but this difference is expecteddue to the different diets of males and females. We found an important difference between the different mosquitoes' response to gut damage: we did not detect any mitotic cell in the midguts of *A. gambiae* mosquitoes, neither in normal situation nor after the induction of gut damage, while in *C. pipiens,* dividing cells exist in the digestive tract and proliferate in the midgut after bacterial or chemical damage, similarly to what was previously observed in *A. albopictus* and *A. aegypti*.

We showed that *A. gambiae* mosquitoes are more sensitive to oral bacterial infections when compared to *A. albopictus* and *C. pipiens.* We propose that the high sensitivity of *A. gambiae* mosquitoes to oral infections is probably due – at least partly- to the incapacity of *A. gambiae*to activate cell division to repair gut damage. Taracena et al. suggested that fast midgut regeneration is a contributing factor to the refractoriness of certain strains to arboviruses infection, while permissive strains lack the capacity to

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quickly activate the program of gut cell division[334]. It will be interesting to examine

this by comparing gut regeneration of different *A. gambiae*strains, especially that the G3

strain we studied is known to be permissive to *Plasmodium*[344, 345].

5. *Conclusion*

We investigated the presence of mitotic cells in the guts of different mosquito

species and studied the response of the gut cells to bacterial and chemical damage. We

foundthat major differences in gut physiological responses exist among different

mosquitoes. The presence of regenerative cells in the mosquito guts and their ability to

multiply after gut damage probably affect the mosquito survival to oral infections and

could also affect its vectorial capacity. These results, with a more in

depthcharacterization of mosquito's immune responses, and with an analysis of the

genetic pathways that control the differences between different mosquito species should

contribute to the development of alternative control strategies of theses disease vectors.

3. Characterization of the role of microflora in the regeneration of *Culex pipiens* mosquitoes

While performing the experimental work for the previous part related to the comparative analysis of midgut regeneration in three mosquito species, we observed interesting results corresponding to the effect of the microflora on gut regeneration in *C. pipiens* mosquitoes. In *C. pipiens* larvae that were not treated with antibiotics, we observed a high number of proliferating cells at the level of the guts of control sucrose fed mosquitoes. Since this effect was only reversed upon administration of antibiotics in the trays where larvae were being maintained, we decided to investigate whether the microflora is inducing this effect.

1. *Antibiotic treatment of Culex pipiens larvae*

Immunohistochemistry analysis using the α-PH3 antibodies on *Culex pipiens* guts fed on sucrose revealed the presence of a relatively high number of PH3+ cells. To further understand this result, we decided to treat the larvae with a mixture of kanamycin, penicillin and streptomycin. Upon emergence of the treated adults, we performed immunohistochemistry analysis using the α-PH3 antibodies, on sucrose fed non-treated *C. pipiens* mosquito guts, SDS fed non-treated mosquito guts, sucrose fed antibiotic-treated mosquito guts and SDS fed antibiotic-treated mosquito guts.
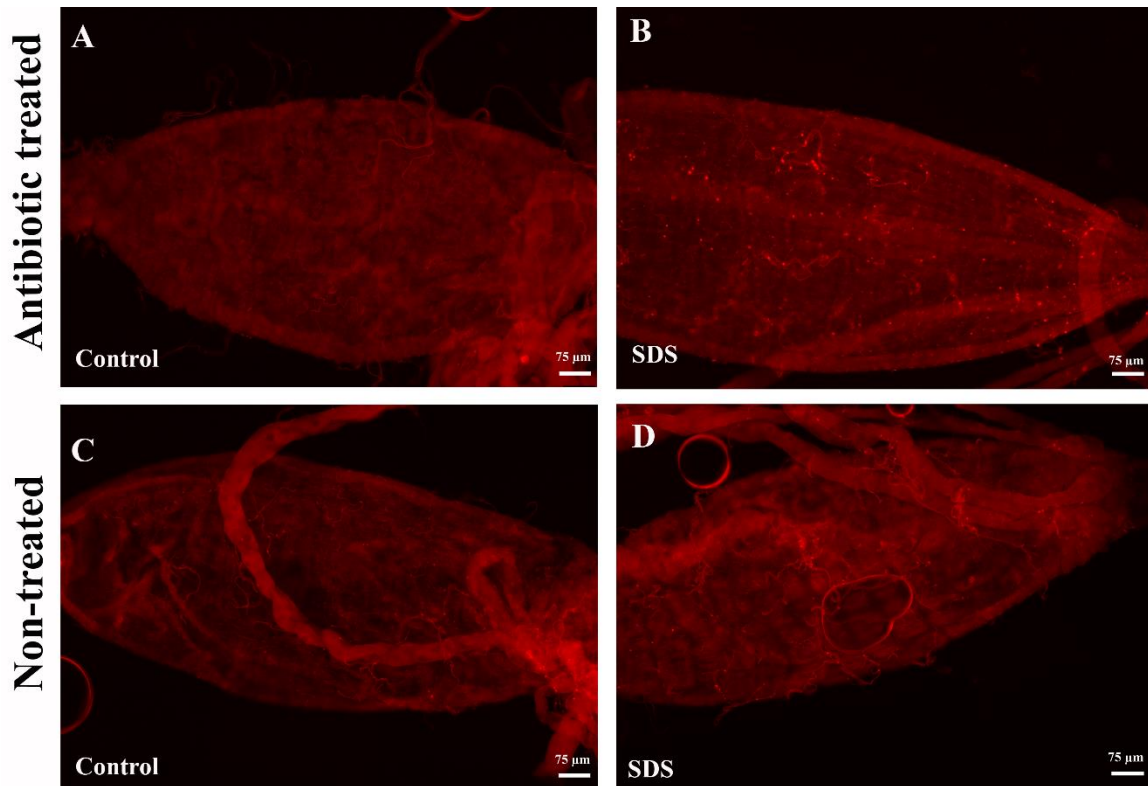
Figure 28: Immunohistochemistry staining of *C. pipiens* mosquito guts reveal a high number of proliferating cells at the level of control non-treated mosquito guts. Antibodies raised against phospho-histone H3 protein (anti-PH3) show that replicative cells are present in the midguts of control *C. pipiens* female mosquitoes (28C). These cells are less abundant in control antibiotic-treated *C. pipiens* mosquitoes (28A). These replicative cells seem more abundant after feeding the mosquitoes on sucrose solutions supplemented with SDS (28C and 28D).

A relatively high number of PH3+ cells was observed in both sucrose fed *C. pipiens* mosquito guts (28C) and SDS fed mosquitoes with no statistical difference (28D). Following antibiotic treatment of the larvae, the sucrose fed antibiotic-treated mosquito guts revealed a very low number of PH3+ cells (28A), which increased significantly upon ingestion of SDS (28D). This indicates that the high number of PH3+ cells at the level of sucrose fed non treated mosquitoes might be due to microflora presence.

2. *Quantification of PH3+ cells in control non-treated and antibiotic-treated C.pipiens guts*

We then quantified the number of PH3+ cells at the level of control sucrose fed

antibitotic-treated and non-treated *C. pipiens* mosquito guts, to check whether the

difference observed is a significant one.
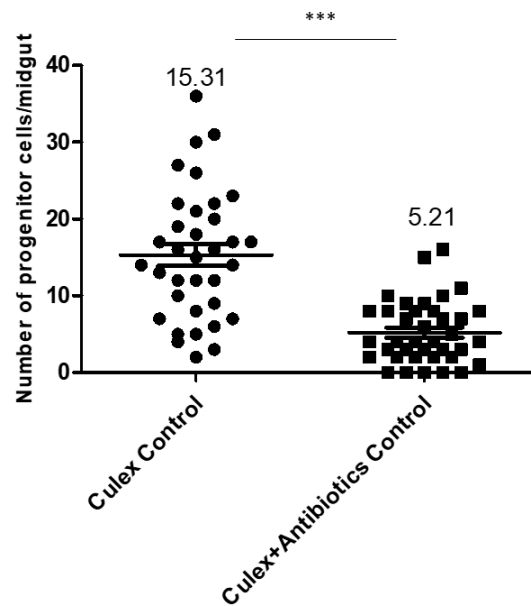
Figure 29: The number of PH3+ cells is significantly higher in control non-treated mosquito guts as compared to antibiotic-treated control mosquito guts.
The number of dividing cells stained by anti-PH3 antibodies per midgut is counted and statistical analysis confirms a significant difference in the number of proliferating cells in control non-treated guts as compared to control antibiotic-treated ones. The number of guts analyzed is n = 35 for non-treated guts and n=38 for antibiotic-treated ones. ***P < 0.001

The average of PH3+ cells detected at the level of control non-treated *C. pipiens* guts is

15.31, whereas it is 5.21 at the level of control antibiotic-treated *C. pipiens* mosquitoes.

The difference observed is statistically significant with a p-value < 0.0001. This suggests that the gut microflora might be inducing the proliferation of mitotic cells.

3. *Phalloidin staining of control antibiotic-treated and non-treated C. pipiens mosquito guts*

Upon the observation that non-treated control mosquito guts presented a significantly higher number of PH3+ cells, we wanted to check the gut integrity and whether physically compromised guts were causing this proliferation of mitotic cells. To do so, we performed phalloidin staining on the guts of control antibiotic-treated and non-treated mosquito guts.
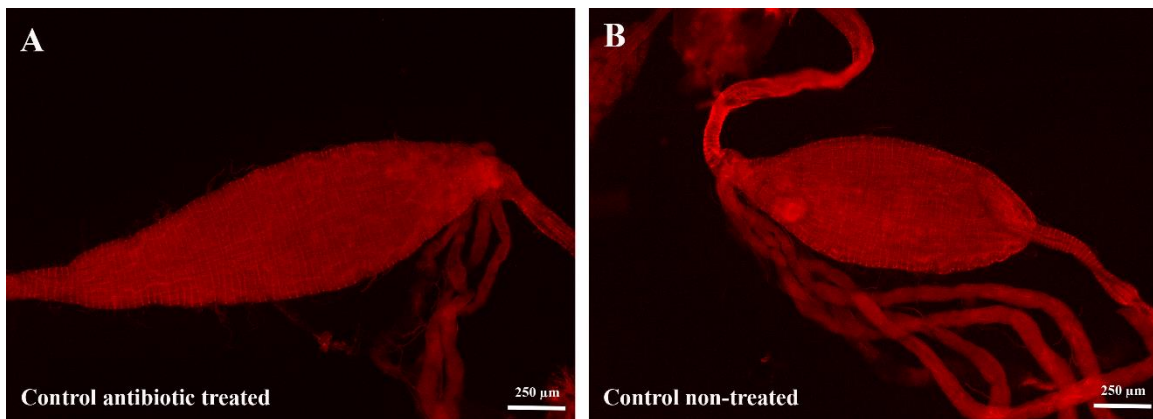
Figure 30: Phalloidin staining of *C. pipiens* antibiotic-treated and non-treated guts does not reveal any difference in gut integrity.

Phalloidin staining of dissected guts shows that the guts of antibiotic-treated mosquitoes (30A) do not have an altered structure as compared to non-treated mosquito guts (30B).

Phalloidin staining revealed that both antibiotic-treated and non-treated *C. pipiens* mosquito guts show a homogenous structure of the F-actin filaments, with no apparent distortion of the gut. No apparent difference can be observed between 30A and 30B. This suggests that the increase in proliferation of mitotic cells is not due to any structural damage of the guts.

4. ***Antibiotic-treated and non-treated C. pipiens mosquitoes show different survival trends in response to microbial and chemical feeding***

Next, we wanted to see whether the difference in the number of mitotic cells could be translated in a difference in survival rates in response to microbial and chemical feeding between the antibiotic-treated and non-treated mosquitoes.
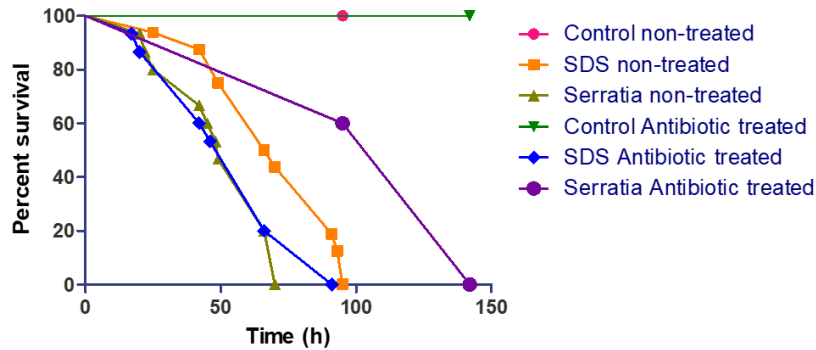
Figure 31: Survival rates of *Culex pipiens* mosquitoes in response to SDS and *S. marcescens* feeding.
The survival of antibiotic-treated and non-treated *C. pipiens* mosquitoes was monitored after feeding on sucrose solutions supplemented with SDS and *S. marcescens*. The experiments were done in triplicates with 15 females for each mosquito species per experiment, and the rates of survivals were plotted as function of time. One representative graph is shown.

Antibiotic-treated and non-treated *C. pipiens* mosquitoes were fed sucrose solutions supplemented with SDS or *S. marcescens*, and their survival rate was monitored over a span of several days. Both antibiotic-treated and non-treated *C. pipiens* mosquitoes fed sucrose solutions showed a similar survival trend, with no death observed over the monitored period of time. However, a significant difference was observed in the survival rates after SDS feeding, with the non-treated mosquitoes surviving better (p=0.04). The opposite was observed with *S. marcescens* feeding, with the antibiotic-treated mosquitoes surviving significantly better than the non-treated mosquitoes (p<0.0001).

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Future experiments will focus on the sequencing of the flora to identify the different bacterial strains present. We will also try to reintroduce flora into antibiotic-treated mosquitoes to see whether we could reproduce the same effect as with the non-treated mosquitoes. We will also quantify the levels of expression of antimicrobial peptides in antibiotic-treated and non-treated mosquitoes to see if the differences in the number of PH3+ cells are accompanied by a difference in the activation of AMPs at the basal levels and after infection.

4. **Characterization of the effect of different microbial infections on *A. albopictus* mosquitoes**

In order to study the immune responses of *A.albopictus* mosquitoes after microbial infections, we proceeded to infect female *A. albopictus* mosquitoes with a selection of microbes, either by microinjection into the thorax, or by natural feeding, then we characterized their tolerance and resistance to the infections. This was also followed by a quantification of the level of AMP activation after microbial infection.

1. ***A. albopictus mosquitoes show different tolerance to microbial infections depending on the method of infection and strain of bacteria used***

In order to study the tolerance of *A. albopictus* mosquitoes to bacterial infection, the mosquitoes were infected with different strains of bacteria, and the survival rate was monitored over a period of 12 days. Different ways of infection were used, either by microinjection through the thorax, or by feeding through the oral route to mimic the natural way of infection. *Staphylococcus aureus* (*S. aureus*) and *Enterococcus faecalis* (*E. faecalis*) were used as Gram-positive bacteria, *Erwinia carotovora carotovara 15* (*Ecc 15*), *Serratia marcescens* (*S. marcescens*) and *Escherichia coli* (*E. coli*) as gram-negative bacteria and *Beauveria bassiana* (*BB*) as fungi for microbial infections.

When infected by means of microinjection, infected mosquitoes succumbed to the infection by all types of microbes in a significant manner when compared to control mosquitoes, except after infection with the bacterium *E. coli*, where no significant mortality was observed after infection.
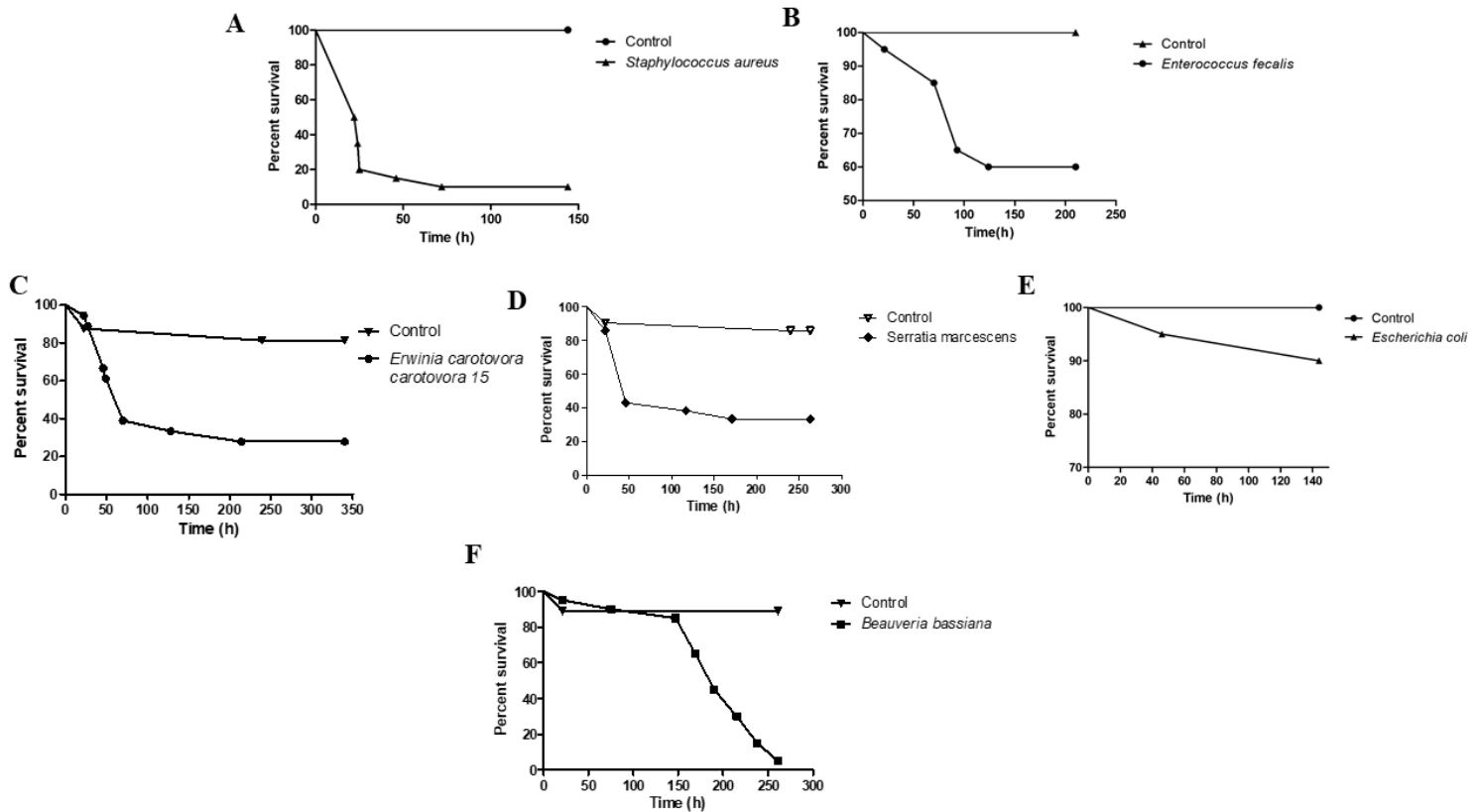
Figure 32: *A. albopictus* **survival rates vary in response to different bacterial strains.**
*A. albopi*ctus mosquitoes injected with the gram-positive bacterium *Staphylococcus aureus*(OD_{600nm} = 1) significantly compromised the survival of infected mosquitoes as compared to the control PBS injected mosquitoes (p<0.0001) **Figure 32A**. The same thing could be observed after injection of the gram-positive bacterium *Enterococcus fecalis* (OD_{600nm} = 0.5), with significant difference in the survival of *A. albopictus* mosquitoes (p = 0.0018) **Figure 32B**. Injection with the gram-negative bacterium *Erwinia carotovora carotovora 15*(OD_{600nm} = 0.5) **Figure 32C** resulted in a decrease of survival for the infected mosquitoes as compared to control mosquitoes (p= 0.0030). Injecting *A. albopictus* mosquitoes with the gram-negative bacterium *S. marcescens* (OD_{600nm} =0.001) leads to a significant decrease in the survival of infected mosquitoes as compared to control mosquitoes (p= 0.0009) **Figure 32D.** The gram-negative bacterium *Escherichia coli*(OD_{600nm} = 1) did not affect the survival of affected mosquitoes (p = 0.01546++) **Figure 32E.** Injection with the fungus *Beauveria bassiana* results in a significant decrease in the survival of infected mosquitoes as compared to control

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mosquitoes (p<0.0001)**Figure 32F.**The graphs are a representative of three independent biological replicates (n=20). The Kaplan-Meier survival test was used to calculate the percent survival. Statistical significance of the observed differences was calculated using the Log-rank test.

Mosquitoes were also fed *S.aureus*, *S. marcescens* and *Ecc 15* through the oral route to mimic the natural way of infection. Feeding the mosquitoes the microbes in a sucrose suspension also led to a significant decrease in the survival of infected mosquitoes **Figure 33.**
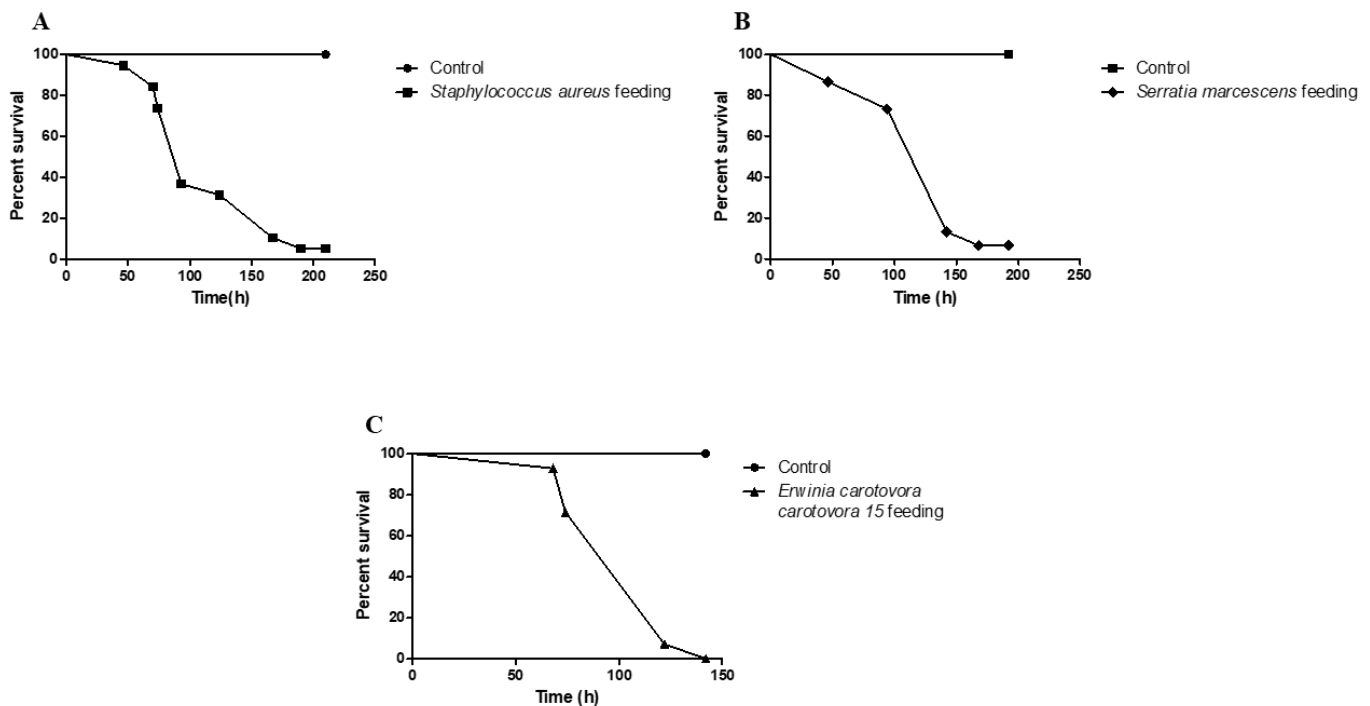
Figure 33: Bacterialfeeding induces death of infected mosquitoes.
Mosquitoes supplemented with sucrose solutions containing a suspension of *S. aureus* (OD_{600nm}=50) **Figure 33A** showed a decrease in survival as compared to control sucrose fed mosquitoes (p<0.0001). Sucrose solutions supplemented with *S.marcescens* also led

to a significant decrease in the survival of infected mosquitoes **Figure 33B** (p<0.0001). Feeding the mosquitoes with *Ecc 15* also led to similar results, with a significant decrease in the survival of infected mosquitoes **Figure 33C** (p=0.003) The Kaplan-Meier survival test was used to calculate the percent survival. Statistical significance of the observed differences was calculated using the Log-rank test. A representative of three independent biological replicates is shown.

2. ***A. albopictus* mosquitoes show different resistance trends to microbial infections depending on the bacteria used**

Survival assays reflect the mosquitoes' ability to tolerate an infection, however they do not show the mosquitoes' ability to eliminate the microbes. This is why we decided to look at the bacterial amount that resides within the mosquitoes using the bacterial proliferation assays. To do so, we infected the mosquitoes with the bacteria *S. aureus*, *S. marcescens* and *Ecc15*, all of which present antibiotic resistance, and we performed bacterial proliferation assays (colony forming units) at different timepoints after the infection (3h, 6h, 12 h, 24h and 48h) to reflect how the bacteria proliferate within the mosquito after infection.

Following *S .aureus* infection, the CFU count was low at 3h, 6h and 12h post infection, and it only started to increase at 24h post infection, reaching the highest count at 48 hours post infection **Figure 34.**
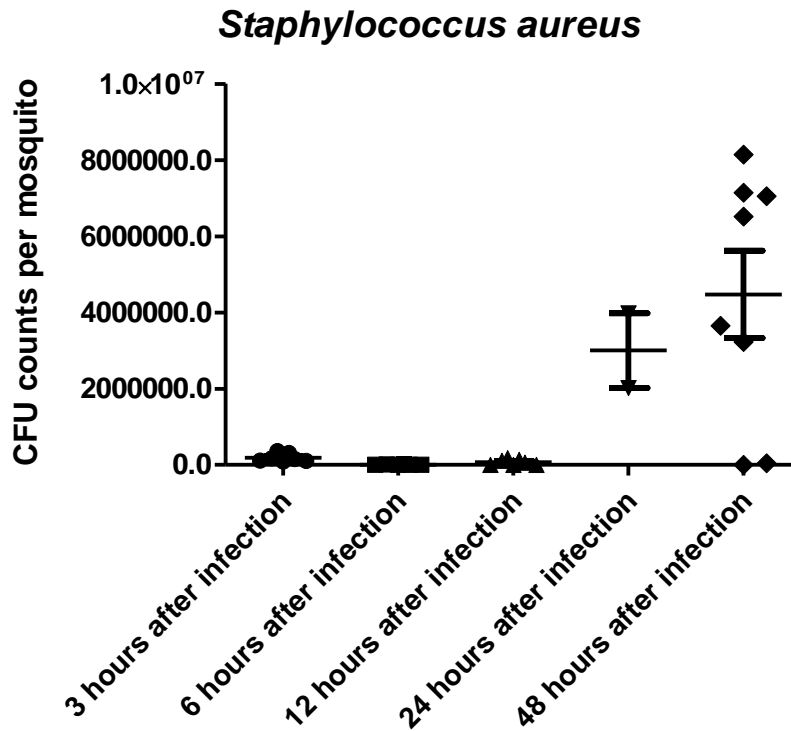
Figure 34: The resistance of *A. albopictus* mosquitoes to *S. aureus* infections over a span of 48 hours.

Bacterial CFU were counted in *A. albopictus* mosquitoes at 3 hours, 6 hours, 12 hours, 24 hours and 48 hours after *S. aureus* infection. For each timepoint, batches of 7 mosquitoes were grinded in LB medium and CFU were counted on LB plates supplemented with tetracycline. Each point on the scatter plot represents the mean CFU per mosquito per batch. Statistical analysis was performed using the Mann-Whitney test.
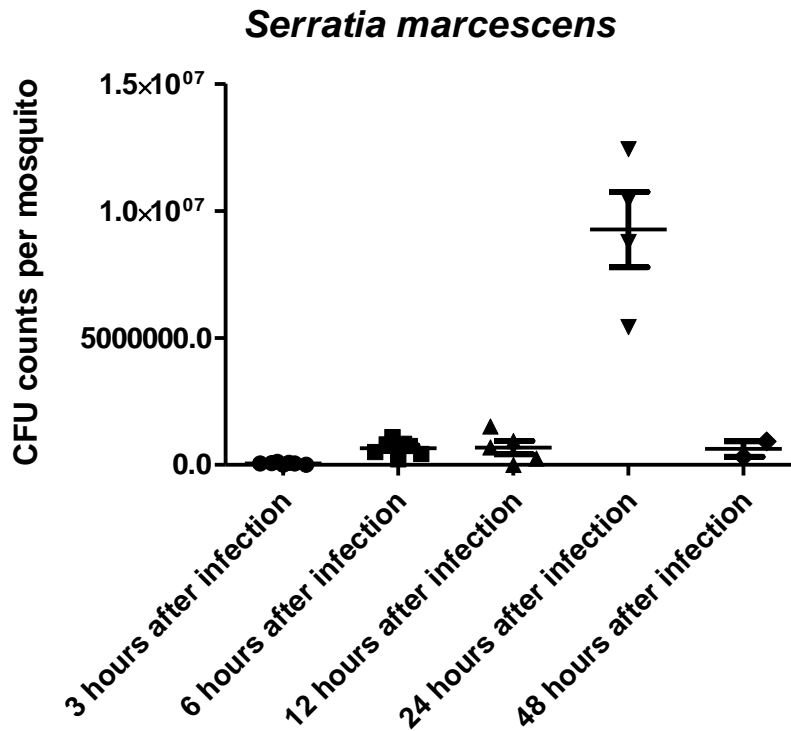
Figure 35: The resistance of *A. albopictus* mosquitoes to *S. marcescens* infections over a span of 48 hours.
Bacterial CFU were counted in *A. albopictus* mosquitoes at 3 hours, 6 hours, 12 hours, 24 hours and 48 hours after *S. marcescens* infection. For each timepoint, batches of 7 mosquitoes were grinded in LB medium and CFU were counted on LB plates supplemented with tetracycline. Each point on the scatter plot represents the mean CFU per mosquito per batch. Statistical analysis was performed using the Mann-Whitney test.

After *S. marcescens* infection, bacterial CFU reached a maximum at 24 hours post

infection, and then started to decrease at 48 hours post infection **Figure 35.**
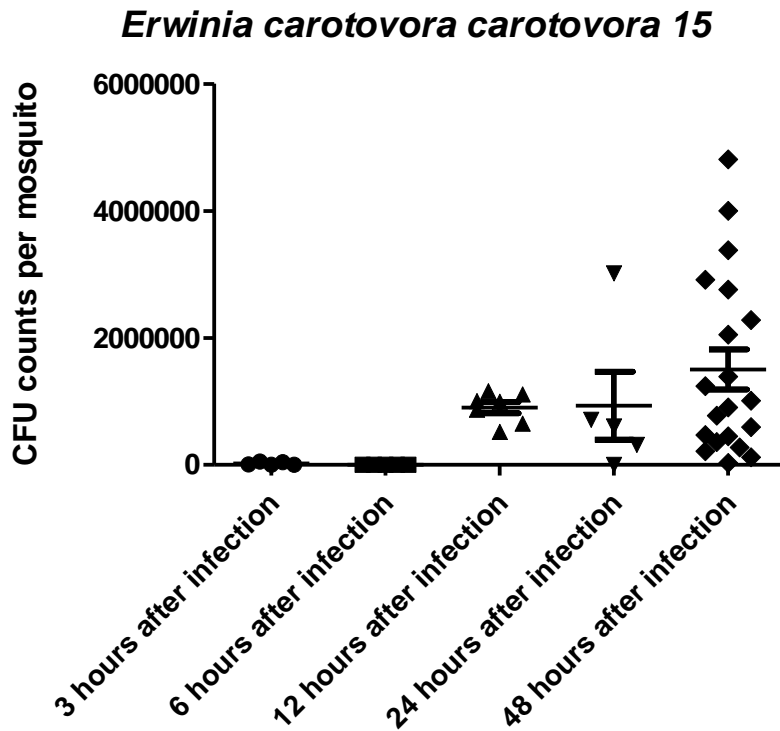
Figure 36: The resistance of *A. albopictus* mosquitoes to *Ecc 15* infections over a span of 48 hours.
Bacterial CFU were counted in *A. albopictus* mosquitoes at 3 hours, 6 hours, 12 hours, 24 hours and 48 hours after *Ecc 15* infection. For each timepoint, batches of 7 mosquitoes were grinded in LB medium and CFU were counted on LB plates supplemented with tetracycline. Each point on the scatter plot represents the mean CFU per mosquito per batch. Statistical analysis was performed using the Mann-Whitney test.

Following *Ecc 15* infection, CFU counts started to increase at 12 hours post infection and

reached a maximum at 48 hours post infection **Figure 36.**

3. *Antimicrobial peptide activation after infection*

We next opted to look at antimicrobial peptide activation after infection. To do so, we

chose a panel of gram-positive and gram-negative bacteria, as well as the fungus

Beauveria bassiana, and we infected *A. albopictus* mosquitoes both by microinjection

and by feeding (or spraying for *Beauveria bassiana*), then we looked at the expression of the antimicrobial peptide Cecropin A1 by real time PCR to see whether microbial infection induced the expression of this AMP.
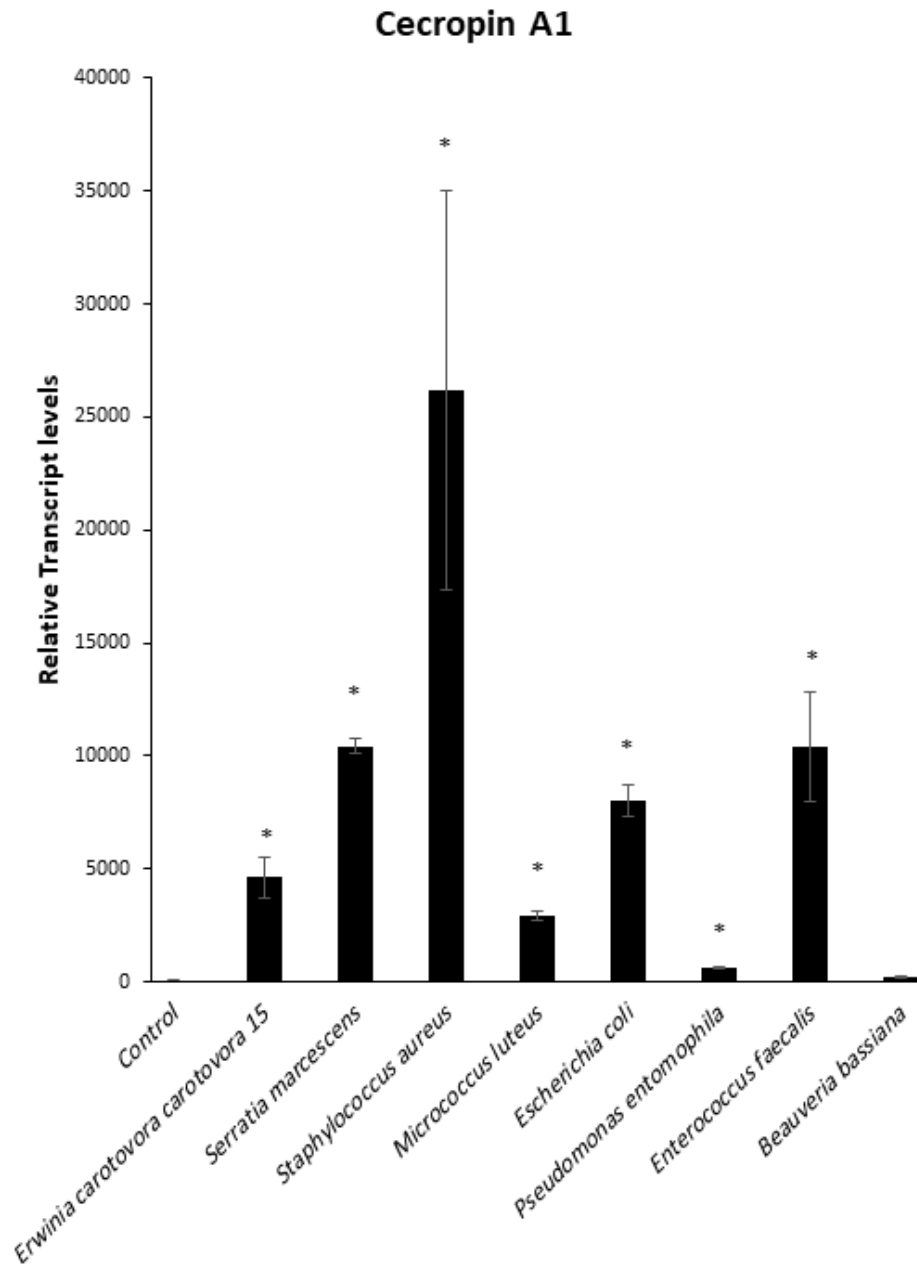
Figure 37: Transcriptional level of the gene encoding Cecropin A1 after microbial infection by injection.
Real time PCR reveals a significant upregulation of the transcription of CecA1 after injection of a panel of gram-negative and gram-positive bacteria. This upregulation however was not apparent after injection with the fungus *Beauveria bassiana*. *P<0.05

Injection of *A. albopictus* mosquitoes with the gram-negative bacterium *Ecc 15* resulted in a 46-fold increase in the expression of CecA1 when compared to the control non-infected mosquitoes. The gram-negative bacterium *S. marcescens* induced a 100-fold increase in the expression of CecA1. *Staphylococcus aureus*, a gram-positive bacterium, resulted in a 260-fold increase in the expression of the AMP. Injection with *M. luteus*, another gram-positive bacterium, induced a 29-fold increase in the expression of CecA1. When injected with the gram-negative bacterium *E.coli*, the expression of CecA1 was upregulated 80 folds. *E. faecalis*, a gram-positive bacterium induced a 104-fold increase in the expression of CecA1. All the bacteria used in this experiment resulted in a significant increase in the expression of CecA1 when injected into *A. albopictus* mosquitoes. Injection with the fungus *BB*, on the other hand, induced a non-significant two-fold increase in the expression of the AMP.

To mimic the natural way of infection, we next decided to look at the expression of CecA1 after bacterial feeding and after *BB* spraying.
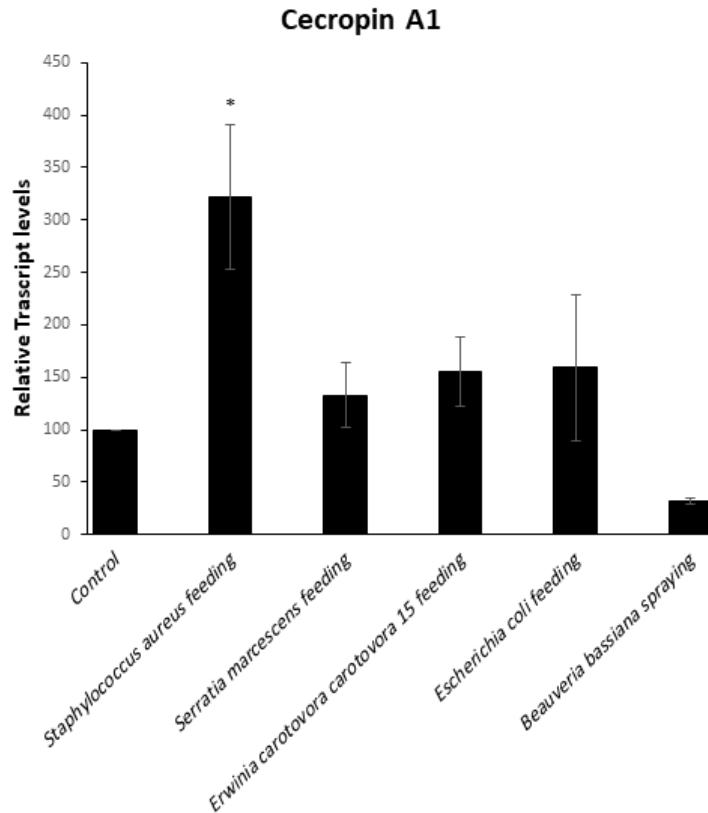
Figure 38: Transcriptional level of Cecropin A1 gene vary after microbial infection by feeding and spraying.
Real time PCR analysis reveals the levels of CecA1 expression after bacterial feeding and fungal spraying. *P<0.05

Feeding the mosquitoes sucrose solutions supplemented with *S. aureus* resulted in a

significant three-fold increase in the transcriptional levels of CecA1. *S. marsescens*

feeding, on the other hand, did not induce a significant increase in the transcription of

CecA1. Both *Ecc 15* and *E.coli*, when supplemented in the sucrose solutions, caused a 1.5 fold increase in the levels of CecA1, though not in a significant manner. Finally, *BB* spraying did not alter the expression of CecA1 in a significant manner, though we see a light decrease in its expression.

4. *Establishment of Wolbachia-free strains*

The α-proteobacterium *Wolbachia* is one of the most abundant endosymbionts, found in about 60% of all insect species [207]. *Wolbachia* has been shown to have a wide range of effects on the host, including the manipulation of reproduction, fitness, mating and immune responses [209, 210, 263, 265]. The manipulation of host reproduction has been shown to offer a selective advantage for the endosymbiont and its propagation through the population [209]. As for the host, the bacteria's presence can induce positive or negative effects, depending on the strain of *Wolbachia* and the species it infects. In the case of the parasitoid wasp *Leptopilina heterotoma,* for example, *Wolbachia* has been shown to have a negative impact on fecundity and longevity [254]. In the case of the parasitic wasp *Asobara tabida,* oogenesis is dependent on the presence of *Wolbachia*[346]. In *A. albopictus*, an infection of two or more strains of *Wolbachia* improves fecundity [253]. As for the effect of *Wolbachia* on host immunity, it has been shown that in some cases the endosymbiont protects the host against some pathogens. In *Drosophila melanogaster*, *Wolbachia*increased the host resistance to West Nile Virus infection [255], Drosophila C virus, cricket paralysis virus and Flock House virus [257]. However, in some native hosts such as *Drosophila melanogaster, Drosophila simulans, Tetranychus urticae*, *Wolbachia* infection did not affect, neither positively not negatively,

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AMP production [266-268]. This wide range of opposing effects that *Wolbachia* infection induces made it interesting for us to explore the effects of a natural *Wolbachia* infection in two Lebanese strains of mosquitoes that we maintain in our lab (*A. albopictus* and *C. pipiens*). To do so, we need to establish *Wolbachia*-free mosquito strains using antibiotics. The generation of these strains will allow us to compare the physiological and immunological responses of the original strain to the *Wolbachia*-free strain. This would allow us to characterize the effect of *Wolbachia* infection on mosquito physiology and immunity.

Culex pipiens mosquitoes were treated with tetracycline for two generations, after which DNA was extracted from adult mosquitoes to test for the presence of *Wolbachia* using PCR. This method proved to be successful in the clearance of *Wolbachia* from these mosquitoes. Before proceeding with the comparative experiments, we wait for 3 generations until the normal microbiota is reestablished. Different methods have been used to treat *Aedes* mosquitoes, such as treatment of larvae with doxocycline, treatment of adults by supplying them with sugar pads supplemented with tetracycline, treatment of adults by introducing tetracycline solutions into the cage. All of these methods did not prove to be successful. *Aedes* mosquitoes are currently undergoing a new tetracycline treatment at the larval stage, which will be followed by a treatment with rifampicin for the adults.*Wolbachia* clearance from *Aedes* mosquitoes has proven to be difficult, which is in agreement with the literature as it has been shown that treatment of *Aedes albopictus* mosquitoes in order to remove *Wolbachia* infection is challenging [347, 348]. We are

currently treating the third generation and will be testing the newly emerged generation

for *Wolbachia* presence using PCR.
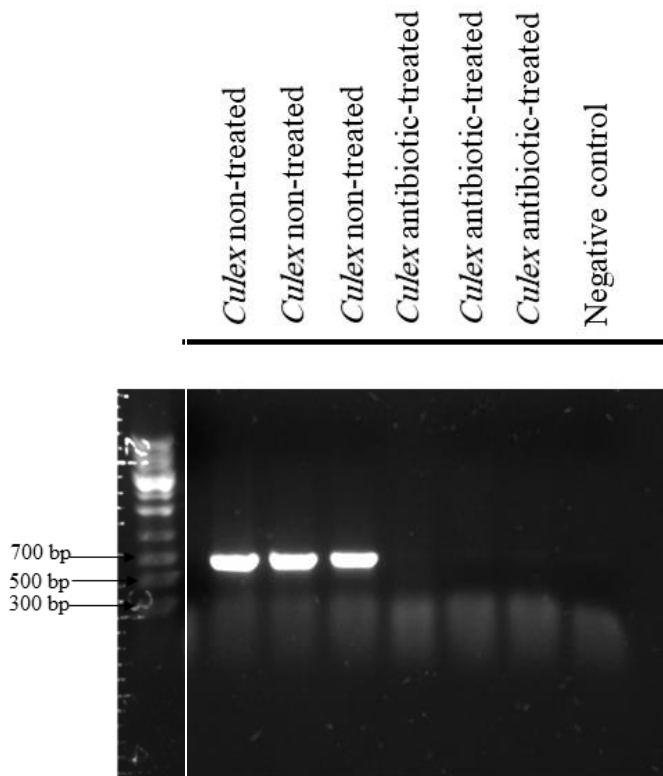
Figure 39: Antibiotic treatment of *Culex* mosquitoes is successful in the clearance of *Wolbachia*.
Gel electrophoresis imaging show the removal of *Wolbachia* from *Culex* mosquitoes after

treatment with tetracycline.
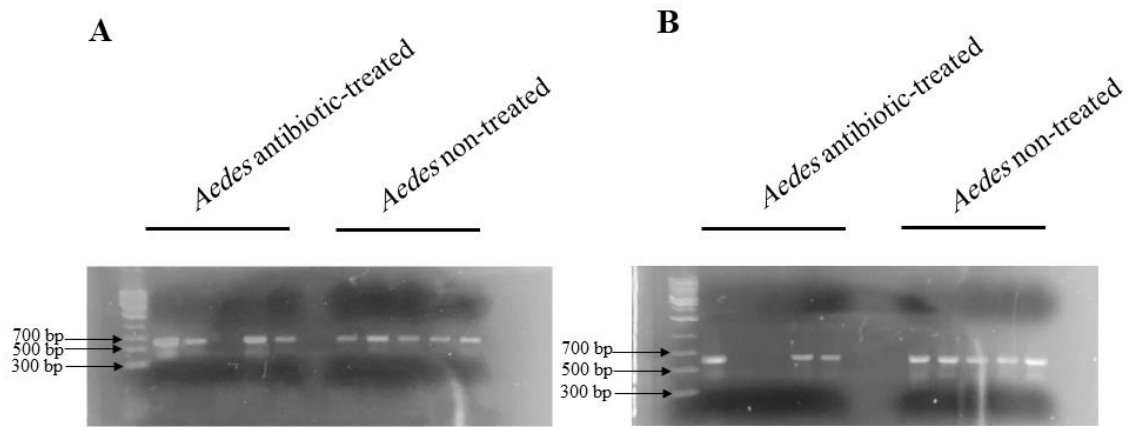
Figure 40: Different antibiotic treatments proved to be unsuccessful in clearing *Wolbachia* from *A. albopictus* mosquitoes.

Gel electrophoresis images of larval doxocycline treatment (Figure 40A) and tetracycline treatment administered to adults through sugar pads (Figure 40B) show that the density of *Wolbachia* in treated mosquitoes was not reduced as compared to the control non-treated *Aedes* mosquitoes.

CHAPTER 4

MATERIALS AND METHODS

1. Ethics Statement

All animal procedures used in this study were carried according to protocols approved by the Institutional Animal 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.

2. Materials

1. *Aedes albopictus and Culex pipiens mosquito strains*

Local strains of *Aedes albopictus* mosquitoes (originally captured from Sarba in the suburbs of Beirut, Lebanon) and *Culex pipiens* mosquitoes were maintained in the insectary at 28°C and 75% humidity using a 12:12 light:dark photocycle. Adults were continuously supplied with cotton pads soaked in a 10% sucrose solution and had access to water cups containing clean tap water. For *Aedes albopictus* mosquitoes, feeding was allowed on anesthetized mice and eggs were collected on filter paper four days after the blood meal. Eggs were dried for two weeks before hatching was attempted by immersion in aged tap water. After hatching, 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. *Culex pipiens* mosquitoes had access to clean water cups where they laid egg rafts. 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.

2. *Bacterial strains*

A GFP-expressing Ampicillin-resistant strain of *Escherichia coli* (DH5 alpha laboratory strain), *Serratia marcescens* pGEN222, *Staphylococcus aureus, Erwinia carotovora carotovra 15, Enterococcus fecalis* were either cultured in Luria-Bertani (LB) broth or plated on LB plates.

3. *Fungal strains*

Beauveria bassiana funguswas plated on Potato Dextrose plates (PDA). A suspension of fungal spores was prepared from the plates when needed to infect mosquitoes either by microinjection or spraying[349].

3. Mosquito survival and proliferation bioassays after microbial proliferation

Bacteria were grown overnight at 37°C in Luria Broth medium containing the appropriate antibiotic. The next day, bacterial cultures were pelleted by centrifugation. The pellets were then resuspended in LB, and the O.D. $_{600nm}$ measured using a spectrophotometer. The O.D. $_{600nm}$ was then adjusted to the appropriate value depending on the bacteria. Female mosquitoes were either injected with the prepared bacterial culture or fed with

sugar solutions containing bacterial suspensions. Mosquito survival rates were followed on a daily basis over a span of 10 days. The Kaplan-Meier survival test was used to calculate the percent survival. Statistical significance of the observed differences was calculated using the Log-rank test. Survival assays were repeated at least three times. A P-value < 0.05 was considered significant.

As for the bacterial proliferation assays, female mosquitoes were injected with bacterial suspensions. At different timepoints, 10 mosquitoes for each replicate were grinded in 500 µL 1xPBS on ice. The lysates were then serially diluted, and plated on LB agar plates containing the appropriate antibiotic (depending on the bacteria used). The plated were then incubated at 37°C overnight to score for bacterial CFUs. All bacterial CFU assays were performed three times and statistical significance was calculated using the Mann-Whitney test. Medians were considered significantly different if P < 0.05.

4. Molecular Biology

1. DNA Extraction

DNA was extracted from whole mosquitoes using one of two different methods: The Livak method and the Sodium-Tris-EDTA (STE) method.

Using the Livak protocol, mosquito samples were homogenised in 100 µL of pre-heated Livak grind buffer (80mM NaCl, 0.16M sucrose, 130mM Tris Base, 50.8 mM EDTA pH=8 and 5 mM SDS). This was followed by an incubation at 65°C for 30 minutes, after which potassium acetate was added to obtain a 1 M solution. After gentle mixing, the samples were incubated on ice for 30 minutes, then centrifuged at 20000 g for 20

minutes. The supernatant was added to 200 µL of ice cold 100% ethanol and mixed.

Pelleting of the DNA was achieved by centrifugation at 20000g for 15 min at 4°C. The

obtained pellet was then washed in 100µL of 70% ethanol and suspended in 50 µL of

water.

Using the STE method, mosquito samples were homogenised in 100 µL of STE buffer

(0.1 M NaCl, 10 mM Tris-HCl pH=8 and 1 mM EDTA, pH=8). This was followed by an

incubation at 95°C for 10 min and centrifugation at 20000 g for 5 min. The resulting

supernatant was removed and used as template for subsequent PCR reactions.

2. *Polymerase Chain Reaction (PCR)*

Extracted DNA was used as a template for PCR reactions to amplify the DNA sample.

Mixtures containing 10x Buffer, $MgCl_2$, dNTPs, forward and reverse primers, Taq

polymerase were prepared and added to the DNA template. Samples were then placed in

a thermal cycler where they underwent cycles of denaturation, annealing and elongation.

The primers used were:

Wolbachia surface protein 328 Forward: 5'-CCAGCAGATACTATTGCG-3'

Wolbachia surface protein 81 Forward: 5'-TGGTCCAATAAGTGATGAAGAAAC-3'

Wolbachia surface protein 691 Reverse: 5'-AAAAATTAAACGCTACTCCA-3'

3. *RNA extraction, reverse transcription for cDNA synthesis, and real-time PCR*

Whole mosquitoes or dissected guts were directly placed and homogenized in TRIzol®. RNA was extracted using choloroform and precipitated with isopropanol according to the manufacturer's instructions (Invitrogen). The extracted RNAs were quantified using a nanodrop spectrophotometer (Thermo) and 500 ng were retrotranscribed into cDNA (iScript Biorad) for each sample. Real-time PCR was performed in presence of SYBR green (Qiagen) on 1/20 dilutions of thecDNA obtained from the RT reactions using a BIO RAD thermocycler (CFX 96 Real-time System, C1000). Ct values for target genes were normalized to Rp49 and compared to controls using the delta Ct method. A minimum of three independent experiments were averaged and unpaired t tests were performed. Primers were designed using Primer3 online software, except for CecA1, adapted from [350].

Primers used were:

Aedes albopictus

Rp49 Forward: 5'-AGTCGGACCGCTATGACAAG-3'

Rp49 Reverse: 5'-GACGTTGTGGACCAGGAACT-3'

CecA1 Forward: 5'-GAGTCGGCAAACGAGTCTTC-3'

CecA1 Reverse: 5'-TTGAACCCGGACCATAAATC-3'

Socs Forward: 5'-TCGACTTCATCCACTGCTTG-3'

Socs Reverse: 5'-ACGACACGGAAAACAGGAAC-3'

Keren Forward: 5'-TGATGATCCATTTCGCAAGA-3

Keren Reverse: 5'-CTTATCCGTCTCCTGCCTGA-3'

Culex pipiens

Rp49 Forward: 5'-AAGAAGCGCAAGCTGATTGT-3'

Rp49 Reverse: 5'-CGACGGGTAATCGAATTTGT-3'

Socs36E Forward: 5'-TTGCCCAAGCAACCAGATT-3'

Socs36E Reverse: 5'-TGAAGTCGACCTGTGAGTGC-3'

Keren Forward: 5'-CGCAGCCTGGTATTGTCTGA-3'

Keren Reverse: 5'-AGGCCGAGTTGGCAGATAAG-3'

Cecropin B2 Forward: 5'-TTGCAATTGTCCTGTTGGCC-3'

Cecropin B2 Reverse: 5'-AGTGCATTAATTCCAGCAACCA-3'

Anopheles gambiae

Rps7 Forward: 5'-TTCAACAACAAGAAGGCGATCA-3'

Rps7 Reverse: 5'-CTTGTACACCGACGCAAAAGTG-3'

STATA Forward: 5'-TACAACGAAACGACCAAGCA-3'

STATA Reverse: 5'-GGTCCATACCGAAAAGACGA-3'

STATB Forward: 5'-ACCGCGGCAACAGGAAACTAA-3'

STATB Reverse: 5'-GATAATGGTTGTGCATGCCAGTTG-3'

Socs Forward: 5'-GTTTTCCGTCTCCTTCCGCAAGTA-3'

Socs Reverse: 5'-CTTCGGTAGCGTCAGCTCGTTGAT-3'

Keren Forward: 5'-CTCGTCCTCCCAGTCCTACA-3'

Keren Reverse: 5'-TCGAACAAAACCAGGGTCTGA-3'

Cecropin A Forward: 5'-GCTGAAGAAGCTGGGAAAGA-3'

Cecropin A Reverse: 5'-ATGTTAGCAGAGCCGTCGTC-3'

Wolbachia pipientis

Wolbachia surface protein Forward: 5'-AGATAGTGTAACAGCGTTTTCAGGAT-3'

Wolbachia surface protein Reverse: 5'-CACCATAAGAACCAAAATAACCAG-3'

5. Protein Biochemistry

1. *Mosquito hemolymph extraction*

Mosquito hemolymph was extracted from mosquitoes (after clipping the mosquito

proboscis) into 1xPBS containing EDTA-free protease inhibitor cocktail (Roche).

6. Immunohistochemistry and microscopy

1. *Chemical and bacterial treatments*

Mosquitoes were starved for 2 hours before their cups were supplemented with cotton

pads soaked in 10% sucrose (for controls), 2% SDS - 10% sucrose, or 0.3% H2O2 -10%

sucrose, or 4mM Paraquat (Sigma-Aldrich, USA) -10% sucrose, or 25μg/ml Bleomycin

(Cell Pharm, Germany) - 10% sucrose (for the induction of chemical damage) or a

bacterial suspension (OD=50) in 10% sucrose (for infection experiments). The

mosquitoes were allowed to feed continuously until the guts were dissected 24 hours after the treatment for immunohistochemistry. The bacterial strains used in this experiment were *Serratia marcescens* pGEN222 and *Erwinia carotovora carotovora 15* (*Ecc15*).

2. *Fixation and staining*

Isolated guts were fixed for 30 minutes using a 4% Parafolmadehyde (VWR, USA) solution in 1X PBS. This was followed by three 15-minute washes in PBS-Triton 0.1 % to allow permeabilization of the guts. Blocking was then performed for 30 minutes by adding a solution of 1X PBS -Triton 0.1%-BSA 1%. After blocking, the primary rabbit α-PH3 antibodies (ABCAM, UK) were added (1:800 in 1X PBS-Triton 0.1%-BSA 1%) overnight at 4ºC. Samples were then washed for 15 minutes in PBS-Triton 0.1% three times, then the secondary antibodies Alexa Fluor® 555 (ABCAM, UK) were added (1:1000 in PBS-Triton 0.1%-BSA 1%) for three hours at room temperature. In other cases,samples were incubated with phalloidin coupled to Alexa Fluor® 647 (ABCAM, UK) for one hour at room temperature (1:500 in PBS-Triton 0.1%-BSA 1%). After secondary antibodies or phalloidin removal, DAPI stain was applied at a concentration of 1:10 000 for 2 minutes, then three final washes in PBS-Triton 0.1% were performed, guts were mounted on microscope slides in anti-fade medium (Immu-Mount, Thermo Scientific) and coverslips were sealed with colorless nail varnish.

3. Microscopy, cell counting and statistical analysis

The slides prepared were observed under an inverted fluorescence microscope (Zeiss Axiovert 200, Source: AttoArc2 HBO 100W) for the counting of proliferating cells and an upright fluorescence microscope (Leica DM6 B) was used for image acquisition using the image stitching option. Cell counts were analyzed using the Graphpad Prism software and an unpaired t test was performed.

4. Scanning electron microscopy

Midguts were dissected and incubated for two hours at room temperature using a PBS fixative solution containing 25% glutaraldehyde and 4% parafolmadehyde. After three 5 minute washes in 1X PBS, the guts were dehydrated using increasing concentrations of ethanol in the following steps: 2 hours in 30% ethanol, overnight in 50% ethanol, 6 hours in 70% ethanol and finally overnight in 100% ethanol. The guts were then dried in a critical point dryer (EMS Quorum 850), coated in gold and observed under the MIRA3 LM TESCAN scanning electron microscope (SEM High Voltage: 15 kV, Detector Oxford Instruments X-Max: SE).

CHAPTER 5

GENERAL DISCUSSION

Mosquitoes are one of the deadliest animals on earth, causing millions of deaths yearly. Different species of mosquitoes are spread over the world, making these insects a global health threat. The most known mosquito species are *Anopheles*, *Aedes* and *Culex*. In our lab, we maintain local Lebanese strains of *Aedes albopictus* and *Culex pipiens* mosquitoes. These two species are vectors of numerous diseases such as Dengue, Chikungunya and Zika virus transmitted by *A. albopictus*, and West Nile Virus, Japanese encephalitis and filariasis transmitted by *C. pipiens*. Until this date, no incident of mosquito-transmitted disease has been recorded in Lebanon; however, this situation might change soon, as we face environmental changes that facilitate the development and spread of vector-borne diseases. This calls the need for the development of preventive measures that might reduce the threat imposed by these local mosquito strains. To do so, an in-depth knowledge of mosquito physiology and immunity is required. The aim of this study is to provide some insight into the immune responses and physiology of *A. albopictu*s and *C. pipiens*, allowing for a better understanding of how these mosquitoes might interact with invaders, and therefore contribute to the transmission of diseases.

Looking at different aspects of the immune system of mosquitoes, we decided to focus in particular at the gut, as guts present the first line of defense against invading pathogens, acquired mostly through the oral route. We were specifically interested in how the

mosquito maintains homeostasis of its gut, especially when continuously confronted to damage-inducing conditions. Even though this subject had been widely explored in the model organism *Drosophila melanogaster*, it was very poorly characterized in mosquitoes. This was peculiar, as mosquitoes transmit pathogens through blood meals, and their gut is therefore continuously confronted to stress-inducers. How the gut maintains its integrity would therefore be of great interest and importance in the aspect of mosquito immunity and disease transmission.

Due to the lack of data throughout the literature for mosquitoes, we decided to establish our own model and build our experiments and results based on that. The first step for us was to characterize the structure of *A. albopictus* male and female guts. We used the scanning electron microscope to do so. We could observe a difference in the structure of male and female guts, the latter having a more developed structure. This could be related to the feeding habits of female mosquitoes, which require blood meals, whereas male mosquitoes only feed on sugar. Because of this difference observed between male and female mosquito guts, and since female mosquitoes are of interest due to their blood feeding and disease transmission, we chose to continue our study with female mosquitoes. We established our model for gut damage using chemicals and bacteria widely used in *D. melanogaster* studies. After confirming that our model did indeed recapitulate gut damage scenarios, we were able to detect through immunohistochemistry analysis the presence of mitotic cells which seem to proliferate after we induced damage to the gut. Using real time PCR studies, we looked into the molecular pathways which might involved in this response, and we gave insight into the possible involvement of the

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Jak-Stat and EGFR pathways in the response of mosquito gut to damage. This study done on mosquito gut homeostasis and renewal after damage was novel in the literature, and it provided very important information which could be explored for the prevention of disease transmission. This could be done, for example, by applying population replacement strategies, where we replace a mosquito population with another having different gut regeneration capacity, and that is refractory to a certain disease.

Having gained some insight into the physiology of *A. albopictus* guts, we were curious to see whether the response we observed is a general response for different mosquito species, or a more specific one for *A. albopictus*. In fact, in a study done by the *Barillas-Mury* group in 2005, the in vivo responses of midgut epithelial cells to ookinete invasion of three different vector-parasite combinations were characterized, and it was shown that the epithelial responses of different mosquito-parasite combinations are not universal, with different mechanisms of repair activated after ookinete invasion [351]. This is why we decided to extend our study to two other mosquito species, *Culex pipiens* and *Anopheles gambiae*, both of which are important disease-vectors. To perform this comparative analysis, we started by comparing the structure of the different species' guts to each other. This was done using the scanning electron microscopy. As with the first study, we were also able to see a difference between male and female guts for each species, with the female gut having a more developed structure. We were also able to observe a difference among the gut structure of three species. Having already established a model for gut damage in the previous study, we used the same method for inducing damage to the guts of female mosquitoes of the three species, and we used

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immunohistochemistry to detect the presence of mitotic cells after damage. We observed

a clear difference in the response of the three different species to gut damage. While *C.*

pipiens mosquitoes exhibited a similar response to *A. albopictus* mosquitoes, with an

increase in the number of mitotic cells after damage, no mitotic cells could be detected at

the level of *A. gambiae* mosquitoes, neither in control conditions nor after inducing

damage, in the same conditions tested for all three species. To make sure that the inability

to detect PH3+ cells in *Anopheles gambiae* guts was not due to any technical issue, we

stained the ovarian tissue as a control, and indeed we were able to detect PH3+ cells at

the level of this tissue, thus proving that the antibodies and the method of staining did

indeed successfully stain tissue in this mosquito species. We also attempted to try less

aggressive stresses (0.5% SDS, 1% SDS) and we tried different timepoints (6,12,24 and

48 hours after feeding the stressor), and in all cases we were not able to detect any PH3+

cells at the level of *A. gambiae* mosquito guts. To confirm that our model is indeed

working, and damage is being inflicted to the gut, we performed experiments to check for

the permeability and leakiness of the gut after damage. Upon feeding *A. gambiae*

mosquitoes on SDS solutions supplemented with blue food colorant, we could observe 24

hours later the blue color diffused all over the body of the mosquito, even at the level of

the legs, proboscis and wings, thus giving the mosquito the appearance of a "smurf". This

allowed us to conclude that the damage is causing damage to the gut, rendering it leaky

and permeable. We also performed another experiment proving the same concept. In this

case, we fed mosquitoes on SDS overnight, then on the bacterium *E.coli*for 2 hours,

which in normal cases would not cross the midgut epithelial barrier. Six hours after the

end of the feeding, we extracted hemolymph from the infected mosquitoes, and plated the

extract on LB plates overnight. We could indeed observe the presence of *E. coli* colonies on the plates, thus demonstrating that the bacteria crossed the gut into the hemolymph, and this was due to the permeability of the gut inflicted by SDS damage.

The differences observed in midgut epithelial responses to damage among the three species was reflected in survival assays, with *A. gambiae* mosquitoes presenting a higher sensitivity to oral infections. Given that we could not detect any mitotic cells in *A. gambiae* mosquitoes, we hypothesized that this higher sensitivity to oral infections might be in part due to the inability of *A. gambiae* to activate cell division to repair gut damage. The reason for this could be the activation of a different repair mechanism after damage. In *Anopheles stephensi*mosquitoes, the epithelial damage inflicted upon *Plasmodium berghei* ookinete invasion is repaired by an actin purse-string-mediated restitution mechanism, which allows the epithelium to 'bud off' the damaged cells without losing its integrity [352].

 It would be interesting to see whether this difference in gut regeneration could be associated with the vectorial capacity of mosquitoes. In fact, in a study published in 2018, in which they used our established model on *Aedes aegypti* mosquito strains with different susceptibilities to dengue viral infection, they were able to show that strains presenting different susceptibilities have different time courses of cell regeneration in response to viral infections, with the susceptible strain delaying the activation of the regeneration process compared to the refractory strain, thus showing that the effectiveness of midgut cellular renewal during viral infection is an important factor in vector competence [334].This is interesting, as the *Anopheles* strain we used for our study

is the G3 permissive strain. We need to repeat the same experiments using a refractory strain of *Anopheles,* to see whether we would get the same results or not.

Our study of gut regeneration capacity in mosquitoes is still limited, as unlike for *Drosophila*, cell markers and transgenic lines are not available, which limits the analysis. The development of tools for mosquito studies would allow further understanding of how the mosquito gut responds to damage, and it would allow us to distinguish between cell types which are undergoing damage.We also still need to repeat the experiments for the G3 *A. gambiae* strain using late timepoints (5 days after the damage), to check whether the proliferation of mitotic cells is being delayed after damage. Another future perspective would be to look into the possible mechanisms by which damage is being induced to the epithelial cells, and this could be done by looking into apoptotic markers, or ROS levels, and that is because the damage might be induced because of the direct physical disruption of the midgut epithelium, or through an oxidative burst.

While performing the experiments for this study, we made interesting observations related to the effect of microflora on the regeneration of the gut in *Culex pipiens* mosquitoes. We decided to further explore this, by treating *C. pipiens* mosquitoes with antibiotics, and characterizing the effect of this treatment on the regeneration of the gut. These results show that different factors come into play in the process of gut regeneration, be it the mosquitoes' microflora, or other external factors such as chemical or bacterial damage.

Moving on unto a different aspect of mosquito immunity, we started by establishing microbial infections through microinjection, or by ways mimicking the natural way of

infection, be it feeding or spraying. This was done using a panel of gram-positive and gram-negative bacteria, as well as fungi. We then looked at the tolerance of infected mosquitoes using survival assays. The results obtained with these experiments allowed us to deduce that mosquitoes are sensitive to microbes introduced through both microinjection and feeding (or spraying). These two methods of infection could therefore be used to establish infections for further studies. The next step for us was to look at the mosquitoes' resistance to infection, as tolerance studies do not accurately reflect the bacteria's proliferation within the mosquito. To do so, we proceeded to infect the mosquitoes with bacteria presenting antibiotic resistance, both by microinjections and by feeding/spraying, and then we looked at bacterial proliferation using the colony forming unit assay, at different timepoints after infection. The three strains of bacteria used for this assay (*S. marcescens*, *Ecc 15* and *S. aureus*) presented a different dynamic of proliferation after infection, as *S. marcescens* proliferation reached a maximum at 24 hours post infection, then started to decrease at 48 hours post-infection. *Ecc 15* proliferation increased consistently starting 12 hours post-infection until 48 hours post-infection. *S. aureus* on the other hand started to proliferate increasingly at 24 hours post infection and this was also observed at 48 hours post-infection. The bacterial proliferation assays performed allowed us to conclude that though the survival assays might show similar results with different types of microbes (meaning that the mosquitoes succumbed to the infection), bacterial proliferation assays highlighted the difference in the behavior and the interaction of the bacteria residing within the mosquito and the host itself.

Mosquitoes are equipped with a plethora of immune responses allowing them to combat invading microbes. One of these responses is the production of antimicrobial peptides. Cecropin A1 is an example of antimicrobial peptide produced after immune activation. To look further into this aspect of immune responses, we decided to look at the levels of transcriptional activation of CecA1 after infection. Using both microinjection and feeding/spraying, we screened for the transcriptional levels of CecA1 in response to infection with a panel of microbes. Comparing the results obtained through real time PCR, we observed that infection by microinjection induced a potent immune upregulation of CecA1, whereas feeding/spraying resulted in a less powerful immune response.

The mosquito strains we worked with are naturally infected with the endosymbiont *Wolbachia.* As a next step, we decided to establish *Wolbachia*-free strains, as a lot of data in the literature show that this endosymbiont may affect the mosquito host physiology and immunity, and it would be interesting whether the same is true for the local strains we work with. We were successful in clearing the endosymbiont from *C. pipiens* mosquitoes, but the same proved to be challenging for *A. albopictus* mosquitoes. Several studies have indeed focused on the generation of new techniques for the removal of *Wolbachia* from *Aedes albopictus* mosquitoes, as the effective clearance of this endosymbiont from *A. albopictus* has not been possible without generating harmful effects on the fertility, fitness and egg hatch [347, 348]. Generating the *Wolbachia*-free strains will be an important tool for the characterization of the effect of this endosymbiont on the immune responses and physiological traits of the local mosquito strains.

We started our study with a characterization of the immune responses of a local strain of *A. albopictus*, in order to provide insights for the development of preventive measures and more effective control strategies, in order to minimize the threat of emergence and spread of vector-borne diseases. Our experiments led us to investigate the function of the gut in the defense of mosquitoes against invading pathogens. To do so, we decided to look at the mechanism lying behind the renewal and homeostasis of the gut, in normal and damaging conditions. This is because most pathogens are acquired through the oral route, and the gut therefore is the first line of defense, and would be involved in the spread of pathogens acquired through the oral route. This study was the first of its kind in the literature, and the results we obtained led us to investigate whether this response could also be observed in other species of mosquitoes. As we extended our study, we observed clear differences in the response of the three species of mosquitoes: *A. albopictus*, *C. pipiens* and *A. gambiae*, all of which are important vectors of diseases.

We hope that the results of this study might contribute to a better understanding of immunity and physiology of mosquitoes. This would allow for the development of preventive measures and more effective control strategies, in order to limit the threat of spread of mosquito-borne diseases in the region.

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