Impact of mating on *Anopheles coluzzii* in response to infection

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Thesis submitted in accordance with the requirements of Imperial College London for the degree of Doctor of Philosophy.

DECLARATION OF OWN WORK

I certify that this thesis and the research to which it refers is the product of my own work and that any ideas or quotations from the work of other people, published or otherwise, are fully acknowledged in accordance with standard referencing practices.

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I was told that I should be writing an emotional and nice acknowledgement by some friends, whilst some other friends suggested that I should be funny. I guess I'm neither emotional (-ly happy) nor funny. I can't be either one until my viva is completed... for now, I am just happy that finally my thesis is done! :) Alhamdulillah…

ABSTRACT

Mating causes dramatic changes of female physiology, behaviour, and immunity in many insects, often inducing oogenesis, oviposition, and refractoriness to further mating. Females from the *Anopheles gambiae* species complex typically mate only once in their lifetime during which they receive sperm and seminal fluid proteins as well as a mating plug that contains the steroid hormone 20E. This hormone, also induced by bloodfeeding, plays a major role in activating vitellogenesis for egg production. In this thesis, I present data showing that the mating status of an *Anopheles coluzzii* female influences her midgut bacterial load and bacterial composition. Furthermore, I show that her susceptibility to *Plasmodium falciparum* infection is also enhanced upon mating especially when infection intensity is high. I find that mating status has a major impact on the midgut transcriptome, but only under sugar-fed conditions; once females have bloodfed, the transcriptional changes that are still observable as induced by mating are masked. To determine whether increased susceptibility to parasites could be driven by the additional 20E that mated females receive from males, I mimicked mating by injecting 20E into virgin females, finding that these females have significantly increased infection intensity compared to controls. I carried out further RNAseq to examine whether the genes that change upon 20E injection in the midgut are similar to those that change upon mating. I find that 11% of the genes upregulated by 20E are in common with genes upregulated by mating. Together, these findings suggest that male *Anopheles* mosquitoes might contribute to malaria transmission by influencing female midgut bacterial loads and by potentially increasing *P. falciparum* susceptibility in females.

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ABBREVIATIONS

16S	16S ribosomal RNA (or 16S rRNA)
20E	20-hydroxyecdysone
A	
ACT	Artemisinin-Combination-Therapy
An.	*Anopheles*
AMP	Antimicrobial peptide
B	
BP	Blood pressure
C	
cDNA	Complementary DNA
CHIKV	Chikungunya virus
CQ	Chloroquine
D	
DBH	Dibenzoylhydrazine
DNA	Deoxyribonucleic acid
dsRNA	Double stranded RNA
E	
EcR	Ecdysone receptor
EtOH	Ethanol
G	
G6PD	Glucose-6-phosphate deficiency
H	
HISAT	Hierarchical Indexing for Spliced Alignment of Transcripts
HPX	Heme peroxidase
I	
ITN	Insecticide-treated mosquito nets
IRS	Indoor residual spraying
J	
JH	Juvenile hormone

L

Lp	Lipophorin

M

MAG	Male accessory gland
MISO	Mating-Induced Stimulator of Oogenesis
mRNA	Messenger RNA

N

NGS	Next-generation sequencing

O

ONNV	O'Nyong Nyong virus

P

P.	*Plasmodium*
PBS	Phosphate buffer saline
PCA	Principal Component Analysis
PCR	Polymerase Chain Reaction
Pf	*Plasmodium falciparum*
PFA	Paraformaldehyde
PGRPS	Peptidoglycan recognition protein (short)
pH	Potential of hydrogen
PM	Peritrophic Matrix

Q

qPCR	quantitative PCR

R

RNA	Ribonucleic acid
RNAi	RNA interference
RNASeq	RNA sequencing
ROS	Reactive oxygen species
rpm	Revolutions per minute
RPMI	Roswell Park Memorial Institute medium

S

S7Uni	S7 ribosomal protein
SP	sulfadoxine-pyrimethamine
ss	sensu stricto

T

TEM	Transmission Electron Microscope

U

USP	Ultraspiracle protein

V

Vg	Vitellogenin

W

WHO	World Health Organization

Y

YPP	Yolk protein precursor

CHAPTER 1

Introduction

Malaria: Historical and current perspective

Malaria acquired its name from its association with inhaling "bad air" from low-lying swamps and marshes (Hempelmann & Krafts, 2013). In fact, malaria was associated with swamps and wetlands because as these bodies of water filled up, mosquitoes increased in numbers. Malaria is a mosquito-transmitted parasitic disease, which still kills more than 400,000 people and infects more than 200 million people worldwide yearly with 90% of the cases in Africa (Figure 1.1; (WHO, 2016)). In areas where malaria is endemic, this disease is the one of the most common causes of death among infants and children. Malaria in pregnant women causes severe symptoms with higher rates of miscarriage, intrauterine demise, premature delivery, low-birth-weight neonates, and neonatal death (Schantz-Dunn & Nour, 2009).

It was not until 1880 that Charles Louis Alphonse Laveran identified the parasites that cause malaria. Later on in 1886, Camillo Golgi further studied malaria parasites and found merozoites released from ruptured infected red blood cells. There are more than 100 *Plasmodium* species in the world that are able to infect animals. So far only 5 *Plasmodium* species have been reported to be able to infect humans. Among these five species, *P. falciparum* is responsible for the highest mortality especially in sub-Saharan Africa (Gething et al., 2016). Another two *Plasmodium* species; *P. vivax* and *P. ovale* are usually found in Southeast Asia and South America (Collins & Jeffery, 2005; Cruz et al., 2013; Feng et al., 2015; Gonzalez-Ceron et al., 2013) with *P. ovale* causing fewer cases and less mortality similar to another species, *P. malariae* (Mendis et al., 2001). Just ten years ago, *P. knowlesi* was found to be able to infect human despite it being a zoonotic species (Singh & Daneshvar, 2013). However, *P. knowlesi* is not able to transmit between humans so transmission from primate to human is likely how transmission occurs (Singh & Daneshvar, 2013).

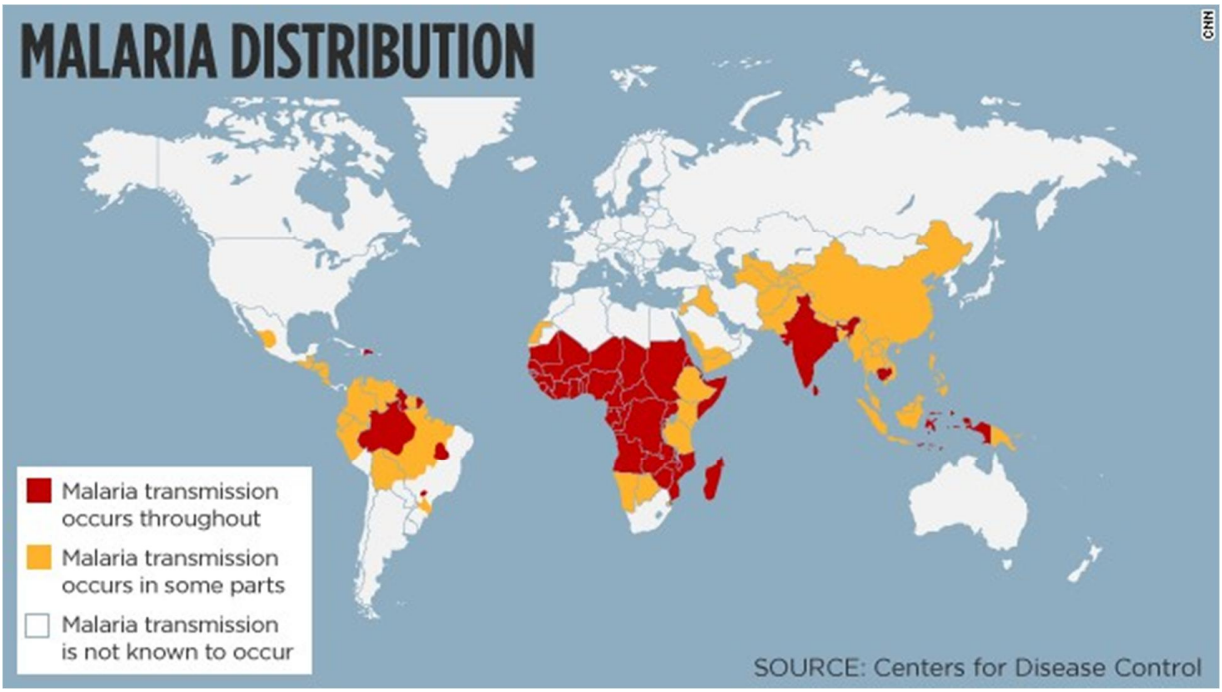

Figure 1.1 Figure taken from the Centers for Disease Control website (https://www.cdc.gov/malaria/) shows the world map summarising the endemic malaria distribution in 2015.

P. falciparum life cycle

Plasmodium parasites require two hosts to complete their lifecycle: a vertebrate host, in which the parasite replicates asexually, and a mosquito host in which the parasite replicates sexually and is able to be transmitted to a new vertebrate host. Figure 1.2 summarizes the *P. falciparum* stages in the human body. When a mosquito bites a human for her blood meal, *P. falciparum* parasites of a life-stage called sporozoites will enter the human body. These sporozoites will migrate to the liver where they will infect the Kupffer cells lining the liver before egressing into the hepatocytes (Sinden & Smith, 1982; Verhave et al., 1980). In the hepatocytes, several rounds of multiplication will form thousands of copies of a stage called merozoites during this pre-erythrocytic stage also known as the exo-erythrocytic cycle. The infected liver cells will burst and release thousands of merozoites that will enter the bloodstream and infect red blood cells. In infected red blood cells, each parasite will initially form a ring-shaped morphology, followed by a trophozoite stage, and then a schizont stage before the infected red blood cell ruptures and

releases as many as 36 merozoites, each of which are able to infect another red blood cell. This erythrocytic cycle of *P. falciparum* takes 48 hours to complete. And it is this part of the life cycle, as well as the defense mechanisms of the host's immune system (Miller et al., 2002) causes symptoms such as headaches, fever, and anemia (Bartoloni & Zammarchi, 2012). At its worst, *P. falciparum* can cause neurological complications by blocking small blood vessels in the brain, leading to swelling and damage to the brain, and cerebral malaria (Idro et al., 2010).

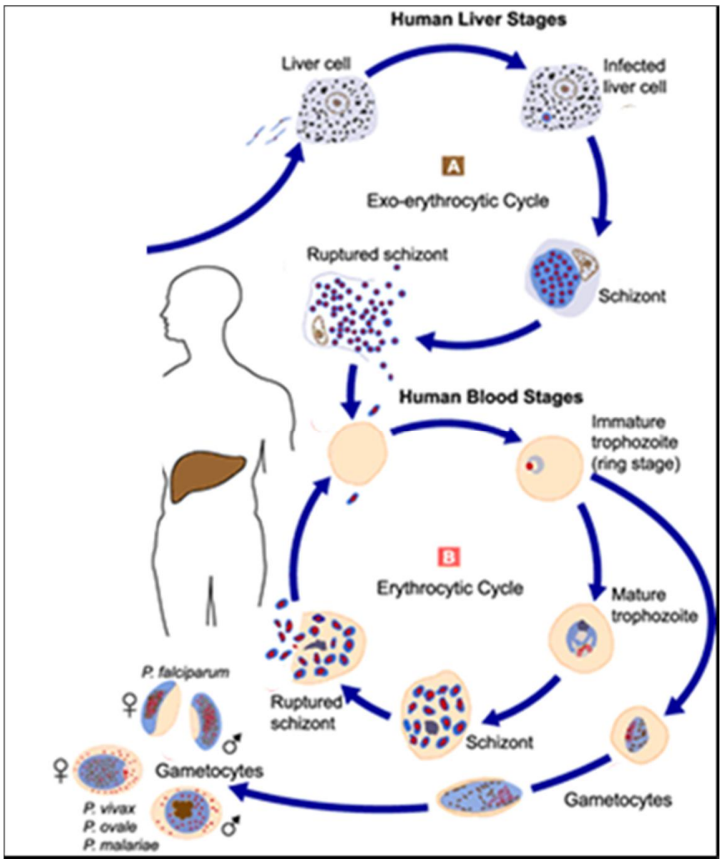

Figure 1.2 ***P. falciparum* life cycle in the human.** Figure taken from Center for Disease Control website (https://www.cdc.gov/malaria/about/biology/index.html) shows sporozoites released from the mosquito bite travel to the liver and proliferate there to produce thousands of merozoites. The infected liver cells rupture and release merozoites into the circulation and start the erythrocytic asexual life cycle. The life cycle in the red blood cells consist of *P. falciparum* parasites at ring, trophozoite and schizont stages and the cycle continues whilst some trophozoites will mature into gametocytes.

A subset of the asexually replicating parasites are able to commit into sexual forms, known as the male and female gametocytes (Bruce et al., 1990). Gametocytes take around 7-10 days to develop (Gardiner & Trenholme, 2015). They are able to avoid the host immune system by avoiding being present in peripheral blood except at mature stage (stage V gametocytes) (Gardiner & Trenholme, 2015). Higher levels of younger gametocytes were found in the bone marrow (44.9%) in comparison to the gut (12.4%), the brain (4.8%) and other organs of the infected host (Gardiner & Trenholme, 2015). These female and male gametocytes are terminally differentiated in the host, and will circulate for at least several weeks (Day et al., 1998; Eichner' et al., 2001) waiting to be taken up in the bloodmeal of a mosquito. *P. falciparum* gametocyte development morphology is easily distinguished by the stages, (stage I-V) using Giemsa stained blood films, and the differences between male and female gametocytes are clear at the mature stage V gametocyte. Gametocyte morphology is different depending on the *Plasmodium* species.

The triggers that parasites detect or employ to either remain in the asexual cycle or to head down the irreversible sexual path towards gametocytes remain poorly understood. Gametocytes may have been programmed in the previous schizont cycle in which it is thought that all resulting merozoites are sexually committed, perhaps even to the same sex (Bruce et al., 1990; Carter & Miller, 1979; Inselburg, 1983). But what causes this subset of parasites to commit is unclear; *in-vitro* studies suggest that factors such as high parasitemia, lack of nutrients in the medium, or antimalarial drug in the culture can induce commitment (Buckling et al., 1999; Carter & Miller, 1979; Dyer & Day, 2003; Williams, 1999). Determining the factors that influence sexual commitment and sex ratio are important and active areas of research due to the potential to target these factors to reduce transmission.

Antimalarial strategies and challenges

Malaria cases and mortality were reduced by 37% and 60% respectively between year 2000 and 2015 (Bhatt et al., 2015). Currently, ongoing research for combating malaria involves developing antimalarial drugs, vaccine development, and vector control. Due to the complex life cycle of the parasite, research in all three areas continues to be very active and it is likely that all methods of

malaria control will be needed in order to eradicate the disease. Below I briefly summarise the current state of each area of malaria control area and the challenges faced.

(i) Parasite resistance to antimalarial drugs

Quinine, a chemical compound of the cinchona tree bark, was the first antimalarial used starting in 1800 and it was used until World War II (Meshnick & Dobson, 2001). Quinine was predominantly used until the synthetic antimalarial chloroquine (CQ) was introduced and then this was used widely from the 1930s to the 1970s. CQ was cheap and easy to obtain, however *P. falciparum* resistance to CQ was first reported in the late 1950s in Southeast Asia followed by India and Africa in the 1970s, resulting in millions of deaths (Sehgal et al., 1973; Trape, 2001). In 1950s, primaquine was introduced, and it remains the only antimalarial that targets both asexual and sexual stages. It also has the ability to prevent relapse in *P. vivax* (Vale et al., 2009). However, this drug has been reported to have a side effect of hemolysis in people with glucose-6-phosphate deficiency (G6PD) (Alving et al., 1960; Watson et al., 2017). In 1960, *P. vivax* was reported to be resistant to primaquine making it a less favoured antimalarial (Arnold et al., 1961; Baird & Hoffman, 2004). Sulfadoxine and pyrimethamine (SP) were then used as a combination drug to replace CQ. These two compounds work better together than alone (Laing, 1970). However, in the late 1970s, resistance towards SP was reported in South East Asia and this spread to sub-Saharan Africa (Verdrager, 1986). Mefloquine was introduced for uncomplicated malaria caused by *P. falciparum* in the early 1980s. However, after 6 years, resistance towards mefloquine appeared in Thailand, Cambodia, Vietnam and in India in 1996 (reviewed in (Bharati & Ganguly, 2013) possibly driven by mutation of *Pfmdr1* (*P. falciparum* multidrug resistance protein) (Price et al., 1999). Although it was reported that mefloquine is efficient against chloroquine-resistant *P. vivax* and *P. falciparum* in Papua, Indonesia (Maguire et al., 2006), there were no other reports supporting this in other malaria-affected areas. Artemisinin Combination Therapies, or ACTs, are currently the frontline treatments against uncomplicated *P. falciparum* malaria (WHO, 2016). Although these treatments are working well in many parts of the world, *P. falciparum* resistance against ACTs has been reported in five countries of the Greater Mekong Subregion: Cambodia, Laos, Myanmar, Thailand and Vietnam (Imwong et al., 2015; Myint et al., 2017; Noedl et al., 2008; Thanh et al., 2017). Looking back at the history of drug resistance,

resistance towards antimalarial drugs typically emerges from Greater Mekong Subregion and then spreads worldwide (Bharati & Ganguly, 2013; Cui et al., 2012). This is alarming because if the trend continues, resistance towards ACTs might spread worldwide.

More generally, it is likely that this battle will never be won with our current arsenal of drugs, and we will continue to need new drug development. Parasite numbers in an infected human host are enormous, easily in the billions. It is possible that every potential mutation exists in the context of a single infection (Hamilton et al., 2017), and thus we should expect that resistance to drugs will evolve eventually. This is one benefit that transmission blocking drugs and vaccines may have; because the numbers of parasites that are targeted at the sexual stage are many fewer (more on this below), resistance may arise more slowly.

(ii) Vaccine Development

The efforts to develop a vaccine against the malaria parasite have been ongoing for decades (Arama & Troye-Blomberg, 2014). Three types of potential vaccines are under development (Figure 1.3). Pre-erythrocytic vaccines target the parasite before it enters the red blood cell stages. Blood-stage malaria vaccines aim to stop parasites from replicating asexually in the blood stage. Transmission blocking vaccines aim to block malaria transmission by targeting either the sexual stages of the parasite, or critical factors in the mosquito that support transmission.

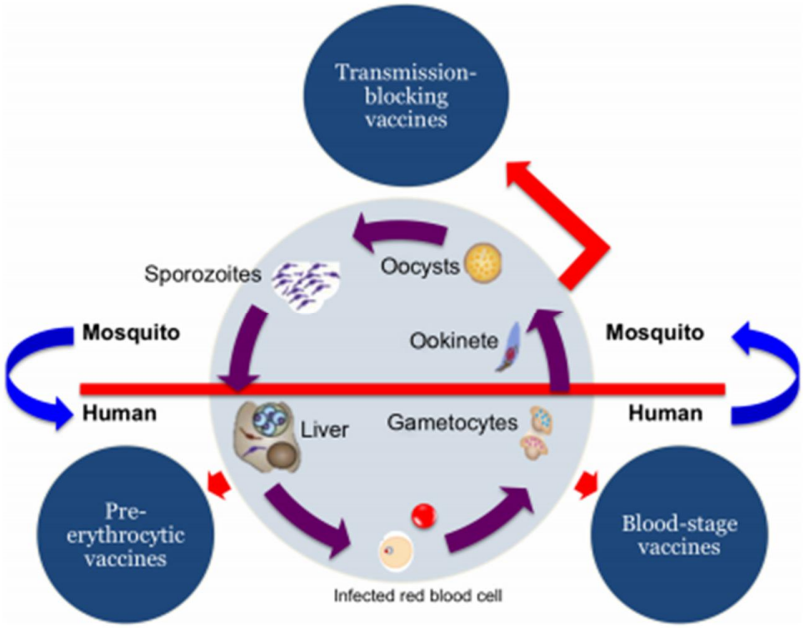

Figure 1.3 Figure taken from (Arama & Troye-Blomberg, 2014) summarises three areas of malaria vaccine development: targeting the pre-erythrocytic stage which involves liver stage vaccine development to prevent malaria in the human host; targeting the erythrocyte stage to block the invasion of erythrocytes by merozoites; or the transmission blocking vaccines that target antigens on gametes, zygotes and ookinetes to prevent parasite development in the mosquito midgut.

All three of these areas of vaccine development are active and although there are indications of success (Arama & Troye-Blomberg, 2014), to date there has never been a successful vaccine deployed against a eukaryotic parasite. Two of the reasons are the complicated genetics which involves genetic polymorphism of *P. falciparum* (Farooq et al., 2012) and complex life cycle of *P. falciparum* parasite which involves multiple hosts make it hard to identify ideal *P. falciparum* antigens (Crompton et al., 2010).

Currently, Mosquirix or better known as RTS,S/AS01 is the most advanced malaria vaccine candidate, and it is now in phase 3 evaluation in Africa (WHO, 2016). RTS,S/AS01 consists of hepatitis B surface antigen virus-like particles, incorporating a portion of the *P. falciparum* derived circumsporozoite protein and a liposome-based adjuvant. This vaccine has the highest protection against parasites with a genotype that matches the circumsporozoite protein allele on

which the vaccine is based (Neafsey et al., 2015). RTS,S/AS01 has been approved by the European Medicines Agency for active immunization of children aged 6 weeks to 17 months against malaria (RTS,S Clinical Trials Partnership, 2015). Although this vaccine provides some protection in the first year after vaccination, the efficacy is reduced subsequently in the following years and is close to zero in the fourth year and beyond of vaccination (Olotu et al., 2016). This might be because the vaccine targets malaria sporozoites but does not induce clinical immunity against the *P. falciparum* asexual stages (Bejon et al., 2011; Campo et al., 2015). However, this vaccine clinical trial is now being brought to the Southeast Asian regions, to see the impact of the vaccine on the Great Mekong Subregion area which has high antimalarial drug resistance but fewer malaria cases (Gosling & von Seidlein, 2016).

(iii) Vector control strategies

As the threat of antimalarial drug resistance grows and vaccine development still has a poor record of success, there is continued pressure to sustain the efficacy of existing vector control methods and to develop alternative methods.

To date, the major intervention strategies aimed at the vector are indoor residual spraying (IRS) of insecticides and long-lasting insecticide treated nets (LLINs) (Rivero et al. 2010; World Health Organization, 2011). It is estimated that these two factors contributed far more substantially to the reduction in malaria that has been observed in the last 15 years than drugs have (Bhatt et al., 2015). These methods both rely in part on the continued susceptibility of mosquitoes to the insecticides we use to kill them. Four classes of insecticides that have been approved and are currently used in the control of malaria carrying mosquitoes by WHO are the organochlorines, organophosphates, carbamates and pyrethroids. IRS involves spraying insecticides which are able to remain on the surface of the internal walls and ceilings in the house. The spraying is usually done before malaria transmission season with the aim of killing the vector before the parasites are able to complete their asexual cycle in the vector which is approximately 14 days from the first bite of infected person carrying *P. falciparum* gametocytes. LLINs insecticide (pyrethroid, typically) treatment lasts for about three years. It acts by killing mosquitoes that come in contact to the nets. Because mosquitoes usually bite between dusk and dawn, sleeping under these LLINs helps to protect people and reduce malaria transmission

(Kawada et al., 2014). Not surprisingly, given the dire consequences for mosquitoes encountering these control measures, insecticide resistance has evolved to all four classes of insecticide currently in use (Karaa, 2012).

In 2010, increased resistance to at least one class of insecticide has been reported from 60 countries and to two or more insecticides from 49 countries (World Health Organization, 2015). The increase of resistance could be partially averted by monitoring and reporting on the resistance in local mosquito vectors (World Health Organization, 2014). Resistance to pyrethroids, the only class currently used in LLINs, is the most commonly reported (Ranson et al., 2011). The rapid rise in resistance is due to extensive and repeated use of these products (Sparks & Nauen, 2015). Apart from this, climate change also has an impact on variation of malaria incidence by increasing mosquito survival (Reiter, 2001). The development of *Anopheles* larvae and adult, the female biting preferences and pathogen development rate are dependent on temperature (Afrane et al., 2012). When the temperature increases, the increased geographic range of mosquitoes could also promote the faster spread of drug resistance (Artzy-Randrup et al., 2010; Soko et al., 2015).

In spite of these ongoing challenges, the vector control strategy is the most promising approach in combating malaria transmission. Beyond the use of insecticides, other methods of vector control include the development of paratransgenesis (Mancini et al., 2016; Villegas & Pimenta, 2014; Wilke & Marrelli, 2015) that aims to use bacteria to block malaria transmission, and the development of transgenic mosquitoes to reduce or replace the population (Knols et al., 2007). A deep understanding of *Anopheles* biology is critical to the further development of vector control strategies. In my research, I have focused specifically on understanding the intersection of *Anopheles* reproductive biology and malaria transmission. This research is potentially relevant for novel vector control approaches by manipulating the reproductive biology of both female and male *Anopheles*.

Anopheles background and current perspective

Mosquitoes belong to the *Culicidae* family, which contains three subfamilies: *Toxorhynchitinae, Anophelinae* and *Culicinae*. There are thousands of mosquito species but among these, those in the genera *Anopheles, Aedes* and *Culex* transmit the majority of dangerous diseases in humans. These species need to blood feed to reproduce which increases their ability to transmit disease. *Aedes* mosquitoes are able to transmit the viruses and parasites that cause yellow fever, dengue fever, and lymphatic filariasis. *Culex* mosquitoes transmit diseases such as encephalitis and filariasis. As for now, the only arbovirus that is transmitted by *Anopheles* is the O'Nyong Nyong virus (ONNV), a virus closely related to Chikungunya virus (CHIKV) (Saxton-Shaw et al., 2013), which causes a severe joint pain (Brault et al., 2004; Waldock et al., 2012).

However, it is only *Anopheles* mosquitoes that are responsible for human malaria transmission. There are over 420 *Anopheles* species in the world but only 50 are thought to be major vectors for human malaria (Figure 1.4) (Sinka et al., 2012). *An. gambiae* and *An. coluzzii* are the most prominent malaria vectors, together causing the most lethality, with *An. funestus* and *An. arabiensis* also proficient malaria vectors of *P. falciparum* in sub-Saharan Africa (Coetzee et al., 2013). In Figure 1.4, *An. gambiae* actually represents both *An. gambiae* and its closely related sister species, *An. coluzzii*. This species was formerly known as *An. gambiae* M form (Coetzee et al., 2013) and is restricted to West and Central Africa compared to *An. gambiae* (formerly known as the S form) which exists across the entire range of *An. gambiae* ss (Crawford & Lazzaro, 2010).

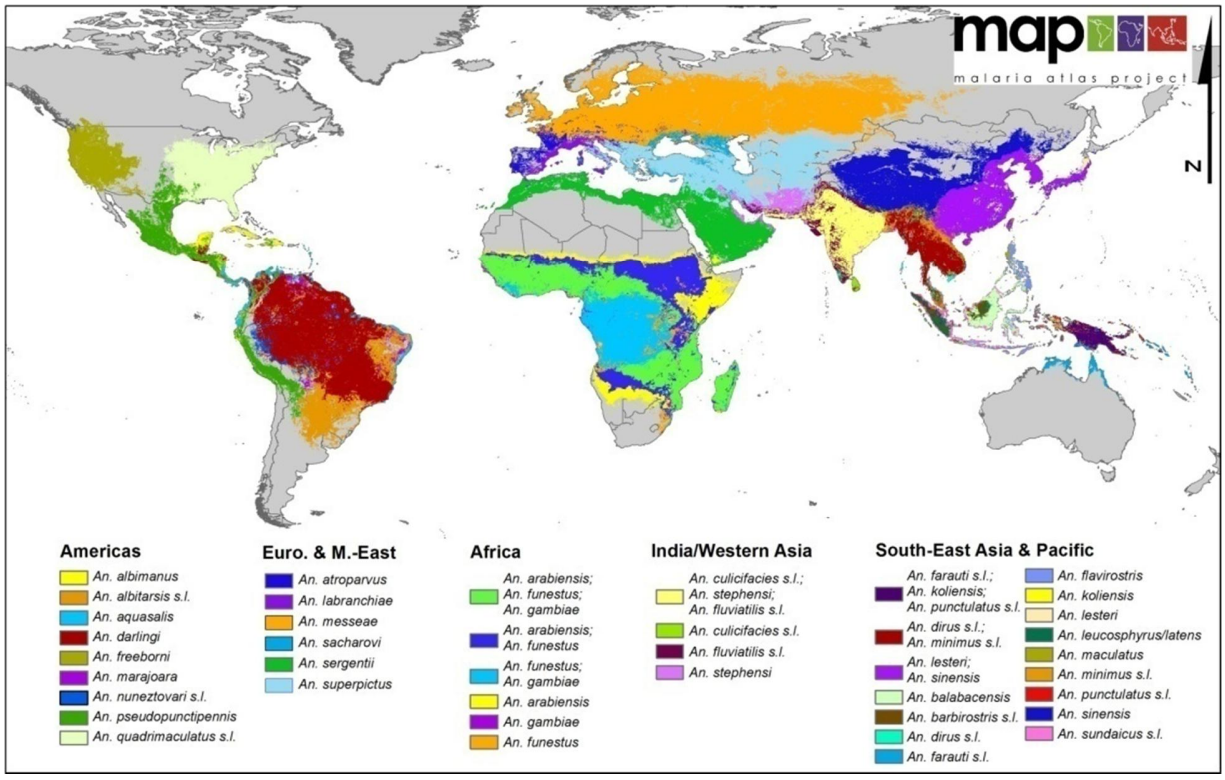

Figure 1.4 This map, taken from (Sinka et al., 2012), illustrates the global distribution of the dominant vector species of malaria in each region.

An. coluzzii dominates disturbed habitats and shows the ability to adapt to new breeding sites created by urbanization while *An. gambiae* is less competitive (della Torre et al., 2005; Simard et al., 2009). The ability to adapt suggests *An. coluzzii* will have the advantage to breed throughout the year which could lead to year round malaria transmission (Caputo et al., 2011). In Senegal Africa, it was reported that susceptibility of *P. falciparum* infection prevalence is higher in *An. gambiae* compared to *An.coluzzii* (Ndiath et al., 2011). However, *An. coluzzii* has higher prevalence in Cameroon (Boissière et al., 2013). In Burkina Faso, it was reported that the susceptibility of *An. gambiae* and *An. coluzzii* to *P. falciparum* is equal (Gnémé et al., 2013). More work is necessary to further compare the roles in malaria transmission across the major vectors in Africa.

Anopheles lifecycle

Anopheles mosquitoes breed by laying eggs in still and clean water. One female *An. gambiae* is able to produce around 50 eggs in a single lay (Sumba et al., 2004). These eggs typically hatch in the next 48 hours to produce L1 larvae. *Anopheles* larvae have four stages to complete. These stages differ in sizes and number of thoracic segments (Savignac & Maire, 1981). After about 10 days, the larvae form pupae. At the pupal stage, one can differentiate each sex by looking at the terminal segments of the pupae under a dissecting microscope. These three stages (egg, larva, pupa) are the aquatic stages of *Anopheles*. Typically 24 hours after pupation, the adults will eclose from the pupae. The optimal temperature for *Anopheles* is 27 to 33°C. Temperature also plays an important role for larval development and impacts the final adult size (Beck-Johnson et al., 2013; Christiansen-Jucht et al., 2015). Adult *Anopheles* of both sexes feed on nectar but only female *Anopheles* feed on animal blood, and both mating and bloodfeeding are essential for fertile egg production in this genus (Figure 1.5).

It is essential for mosquitoes to blood feed in order to provision their eggs. This egg provisioning is regulated by a variety of different hormones. In general, upon blood feeding, the fat body in the mosquito will synthesize and secrete ovary ecdysteroidogenic hormone (OEH). OEH stimulates the ovaries to secrete ecdysone which will hydroxylate to 20-hydroxyecdysone (20E) in the female fat body (Hagedorn et al., 1975; Swevers et al., 1995). 20E will next activate yolk precursor proteins (YPP) by binding to the ecdysone receptor (EcR) / ultraspiracle (USP) dimer. Upon activation, both Vitellogenin (Vg) and Lipophorin (Lp) are secreted into the hemolymph and incorporated into growing oocytes that will mature within 2-3 days (Hagedorn et al., 1975; Swevers et al., 1995).

In *Anopheles* as well as other insects, 20E is also important to complete metamorphosis. Metamorphosis in insects is regulated by Juvenile Hormone and (20E) hormone that work antagonistically (Bai et al., 2010; Royer et al., 2002; Wu et al., 2006; Zhou & Riddiford, 2002).

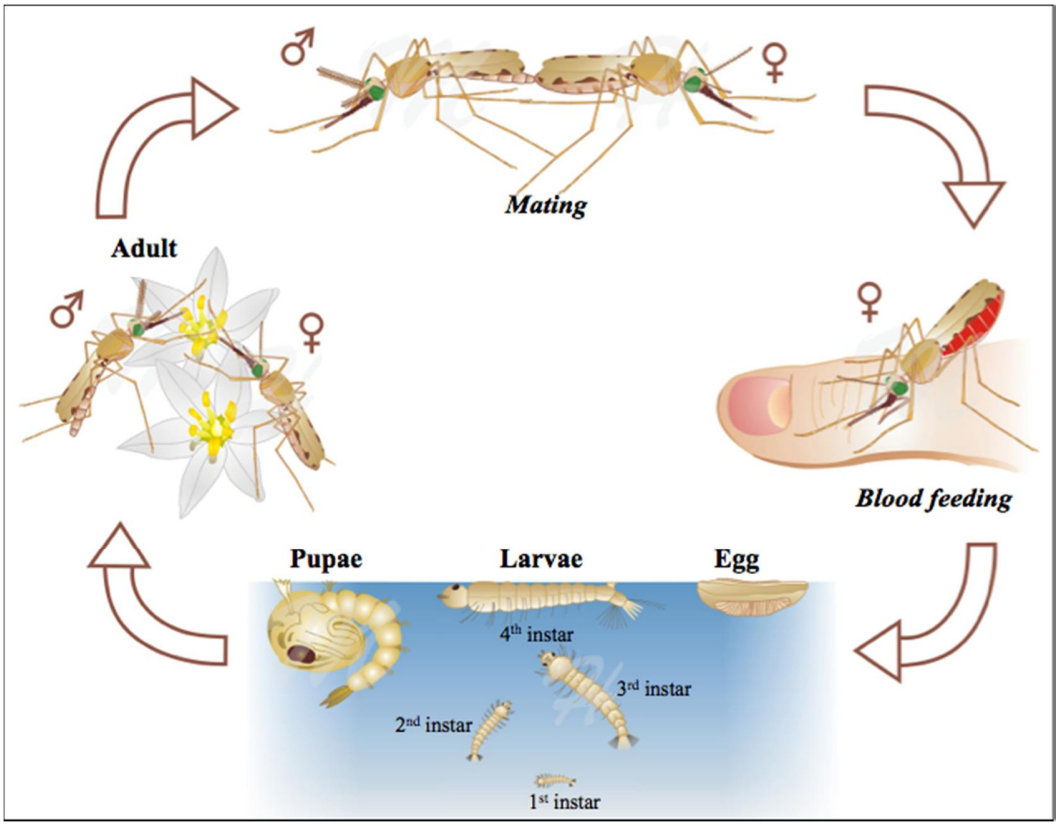

Figure 1.5 Life cycle schematic of *An. gambiae* which includes eggs, larvae, pupae and adult. Figure was taken and edited from http://www.biographix.cz/portfolio/schemes-models/life-cycle-of-the-mosquito-anopheles-gambiae/

Upon blood feeding, the transcriptional profile of *An. gambiae* midguts changes abruptly and likely influences blood digestion, egg production, and the response to oxidative stress (Dana et al., 2005). An important structure secreted by the midgut epithelial cells upon taking in a blood meal is the type I peritrophic matrix (PM). The PM is a layer containing chitin, proteoglycans and proteins that surrounds the blood bolus in the midgut and separates it from the midgut epithelium cells (Freyvogel & Jaquet, 1965; Shen & Jacobs-Lorena, 1998; Tellam et al., 1999). The PM is important in blood digestion and protection against toxic products and parasites in mosquitoes (Huber et al., 1991; Pascoa et al., 2002).

In order to develop and lay fertile eggs, *Anopheles* females must have both taken a bloodmeal and mated. The order of whether they mate first or take a bloodmeal first appears to be flexible (Charlwood et al., 2003; Gabrieli et al., 2014). *Anopheles* typically mate in a swarm at dusk

(Charlwood et al., 2003; Charlwood & Jones, 1979). In short, the females will enter the swarm which consists mainly of the males, form mating couples and leave the swarm in copula. While males may mate multiply, females are monandrous, mating only once and storing sperm for life in an organ known as the spermatheca. Female monogamy in *Anopheles* is induced by male accessory gland proteins (Shutt et al., 2010). However, the evolutionary pressures that have resulted in female monandry are poorly understood.

In *An. gambiae,* during mating, along with sperm, the male transfers 20E hormone and a gelatinous mating plug which consists of seminal fluid proteins that is digested over the following 24 hours inside the female atrium (Giglioli & Mason, 1966; Pondeville et al., 2008; Rogers et al., 2009) (Figure 1.6). Females that do not receive the mating plug fail to store sperm and thus do not become inseminated (Rogers et al., 2009). The formation of the mating plug is mediated by a male accessory gland (MAG) specific transglutaminase (TGase) exclusively found in *Anopheline* mosquitoes (Rogers et al., 2009). The male derived 20E induces oviposition and refracts the females from further mating. Heme peroxidase-15 (HPX-15) is expressed in the spermatheca to ensure sperm stability. HPX-15 expression is regulated by the male-derived 20E hormone (Shaw et al., 2014). There is a high correlation of gene expression between uninjected mated females and 20E-injected virgin females in the atrium and spermatheca tissue which suggest that 20E could mimic the transcriptional impact of mating (Gabrieli et al., 2014) and that 20E is thus ultimately responsible for many of the changes that females experience post-mating.

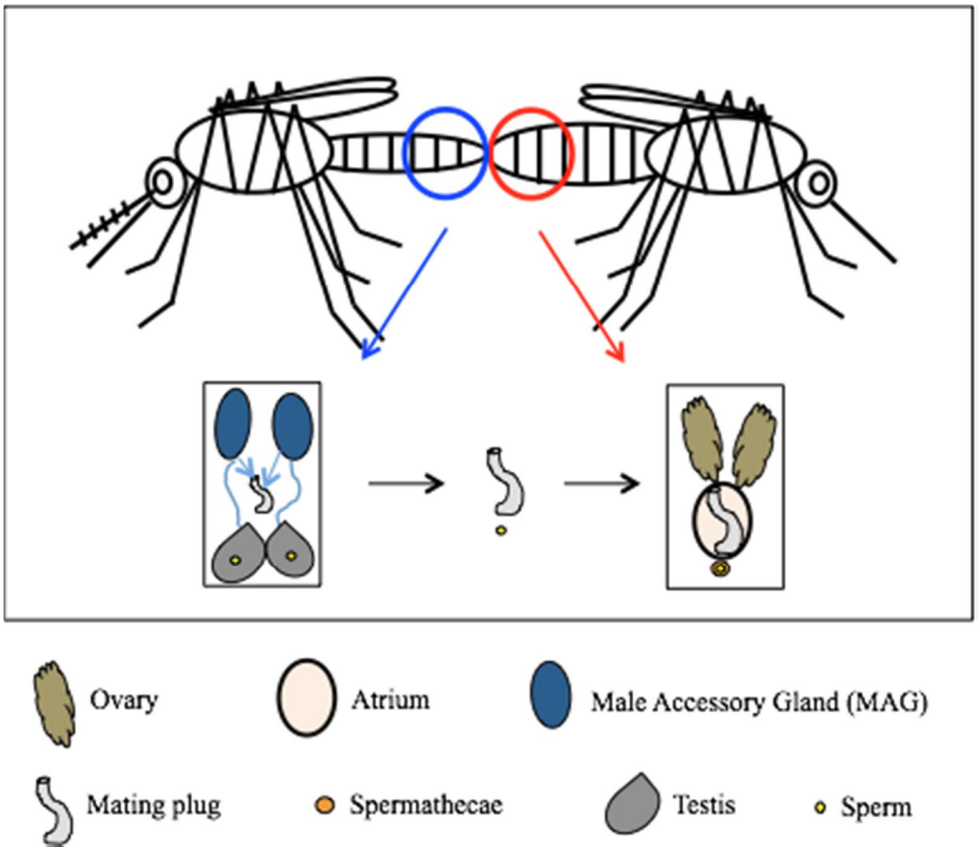

Figure 1.6 Anatomy of reproductive tracts of male and female *Anopheles*. Male *Anopheles* (left) transfer sperm together with a mating plug during copulation. Sperm are stored in the spermatheca whilst the mating plug is dissolved within 24 hours in the female atrium.

In many insects, mating has a huge impact on female physiology, behaviour, and immunity (McGraw et al., 2004; Ram & Wolfner, 2007). For example, induction of oogenesis, oviposition, and refractoriness to further mating are common results of mating (Klowden & Russell, 2004; Rogers et al., 2008; Uchida et al., 2003). In *Drosophila melanogaster,* mated females are immunosuppressed compared to virgin females: they have lower antimicrobial peptide (AMP) gene expression as compared to virgins and this correlates with higher systemic bacterial loads (Short & Lazzaro, 2010). These effects are due to the seminal fluid components transferred by the male that alter female *D. melanogaster* humoral immune system activity. This response is dependent on the presence of female germline cells such that females with ablated ovaries do not have immunosuppression upon mating (Short et al., 2012). Both reproduction and immune

responses require high energy usage and demanding physiologically. When the reproduction increases, immunity reduces and the same is found when immune response is higher, reproductive output decreases. These findings suggest that there may be a tradeoff between reproduction and immunity (Schwenke et al., 2016).

Similar to other insects, female *Anopheles* undergo behavioural changes after mating, such as induction of egg laying and refractoriness to further insemination (Gabrieli et al., 2014; Klowden & Russell, 2004; Rogers et al., 2008, 2009; Tripet et al., 2001). They also undergo major physiological changes. Transmission Electron Microscope experiments of the atrial cells of a virgin female show smooth endoplasmic reticulum and storage vacuoles populating the apical cytoplasm, mitochondria alongside the smooth endoplasmic reticulum and abundant rough endoplasmic reticulum at the basal pole. However after mating, the structure changes where the apical surface contains electron-dense vacuoles with collapsed smooth endoplasmic reticulum membranes. Atrial cells of mated females that have direct contact with the mating plug show endosomes and lysosomes occurring densely at the apical pole and the basal labyrinth is expended and the basal lamina is distinct (Rogers et al., 2008). Mated female atrial cells are permanently altered in ways that suggest these cells function to support the uptake of male material transferred during copulation (Rogers et al., 2008). Comparison of post mating gene expression between whole virgin and mated *An. gambiae* females suggested that mating causes permanent changes in gene expression although immune genes do not appear to be regulated by mating like they are in *Drosophila* (Rogers et al., 2008; Short & Lazzaro, 2013).

Anopheles and *P. falciparum* transmission : *P. falciparum* sexual life cycle in *Anopheles* midgut

After a female Anopheline bites an infected human, the sexual stage male and female gametes emerge from the red blood cells inside the gut of the mosquito. The male gametocyte undergoes 3 rapid DNA replications during a process called exflagellation to form 8 motile haploid microgametes. These motile microgametes (male) will search the bloodmeal to find a macrogamete (female) and then fertilization will occur, forming a diploid zygote that develops

over the next 24 hours into a motile ookinete. This occurs inside the bloodmeal as it is being digested, and once mature, the ookinete must penetrate the *Anopheles* PM that has fully formed during this time. After penetrating the PM, the ookinete must also pass through the midgut epithelium, comprising a single layer of cells, before it exits into the basal lamina where it then matures over the next 7-10 days into an oocyst containing thousands of haploid sporozoites. When the oocyst bursts around 16 days post blood feed, these sporozoites migrate around the mosquito body and some enter the salivary gland where they are then poised to infect any humans the mosquito bites after this stage (Figure 1.7).

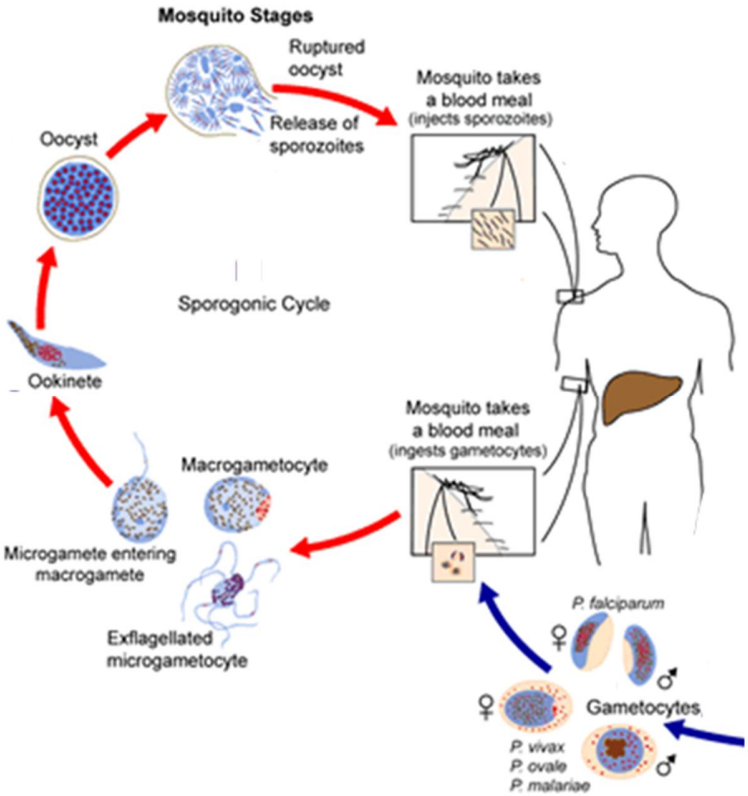

Figure 1.7 *P. falciparum* life cycle in the ***Anopheles*** **mosquito**. Figure taken from Center for Disease Control website (https://www.cdc.gov/dpdx/malaria/index.html).

Anopheles and microbiota

The microbiota comprise the resident bacteria that live in an *Anopheles* mosquito midgut. Although there are no known obligate bacterial species in *Anopheles,* there is a great diversity of bacteria that are likely to enhance mosquito fitness by their contribution to digestion of plant-derived polymers from nectar, digestion of lipid and protein from the blood meal for nutrient absorption, and/or their contribution to pathogen protection (Douglas, 2009; Engel & Moran, 2013; Gaio et al., 2011; Minard et al., 2013). *An. gambiae* and *An. coluzzii* harbor similar bacterial communities during the larval stage, and the species composition of microbiota reduces when they are adults (Gimonneau et al., 2014). Four prominent bacterial classes, *Gammaproteobacteria, Alphaproteobacteria, Betaproteobacteria* and *Actinobacteria,* are commonly found in newly emerged *An. gambiae* and *An. coluzzii* midguts, ovaries and salivary glands (Gimonneau et al., 2014). These observations suggest that these bacteria may represent *An. gambiae* and *An. coluzzii* symbionts (thought not obligate) that are environmentally and/or transstadially transmitted.

The bloodmeal, which is critical both for malaria transmission and mosquito reproduction, has a massive impact on mosquito midgut bacterial proliferation. Twenty four hours upon blood feeding, the microbiota in *An. gambiae* midguts dramatically increased by 100 times compared to unfed midguts (Dong et al., 2009; Meister et al., 2009). The resident microbiota play a key role in digestion as well as immune regulation (Gaio et al., 2011; Rodgers et al., 2017) and thus there are multiple potential direct and indirect interactions in the midgut between the mosquito, the midgut bacteria, and the malaria parasites. Microbiota can have a negative impact on mosquito susceptibility to *P. falciparum* (Dong et al., 2009), potentially due to competition for nutrients (Hentschel et al., 2003; Ivanov & Littman, 2011; Reid et al., 2001), through mechanisms that involve oxidative stress that kill the parasites (Cirimotich et al., 2011; Luckhart et al., 1998; Ngwa et al., 2013), or by inducing immune responses that trigger activation of the antibacterial pathway (Dennison et al., 2015; Stathopoulos et al., 2014; Tchioffo et al., 2013). Removing bacteria through antibiotic treatment from the midgut increases parasite infection suggesting that the microbiota can reduce *P. falciparum* infection (Dong et al., 2009; Gendrin et al., 2015; Rodgers et al., 2017). Some of the bacterial species found in *Anopheles* are of interest

in malaria research such as *Serratia* (Dong et al., 2009), *Pantoea* (Straif et al., 1998), *Klebsiella* (Gonzalez-Ceron et al., 2003; Pumpuni et al., 1993; Harutyunova et al., 2013), *Elizabethkingia* (Rani et al., 2009) and *Asaia* (Capone et al., 2013).

Studies in *Drosophila* suggest that mating has an impact in increasing microbiota in the whole female (Short et al., 2012). It is not known if this is the case in *Anopheles*. It is important to understand if the mating status of *Anopheles* influences bacterial loads and bacterial diversity in the midgut because the midgut is also an important place for *Plasmodium* parasites to complete their sexual stage to ensure successful transmission. Microbiota research is widely studied with respect to mosquito borne diseases because of the potential for bacteria to influence disease transmission. One bacterial species that has received much attention in studies concerning mosquito transmitted disease is *Wolbachia*. This bacteria was shown to be able to reduce vector competence in *Aedes* towards dengue and Zika virus (Dutra et al., 2016; McMeniman et al., 2009). In *Anopheles* however, there have been inconsistent impacts of *Wolbachia* on malaria parasites (Hughes et al., 2014). Recently, *Wolbachia* was reported to be able to infect *An. coluzzii* and manipulate reproduction by reducing the time for egg laying (Shaw et al., 2016). Furthermore, there is a negative correlation between the presence of *Plasmodium* parasites and *Wolbachia* infection (Shaw et al., 2016). However, the presence of *Wolbachia* does not induce cytoplasmic incompatibility nor distort the sex ratio like it can in *Aedes* and *Culex* which suggests a different response in *An. coluzzii* compared to other mosquito species that are able to transmit diseases (Shaw et al., 2016).

Understanding the impact of a female's mating status on her bacterial abundance and diversity might aid in efforts to tackle the spread of mosquito borne diseases. Also relevant is whether the mating status of *Anopheles* has an impact on malaria transmission. Looking at the transcriptome of the midgut in response to mating, blood feeding, and antibiotic treatment will aid in understanding these factors might influence transmission. Although the 20E hormone that males transfer during mating has been shown to increase egg production and stop the female from further insemination (Gabrieli et al., 2014), the male transfer of 20E in all of the major human malaria vector species raises the question of the hormone's role, and thus the role of mating, in increasing malaria transmission (Mitchell et al., 2015). Furthermore, it is not known if 20E has a

direct impact on the midgut of *Anopheles*, thus it is important to understand the transcriptome of the midgut of female *Anopheles* experiencing 20E hormone independent of mating.

CHAPTER 2

Aim of the thesis

Around 663 million malaria cases have been prevented from 2000-2015 due to malaria control interventions. Of these, it is estimated that 69% were averted due to the use of insecticide-treated mosquito nets (ITNs) (UI: 63–73%), 21% due to artemisinin based combination therapy (ACT) (UI: 17–29%), and 10% due to indoor residual spraying (IRS) (UI: 6–14%) (Bhatt et al., 2015). These numbers clearly show that targeting vectors has the greatest impact on reducing malaria transmission. However, insecticide resistance now exists against all four classes of insecticides and it is on the rise in many wild populations (Bhatt et al., 2015). New methods of vector control are sorely needed to maintain the gains achieved in reducing malaria transmission.

My thesis aims to explore several facets of mosquito biology that are critical to malaria transmission. Female mosquitoes depend on bloodmeals to develop their eggs. These bloodmeals are where the mosquitoes acquire the pathogens and parasites that they can transmit. The bloodmeals also have a large impact on the microbiota present in the midgut. Mating is known in many insects to influence facets of female physiology and behaviour, so the overall aim of my thesis is to examine whether mosquito mating status influences the abundance and diversity of microbiota and susceptibility to human malaria parasites. I also explore the transcriptional patterns that distinguish virgins from mated females under a variety of circumstances, including whether they are sugar fed or blood fed or with or without microbiota.

Given the complexity of the bacterial composition in the *Anopheles* midgut, I first investigated whether mating status affects bacterial abundance and composition in the midgut of female *An. coluzzii*. From this thesis, bacterial abundance is higher in the midgut of mated females than virgin females. Bacterial composition is altered due to both mating and antibiotic treatment.

Secondly, I am to investigate the impact of mating status, blood feeding, and antibiotic treatment on *An. coluzzii* female midgut transcriptomes. The RNASeq analysis shows that the transcriptome of the *An. coluzzii* sugar fed female midguts show many genes are regulated by mating. However this impact is less apparent once females are bloodfed. Many genes were

significantly differentially expressed in response to antibiotic treatment on the *An. coluzzii* female *P. falciparum* fed midgut transcriptome which is consistent with bacteria having an important role in midgut dynamics.

Next, I test whether the mating status of *An. coluzzii* females influence their susceptibility to *P. falciparum*. From my experiment, independent of bacteria, mating frequently has an impact on mosquito susceptibility to *P. falciparum* especially when *P. falciparum* infection levels are high.

To further explore the impact of mating status on *P. falciparum* susceptibility, I specifically mimic the male transfer of 20E by injecting it into virgins to test the impact of 20E on *An. coluzzii* susceptibility to *P. falciparum*. 20E injection in virgins results in an increased *P. falciparum* infection intensity. However, the same result is not seen when 20E injection is administered to mated females, suggesting 20E acts once, perhaps to remodel the gut and prepare it for digestion and egg provisioning.

Finally, I investigate if the impact of 20E-injection on virgin *An. coluzzii* female midgut transcriptomes resemble the mated females midgut transcriptome, as is true for the lower reproductive tract. The impact of 20E injection on the virgin's midgut transcriptome shares 11% of the differentially expressed genes observed between mated and virgin females. This amount of overlap suggests that 20E injection does not fully mimic the mated females midgut transcriptome, but that 20E might underlie some of the changes observed.

CHAPTER 3

The impact of mating on bacterial abundance and composition in the midgut of female *An. coluzzii* mosquitoes

Introduction

While there are no known obligate bacteria in *Anopheles,* diverse species of bacteria live and multiply in the midgut of mosquitoes, enhancing mosquito fitness by contributing to the digestion of plant-derived polymers from nectar, the digestion of lipids and proteins from the blood meal, or by protecting them from pathogens (Douglas, 2009; Engel & Moran, 2013; Gaio et al., 2011; Minard et al., 2013). Adult mosquitoes likely acquire microbiota in their midgut from environmental exposure (Gimonneau et al., 2014). Adult female *Anopheles* mosquitoes require a bloodmeal to develop their eggs, and these bloodmeals also have a dramatic impact on the resident microbiota: twenty four hours upon blood feeding, the microbiota in *An. gambiae* midguts increases by 100 times compared to unfed midguts (Dong et al., 2009). If the female has taken up sexual stage *P. falciparum* parasites in her meal, many parasites will be ookinetes at this 24 hour time point post bloodmeal, penetrating the midgut epithelium.

The resident microbiota play a key role in digestion as well as immune regulation (Gaio et al., 2011) and thus there are multiple potential direct and indirect interactions between the mosquito, the midgut, and the malaria parasites. Microbiota can have a negative impact on mosquito susceptibility to *P. falciparum* (Dong et al., 2009), potentially due to competition for nutrients (Hentschel et al., 2003; Ivanov & Littman, 2011; Reid et al., 2001) through mechanisms that involve oxidative stress that kills parasites (Cirimotich et al., 2011; Luckhart et al., 1998; Ngwa et al., 2013) or by inducing immune responses that trigger activation of the antibacterial pathway (Dennison et al., 2015; Stathopoulos et al., 2014; Tchioffo et al., 2013).

Studies on mosquito bacterial diversity are of interest because of the potential role of bacteria in developing paratransgenesis to control the vector (Gendrin et al., 2015; Minard et al., 2013; Wilke & Marrelli, 2015). Paratransgenesis utilizes the symbiont to deliver anti-Plasmodial

transgenes that eliminate the parasite (Wilke & Marrelli, 2015). Several species of bacteria are under consideration for their utility in paratransgenesis (Bahia et al., 2014). Table 3.1 summarizes the bacteria found in *Anopheles* and some studies of these bacteria and *Plasmodium* infection. For example, *Serratia* bacteria are dominant in some disease vector species, such as *Anopheles*, tsetse fly, sandflies and *Aedes* (Azambuja et al., 2005; Bando et al., 2013; Dong et al., 2009; Geiger et al., 2010; Gusmão et al., 2010). *Serratia marcescens* strain HB3 interrupts ookinete invasion in *An. stephensi* midguts (Bahia et al., 2014; Bando et al., 2013) and is also able to block *P. vivax* sporogonic development in *An. albimanus* (Gonzalez-Ceron et al., 2003). Recently, a particular *Serratia* bacterium strain (AS1) which was isolated from *Anopheles* ovaries was engineered to carry anti-*Plasmodium* effector proteins. This genetically engineered bacterium is able to spread rapidly in the midgut and inhibit *P. falciparum* development (S. Wang et al., 2017) *Serratia* and *Pantoea* are the most common species identified from field caught *An. gambiae* (Straif et al., 1998). Another example is a *Pantoea agglomerans* strain that has been engineered to secrete antimalarial proteins in the mosquito midgut that suppress *P. falciparum* and *P. berghei* development (Wang et al., 2012).

Furthermore, *Asaia* bacteria are resident not only in the midgut, but also in the salivary glands and reproductive organs of *An. gambiae* which makes it a potentially promising bacterial species to be used in vector control strategy given that parasites must also invade the salivary gland to infect the next person, and that the presence of these bacteria in the reproductive tissues might enhance vertical transmission (Capone et al., 2013; Damiani et al., 2010; Mancini et al., 2016). Studies in *An. stephensi* showed that *Asaia* bacteria replication remains the same whether or not the mosquitoes were given *P. berghei* blood meal (Damiani et al., 2010). This suggests that the immune response upon parasite invasion does not interfere with *Asaia* replication (Capone et al., 2013). Another interesting bacterial species is *Elizabethkingia* from the *Flavobacteriaceae* family, which is dominant in *Anopheles* from South East Asia (Rani et al., 2009) and possesses anti-Plasmodial activity (Ngwa et al., 2013). *Klebsiella* is another bacteria found in midgut of *Anopheles* (Harutyunova et al., 2013) but there are no studies examining the impact of *Klebsiella* on *P. falciparum*. All together, while there are no obligate species of bacteria present in *Anopheles* mosquitoes, there are many that are commonly found, and many of these have potential consequences on mosquito susceptibility to parasites.

Bacteria	Found in	Interaction with *Plasmodium*
Serratia	*An. gambiae* (Dong et al. 2009)	- Interrupts ookinete invasion in *An.stephensi* (*P. berghei) and An.gambiae (P. falciparum)* midguts - Block *P. vivax* sporogonic development in *An. albimanus* - *Serratia* bacterium srain (AS1) genetically engineered to secrete anti-*Plasmodium* effector protein (Bando et al. 2013; Bahia et al. 2014; Gonzalez-Ceron et al. 2003, Wang et al. 2017)
Pantoea	*An. gambiae* (Straif et al. 1998)	Secrete antimalarial proteins in the mosquito midgut suppressing *P. falciparum* and *P. berghei* development (Wang et al. 2012)
Klebsiella	Dominant in many *Anopheles* mosquitoes species (Pumpuni et al. 1993; Gonzalez-Ceron et al. 2003) (Harutyunova et al. 2013)	No report
Asaia	Able to localize in *An. stephensi* (Capone et al. 2013)	An interesting bacteria for paratransgenesis (Capone et al. 2013; Damiani et al. 2010; Mancini et al. 2016)
Elizabethkingia	Dominant in *Anopheles* in the wild and lab-reared colony (Rani et al. 2009)	Possess anti-Plasmodial activity (Ngwa et al. 2013)

Table 3.1 List of some known bacterial species which will be discussed in this chapter and later chapters and studies on interaction with *Plasmodium* parasites in order to develop paratransgenesis for combatting malaria.

One possible problem with studying the microbiota of lab mosquitoes is the potential difference in species composition or diversity compared to wild mosquitoes. There are significant differences between field-collected and laboratory reared mosquitoes where there will be some loss in bacterial species over time with the controlled environment and food in laboratory-reared mosquitoes (Boissière et al., 2012; Wang et al., 2011). However, most frequent bacterial genera are present in laboratory-reared and field-collected adult *Anopheles* midgut which suggests that the bacterial diversity is still preserved in the laboratories colonies (Gendrin & Christophides, 2013; Wang et al., 2011).

In addition to blood feeding, which influences the microbiota of the female, females must also mate to successfully reproduce. In insects, mating induces physiological and behavioural changes in females such as an increase in egg production, food intake, and refractoriness to additional matings (Herndon & Wolfner, 1995; Rogers et al., 2009; Rolff & Siva-Jothy, 1999;

Tsukamoto et al., 2014). Mating can also expose females to bacterial, viral, fungal and sexual transmitted diseases (STDs) (Knell & Webberley, 2004; Miest & Bloch-Qazi, 2008; Nalepa & Weir, 2007). In *Drosophila*, mated females have reduced resistance to bacterial infection relative to virgin females (Short & Lazzaro, 2010). This was shown to be driven by the seminal fluid proteins transferred by the males during mating which results in the suppression of antimicrobial peptide (AMP) gene expression (Short et al., 2012). In *An. gambiae*, mating induces refractoriness to further copulation and increases egg production and oviposition (Klowden & Russell, 2004; Rogers et al., 2008, 2009; Tripet et al., 2003). However, one important difference between *Anopheles* and *Drosophila* is that *Anopheles* requires a blood meal to produce eggs which will increase the bacteria abundance in the gut. Therefore, discerning whether mating also influences the microbiota abundance or diversity in the gut is the aim of the work presented in this chapter. First, I examine whether mating has an impact on bacterial abundance in sugar fed and blood fed female midguts. Secondly, I investigate whether mating changes the diversity of microbiota in *An. coluzzii* female midguts upon mating. This work is of general interest because of the known relationship between the microbiota and the outcome of *Plasmodium* infection. Furthermore, this work is important for later chapters in which I explore the impact of mating on transmission because it is important to understand whether microbiota are influenced by mating as well, and thus could indirectly explain outcomes of *Plasmodium* infection.

Materials and Methods

There are some similarities of the sample preparation in every chapter in this thesis. Minor changes such as number of mosquitoes, age of *P. falciparum* gametocyte culture and other related metadata are summarized in Appendix S1 and Appendix S2.

Mosquitoes

Mosquito rearing

The Ngousso strain of *An. coluzzii*, which originates from Cameroon was reared under standard conditions (26°C-28°C, 65%-80% relative humidity, 12hr:12hr light/darkness photoperiod). Eggs were floated in a pan filled with deionized water and once larvae hatched, around 1200 larvae were reared in a 32L plastic pan (66cm x 45cm x 17cm). The larvae were fed on one mL of ground fish food each day and 2 pieces of cat food on Friday evening for the weekend. When the adults emerged, they were maintained on 10% autoclaved fructose solution.

Collecting and separation of Ngousso pupae

Adult mosquitoes were separated by sex as pupae and placed in separate cages in dishes filled with deionized water. Cages were inspected when adults emerged and any of the wrong sexed adults were removed. Some females were introduced to the males via aspirator after emergence and remaining females were retained in the original cage and labelled as virgin. The aspirator was cleaned with 70% ethanol prior to use.

Ngousso mating

Mating assays were performed to determine the percentage of females mating over the course of one night. The spermatheca of females housed overnight with males were dissected the following morning and viewed under light microscope and 80% were found to have mated. After that, we use a single overnight mating method in all of our experiments. Mated females and males were separated after they were left overnight by putting the cage on ice.

Ngousso treatment with antibiotics

Female Ngousso mosquitoes were separated into two sets from eclosion. Set one is fed on normal sugar whilst the second set of mosquitoes were maintained with antibiotic-containing sugar solution (25µg/mL gentamycin, 10µg/mL penicillin and 10 unit/mL streptomycin) to remove most of the bacteria from their midgut. Introducing antibiotics in the sugar meal has been reported not to interfere with the mosquitos' longevity as it does if antibiotics were added into blood meal. This might be due to microbiota expansion upon blood feeding (Dennison et al.,

2016). Both untreated and antibiotic treated sets of mated females and virgin females were placed in four different cups and left overnight before blood feeding.

Ngousso blood feeding on *P. falciparum*-infected blood

Information on preparing *P. falciparum* infected blood will be discussed in Chapter 5 Materials and Methods. Cotton was removed 3 hours before blood feeding. Each cup contained Ngousso females with different treatments: mated untreated, mated treated with antibiotics, virgin untreated, and virgin treated with antibiotics. They were left to blood feed on *P. falciparum*-infected blood for 5-10 minutes using the membrane feeding assay. Blood was then removed, and new sugar-soaked cotton was placed on each cup and the cups were kept in an incubator at 26°C and 80% humidity.

24 hours after blood feeding, mosquitoes were killed with 70% ethanol and washed thrice with 1x PBS. The mosquitoes were kept in 1x PBS on ice. Each mosquito's midgut was dissected on a glass slide on a drop of 1x PBS and put in a homogenizer tube on dry ice.

Molecular Biology Methods

RNA Extraction

Total RNA from female Ngousso midguts was extracted using TRIzol (Life Technologies) and chloroform (Sigma). For sugar fed midguts, five midguts were pooled together whilst for blood fed midguts, each midgut was prepared individually. Figure 3.1 summarizes the samples used in this chapter (Chapter 3) and in the RNASeq experiment (orange box) discussed in Chapter 4. Each sample was homogenised using the Precellys 24 homogenizer at maximum speed, 6,800rpm for 30 seconds and let to rest for 5 minutes at room temperature (RT). Samples were then centrifuged at 13,200 rpm for 15 minutes at 4°C. The aqueous phase was collected into a tube that was filled with 250µL isopropanol (Sigma) and 1µg glycogen (Sigma) to precipitate the RNA. The pellet was kept to extract DNA using the back extraction buffer (described below). Samples were centrifuged at 13,200rpm for 15 minutes at 4°C followed by washing using 70% ethanol (Sigma) and concentrated using the DNA concentrator for 10 minutes. 10µL molecular

biology grade water was added to each tube and was placed on a heat block (37°C) for 15 minutes. Total RNA was measured using Nanodrop.

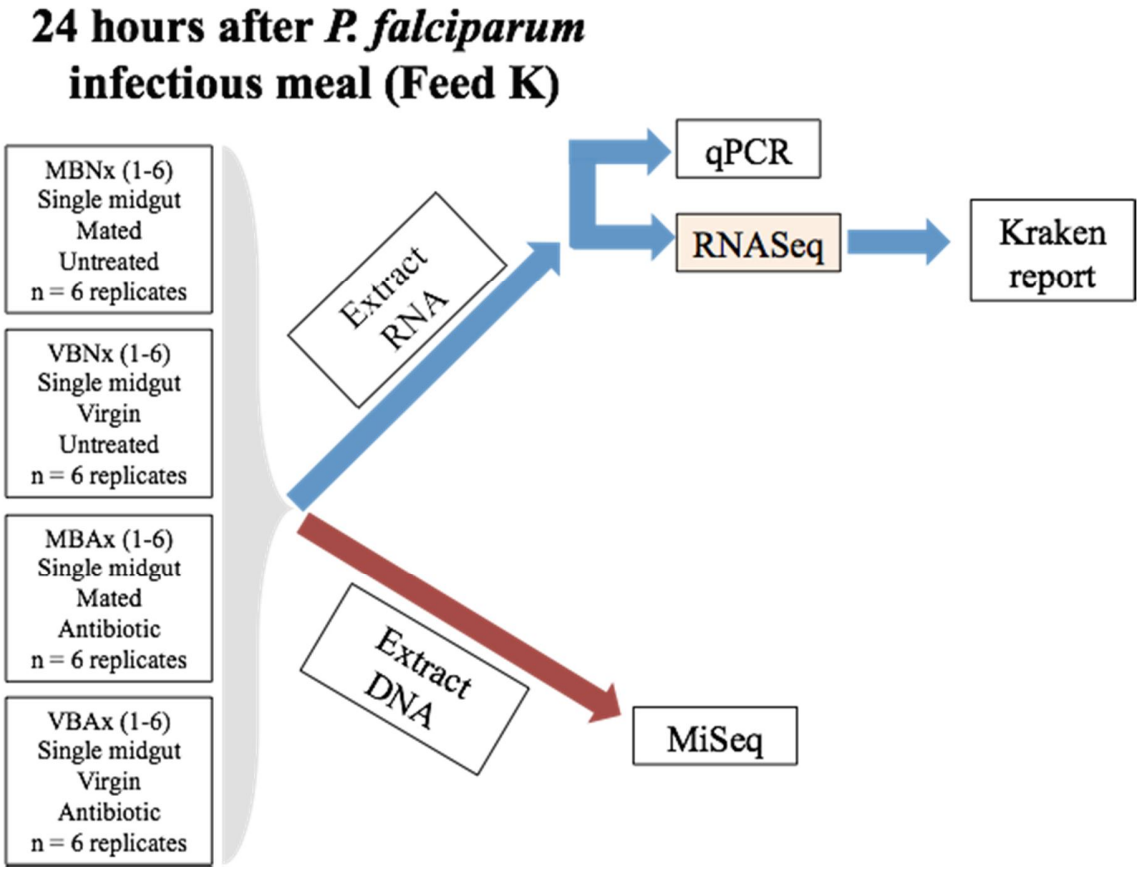

Figure 3.1 Six midguts from each treatment and mating status were dissected from 24 hours post *P. falciparum*-infected blood fed mosquitoes. This experiment [K] had 100% *P. falciparum* infection prevalence. RNA was extracted from each midgut and used in qPCR in this chapter as well as for RNASeq in Chapter 4 (total = 24 samples). The Kraken analysis, derived from the RNASeq datasets, provides information on the microbiota load and composition. Whilst extracting RNA, DNA from each sample was also extracted using the back extraction buffer. From these DNA extractions, PCR to amplify the 16S genes from the microbiota was carried out, and sequenced, and these were also used to analyse microbiota loads and composition. (M=mated, V=virgin, B=blood fed, N=untreated, A=antibiotic, x=experiment [K] with 100% *P. falciparum* infection)

cDNA production

An equal amount of RNA (100ng) from each sample was used for cDNA synthesis. cDNA preparation was done using the Takara reverse transcription kit (RR037B), according to the manufacturer's instructions (37°C for 15 minutes, 85°C for 5 seconds followed by a 4°C hold). Reverse transcription reactions were performed in 10µL volumes by using 0.5µL of Oligo dT Primer (50µM), 0.5µL of Random hexamer (100µM), 2µL of 5x PrimeScript Buffer, 0.5µL PrimeScript RT Enzyme Mix (100µM), 100ng/µL RNA (all reagents from Takara). Reactions were done using Veriti 96 well Thermal Cycler.

qPCR

Using the Primer3 software, I also designed a primer pair for for an *Anopheles* reference gene, the ribosomal protein (S7 Universal – S7Uni) that amplifies an exon that is conserved across all *Anopheles* in order to be able to use this primer in further species of mosquitoes if desired. All primer pairs are listed in Appendix S3.

Quantification of genes of interest were performed using duplicate 10µL reactions prepared using 2µL of 30-fold diluted cDNA, 5µL SYBR Premix Ex Taq (2x), 0.04µL ROX Reference Dye (50x), 0.2µL forward primer (10µM), 0.2µL reverse primer (10µM), 2.6µL molecular grade water (all reagents from Takara). The 7500 Fast Real-Time PCR System machine and 7500 Software v2.0.6 were used with conditions of 30 seconds at 95°C, followed by 40 cycles of 3 seconds at 95°C, and 30 seconds at 60°C.

Primer efficiency values were generated to check the primer quality by creating standard curves from serial dilution of the primers. S7Uni was used for normalization of the cDNA template and to calculate threshold values. The threshold values were calculated by multiplying S7Uni efficiency and S7Uni Ct value divided by the respective bacterial primer efficiency estimate multiplied by its Ct value.

The ratio derived from the Ct values of each bacterial species of interest compared to the S7Uni estimate of total mosquito RNA was plotted using ggplot2 to generate a boxplot graph of mated versus virgin bacterial load. Linear mixed-effects models using the packages Eigen and S4

(lme4) were used to perform the ANOVA in R to assess the significance of differences between treatments.

Primers used to examine patterns of bacterial proliferation included a generic 16S primer, which amplifies all bacteria, but also specific primers that amplify *Klebsiella, Pantoea,* and *Serratia* which belong in the family of *Enterobacteriaceae*. These three bacterial species have been reported to be dominant in *An. gambiae* field and lab-reared colonies (Gendrin et al., 2015). Additionally, *Elizabethkingia* and *Asaia* primers which belongs in the *Flavobacteriaceae* and *Acetobacteriaceae* family respectively were also used.

Assessing bloodmeal volumes

In order to assess whether mated and virgin females take in different blood volumes, two experiments were performed, one which weighed females after their bloodmeal and another which used the Drabkin reagent to assess the amount of hemoglobin in each female's gut (hemoglobinometry) (Briegel et al., 1979). Ngousso mosquitoes were fed using the membrane feeder on 1mL washed red blood cells (x3) mixed with 1mL A+ serum. After 10 minutes of feeding, unfed females were removed on ice. Twenty females were separated and placed in a new cup on dry ice. They were then weighed individually and their mating status was scored by spermathecal dissection as described above. Remaining females were kept in a container in an incubator for 24 hours at 26°C and 80% humidity, similar to the condition with mosquitoes fed with *P. falciparum*-infected blood. These females were then weighed and mating status was assessed. This resulted in weights and Drabkin assessments of bloodmeal size for mated and virgin females both immediately after a bloodmeal, and 24 hours later to examine whether females may digest at different rates depending on their mating status. The Drabkin reagent (Sigma D5941) was prepared by mixing one vial of Drabkin reagent to 1L of water. Each mosquito abdomen was added to 0.5mL of Drabkin and mixed well, 200µL of mixture was pipetted into a 96-well plate and examined together with standard curve using normal blood; 0.5µL, 1.0µL, 2.0µL and 4µL. Controls of unfed mosquito abdomens with Drabkin and also Drabkin reagent alone were also included. Plates were read at 540nm and the standard curve was used to convert the OD value to blood volume in µL.

Kraken analysis

The Kraken results presented in this chapter were generated using RNASeq data on the midgut samples described in Figure 3.1. The RNAseq analyses on the midguts themselves will be discussed in Chapter 4. Kraken is a software package that assigns taxonomic labels to short DNA sequences and is commonly used for microbiome detection (Wood & Salzberg, 2014; Zhang et al., 2015). In this case, Kraken was used to identify contaminant reads of prokaryote or virus from an RNASeq dataset I generated on mosquito midguts. Kraken classifies 100 base pair reads at a rate of over 4.1 million reads per minute (Wood & Salzberg, 2014). Kraken identifies bacterial species by matching k-mers to a database of bacterial k-mers. The database used in my Kraken analysis consists of all genomes from Bacteria RefSeq, Virus RefSeq, Plasmid RefSeq and Mouse and Human v38 reference genomes. I used read counts for each bacterial species as estimated by Kraken normalized by total read counts achieved in the sample to quantify the relative bacterial loads overall and for several specific groups of bacteria. These analyses were carried out on all 24 individual midgut samples depicted in Figure 3.1.

16S Sequencing by MiSeq

Illumina MiSeq sequencing of 16S rRNA gene amplicons was also applied to investigate the microbial community structure and composition regardless of whether there was a reference genome available for mapping (which Kraken depends on). Methods for preparing midgut DNA for MiSeq analysis are explained below.

DNA Extraction

A Back Extraction Buffer protocol obtained from Thermo Fisher website (www.thermofisher.com), which consists of 4M guanidine thiocyanate (Sigma), 50mM sodium citrate (Sigma) and 1M Tris pH8 (Sigma), was added to the pellet from the first round of centrifugation of midguts with TRIzol (Life Technologies) and chloroform (Sigma) during RNA extraction. Samples were centrifuged at 13,200rpm, 4°C for 15 mins. The aqueous layer was collected into new tube which contains isopropanol and left to rest at RT for 5 minutes before centrifugation at 13,200rpm, 4°C for 15 mins. Isopropanol was then removed and 400µL of 70% ethanol was added to the pellet of the samples. Samples were vortexed and centrifuged at

13,200rpm, 4°C for 10 mins. The washing step was repeated three times. DNA was used for the MiSeq experiment.

PCR amplification

Each DNA sample was subjected to a 16S amplification by PCR using bespoke MiSeq primers. The forward primer for each sample is the same, whilst the reverse primer is different for each sample and contains a unique Golay barcode. The PCR reaction kit from New England Biolabs (NEB) consists of 5x Q5 buffer, 10mM dNTP, 10mM forward primer, 10mM reverse primer, DNA template, Q5 Taq and NF water with total reaction volume of 25µL. Amplification was done by 98°C for 2 minutes, 20 cycles of 30 seconds at 98°C, 30 seconds at 50°C, 1 ½ minutes at 72°C and 72°C for 5 minutes. 2µl of each PCR product were run on a 1% agarose gel to check the quality where expected band should be approximately 350bp. All four PCR products from each sample were pooled in a new 1.5ml tube. DNA was quantified using picogreen.

One hundred µL of the equimolar mix was run on 1% agarose gel and the appropriate-sized band was cut and placed into a 1.5mL tube. Membrane binding solution (10µL) was added per 10mg of gel slice and vortexed. Tubes were placed in a water bath at 60°C to dissolve the gel slice. 700µL of dissolved gel mixture were pipetted into a SV Minicolumn and centrifuged at 13,200rpm for one minute. Supernatant was discarded and the SV Minicolumn was reinserted into the collection tube. These steps were repeated until no dissolved gel mixture remained. 700µL of membrane wash solution containing ethanol was added and centrifuged at 13,200rpm for 1 minute. The supernatant was discarded and the SV Minicolumn was reinserted into the collection tube and 500µL membrane wash solution was added. Tubes were centrifuged at 13,200rpm for 1 minute and supernatant was discarded. The SV Minicolumn was transferred to a 1.5mL tube and 100µL of nuclease-free water was added directly onto the membrane in SV Minicolumn and left for a minute. The tubes were centrifuged at 13,200rpm for one minute and half of the DNA collected was sent for MiSeq sequencing.

MiSeq sequencing

16S sequencing (MiSeq) was done using a protocol developed in the Lawley lab at the Wellcome Trust Sanger Institute and analysed using the software package Mothur (Schloss et al., 2009).

The process starts at “make.contigs” step. The paired fastq files from sequencing were used as input.

Results

One source of variation could be differences in the volume of blood taken by the mosquitoes for example if mating status influences bloodmeal volume. Indeed there is evidence in *Drosophila* that females eat more after mating (Barnes et al., 2008). In order to be certain that the trend was not driven by differences in the size of a bloodmeal being different between virgin and mated females, I tested whether they took different volumes of blood using two different measures. From three independent experiments, mated and virgin females appear to take equal bloodmeal sizes and digest at similar rates, comparatively (Figure 3.2a and Figure 3.2b). Mosquitoes are difficult to weigh accurately using our scale because they are so light, so I repeated another set of experiments to assess the correlation between weight and the Drabkin method. The Drabkin method is a hemoglobin-based estimation of the blood meal taken by each mosquito (Chagas et al., 2014). Results from weighing and the Drabkin method are highly correlated (Correlation Test : $R^2 = 0.67$), suggesting weight accurately represents bloodmeal size, and either method can be used to quantify the blood meal size taken in the future.

(a) Weight (b) Hemoglobinometry

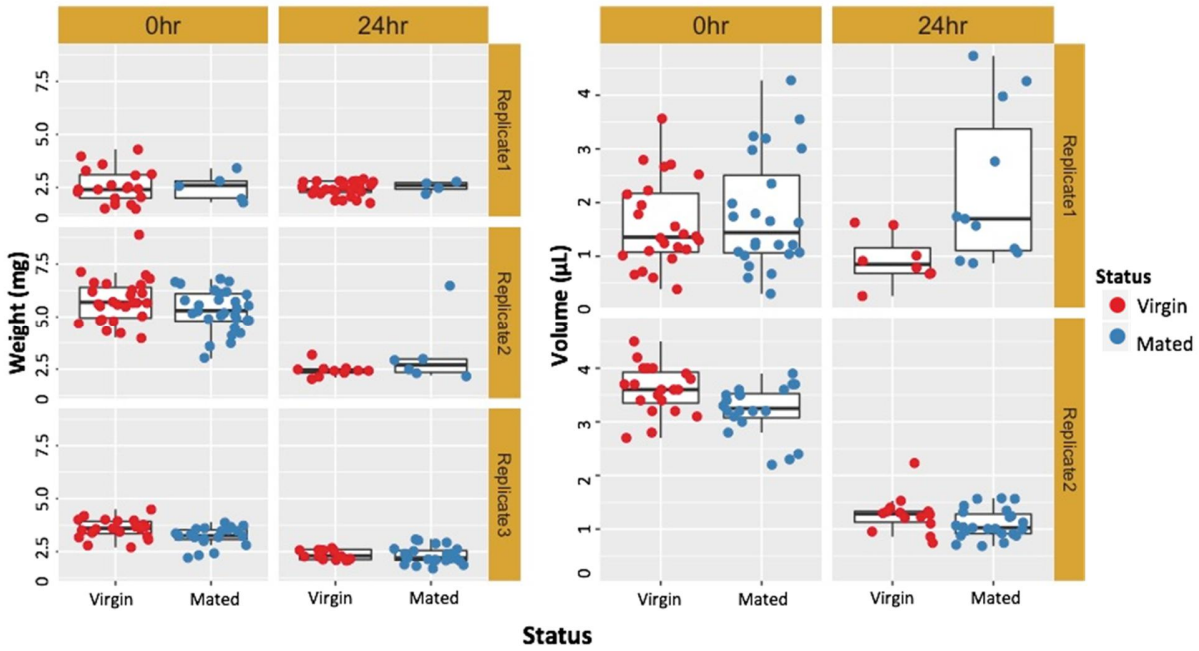

Figure 3.2 Virgin and mated female Ngousso appear to take similar volumes of blood when feeding. Each dot represents a measurement from an individual midgut. Boxplots indicate the median and 25-75 percentiles. (a) Weight of naive blood fed virgin and mated Ngousso at 0hr and 24hr post blood feed. (b) Hemoglobinometry using Drabkin reagent to assess the volume taken by virgin and mated females.

Effect of mating on mosquito midgut microbiota abundance

I first examined whether mating status influences bacterial abundance in the midgut under sugar fed conditions. Using similarly sized females, whose mating status was confirmed by visual inspection of the spermatheca, bacterial abundance in mated and virgin pools of five midguts as estimated by qPCR on 16S is not statistically significantly different, but strongly trends towards mated females showing higher bacterial loads than virgin females (ANOVA : n=12, p=0.06; Figure 3.3a). Female Ngousso that were given antibiotics in the sugar solution from eclosion onwards have almost all of the bacteria removed from their midgut. Sugar fed midgut samples used for the 16S qPCR experiment are a pool of five midguts, which might underlie the high levels of variation. For example, if a single midgut in the pool of 5 had a very high bacterial load,

this could skew the whole pool. For later work on blood fed guts, I moved to analysing single guts.

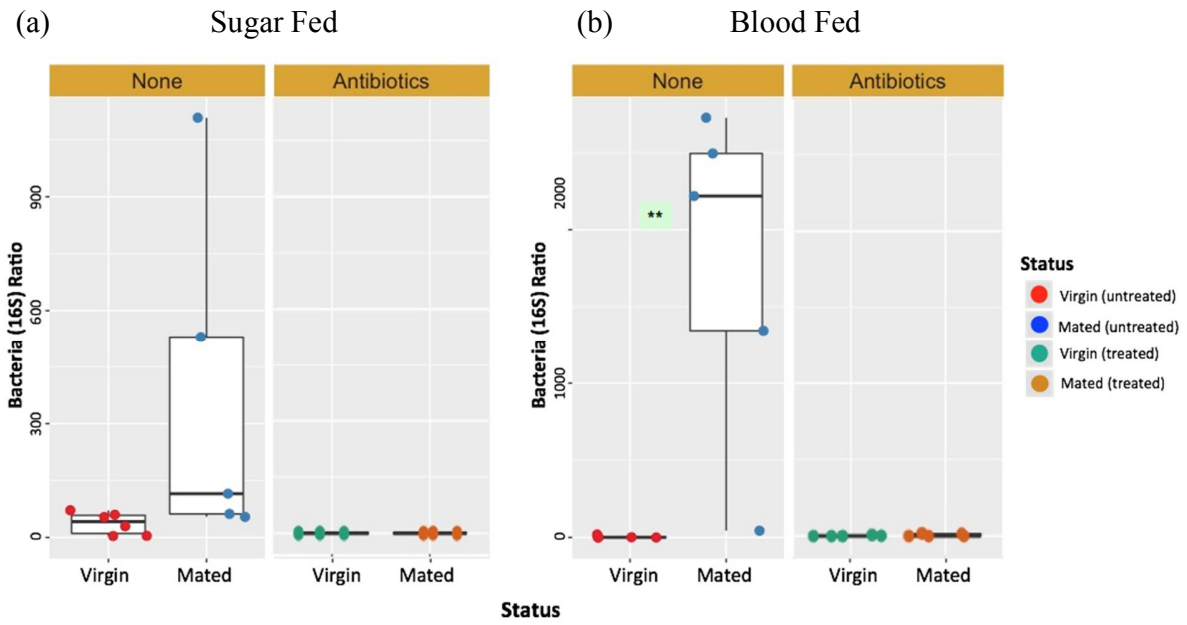

Figure 3.3 qPCR quantification of midgut bacterial 16S rRNA abundance in (a) normal sugar fed mosquitoes without antibiotics in their sugar meal and with antibiotics in their sugar meal. Each dot represents a pool of 5 midguts (p = 0.06). Boxplots indicate the median and 25-75 percentiles.(b) 24 hours post blood fed mosquitoes midgut given normal sugar or an antibiotics cocktail in the sugar meal since eclosion. Each dot represents a single midgut. Boxplots indicate the median and 25-75 percentiles. (** = p-value <0.005)

I next examined whether mating status has an impact on bacterial proliferation in blood fed mosquitoes because bacteria proliferate massively upon a blood meal and microbiota are known to have both direct and indirect impacts on malaria susceptibility (Dong et al., 2009). Upon blood feeding, individual mated female midguts contain a significantly higher bacterial load than individual virgin female midguts (ANOVA : n=12, p = 0.002; Figure 3.3b). When females were given antibiotics in their sugar meal, no difference in bacterial abundance due to mating status in the blood fed midguts was observed as most bacteria were removed. Blood feeding causes a massive increase in overall levels of bacteria. The impact of mating status on bacterial proliferation in individual blood fed females strongly suggests that males may transfer something that leads to higher levels of bacteria in the female gut, especially upon bloodfeeding.

Next, I carried out qPCR to examine several specific bacterial species in mated and virgin Ngousso midguts. I found that mated females have higher *Klebsiella* (ANOVA : n=12, $p = 0.05$), *Pantoea* (ANOVA : n=12, $p = 0.05$), *Serratia* (ANOVA : n=12, $p = 0.02$) and *Asaia* (ANOVA : n=12, $p = 0.002$) compared to virgin females (Figure 3.4). In spite of much lower overall levels of bacteria in antibiotic treated females, similar trend was also observed, with an increase in *Klebsiella* (ANOVA : n=12, $p = 0.05$) and *Serratia* (ANOVA : n=12, $p = 0.03$) in response to mating status and a trend towards higher *Pantoea* levels in mated females ($p = 0.08$).

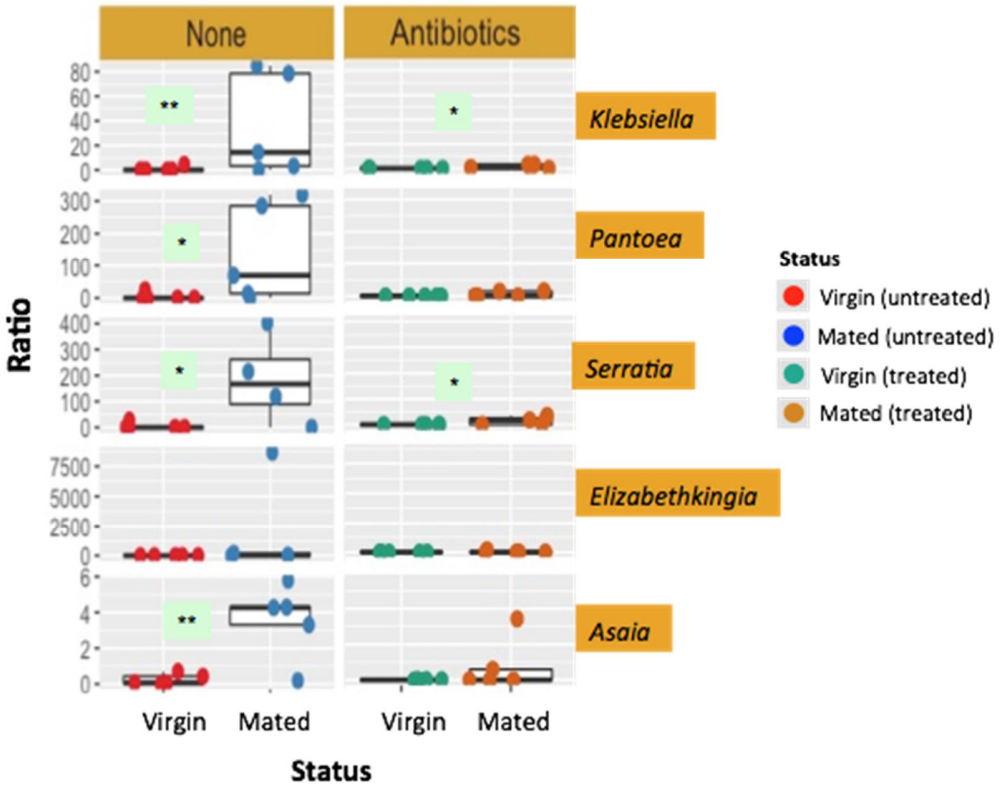

Figure 3.4 Relative bacterial loads as estimated by qPCR for *Klebsiella*, *Pantoea, Serratia, Elizabethkingia* and *Asaia* on virgin and mated midguts. Each dot represents RNA extracted from single midgut from *P. falciparum*-infection experiment [K] with 100% prevalence. "None" refers to untreated mosquitoes while "Antibiotics" refers to mosquitoes treated with Pen/Strep/Gent. Boxplots indicate the median and 25-75 percentiles. (** = p-value <0.005, * = p-value <0.05)

Altogether, the qPCR based analysis on 16S rRNA strongly suggests that mated females have higher midgut bacterial loads than virgin females, especially when bloodfed. To confirm this finding and explore whether mating influences bacterial composition, I further analysed the patterns using Kraken to evaluate bacterial reads in the RNAseq data from these same samples. Because the samples are the same, I expected the estimates of the bacterial loads to be similar for both qPCR on 16S and Kraken. The estimated bacterial loads from the Kraken analysis shows mated females have more bacteria than virgin females (p = 0.04; Figure 3.5a). Antibiotic-treated females show lower bacterial levels compared to untreated females, which is what should be observed and has been reported previously (Demaio et al., 1996; Vaughan et al., 1994). No significant differences were found between antibiotic-treated mated and treated virgin females, though given most bacteria are likely eliminated, this is expected.

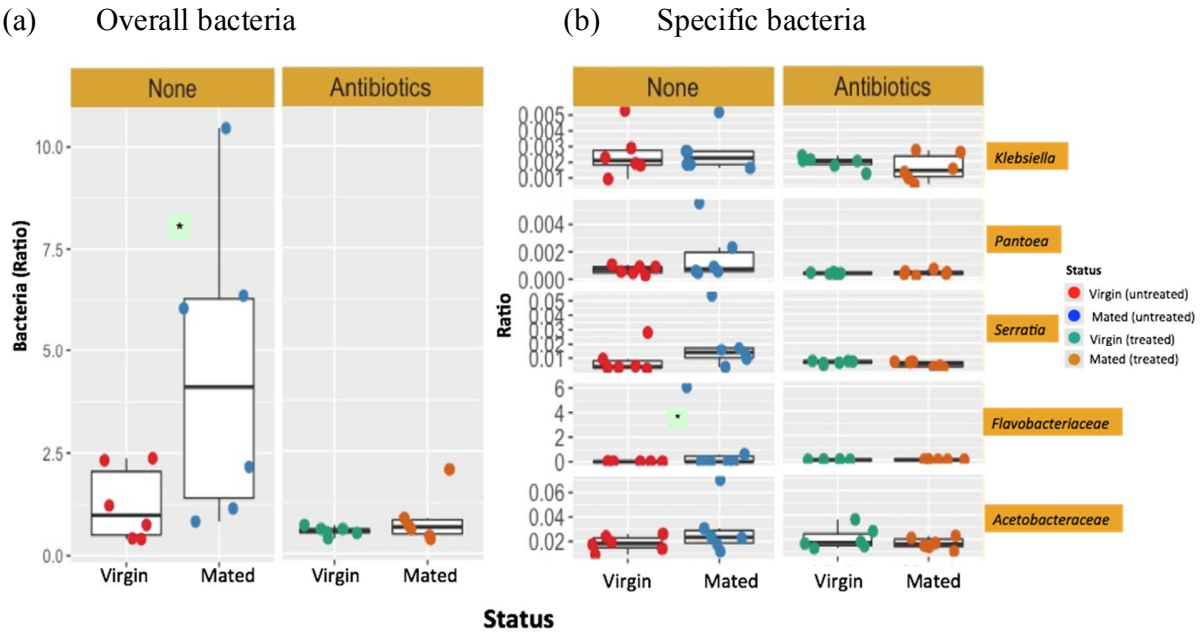

Figure 3.5 Kraken analysis of (a) overall bacteria (b) specific bacterial loads as estimated by Kraken report for *Klebsiella*, *Pantoea, Serratia, Flavobacteriaceae* (taxonomic family of *Elizabethkingia*) and *Acetobacteraceae* (taxonomic family for *Asaia*) on virgin and mated midguts. Each dot represents RNA extracted from single midgut from *P. falciparum*-infection experiment [K] with 100% prevalence. "None" refers to untreated mosquitoes while "Antibiotics" refers to mosquitoes treated with Pen/Strep/Gent. Boxplots indicate the median and 25-75 percentiles. (* = p-value <0.05).

Klebsiella, Pantoea and *Serratia* (taxonomic family of *Enterobacteriaceae*) were all detected in the Kraken analysis, but *Elizabethkingia* and *Asaia* were not. Therefore, I examined one taxonomic level higher for both *Elizabethkingia* and *Asaia*. For *Elizabethkingia*, this is the family *Flavobacteriaceae* and for *Asaia*, this is the family *Acetobacteraceae*. While mated females tend to have higher levels of each of these bacterial species, it is only *Flavobacteriaceae* that is significantly higher using the results from the Kraken analysis. In antibiotic treated midguts, there is no difference between mated and virgin female bacterial abundance for any of the bacterial groups (Figure 3.5b). Overall, mated untreated midguts contain much more bacteria than virgin untreated, mated treated and virgin treated (Figure 3.6) which correlates with total bacteria found from Kraken analysis (Figure 3.5a).

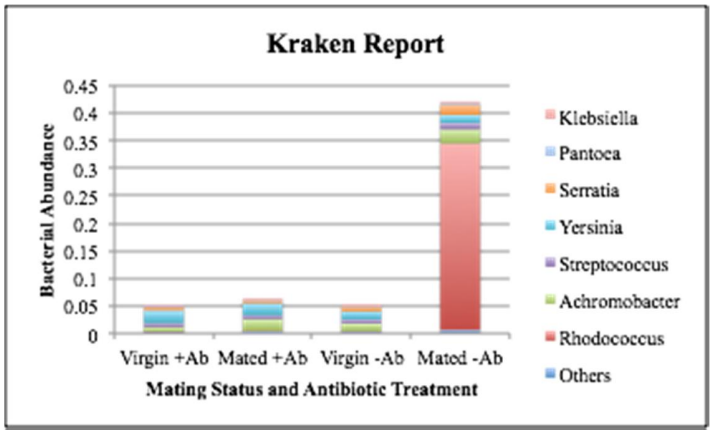

Figure 3.6 Some bacterial groups including *Rhodococcus, Achromobacter, Streptococcus, Yersinia, Serratia, Pantoea, Klebsiella* and 'Others' which represents mixtures of other bacteria with lower abundance. This graph was generated from the read counts of each specific bacteria from Kraken report.

Overview of microbial community diversity

The analyses presented above investigated the impact of mating on bacterial abundance as estimated by two separate measures, qPCR and Kraken. These analyses show that mated females that have not been treated with antibiotics have a greater abundance of microbiota in their midguts than virgin females.

In addition to investigating bacterial abundance, Kraken and 16S sequencing also enable the investigation of bacterial species composition, whereas qPCR requires specific primers targeting each species of interest. Therefore, I also examined the composition of the midgut microbiota using two methods: the Kraken based method that used RNA, and the MiSeq based method in which I sequenced the the 16S amplicon from the DNA of each sample.

At 24 hours post *P. falciparum* infectious blood feed, using 16S sequencing, some bacterial reads were found in the negative control, and after subtracting these because they might be from contamination, *Klebsiella, Pantoea* and *Serratia* bacteria data were no longer found in this analysis. Therefore, these three bacteria were not added in Figure 3.7b. Quantification was done using the number of reads for each group normalized against the total library size.

(a) (b)

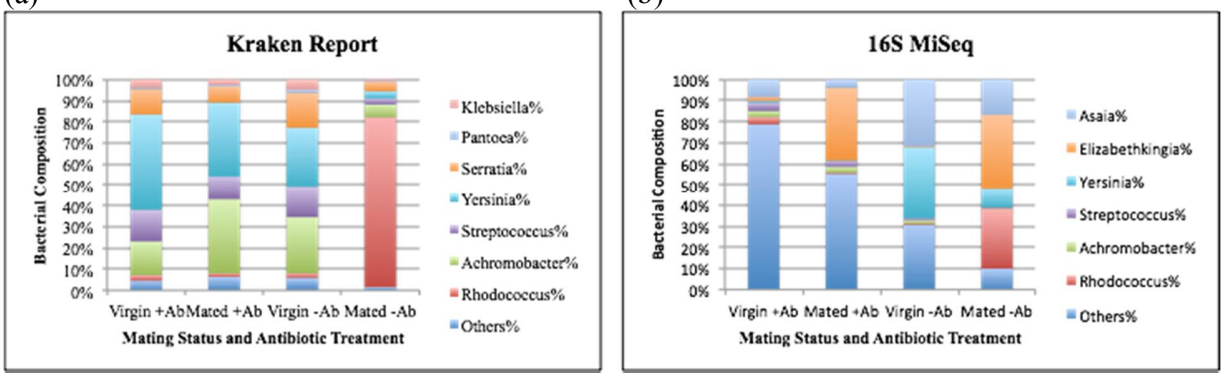

Figure 3.7 Bacterial composition in midguts obtained using the Kraken analysis and 16S MiSeq. (a) the Kraken analysis analysis shows the bacterial composition of some bacterial groups including *Rhodococcus, Achromobacter, Streptococcus, Yersinia, Serratia, Pantoea, Klebsiella* and 'Others' which represents mixtures of other bacteria with lower abundance. (b) 16S MiSeq sequencing analysis summarizing the bacterial composition of *Rhodococcus, Achromobacter, Streptococcus, Yersinia, Elizabethkingia* and *Asaia*.

Both analyses also indicate that the species composition is distinct in mated guts with a higher relative abundance of *Rhodococcus* (Figure 3.7 a and b) and a specific presence of *Elizabethkingia* (Figure 3.7b). In both the Kraken (Figure 3.7a) and the 16S MiSeq analysis (Figure 3.7b), mated females untreated with antibiotics have more bacteria and show a specific expansion of the *Rhodococcus* group and relatively less of *Yersinia* and *Achromobacter* bacterial group. However, in both Kraken and 16S MiSeq analysis, no significant differences were found for *Rhodococcus* bacteria (Kraken: *p*-value = 0.25 and 16S MiSeq *p*-value = 0.08) although the composition seems tremendous in mated untreated females (Figure 3.7). This could be driven by 3 out of 6 mated samples which have high level of *Rhodococcus* (Figure 3.8). Raw data for Kraken report and MiSeq could be found online :

Farah-MiSeq&Kraken raw data

https://docs.google.com/spreadsheets/d/1yQ8_Ziz89Ab-djwoC2eTrwpzeyhiXZ0CaG6Tz7C1sFY/edit?usp=sharing

(a) Kraken analysis (b) 16S Sequencing

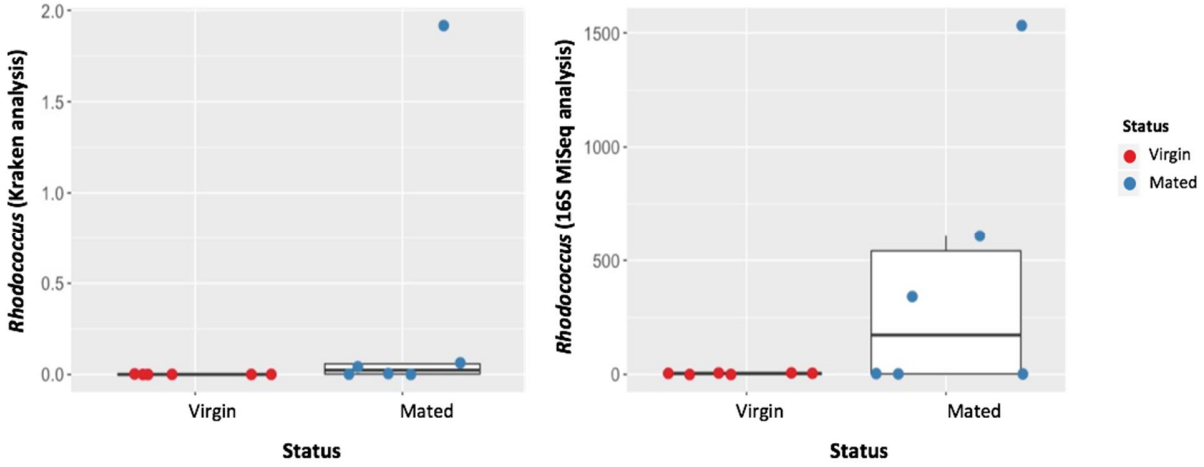

Figure 3.8 *Rhodococcus* bacterial loads obtained from (a) Kraken and (b) 16S MiSeq analysis. Each dot represents one midgut sample. Boxplots indicate the median and 25-75 percentiles.

Discussion

While it is well established that blood feeding increases bacterial proliferation, from the experiments that I carried out here, it is also clear that mating has an impact on bacterial abundance in the midgut. Mating did not induce immune response genes in whole female *An. gambiae* (Rogers et al., 2008). However, mating changes the female *An. gambiae* atrium ultrastructure (Rogers et al., 2008) which leads us to hypothesize that perhaps mating also induces changes in the *Anopheles* midgut. From this chapter, mated females show a trend to have more bacteria than virgin females in sugar fed midguts. Upon blood feeding, the difference between mated and virgin bacterial loads is significant. This result was shown by qPCR, which is perhaps the most reliable of the measures, and also found in the Kraken analysis. Antibiotic treatment removed the majority of the bacteria from midguts and differences of bacterial abundance between mated and virgin female midguts are no longer seen.

Looking at specific bacterial species abundance by qPCR, mated females have more of *Enterobacteriaceae* family bacteria (*Klebsiella, Pantoea* and *Serratia*) and *Asaia* (*Acetobacteraceae* class), whereas the *Flavobacteriaceae* family (*Elizabethkingia*) shows no significant difference. The overall pattern of these bacterial groups is similar with the Kraken analysis although it is only significant for the *Flavobacteriaceae* family. Although the signal is weaker, the similarity suggests that Kraken analysis is reliable for further investigating the composition of many bacteria in the sample, whereas we need specific primers to detect other bacteria using qPCR.

I also used the MiSeq sequencing of the 16S amplicon from each sample's DNA to evaluate the bacterial species compositions of these samples. These two analyses, Kraken and 16S MiSeq, give some insight into the impact of both antibiotics and mating on midgut bacterial composition. *Elizabethkingia* in the *Flavobacteriaceae* family whilst *Rhodococcus* in *Nocardiaceae* family. In my experiment, examining midguts fed on *P. falciparum* infected blood, overall levels of bacteria are significantly higher in mated females. When I explored specific groups of bacteria, I found that the composition of *Flavobacteriaceae* (namely *Elizabethkingia), Enterobacteriaceae* (*Klebsiella*, *Pantoea* and *Serratia*) and *Nocardiaceae,* a

family not tested using qPCR but which contains a bacterial species, *Rhodococcus,* found to be abundant in mated females. Family trend is more in mated female midguts than virgin female midguts. Other than that, *Yersinia* which belongs to *Yersiniaceae* family composition is more in untreated virgin female midguts than mated female midguts.

Enterobacteriaceae and *Flavobacteriaceae* were reported to dominate the *An. gambiae* adult midgut (Wang et al., 2011). The abundance of *Enterobacteriaceae* family is higher in *P. falciparum*-infected mosquitoes midguts than uninfected females (Boissière et al., 2012).

Nocardiaceae family bacteria are found in *An. coluzzii* and *An. funestus* mosquitoes (Lindh et al., 2005). Although the composition of *Nocardiaceae* was shown to be very high among mated females, the result was not significant and was potentially driven by several females with exceptionally high levels rather than by a consistent impact of mating. However, given the possibility of different infection per midgut, it is possible that *Nocardiaceae* may have an impact on parasites and further analysis of *Nocardiaceae* and *P. falciparum* infected mosquitoes could potentially aid in understanding if this bacteria has a direct impact in assisting the infection.

Antibiotics added in the sugar meal reduce bacterial abundance. *Nocardiaceae* were found to be removed from the midgut bacterial composition upon antibiotic treatment. The increase of *P. falciparum* infection in regards to removal of some bacteria suggests that these bacteria might have a direct interaction or competition with *P. falciparum* which might reduce the possibility of *P. falciparum* to mature in the midgut. One way to test the impact on *P. falciparum* development is by introducing specific bacteria in the *Anopheles* by injection or food intake, and feeding these *Anopheles* on *P. falciparum* infected blood. Assessments could be done such as survival assay and immune system activity of the vector, bacterial load assay and phenotypic analysis of *P. falciparum* infection.

Yersiniaceae family bacteria was found composition is higher in virgin females. It is one of the newly described bacteria found in *Anopheles* microbiota (Ngo et al., 2016), and in fleas, *Yersiniaceae* release a toxin in order to survive in the flea midgut (Hinnebusch et al., 2002). One of the important components of the immune response of *Anopheline* mosquitoes to limit

Plasmodium development is by synthesis of nitric oxide metabolites (Peterson et al., 2007) or inducing the antimicrobial pathway (Dennison et al., 2015). Whether or not *Yersiniaceae* has an impact on *Anopheles* fitness or releasing nitric oxide that could impair *Plasmodium* development remains unknown and will be interesting to explore given findings I report in Chapter 5.

Comparing these three methods, qPCR is the most reliable method in detecting bacteria as it is using the RNA extracted and primer specifically targeting the bacteria. Both Kraken and 16S MiSeq analyses are exploratory methods that I used to investigate bacterial composition beyond the five specific bacterial species that I ran qPCR on. Perhaps in the future some changes could be made in preparation of samples in order to maximize the potential of using these three methods simultaneously, such as running dual-RNASeq by keeping some rRNA for Kraken analysis and potentially do RNASeq analysis on the bacteria sequence as well. For the MiSeq experiment, perhaps by reducing the possibility of contamination in negative control would help in getting a much more solid data.

Overall, the results suggest that mating status does not only increase the overall bacterial abundance in sugar fed and blood fed midguts, it is also able to increase certain bacteria species in the midgut. Furthermore, mated females also have different bacterial composition compared to virgin females, such as *Enterobacteriaceae, Acetobacteraceae* and *Nocardiaceae* family. Differences in bacterial abundance and composition in response to mating suggests that there is something that was transferred by males to the females and induced the response of microbiota in the mated females midgut.

The abundance of natural microbiota in the midgut increases dramatically upon a blood meal (Kumar et al., 2010; Pumpuni et al., 1996; Wang et al., 2011). This bacterial growth after a bloodmeal is reported to trigger an immune response via the Immune-deficiency (Imd) pathway, which causes synthesis of antimicrobial peptides and other immune effectors (Meister et al., 2009). In *Drosophila*, it has been reported that mating increased bacterial loads in females (Short & Lazzaro, 2010). This was supported by the reduction of AMP in mated females, which corresponds to higher bacterial loads in mated females. This phenomenon was shown to be driven by seminal fluid proteins transferred through mating (Short et al., 2012).

In *Drosophila*, the female midgut is dramatically remodelled via hormonal signals to enhance egg production (Reiff et al., 2015). The midgut cell number increases resulting in bigger midgut after mating. This effect is caused by JH which also involved in lipid metabolism, preparing the mated female for the increased nutritional demand required for egg production (Reiff et al., 2015). It could be hypothesized that mating might also increases the number of cells in *Anopheles* females midgut. An expansion of the midgut size in mated females could facilitate the proliferation of bacteria in the midgut in *Anopheles*. A bigger midgut and more midgut cells could also mean that greater *P. falciparum* infection is possible. Further studies on midgut structure and gut cell number upon mating is required to test these hypotheses.

Using bacteria to help control malaria parasites inside of mosquitoes will benefit from a deeper understanding of the factors that influence both bacterial proliferation and diversity as well as parasite development. If the bacterial load is higher and the parasite infection is low, it may be caused by the immune response from either host or bacteria itself. For example Esp_Z from *Enterobacter* prevents *Plasmodium* development, and this is mediated by reactive oxygen species (ROS) which was produced by the bacteria (Cirimotich et al., 2010). *Serratia* is able to reduce *Plasmodium* loads in *An. stephensi* (Bando et al., 2013). Recently, *Serratia* bacteria was successfully used in developing paratransgenesis to deliver anti-Plasmodial components in the *An. gambiae* midgut (Wang et al., 2017). Another possible interaction is that bacteria could have a positive impact on parasite development as might be the case with *Rhodococcus* here. Targeting these bacterial species could potentially reduce transmission. One of the promising bacteria in paratransgenesis development is *Asaia*. From my data, *Asaia* too seem to be influenced by mating status of the vector and could potentially be a vector in delivering anti-malarials into the midguts of Anophelines because of its stability and ability to transmit via vertical or horizontal transmission route (Capone et al., 2013; Favia et al., 2007).

Microbiota may prove as effective agents for manipulating vector competence of malaria parasites and other important human pathogens (Chouaia et al., 2010; Cirimotich et al., 2011; Favia et al., 2007; Rani et al., 2009). But as I have shown here, mating status has a significant impact on bacterial abundance and composition and therefore mating status of the vector should be taken into consideration when developing microbiota based vector control strategies.

CHAPTER 4

Transcriptomic responses in the midgut in response to mating

Introduction

Mating changes the behaviour and physiology of female insects such as inducing oogenesis and refractory to further insemination. In *Drosophila*, immune gene expression is activated as a result of mating (Lawniczak & Begun, 2004; McGraw et al., 2008). However, immune genes were not found to be differentially expressed upon mating in *Anopheles* (Rogers et al., 2008). Whilst there is evidence of a mating impact on inducing transcriptional changes in the LRT, head, and carcass, mating also induced some genes expressed primarily or exclusively in the gut (Rogers et al., 2008). Mating induces permanent changes to the atrial structure (Rogers et al., 2008) and long lasting transcriptional responses in whole females (Rogers et al., 2008) and blood feeding induces transcriptomic and metabolomic responses in *An. gambiae* female midguts (Champion et al., 2017; Rodgers et al., 2017).

Mosquito guts are critical for nutrient absorption. Nectar from plants is a common food for male and female mosquitoes. Ingestion of nectar or sugar is important for other metabolic needs and to increase longevity and fecundity of the mosquitoes (Souza-Neto et al., 2007). Sugar is composed of various hexoses such as glucose, fructose, and sucrose. The nectar, once eaten, is stored in a sac-like crop which is connected to the gut. In the midgut, secreted alpha glucosidase enzymes digest the sugar (Souza-Neto et al., 2007). It is known that insects utilize microorganisms to break down plant derived food (Douglas, 2009), however it has not been shown before in *Anopheles* if microbiota aids in digestion of sugar. Glucose absorbed from the gut is converted by the fat body to trehalose that is secreted into the hemolymph and to glycogen that is stored. This will then provide the mosquitoes with energy to fly (Handel, 1984).

Additionally, in *Anopheles* females, the midgut serves as the host for many different type of microbiota. Upon ingesting the *P. falciparum* gametocytes, it could also be the tissue that is

needed for the parasites to complete their sexual cycle. Female *Anopheles* needs to take in blood which is the source of protein and lipid to produce eggs. Blood digestion is directed to the posterior end of the midgut which is capable of expansion (Handel, 1984). Heme is released upon hemoglobin digestion and protein derived from blood is broken down to obtain amino acids that are transformed by the fat body into other products, mainly vitellogenin which is important for egg provisioning (Handel, 1984). The blood digestion turns the midgut into an oxidative environment where heme can induce oxidative stress by generating hydroxyl radicals (Sadrzadeh, et al., 1984). This oxidative environment imposes pressure on mosquito fecundity and other physiological traits (Champion & Xu, 2017). Bacteria in the midgut contribute in the process of blood digestion in mosquitoes (Gaio et al., 2011; Minard et al., 2013).

The insect gut is an important endocrine tissue that modulates the secretion of peptides that can influence reproductive physiology, similar to the gut-brain of the vertebrates (Žitňan et al., 1993). In *Drosophila,* mating has been shown to restructure the female midgut which is important for their reproduction (Reiff et al., 2015). This leaves an open question as to whether the sugarfed or bloodfed gut has a transcriptional response to mating? It could be hypothesized that in *Anopheles*, mating could induced some transcriptional changes in the midgut to ensure successful reproduction. Additionally, given that mating influences the microbiota as I have shown in Chapter 3, it is also important to understand what transcriptomic changes might result in the gut upon mating and bloodfeeding that are independent of the microbiota. This raises another question if mating status of *An. coluzzii* females induce transcriptional changes in the midgut which might facilitate *Plasmodium* infection?

To further investigate the differences that might be present specifically in the midgut depending on mating status, blood feeding, and antibiotic treatment, I carried out RNASeq on a variety of midgut samples (summarized in Figure 4.1). These include 12 sugar fed midguts (pools of 7 midguts) and 12 single guts from an experiment that resulted in 80% *P. falciparum* infection prevalence. Furthermore, I also examined 24 single guts (12 untreated and 12 treated with antibiotics) from the experiment in which 100% *P. falciparum* infection prevalence was achieved. These latter samples were used previously in the experiments in Chapter 3, where I showed that mated females had higher bacterial loads than virgin females (Chapter 3 Figure 3.1).

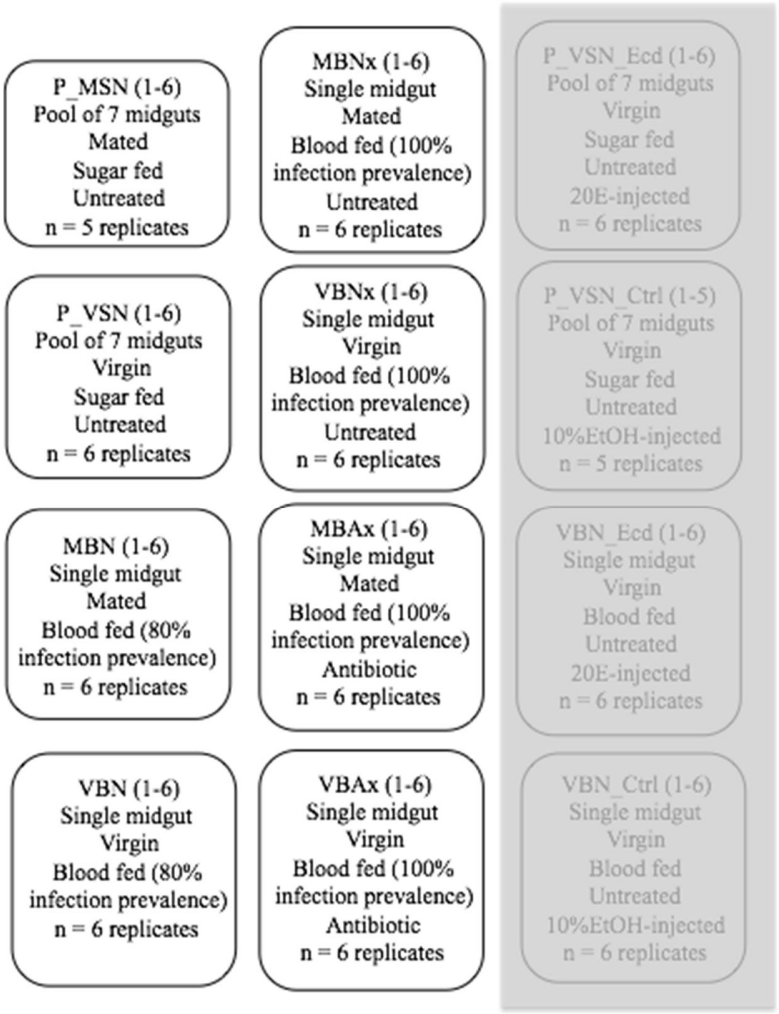

Figure 4.1 Description of 47 samples of midguts used for RNASeq analyses in this chapter. Midguts were dissected at 24 hours post *P. falciparum*-infected bloodfeed. RNA was extracted from each midgut and used in the RNASeq experiments presented in this chapter. (P=pooled of 7 midguts, M=mated, V=virgin, B=blood fed, S=sugar fed, N=untreated, A=antibiotics treated, x=experiment with 100% *P. falciparum* infection prevalence, experiment [K] from a later chapter). Experiment with 80% *P. falciparum* infection prevalence will be referred to as experiment [I]. The greyed out samples will be discussed in a later chapter.

Materials and Method

Mosquito preparation

Ngousso rearing, collection and separation of Ngousso pupae and Ngousso adult mating were performed as described in earlier chapters. See Appendix S1 and Appendix S2 for more information.

P. falciparum Infection (Membrane Feeding Assay)

Cotton was removed from mosquito cages six hours before blood feeding. 15 and 18 day old *P. falciparum* gametocyte cultures were pooled and spun down at 38°C, 2000rpm, for 5 minutes. Supernatant was removed and 1mL serum was added into the tube and topped up with fresh washed O+ blood to obtain a haematocrit of 45%. The mixture was always kept on a heat block (38°C) during the process to avoid inducing activation. The mixture was then added to a glass membrane feeder using a blunt syringe. Each cup of mosquitoes fed for 7 minutes and sugar-soaked cotton was placed on each cup after feeding was completed. Cups were kept in a secure container in an incubator at 26°C and 80% humidity. 24 hours after feeding, unfed females were removed. Some females were put aside for midgut dissection and scoring mating status, and leftover fed females were retained to assess infection rates at day 10 post blood-fed (Chapter 5). Sugar-soaked cotton was changed every two days with normal sugar for all mosquitoes.

In this chapter, two sets of samples from two different experiments were prepared. The first set was from experiment [I], with two groups of virgin and mated females. One group was given *P. falciparum*-infected blood meal and another group was not given a blood meal (referred to as sugarfed). Another set was from experiment [K], with two groups of virgin and mated females prepared. One group was given antibiotics in their sugar meal since eclosion and another group was not given antibiotics. These were all fed on *P. falciparum*-infected blood. 50 sugar fed and 50 bloodfed females from each of 24 samples from experiment [I] (virgin and mated) and from 24 samples from experiment [K] (virgin, mated, virgin treated with antibiotics, mated treated with antibiotics) were kept for 24 hours after the bloodmeal. Twenty-four hours post bloodfeed, midguts were dissected and 6 replicate pools of 7 midguts from each sugarfed treatment were

pooled together, whereas blood fed midguts were collected individually, also in 6 replicates. These samples are described in Figure 4.1.

Molecular Biology Method

RNA Extraction

Total RNA was extracted using TRIzol (Life Technologies) and chloroform (Sigma). Each biological condition was represented by six replicates each for single midgut for all blood fed mosquitoes and 7 pooled midguts of sugar fed mosquitoes. The midguts were homogenised using Precellys 24 homogenizer at maximum speed, 6,800rpm for 30 seconds and let to rest for 5 minutes at room temperature. Samples were then centrifuged at 13,200 rpm for 15 minutes at 4°C. The aqueous phase was collected into a tube that was filled with 250µL isopropanol and 1µg glycogen to precipitate the RNA. Samples were centrifuged at 13,200rpm for 15 minutes at 4°C followed by washing using 70% ethanol (Sigma) and concentrated using the DNA concentrator for 10 minutes. 10µL molecular biology grade water was added in each tube and was placed on a heat block (37°C) for 15 minutes. Total RNA was measured using the Nanodrop.

RNA Sequencing

Subsets of mosquitoes as described above were kept aside specifically for RNA sequencing (RNASeq; Figure 4.1). The details are summarized in Appendix S1 and Appendix S2. At 24 hours post blood fed, mosquito guts were dissected in RNAlater. Individual guts were placed in PCR tubes on dry ice as described above and RNA extraction was carried out as described above, on single or pooled guts depending on the experiment. These samples were assessed for RNA quality and quantity using the Agilent Bioanalyzer. Briefly, RNA samples were denatured by incubating at 70°C for 2 minutes and then placed on ice. 9µL of gel-dye mix was pipetted into bottom of nanochip. 1µL of samples, 1µL of RNA 6000 Nano Marker and 1µL of RNA 6000 Ladder were pipetted in assigned well, vortex and run using Eukaryote Total RNA Nano Series II programme. Total RNA was diluted to concentration of 500ng in 50µL and was sent to the Wellcome Trust Sanger Institute for RNA library preparation and sequencing.

The kit used for library prep was Illumina TruSeq Stranded mRNA Library Prep Kit. mRNA was purified from total RNA using an oligo dT magnetic bead pull-down. A random-primed cDNA library was synthesized. During second strand synthesis dUTP was incorporated in place of dTTP. The incorporation of dUTP quenches the second strand during amplification because the polymerase does not incorporate past this nucleotide resulting in a strand specific library. Ends were repaired with a combination of fill-in reactions and exonuclease activity to produce blunt ends. A-tailing was performed, whereby an "A" base was added to the blunt ends. Illumina paired-end sequencing adapters containing unique index sequences, allowing samples to be pooled, were ligated. The libraries then went through 10 cycles of PCR amplification using KAPA Hifi Hot Start Polymerase rather than the kit-supplied Illumina PCR Polymerase due to better performance (especially with AT rich DNA). Libraries were quantified and pooled based on a post-PCR Agilent Bioanalyzer. Sequencing was done on the HiSeq v4, 75bp paired end reads, and the data was analysed using Illumina RTA software version 1.18.61. Automatic and manual quality control (QC) was performed and then the data was archived in iRODS as CRAM files. Data mapping to reference genome was done using TopHat2 (v.2.0.9) (Kim et al., 2013) which makes use of the aligner Bowtie2 (v.2.1.0) (Langmead & Salzberg, 2012), to the *An. gambiae* PEST genome (AgamP4) obtained from VectorBase.

Data received were run and mapped to reference genome of *An. gambiae* which was obtained from VectorBase. HTSeq (v.0.6.1) (Anders et al., 2015) was used to count transcripts for each gene. Differentially expressed genes were determined using DESeq2 (v.1.8.2) (Anders et al., 2015; Love, et al., 2014) in R (v.3.2.5) (R Core Team 2014). Functional interpretation of each gene set was performed by doing Gene Ontology analysis using web server, TopGO (Alexa & Rahnenführer, 2009; Alexa et al., 2006). The top 10 functional analysis of biological process (BP) were extracted. Other significant gene ontology results (molecular function and cellular compartment) can be found in Appendix S5.

Results

For sugar fed mosquito midguts it is challenging to get enough RNA from a single midgut for RNA-sequencing. Therefore, 7 midguts from mated female *An. coluzzii* and 7 midguts from virgin female *An. coluzzii* were pooled into 6 virgin and 6 mated sugarfed gut pools. From the PCA plot, one of the replicates of mated females was an outlier and this sample was removed. There is a clear impact of mating on sugarfed midgut transcriptomes as shown in the Principal Component Analysis (PCA) plot (Figure 4.2a). Using DESeq2, 479 genes were reported to be significantly differentially expressed (*padj* <0.05) between virgin and mated female sugarfed midguts. 312 genes were upregulated and 167 genes were downregulated upon mating. The genes that were upregulated by mating are involved in diverse processes including glucose import, hexose transmembrane transport, flavonoid biosynthetic and glucuronidation processes, defence towards bacteria, innate immune response, transmembrane transport, peptide and insecticide catabolic processes and response to DDT (Table 4.1). Genes that were downregulated upon mating are mostly involved in translational process (Table 4.2). List of significantly differentially expressed genes can be found online:

Farah-RNASeq gene list

https://docs.google.com/spreadsheets/d/1EXtON0oqwrKjiuz_lT2Th6PMh2H0etmn-BJ3cJiAENg/edit?usp=sharing

(a) Sugarfed (b) Bloodfed

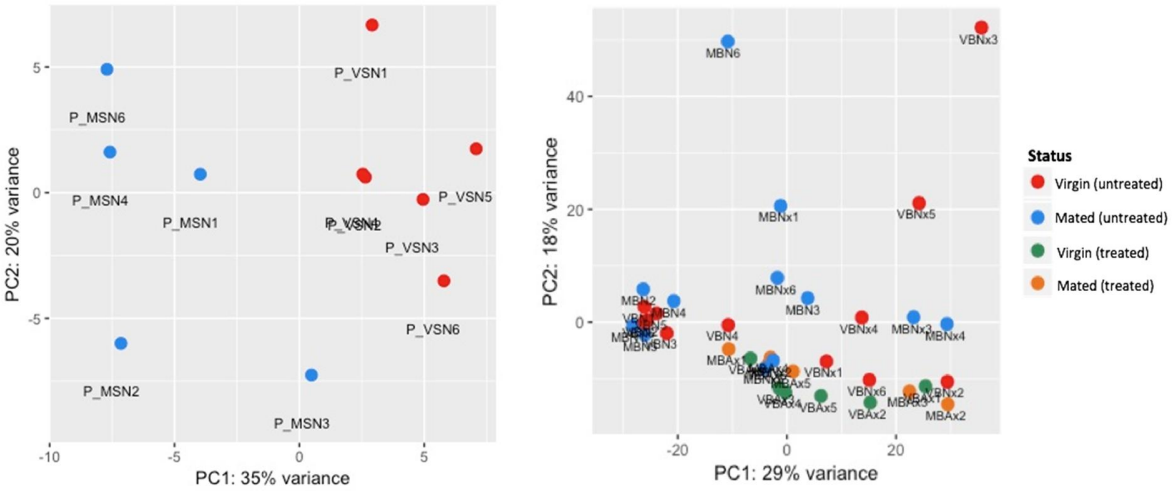

Figure 4.2 Principal Components Analyses on global RNAseq patterns for (a) mated [M] and virgin [V] female midguts fed only on sugar [S] and untreated with antibiotics [N] with each dot comprising data from a pool [P_] of 7 midguts, and (b) single midguts of mated and virgin females that were bloodfed [B] on *P. falciparum* infective blood, and either untreated [N] or treated [A] with antibiotics. Most samples came from an experiment that achieved 80% prevalence after *P. falciparum* infection (experiment [I]), but samples labeled with an [x] were from an experiment with 100% *P. falciparum* infection prevalence (experiment [K]).

GO.ID	Term	Annotated	Significant	Expected	weight01
GO:0046323	glucose import	17	6	0.56	1.1e-05
GO:0035428	hexose transmembrane transport	17	6	0.56	1.1e-05
GO:0009813	flavonoid biosynthetic process	19	6	0.63	2.3e-05
GO:0052696	flavonoid glucuronidation	19	6	0.63	2.3e-05
GO:0042742	defense response to bacterium	10	4	0.33	0.00021
GO:0045087	innate immune response	31	6	1.03	0.00046
GO:0055085	transmembrane transport	423	31	14.03	0.00121
GO:0043171	peptide catabolic process	29	5	0.96	0.00237
GO:0046701	insecticide catabolic process	21	4	0.70	0.00451
GO:0046680	response to DDT	21	4	0.70	0.00451

Table 4.1 Functional enrichment analysis of differentially expressed genes (*padj*<0.05) that were upregulated in response to mating in sugar fed midguts.

GO.ID	Term	Annotated	Significant	Expected	weight01
GO:0000027	ribosomal large subunit assembly	15	4	0.26	9.8e-05
GO:0000479	endonucleolytic cleavage of tricistronic..	8	3	0.14	0.00026
GO:0000055	ribosomal large subunit export from nucl..	5	2	0.09	0.00284
GO:0031126	snoRNA 3'-end processing	6	2	0.10	0.00422
GO:0006611	protein export from nucleus	6	2	0.10	0.00422
GO:0002181	cytoplasmic translation	21	3	0.36	0.00528
GO:0006414	translational elongation	21	3	0.36	0.00528
GO:0006783	heme biosynthetic process	7	2	0.12	0.00584
GO:0000154	rRNA modification	17	3	0.29	0.00758
GO:0000463	maturation of LSU-rRNA from tricistronic...	8	2	0.14	0.00770

Table 4.2 Functional enrichment analysis on genes that were downregulated upon mating (*padj*<0.05) in sugar fed female midguts.

Next I looked at the impact of mating on the midgut transcriptomes of females fed on a *P. falciparum* infected blood meal. Single midguts all untreated with antibiotics with 80% overall *P. falciparum* infection prevalence (n=12 midguts, 6 biological replicates of the two treatments, experiment [I]). These females are from the same batch as the sugar fed females, reared in the same way, and housed together until the time of infectious blood feeding, which led to an 80% *P. falciparum* infection prevalence overall. The single midguts I examined using RNA-seq were at 24 hours post feed and included 6 virgin and 6 mated female midguts. However, in contrast to the sugarfed guts from the same experiment, there is no clear separation of mated and virgin females on the PCA plot (Figure 4.2b). Only 6 genes were found to be significantly differentially expressed in blood fed midguts comparing mating status. The signal that was found from mating in sugar fed as shown before is gone with only one gene remaining found in both sugar fed and blood fed, AGAP005498, a phospholipid scramblase 2 involved in apoptotic cell removal.

I have also another set of untreated single midguts that had 100% *P. falciparum* infection prevalence (n=12 midguts, 6 biological replicates of the two treatments, experiment [K]). These blood fed samples are from a different timing than the sugar fed samples. From these samples, I've found 33 genes that were significantly differentially expressed between mated and virgin female midguts. However, none of these genes are common with the 6 genes found to be differentially regulated by mating in the midgut in the previous RNAseq analysis. However, there are 7 genes that are significantly differentially expressed genes in both bloodfed from experiment [K] and sugarfed midguts (Table 4.3). Two of these genes (AGAP011052 and AGAP012852), predicted to be involved in oxidoreductase activity, were downregulated upon mating in sugar fed, but upregulated in mated female midguts upon bloodfeeding. This suggests that upon blood feeding, the massive microbiota abundance increase could have induced ROS rich environment which resulted in higher oxidoreductase in mated female midgut.

Gene ID	Gene Name	Function	*padj* – value (MvsV sugar)	Higher in	*padj* – value (MvsV blood) experiment [K]	Higher in
AGAP010935	Porphobilinogen synthase	Biosynthetic process	0.0001	Mated	0.012	Mated
AGAP010413	SH3 and multiple ankyrin repeat domains protein	Protein binding	0.001	Mated	0.025	Virgin
AGAP003343	Cytochrome P450	Oxidoreductase activity	0.002	Mated	0.035	Mated
AGAP011244	rRNA 2'-O-methyltransferase fibrillarin	rRNA processing	0.003	Virgin	0.032	Virgin
AGAP011052	Aldose reductase	Oxidoreductase activity	0.022	Virgin	0.034	Mated
AGAP012852	Unknown	Oxidoreductase activity	0.024	Virgin	0.039	Mated
AGAP006342	Short peptidoglycan protein 3 (PGRPS3)	Innate immune response	0.035	Mated	0.001	Mated

Table 4.3 List of 7 significant genes upregulated by mating found in common between sugar fed (experiment [I]) and blood fed (experiment [K]) (*padj*<0.05).

I ran a multi factor analysis on all the midguts that were not treated with antibiotics, controlling for the impact of blood or sugar feeding as an additional factor (24 single midguts fed with *P. falciparum* infected blood, 12 pools of 7 sugar fed midguts). I found 14 genes significantly differentially expressed between mated and virgin midguts when all three experiments were included (Table 4.4).

Gene ID	Gene Name	Function	*padj* –value (MvsV sugar)	Higher in
AGAP006508	Small calcium-binding mitochondrial carrier protein 3	Transport	0.01	Mated
AGAP005656	Cytochrome P450	Oxidoreductase activity	0.01	Mated
AGAP003523	Hypoxia-inducible factor prolyl hdyroxylase	Oxidoreductase activity	0.01	Mated
AGAP005242	Unknown	Unknown	0.01	Mated
AGAP008931	Inorganic phosphate cotransporter	Transport	0.02	Virgin
AGAP005498	Phospholipid scramblase 2	Phospholipid scramblase activity	0.02	Virgin
AGAP001874	Ras-related protein Rap-1A	Signal transduction	0.02	Mated
AGAP010682	Unknown	Unknown	0.02	Virgin
AGAP007801	Vrille	Regulation of transcription	0.02	Mated
AGAP002114	Cell division cycle 20-like protein 1	Positive regulation of ubiquitin protein ligase activity	0.03	Mated
AGAP005163	Glucosyl transferases	Metabolic process	0.04	Mated
AGAP012018	Unknown	Unknown	0.04	Mated
AGAP000693	Cecropin anti microbial peptide	Antibacterial humoral response	0.04	Mated
AGAP008926	Shingomyelin synthase	Biosynthetic process	0.04	Mated

Table 4.4 List of significant genes upregulated by mating found from a multi factor analysis on untreated sugar fed, blood fed experiment[I] and blood fed experiment [K] midguts (*padj*<0.05)

I next analysed differences between sugar fed and blood fed midguts. From this comparison, 5814 genes are significantly differentially expressed between sugar fed and blood fed midguts. 3091 genes were upregulated upon blood feeding and most of the genes are involved in different activities such as threonine endopeptidase, heterodimerization, ubiquinone, cytochrome-c, structural constituent of ribosome, SNAP receptor, apoptosis, TBP-class protein binding and proteosome activating ATPase activity (Table 4.5). Whilst the remaining genes were downregulated upon blood feeding which are involved mainly in activities involving in processes involving ATP (energy) and protein binding activity (Table 4.6).

GO.ID	Term	Annotated	Significant	Expected	weight01
GO:0004298	threonine-type endopeptidase activity	15	15	3.55	1.0e-10
GO:0046982	protein heterodimerization activity	57	31	13.50	5.3e-07
GO:0008137	NADH dehydrogenase (ubiquinone) activity	24	17	5.68	1.3e-06
GO:0004129	cytochrome-c oxidase activity	14	12	3.32	1.7e-06
GO:0031625	ubiquitin protein ligase binding	26	17	6.16	7.2e-06
GO:0003735	Structural constituent of ribosome	173	66	40.98	1.2e-05
GO:0005484	SNAP receptor activity	19	13	4.50	4.4e-05
GO:0097200	cysteine-type endopeptidase activity (apoptosis)	14	10	3.32	0.00021
GO:0017025	TBP-class protein binding	12	9	2.84	0.00025
GO:0036402	proteasome-activating ATPase activity	8	7	1.89	0.00026

Table 4.5 Functional enrichment analysis on upregulated genes (*padj*<0.05) in response to blood feeding.

GO.ID	Term	Annotated	Significant	Expected	weight01
GO:0005524	ATP binding	684	229	162.19	9.0e-10
GO:0005515	protein binding	2327	653	551.77	2.0e-09
GO:0005488	binding	5521	1441	1309.13	9.1e-09
GO:0004812	aminoacyl-tRNA ligase activity	43	23	10.20	2.3e-05
GO:0005096	GTPase activator activity	58	28	13.75	3.7e-05
GO:0016758	transferase activity, transferring hexos..	89	35	21.10	0.00016
GO:0003723	RNA binding	346	122	82.04	0.00057
GO:0008026	ATP-dependent helicase activity	56	29	13.28	0.00072
GO:0003924	GTPase activity	61	26	14.46	0.00082
GO:0043015	gamma-tubulin binding	7	6	1.66	0.00099

Table 4.6 Functional enrichment analysis on genes that were significantly downregulated upon blood feeding in female midguts (*padj*<0.05)

There is no overlap among the differentially expressed genes I detected between virgin and mated bloodfed females from feed [K] and feed [I]. Factors that contribute to this lack of overlap include different *P. falciparum* parasite infection success rates and infection intensity levels and potentially different bacterial abundances and compositions as these experiments were carried out months apart. To test this, I compared the bacterial abundance from both experiments [I] and [K] without looking at the mating status. Total bacterial loads are estimated as slightly higher by Kraken analysis in experiment [K] although this is not significant (Figure 4.3a). Looking at other bacterial species, Experiment [I] has more *Pantoea* (ANOVA: n=12, p = 0.001) and *Serratia* (ANOVA: n=12, p = 0.02) whilst *Klebsiella* and *Flavobacteriaceae* had similar abundances in both experiments and *Acetobacteraceae* is significantly more in experiment [K] (ANOVA: n=12,

p = 0.02) (Figure 4.3b). This suggests that there might be some other bacterial species that are higher in experiment [K] that might have an impact on the midgut transcriptome and infection phenotype. I found that bacteria from *Rhodobacteraceae* (ANOVA: n=12, p = 0.01), *Propionibacteriaceae* (ANOVA: n=12, p = 0.02), *Staphylococcaceae* (ANOVA: n=12, p = 0.01), and *Micrococcaceae* (ANOVA: n=12, p = 0.06) are significantly higher in experiment [K] than experiment [I] (Appendix S4).

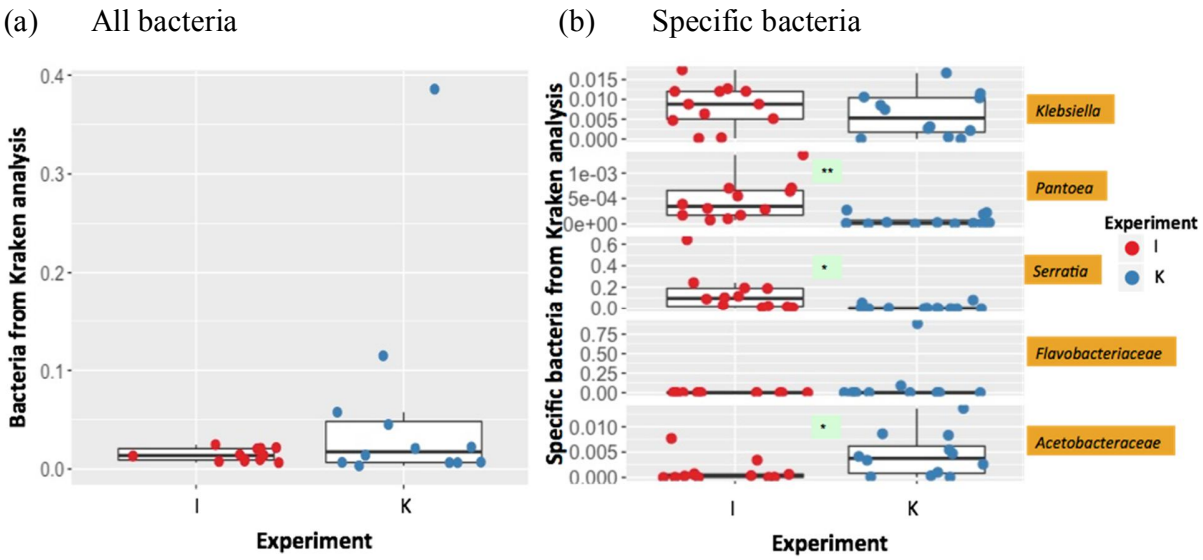

Figure 4.3 Estimates of bacterial abundance from the Kraken analysis on RNAseq data from single midguts from experiments that resulted in 80% *P. falciparum* infection prevalence (experiment[I]) and 100% *P. falciparum* infection prevalence (experiment[K]). Analyses were done on (a) total bacteria (b) specific bacterial species, *Klebsiella, Pantoea, Serratia* and bacterial family *Flavobacteriaceae* and *Acetobacteraceae*. Each dot represents one midgut. Boxplots indicate the median and 25-75 percentiles.

To further explore whether the microbiota might contribute to the signature of mating on the midgut, I carried out RNAseq on 6 virgin and 6 mated single guts post bloodfeed from females that were treated all through adulthood with antibiotics (single midguts treated with antibiotics that had 100% *P. falciparum* infection prevalence (n=12 single guts in total, 6 biological replicates of each of the 2 treatments, mated and virgin). These females were reared and given the same infected blood that led to 100% *P. falciparum* infection prevalence. Only one gene was significantly differentially expressed in response to mating in antibiotic treated midguts. This gene is AGAP009784 which is involved in protein kinase activity.

To understand if the antibiotic treatment has an impact on the midgut transcriptome at 24 hours post blood feed, I repeated the analysis comparing antibiotic treated midguts and untreated midguts from the same batch (n=24 single guts in total which consists of 6 mated and 6 untreated virgin female guts, 6 mated and 6 virgin female guts which were given antibiotics Pen/Strep/Gen in their sugar meal). In the RNASeq analysis, I've added mating status as an additional factor (multi factor analysis) to be counted for as mating has some impact on the transcriptome with antibiotic treatment as the main factor for the analysis. From this analysis, there are 1507 genes significantly differentially expressed (*padj* <0.05), 574 of which were upregulated upon antibiotic treatment, indicating that these genes have higher expression in midguts with less bacteria. The genes are mainly involved in RNA processing activity (Table 4.7). Of the 933 genes that were significantly downregulated upon antibiotic treatment, the majority of these are involved in multiple activities such as protein binding, serine/threonine kinase, protein tyrosine phosphatase, histone-lysine N-methyltransferase, zinc ion and amide binding, transcription factor, transcription regulatory region sequence, modified amino acid binding and chromatin binding. This suggests upon antibiotic treatment, with lesser bacterial abundance, midgut cells could focus the function in breaking down blood meal. Another function that was regulated is the binding activity to modulate transcription and protein kinase activities (Table 4.8).

GO.ID	Term	Annotated	Significant	Expected	weight01
GO:0006457	protein folding	87	19	4.80	1.6e-07
GO:0010501	RNA secondary structure unwinding	29	10	1.60	1.8e-06
GO:0034314	Arp2/3 complex-mediated actin nucleation	12	6	0.66	1.9e-05
GO:0006364	rRNA processing	102	28	5.62	3.6e-05
GO:0043623	Cellular protein complex assembly	98	27	5.40	0.00044
GO:0008535	respiratory chain complex IV assembly	8	4	0.44	0.00053
GO:0000470	maturation of LSU-rRNA	22	8	1.21	0.00063
GO:0031167	rRNA methylation	9	4	0.50	0.00092
GO:0018298	protein-chromophore linkage	9	4	0.50	0.00092
GO:0035069	larval midgut histolysis	9	4	0.50	0.00092

Table 4.7 Functional enrichment analysis on genes that were significantly upregulated upon antibiotic treatment in female midguts (*padj*<0.05)

GO.ID	Term	Annotated	Significant	Expected	weight01
GO:0005515	protein binding	2327	234	184.68	6.5e-06
GO:0004674	protein serine/threonine kinase activity	118	24	9.37	2.5e-05
GO:0018024	histone-lysine N-methyltransferase activ...	11	5	0.87	0.00096
GO:0008270	zinc ion binding	637	71	50.56	0.00177
GO:0033218	amide binding	54	10	4.29	0.00185
GO:0003700	transcription factor activity, sequence-...	204	32	16.19	0.00418
GO:0000976	transcription regulatory region sequence...	34	6	2.70	0.00439
GO:0072341	modified amino acid binding	5	3	0.40	0.000441
GO:0004725	protein tyrosine phosphatase activity	37	8	2.94	0.00738
GO:0003682	chromatin binding	30	7	2.38	0.00778

Table 4.8 Functional enrichment analysis on differentially expressed genes (*padj*<0.05) down regulated in response to antibiotic treatment in single blood fed midguts.

Discussion

Mating has been shown in other organisms to influence female gut physiology (McGraw et al., 2008; Reiff et al., 2015), however this has not been explored in much detail in *Anopheles*. It is particularly relevant for *Anopheles* and other blood feeding mosquitoes that transmit disease. If mating influences the outcome of infections by the pathogen/parasite, it is of medical relevance and reducing mating opportunities by, for example, targeting males could be a potential control strategy that impacts more than just fertilization rates. Previous work examining whole carcasses of mated females using microarrays shows that mating induced changes and these changes are long lasting (Rogers et al., 2008b). This is expected as *An. gambiae* females mate only once and they may need to maintain these transcriptional changes for successful reproduction. However, these previously observed differences are mainly in the lower reproductive tract of *An. gambiae*.

In this chapter, my RNASeq analysis shows that mating induces many transcriptional changes in the sugar fed female *An. coluzzii* midgut. Many of the functional categories that are overrepresented by genes that were upregulated by mating reflect an impact on digestion and the immune response (Table 4.1). In Chapter 3, I have shown that mated females have higher bacterial loads compared to virgin females sugar fed midguts. The digestion of nectar is usually performed by the enzymes available in the midguts (Souza-Neto et al., 2007). Higher digestion activity in the midguts of mated females suggests that mated females might channel their efforts towards digesting the nectar for energy more than virgin females. This might be due to the fact that they are already mated and thus poised to lay eggs as soon as they receive a bloodmeal. Although it is unknown in *An. coluzzii*, another possibility is that the microbiota might be able to help speed the digestion in the mated female midgut. In *Ae. aegypti,* microbiota was reported helped to digest blood meal (Gaio et al., 2011) and gut clearance has been reported to affect blood digestion by less hematin excretion by *An. stephensi* (Sharma et al., 2013). All together, these newly discovered patterns suggest that a male transfers something to the female that increases her midgut microbiota loads, and shifts her digestive and immune gene regulation perhaps to improve digestion and cope with higher bacterial loads.

However, upon blood feeding, the strong transcriptional signal observed upon mating in the sugarfed samples is absent, and the weak signal that remains is inconsistent between experiments. This finding suggests that mating might have increased the expression of certain genes in order to facilitate blood digestion. No genes are differentially expressed between mated and virgin midguts consistently across the two RNA sequencing experiments I carried out on bloodfed females (80% and 100% *P. falciparum* prevalence), and in general very few genes remain regulated by mating after a bloodmeal. These different midgut transcriptomic responses to mating in the two experiments could be driven by different microbiota in the midguts. Indeed, using Kraken to identify bacterial loads and composition, it is clear that that experiment [K] shows a trend of higher bacterial abundance than experiment [I] and also the two experiments have different bacterial species composition (Figure 4.3).

From the comprehensive multifactor analysis I carried out in which I analysed all samples together from both experiments, there are 14 genes that are significantly differentially expressed in response to mating. These genes are mainly involved in oxidoreductase activity, binding activity, activity involving energy, antimicrobial activity, apoptotic cell removal and some genes are with unknown function. This finding suggests that mated females might respond more intensely to produce energy and digest blood for egg provisioning than virgin females. This activity might be mediated by the microbiota in the midgut.

Both sugar fed and blood fed (experiment [I]) mosquitoes were reared the same way and midguts were dissected at the same time, which is at 24 hours post blood feed. At this time, all of the mated females (both sugar fed and blood fed) are at 3 days post mating. Although there were not many genes that were differentially expressed between bloodfed mated and virgin female midguts, the RNASeq analysis on the sugar fed midguts show that there is a major impact of mating on the midgut transcriptome. Previously, the impact of mating on the whole female transcriptome at 2 hours, 6 hours and 24 hours post mating revealed that the most genes are differentially regulated at 24 hours post mating (Rogers et al., 2008). From my data, it shows that at 4 days post mating, there is a huge difference between mated and virgin female midgut, many more genes differentially regulated just in the gut than were detected in the whole mosquito at 24 hours post mating (479 genes vs 141 genes from (Rogers et al., 2008)) which suggests the

possibility that overtime, the number of changes at the transcriptomic level due to mating might increase. One hypothesis is that perhaps with this impact of mating in the midgut prior to blood feeding in the midgut, there might be changes in such as way as to better support microbiota and facilitate *P. falciparum* development. Among the 141 genes from (Rogers et al., 2008), 41 genes were upregulated and 96 genes were downregulated at 24 hours post mating. 7 genes from these 41 genes were found to be in common with genes that were upregulated upon mating in the midgut. These genes are mainly involved in proteolysis activity and protein transport. None of these genes were reported to be expressed in the midgut upon mating before (Rogers et al., 2008) perhaps because the qPCR was only done on the top 20 genes.

Many genes that were upregulated upon blood feeding in the midguts are involved in activities which involves breaking down blood component. This corresponds with the higher bacterial loads upon blood feeding (Dong et al., 2009; Gendrin & Christophides, 2013). In *Aedes* mosquitoes, the microbiota aid in digestion of the blood in the midgut (Gaio et al., 2011). The blood digestion turns the midgut into an oxidative environment where heme can induce oxidative stress by generating hydroxyl radicals (Sadrzadeh et al., 1984). This oxidative environment imposes pressure on mosquito fecundity and other physiological traits (Champion & Xu, 2017).

The impact of antibiotics on the midgut transcriptome was examined previously. In this experiment, RNASeq was carried out on pools of 20 midguts at 0hr (sugar fed), and then 5 hr, 24hr and 72 hr post naive bloodfeed. They found the highest number of differentially expressed genes at 0hr and 72 hours. However, at these two time points, microbiota exhibits the least abundance as compared to 24 hours post blood feed (Rodgers et al., 2017). From my analysis, removing microbiota from the midgut by antibiotics has a huge impact on the transcriptome of the midgut at 24 hours post *P. falciparum* infected blood fed. Most of the genes in the midgut were downregulated upon antibiotic treatment. The genes that were upregulated upon antibiotic treatment (microbiota absent) are mainly involved in activities involving RNA such as RNA secondary structure unwinding, rRNA processing, maturation of LSU-rRNA, rRNA methylation and protein functions such as protein folding, cellular protein complex assembly and protein - chromophore linkage. This suggests that when the microbiota is absent, the midgut focuses more in RNA processing and translational processes. The genes that were downregulated upon

antibiotic treatment (microbiota present) are mainly involved in catalysis activity. This is almost the same as the upregulation impact upon blood feeding, when microbiota loads increase tremendously. Studies using *Ae. aegypti* showed that reduction of bacteria affected red blood cell lysis which subsequently retarded protein digestion and affected oocyte maturation (Gaio et al., 2011).

In Chapter 3, I found that mated females typically have more microbiota than virgin females in sugar fed and blood fed females. In this chapter, my RNASeq analysis shows that mating has an impact on the midgut transcriptome in sugar fed females. Most of the responses are towards digestion of the blood and immune protection towards bacteria (Table 4.1). When the females have their blood meal, the impact of mating on the midgut transcriptome seems to disappear, and I found very few genes that are differentially expressed due to mating even when I performed a multifactor analysis using all the data. However, upon removing bacteria by using antibiotics, the impact of mating on the midgut transcriptome is no longer seen, perhaps overwhelmed by the massive gene expression changes that occur due to blood feeding, but these changes that were observed in sugarfed guts would still be there at the time of bloodfeeding and may have had an impact even prior to bloodfeeding. This is consistent with the phenotypic differences observed suggesting that mated females frequently have different microbiota and parasite infection levels than virgin females, even though they don't seem to have major gene expression changes at the 24 hour post bloodfeeding time point.

Overall, my RNASeq findings suggest that mating has an impact on sugar fed midgut transcriptome. Microbiota in the midgut play a big role especially upon blood feeding by channeling most of the function in the midgut in breaking down the blood for nutrient absorption and to provision eggs. These results create a platform for us to proceed with the hypothesis that the huge impact on the transcriptome from mating is probably microbiota dependent. Furthermore, it could also be hypothesized that microbiota loads in the female midguts might be contributing to the impact of mating status on susceptibility to malaria transmission by mediating the immune response of the vector towards *P. falciparum* infection.

CHAPTER 5

The impact of mating on *P. falciparum* infection

Introduction

Similar to other insects, female *Anopheles* undergo behavioural changes after mating, such as induction of egg laying in blood fed females and refractoriness to further insemination (Gabrieli et al., 2014; Klowden & Russell, 2004; Rogers et al., 2008, 2009; Tripet et al., 2003). They also undergo major physiological changes. Transmission Electron Microscope (TEM) experiments show mated female atrial cells are permanently altered as compared to virgin cells in ways that suggest these cells function to support the uptake of male material transferred during copulation (Rogers et al., 2009). Mating also influences gene regulation in *Anopheles* females, although immune genes do not appear to be regulated by mating like they do in *Drosophila* (Rogers et al., 2008; Short & Lazzaro, 2010). Comparison of post mating gene expression between whole virgin and mated *An. gambiae* females suggests that mating causes permanent changes in gene expression (Rogers et al., 2009).

In female mosquitoes, lipid and proteins derived from blood feeding and sperm from mating are crucial for a successful egg production. 20E hormone is involved in stimulating egg production. In *An. gambiae*, the 20E titre in females is influenced by two major factors: blood feeding and mating (Bai et al., 2010; Hagedorn et al., 1975; Pondeville et al., 2008). 20E is synthesized by the ovaries after a bloodmeal, and these levels are highest at 18 and 24 hours post blood feed(Pondeville et al., 2008). Blood feeding stimulates the ovary to secrete ecdysone which will next be hydroxylated to 20E by the fat body where it binds to the ecdysone receptor (EcR), which forms a dimer with USP and activates yolk protein precursors (YPP) such as Vg and lipophorin (Lp) (Hagedorn et al., 1975; Raikhel et al., 1999; Swevers et al., 1995). Both Vg and Lp are lipid transporters involved in egg production.

In addition to blood feeding, mating also increases the titre of 20E in females. In *An. gambiae*, the male accessory gland (MAG) produces a high titre of 20E that it is transferred to the females during mating. The 20E hormone will be replenished in the MAG within a few hours of mating (Pondeville et al., 2008). The 20E derived from males interacts with female protein known as Mating-Induced Stimulator of Oogenesis (MISO) in the atrium to regulate oogenesis and influence lipid accumulation in oocytes (Baldini et al., 2013). Apart from oogenesis, 20E also has a function in preserving the sperm in spermatheca by inducing HPX-15 to make sure that the sperm remain functional as females typically only mate once in their life (Shaw et al., 2014).

A microarray analysis of the atrium and spermathecae of *An. gambiae* females injected with 2.5µg 20E in *An. gambiae* at 24 hours post injection showed that 20E injection results in gene expression patterns in the atrium and spermatheca that are very similar to gene expression patterns seen in these tissues 24 hours post mating (Gabrieli et al., 2014). The induction of egg laying and refractoriness to further mating that occurs after mating are primarily driven by this hormone and not by the sperm (Gabrieli et al., 2014; Thailayil et al., 2011).

20E is transferred to the female together with sperm and seminal fluid proteins in the form of a mating plug during copulation (Pondeville et al., 2008). There is variability in the mating plug phenotypes of *Anopheles* species across the world. Major malaria vector species such as *An. gambiae, An. arabiensis, An. funestus* and *An. stephensi* have a solid and fully coagulated plug. *An. farauti, An. dirus* and *An. sinensis* which are the east and southeast Asian species and *An. atroparvus*, the European species, have a less coagulated, amorphous plug phenotype (Mitchell et al., 2015). *An. albimanus,* which is the major malaria vector in South America, does not have a mating plug and females from this species are able to mate multiply (Mitchell et al., 2015). Intriguingly, this geographic region has fewer malaria cases compared to areas where malaria vectors have a solid and structured mating plug, leading the authors to speculate that the mating plug may have an impact on transmission rates (Mitchell et al., 2015). Furthermore, ancestral reconstruction using maximum parsimony of mating plug phenotype and 20E titre shows a high correlation between the solid and fully coagulated plug and a high titre of 20E produced in the MAG in all four major malaria vector species; *An. gambiae*, *An. arabiensis, An. stephensi*, and

An. funestus (Mitchell et al., 2015) which suggests that 20E in particular might play a critical role in malaria transmission.

In Chapter 3, I have shown that female mating status impacts the abundance and composition of the microbiota in the midgut of *An. coluzzii* mosquitoes. Work by others has shown that the microbiota can influence the outcome of infection with the malaria parasite, *P. falciparum* (Gendrin & Christophides, 2013). Together, these findings led us to examine whether mating status influences the susceptibility of *Anopheles* to *P. falciparum*, and if so, whether this is dependent on bacteria. Furthermore, 3 genes which were significantly differentially expressed from my RNASeq analysis from experiment [K] in Chapter 4 were chosen for dsRNA based knock down to evaluate whether they had an impact on *P. falciparum* infection. Finally, I also ask whether 20E in particular influences the outcome of parasite infection in female mosquitoes.

Materials and Methods

Detailed information on number of mosquitoes and *P. falciparum*-infection feeds information can be found in Appendix 4.

Mosquitoes

Mosquito rearing

The Ngousso strain of *An. coluzzii*, which originates from Cameroon was reared under standard conditions (26°C-28°C, 65%-80% relative humidity, 12 h:12 h light/Darkness photoperiod). Eggs were floated in a pan filled with deionized water and once larvae hatched, around 1200 larvae were reared in a 32L plastic pan (66cm x 45cm x 17cm). The larvae were fed on one mL of ground fish food each day and 2 pieces of cat food on Friday evening for the weekend. When the adults emerged, they were maintained on 10% autoclaved fructose solution.

Adult mosquitoes were separated by sex as pupae and placed in separate cages in dishes filled with deionized water. Cages were inspected when adults emerged and any of the wrong sexed

adults were removed. A subset of females were introduced to the males via aspirator three days after emergence and remaining females were retained in the original cage and labelled as virgin. The aspirator was cleaned with 70% ethanol prior to use. One day after females were introduced to the males, the males were removed from the cage and these females were known as mated females. This status was always later confirmed by visual inspection of the spermatheca upon dissection, or molecular detection of the presence of Y bearing sperm in the spermatheca (Krzywinski, Nusskern, Kern, & Besansky, 2004), and any females not found to have mated were discounted.

Ngousso treatment with antibiotics

Female Ngousso mosquitoes were separated into two sets in three separate *P. falciparum* infection experiments [H],[J],[K]. Set one is fed on normal sugar whilst the second set of mosquitoes was maintained with antibiotic-containing sugar solution (25μg/mL gentamycin, 10μg/mL penicillin and 10 unit/mL streptomycin) to remove most of the bacteria from their midguts. For each of the sets, females were split into two further groups, virgin and mated females (mixed with males overnight). Mated females and virgin females were collected as described previously.

Double stranded RNA (dsRNA)

Three genes significantly regulated by mating in the midgut from Chapter 4 (Vg, PGRPS3 and PM) were amplified by PCR using 25μL of High Fidelity Phusion master mix (NEB M0531S), 2.5 μL of 10μM forward and reverse primer, respectively, and genomic DNA template in a 50uL reaction volume. The list of primers is available in Appendix S3. Reactions were done using the Veriti 96 well Thermal Cycler with conditions of 98°C for 2 mins, followed by 30 cycles of 30 seconds at 98°C, 30 seconds at 60°C, 1 ½ minutes at 72°C and 72°C for 5 minutes. PCR products were run on 1% agarose gel (120V, 20 minutes) to confirm that the right product was amplified and also to look at the band intensity. Qiagen Qiaquick PCR Purification kit (Qiagen 23104) was used to purify the PCR product. Following the manufacturer's protocol, buffer PB was added at 5 times volume of PCR product followed by 10μL of sodium acetate. The mixture was mixed well and loaded into a column. The column was spun for 60 seconds and the solution collected in the collection tube was removed. 750μL of PE buffer was next added and spun down

for 30 seconds twice to remove the solution completely. Using a new 1.5mL eppendorf tube, 50µL of RNase-free water was added into the column and spun for a minute to wash out the DNA into the new collection tube. The DNA concentration was checked using Nanodrop. HiScribe T7 High Yield RNA Synthesis Kit (E2040S) was used to synthesize dsRNA. Using the manufacturer's protocol for a 20µL reaction, 2µL T7 RNA Polymerase was added and mixed into NTP buffer mix and 1µg DNA and incubated overnight at 37°C. Using RNeasy Plus Mini Kit (74134), 80µL RNase free water was added and mixed well into 20µL of dsRNA to dilute, followed by 350µL RLT buffer, 250µL ethanol and transferred into RNeasy mini column. The columns were spun down at 8000g for 15 seconds and the collection tubes were replaced. 50µL of RPE buffer were next added and spun for 15 seconds at 8000g and flow through was discarded. This step was repeated again before changing the collection tube. Columns were spun at a full speed for 1 minute to remove ethanol traces. RNA was collected in a new RNAse free eppendorf tube eluted in 30µL of RNase free water. The concentration of dsRNAs were checked by Nanodrop.

Double stranded RNA (dsRNA) Injection

A set of females (mixed mating status) were injected with 3µg/µL of dsRNA at the thorax.

Assessing Knock Down Effect of dsRNA via PCR

A set of females (mixed mating status) were injected with 3µg/µL of each dsRNA, 3 days before blood feeding. They were blood fed, either by naive blood or *P. falciparum* infected blood, and dissected at 24 hours post blood feed. RNA were extracted from each sample as mentioned in RNA extraction methods and qPCR were performed on each sample with their respective gene primer. The list of primers sequences is available on Appendix S3. The knock down efficiency was calculated by below formula =

$$\text{Knockdown efficiency} = 1 - 1 / \left(\frac{\text{Average dsLacZ}}{\text{Average dsRNA}} \right)$$

20-Hydroxyecdysone Injection

A set of virgin females and mated females were injected in the thorax with 2.5µg 20-hydroxyecdysone (H5142 Sigma) diluted in 10% ethanol at the thorax to mimic mating in virgins (Baldini et al., 2013) and to see the impact on mated females. Another set of virgins and mated females were injected with 10% ethanol as controls. These injections were performed either 2 or 3 days before blood feeding, typically early in the morning the day after males were introduced to some females overnight to have mated female set. Furthermore, after injection, the mosquitoes were given some time to recover so that they will blood feed well.

Plasmodium falciparum

P. falciparum Gametocyte Culture

P. falciparum strain NF54 was cultured in complete RPMI 1640 (Invitrogen) supplemented with 10% human serum (mixed pooled serum from more than 4 individuals), sodium bicarbonate (Sigma), hypoxanthine (Sigma) and D-glucose (Sigma). To induce *P. falciparum* gametocytes, cultures were set up to 0.75% - 1% parasitemia at 6% hematocrit in complete RPMI 1640 medium. NHS Blood and Transplant (NHSBT) non-clinical use O+ blood was used after washing three times with incomplete medium (RPMI 1640 without serum). Culture medium was changed everyday and gassed for 15 seconds with 1% oxygen, 3% carbon dioxide and 96% nitrogen. *P. falciparum* gametocytes matured from day 14 to 18. *P. falciparum* morphology and stages on day 7 and day 14 were monitored under the microscope by Giemsa staining. In short, 3µL of *P. falciparum*-infected red blood cells were thin-smeared on a glass slide and fixed with methanol. Slides were stained with 20% Giemsa for 20 minutes, rinsed with water and dried before being assessed under 100x oil magnification light microscope. An exflagellation assay was performed on day 14 and 17 to assess male gametocyte maturity. For these assays, 50µL of culture was placed on a glass slide and covered with a glass coverslip. Following 20 minutes of incubation at room temperature, the numbers of exflagellation centers were counted at magnification of 10x. Exflagellation is a process when the temperature drops and pH increases which induce the male gametes to undergo three times DNA replication and form eight highly motile flagellated microgametes. These microgametes will then fuse with female gametes to

form zygotes in the vector's gut. Only parasite cultures that showed exflagellation were used for feeds.

P. falciparum Infection (Membrane Feeding Assay)

Virgin and mated female Ngousso mosquitoes were prepared as described above. Cotton was removed six hours before blood feeding. 14 or 15 and 17 or 18 day old *P. falciparum* gametocyte cultures were pooled and spun down at 38°C, 2000rpm, for 5 minutes. Supernatant was removed and 1mL serum was added into the tube and topped up with fresh washed O+ blood to obtain a haematocrit of 45%. The mixture was always kept on a heat block (38°C) during the process to avoid inducing activation. The mixture was then added to a glass membrane feeder using a blunt syringe. Each cup of mosquitoes fed for 7 minutes and new sugar-soaked cotton was placed on each cup after feeding was completed. Cups were kept in a secure container in an incubator at 26°C and 80% humidity. 24 hours after feeding, unfed females were removed. Some females were put aside for midgut dissection and scoring mating status, and leftover fed females were retained to assess infection rates at day 10 post blood-fed. Sugar-soaked cotton was changed every two days with normal sugar for all mosquitoes.

Assessing *P. falciparum* Infection

Two assessments of *P. falciparum* infection were performed: infection intensity, which is the number of oocysts in each midgut and infection prevalence, which scores whether the mosquito is infected or not.

At day 10 post blood feed, mosquitoes were killed using 70% ethanol and they were rinsed with 1X PBS and dissected in 0.5% mercurochrome (Sigma) diluted in water with 1µL Hoescht added (1µg/ml). The guts were fixed in 4% Paraformaldehyde (PFA) for 30 minutes, then dipped in 1X PBS, and then left in another container of 1X PBS for 30 more minutes. Fixed and stained guts were mounted in Vectashield (Mounting Medium for fluorescence Vector Lab) and kept at 4^{o}C until viewed. For each gut, the number of oocysts was counted under the microscope and recorded. Within R, the library 'ExactRankTests' was used to perform Wilcoxon rank statistical tests to assess whether there were significant differences in infection levels due to mating status,

antibiotic treatment, dsRNA injection, and 20E injection treatment. Chi Square tests were used to assess the impact of these treatments on infection prevalence.

Assessing Mating Status via light microscopy or Y-PCR

Mating status for each female was assessed. Dissected spermathecae were either visually examined using light microscopy or using the Y-PCR method to confirm mating status on each spermatheca. 50µL lysis buffer (1M Tris HCl, 1M KCl, Proteinase K, Tween 20) were added into the PCR strips which contain spermatheca. PCR tubes were placed in the thermocycler (65^{o}C for 1 hour, 95^{o}C for 10 minutes followed by a 4^{o}C hold). PCR was then performed using OneTaq 2 x master mix (Qiagen), 23S forward and reverse primer which is the Y-chromosome specific PCR markers (Krzywinski et al. 2004, 2005), the primer sequences can be found in Appendix S3. and DNA template in the condition of (94^{o}C for 3 minutes, 94^{o}C for 20 seconds 60^{o}C for 30 seconds 72^{o}C for one minute for 35 cycles followed by 72^{o}C for 10 minutes and 4^{o}C hold). PCR products were then run on 3% agarose gel at 110V and viewed using Imagelab 4.0.1 software.

Results

To understand if mating status has an impact on female mosquito susceptibility to *P. falciparum*, I carried out a total of 11 successful *P. falciparum* infection experiments (14 additional feeds were carried out that did not lead to any infection). The infection intensity between experiments varies and is very difficult to control. Some infection feeds have low oocyst counts (0-10/gut), while in other experiments, the oocyst counts are relatively high (>20/gut). Every *P. falciparum* infection experiment is represented by a letter, and these letters are in order of increasing prevalence with infection [A] having the lowest infection prevalence to infection [K] which had 100% infection prevalence. Generally, by looking at the graphs, the trend suggests that mated females have higher infection intensity compared to virgin females. However, only 6 feeds out of 11 feeds show significant differences (in bold) that mated females have higher infection intensity than virgin females; Wilcoxon Rank Test: A (p = 0.09), **B (p = 0.05)**, C (p = 0.99), **D (p = 0.04)**, **E (p = 0.05)**, F (p = 0.2), G (p = 0.85), **H (p = 0.0002)**, **I (p = 0.003)**, **J (p = 0.004)**, K (p = 0.06) (Figure 5.1).

The phenotype of infection intensity (oocysts per gut) is correlated with the phenotype of prevalence (did the mosquito get infected or not) where higher oocyst counts per gut are typically associated with higher overall prevalence (Figure 5.1). Therefore I also examined whether infection intensity still shows a relationship with mating status after all uninfected females are removed. Three experiments retain a significant association of mating status with infection intensity; ; Wilcoxon Rank Test: A (p = 0.12), **B (p = 0.02)**, C (p = 1), D (p = 0.07), E (p = 0.41), F (p = 0.35), G (p = 0.96), **H (p = 0.01)**, I (p = 0.92), **J (p = 0.012)**, K (p = NA).

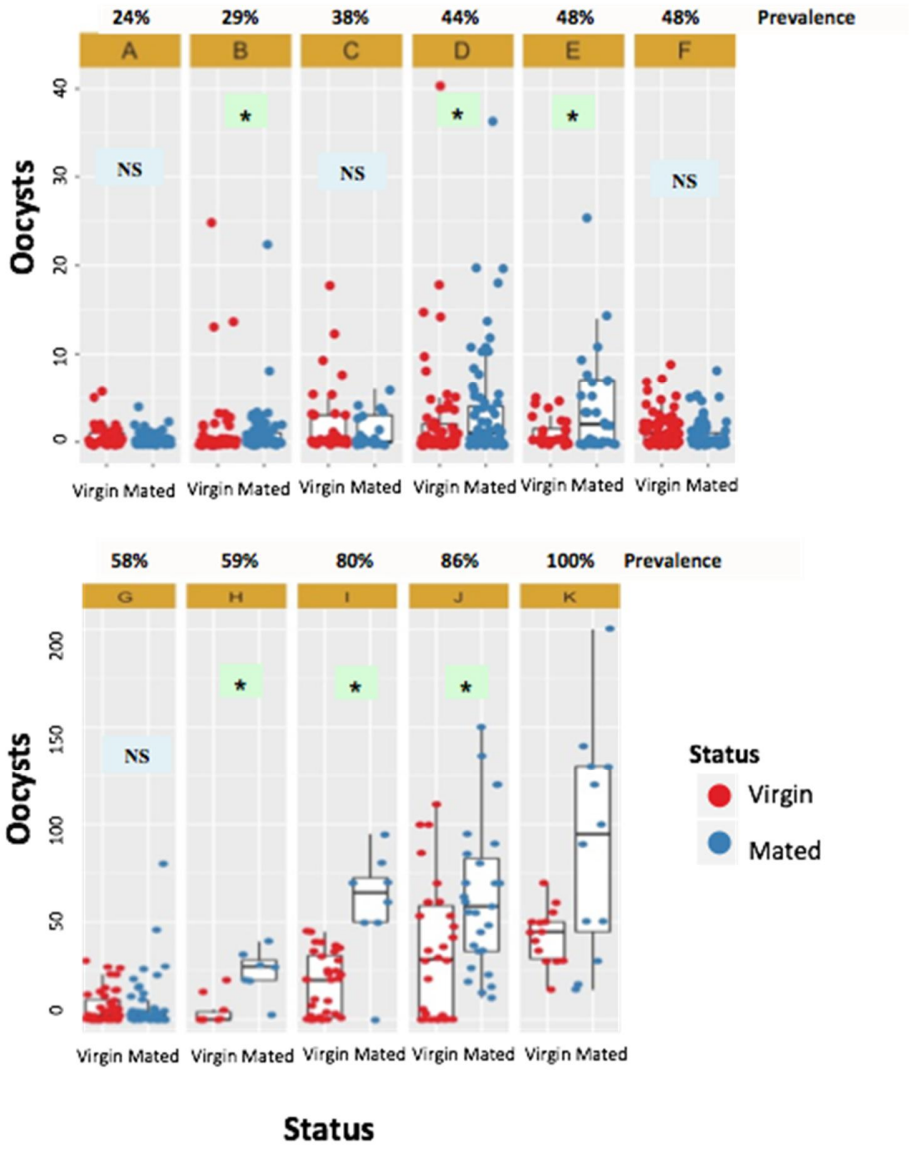

Figure 5.1 Overall *P. falciparum* infection intensity in virgin and mated female midguts from the lowest to highest infection prevalence. Each dot represents number of oocysts per single midgut dissected on day 10 post blood feed. Note the difference in scale between the upper panel, which had lower typical infection outcomes, and the lower panel which had very high infection intensities. Boxplots indicate the median and 25-75 percentiles.

What is even more striking is that all females that took in a *P. falciparum*-infected blood meal but did not become infected were virgins leading to a very significant impact of mating status on prevalence (Table 5.1). Among untreated females, mated females have a higher prevalence of *P. falciparum* infection compared to virgin females in three experiments. Experiments [B], [H] and [J] showed significantly different infection intensity and infection prevalence (Figure 5.1, Table 5.1) whilst Experiments [D], [E] and [I] only have a significant impact of mating on infection intensity (not prevalence) (Figure 5.1). In three of the experiments, mating status is impacting infection intensity, and another three experiments is impacting prevalence (Table 5.1) which suggests that mating can frequently affect *P. falciparum* susceptibility.

Experiment	Virgin		Mated		Chi square (p-value)	Oocyts (median)
Untreated	Infected (n)	Uninfected (n)	Infected (n)	Uninfected (n)		
A	16	35	10	49	0.12	0
B	12	51	22	34	**0.02***	0
C	13	23	8	12	1	0
D	23	44	35	34	0.07	0
E	11	16	16	13	0.42	0
F	40	37	26	35	0.36	0
G	24	32	28	21	0.97	0
H	2	7	7	0	**0.01***	5
I	25	7	7	1	0.92	23
J	22	8	27	0	**0.01***	45
K	14	0	12	0	*NA*	50
Antibiotics treated						
H	5	1	22	1	0.87	15
J	16	9	30	4	**0.05***	32
K	21	0	20	0	*NA*	35

Table 5.1 *P. falciparum* infection prevalence statistical analyses using Chi Square test on untreated and antibiotic treated experiment. (* = $p < 0.05$). The rightmost column is the median of oocysts count from each experiment that was used to separate the experiments.

Infection with the NF54 strain of *P. falciparum* generally results in a higher infection intensity than is found in the field. One interesting observation is that in experiment [H] and [J], all of the uninfected females are virgins (Table 5.1). To understand if the outcomes depend on infection intensity, the *P. falciparum* infection feeds were grouped into experiments with median oocyst counts of less than 10 per gut (Experiments [A-H]; Figure 5.2; Table 5.1), and experiments with median oocyst counts of more than 10 per gut (Experiments [I-K]; Figure 5.3; Table 5.1). When infection intensity is low, there are no significant differences found between mated and virgin female Ngousso (p = 0.11) (Figure 5.2). Even when the uninfecteds (oocysts with zero counts) were removed, no significant difference between mated and virgin females was seen. However in high *P. falciparum*-infection intensity experiments with oocysts count median of more than 11, mated females having significantly higher infection intensity compared to virgin females (Wilcoxon Rank Test: p = 9.24e-08; Figure 5.3). This is true even when the uninfected midgut counts were removed (Wilcoxon Rank Test: p = 7.78e-08).

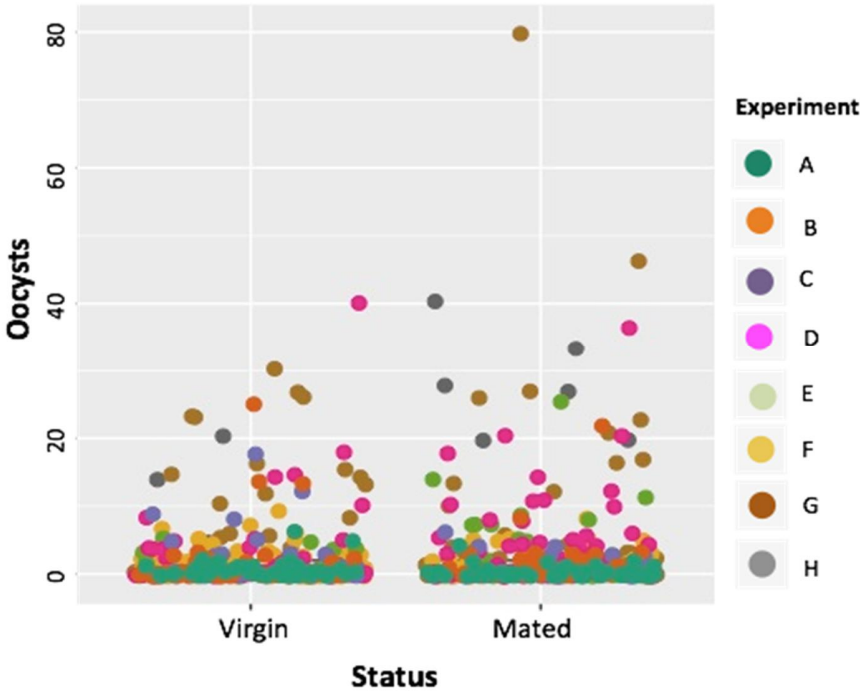

Figure 5.2 Evaluation of the impact of mating status on infection intensity among experiments that lead to low infection intensities shows, i.e., median oocyst count < 10, shows no consistent impact of mating status on infection level.

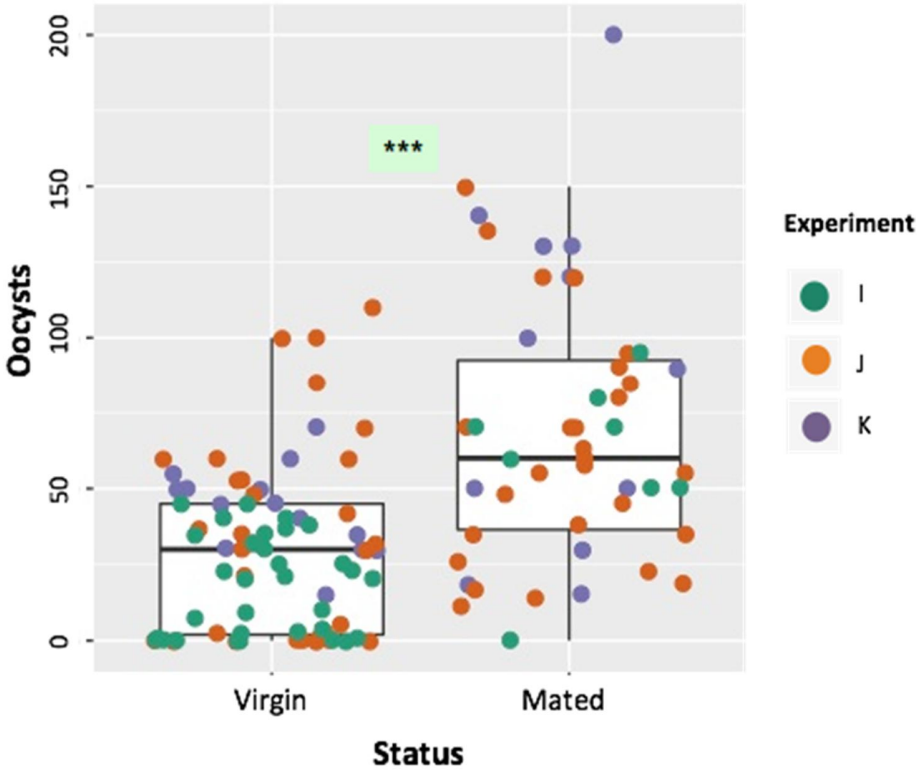

Figure 5.3 Evaluation of the impact of mating status on infection intensity among experiments that lead to high infection intensities shows, i.e., median oocyst count of more than 11, shows a dramatic impact of mating status on infection level. Each dot represents oocysts count in a single midgut. Boxplots indicate the median and 25-75 percentiles. (*** = p<0.0005).

Because bacteria can have a negative impact on *P. falciparum* infection susceptibility (Boissière et al., 2012; Dong et al., 2009), I ran three independent experiments comparing *P. falciparum* infection in females given normal sugar compared to females that were given antibiotics in their sugar meal. This facilitates investigating the impact of mating status on susceptibility to *P. falciparum* independent of bacteria.

These three experiments, [H], [J], and [K], on untreated females were shown earlier (Figure 5.1) but now I am also showing the results on the antibiotic treated virgin and mated females that were fed on the same infectious blood. Looking at all three experiments together, I found that

mated females are dramatically and significantly more susceptible to *P. falciparum* than virgins and this is independent of the presence of the microbiota because the same result is found in antibiotic treated females (Figure 5.4). As reported before in Figure 5.1, Figure 5.4 shows mated females have significantly higher oocyst intensity compared to virgin females in two out of three untreated *P. falciparum* infection experiments; Wilcoxon Rank Test: **H (p = 0.001), J (p = 0.004)** and K (p = 0.06). A similar pattern was observed in females given antibiotics in their sugar meal where the infection intensity is significantly higher in mated females compared to virgin females in two out of three independent experiments; Wilcoxon Rank Test: H (p = 0.12), **J (p = 0.05) K (p = 0.0001)** (Figure 5.4 right panel). This suggests that when infection intensity is high, mated females are more susceptible than virgin females both in the presence and absence of microbiota.

Differences in *P. falciparum* infection intensity in mated and virgin female Ngousso was seen to be driven predominantly by the uninfected midguts. This pattern is slightly different in antibiotic treated females, but the same trend that mated females are more likely to become infected is observed and is independent of the microbiota.

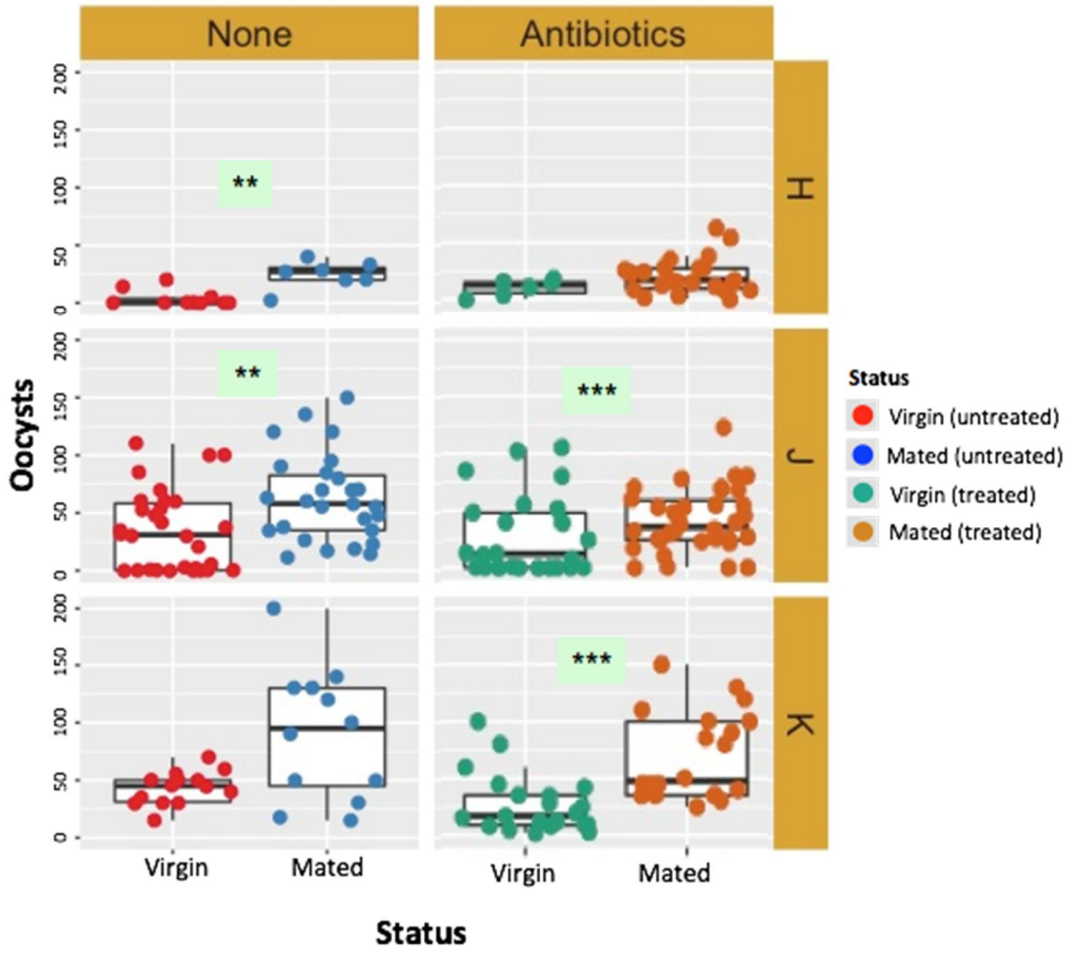

Figure 5.4 *P. falciparum* infection intensity between virgin and mated females in three independent experiments without ("None") and with ("Antibiotics") antibiotics. Each dot represents the number of oocysts found in an individual midgut. Boxplots indicate the median and 25-75 percentiles. (Statistics * = $p<0.05$, ** = $p<0.005$, *** = $p<0.0005$).

The “x” samples for which I have RNASeq data, discussed in Chapter 4, were from Experiment [K] which resulted in 100% prevalence. To follow up on the most differentially expressed genes between mated and virgin female midguts in that experiment, I selected the three most differentially expressed genes for functional genetic analysis. These 3 genes, Vitellogenin (Vg also known as AGAP004203), short peptidoglycan protein 3 (PGRPS3, also known as AGAP006342) and a peritrophin (referred to hereafter as PM, also known as AGAP006796) were each found to be higher in mated female midguts. Using dsRNA injection in 3 different experiments [D, E and I], I knocked these genes down. However, dsVg knock down was only found in the carcass and not in the midgut. For dsPGRPS3, There is no consistency in the knock down effect in either midgut or carcass. dsPM knock down is found in both midgut and carcass in almost all the samples (Table 5.2).

	Trial 1 - inject 3d before blood feeding - sampling 24 hr pbf		**Experiment D** - inject 3d before blood feeding - sampling 24 hr pbf		**Experiment E** - inject 1day before blood feeding - sampling at 24 hr pbf)		**Trial 2** - inject 1day before blood feeding - sampling 3 days pbf (double concentration)	
	Midgut	**Carcass**	**Midgut**	**Carcass**	**Midgut**	**Carcass**	**Midgut**	**Carcass**
dsVg	No kd	99%	No kd	37%	No kd	91%	No kd	90%
dsPGRPS3	No kd	No kd	24%	No kd	66%	67%	No kd	59%
dsPM	No kd	70%	75%	76%	48%	81%	85%	98%

Table 5.2 dsRNA knock down efficiency on midgut and carcass. Each mosquitoes were injected 3 days before blood feeding, blood fed, and dissected 24 hours post blood fed. Trial 1 and 2 were fed on naive blood with Trial 2 using double concentration of the dsRNA (6µg/µL). Whilst some females from Experiment [D] and [E] were sacrificed to assess the knock down efficiency. Knockdown efficiency is represented by percentage value. No kd = no knock down effect were observed.

dsRNA was injected into females, many of which were expected to be mated because the females were left with males for two nights. None of these three genes had a consistent impact on the outcome of infection when knocked down in mated females although dsVg showed a significant difference in experiment [D], increasing infection intensity and prevalence when knocked down (Figure 5.5 and Table 5.3). This is opposite to what would have been expected given that Vg expression was determined to be higher in mated female guts in Experiment [K], and given mated females were more susceptible, knocking down this gene should have resulted in females having lower infection levels or rates. However, experiment [D] was carried out at a different time than experiment [K] and thus these females may have had different microbiota. If Vg is responding in part to the specific microbiota, which is possible given that Vg was not detected as differentially expressed in the repeated RNA seq experiment, then it may also result in different outcomes of infection when removed, depending on the microbes present.

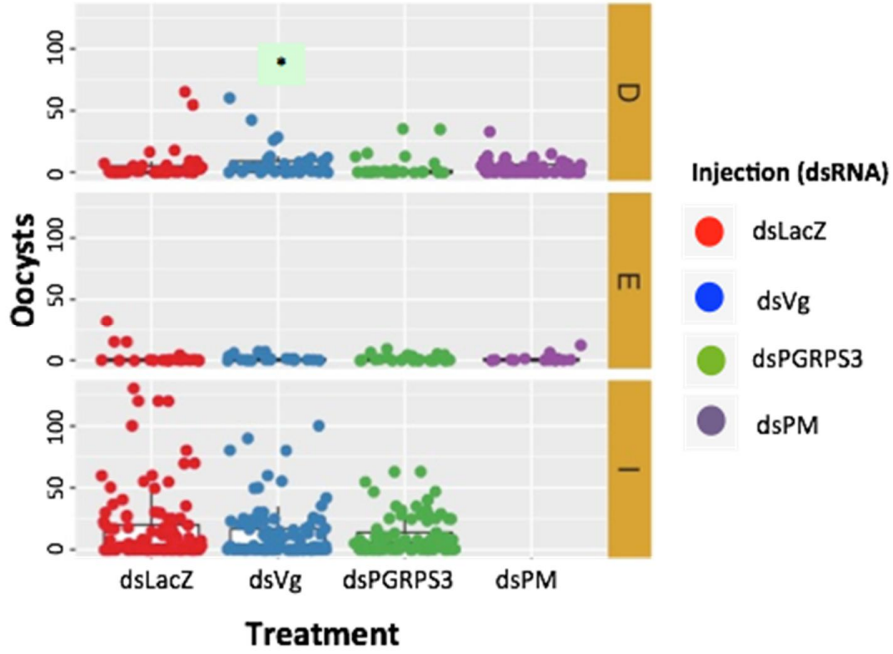

Figure 5.5 Impact on infection intensity of knocking down three different genes (dsVg, dsPGRPS3 and dsPM) using dsRNA injection in three different *P. falciparum* infection experiments: [D], [E] and [I]. Boxplots indicate the median and 25-75 percentiles.

Experi-ment	dsLacZ		dsVg		Chi square	dsPRGPS3		Chi square	dsPM		Chi square
	Infected (n)	Uninfected (n)	Infected (n)	Uninfected (n)	(p-value)	Infected (n)	Uninfected (n)	(p-value)	Infected (n)	Uninfected (n)	(p-value)
D	17	23	26	10	0.01	10	18	0.75	25	31	1
E	5	15	9	12	0.38	8	17	0.85	5	9	0.77
I	60	48	53	53	0.49	59	35	0.37	NA	NA	NA

Table 5.3 Impact of knocking down using dsRNA injection of three different genes (dsVg, dsPGRPS3 and dsPM) in three independent *P. falciparum* infection experiments: [D], [E] and [I] infection prevalence.

Mated females tend to have higher infection intensity compared to virgin females especially when the intensity is high. This observation is independent of microbiota in the midgut. This suggests that some other molecules are able to influence the increase in *P. falciparum* infection intensity. The hormone 20E is transferred in the mating plug to the female during mating (Pondeville et al., 2008) and it is related to the vector competency (Mitchell et al., 2015). Therefore, I next tested the hypothesis that 20E might influence the outcome of infection.

20E injection impact on *P. falciparum* infection

Virgin females injected with 20E to mimic mating have significantly higher infection intensity compared to virgin females injected with 10% EtOH as a control across 4 independent *P. falciparum* experiment (Figure 5.6). Two of these experiments [A] and [G] also showed a significant impact of 20E injection increasing prevalence (Wilcoxon Rank Test: **A: $p = 0.02$**, D: $p = 0.09$, **G: $p = 0.005$**, I: $p = 0.5$; Table 5.4). This suggests that 20E injection on virgin Ngousso increases the infection intensity. Mated females injected with 20E show no differences in infection intensity compared to mated females injected with 10%EtOH, suggesting that 20E may only be able to act once to increase susceptibility (Figure 5.6).

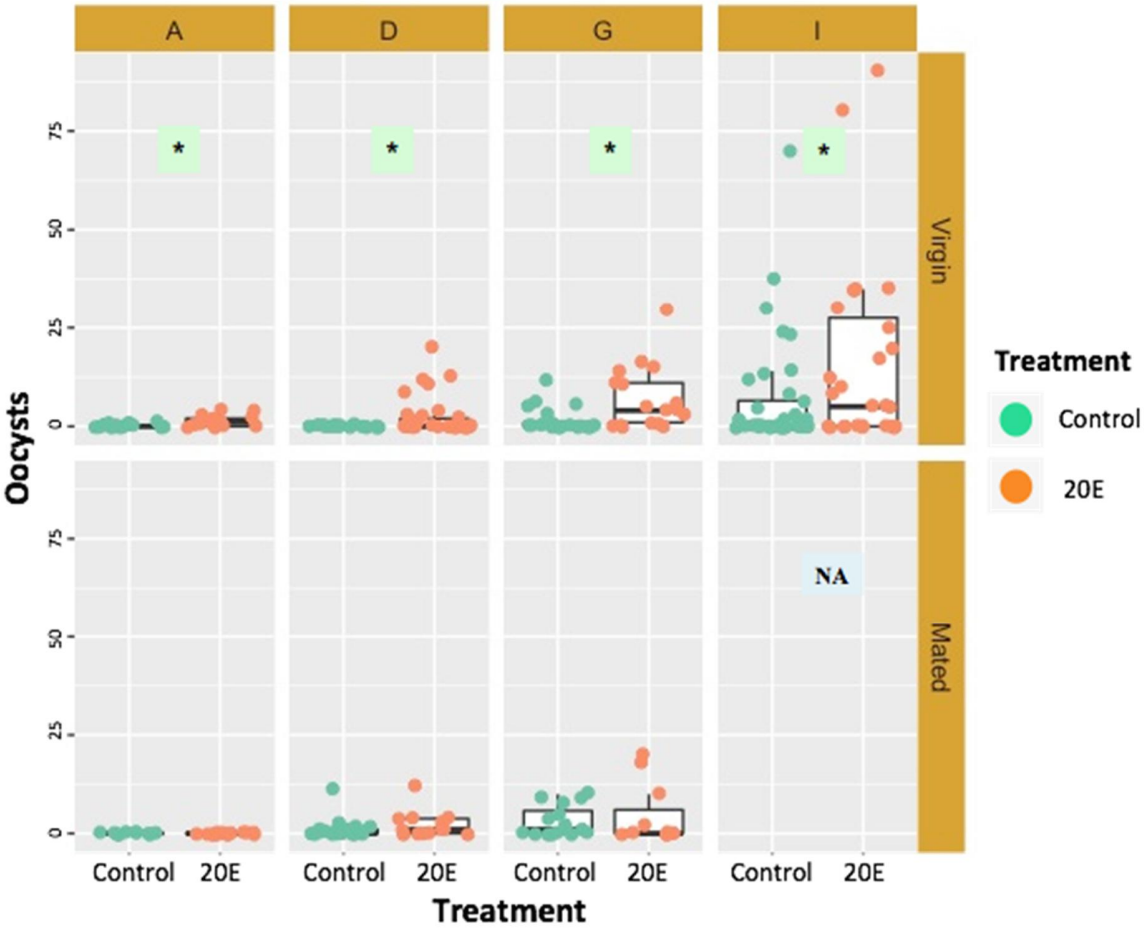

Figure 5.6 Each dot represents the oocyst count in a single midgut from 4 different *P. falciparum* infectious feeds where females were either virgin or mated, and either injected in the thorax with the control 10% EtOH carrier or with the hormone 20E in 10% EtOH. Boxplots indicate the median and 25-75 percentiles.

Experiment	Status	Control		20E		Chi square (p-value)
		Infected (n)	Uninfected (n)	Infected (n)	Uninfected (n)	
A	Virgin	3	15	9	5	**0.01***
A	Mated	0	10	0	13	NA
D	Virgin	1	18	10	25	0.09
D	Mated	7	15	8	6	0.24
G	Virgin	6	15	13	4	**0.009****
G	Mated	9	7	4	7	0.53
I	Virgin	16	20	16	11	0.36

Table 5.4 *P. falciparum* infection prevalence in virgin Ngousso injected with 20E and control; 10%EtOH from four different *P. falciparum*-infection experiments. Infection experiment was not repeated for mated females in experiment [I]. (* = $p < 0.05$, ** = $p < 0.005$)

Discussion

To explore the impact of mating status on parasite development, I fed laboratory reared mosquitoes via a membrane feeder on lab cultured *P. falciparum* NF54 gametocytes and evaluated their infection rates and oocyst counts across 11 independent experiments. Because the development of malaria parasites in *Anopheles* mosquitoes is also influenced by the microbiota present in the midguts of exposed females (Dong et al., 2009; Gendrin & Christophides, 2013), it is possible that mosquito mating status could influence malaria transmission via the impact on the microbiota. Indeed, in Chapter 3, I have shown that mating increases the midgut bacterial load and alters the bacterial composition so the possibility that mating influences the outcome of parasite infection via an impact on the microbiota is very realistic. It is also possible that the mating status of females could have an impact on parasite development independent of the impact on microbiota. My work in this chapter supports the idea that mating status influences the outcome of infection and that this is dependent to some extent on the intensity of the infection but potentially also the microbiota that are present.

The number of oocysts found in wild type female *Anopheles* mosquitoes caught in the field is usually low, around 1-10 oocysts per midgut (Muirhead-Thomson, 1954). Among my replicate feeds, I have experiments that resulted in infection intensities more typical of a natural infection and also several experiments that resulted in high infection intensities (>10 oocysts/gut). In general, mated females tend to show increased susceptibility to parasites compared to virgin females, but this is more consistently significant when the infection levels are high. The power to detect differences is increased when more individuals are infected or when they are infected more heavily, so this could be partly responsible for the difference. On the other hand, in the laboratory environment females do not have difficulties in finding food or mates as they might in a natural environment. Likewise, the colony mosquitoes are kept in a healthy state. This reduction of stress might enable mosquitoes to handle infections differently than they would under natural conditions. It is possible for example that the infection levels that are observed more typically in nature would result in different outcomes of infection between more stressed virgin and mated females. This remains to be tested.

It is also possible that the microbiota differ considerably between experiments and that this influences the outcome of infection and the impact of mating status. My attempts to understand this more conclusively suggest that the microbiota are likely contributing but also that, at least at high infection levels which is when I happened to test the impact of removing the microbiota, that these do not play a critical role. At high infection intensities, removing the microbes leads to the same result as when they are present, which is that mated females have significantly higher levels of infection. However, at lower infection intensities, when the outcomes are more variable, it may be that microbiota underlie this variation which was shown in Chapter 4.

To explore the impact of some of the genes that I detected as dramatically differentially expressed in Chapter 4, I generated dsRNA constructs to target these genes via RNA interference (RNAi). In three independent experiments (experiment [D], [E] and [I]), I did not detect an impact of gene knockdown on either infection intensity or infection prevalence (Figure 5.5 and Table 5.3). These three experiments were shown to have significantly higher infection intensity in mated females than virgins (Figure 5.1). However, their overall infection prevalences (40%, 48% and 80%, respectively), were lower than the experiment in which the differential expression

was originally identified. Perhaps if the dsRNA was injected in a highly infectious *P. falciparum* intensity and 100% infection prevalence experiment, we could possibly see the phenotypic differences driven by knocking down the genes. Alternatively, perhaps the microbiota were different between these experiments, and the genes that I targeted via dsRNA were responding more directly to the microbiota than to the mating status per se. Another possible reason is the knockdown efficiency. The knockdown efficiency shows that the knock down of these genes is not necessarily in the midgut. I injected dsRNA on mosquitoes and fed them on *P. falciparum* blood. For Trial 1, I fed them on naive blood, and in trial 2 I injected twice as much dsRNA as usual (6μg/μL) to get the knockdown in the midgut, but this still did not improve the knock down efficiency, so I didn't pursue further knockdown experiments.

To further investigate what the male might be transferring that could increase susceptibility to parasites among mated females, I injected 20E into both virgins and mated females and I show that 20E injection makes virgin females more susceptible to *P. falciparum* infection, and indeed brings them to a level of infection seen in mated females. However, enhanced susceptibility to *P. falciparum* was not found among already mated females that were then injected with additional 20E (Figure 5.6). These latter experimental females received 20E from mating, from blood feeding, and also from the injection of 20E yet did not show increased susceptibility compared to control injected mated females. This suggests that the additional amount of 20E does not make the female more or less susceptible to infection. Therefore I hypothesize that 20E transferred by males during mating acts to change the female mosquito midgut in ways that frequently make it more susceptible to parasite infection. Not much is known about 20E and *Anopheles*. However, it is possible that each male's ability to produce 20E and transfer to the female during mating is different. Perhaps in lower infection intensity experiments, the mated female received lower amounts of 20E from the male. This remains to be tested.

Another hypothesis is that perhaps 20E injection, which mimics mating, could remodel the midgut of the female adult as it does the atrium (Rogers et al., 2008). In *Drosophila,* mating has been shown to increase the size of the midgut which is modulated by JH. The genes which are involved in lipid metabolism were increased upon mating which then increases the egg production. These findings suggest that mating increases reproductive success in *Drosophila*

(Reiff et al., 2015). However, the impact of mating on other tissues in *Anopheles* especially the midgut is poorly understood. Perhaps upon mating, the 20E titre increases which then remodels the gut and prepares it for diverting resources to egg production. The midgut could then have more midgut cells potentially allowing more parasites to reside in the midgut and causing higher infection intensity. Otherwise, upon remodeling the gut for nutrient absorption for egg development, the parasites could benefit from a reduced immune response resulting from most resources shifting towards egg provisioning. If true, this could also result in a higher parasite count.

In *Drosophila*, starvation and 20E injection increase the titre of 20E which is able to induce apoptosis of ovarian follicles when nutrition is too low for oocyte development (Soller et al., 1999; Terashima et al., 2005). Perhaps in *Anopheles*, mated females (which have 20E induced upon blood feeding and mating) can maximize their fitness by investing in egg production rather than in clearing infection. This lower investment in immunity could result in higher infection intensities. However, when females receive or produce more 20E than needed, it could induce apoptosis in the ovary which now will direct the resources in immune defence rather than egg production. This is one hypothesis for why no differences in *P. falciparum* infection were observed between mated females injected with 20E and mated females injected with control.

Mimicking mating to disrupt the vector population could be an effective vector control strategy because of female monandry: if virgin females "think" they have been mated, this would effectively sterilize the population. Recently (Childs et al., 2016) the use of a 20E agonist Dibenzoylhydrazine (DBH) in the lab was able to block *P. falciparum* infection, prevent insemination, reduce egg laying and female lifespan. DBH mimics the action of 20E by competitively binding to the ecdysteroid receptor and enhancing ecdysteroid activity (Dhadialla et al., 1998). Whether DBH binding to ecdysteroid receptor is permanent or temporal is not known. Furthermore, DBH impact on egg laying is opposite of mating impact which would be an increase in egg production. This might increase the urge of females to take in blood again and possibly be able to remate if DBH effect is temporal. These events will increase 20E titre in the mosquitoes. Caution is urged as 20E injection shown to increase the infection intensity, there might be an impact of 20E activating pathway which would enhance malaria transmission.

CHAPTER 6

The impact of 20-hydroxyecdysone injection on the female midgut transcriptome

Introduction

Hormones such as 20-hydroxyecdysone (20E) and juvenile hormone (JH) play an important role in coordinating development, growth, reproduction and aging in insects (Hagedorn et al., 1975; Li et al., 2000; Shapiro & Hagedorn, 1982). In adult *An. gambiae*, 20E is produced in the ovary and secreted to the fat body to function (Pondeville et al., 2008). Blood feeding has an impact on ecdysteroid levels in females: upon blood feeding, ovarian follicle cells release ecdysteroids into the hemolymph which are hydroxylated into 20E, an important hormone for oogenesis (Pondeville et al., 2008). Virgin females are able to perform oogenesis if they have a bloodmeal but they will not typically lay their eggs until they mate (Gabrieli et al., 2014; Klowden & Russell, 2004).

Microarray data on whole sugar fed females showed at 2, 6 and 24 hours post mating, in total 141 genes were regulated (Rogers et al., 2008). 20E injection has been shown to mimic mating (Baldini et al., 2013). Injection of 20E on virgin *Anopheles* shows similarity between the LRT with mated females(Gabrieli et al., 2014) refracts females from further inseminations (Gabrieli et al., 2014). Some of the genes that were upregulated in the LRT are genes that involved in cytoskeleton or musculature-associated genes which may trigger remodelling of the atrium and makes the mated female tissue refractory from further insemination by males (Gabrieli et al., 2014). Furthermore, it also increases egg laying upon blood feeding and thus mimics the impact of mating (Gabrieli et al., 2014). To further investigate whether 20E injection mimics mating at a transcriptional level in the lower reproductive tract (LRT), a previous study identified 628 genes that were regulated upon mating in the LRT and upon 20E injection in virgins, 459 out of these 628 genes were also found to be regulated in the (LRT) (Gabrieli et al., 2014). This suggests that much of the transcriptional change that occur upon mating may be driven by 20E in particular,

and this is consistent with the phenotypic changes of induced egg laying and increased refractoriness to remating being driven by this hormone.

The use of 20E agonists as a vector control strategy has been proposed: females exposed to the 20E agonist will behave as if mated, so although they will blood feed and lay eggs, those eggs will be unfertilised because the females would not have mated. I showed in Chapter 5 that 20E injection in virgin *An. coluzzii* increases *P. falciparum* infection intensity compared to control-injected virgins. Given this result, I next wanted to examine how 20E may be influencing the gut environment where infection with parasites occurs. The impact of mating or 20E has been reported on whole mosquitoes (Rogers et al., 2008) and in the LRT of *An. gambiae* (Baldini et al., 2013) but never on the midgut tissue. Very little is known about the transcriptome in the midgut upon mating or 20E injection, and an examination of transcriptional changes in this tissue might help reveal the underlying cause of the frequently observed increase in susceptibility to *P. falciparum* infection among mated females and among 20E injected virgin females. One hypothesis is that 20E injection might have transformed the midgut transcriptome to be more similar to mated females' midguts, as has been observed for the LRT (Gabrieli et al., 2014). To further investigate impact of mating and 20E injection on the midgut transcriptome, I carried out RNASeq on 20E injected and control injected virgin Ngousso midguts, both sugar fed and blood fed with *P. falciparum* infectious blood (experiment [I]). Alongside, I also examined mated and virgin female midgut transcriptomes in order to understand if the impact of 20E resembles the impact of mating.

Materials and Method

Mosquito preparation

Ngousso rearing, collection and separation of Ngousso pupae and Ngousso adult mating were performed as described in earlier chapters. See Appendix S1 and Appendix S2 for more information. Two groups of virgin and mated females were prepared. One group is without injection and another group is injected.

20-Hydroxyecdysone Injection

Experimental females were injected with 2.5µg 20-hydroxyecdysone (H5142 Sigma) diluted in 10% ethanol in the thorax (as described in (Baldini et al., 2013)) and control females were injected with 10% ethanol, the carrier used for 20E.

P. falciparum Infection (Membrane Feeding Assay)

Cotton was removed from mosquito cages six hours before blood feeding. 15 and 18 day old *P. falciparum* gametocyte cultures were pooled and spun down at 38°C, 2000rpm, for 5 minutes. Supernatant was removed and 1mL serum was added into the tube and topped up with fresh washed O+ blood to obtain a haematocrit of 45%. The mixture was always kept on a heat block (38°C) during the process to avoid inducing activation. The mixture was then added to a glass membrane feeder using a blunt syringe. Each cup of mosquitoes fed for 7 minutes and sugar-soaked cotton was placed on each cup after feeding was completed. Cups were kept in a secure container in an incubator at 26°C and 80% humidity. 24 hours after feeding, unfed females were removed. Some females were put aside for midgut dissection and scoring mating status, and leftover fed females were retained to assess infection rates at day 10 post blood-fed (Chapter 5). Sugar-soaked cotton was changed every two days with normal sugar for all mosquitoes.

50 sugar fed and 50 bloodfed females from each of 4 treatments (virgin, mated, virgin injected with 20E, and virgin injected with 10%EtOH) were kept for 24 hours after the bloodmeal. Twenty-four hours post bloodfeed, midguts were dissected and 6 replicate pools of 7 midguts

from each sugarfed treatment were pooled together, whereas blood fed midguts were collected individually, also in 6 replicates. These samples are described in Figure 6.1.

Molecular Biology Method

RNA Extraction

Total RNA was extracted using TRIzol (Life Technologies) and chloroform (Sigma). Each biological condition was represented by six replicates each for single midgut for all blood fed mosquitoes and 7 pooled midguts of sugar fed mosquitoes. The midguts were homogenised using Precellys 24 homogenizer at maximum speed, 6,800rpm for 30 seconds and let to rest for 5 minutes at room temperature. Samples were then centrifuged at 13,200 rpm for 15 minutes at 4°C. The aqueous phase was collected into a tube that was filled with 250µL isopropanol and 1µg glycogen to precipitate the RNA. Samples were centrifuged at 13,200rpm for 15 minutes at 4°C followed by washing using 70% ethanol (Sigma) and concentrated using the DNA concentrator for 10 minutes. 10µL molecular biology grade water was added in each tube and was placed on a heat block (37°C) for 15 minutes. Total RNA was measured using the Nanodrop.

RNA Sequencing

Subsets of mosquitoes as described above were kept aside specifically for RNA sequencing (RNASeq; Figure 6.1). The details are summarized in Appendix S1 and Appendix S2. At 24 hours post blood fed, mosquito guts were dissected in RNAlater. Individual guts were placed in PCR tubes on dry ice as described above and RNA extraction was carried out as described above, on single or pooled guts depending on the experiment. These samples were assessed for RNA quality and quantity using the Agilent Bioanalyzer. Briefly, RNA samples were denatured by incubating at 70°C for 2 minutes and then placed on ice. 9µL of gel-dye mix was pipetted into bottom of nanochip. 1µL of samples, 1µL of RNA 6000 Nano Marker and 1µL of RNA 6000 Ladder were pipetted in assigned well, vortex and run using Eukaryote Total RNA Nano Series II programme. Total RNA was diluted to concentration of 500ng in 50µL and was sent to the Wellcome Trust Sanger Institute for RNA library preparation and sequencing.

The kit used for library prep was Illumina TruSeq Stranded mRNA Library Prep Kit. mRNA was purified from total RNA using an oligo dT magnetic bead pull-down. A random-primed cDNA library was synthesized. During second strand synthesis dUTP was incorporated in place of dTTP. The incorporation of dUTP quenches the second strand during amplification because the polymerase does not incorporate past this nucleotide resulting in a strand specific library. Ends were repaired with a combination of fill-in reactions and exonuclease activity to produce blunt ends. A-tailing was performed, whereby an "A" base was added to the blunt ends. Illumina paired-end sequencing adapters containing unique index sequences, allowing samples to be pooled, were ligated. The libraries then went through 10 cycles of PCR amplification using KAPA Hifi Hot Start Polymerase rather than the kit-supplied Illumina PCR Polymerase due to better performance (especially with AT rich DNA). Libraries were quantified and pooled based on a post-PCR Agilent Bioanalyzer. Sequencing was done on the HiSeq v4, 75bp paired end reads, and the data was analysed using Illumina RTA software version 1.18.61. Automatic and manual quality control (QC) was performed and then the data was archived in iRODS as CRAM files. Data mapping to reference genome was done using TopHat2 (v.2.0.9) (Kim et al., 2013) which makes use of the aligner Bowtie2 (v.2.1.0) (Langmead & Salzberg, 2012), to the *An. gambiae* PEST genome (AgamP4) obtained from VectorBase.

Data received were run and mapped to reference genome of *An. gambiae* which was obtained from VectorBase. HTSeq (v.0.6.1) (Anders et al., 2015) was used to count transcripts for each gene. Differentially expressed genes were determined using DESeq2 (v.1.8.2) (Anders et al., 2015; Love et al., 2014) in R (v.3.2.5) (R Core Team 2014). Functional interpretation of each gene set was performed by doing Gene Ontology analysis using web server, TopGO (Alexa & Rahnenführer, 2009; Alexa et al., 2006). Functional analysis of 3 biological process, 3 cellular compartment and 3 molecular function were reported in this chapter. The top 10 significant functional analysis for each biological process, cellular compartment and molecular function were extracted and available in Appendix S5.

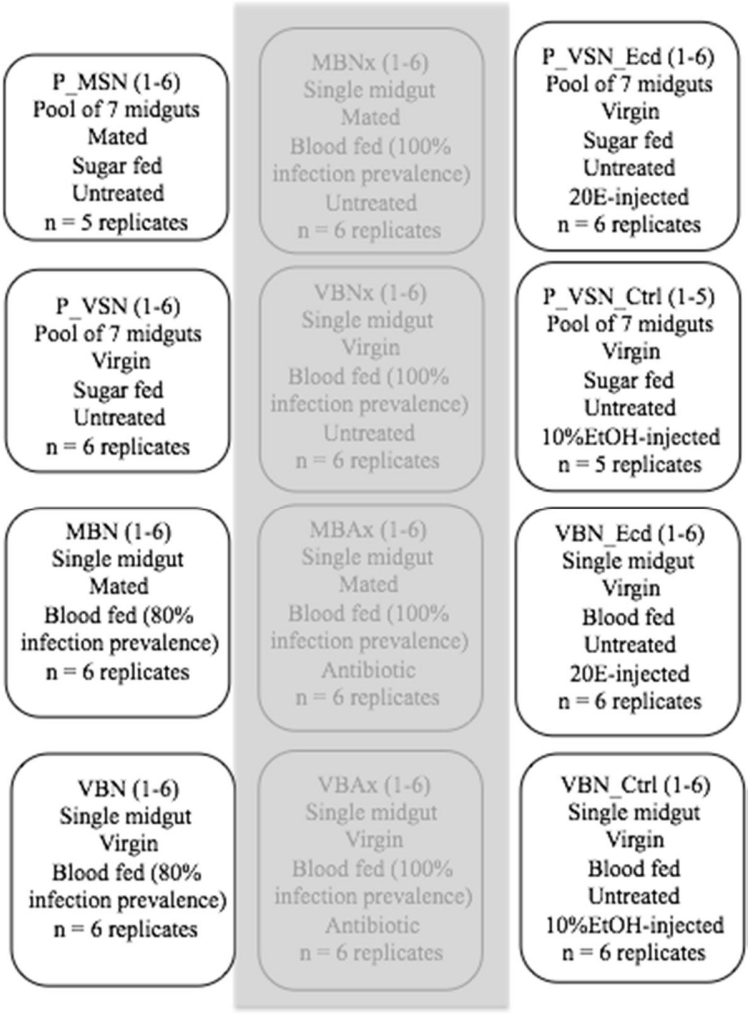

Figure 6.1 Overview of 46 samples of midguts used for RNASeq analysis in this chapter. Midguts were dissected at 24 hours post blood feed from experiment [I] (80% *P. falciparum* infection prevalence). RNA was extracted from each midgut and used for RNASeq experiment in this chapter. (P= pooled of 7 midguts, M= mated, V= virgin, B= blood fed, S= sugar fed, N= untreated, Ecd= ecdysone/20E injected, Ctrl= control, 10%EtOH injected). The greyed out samples were already discussed in chapter 4 and are not referred to in this chapter.

Results

The PCA on sugar fed samples showed a clear separation between 20E injected virgin midguts (n=6 pools of 7 midguts) vs control injected virgin midguts (n=6 pools of 7 midguts) (Figure 6.2a). Around 1076 genes were found to be significantly differentially expressed (*padj* < 0.05) between these treatments. There are 445 genes were upregulated upon 20E injection and the rest were down regulated compared to the control Among these, 79 genes overlap with the genes which were significantly differentially expressed due to mating in sugar fed Ngousso midgut which was discussed in Chapter 4. In general, 20E injection induced activity involving ATP hydrolysis coupled protein transport, process that modulated the frequency, rate or extent of retrograde vesicle-mediated transport from golgi to endoplasmic reticulum and ATP synthesis coupled proton transport activity (Table 6.1a). Whilst genes that were down regulated upon 20E injection are mainly involved in protein phosphorylation activity, regulation of transcription process and transmembrane receptor protein tyrosine kinase activity (Table 6.1b). These findings suggest that there is an impact of 20E injection as compared to 10% EtOH in the midgut. The top 10 most significant functional categories for biological process (BP), cellular compartment (CC) and molecular function (MF) of both upregulated and downregulated genes in response to 20E injection in the midgut can be found in Appendix S5.

(a)

GO.ID	Term	Annotated	Significant	Expected	weight01
GO:0015991	ATP hydrolysis coupled proton transport	22	7	0.85	1.2e-05
GO:0006890	retrograde vesicle-mediated transport, G...	11	5	0.43	3.2e-05
GO:0015986	ATP synthesis coupled proton transport	21	6	0.81	0.00010

(b)

GO.ID	Term	Annotated	Significant	Expected	weight01
GO:0006468	protein phosphorylation	263	44	15.91	2.6e-07
GO:0006355	regulation of transcription, DNA-templat..	532	64	32.18	9.3e-06
GO:0007169	transmembrane receptor protein tyrosine ...	32	9	1.94	0.00013

Table 6.1 Functional enrichment analysis on genes that were significantly (a) upregulated and (b) downregulated upon 20E injection in sugar fed virgin midguts.

Similar to the PCA on blood fed midguts shown in Chapter 4, the PCA on bloodfed virgin samples injected with 20E or ethanol do not show as clear a separation as compared to the sugar fed samples (Figure 6.2b). Only nine genes are detected as differentially expressed between bloodfed female Ngousso virgins that were injected with 20E (n=6 single midguts) or injected with 10%EtOH (n=6 single midguts).

(a) Sugar fed (b) Blood fed

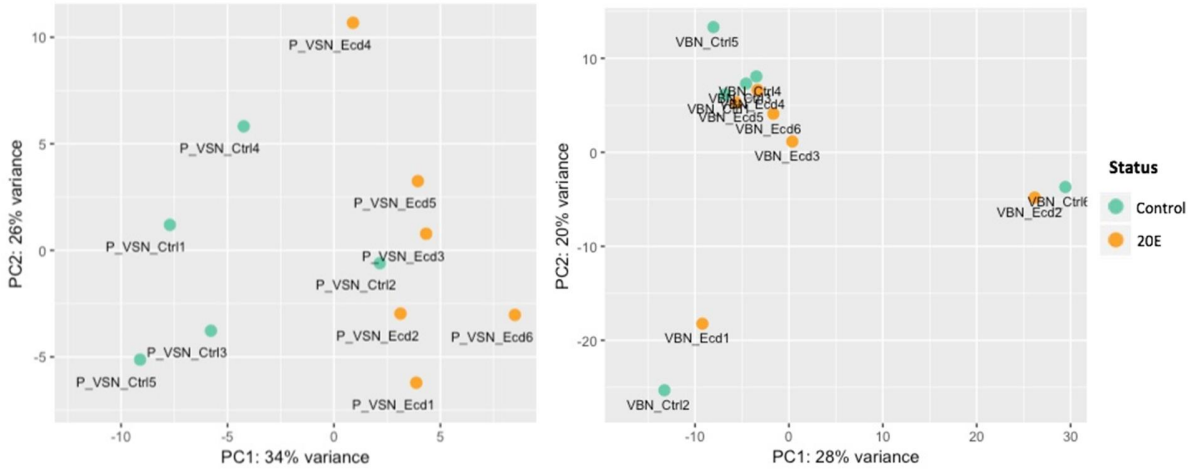

Figure 6.2 Principal Components Analyses on RNAseq data for virgin females injected with (a) 20E [Ecd] and control [Ctrl] female midguts fed only on sugar [S] and untreated with antibiotics [N] with each dot comprising data from a pool [P_] of 7 midguts, and (b) single midguts virgin females that were injected with 20E and 10%EtOH, bloodfed [B] on *P. falciparum* infective blood, and untreated [N] with antibiotics. These samples came from experiment [I] that achieved 80% prevalence after *P. falciparum* infection.

Two genes were found to be differentially expressed in both sugar and bloodfed guts, including AGAP007567, a membrane bound O-acyl transferase which is an enzyme involved in lipid biosynthesis, phospholipid remodelling and protein/peptide acylation. The other gene, AGAP001321, has no known or predicted function.

In Chapter 5, 20E injection into virgin females resulted in a significant increase in *P. falciparum* infection intensity. I hypothesized the 20E injection could have transformed the midgut to mimic a mated female midgut, and thus enhanced susceptibility. In comparing the similarity of virgin and mated sugarfed guts to virgin 20E and virgin ethanol injected guts (Figure 6.3a), while it is clear virgin sugar fed guts are distinct from mated sugarfed guts, and 20E injected guts are distinct from ethanol injected guts, the overlap between the 20E injected samples is not with the mated guts, as predicted, but with the virgin guts. This result suggests that 20E injected transcriptome in the midgut might not mimic the impact on the mated midgut.

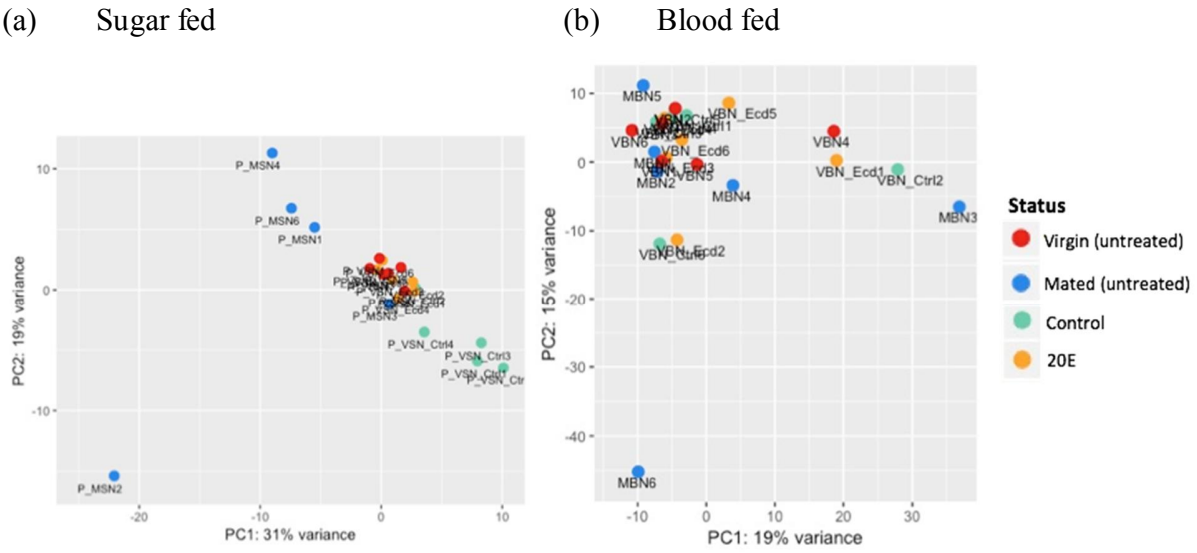

Figure 6.3 Principal Component Analyses on RNAseq data for virgin females injected with (a) 20E [Ecd], control [Ctrl] females, uninjected mated females and virgin females midguts fed only on sugar [S] and untreated with antibiotics [N] with each dot comprising data from a pool [P_] of 7 midguts. (b) single midguts virgin females that were injected with 20E and 10%EtOH, uninjected mated females and virgin females bloodfed [B] on *P. falciparum* infective blood, and untreated [N] with antibiotics. These samples came from experiment [I] that achieved 80% prevalence after *P. falciparum* infection.

To further explore this, I compared the significantly differentially expressed genes detected in the 20E and 10% EtOH experiment with those detected as differentially expressed due to mating in sugarfed guts as described in Chapter 4. Of the 445 genes upregulated by 20E, and 312 genes upregulated by mating, 53 genes were found in both analyses (Figure 6.4). Functional enrichment analysis of the common genes upregulated by 20E injection and mating shows that these genes are mainly involved in biosynthetic process, tRNA aminoacylation for protein translation and peptide catabolic process. The majority of the genes are predicted to function in the cytoplasm, some in the endoplasmic reticulum membrane and rough endoplasmic reticulum. The top 3 molecular functions are metalloaminopeptidase activity, aminoacyl-tRNA ligase activity, and peptide binding (Table 6.2). This suggests that, perhaps the common function between 20E and mating are in synthesizing some biological compound, producing protein and breaking down peptides in order to facilitate egg production or perhaps remodelling the midgut.

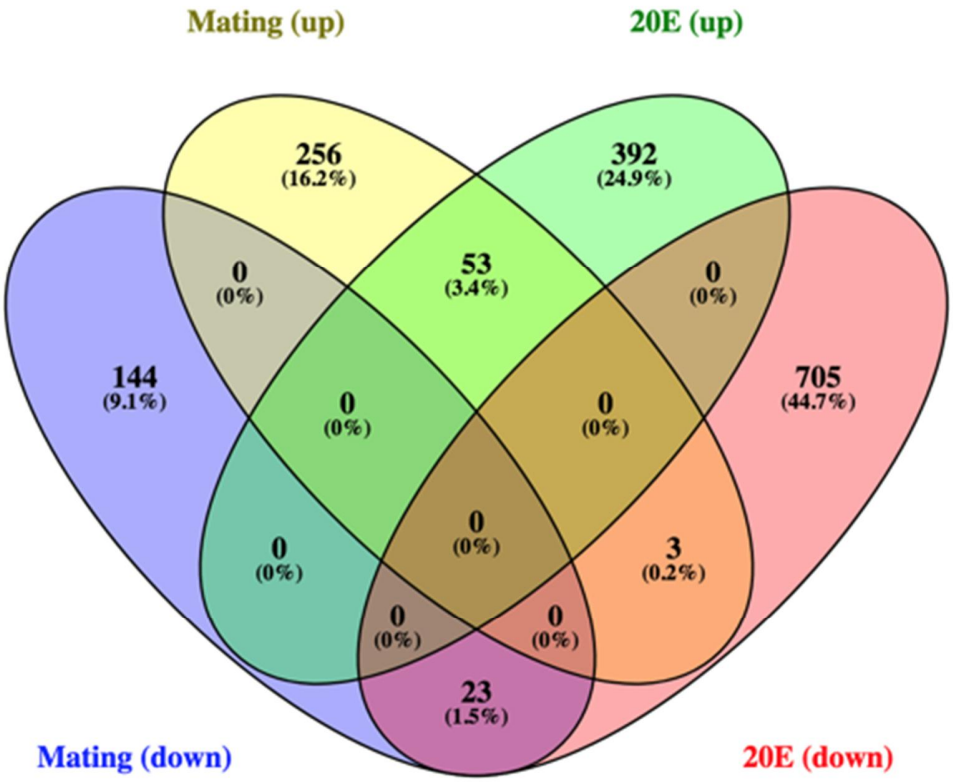

Figure 6.4 Venn Diagram on genes that were up-regulated (up) and down-regulated (down) by mating status (Mating) and 20E injection (20E) in sugar fed midgut.

(a)

GO.ID	Term	Annotated	Significant	Expected	weight01
GO:0043171	peptide catabolic process	29	4	0.17	2.4e-05
GO:0006418	tRNA aminoacylation for protein translat...	44	3	0.26	0.0022
GO:0009058	biosynthetic process	1484	13	8.93	0.0076

(b)

GO.ID	Term	Annotated	Significant	Expected	weight01
GO:0005791	rough endoplasmic reticulum	5	2	0.03	0.0003
GO:0005737	cytoplasm	1714	18	9.62	0.0011
GO:0005789	endoplasmic reticulum membrane	94	3	0.53	0.0153

(c)

GO.ID	Term	Annotated	Significant	Expected	weight01
GO:0070006	metalloaminopeptidase activity	26	3	0.11	0.00017
GO:0004812	aminoacyl-tRNA ligase activity	43	3	0.18	0.00078
GO:0042277	peptide binding	50	3	0.21	0.00122

Table 6.2 Functional enrichment analysis on 53 genes found to be significantly upregulated in response to 20E injection (20E vs control) and mating status (mated vs virgin) in sugar fed midgut samples according to (a) biological process [BP], (b) cellular compartment [CC] and (c) molecular function [MF].

Surprisingly, although the PCA plot showed that 20E and virgin cluster are close to each other, there are no genes which were found to overlap between genes upregulated by 20E injection and genes upregulated in virgin as compared to mated. However, among 731 genes downregulated by 20E injection and 167 genes downregulated by mating, there were 23 genes overlapping between these analyses (Figure 6.4). Functional enrichment analysis on the genes significantly downregulated by 20E injection and mating are involved in biological processes such as digestion, phosphatidylglycerol metabolism and negative regulation of ribosomal large subunit export from nucleus. These overlapping genes are mainly functional in preribosome, a small

subunit precursor, in the nucleolus and cytoplasmic exosome. The top 3 molecular functions are 3'-5'-exoribonuclease activity, prenyltransferase activity and protein kinase binding (Table 6.3). This finding suggests that the function of commonly found genes between virgin and control are involved in breaking down nectar compound in the midgut. One hypothesis could be that these genes are involved in maintaining the homeostasis in the midgut.

(a)

GO.ID	Term	Annotated	Significant	Expected	weight01
GO:0007586	digestion	10	2	0.03	0.00032
GO:0046471	phosphatidylglycerol metabolic process	5	1	0.01	0.01375
GO:0000055	ribosomal large subunit export from nucl...	5	1	0.01	0.01375

(b)

GO.ID	Term	Annotated	Significant	Expected	weight01
GO:0030688	preribosome, small subunit precursor	5	1	0.01	0.014
GO:0005730	nucleolus	87	2	0.25	0.025
GO:0000177	cytoplasmic exosome (RNase complex)	9	1	0.03	0.026

(c)

GO.ID	Term	Annotated	Significant	Expected	weight01
GO:0000175	3'-5'-exoribonuclease activity	7	1	0.02	0.019
GO:0004659	prenyltransferase activity	8	1	0.02	0.021
GO:0019901	protein kinase binding	12	1	0.03	0.032

Table 6.3 Functional enrichment analysis on 23 genes found to be significantly downregulated in response to 20E injection (20E vs control) and mating status (mated vs virgin) in sugar fed midgut samples according to (a) biological process [BP], (b) cellular compartment [CC] and (c) molecular function [MF].

In sugar fed 20E injected midguts, 53 genes were found to be in common with mating impact. Whilst in the LRT, 435 upregulated genes upon 20E injection are in common with mating impact (Gabrieli et al., 2014). Venn Diagram shows only 3 genes overlap between the midgut and LRT mating regulated genes: AGAP008265, AGAP001874 and AGAP007088 which are involved in protein translation, signal transduction and protein folding. This finding suggests that the remaining 50 genes might be specifically expressed in the midgut.

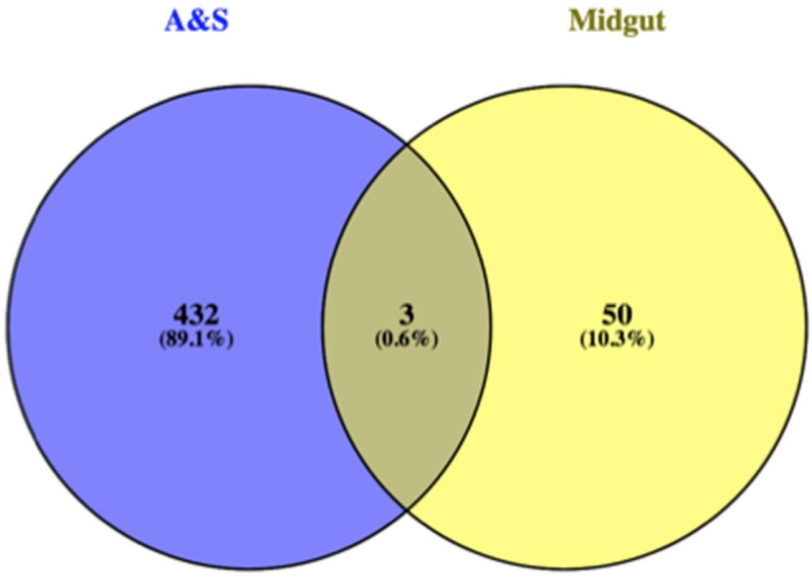

Figure 6.5 Overlap of genes which were up regulated and commonly found between 20E injection and mating in the midgut (yellow), from my RNASeq experiment and in the LRT which consist of atrium and spermathecae (A&S). The gene list for common genes between 20E and mating in LRT is from Gabrieli et al. 2014.

Microbiota has been shown to have an impact on *P. falciparum* susceptibility (Bahia et al., 2014; Dong et al., 2009; Gendrin & Christophides, 2013). As previously described in Chapter 3, mating status has an impact in increasing bacterial loads in the gut which was confirmed by qPCR and Kraken analysis. Since the RNAseq data contains information on the abundance and composition of bacteria present in these midguts. Therefore, I used the Kraken analysis to explore whether 20E injected midguts resembled mated midguts in bacterial abundance and/or composition. In sugar fed females, 20E injected midguts actually have significantly lower bacterial loads than control injected midguts (ANOVA : n=12, p = 2.727 e-05, Figure 6.6a, left panel). This is the opposite of what would be expected based on patterns in sugarfed virgin and mated midguts, which shows a significantly higher bacterial load in mated midguts as previously described (Figure 6.6b, left panel). Upon blood feeding, bacterial loads are significantly higher in 20E injected compared to control injected midguts, consistent with what has been observed in bloodfed midguts (ANOVA : n=12, p = 0.05, Figure 6.6a, right panel). It is unclear why the overall bacterial levels don't appear much higher in bloodfed midguts than sugar fed midguts, but this may be in part driven by improved detection of bacteria in the sugar fed samples due to the fact that 7 midguts were pooled together. Bacterial levels in the sugar fed midguts of normal mated and virgin females are, as reported before (using qPCR data on different samples) borderline significantly higher in mated than virgin midguts (ANOVA : n=12, p = 0.08, Figure 6.6b left panel). However, upon blood feeding, the bacterial loads seems to be similar between mated and virgin midguts (Figure 6.6b right panel) and this is very different than what was previously observed in Experiment [K] and reported in Chapter 3. Perhaps different bacteria were present in the midguts of these mosquitoes. I used Kraken to examine this question.

(a) 20E injection impact (b) Mating Impact

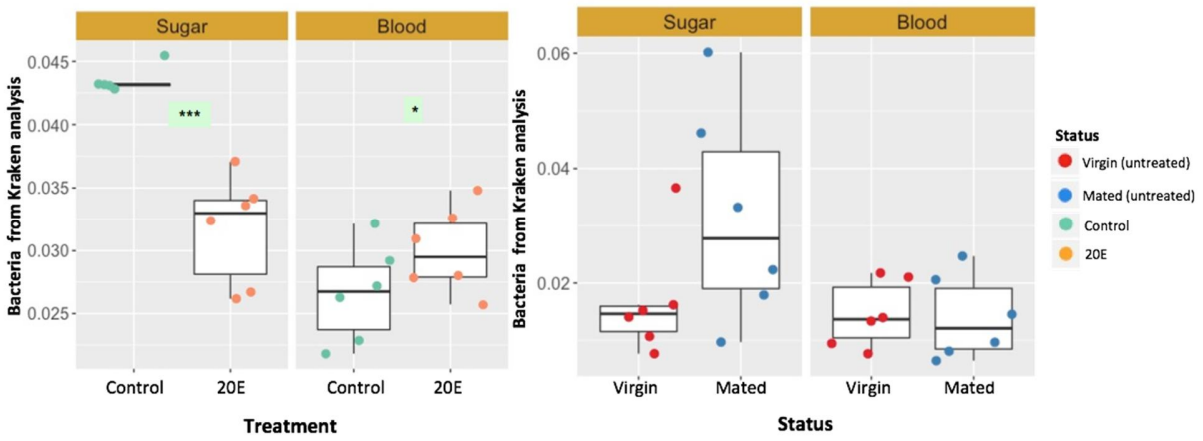

Figure 6.6 Overall Kraken analysis on total bacteria on (a) 20E and control injected and (b) virgin and mated uninjected midguts. Each dot in the sugar fed column represents RNA extracted from a pool of 7 midguts. Each dot in the blood fed column represents RNA extracted from single midgut from a *P. falciparum*-infection experiment with 80% prevalence. Boxplots indicate the median and 25-75 percentiles. (*** = p-value < 0.005, * = p-value <0.05)

First, I looked at the specific bacterial loads that I have qPCR data on from separate samples discussed in Chapter 3. Very few bacteria showed different abundances depending on 20E injection or mating status. Among sugar fed midguts, only *Acetobacteraceae* was significantly higher in 20E (ANOVA : n=12, p = 0.005; Figure 6.7a), and only *Flavobacteriaceae* was significantly higher in mated midguts (ANOVA : n=12, p = 0.04; Figure 6.7b). This is consistent with what was observed in Chapter 3 (Figure 3.5) where *Flavobacteriaceae* was significantly different between mated and virgin and although for *Acetobacteraceae* the difference is not significant, but the trend shows to be higher in mated female. These suggests this two groups of bacteria may frequently be increased in mated females. *Flavobacteriaceae* is not found in any of the injected midguts, 20E and control in both sugar and blood fed midguts. Among bloodfed midguts, only *Klebsiella* was responsive to mating and 20E injection, but it was responsive in opposite directions, being significantly higher in 20E injected as compared to control injected guts, but significantly lower in mated as compared to virgin guts (ANOVA : n=24, p = 0.04; p = 0.01; Figure 6.7 a & b).

(a) 20E injection impact (b) Mating impact

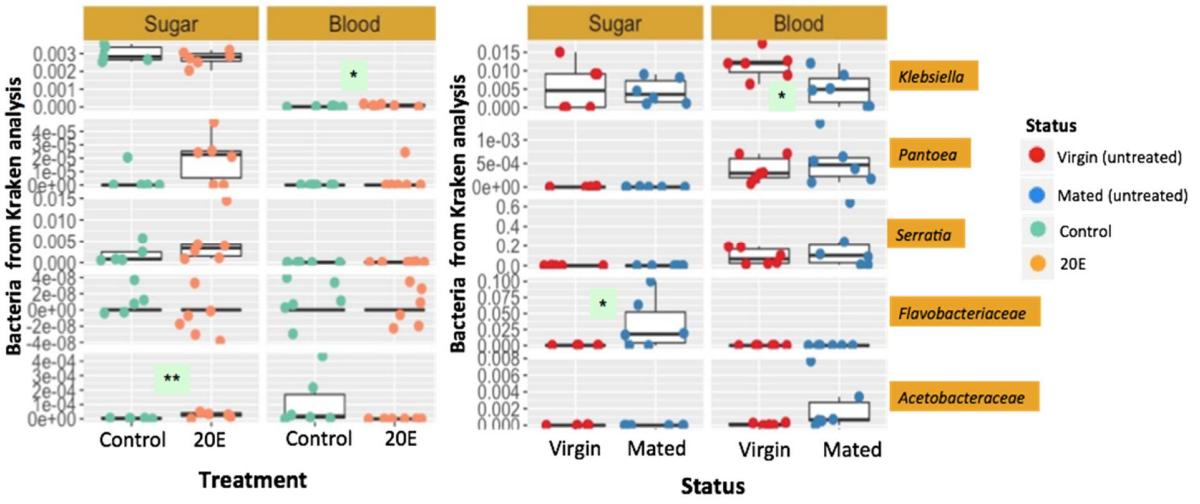

Figure 6.7 Overall Kraken analysis on (a) bacterial loads of *Klebsiella, Pantoea* and *Serratia* on 20E and control injected midguts. (b) bacterial loads on *Klebsiella, Pantoea, Serratia* and *Flavobacteriaceae* on uninjected mated and virgin midguts. Each dot in the sugar fed column represents RNA extracted from pool of 7 midguts. Each dot in blood fed column represents RNA extracted from single midgut from a *P. falciparum*-infection experiment [I] with 80% prevalence. Boxplots indicate the median and 25-75 percentiles. (* = *p*-value <0.05).

I also used Kraken to look at relative abundances of the bacteria detected in the RNAseq data. As seen in Chapter 3, *Yersinia* is found in both sugar and bloodfed virgins, but not in injected females (Figure 6.8). In sugar fed female midguts, *Klebsiella* is dominant, especially among mated females. Whereas in 20E and control injected midguts, *Serratia* and *Klebsiella* are dominant (Figure 6.8a). Upon blood feeding, all four treatment show *Serratia* bacterial abundance seems to be dominant. In blood fed injected 20E and control midguts, some other bacteria compositions were spotted, unlike in sugar fed samples, such as *Achromobacter, Streptococcus, Pantoea* (Figure 6.7b). This suggests that these bacteria proliferate more upon blood feeding.

(a) Sugarfed (b) Bloodfed

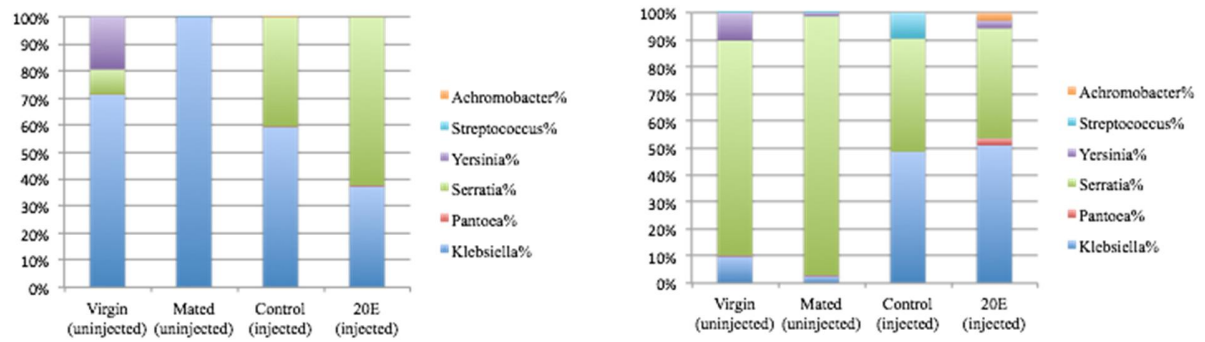

Figure 6.8 Bacterial composition over total percentage in midguts obtained using the Kraken analysis which shows the bacterial composition load of some bacterial groups including *Achromobacter, Streptococcus, Yersinia, Serratia, Pantoea* and *Klebsiella* in virgin and mated (uninjected) and 20E and control injected Ngousso midguts (a) sugar fed (b) given *P. falciparum* infectious blood (experiment [I]). All of these midguts are from the same batch of mosquitoes.

Discussion

My RNASeq analysis on sugar fed females shows that 20E injection has an impact on the midgut transcriptome of injected virgin females. My 20E injection was done 2 or 3 days prior to feeding on *P. falciparum* blood. This is because the injected females would take some time to recover before they are able to blood feed properly. Furthermore, the injection was done early in the morning to mimic mated females timing (that were given males the night before would have mated). Dissections were done 24 hours post blood feed, which will be 3 or 4 days post injection. 20E injection was reported previously to mimic the impact of mating on the female *An. gambiae* LRT transcriptome using microarray method (Gabrieli et al., 2014). The virgin females were injected at 3 days old and dissected at 1 day post injection. The genes that were upregulated upon 20E injection are involved in remodelling the atrium which prevent the female from further insemination (Gabrieli et al., 2014).

However, although 20E injection mimics mating in the LRT of the females (Gabrieli et al., 2014), the pattern is not as clear for the midgut. Only 53 genes out of 445 genes which were upregulated by 20E injection are common with mating. From these 53 genes, 3 genes were also found to be upregulated in the LRT as reported by Gabrieli et al. 2014. This finding suggests that the remaining 50 genes might be specifically activated in the midgut upon 20E injection. Although the biological process is poorly understood, the midgut function or contribution upon 20E and mating to reproduction remains an interesting area to be tested.

The impact of male-derived 20E might also be influenced by the microbiota in the midgut. The kraken analysis in this chapter was done using samples which were fed on *P. falciparum* infected blood that resulted in 80% infection prevalence (Figure 6.1). In sugar fed midguts, control injected females had significantly higher bacterial loads compared to 20E injected midguts. This could be due to the immune response being activated upon 20E injection which could have reduced the bacterial abundance (Upton et al., 2015). In the silkworm *Bombyx mori,* 20E injection into larvae induced changes in the number of cultivable bacteria, DNA synthesis, and gene expression in the insect midgut, all of which were similar to the changes that occurred during the larva-to-larva molting stage (Yang et al., 2016). Additionally, the increased 20E titer

in the hemolymph likely induced the noted physiological changes within the midgut (Yang et al., 2016). Perhaps 20E injection reduced the bacterial loads, similar to using antibiotics in clearing bacteria abundance in the midguts. This could possibly lead to an increase in *P. falciparum* susceptibility as found in Chapter 5.

In some cases, detection of *P. falciparum* RNA by qPCR in the same sample used for RNASeq would allow the identification of single midgut that might have been infected and responsible for variance in the data (Figure 6.2b). However, there is no leftover RNA to be used to answer this question hence this is one of the limitation of the study. Upon blood feeding, the bacterial loads in 20E injected females increases and are significantly higher than found in control injected blood fed females. 20E injection followed by blood feeding could have activated oogenesis in the female. Bacteria has been shown to proliferate massively upon blood feeding. This result suggests that perhaps 20E injection has an impact in a trade off with immunity where it could have channeled their energy in producing eggs which resulted in higher bacterial loads.

Microbiota loads of mated and virgin midguts from this (experiment [I] with 80% *P. falciparum* infection prevalence) are clearly different compared to microbiota loads from experiment K (100% *P. falciparum* infection prevalence). In experiment [K] (discussed in Chapter 3, Figure 3.3b), the mated midguts clearly have much higher bacterial loads than virgin midguts. This impact was confirmed using qPCR, Kraken analysis and MiSeq. Unfortunately, I did not examine sugarfed virgin and mated females in that experiment. There are some similarity between experiment [I] and [K] where the trend of these bacteria are higher in mated female midguts; *Pantoea, Serratia* and *Acetobacteraceae* whilst *Klebsiella* is higher in virgin. This suggests that there might be some other bacterial species that could have driven the result observed in the samples from experiment [I] which could have an impact on the infection prevalence as well.

Overall, 20E injection on virgin female has an impact on the midgut transcriptome. Although the impact does not entirely mimic mating, 20E injection which remodelled the female atrium (Gabrieli et al., 2014) could have an impact on the midgut which needs further exploration. Furthermore, 20E injection is able to alter the bacterial abundance and composition suggests that

it might be involved in a trade off with immunity which might also affect the female susceptibility of *P. falciparum.*

CONCLUSIONS

In mosquitoes, a mating plug is found exclusively in some *Anopheles* species (Baldini et al., 2012; Mitchell et al., 2015). The exclusivity of the mating plug in *Anopheles* suggest the importance of it in reproduction and also the probability of its function in *P. falciparum* infection. Transfer of sperm and mating plug which contains 20E hormone from males to females ultimately influence the female immunity response. The response of *An. coluzzii* on the existence of natural microbiota in the midgut together with the *P. falciparum* is poorly studied thus the link between reproduction system of *An. coluzzii* and *P. falciparum* susceptibility is an interesting area to be looked at. By studying the mating status impact on infection, we were able to identify the possibility of male involvement in *P. falciparum* infection.

Microbiota in the midgut has been an important area of study in malaria vector control. Bacteria are usually ingested when the mosquitoes feed. In my thesis, I have shown that mating increases bacterial abundance in the midgut in sugar fed and 24 hours post infectious blood feed. Blood feeding increases the bacterial abundance in the midgut of *An. coluzzii*. Antibiotic treatment consists of penicillin, streptomycin, and gentamicin given in the sugar solution since eclosion and this removes the majority of bacteria in *An. coluzzii* females midguts. Mating too has an impact in altering some bacterial composition whereby some bacteria were found to be higher in mated females compared to virgin females. This suggests that mating is a factor that should be taken into consideration in developing paratransgenesis in combating malaria.

RNASeq analysis on sugar fed midguts showed that mating has an impact in altering the transcriptome of midguts. However, upon blood feeding, mating impact on the transcriptome seems lesser. This could be due to the timing of sampling which is at 24 hours post blood feed. At this time, microbiota massively proliferate and this could have increased a lot more gene expression in virgin females midgut which covers the impact of mating. Furthermore, upon blood feeding, there is no common genes found to be significantly differentially expressed between two different experiments, experiment with 80% and 100% *P. falciparum* infection prevalence. Apart from that, these two experiment contains different level of bacteria as well.

Besides mating, blood feeding and antibiotics treatment has an impact in changing the midgut transcriptome as well.

Next, I looked at the impact of mating status on *P. falciparum* susceptibility. Mating has an impact in increasing *P. falciparum* infection intensity especially in higher intensity *P. falciparum* infection. This impact of mating on *P. falciparum* is believed to be independent of microbiota as the same trend is found in antibiotic treated females. However, at lower *P. falciparum* intensity, this impact is not significant although the trend is there. This might be due to the inability to quantify the impact when the oocysts counts are low. Although in the wild, normally the oocysts counts are 0-10 oocysts, the laboratory *P. falciparum* infection is in a controlled condition where the females would not have a stressful condition to find mating partner and find food. This experiment suggests that mating has an impact in increasing *P. falciparum.*

One of the hypothesis of this observation is due to the unique mating system of *Anopheles*. The females are usually monandrous and they receive not only sperm, but also mating plug and seminal fluid proteins which consists of 20E hormone. Mating plug and 20E hormone have been correlated with the vectorial capacity which got me to test if 20E might have increased *Pf* susceptibility. 20E injection on virgin *An. coluzzii* does increases *P. falciparum* infection intensity as compared to virgins injected with control, 10%EtOH which is the solution to dilute 20E. However, it is not the case in mated *An. coluzzii*.

20E injection has been reported to mimic mated female in increasing egg laying, refracting the females from further copulation and microarray on LRT of 20E injection shows similarity with mated females LRT. This next suggests that perhaps 20E injection could also mimic the midgut of mated female. 20E injection on virgin *An. coluzzii* changes the midgut transcriptome compared to the control virgin. From PCA plot of RNASeq, 20E injection in virgin *An. coluzzii* showed that 20E does not cluster near mated females. However, from RNASeq analysis, 11% from the genes which were upregulated by 20E injection in virgin *An. coluzzii* are in common with the genes which were upregulated by mating. In sugar fed samples, 20E injected females have lower bacteria than control injected female midguts. This event appear to be similar to the

impact of using antibiotic to clear bacteria from the midguts, which will increase *P. falciparum* infection.

Future works/ directions:

1) Metagenomic studies on midgut microbiota comparing mated and virgin could enhance the knowledge of interaction between mating, bacteria and *P. falciparum* infection.
2) Functional genetic experiment by knocking down some genes in the midgut followed by blood feeding on infectious *P. falciparum* could give an idea on the importance of certain genes in the midgut for parasite survival.
3) Very little is know about male derived-20E pathway. Perhaps the pathway between 20E activated by blood feeding and mating are slightly different which suggests the importance of 20E from blood feeding and mating, separately.
4) Mating could remodel the gut, and it could be driven by the hormone transferred by the male. Studies on the midgut structure and cell composition in the presence/absence of hormone would give an insight on the hypothesis.

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APPENDICES

Chapter	Number of males per experiment	Number of females per experiment	Mating status	Sample	Samples obtained							
					BF/SF	Treatment (sugar)	Injection	*P. falciparum* infection	qPCR	*P. falciparum* oocyst	RNASeq	MiSeq
Ch. 3	30	60	M	P_5	SF	Ab+/Ab-	No	No	Yes	No	No	No
Ch. 3	200	400	M & Y	S	BF	Ab+/Ab-	No	Yes	Yes	No	No	Yes
Ch. 4	-	3 x 5 infected	M & Y	P_5	BF	Ab+/Ab-	No	Yes	No	No	Yes	No
Ch. 4	-	12 infected	M & Y	S	BF	Ab+/Ab-	No	Yes	No	No	Yes	No
Ch. 5	300	500	M & Y	S	BF	Ab+/Ab-	Yes	Yes	No	Yes	No	No
Ch. 6	300	500	M	P_7	SF	Ab-	Yes	No	No	No	Yes	No
Ch. 6	-	24 infected	M	S	BF	Ab-	Yes	Yes	No	No	Yes	No

M – light microscope	P – pool of 5 midguts	BF – blood fed	A+ – antibiotic treated
Y – Y-PCR	S – single midgut	SF – sugar fed	A- – untreated

APPENDIX S1 Summary of approximate number of female and male mosquitoes used for bacteria abundance and *P. falciparum* infection experiment. Females were fed with naïve whole blood or with *P. falciparum* infected blood depending on experiment (BF), whilst some midguts were collected from unfed females and labeled as sugar fed (SF). Mating status of females were confirmed by light microscope (M) and Y-PCR (Y) for some experiment. Samples obtained were processed as individual midgut (S) or/and pooled of 5 or 7 midguts (P_5/P_7). Females were further brought to downstream work after feeding either for bacteria work (qPCR of 16S) or *P. falciparum* infection work (RNASeq and oocysts count).

Prevalence	**Feed 2**	**Feed 3**	**Feed 4**	**Feed 8**	**Feed 16**	**Feed 21**	**Feed 22**	**Feed 23**	**Feed 24**	**Feed 28**	**Feed 30**
Virgin	30%	73%	100%	36%	50%	37%	19%	16%	55%	41%	78%
Mated	100%	100%	100%	40%	43%	51%	40%	17%	57%	54%	88%
Overall	59%	86%	100%	38%	48%	44%	29%	17%	56%	47%	80%
Gametocytaemia	Low	Low	Low	Low	High	High	High	High	Low	Low	High
Order (prevalence)	H	J	K	C	F	D	B	A	G	E	I
Treatment	Ab	Ab	Ab	-	-	20E	-	20E	20E	-	20E
Age of mosquitoes	**Status**										
Day0	Pupae	Pupae	Pupae	Pupae	Pupae	Pupae	Pupae	Pupae	Pupae	Pupae	Pupae
Day1	Adult	Adult	Adult	Adult	Adult	Adult	Adult	Adult	Adult	Adult	Adult
Day2	-	Mate	-	-	Mate	-	-	Mate	Mate	Mate	Mate
Day3	Mate	-	Mate	Mate	-	Mate	Mate	-	-	-	20E

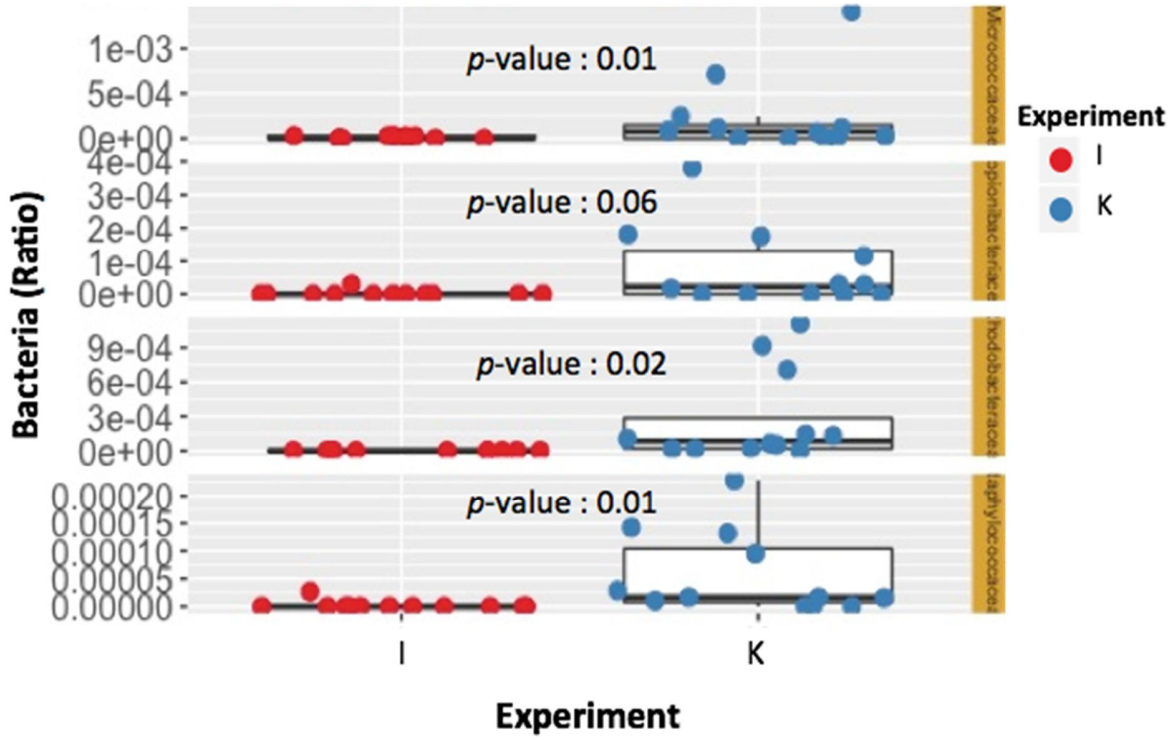
Day4	-	-	-	-	-	20E	-	20E	20E	-	-
Day5	*Pf* Feed (d17,d14)	-	-	*Pf* Feed (d17,d14)	*Pf* Feed (d18,d15)	-	-	20E	-	-	-
Day6	-	*Pf* Feed (d17,d14)	*Pf* Feed (d17,d14)	-	-	*Pf* Feed (d18,d15)	*Pf* Feed (d18,d15)	*Pf* Feed (d18,d15)	*Pf* Feed (d18,d15)	*Pf* Feed (d18,d15)	*Pf* Feed (d18,d15)

APPENDIX S2 Information on the *P. falciparum* infection from all successful *P. falciparum* infection feeds. Total of 11 feeds with 3 additional experiments with antibiotic added in their sugar meal to look at the impact of mating upon removal of bacteria. Four experiments injecting 20E or 10%EtOH on virgin female Ngousso thorax were carried out to look at the impact of 20E on *P. falciparum* infection. *P. falciparum* feeds were a mixture of gametocyte either day 17 and day 14 (d17, d14) or day 18 and day 15 (d18, d15) to make sure the proportion of males and females are similar. Infection prevalence were calculated by dividing number of affected females over total number of infected and uninfected females times with 100. Mated females were confirmed by scoring the sperms in the spermathecae using light microscopy. Mating sourcing shows the way the females were mixed after some females were introduced to the males (mated females).

Primer	**Sequence**	**Species/ Gene**	**Usage**
S23 (F) S23 (R)	CAAAACGACAGCAGTTCC TAAACCAAGTCCGTCGCT (Ref : Krzywinski et al. 2004; Krzywinski et al. 2005)	Y-chromosome	Y-PCR
S7Uni_F S7Uni_F	CCAACAAGCAGAAGAGACCG CCAGGATGGCATCGTACAC	*Anopheles* (universal)	qPCR bacterial load (S7 Uni)
16S_F 16S_R	TCCTACGGGAGGCAGCAGT GGACTACCAGGGTATCTAATCCTGTT	16S	qPCR bacterial load (overall bacteria)
As_16S_F As_16S_R	GTGCCGATCTCTAAAAGCCGTCTCA TTCGCTCACCGGCTTCGGGT	*Asaia*	qPCR bacterial load (*Asaia*)
Em_16S_F Em_16S_R	TAAGGTTGAAGTGGCTGGAATAA GTCCATCAGCGTCAGTTAAGACT	*Elizabethkingia*	qPCR bacterial load (*Elizabethkingia*)
Kp_16S_F Kp_16S_R	CGTGCTACAATGGCATATACAAAGAGAAG AGCATTCTGATCTACGATTACTAGCGATTC	*Klebsiella*	qPCR bacterial load (*Klebsiella*)
Pa_16S_F Pa_16S_R	GTTAATAACCTTGCCGATTGACGTTAC GGGATTTCACATCTGACTTAACAGAC	*Pantoea*	qPCR bacterial load (*Pantoea*)
Sm_16S_F Sm_16S_R	ACGTTCATCAATTGACGTTACTCGCA AACCGCCTGCGTGCGCTTTA	*Serratia*	qPCR bacterial load (*Serratia*)

AGAP004203:F2T7 AGAP004203:R2T7	taatacgactcactatagggGACGAGAAGGAGCGT taatacgactcactatagggGACGAATTTGTTCGA (Ref : Rono et al. 2010)	*Vitellogenin* gene	T7 – dsRNA (dsVg)
AGAP006342F2T7 AGAP006342R2T7	taatacgactcactatagggGTGGGTCGTGATGCA taatacgactcactatagggCCAGTTTCGGATCTC	*PGRPS3* gene	T7 – dsRNA (dsPGRPS3)
AGAP006796FT7 AGAP006796RT7	taatacgactcactatagggACGGAACGGTACGTCTTAGC taatacgactcactatagggTGGCTGCAGATCAGGAACTT	*Peritrophin* gene	T7 – dsRNA (dsPM)
AGAP004203:F2qPCR AGAP004203:R2qPCR	CCGACTACGACCAGGACTTC CTTCCGCGTAGTCAGACGAA (Ref : Baldini et al. 2013)	*Vitellogenin* gene	qPCR- kd efficiency (Vg)
AGAP006342F2qPCR AGAP006342R2qPCR	CAACTTCCTGGTCGGTGAGA GACACACCACAGCTGATCAG	*PGRPS3* gene	qPCR- kd efficiency (PGRPS3)
AGAP006796FqPCR AGAP006796RqPCR	TGCTTTTGGTGGGTTCAGTG ATAGTCACACAGCTTCCGGG	*Peritrophin* gene	qPCR- kd efficiency (PM)

APPENDIX S3 List of bacteria primer sequences used in qPCR analysis for Y-PCR, bacterial load assessment, dsRNA production and qPCR analysis for dsRNA efficiency.



APPENDIX S4 Bacterial loads from some family, *Micrococcaceae, Propionibacteriaceae, Rhodobacteraceae and Staphylococcaceae* obtained from experiment [K] and [I] analysed in Kraken analysis. Each dot represents one midgut sample.

Functional enrichment analysis on genes that were significantly upregulated by mating

a.

GO.ID	Term	Annotated	Significant	Expected	weight01
GO:0046323	glucose import	17	6	0.56	1.1e-05
GO:0035428	hexose transmembrane transport	17	6	0.56	1.1e-05
GO:0009813	flavonoid biosynthetic process	19	6	0.63	2.3e-05
GO:0052696	flavonoid glucuronidation	19	6	0.63	2.3e-05
GO:0042742	defense response to bacterium	10	4	0.33	0.00021
GO:0045087	innate immune response	31	6	1.03	0.00046
GO:0055085	transmembrane transport	423	31	14.03	0.00121
GO:0043171	peptide catabolic process	29	5	0.96	0.00237
GO:0046701	insecticide catabolic process	21	4	0.70	0.00451
GO:0046680	response to DDT	21	4	0.70	0.00451

(b)

GO.ID	Term	Annotated	Significant	Expected	weight01
GO:0005887	integral component of plasma membrane	270	18	7.39	0.00066
GO:0043231	intracellular membrane-bounded organelle	2102	43	57.54	0.00161
GO:0005791	rough endoplasmic reticulum	5	2	0.14	0.00705
GO:0005885	Arp2/3 protein complex	7	2	0.19	0.0142
GO:0016020	membrane	2818	99	77.15	0.02403
GO:0000139	Golgi membrane	48	4	1.31	0.04144
GO:0005737	cytoplasm	1714	55	46.92	0.04454
GO:0030119	AP-type membrane coat adaptor complex	12	2	0.33	0.05377
GO:0005576	extracellular region	426	14	11.66	0.08671
GO:0016021	integral component of membrane	2381	79	65.18	0.11228

(c)

GO.ID	Term	Annotated	Significant	Expected	weight01
GO:0005355	glucose transmembrane transporter activi...	17	6	0.44	2.9e-06
GO:0005351	sugar:proton symporter activity	20	6	0.52	8.5e-06
GO:0015171	amino acid transmembrane transporter act...	31	5	0.81	0.0013
GO:0016671	oxidoreductase activity, acting on a sul...	9	3	0.24	0.0013
GO:0016831	carboxy-lyase activity	25	5	0.65	0.0016
GO:0004602	glutathione peroxidase activity	21	4	0.55	0.0019
GO:0070006	metalloaminopeptidase activity	26	4	0.68	0.0043
GO:0008194	UDP-glycosyltransferase activity	41	6	1.07	0.0048
GO:0003824	catalytic activity	3383	126	88.40	0.0048
GO:0004364	glutathione transferase activity	27	4	0.71	0.0050

Functional enrichment analysis on genes that were significantly downregulated by mating in sugar fed midguts

(a)

GO.ID	Term	Annotated	Significant	Expected	weight01
GO:0000027	ribosomal large subunit assembly	15	4	0.26	9.8e-05
GO:0000479	endonucleolytic cleavage of tricistronic..	8	3	0.14	0.00026
GO:0000055	ribosomal large subunit export from nucl..	5	2	0.09	0.00284
GO:0031126	snoRNA 3'-end processing	6	2	0.10	0.00422
GO:0006611	protein export from nucleus	6	2	0.10	0.00422
GO:0002181	cytoplasmic translation	21	3	0.36	0.00528
GO:0006414	translational elongation	21	3	0.36	0.00528
GO:0006783	heme biosynthetic process	7	2	0.12	0.00584
GO:0000154	rRNA modification	17	3	0.29	0.00758
GO:0000463	maturation of LSU-rRNA from tricistronic...	8	2	0.14	0.00770

(b)

GO.ID	Term	Annotated	Significant	Expected	weight01
GO:0005730	nucleolus	87	11	1.35	0.0001
GO:0044452	nucleolar part	30	5	0.46	0.00025
GO:0022625	cytosolic large ribosomal subunit	43	5	0.67	0.00048
GO:0005732	small nucleolar ribonucleoprotein comple...	12	3	0.19	0.00071
GO:0030688	preribosome, small subunit precursor	5	2	0.08	0.00230
GO:0032040	small-subunit processome	29	3	0.45	0.00979
GO:0005654	nucleoplasm	149	4	2.31	0.02471
GO:0030686	90S preribosome	22	2	0.34	0.04855
GO:0030687	preribosome, large subunit precursor	23	2	0.36	0.04855
GO:0043292	contractile fiber	5	1	0.08	0.07503

(c)

GO.ID	Term	Annotated	Significant	Expected	weight01
GO:0003746	translation elongation factor activity	13	3	0.21	0.0010
GO:0008422	beta-glucosidase activity	6	2	0.10	0.0037
GO:0003735	structural constituent of ribosome	173	8	2.77	0.0064
GO:0004659	prenyltransferase activity	8	2	0.13	0.0067
GO:0008745	N-acetylmuramoyl-L-alanine amidase activ...	9	2	0.14	0.0085
GO:0005548	phospholipid transporter activity	9	2	0.14	0.0085
GO:0030515	snoRNA binding	14	2	0.22	0.0204
GO:0015020	glucuronosyltransferase activity	14	2	0.22	0.0204
GO:0004004	ATP-dependent RNA helicase activity	38	3	0.61	0.0224
GO:0004521	endoribonuclease activity	18	2	0.29	0.0329

Functional enrichment analysis on genes that were significantly upregulated by 20E injection in sugar fed midguts

(a)

GO.ID	Term	Annotated	Significant	Expected	weight01
GO:0015991	ATP hydrolysis coupled proton transport	22	7	0.85	1.2e-05
GO:0006890	retrograde vesicle-mediated transport, G...	11	5	0.43	3.2e-05
GO:0015986	ATP synthesis coupled proton transport	21	6	0.81	0.00010
GO:0051603	proteolysis involved in cellular protein...	157	14	6.08	0.00013
GO:0034314	Arp2/3 complex-mediated actin nucleation	12	4	0.46	0.00085
GO:0006418	tRNA aminoacylation for protein translat...	44	7	1.70	0.00134
GO:0045047	protein targeting to ER	12	5	0.46	0.00146
GO:0006465	signal peptide processing	7	3	0.27	0.00178
GO:0006122	mitochondrial electron transport, ubiqui..	8	3	0.31	0.00277
GO:0015031	protein transport	229	20	8.86	0.00402

(b)

GO.ID	Term	Annotated	Significant	Expected	weight01
GO:0033179	proton-transporting V-type ATPase, V0 do...	9	5	0.42	2.2e-05
GO:0005737	cytoplasm	1714	133	79.27	7.6e-05
GO:0005885	Arp2/3 protein complex	7	4	0.32	0.00014
GO:0019773	proteasome core complex, alpha-subunit c...	7	4	0.32	0.00014
GO:0005789	endoplasmic reticulum membrane	94	14	4.35	0.00034
GO:0005739	mitochondrion	353	43	16.33	0.00043
GO:0030126	COPI vesicle coat	6	3	0.28	0.00176
GO:0016471	vacuolar proton-transporting V-type ATPa...	6	3	0.28	0.00176
GO:0000276	mitochondrial proton-transporting ATP sy...	7	3	0.32	0.00298
GO:1905368	peptidase complex	45	11	2.08	0.00431

(c)

GO.ID	Term	Annotated	Significant	Expected	weight01
GO:0004298	threonine-type endopeptidase activity	15	7	0.48	1.7e-07
GO:0046961	proton-transporting ATPase activity, rot...	16	6	0.51	6.3e-05
GO:0015078	hydrogen ion transmembrane transporter a...	57	17	1.83	1.0e-05
GO:0051117	ATPase binding	5	3	0.16	0.00031
GO:0060590	ATPase regulator activity	5	3	0.16	0.00031
GO:0051087	chaperone binding	12	4	0.39	0.00042
GO:0008233	peptidase activity	625	30	20.07	0.00056
GO:0004129	cytochrome-c oxidase activity	14	4	0.45	0.00081
GO:0030554	adenyl nucleotide binding	688	25	22.09	0.00102
GO:0004812	aminoacyl-tRNA ligase activity	43	6	1.38	0.00233

Functional enrichment analysis on genes that were significantly downregulated by 20E injection in sugar fed midguts

(a)

GO.ID	Term	Annotated	Significant	Expected	weight01
GO:0006468	protein phosphorylation	263	44	15.91	2.6e-07
GO:0006355	regulation of transcription, DNA-templat..	532	64	32.18	9.3e-06
GO:0007169	transmembrane receptor protein tyrosine ...	32	9	1.94	0.00013
GO:0071526	semaphorin-plexin signaling pathway	6	4	0.36	0.00018
GO:0048841	regulation of axon extension involved in...	6	4	0.36	0.00018
GO:0006470	protein dephosphorylation	51	12	3.08	0.00026
GO:0006606	protein import into nucleus	34	12	2.06	0.00027
GO:0051056	regulation of small GTPase mediated sign..	42	12	2.54	0.00037
GO:0030010	establishment of cell polarity	7	4	0.42	0.00040
GO:0043547	positive regulation of GTPase activity	30	7	1.81	0.00043

(b)

GO.ID	Term	Annotated	Significant	Expected	weight01
GO:0005856	Cytoskeleton	192	31	11.33	1.1e-6
GO:0005622	intracellular	3296	250	194.48	1.1e-05
GO:0005634	nucleus	1328	111	78.36	0.00030
GO:0005737	Cytoplasm	1714	111	101.13	0.00035
GO:0016459	Myosin complex	18	6	1.06	0.00041
GO:0005643	Nuclear pore	30	7	1.77	0.00146
GO:0030864	Corticol actin cytoskeleton	5	3	0.30	0.00186
GO:0034399	Nuclear periphery	5	3	0.30	0.00186
GO:1902554	Serine/threonine protein kinase complex	15	5	0.89	0.00589
GO:0071004	U2-type prespliceosome	8	3	0.47	0.00913

(c)

GO.ID	Term	Annotated	Significant	Expected	weight01
GO:0005515	protein binding	2327	236	153.43	4.0e-14
GO:0005488	binding	5521	454	364.02	2.1e-07
GO:0004674	protein serine/threonine kinase activity	118	26	7.78	5.7e-06
GO:0005096	GTPase activator activity	58	14	3.82	1.7e-05
GO:0008536	Ran GTPase binding	16	7	1.05	3.5e-05
GO:0003700	transcription factor activity, sequence-...	204	28	13.45	6.2e-05
GO:0008139	nuclear localization sequence binding	13	6	0.86	9.2e-05
GO:0004725	protein tyrosine phosphatase activity	37	10	2.44	9.8e-05
GO:0005524	ATP binding	684	69	45.10	0.00019
GO:0019901	protein kinase binding	12	5	0.79	0.00066

APPENDIX S5 List of functional enrichment analysis upon mating and 20E injection on sugar fed *An. coluzzii* midgut transcriptome. (a) biological process (BP), (b) cellular compartment (CC) and (c) molecular function (MF).