

Reciprocal Host-Microbiota Interactions In The Malaria Vector Mosquito *Anopheles coluzzii*

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Declaration of Originality

The work described in this thesis is my own. All material contributions and ideas of others are duly attributed.

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Abstract

The gut microbiota of the malaria vector mosquito *Anopheles coluzzii* are understood to have wide reaching influence on mosquito physiology and basal immunity. Here, it is shown that the microbiota proliferation that is associated with a mosquito blood meal causes a reduction in mosquito life span and fecundity, both of which translate to reduced mosquito vectorial capacity. A transcriptional analysis is employed to explore the responses of the mosquito midgut to the dynamic changes in microbial load occurring over the blood feeding and egg laying cycle, leading to the identification and characterisation of a key mosquito resistance response that reduces the load of specific bacterial families within the gut, abrogates the stimulation of epithelial immunity and prevents the gut microbiota from seeding a systemic infection. Finally, the role of the variable transmembrane receptor PGRPLC in microbiota-dependent immune stimulation is characterised. Altogether, these data contribute to our understanding of mosquito gut homeostasis, supporting the potential of exploiting microbiota-immune interactions for novel transmission blocking applications.

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Abbreviations

ACT	Artemisin-based Combination Therapy
AMP	Antimicrobial Peptide
BSA	Bovine Serum Albumin
CBD	Chitin Binding Domain
CTL	C-type Lectin
DAP	(<i>meso</i> -)Diaminopimelic Acid
DDT	Dichlorodiphenyltrichloroethane
DUOX	Dual Oxidase
FBN	Fibrinogen-like Domain
FCS	Foetal Calf Serum
FREP	Fibrinogen-Related Protein Family
GlcNAc	N-acetylglucosamine
GLMM	Generalised Linear Mixed Model
GNBP	Glucan Binding Protein (formerly Gram Negative Binding Protein)
GO	Gene Ontology
GPCR	G Protein Coupled Receptor
GPI	Glycophosphatidylinositol
GPX	Glutathione peroxidase
GR	Gustatory Receptor
GRX	Glutathione Reductase
H&E	Hematoxylin and Eosin
HEG	Homing Endonuclease Gene
HPX	Haem Peroxidase
ILP	Insulin Like Peptide
IMD	Immune Deficiency
IRS	Indoor Residual Spraying
ITN	Insecticide Treated Net
JAK/STAT	Janus Kinase/Signal Transducers and Activators of Transcription
JH	Juvenile Hormone
KEGG	Kyoto Encyclopedia of Genes and Genomes

LPS	Lipopolysaccharide
LRIM	Leucine Rich Immune Protein
LRR	Leucine Rich Repeat
MAPK	Mitogen Activated Protein Kinase
ML	MD2-like
MurNAc	N-acetylmuramic acid
NOS	Nitric Oxide Synthase
OTU	Operational Taxonomic Unit
PAMP	Pathogen Associated Molecular Pattern
PBS(T)	Phosphate Buffered Saline (Tween)
PCA	Principle Components Analysis
PGRP	Peptidoglycan Recognition Protein
PLC- β	Phospholipase C β
PM	Peritrophic Matrix
PO	Phenoloxidase
PRR	Pattern Recognition Receptor
PS	Penicillin-Streptomycin
RBC	Red Blood Cell
RHIM	Rip Homotypic Interaction Motif
RIDL	Release of Insects carrying a Dominant Lethal
ROMO1	Reactive Oxygen Species Modulator 1
ROS	Reactive Oxygen Species
SNP	Single Nucleotide Polymorphism
SOD	Superoxide Dismutase
SP	Signal Peptide
TALEN	Transcription Activator Like Effector Nuclease
TCT	Tracheal Cytotoxin
TEP	Thioester-containing Protein
TEV	Tobacco Etch Virus
TPX	Thioredoxin Peroxidase
TRXR	Thioredoxin Reductase
WHO	World Health Organisation
ZFN	Zinc Finger Nuclease

Chapter 1: Introduction

1.1 The Biology of Mosquitoes as Vectors

Mosquitoes as vectors of infectious disease

As obligate blood feeders, female mosquitoes are responsible for the transmission of protozoan and viral pathogens that have an incredible impact on human mortality and morbidity throughout the world. Malaria, transmitted by mosquitoes of the *Anopheles* genus, was responsible for approximately 438,000 deaths in 2015, with most of those at risk being in Africa and children under the age of five (WHO, World Malaria Report 2015). Similarly, *Aedes* mosquitoes transmit viral diseases such as dengue, yellow fever and chikungunya, whilst the *Culex* genus is responsible for the transmission of lymphatic filariasis, Japanese encephalitis and West Nile fever. All of these diseases impose considerable burdens on human populations.

It was understood that these infections require a blood sucking arthropod for their transmission in the late 19th century, after which vector control became a primary means of tackling such diseases. The case of malaria in early 20th century Italy exemplifies the early success of this approach. Here, malaria was eliminated through the disruption of *Anopheline* larval habitats as well as the extensive distribution of quinine (Majori, 2012). Resurgence after World War II was efficiently and successfully tackled through targeting adults with dichlorodiphenyltrichloroethane (DDT). Today, control of mosquito populations through insecticides and larvicides remains an essential means of control, though its efficacy is receding at an alarming rate due to the evolution of insecticide resistance. The same is true for the efficacy of drugs that target the parasite. As such, the development of a host of novel interventions, derived from both new and old technologies, is of paramount importance if global malaria eradication is to be conceivable in the coming years.

Anopheles and *Plasmodium* life cycles

There are approximately 400 known species of *Anopheles* mosquito, of which around 30 are able to transmit malaria (WHO, World Malaria Report 2015). Malaria is caused by protozoan parasites of the genus *Plasmodium*, of which five species (*falciparum*, *vivax*, *ovale*, *knowlesi* and *malariae*) are able to

infect humans, with *P. falciparum* causing the highest mortality in the African continent. Six of the main African vectors of *P. falciparum* are members of the *Anopheles gambiae sensu lato* species complex, a complex of eight morphologically indistinguishable sibling species. *A. gambiae* (formerly *A. gambiae* S molecular form), *A. coluzzii* (formerly *A. gambiae* M molecular form) and *A. arabiensis* constitute the main African vectors¹. *Anopheles melas*, *A. merus* and *A. bwambae* are competent but geographically limited, rendering them less significant as vectors (Sinka et al., 2010; White, 1985). *Anopheles funestus* is not a member of the species complex but is also a significant African vector species.

Anophelines have 4 stages to their life cycle (Fig 1.1.1). Mated and blood fed females lay approximately 50-200 eggs per oviposition, which hatch into larvae 48 -72 h later. The aquatic larvae go through 4 instar stages over the course of approximately 7-10 days, during which they feed on organic detritus and microorganisms. The pupal stage then lasts for approximately 24 h before the adult emerges. Adult males acquire energy by feeding on nectar and honeydew, whilst females also take sugar (Foster, 1995), but additionally require one or more vertebrate blood meals to develop a batch of eggs (Scott and Takken, 2012). Following the first blood meal, females take 72 h to develop their eggs, with a female then completing gonotrophic cycles every 2-3 days throughout her lifetime (Clements, 2000).

¹*A. gambiae* and *A. coluzzii* have only recently been recognised as distinct species (Coetzee et al., 2013). In most pre-2013 publications, it is not clear whether the author is referring to *A. gambiae* S molecular form or *A. gambiae* M molecular form (latterly *A. coluzzii*). Therefore, in this literature review we refer solely to '*A. gambiae*' for the sake of consistency, where this could refer to either species. In the results section we refer to *A. coluzzii*, as the colony on which these experiments were performed is known to be this species.

Figure 1.1.1

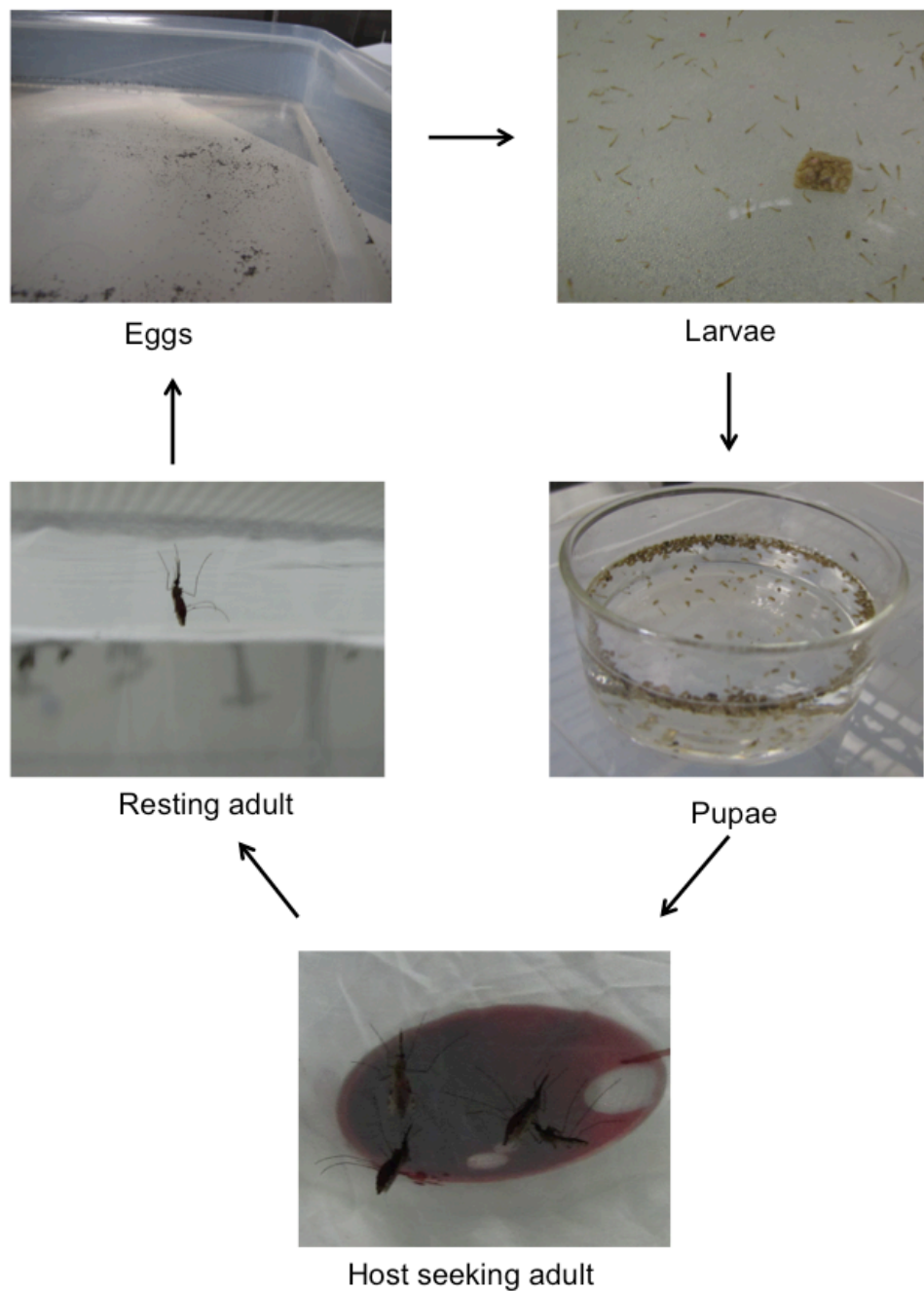


Figure 1.1.1. The mosquito life cycle. Upon taking a bloodmeal, female mosquitoes take 48-72 h to mature their eggs. These are laid in standing pools of water. Over approximately 7-10 days the larvae mature into pupae that emerge into adults, starting the cycle again.

It is during the obligate blood feeding stage of the female *Anopheles* life cycle that a mosquito may become infected with the *Plasmodium* parasite, with the ingestion of mature gametocyte stage parasites (the sexual stage). Triggered by a drop in temperature and chemical cues in the mosquito (Billker et al., 1998), gametocytes escape from their erythrocyte host cells and mature into micro and macrogametes which fuse to form a motile ookinete (reviewed in Angrisano et al., 2012). Upon migration out of the blood bolus, the ookinete must first traverse the peritrophic matrix (PM), an extracellular layer of chitin and protein that is induced upon blood feeding to control the exposure of the gut epithelium to blood-derived molecules and cells (Dinglasan et al., 2009). The ookinete then travels through the midgut epithelium by penetrating the apical end of a midgut epithelial cell. Upon exit at the basal end of the cell the ookinete transforms into a non-motile, spherical cell that is attached to the midgut wall (Aly et al., 2009). This transition from the ookinete to the sessile oocyst marks a major bottleneck in the *Plasmodium* life cycle whereby parasite numbers are reduced from the thousands to the tens, in part thanks to local and systemic mosquito immune activity. The oocyst matures on the wall of the midgut for 10 to 12 days, undergoing multiple cycles of mitotic division. Within the oocyst, sporozoites develop and acquire motility, before finally the oocyst ruptures and sporozoites are released into the hemocoel (the open circulatory system of the mosquito). Upon encountering the salivary glands, a specific interaction between the parasite and the salivary gland basal lamina mediates adherence and invasion. Only when they harbour salivary gland sporozoites are mosquitoes considered infectious; from *P. falciparum* gametocyte ingestion to the presence of sporozoites in the salivary glands takes between 10 and 18 days.

Within the human host, injected skin stage parasites migrate immediately to the liver where they multiply asymptotically for 1-2 weeks. Merozoite stage parasites then escape from the liver, entering the blood stream where they undergo repeated 1-3 day cycles of erythrocyte invasion and egress; it is these asexual blood stages that cause the symptoms characteristic of febrile malaria. A proportion of infected blood cells leave the cycle of asexual

replication and enter into sexual development, with mature, infectious gametocytes restarting the infectious cycle when they are taken up in the next mosquito bloodmeal.

Mosquitoes as targets for intervention against disease transmission

There are broadly three types of intervention currently in use that aim to reduce malaria-associated mortality: drugs and prophylactic treatments (for children, pregnant women and travellers) that target the *Plasmodium* parasite, insecticides that reduce mosquito populations by killing adults or larvae, and other interventions that reduce contact between mosquitoes and human hosts, such as repellents and decoy traps. The current first line treatment in the majority of *P. falciparum* endemic countries is artemisinin-based combination therapy (ACT), which, critically, has activity against both asexual blood stages of the parasite and gametocytes, reducing onward transmission (Makanga, 2014). A major issue with the dependence on ACTs is the development of drug resistance, with this now being reported in five countries in South East Asia (WHO, World Malaria Report 2015). What's more, whilst ACT remains an effective treatment in Africa, only 20 % of *P. falciparum* confirmed children currently access treatment.

The mosquito itself is also a key target in reducing malaria transmission. Insecticides are deployed primarily against adult mosquitoes through indoor residual spraying (IRS) and insecticide treated nets (ITNs); they benefit from better coverage than ACT treatment, with 67 % of the at risk population estimated to have access to an ITN (WHO, World Malaria Report 2015). Whilst there are four classes of insecticide available, only pyrethroids are licensed for use on ITNs and these are also used widely in IRS programmes. Resistance to at least one insecticide has been reported in over 75 % of countries that submit monitoring data. Similarly, of the countries monitoring pyrethroid resistance, 75 % have detected resistance (WHO, World Malaria Report 2015) . As for ACTs, resistance threatens to severely compromise the efficacy of these interventions. Indeed, it is not clear whether the current tools alone are capable of completely eliminating malaria in high transmission settings (Mendis et al., 2009).

As such, efforts are underway both to effectively manage the spread of resistance and to develop new active compounds. Novel drugs (Baragaña et al., 2015), insecticides (Ngufor et al., 2014) and vaccines (Moreno and Joyner, 2015) are in development, all of which will be crucial for maintaining the decline in malaria incidence. In addition, two new technologies promise to transform the antimalarial arsenal: transgenic mosquitoes and bacterial symbionts that allow the manipulation of mosquito fertility, sex ratio, longevity or pathogen resistance.

Two approaches are currently under investigation for transgenic mosquitoes: engineering population crashes or producing malaria refractory strains. Release of Insects carrying a Dominant Lethal (RIDL) is an approach whereby male mosquitoes of a strain homozygous for a dominant lethal transgene are released, mating with wild type females of the natural population such that they have no viable offspring (Thomas et al., 2000). As female mosquitoes mate only once in their lifetime, this effectively sterilises them. The RIDL strain can be reared in the laboratory as the dominant lethal is under the control of a tetracycline repressible promoter. Development of this technique for *A. aegypti* is now advanced to field trials (Carvalho et al., 2015; Wise de Valdez et al., 2011), and is also under investigation for the Asian vector *Anopheles stephensi* (Marinotti et al., 2013). Self-sustaining genetic technologies that exploit selfish genetic elements known as homing endonuclease genes (HEGs) (Windbichler et al., 2007) are also under development. A naturally occurring HEG has been identified that has specific activity against an rRNA sequence present repetitively and exclusively on the X chromosome; restricting the activity of this gene to spermatogenesis successfully biases the sex ratio such that a female mating with a HEG-bearing male produces more than 95% male offspring due to 'X shredding' (Galizi et al., 2014). This sex ratio distortion has the potential to cause population crashes, and could be made even more powerful with the introduction of the HEG onto the Y chromosome. Similarly, engineer-able nucleases such as zinc finger nucleases (ZFNs) and transcription activator like effector nucleases (TALENs) can be used as HEGs targeted against specific sequences, such as genes involved in fertility (Simoni et al., 2014). Finally, a CRISPR-Cas9 based gene

drive mechanism has recently been developed; using this promising technology, a recessive female fertility gene was disrupted, and this mutation was transmissible to progeny rates of up to 99.6 % (Hammond et al., 2016).

An alternative transgenic control approach depends on the introduction of transgenes, either native or synthetic, that render the mosquito resistant to parasite establishment when overexpressed. Many such genes have been described and demonstrated to be effective at reducing *Plasmodium* infection load, including immune effectors (Abraham et al., 2005; Isaacs et al., 2011; Meredith et al., 2011; Moreira et al., 2002; Yoshida et al., 2007), signal transducers (Corby-Harris et al., 2010; Dong et al., 2011) and mosquito receptor binding peptides (Ito et al., 2002). These genes have varied effects on mosquito fitness, and would likely need to be combined with a gene drive mechanism to be stably introduced into a population, a prospect that has not been demonstrated to date. A potential advantage of transgenic methods over insecticides is their species specificity, limiting potential environmental consequences of insecticide spraying. Equally, however, gene flow boundaries mean that transgenic technologies would have to be individually developed and implemented for each vector species.

Rather than introducing a transgene into the mosquito genome, an alternative approach involves introducing transgenes via the genome of a microbial symbiont, known as paratransgenesis (reviewed in Wilke & Marrelli, 2015). This approach has two important advantages: first, genetic manipulation of bacteria is technically more straightforward than mosquitoes, and secondly one bacteria species is likely able to colonise multiple host species. The potential of this technology has been demonstrated using *E. coli* (Riehle et al., 2007; Yoshida et al., 2001) and more recently is being developed for microorganisms that naturally occur as part of the mosquito gut microbiota, such as *Pantoea agglomerans* (Bisi and Lampe, 2011; Wang et al., 2012), or that are natural entomopathogens, such as the fungus *Metarhizium anisopliae* (Fang et al., 2011). Perhaps most promisingly, bacteria of the genus *Asaia* have been shown to associate with diverse species including *A. stephensi* (Favia et al., 2007), *A. aegypti* (Crotti et al., 2009), *A. gambiae* (Damiani et al.,

2010), *A. albopictus* (Minard et al., 2013), *C. pipiens* and *C. quinquefasciatus* (De Freece et al., 2014), with so far no evidence that they circulate in human populations (Epis et al., 2012), making them ideal candidates for paratransgenesis. *Asaia* is associated with several key tissues in *Plasmodium* development, including the female gut and salivary glands and, critically, is transmitted both horizontally and vertically (Damiani et al., 2010; Favia et al., 2007, 2008). This transmission has recently been shown to be maintained in large cage trials (Mancini et al., 2016). What's more, it is amenable to genetic manipulation (Favia et al., 2007), and expression of efficient anti-*Plasmodium* factors has been demonstrated in this genus (Bongio and Lampe, 2015).

In addition to using symbionts as a delivery tool, several microorganisms have inherent characteristics that make their introduction to mosquito populations desirable. For instance, *Wolbachia* is a genus of intracellular, maternally inherited bacteria that naturally infects about 40 % of all terrestrial arthropod species (Zug and Hammerstein, 2012). It has great potential as a biological control agent for two reasons: it can be reproductively manipulative, inducing cytoplasmic incompatibility and therefore ensuring its persistence in a population, and it can affect its host's ability to transmit pathogens via effects on longevity and resistance (reviewed in Bourtzis et al., 2014). *Wolbachia* infections do not occur naturally in many disease vector mosquitoes, however they have been stably introduced in *A. aegypti* (McMeniman et al., 2009), and shown to be able to invade a wild Australian population (Hoffmann et al., 2011). *Wolbachia* has also been stably introduced to an *A. stephensi* colony, where it confers resistance to *P. falciparum* infection (Bian et al., 2013). Interestingly, it was long thought that anophelines do not harbour *Wolbachia* infections, however, recently, West African populations of *A. gambiae* and *A. coluzzii* were found to have maternally transmissible *Wolbachia* infections in their reproductive tissues (Baldini et al., 2014). This finding further supports the prospect of exploiting *Wolbachia* for population replacement or suppression strategies in African anophelines.

Finally, several bacterial genera that are common components of the anopheline gut microbiota have been identified as having the potential to

reduce vector competence via effects on mosquito longevity, immune induction and direct parasite killing (Bahia et al., 2014; Bando et al., 2013). In particular, an *Enterobacter* sp. isolated from the guts of a Zambian *A. gambiae* population confers resistance to *P. falciparum* infection by producing reactive oxygen species (ROS) in the gut lumen (Cirimotich et al., 2011). Similarly, a *Chromobacterium* species is able to dominantly colonise both *A. aegypti* and *A. gambiae* guts when introduced in a sugar meal, where it reduces mosquito life span and increases resistance to dengue and *Plasmodium* infection, respectively (Ramirez et al., 2014a). The manipulation of gut bacteria as a control intervention is exceptionally appealing; this method is simple (bacteria can be introduced via sugar baited traps), safe (the species in question are already naturally present in the environment) and avoids the necessity of ethical and regulatory approval associated with transgenic mosquito releases.

With this in mind, a renewed focus on the mosquito immune system is now paramount to understand how the native gut bacteria population is shaped and maintained, and, reciprocally, how the gut bacteria affect immune induction and host physiology in a manner that influences parasite load and vector competence.

1.2 The Mosquito Immune System

Overview of Mosquito Immunity

For the purposes of this discussion, there are two main compartments in the mosquito: the nominally sterile haemocoel, which houses the fat body, the main metabolic and immune organ of the insect, and the gut, which is open to the environment, receives and digests blood and sugar meals and houses the microbiota. Immunosurveillant blood cells known as haemocytes circulate within the haemocoel. Both compartments are vulnerable to infection, the former either via cuticular damage or pathogen penetration through the gut epithelium, and the latter via microbe ingestion. As in all metazoans, mosquitoes recognise non-self from self through the binding of pathogen-associated molecular patterns (PAMPs) to pattern recognition receptors

(PRRs), resulting in an intracellular or extracellular signalling cascade. The main intracellular signalling cascades in the mosquito immune response are the TOLL, IMD (Immune deficiency) and JAK/STAT (Janus kinase/ signal transducers and activators of transcription) pathways, which result in broad transcriptional responses that direct either pathogen killing (eg antimicrobial peptide (AMP) or ROS production) or the host's tolerance to infection (eg epithelial stem cell renewal). The extracellular signalling cascades, by contrast, result in the localised killing of the pathogen that triggered the pathway. In all cases, the outcome for the pathogen is one of three methods of killing: lysis, melanisation or phagocytosis.

Recognition and transduction: the TOLL, IMD and JAK/STAT pathways

The primary PAMP in insect immunity is peptidoglycan, the major cell wall component of both Gram types of bacteria. Briefly, peptidoglycan is composed of sugar polymers (alternating residues of β -(1,4) linked N-acetylglucosamine and N-acetylmuramic acid), that are cross linked by peptide chains of three to five amino acids. The Gram types are distinguishable by the molecular composition of their peptide chain, with Gram-negative bacteria (and bacilli) containing a *meso*-diaminopimelic acid (DAP) residue in the third amino acid position and Gram-positive bacteria containing a lysine residue. The insect TOLL and IMD pathways are both stimulated by peptidoglycan.

The intracellular signalling pathways TOLL and IMD were identified upon the sequencing of the *A. gambiae* genome (Holt et al., 2002) by comparison to their homologous pathways in the model organism *Drosophila melanogaster* (Fig 1.2.1) (Christophides et al., 2002). As experimental characterisation of these two signalling pathways is considerably more advanced in *Drosophila*, and the intracellular components of the pathway are highly conserved between the two organisms, this discussion will focus first on the *Drosophila* model for each pathway before considering what is known in *A. gambiae*.

The *Drosophila* Toll pathway recognises Gram-positive bacteria and fungi (Lemaitre et al., 1996). The peptidoglycan recognition protein (PGRP) PGRP-

SA and the glucan binding protein (formerly known as Gram negative binding protein) GGBP1 are extracellular, circulating PRRs. Together they bind a mucopeptide dimer of Lys-type peptidoglycan (Filipe et al., 2005). Genetic evidence additionally points to a role for GGBP3 in binding fungal glucans (Gottar et al., 2006). Ligand binding triggers a proteolytic cascade that culminates in the cleavage of the cytokine Spaetzle, which binds the Toll receptor, inducing Toll dimerisation (Weber et al., 2003). Intracellularly, Toll recruits a protein complex consisting of Tube, Myd88 and Pelle, the latter being a kinase that then phosphorylates the negative regulator Cactus, causing its degradation by the proteasome (reviewed in Belvin & Anderson, 1996). Cactus degradation releases the transcription factors Dorsal and Dif to be translocated to the nucleus where they initiate the transcription of effector genes. The transcription of the AMP-encoding gene *Drosomycin* is typically considered a read out for the Toll pathway in *Drosophila*.

Figure 1.2.1

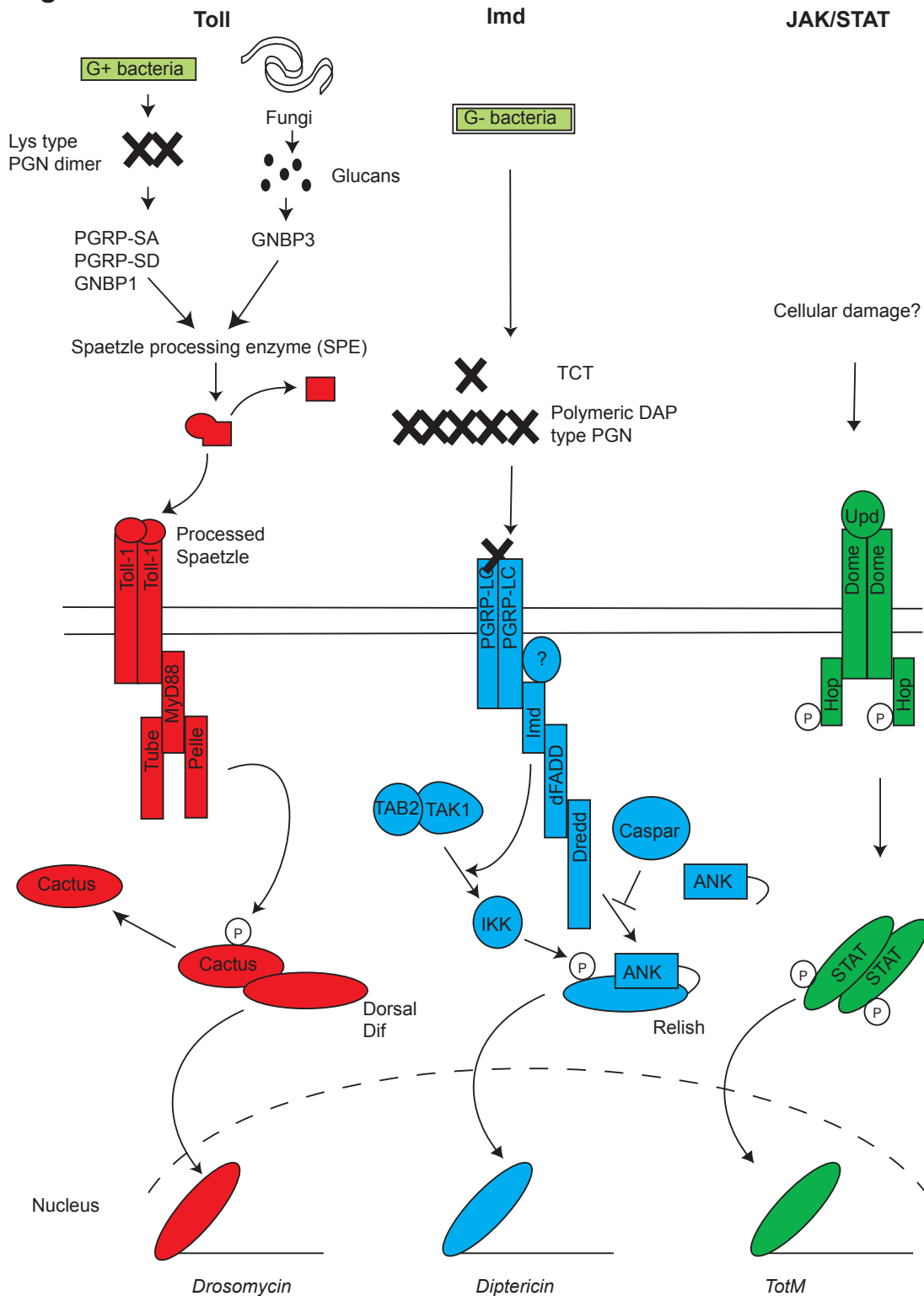


Figure 1.2.1. The Toll, Imd and JAK/STAT pathways in *Drosophila*. The Toll pathway recognises Gram-positive bacteria and fungi, the Imd pathway recognises Gram negative bacteria and the JAK/STAT pathway is likely triggered by cellular damage. All result in the transcription of immune effectors

With the exception of Dif, the *A. gambiae* genome encodes 1:1 orthologues for all of the intracellular components of the Toll pathway, likely reflecting the conserved role of this pathway in dorsal-ventral patterning (Christophides et al., 2002). Extracellularly, *A. gambiae* encodes a 1:1 orthologue of *Drosophila* PGRP-SA, PGRPS1, though the GNBPs have undergone a mosquito specific expansion, with *A. gambiae* encoding seven GNBPs to *Drosophila*'s three (Waterhouse et al., 2007). Similarly, the *Drosophila* Toll that has been recruited for immune function (Toll-1) does not have a clear orthologue, resembling 4 *A. gambiae* TOLLs (1A, 1B, 5A and 5B), whilst both Spaetzle and the proteases that cleave it have no clear equivalent (Waterhouse et al., 2007). Functionally, the situation is better resolved in *A. aegypti*, where TOLL5A and SPZ1C have been characterised as functional equivalents to *Drosophila* Toll-1 and Spaetzle (Shin et al., 2006), whilst the serine protease CLIPB15 likely mediates the proteolytic processing of the Spaetzle cytokine (Zou et al., 2010). The divergence of mosquito and *Drosophila* recognition modules is likely to reflect the different microbial challenges that these genera have evolved with.

Functionally, the *Anopheles* Toll pathway has been implicated in limiting *P. berghei* but not *P. falciparum* infection (Frolet et al., 2006; Garver et al., 2009), via a mechanism that may involve inducing basal levels of the complement-like factor thioester-containing protein 1 (TEP1) in the haemolymph (Frolet et al., 2006) and shaping haemocyte populations (Ramirez et al., 2014b). Although there is no functional evidence for a protective role in *P. falciparum* defence, one study did identify two single nucleotide polymorphisms (SNPs) in the *TOLL5B* gene that are associated with *P. falciparum* susceptibility (Horton et al., 2010). Interestingly, there is so far no evidence for a role for the *A. gambiae* TOLL pathway in antibacterial defence, with knock down of REL2 (the orthologue of the transcription factor Dorsal) having no effect on survival to bacterial infection of either Gram type (Meister et al., 2005). PGRPS1, similarly, has no effect on survival to *E. coli* challenge and a minor agonistic effect against *S. aureus* (Meister et al., 2009).

The *A. gambiae* GNBPs, however, do appear to play a role in the defence against both Gram types of bacteria and *Plasmodium* parasites, suggesting that these PRRs are likely to signal to pathways other than the TOLL pathway in the mosquito (Warr et al., 2008). In *A. aegypti*, the Toll pathway has been implicated in immunity to fungal infection (Shin et al., 2005) and dengue virus infection (Pan et al., 2012; Ramirez and Dimopoulos, 2010; Xi et al., 2008).

The *Drosophila* Imd pathway is responsible for defence against Gram-negative bacteria (reviewed in Lemaitre & Hoffmann, 2007). The stimulating ligand is DAP-type peptidoglycan, which can be recognised in its monomeric form (tracheal cytotoxin, TCT) or as a polymer. Two PRRs are responsible for Imd activation: PGRP-LC and PGRP-LE. The exact functions of *Drosophila* PGRP-LC and PGRP-LE are discussed fully in chapter 3.3, but, briefly, PGRP-LC is a transmembrane receptor that detects extracellular TCT and polymeric peptidoglycan, whilst PGRP-LE is a TCT receptor that is able to act both extracellularly, where it circulates and recruits TCT to membrane bound PGRP-LC, and intracellularly, where it acts independently as a receptor for intracellular TCT (Kaneko et al., 2006; Lim et al., 2006). As with the Toll receptor, ligand binding is thought to induce receptor dimerisation and activate a downstream signalling cascade consisting of the Imd protein, the dFADD adaptor and the caspase Dredd. In parallel, the IKK complex is activated by TAB2/TAK1 in an Imd-dependent manner to phosphorylate the transcription factor Relish. Phosphorylated Relish is a target for cleavage by Dredd, which cleaves off the Ankyrin domain, allowing the Rel domain to translocate to the nucleus and activate transcription. The negative regulator Caspar inhibits Dredd dependent Relish cleavage to suppress the pathway (Kim et al., 2006). Similar to the Toll pathway, the *A. gambiae* genome encodes 1:1 orthologues of all intracellular components of the Imd pathway except TAB2, though it only encodes one receptor, PGRP-LC, with no PGRP-LE orthologue (Christophides et al., 2002).

Functionally, the *A. gambiae* IMD pathway appears to be the main pathway involved in antibacterial defence against both Gram-positive and Gram-negative bacteria in *A. gambiae* and *A. aegypti* (Cooper, Chamberlain, &

Lowenberger, 2009; Dong et al., 2011; Magalhaes, Leandro, & Ayres, 2010; Meister et al., 2005, 2009). The *A. gambiae* genome encodes two isoforms of REL2, the homologue of *Drosophila* Relish; the short isoform, REL2-S, lacks the Ankyrin domain-encoding region and appears to be responsible for the response against Gram-negative bacteria, whilst the full length isoform, REL2-F, encodes the Ankyrin domain and is implicated in resistance to Gram-positive bacteria (Meister et al., 2005). What's more, the IMD pathway is now well established as playing a major role in the defence against *P. falciparum* (Clayton, Cirimotich, Dong, & Dimopoulos, 2013; Dong et al., 2011; Garver et al., 2012, 2009; Meister et al., 2009). REL2-dependent parasite killing occurs in both the gut lumen and the haemocoel (Dong et al., 2011; Garver et al., 2012), and is at least partly dependent on the presence of the gut microbiota for its activation (Meister et al., 2009). The downstream mechanism responsible for parasite killing is unclear, though REL2 is known to induce the transcription of numerous effectors that have been shown to have anti-*Plasmodium* activity, including TEP1 and APL1C (Dong et al., 2011; Meister et al., 2005).

The JAK/STAT pathway was first identified for its role in vertebrate immune signalling (reviewed in Villarino et al., 2015) and was later characterised in *Drosophila* as a key signalling pathway in development, immunity and stem cell maintenance (reviewed in Arbouzova & Zeidler, 2006). Briefly, binding of the Unpaired (Upd) ligand to the transmembrane receptor Dome leads to receptor dimerisation, causing the receptor associated JAKs to phosphorylate both themselves and Dome. Cytoplasmic STAT transcription factors are then recruited to the membrane-associated complex; their phosphorylation induces STAT dimerisation and subsequent nuclear translocation. It is not clear what the trigger of the pathway is, and indeed it may be the case that rather than direct recognition of a microbial elicitor, damaged or infected cells produce the Upd ligand which then acts as a cytokine to trigger the pathway in neighbouring cells (Buchon et al., 2009a).

The *A. gambiae* genome encodes two homologues of the STAT transcription factor, STAT1 and STAT2 (Waterhouse et al., 2007). In *Drosophila*, the

JAK/STAT pathway appears to contribute to antiviral immunity, where it induces a set of effector genes distinct from those induced by the Toll and Imd pathways (Dostert et al., 2005). The same seems to be true in mosquitoes, where JAK/STAT has been shown to play a role in anti dengue defence in *A. aegypti* (Souza-Neto et al., 2009). There is early evidence that the JAK/STAT pathway responds to bacterial infection in *A. gambiae* (Barillas-Mury et al., 1999), though it does not appear to be necessary for survival to systemic infection (Gupta et al., 2009). Interestingly, this pathway plays a dual role in *Plasmodium* infection, with STAT signalling supporting the survival of early stage *P. berghei* oocysts but mediating lysis of late stage oocysts (Gupta et al., 2009).

Pathogen killing: lysis, phagocytosis and melanisation

One of the outcomes of the above pathways is the killing of foreign organisms by lysis, phagocytosis or melanisation (Fig 1.2.2).

Figure 1.2.2

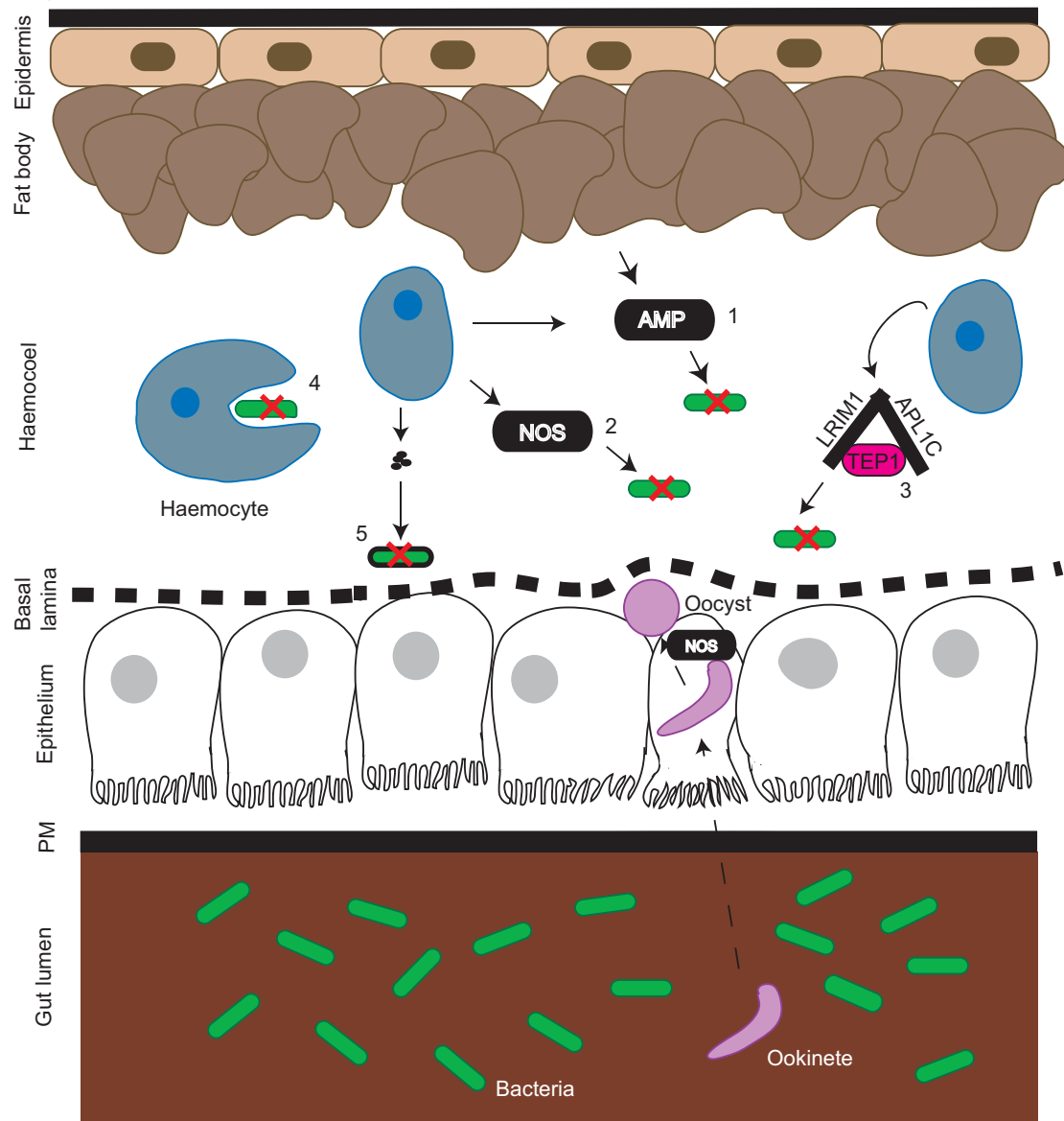


Figure 1.2.2. The major mechanisms of pathogen killing in *Anopheles gambiae*. (1) The fat body and haemocytes produce antimicrobial peptides (AMPs) that cause pathogen lysis. (2) Nitric oxide synthase (NOS) transcription is induced in epithelial cells by invading ookinetes and in haemocytes by systemic bacterial infection. (3) Haemocytes secrete a complement-like complex that is deposited on the surface of bacteria and *Plasmodium* parasites, resulting in their lysis or melanisation. (4) Pathogens are phagocytosed by a subpopulation of haemocytes, the granulocytes. (5) A prophenoloxidase cascade results in the deposition of melanin on the pathogen surface.

AMPs are small, stable peptides that are believed to kill pathogens by acting at the membrane, causing lysis. They can be synthesised by the fat body, haemocytes and epithelia. In *Drosophila* they have specific activity against Gram-negative bacteria, Gram-positive bacteria or fungi (reviewed in Lemaitre & Hoffmann, 2007). The *A. gambiae* genome encodes four families of AMP: four cecropins, four defensins, one attacin and one gambicin (Waterhouse et al., 2007). The cecropins have broad spectrum activity against both Gram types of bacteria, filamentous fungi, yeast, viruses, *Leishmania* and *P. berghei* (Kim et al., 2004; Luplertlop et al., 2011; Vizioli et al., 2000) and gambicin, which is a mosquito specific AMP, is similarly active against both Gram types of bacteria, fungi and *P. berghei* ookinetes (Vizioli et al., 2001a). Defensin seems to be more specific in its activity, killing Gram-positive bacteria and filamentous fungi but with no effect on yeast, Gram-negatives or *Plasmodium* (Blandin et al., 2002; Vizioli et al., 2001b). The *A. gambiae* genome also encodes eight C-type lysozymes that act by hydrolysing peptidoglycan. Interestingly, in addition to its antibacterial role, LYSC1 appears to facilitate *Plasmodium* development and has been shown to bind both *P. falciparum* and *P. berghei* oocysts *in vivo* (Kajla et al., 2011).

In addition to AMPs, immune signalling also induces the production of reactive nitrogen and oxygen species. Nitric oxide synthase (NOS) catalyses the conversion of L-arginine to L-citrulline, producing the nitric oxide free radical in the process. NOS is transcriptionally induced in haemocytes in response to systemic bacterial infection (Hillyer and Estévez-Lao, 2010) and in midgut epithelial cells in response to *Plasmodium* infection (Luckhart et al., 1998). In the latter case this has been reported to be dependent on the activation of kinases associated with insulin signalling by the parasite's glycosylphosphatidylinositol (GPI) anchor and hemozoin (Akman-Anderson et al., 2007; Lim et al., 2005), and, in a separate study, JAK/STAT signalling (Gupta et al., 2009). Nitric oxide limits bacterial proliferation, *Plasmodium* development and dengue infection (Hillyer and Estévez-Lao, 2010; Luckhart et al., 1998; Ramos-Castañeda et al., 2008). Similarly, resistance to both systemic bacterial infection and *Plasmodium* infection correlates with systemic levels of ROS, and the presence of ROS is essential for effective antibacterial

and anti-*Plasmodium* responses (Kumar et al., 2003; Molina-Cruz et al., 2008). Mitochondrial and membrane bound NADPH oxidases produce the highly toxic superoxide anion; levels of superoxide are kept under close control by detoxifying enzymes such as superoxide dismutase (SOD) which converts superoxide to the less toxic hydrogen peroxide, and catalase, which further breaks down hydrogen peroxide. Interestingly, *P. falciparum* infection of *A. stephensi* has recently been shown to activate p38 mitogen activate protein kinase (MAPK) signalling, which has the effect of reducing ROS generation and, broadly, immune gene induction, resulting in longer mosquito life span and greater permissiveness to parasite development (Wang et al., 2015).

One of the best characterised pathogen killing pathways in the mosquito is the complement-like response. TEP1 is a soluble haemolymph protein that is similar in structure to vertebrate C3 (Baxter et al., 2007). In the vertebrate system, proteolytic cleavage leads to deposition of C3 on the pathogen surface, opsonising it for clearance by lysis or phagocytosis. TEP1 appears to play an analogous role in mosquitoes: it is cleaved in response to septic injury and binds bacterial surfaces via a thioester bond, promoting phagocytosis (Levashina et al., 2001). It similarly binds *P. berghei* ookinetes in the midgut basal lamina, resulting in parasite death via lysis and/or melanisation (Blandin et al., 2004). TEP1 deposition is controlled by two leucine rich repeat containing proteins (LRRs), LRIM1 and APL1C (Osta et al., 2004; Riehle et al., 2006, 2008) that circulate in the haemolymph as a disulphide bonded dimer and are necessary for TEP1 binding to *P. berghei* ookinetes (Povelones et al., 2009). The mechanics of this pathway are under continuing investigation, with both positive and negative regulators being recently identified (Povelones et al., 2013; Yassine et al., 2014). Interestingly, the role of the complement response against *P. falciparum* infection has so far been unclear, with one field study reporting no effect of LRIM1 knock down on *P. falciparum* infection (Cohuet et al., 2006) and another laboratory study suggesting that APL1C may be replaced by a paralogue, APL1A, in the context of *P. falciparum* infection (Mitri et al., 2009). However, TEP1 itself does appear to be active against human malaria parasites (Dong et al.,

2006a; Garver et al., 2009). Recent work suggests that *P. falciparum* may evade the complement response by repressing midgut nitration, which 'tags' the parasite for targeting by the complement pathway (Molina-Cruz et al., 2013; Ramphul et al., 2015).

Phagocytosis occurs solely in the haemolymph by a highly abundant population of haemocytes, the granulocytes. The pathogen is either opsonised by circulating humoral factors or recognised directly by transmembrane haemocyte receptors, then internalised by the haemocyte into a phagosome, which fuses with the lysosome before pathogen digestion (reviewed in Julián F Hillyer & Strand, 2014). This defence mechanism is known to be effective against bacteria (Hillyer et al., 2004), yeast (Da Silva et al., 2000) and, to an extent that seems to vary with species, *Plasmodium* sporozoites (Hillyer et al., 2003, 2007). Several mosquito proteins have been implicated in initiating phagocytosis, including the transmembrane IMD pathway receptor PGRPLC, the complement-like factors TEP1, 3 and 4, and their associated LRRs (Moita et al., 2005).

A separate subpopulation of haemocytes, the oenocytoids, produce the enzymes involved in melanisation. Insect melanisation is essential for several physiological processes besides immunity, including cuticle hardening and egg darkening, and involves the deposition of melanin and cross-linked proteins on the surface of a pathogen, causing its death. The rate-limiting step in melanisation is the conversion of tyrosine to melanin precursors, which is catalysed by phenoloxidase (PO). PO exists in zymogen form, ProPO, and a proteolytic cleavage cascade that is activated upon pathogen recognition results in its activation. This cascade involves a multitude of PRRs, CLIP domain serine proteases and serine protease inhibitors (Yassine and Osta, 2010). There was considerable interest in melanisation with the observation that a laboratory strain of *A. gambiae*, L3-5, that was selected for *Plasmodium* refractoriness, was observed to kill ookinetes by melanisation (Collins et al., 1986). However, it has since transpired that non-selected laboratory strains and wild caught mosquitoes rarely melanise ookinetes, and indeed that melanisation may only be a relevant response in allopatric mosquito-parasite

species combinations (Michel et al., 2006; Schwartz and Koella, 2002). Similarly, although mosquitoes are observed to melanise Gram-positive and Gram-negative bacteria in the haemolymph, genetic disruption of the melanisation response does not compromise their ability to resist the infection (Schnitger et al., 2007). More recent evidence, however, suggests that melanisation is an important response in conferring resistance to fungal infection (Yassine et al., 2012).

Despite representing the best characterised components of the mosquito immune system, this discussion is by no means comprehensive. Several characterised genes have a clear role in immunity though it is not clear where they fit in to the above scheme. For example, two C-type lectins, CTL4 and CTLMA2, play an apparently contradictory role whereby they are negative regulators of *Plasmodium* melanisation yet are essential for resistance to Gram-negative bacterial infection (Schnitger et al., 2007). What's more, many studies have focused on pathogen killing in the haemocoel, with little understanding of how microbes in the gut lumen interact with and are controlled by the immune system. A recent genome wide association study using an oral bacterial infection model identified several novel immune modulators that control the outcome of infection, including a gustatory receptor that links immunity to feeding behaviour for the first time in the mosquito (Stathopoulos et al., 2014). This study highlighted that there is much still to understand about the gut immune response, and especially how this response is modulated to tolerate constant exposure to microbial elicitors in the gut, in the form of the microbiota.

1.3 Microbiota

Microbiota in Health and Disease

Recent years have seen rapidly growing interest in microbiota, defined as the complement of microorganisms that live in symbiosis with a host organism (Sommer and Bäckhed, 2013). Studies have focused both on describing the species of microorganisms living in animal hosts (Ellis et al., 2013; Mathieu et al., 2013) and their impact on host physiology (Devaraj et al., 2013; Pope et

al., 2012), resulting in increasingly complex models of the microbiota not as an immunological threat but with an important, if not essential, role in host health. The microbiome (the collective genomes of the microbiota) is a dynamic entity that changes in size and composition in response to changes in host lifestyle (diet, threat of infection, use of antibiotics), unlike the host genome, which must remain static (Rosenberg and Zilber-Rosenberg, 2011). This arrangement is mutually beneficial to host and bacteria, with the host providing a stable, nutrient-rich environment and bacteria aiding in digestion, protection against opportunistic pathogens and maturation of the immune system. Indeed, the impact of the microbiota on host physiology cannot be understated: in humans, microbiota perturbation has been associated with not only gastrointestinal and metabolic disorders (such as obesity, irritable bowel disease, colorectal cancer and diabetes), but also several neuronal disorders such as attention deficit hypersensitivity disorder, multiple sclerosis and depression (Marchesi et al., 2015; Petra et al., 2015). Similarly, in the mosquito the microbiota are increasingly understood to influence host susceptibility to protozoan and viral infection, as well as several other aspects of mosquito physiology with relevance to disease transmission.

The Microbiota of *Anopheles gambiae*

Mosquito microbiota composition has been studied primarily by sequence analysis of the 16S rRNA gene, using both culture dependent and culture independent methods. Over 100 bacterial genera have now been identified in *Anopheles* larval to adult stages in both field caught mosquitoes and laboratory colonies (reviewed in Villegas & Pimenta, 2014), though no genera have been reported in all studies- suggesting that *Anopheles* mosquitoes do not harbour obligate symbionts. The most commonly reported genera are *Pseudomonas*, *Aeromonas*, *Asaia*, *Elizabethkingia*, *Enterobacter*, *Klebsiella*, *Pantoea* and *Serratia* (Gendrin & Christophides, 2013), all of which are Gram-negative bacteria, and all of which are *Proteobacteria* with the exception of *Elizabethkingia*, a *Bacteroidetes*. The frequency with which these genera are reported may suggest that they constitute something of a 'core mosquito microbiota'. Figure 1.3.1, taken from one of the most recent meta analyses of microbiota reported in field mosquitoes (Minard et al., 2013), illustrates the

diversity of bacterial phyla identified in *A. gambiae* and *A. stephensi* larvae and adults.

Figure 1.3.1

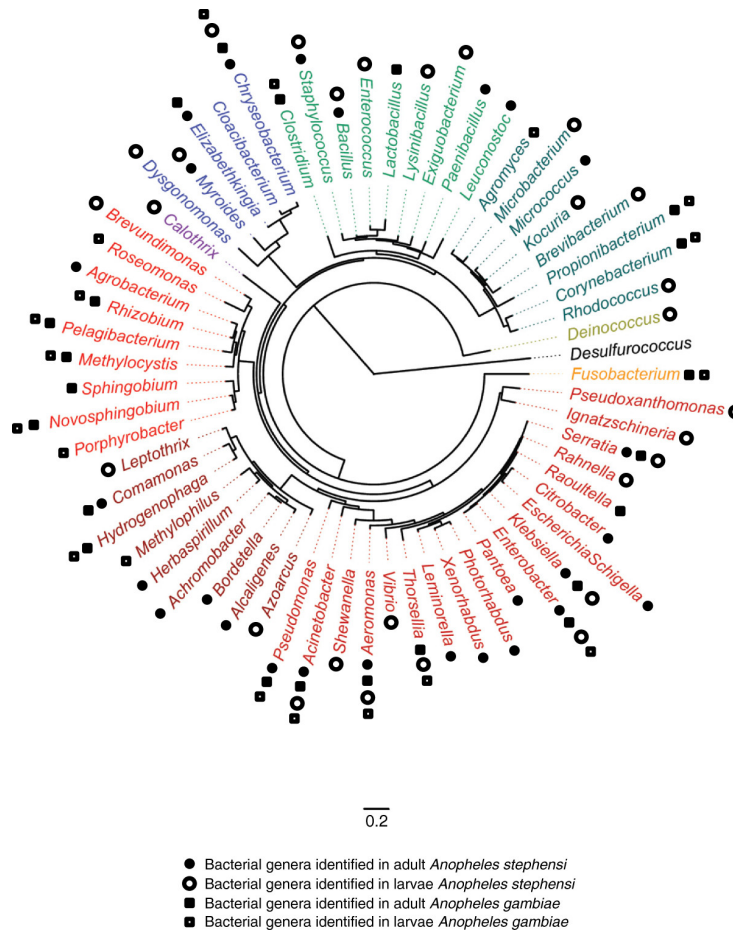


Figure 1.3.1. Phylogenetic dendrogram of genera identified in the larvae and adult stages of *A. stephensi* and *A. gambiae*, taken from Minard et al., 2013. Text colour represents the phylum: red- Proteobacteria, blue- Bacteroidetes, green- Actinobacteria, Firmicutes, Tenericutes, purple- Cyanobacteria, orange- Fusobacteria, yellow- Deinococcus-Thermus. Tree constructed using the maximum likelihood method with HKY model using 100 bootstraps, using *Desulforococcus* as the outgroup. This figure was distributed under a Creative Commons Licence that allows its reproduction.

In all studies to date looking at field or semi-field African anopheline mosquitoes, *Proteobacteria* have dominated the adult gut (Boissière et al., 2012; Gimonneau et al., 2014; Osei-Poku et al., 2012; Tchioffo et al., 2013; Wang et al., 2011). It has also been reported that gut community structure changes drastically upon maturation, with a decrease in microbial diversity from larvae to sugar fed adults, and the lowest diversity in blood fed adult females (Wang et al., 2011). Several studies have noted the large variation in composition between individuals within a mosquito species and the fact that each individual hosts a small number of dominant taxa (Boissière et al., 2012; Osei-Poku et al., 2012). For example, one study in Kenyan populations estimated individuals to harbour a median of 42 operational taxonomic units (OTUs), though on average the four most abundant of these constitute 90 % of the total population (Osei-Poku et al., 2012). Finally, there is some indication that microbiota composition is influenced by the larval breeding site (Boissière et al., 2012; Gimonneau et al., 2014), though collection site did not predict microbiota composition in another study using adult collection, suggesting that breeding site acquired compositions may be masked by bacteria acquired during adulthood (Osei-Poku et al., 2012). The majority of mosquito microbiota research to date has been bacteria centric, with very little known about fungal and viral components of the gut ecosystem. Early evidence does suggest, however, that mosquitos harbour complex viral and fungal communities (Chandler et al., 2015).

Importantly, these studies have additionally given some insight into the extent to which the microbiota of laboratory mosquito colonies reflects that of their field counterparts. Two reports have addressed this directly for *A. gambiae*, with one concluding that although laboratory mosquitoes have lower overall diversity than field mosquitoes, the majority of genera in laboratory mosquitoes are also found in the field (Wang et al., 2011). The other found the N'gousso colony that they analysed to be dominated by *Elizabethkingia* (Boissière et al., 2012). In agreement with both of these conclusions, other microbiota-based analyses from laboratory colonies have reported similar taxa to those identified in field studies, though in some cases with an over

dominance of *Bacteroidetes* (in particular *Flavobacteriaceae*) instead of *Proteobacteria* (Coon et al., 2014; Ngwa et al., 2013; Stathopoulos et al., 2014). The fact that there is some maintenance of the native microbiota upon mosquito colonisation, together with the observation that the same genera tend to be found in separate field collections, suggests that there is to some extent a 'core microbiota'. Whether this is due to host selectivity, bacterial adaptation or bacterial heritability, or some combination of the three, remains unclear. Having said that, it does also appear to be the case that the bacteria found in the mosquito gut are widespread in other environments, so opportunistic colonisation by any species that is able to persist in the gut environment (considering its pH, redox potential, substrate availability etc) is likely to shape the community to a great extent. The variation seen between laboratory colonies in the *Bacteroidetes: Proteobacteria* ratio could stem from differences in husbandry, such as larval diet or water purity, between laboratories.

Indeed, one question that remains poorly addressed is how adult mosquitoes acquire their microbiota. It is known that some bacterial genera, such as *Asaia*, are transmissible both horizontally (between individuals of the same generation) and vertically; in the latter case this may be via smearing of the bacteria onto eggs in the reproductive tract (Damiani et al., 2010). Larvae feed on microorganisms in their aquatic environment, giving a clear mechanism for gut colonisation; however, upon metamorphosis the gut is thought to undergo two processes of sterilisation, leaving the newly emerged adult gut almost free of bacteria. First, 4th instar larvae egest the contents of the alimentary canal and the peritrophic matrix before pupation, and, second, emerging adults ingest exuvial fluid that has bactericidal properties (Moll et al., 2001). Nevertheless, this process is incomplete and adult guts are able to be colonised to some extent trans-stadially (Coon et al., 2014). Adult mosquitoes are also thought to recolonise their guts by feeding on larval water (Lindh et al., 2008), and they likely acquire new bacteria from the environment whilst taking sugar meals from flowers (Manda et al., 2007).

The most striking characteristic of the mosquito microbiota is its behaviour following a bloodmeal, when bacterial load increases from 10 to 100 fold in the first 24 h following the bloodmeal (Kumar et al., 2010; Pumpuni et al., 1996). The cause of this bacterial growth is not clear, though two main, non-mutually exclusive, explanations have been proposed. First, the ingestion of blood may provide gut bacteria with the nutritional sustenance for an increased rate of proliferation. Second, following the blood meal the gut may reduce its level of immune control, essentially permitting bacteria that are under tight control in the non-blood fed gut to proliferate (Kumar et al., 2010; Oliveira et al., 2011). The potential mechanisms behind this are discussed further below. By 48-72 h post bloodmeal, bacterial load has receded back to pre-bloodmeal levels; again, the mechanism that brings bacterial load back to homeostatic levels has not been elucidated.

The contribution of the microbiota to mosquito physiology is also an open question. In other insects, the microbiota have been shown to play diverse roles in host nutrition including digestion of plant polymers (Engel et al., 2012; Kaufman and Klug, 1991), provisioning nutrients such as essential amino acids and B vitamins (Douglas, 1998; Nikoh et al., 2011; Schaub and Eichler, 1998) and neutralising dietary toxins (Dowd and Shen, 1990). The role of the microbiota in providing nutritional sustenance to adult mosquitoes is unclear. In *A. aegypti*, a 90-100 % depletion of the microbiota was found to cause a reduction or delay of red blood cell lysis that was concomitant with a reduction in mosquito fecundity (Gaio et al., 2011). Similarly, in *A. albopictus* two isolated *Acinetobacter* species were found to have specifically gained the capacity to metabolise amino acids and common plant components, suggesting that strains found within the mosquito host have adapted to digest blood and nectar respectively (Minard et al., 2013). What's more, it has been noted in one study that blood feeding induces a shift in microbiota composition to favour the *Enterobacteriaceae*. Genera within this family contain more genes known to be involved in combatting oxidative and nitrosative stress than genera prevalent in the sugar fed gut, appropriate for the environment rich in reactive oxygen and nitric oxide species that is associated with blood meal catabolism (Wang et al., 2011). More broadly

speaking, microbiota clearance by antibiotic treatment has been shown in *A. stephensi* to have a mildly detrimental effect on mosquito longevity and a more severe negative effect on fecundity (Sharma et al., 2013).

The *Anopheles* microbiota additionally have a fundamental impact on larval development. Several studies have shown retardation of larval development with antibiotic treatment (Chouaia et al., 2012; Mitraka et al., 2013), and another that fully axenic larvae cannot survive beyond the 1st instar (Coon et al., 2014). In the latter case this was not due to the loss of bacteria as a food source, as even when provided with heat-killed bacteria that were shown to be ingested, the larvae did not develop. Despite this, the existence of an aseptic colony has been reported; however, the nutritional composition of the larval diet that supports this colony is not publically available (Lyke et al., 2010). A similar developmental delay is observed in *Drosophila* larvae, and has been ascribed to two mechanisms: first, *Acetobacter pomorum* promotes insulin signalling by production and metabolism of acetic acid (Shin et al., 2011) and secondly *Lactobacillus plantarum* facilitates dietary amino acid accumulation, which signal via the TOR kinase, again promoting insulin signalling (Storelli et al., 2011).

Microbiota-Immune Interactions

Despite the microbiota playing a key role in the physiology of the mosquito, at least in the larval stage, the proximity of bacteria to epithelial membranes and their sheer numbers present a challenge to the host immune system, which must develop systems to balance the ability to recognise pathogenic organisms and manage microbiota growth whilst avoiding chronic activation. This conundrum has led to the concepts of immune resistance and tolerance; resistance responses combat infection by reducing microbial load, whilst tolerance responses aim to reduce the impact of the infection on the host. The latter may involve either reducing or avoiding the induction of an immune response, which can itself be harmful to the host, or repairing microbial or immunity derived tissue damage.

An understanding of both resistance and tolerance mechanisms in the *Anopheles* gut could open up novel approaches for transmission blocking. Targeting resistance responses could introduce dysbiosis or uncontrolled microbiota growth in the gut, resulting in shortened mosquito life span. Equally, targeting a tolerance response could also affect host life span- excessive immune activation is known to be detrimental to health- whilst having the added effect of increasing resistance to *Plasmodium* infection. It is paramount to study these responses specifically in the gut for two reasons: first, this is the tissue that houses the microbiota, as well as being the site of a vulnerable stage of parasite development, the ookinete to oocyst transition. Second, any intervention must be able to access its target, rendering molecules that are either secreted into the gut lumen or situated in the luminal membrane of the epithelium ideal targets for orally administered drugs.

Understanding of microbiota-gut interactions remains in its infancy in the mosquito. It is known that midgut bacterial load is limited by the IMD pathway in both sugar fed and blood fed guts (Clayton et al., 2013; Dong et al., 2011; Dong, Manfredini, & Dimopoulos, 2009; Meister et al., 2009). Indeed, microbiota dependent IMD pathway induction is thought to be one of the reasons for the consistent observation that antibiotic treated mosquitoes have increased susceptibility to *Plasmodium* infection (Dong et al., 2009; Meister et al., 2009). There has been one transcriptional analysis looking at the effect of microbiota depletion on whole mosquitoes; this revealed that considerably fewer genes are regulated by the presence of the microbiota than by oral bacterial infection, and identified a host of immune genes that are responsive to the gut microbiota including AMPs, PRRs and signal transducing serine proteases (Dong et al., 2009). Nevertheless, a more complete picture of the gut immune system has emerged in *Drosophila*: this discussion will therefore be based around the *Drosophila* system, with reference to *Anopheles* where information is available.

There are two main resistance mechanisms in gut epithelial immunity in *Drosophila*: AMP production, exclusively driven by the Imd pathway, and ROS

production, driven in the gut by the dual oxidase (DUOX) system. Both of these are now understood to be negatively regulated at multiple levels, preventing their overstimulation by gut commensals.

First, the amidase PGRPs act at the level of the peptidoglycan ligand to reduce Imd pathway stimulation. *Drosophila* PGRP-LB is able to hydrolyse both polymeric DAP-type peptidoglycan and its monomer, TCT, to non-immunostimulatory compounds (Zaidman-Rémy et al., 2006). It is induced by the Imd pathway and has a major role in the gut in preventing excessive microbiota dependent induction of AMPs and intestinal stem cell activity (Paredes et al., 2011; Zaidman-Rémy et al., 2006). PGRP-SC1 and 2 appear to play a similar role, though in the response to systemic infection (Paredes et al., 2011), and have also been implicated in the epithelial response in larvae (Bischoff et al., 2006). PGRP-SB1 and 2 have demonstrated enzymatic activity against DAP-type peptidoglycan, but so far no significant effect *in vivo* has been demonstrated in terms of resistance to infection or immune regulation (Paredes et al., 2011; Zaidman-Rémy et al., 2011). A role as an immune effector is possible, whose significance may so far be masked due to redundancy with other antimicrobial peptides. *A. gambiae* encodes a 1:1 orthologue of PGRP-LB, along with two other catalytic PGRPs, PGRPS1 and S2, though these have not been functionally analysed.

Drosophila PGRP-LF is encoded by a gene in the same cluster as the Imd pathway receptors PGRP-LC and PGRP-LA, and acts as negative regulator of the Imd pathway. It is a transmembrane protein with a small intracellular tail and two extracellular PGRP domains that are not able to bind peptidoglycan (Basbous et al., 2011). PGRP-LF knockdown causes activation of the Imd pathway independently of infection, whilst overexpression causes downregulation of the response to infection (Maillet et al., 2008; Persson et al., 2007). PGRP-LF is able to bind to PGRP-LCx, suggesting a mechanism whereby the stoichiometric ratio of LF to LC determines the pathway's responsiveness to a given level of ligand (Basbous et al., 2011). In addition to PGRP-LF, Pirk (previously known as PIMS and Rudra) is an intracellular protein that interacts with the cytoplasmic domain of PGRP-LC, changing its

subcellular localisation such that it does not associate with the membrane and thereby downregulating signalling (Aggarwal et al., 2008; Kleino et al., 2008; Lhocine et al., 2008). Pirk expression is enriched in the gut in a manner that is dependent on the gut microbiota and Relish signalling (Lhocine et al., 2008). Interestingly, *A. gambiae* does not encode orthologues of either PGRP-LF or Pirk, raising the question of which mechanisms, if any, are employed to downregulate the IMD pathway.

Finally, Caudal is a homeobox transcription factor that is expressed exclusively in the gut where it specifically represses the NF- κ B dependent expression of AMPs but not of the negative regulators of the Imd pathway (Ryu et al., 2008). Similarly, in *Anopheles* Caudal has been shown to repress AMP expression, support *P. falciparum* development and maintain microbiota load (Clayton et al, 2013).

The other main resistance mechanism in *Drosophila* is ROS production, driven by the DUOX system. DUOX is a membrane bound NADPH oxidase that is indispensable for the defence against oral bacterial infection (Ha et al., 2005) and plays an essential role in limiting microbiota (primarily yeast) proliferation in non-infected flies (Ha et al., 2009a). DUOX is regulated at several levels; the DUOX activation pathway is dependent on phospholipase C- β (PLC β) activity (Ha et al., 2009a), whilst *DUOX* expression is regulated in a PGRP-LC and p38 dependent manner. Furthermore, in non-infected conditions, low PLC β activity inhibits *DUOX* expression via negative regulation of p38 phosphorylation, whilst in infected conditions high PLC β activity activates the *DUOX* expression pathway upstream of p38 (Ha et al., 2009b). Both of these regulatory mechanisms are essential for survival to varying levels of microbial ligand: the former prevents commensal bacteria from inducing excessive oxidative activity, whilst the latter is essential for resistance to high bacterial loads that are indicative of infection. Interestingly, DUOX is regulated by two microbial ligands: peptidoglycan, which is recognised by PGRP-LC, and the nucleobase uracil, which activates the PLC β pathway via hedgehog signalling (Lee et al., 2013, 2015). Incredibly, uracil was found to be secreted by pathogenic bacteria but not bacterial

constituents of the *Drosophila* microbiota, revealing for the first time a mechanism by which the innate immune system is able to discriminate between pathogens and symbionts (Lee et al., 2013, 2015).

In *Anopheles*, DUOX has been characterised as playing a slightly different role in antimicrobial defence; it coordinates with a haem peroxidase, IMPer (also known as HPX15), to catalyse the formation of dityrosine bonds in a mucin protein layer between the peritrophic matrix and the gut lumen following blood feeding (Kumar et al., 2010). This has the dual effect of reducing the elicitation of an immune response to both the gut microbiota and *Plasmodium* parasites, indicating one means by which ROS may partake in a tolerance response. The role of ROS as a microbiota resistance mechanism in the gut lumen is less clear. At least one bacteria strain (an *Enterobacter* species) found in the gut of field caught *A. arabiensis* itself produces ROS, which are detrimental to *P. falciparum* development as well as likely supporting *Enterobacter* sp. dominance within the gut ecosystem (Cirimotich et al., 2011). Interestingly, one study in *A. aegypti* has suggested that DUOX-produced ROS play a key role in preventing microbiota proliferation in the sugar fed gut, but that the presence of haem in the bloodmeal triggers an immediate reduction in ROS generation, via protein kinase C signalling, with this being partially responsible for the microbiota growth observed upon blood feeding (Oliveira et al., 2011). This is proposed to be an adaptation to the pro-oxidant effect of haem, which catalyses the conversion of oxidised molecules to highly reactive intermediates in the lipid peroxidation chain.

All in all, the recent explosion of interest in the mosquito gut microbiota has opened up the possibility of several novel interventions for vector borne diseases. Through introduction of specific bacterial isolates to mosquito hosts, paratransgenesis or development of drugs that target mosquito immune modules, there are several potential means by which manipulation of the microbiota could result in a reduction of mosquito life span or an increase in resistance to *Plasmodium* parasites, either of which should translate to lowered malaria transmission. For such interventions to be feasible, several key questions remain to be addressed. Not least, the contribution of the

microbiota to mosquito physiology and the resistance and/or tolerance responses employed by the gut to allow persistence of the microbiota whilst maintaining vigilance to infection. The work described herein seeks to address both of these questions.

1.4 Aims

1. To elucidate the contribution, if any, of the midgut microbiota to the physiology of the adult mosquito.
2. To elucidate the mosquito epithelial immune response to the gut microbiota, especially with regards to the dynamic changes in bacterial load observed over the blood feeding cycle. In particular, to explore the balance between tolerance and resistance responses induced after blood feeding.
3. To look specifically at the role of IMD pathway receptor PGRPLC in inducing the IMD pathway in the gut, particularly in the light of the absence of many of the modulators of the IMD pathway described in *Drosophila* (PGRP-LE, Pirk, PGRP-LF).
4. To determine whether manipulation of the native microbiota can influence disease transmission. This is to be investigated primarily in terms of the presence of antibiotics in human blood. An effect of microbiota manipulation on vector competence would not only have implications for disease transmission in regions with high antibiotic usage, but would also be suggestive of mechanisms by which mosquito transmission capacity could be manipulated.

Chapter 3.1 addresses aims 1 and 4; here, it is shown that depletion of microbiota growth following a bloodmeal both increases the survival rate and the fecundity of the mosquito. In chapter 3.2, aims 1 and 2 are addressed by an RNA sequencing study; this analysis identifies the peritrophic matrix as playing a key role in maintaining homeostasis in the gut. Finally, chapter 3.3 addresses aim 3 via both *in vivo* and *in vitro* functional analysis of the IMD pathway receptor PGRPLC.

Chapter 2: Materials and Methods

Mosquito colony maintenance

The *A. coluzzii* (formerly *A. gambiae* M molecular form) N'gousso colony, established from field mosquitoes collected in Yaoundé, Cameroon, in 2006, was used in all experiments described here. Eggs were hatched in 0.1 % salt water and larvae fed Tetramin or Nishikoi fish food. All adults were allowed *ad libitum* access to 5 % w/v fructose solution and females were maintained on human blood. The insectary was maintained at 27 °C (± 1 °C), 70-80 % humidity with a 12 h light/dark cycle. Female mosquitoes were used exclusively for all experiments described herein.

Human blood feeding

Human blood for mosquito feeding was acquired from the NHS blood service or from local donors. Wherever possible blood was used within one week of its donation. Blood was maintained at 37 °C on a membrane-feeding device or in a parafilm covered Petri dish warmed with a hand warmer. Mosquitoes were allowed to feed for 1 h and non-engorged mosquitoes removed within 24 h. For experiments involving multiple blood meals, mosquitoes were offered egg dishes for oviposition the night before each subsequent bloodmeal.

Antibiotic treatment

Two antibiotic treatments were employed here: a therapeutic treatment (chapter 3.1), intended to replicate the antibiotic concentration in the blood of a patient undergoing treatment, and a robust treatment (chapters 3.2 and 3.3) intended to completely clear the mosquito guts of all bacteria. In the former case, well-mixed blood from the same donor was supplemented with antibiotics (20 U/ml penicillin and 20 µg/ml streptomycin) or an equal volume of water (as a control) immediately prior to feeding. In the latter case, antibiotics (60 U/ml penicillin, 60 µg/ml streptomycin and 50 µg/ml gentamicin) or an equal volume of water were supplemented in the sugar solution offered from emergence, in the blood feed and in the egg dish provided for oviposition.

***Plasmodium berghei* infection**

TO strain mice were used for mouse blood feeding and *P. berghei* infections. Mice were infected with EF1 strain *P. berghei* parasites by intraperitoneal injection 2-3 days prior to mosquito feeding. Before feeding, parasitemia was estimated with a Giemsa-stained blood smear. Mosquitoes were allowed to feed on anaesthetised naïve or infected mice for approximately 20 minutes and non-engorged mosquitoes removed within 24 h. Fed mosquitoes were maintained at 21 °C, the temperature permissive for *P. berghei* development. For antibiotic treatment of mice, after the control cohort had fed on an infected or naïve mouse the penicillin-streptomycin cocktail was injected intravenously and allowed to diffuse throughout the blood for 10 minutes before the same mouse was offered to the experimental cohort. The volume of antibiotic solution injected was calculated based on the mass of the mouse, considering that a mouse contains 92 ml blood/kg body mass (Riches et al., 1973), to give a final blood concentration of 20 U/ml penicillin and 20 µg/ml streptomycin.

Survival analysis

Age-matched 2-6 day old mosquitoes were offered one mouse blood meal or three consecutive human blood meals. Survival was monitored and carcasses removed on a daily basis.

Fecundity and fertility analysis

Mosquitoes were given a human blood meal and stored in individual cups with filter paper moistened with distilled water for oviposition. Eggs were counted upon laying, with those individuals not laying eggs after at least six days being considered non-egg layers. For fertility analyses, eggs were counted and floated in 0.1 % salt water. Larvae were counted and removed on a daily basis. Any eggs remaining after at least one week were considered unhatched.

Blood meal quantification

For quantification of blood meal size, fed female mosquitoes were stored individually in plastic vials until they laid their faecal pellets. Faecal pellets were dissolved in 1 % lithium carbonate solution and the absorbance of the

resulting solution read at 387 nm, according to (Briegel, 1980). Haematin concentration was calculated based on a standard curve constructed from porcine serum haematin (Sigma).

Midgut and fat body dissection

Prior to dissection mosquitoes were surface sterilised by immersion in 75 % ethanol for 3-5 minutes and washed three times in phosphate buffered saline (PBS). This was to cross link bacteria to the mosquito carcass to minimize environmental contamination of dissected midguts. Midguts were removed under a dissecting microscope and frozen immediately on dry ice in groups of 5-10, then stored at -20 °C until processing. For fat body dissections, the abdominal carcass and attached fat body tissue was dissected, ensuring that all other organs (ovaries, gut, malphigian tubules) were removed. For regional midgut dissections (chapter 3.3), a needle was used to cut the midgut into the specified regions.

RNA extraction and cDNA synthesis for qRT-PCR

Frozen tissues were homogenised in 500 µl Trizol (Invitrogen) and 120 µl chloroform for 30 seconds using a Precellys24 tissue homogenizer (Bertin) with bead beating. After phase separation, RNA was precipitated from the aqueous phase by centrifugation at 4 °C for 30 minutes in isopropanol plus 10 µg/ml glycogen. RNA pellets were washed twice in 70 % ethanol and resuspended in water. Sample RNA concentrations were normalised within experiments and cDNA synthesised from up to 500 ng RNA using the Takara reverse transcriptase kit, according to the manufacturer's instructions. cDNA was diluted 1:5 before further processing.

DNA extraction

Frozen tissue samples were homogenised in 500 µl lysis buffer (100 mM EDTA, 10 mM Tris HCl, pH 7.5) plus 5 µl proteinase K and incubated for 2 h at 55 °C. DNA was extracted in 500 µl phenol-chloroform-isoamyl alcohol and precipitated in ethanol. After an ethanol wash, DNA pellets were resuspended in water.

qRT-PCR

qRT-PCR was used to quantify *A. gambiae* mRNA levels, as well as bacterial load by amplification of the 16S ribosomal RNA gene, employing primers that anneal to a region of the sequence that is common to all eubacteria or to regions specific to certain bacterial families. Primer sequences are listed in Table 6.1 (appendix). In each case the *A. gambiae* ribosomal protein encoding gene S7 was used as an internal control of quantity of input RNA. Expression ratios were then calculated as follows:

$$\text{Expression ratio} = \frac{\text{Primer efficiency } S7^{Ct S7}}{\text{Primer efficiency target gene}^{Ct \text{ target gene}}}$$

Primer efficiencies were determined by amplification of serially diluted targets and are listed in Table 6.1 (appendix). Two negative control reactions were performed: an RNA- control, to control for environmental contamination, and a reverse transcriptase- control, to control for genomic DNA contamination (except where the target was DNA). qRT-PCR amplifications were performed in duplicate using the SYBR premix ex Taq kit (Takara) in a total volume of 10 ul on a 7500 Fast Real Time PCR machine (Applied Biosystems).

RNAseq library preparation and sequencing

All library preparation and sequencing steps described here beyond total RNA purification were performed at the sequencing facility, by facility staff.

Total RNA was prepared as described followed by an additional column purification using the Qiagen RNeasy kit. Libraries were prepared in accordance with the Illumina TruSeq RNA sample preparation v2 guide (Part # 15026495, rev.D, September 2012) for Illumina Paired-End Indexed Sequencing. PolyA mRNA first underwent two rounds of purification using Illumina poly-T oligo-attached magnetic beads. During the second elution, the polyA mRNA was fragmented and primed with random hexamers for cDNA synthesis. After first strand cDNA synthesis the RNA template was removed and a replacement strand synthesised to generate double stranded cDNA. Ends were then repaired, dA base added and Illumina indexing adapters

ligated. cDNA fragments with adapters on both ends underwent 15 cycles of PCR, libraries were validated with the Agilent 2100 bioanalyser to check size distribution. Samples were then quantified by qRT-PCR, the concentrations normalised and samples pooled according to biological replicate (i.e. all time points from replicate one pooled together and so on). Pools were loaded at 10 pM onto four lanes of an Illumina flowcell v3 and sequenced using the Illumina HiSeq 1500, 2 X 100 bp paired end run.

RNAseq quality control and read filtering

Quality control, filtering and alignment were all conducted in the Vectorbase instance of the Galaxy platform (Blankenberg et al., 2010; Giardine et al., 2005; Goecks et al., 2010). Groomed FASTQ files underwent adapter clipping (ILLUMINACLIP with Truseq3 adapter sequences) and were then trimmed by sliding window, averaging a minimum Phred quality score of 20 over 4 bases (Trimmomatic tool version 0.32.1). Only reads with both mate pairs being longer than 20 bp were processed further. These were aligned by Bowtie2 (version 0.4) to a custom built index to filter out non-mRNA reads, composed of all sequences annotated as *A. gambiae* in the SILVA rRNA database (release 119) (Quast et al., 2013), plus all sequences annotated as *A. gambiae* tRNAs and mitochondrially-encoded rRNAs in the AgamP4.2 geneset in Vectorbase.

RNAseq sequence alignment

The splice aware aligner Tophat2 v2.0.9 was used to align paired end reads to the *A. gambiae* PEST genome AgamP4. The mean inner distance between mate pairs was set to -25 with a standard deviation of 60. Default settings were used for alignment with the following exceptions: the maximum number of mismatches allowed between a read and the reference sequence was 5 to allow for the highly polymorphic nature of the *A. gambiae* genome, and the minimum intron length was set to 30 bp. The accepted hits were then filtered such that only reads that were uniquely mapped were accepted for downstream analysis.

Generation of count data

Aligned reads were converted to gene count data using HTSeq, specifying the union mode (Anders et al., 2014). The input gtf file was AgamP4.2 after removal of all features annotated as rRNA.

Differential expression calling

Differential expression analysis was conducted with the DESeq2 package (Love et al., 2014), using HTSeq count tables as input files. For each gene, the DESeq2 package fits a generalised linear model with a negative binomial distribution. For pairwise comparisons at each time point the input parameters were “replicate” and “treatment” (i.e., plus/minus antibiotics), and “treatment” was removed in the reduced model. The Wald test was applied to assess statistical significance followed by a Benjamini-Hochberg adjustment for multiple testing. To identify genes with a significant interaction between time and treatment, a model was specified with the parameters “replicate”, “time”, “treatment” and “time:treatment”, removing the latter in the reduced model. In this case, a likelihood ratio test followed by a Benjamini-Hochberg adjustment for multiple testing was employed to assess statistical significance. In both cases, genes with adjusted p values < 0.1 were considered significantly differentially expressed.

Clustering analysis

Variance stabilising transformation of count data was performed in DESeq2 prior to clustering. A median transformed value of the four replicates was calculated for each condition and soft clustering performed in Mfuzz (Futschik and Carlisle, 2005). Soft clustering does not require *a priori* gene filtering, is noise robust and allows genes to be placed in more than one cluster, making it ideal for time course data. The fuzzifier *m* was chosen with the *mestimate* function, and the optimal number of clusters, 12, was selected based on when the minimum distance between cluster centroids (*Dmin*) declines at a reduced rate.

KEGG/GO enrichment analysis

KEGG and GO term enrichment analysis for cluster cores and differentially expressed genes were performed in g:Profiler (Reimand et al., 2007). For differentially expressed genes, genes were ordered by their fold change for input to the software and a Bonferroni adjustment was made for multiple testing.

Double stranded RNA preparation and injection

Double stranded RNA (dsRNA) was used for transient *in vivo* knock down of target genes by RNAi. The target region was amplified from total *A. gambiae* cDNA using primers flanked with the T7 RNA polymerase promoter sequence (primer sequences listed in Table 6.2). PCR products were verified by size on an agarose gel and/or by sequencing and purified using the Qiagen PCR purification kit. dsRNA was then synthesised from the PCR product by overnight incubation at 37 °C with T7 polymerase and dNTPs from the MEGAscript RNAi kit, according to the manufacturer's instructions. dsRNA was purified using the Qiagen RNeasy kit, adjusted to a concentration of 6000 ng/μl, and stored in aliquots at -20 °C until use. 69 nl of 6000 ng/μl dsRNA (totalling 414 μg) was injected into the thorax of CO₂- anaesthetised 0-2 day old female mosquitoes using the Nanoject II (Drummond Scientific). dsRNA against a region of the *lac* operon (dsLacZ), not present in the *A. gambiae* genome, was injected as a control for the injection process.

Polyoxin D feeding

1 mg of polyoxin D (Calbiochem) was dissolved in 192 μl water to give a 0.01 M (= 100X) solution. Immediately before feeding this was added to human blood at a final concentration of 100 μM, adding an equal volume of water to the control blood meal. After reconstitution polyoxin D was stored at room temperature for no longer than one month before use.

H&E staining

Sectioning and staining were performed by Ms Lorraine Lawrence.

24 h post blood feeding, mosquitoes were decapitated and their legs and thorax removed. Abdomens were fixed overnight at 4 °C in Duboscq's-Brasil

fixative (0.4 % w/v picric acid, 53 % ethanol, 27 % formalin, 7 % glacial acetic acid) and washed in 70 % ethanol. Abdomens were then embedded in 2-3 % agarose cooled to 37-45 °C and sectioned at 5 µm thickness for staining with hematoxylin and eosin (H&E).

Cloning for recombinant protein production

Primer sequences for PGRP ectodomain cloning are listed in Table 6.3 (appendix). The PGRP ectodomains were amplified from an *A. coluzzii* cDNA library by PCR with Phusion polymerase (NEB), according to the manufacturer's instructions. The amplified product of the appropriate size was gel extracted using the Qiagen Gel Extraction Kit to ensure that only spliced product was cloned. The PCR product was cloned by ligation independent cloning using the Ek/LIC cloning kit (Novagen) into the pLEX-10 vector (Novagen) for insect cell expression and its sequence verified. pLEX-10 introduces an N-terminal strep tag II, a C-terminal his tag and a secretion signal peptide.

Sf9 cell maintenance

Sf9 lepidopteran cells were used for expression of mosquito proteins. Cells were maintained at 27 °C in adherent culture in SF900 II serum free medium. Media was refreshed every 2-3 days and cells were split 1:5- 1:10 upon reaching confluency.

Transfection and stable cell line generation

Sf9 cells were transfected with the pLEX-10 vector containing the appropriate insert in 6 well plates, generating three stable cell lines expressing the PGRP ectodomains of LC1, LC2 and LC3 respectively. Cells were seeded at 80 % confluency in 2.5 ml SF900 II medium. 2 µg pLEX-10+insert and 0.2 µg PIE1-neo (Novagen) were added dropwise with 8 µg Cellfectin II reagent (Invitrogen). PIE1-neo expresses the *neo* gene, facilitating selection in the presence of the antibiotic G418. The transfection was performed in a 10:1 ratio of insert plasmid: helper plasmid to maximise the likelihood that resistant cells that have integrated PIE1-neo into their genome have also integrated pLEX-10. 24 h post transfection, the medium was replaced with complete

medium (SF900 II plus 10 % foetal calf serum (FCS)) and cells were re-plated in serial dilutions of 10 %, 5 % and 2.5 %. After 36 h, the medium was changed to complete medium plus 1 mg/ml G418. The selective medium was refreshed every 4 days for approximately 2 weeks, or until resistant colonies were observed. For each transfection, the dilution with single isolated colonies was chosen for further processing. Selection was reduced to 0.3 mg/ml G418 in complete medium and this concentration was maintained throughout. Once the selected cells reached confluency, a sample of conditioned medium was analysed by Western blot for expression of recombinant protein.

Coomassie staining and Western blotting

All blots shown herein are non-reducing, with a 12.5 % resolving gel and 4 % stacking gel. Proteins were separated by SDS-PAGE. For Coomassie staining, gels were washed briefly in distilled water and incubated in Imperial Protein Stain (Fisher), shaking, for 1 h at room temperature, before overnight destaining in distilled water. For Western blotting, proteins were transferred to a nitrocellulose membrane by semi dry transfer (15 V for 30 minutes) and membranes blocked in phosphate buffered saline plus 1 % Tween-20 (PBST) with 3 % bovine serum albumin (BSA), either overnight at 4 °C or for 1 h at room temperature. Primary antibody was added in fresh blocking buffer at the specified concentration and incubated overnight at 4 °C. After three 10 minute washes in PBST, blots were incubated with the specified secondary antibody conjugated with horse radish peroxidase (HRP) at a 1:10000 dilution in PBST with 3 % BSA for 1 hour at room temperature, before three further washes in PBST. Blots were exposed with ECL chemiluminescence substrate (Pierce) and visualised with a Biorad Chemidoc Imager.

Recombinant protein purification

Recombinant proteins were purified from conditioned medium by His tag affinity. TALON beads (Takara) (approx. 250 µl per 150 ml conditioned medium) were equilibrated with two washes in 10 volumes of equilibration buffer (50 mM NaH₂PO₄, 300 mM NaCl, pH 7.0). The beads were incubated with conditioned medium, rotating at room temperature, for 2 h. Beads were

then washed twice with 20 volumes of wash buffer (50 mM NaH₂PO₄, 300 mM NaCl, 10 mM imidazole, pH 7.0) for 15 minutes followed by one wash with 5 volumes of wash buffer for 5 minutes, both at room temperature. His tagged proteins were eluted in 4 X 4 volumes of elution buffer (50 mM NaH₂PO₄, 300 mM NaCl, 150 mM imidazole, pH 7.0). Purified proteins were then dialysed (Amicon Ultra 4 ml dialyser, 10 kDa pore size) overnight at 4 °C into wash buffer to remove the imidazole for further processing.

Protein quantification

Purified proteins were quantified by a BCA assay (Pierce), measuring absorbance at 562 nm in a 96 well plate on a Fluostar plate reader (BMG Labtech). Sample concentration was calculated by comparison to a standard curve constructed from BSA serial dilutions.

His tag cleavage

The C terminal his tag was cleaved with ProTEV plus protease (Promega), using the TEV cleavage site that was introduced with the reverse primers during cloning. Approximately 200 µg recombinant protein was incubated with ProTEV cleavage buffer, 1mM DTT and 100 units of ProTEV protease for 3 h at 30 °C. Cleaved protein was isolated from the His tag and the protease itself, which also has a His tag, using TALON beads as described above, retaining the flow through containing the cleaved protein.

PNGase F treatment

5 µg recombinant protein was incubated in denaturation buffer (0.02 % SDS, 10 mM β-mercaptoethanol) at 100 °C for 10 minutes before being incubated with 2.5 units PNGase F and 0.8 % Triton X-100 at 37 °C for 3 h.

Peptidoglycan binding assay

Polymeric DAP-type peptidoglycan used in this assay is a kind gift from Dr Dominique Mengin-Lecreux.

All peptidoglycan binding assays were conducted with his tag cleaved proteins. Recombinant protein (100 µg/ml) was incubated with insoluble

peptidoglycan from *E. coli* (1 mg/ml) in 50 µl binding buffer (150 mM NaCl, 20 mM HEPES, pH 7.5) for 1 h at room temperature, rotating. The insoluble peptidoglycan was then pelleted by centrifugation (13000 X g, 5 min). The pellet was washed three times in 1 ml binding buffer and resuspended in 50 µl binding buffer. Pellet and supernatant fractions were analysed by Western blot, using a primary antibody against the N-terminal strep tag II.

Protein-protein Interaction Assay

TCT used in this assay is a kind gift from Dominique Mengin-Lecreulx.

For protein dimerisation assays, recombinant protein after cleavage of the His tag (100 µg/ml) was incubated with recombinant protein that retained its His tag (100 µg/ml) in the presence of an approximately 5 fold molar excess of TCT (20 µM) in 50 µl binding buffer for 1 h at room temperature, rotating. His tagged protein and any interacting protein were then purified by His tag affinity, as described above. The flow through and bead bound fractions were analysed for their protein content by Western blot, probing for the N-terminal strep tag II.

Statistical analysis

qRT-PCR expression data (including 16S analysis) and egg counts were analysed by generalized linear mixed models (GLMMs) in R (version 3.1.2). GLMMs fit both fixed-effect parameters and random effects in a linear predictor via maximum likelihood. Mixed effect models were used to account for the use of multiple samples per treatment within each independent replicate, avoiding issues of pseudoreplication. Expression data and egg counts were analysed by an ANOVA test on a linear mixed effect regression model (lmer, in the lme4 package). Binary data, namely the prevalence of egg laying, were analysed by an ANOVA test on a logistic regression model (glmer). Human blood meal survival data were analysed using a log-rank test and mouse blood meal survival data by a Gehan–Wilcoxon test. Different tests were chosen to reflect the experimental setup where human blood meals are given throughout the experiment and a mouse blood meal only on the first day. In both the cases, survival tests were stratified by experimental replicate.

Correlations were analysed with Spearman's rank-order correlation.
Contingency data were analysed with a Chi-square test.

Chapter 3: Results

3.1 Antibiotics in Ingested Human Blood Affect the Mosquito Microbiota and Capacity to Transmit Malaria

Data presented in Figures 3.1.1- 3.1.4 are published in the following article:

“Antibiotics in Ingested Human Blood Affect the Mosquito Microbiota and Capacity to Transmit Malaria” Gendrin M, Rodgers FH, Yerbanga RS, Ouedraogo JB, Basanez MG, Cohuet A, Christophides GK. Nature Communications 6:5921, Jan 2015.

Abstract

Given that the *A. gambiae* microbiota are known to protect their host from the establishment of malaria infection (Dong et al., 2009; Meister et al., 2009), we sought to investigate whether the therapeutic use of antibiotics in a field scenario could impact on the transmission of human malaria, via their effect on the mosquito microbiota. Here it is shown that the presence of a penicillin-streptomycin (PS) cocktail in a human blood meal, at an equivalent concentration to that which would be found in the peripheral blood stream of a human undergoing antibiotic treatment, depletes the mosquito gut microbiota. Microbiota depletion in turn affects several parameters that determine mosquito vectorial capacity.

Introduction

Malaria transmission epidemiology can be effectively described by the basic reproductive number (R_0), which is the number of secondary human infections arising from a single human infection in a wholly naïve and susceptible population (Smith and McKenzie, 2004). For malaria transmission to be maintained in a population, R_0 must be greater than 1. The basic reproductive number can be described by the following Ross-MacDonald model:

$$R_0 = \frac{ma^2bcp^n}{r(-\ln p)}$$

Where:

m = mosquito: man ratio

a = number of bites on humans per mosquito per day

b = the probability that an uninfected human becomes infected when bitten by an infected mosquito

c = the probability that an uninfected mosquito becomes infected when it bites an infected human

p = daily probability of mosquito survival

n = number of days from gametocyte ingestion to a mosquito being infectious (entomological/extrinsic incubation period)

r = daily rate at which infected humans become non-infectious

The mosquito microbiota is already known to influence parameter c , the probability that an uninfected mosquito becomes infected when it bites an infected human (Dong et al., 2009; Meister et al., 2009). We hypothesised that the microbiota may also influence m and p , via effects on mosquito longevity or reproductive capacity, as well as the mosquito biting rate, a , and the extrinsic incubation period, n . Not only would investigating this hypothesis contribute to understanding the role of the microbiota in mosquito physiology, it could also suggest that antibiotic usage may potentially have an impact on malaria transmission in endemic regions.

Data on antibiotic usage in malaria endemic regions are sporadic, but in totality indicate that antibiotic incidence in human blood may be substantial, and is often coincident with malaria infection. Antibiotic prescription to patients presenting at health centres has been reported to be as high as 70.7 % for children diagnosed with clinical malaria in rural Rwanda (Sievers et al., 2008) and 39-43.4 % in Uganda (Batwala et al., 2011). What's more, tuberculosis and leprosy patients are prescribed long courses of antibiotics (WHO Drugs Used in Leprosy; WHO Treatment of Tuberculosis Guidelines); these diseases overlap geographically with malaria. In addition, the WHO recommends or is investigating the mass administration of antibiotics for neglected tropical diseases such as trachoma and filariasis (Taylor et al., 2005; WHO Trachoma Control), whilst doxycycline is prescribed to travellers as a malaria prophylactic (Tan et al., 2011).

Here, as a proof of concept we show that the presence of a PS cocktail in human blood causes an increase in mosquito survival rate, fecundity and susceptibility to *Plasmodium* parasites, all of which may translate to an increase in malaria transmission. Our findings suggest that the effect on mosquito vectorial capacity of regularly used antibiotics should be assessed in order to most effectively allocate malaria control measures to the regions and individuals at highest risk. Furthermore, we identify previously unrecognised roles for the gut microbiota in mosquito survival and reproductive capacity.

Results

Therapeutic PS in a human blood meal depletes the mosquito microbiota

As a proof of concept, we investigated whether the presence of a PS cocktail in a human bloodmeal could significantly change the mosquito gut microbiota load or composition (Fig 3.1.1)². Penicillin and streptomycin were chosen for this as these are both broad-spectrum antibiotics that together target both Gram-positive and Gram-negative bacteria. The blood concentration (hereafter referred to as 'therapeutic concentration'), 20 U/ml penicillin plus 20 µg/ml streptomycin, was chosen to be within the range of reported or expected blood concentrations of patients undergoing treatment (PDRNetwork, 2014a, 2014b; Schulz and Schmoldt., 2003).

² Data presented in Figure 3.1.1A were partially contributed by Dr Mathilde Gendrin and partially by myself. Data presented in figure 3.1.1B and 3.1.2 were wholly contributed by Dr Mathilde Gendrin and are reproduced here, with permission, for the sake of completion. All other data presented in this chapter were generated by myself.

Figure 3.1.1

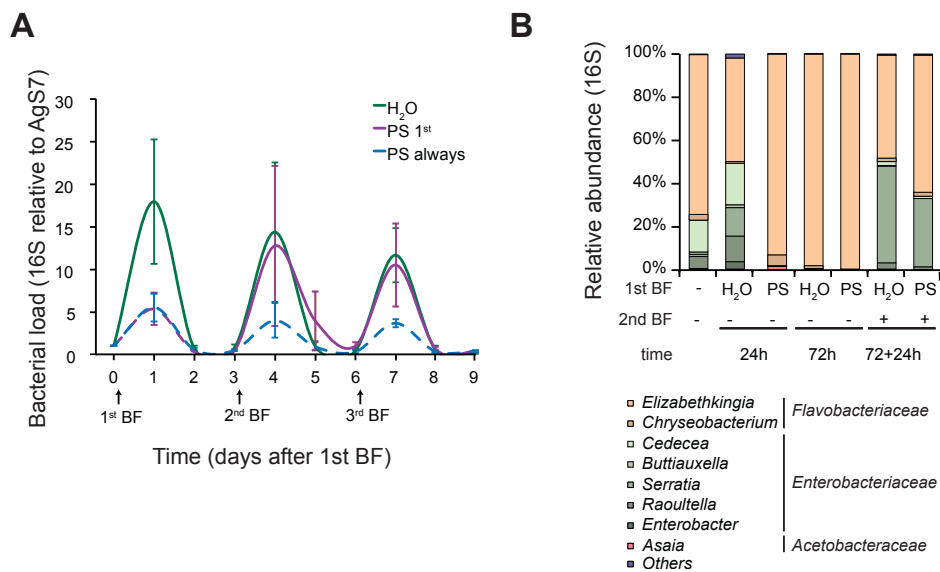


Figure 3.1.1. The effect of therapeutic PS in a human bloodmeal on bacterial load and composition.

Figure modified from Gendrin et al., 2015.

3.1.1A Bacterial load in the midgut over the course of three human blood meals, with the addition of PS in none (H₂O), the 1st (PS 1st) or all three blood meals (PS always). Bacterial load is assessed by qRT-PCR on DNA using universal 16S primers. Data represent the mean $\pm$ standard error of three biological replicates, with each replicate consisting of 3 pools of 15 guts at each time point.

3.1.1B Bacterial composition assessed by 16S 454-pyrosequencing of the 16S rDNA locus. BF= blood feed, time= time post bloodmeal. For full details and methods see Gendrin et al., 2015.

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Indeed, therapeutic PS was found to deplete microbiota load by approximately 70 % following a human blood meal, with this being recurrent following two subsequent PS supplemented blood meals (Fig 3.1.1A, 'PS always'). The presence of PS only in the first blood meal did not cause any reduction in bacterial load in the subsequent two blood meals (Fig 3.1.1A, 'PS 1st'). Bacterial community composition was additionally analysed by 454 pyrosequencing (Fig 3.1.1B), indicating that the microbiota of our *A. coluzzii* colony is dominated by the *Flavobacteriaceae* and *Enterobacteriaceae* families. PS treatment was found to reduce the microbiota diversity by 50 %,

substantially reduce the *Enterobacteriaceae*, increase the *Acetobacteriaceae* and have no significant effect on the *Flavobacteriaceae* (Gendrin et al., 2015).

Therapeutic PS increases mosquito susceptibility to *Plasmodium* infection

It was investigated whether the observed effect of therapeutic PS on bacterial load and composition is sufficient to reproduce the previously observed effect of microbiota clearance on *Plasmodium* susceptibility³ (Dong et al., 2009; Meister et al., 2009). Indeed, the observed 70 % reduction in bacterial load was sufficient to significantly increase the infection intensity (number of oocysts per midgut) of *P. berghei* infection, laboratory *P. falciparum* infection and infection with field *P. falciparum* parasites (Fig 3.1.2). The same was true of infection prevalence (the proportion of mosquitoes harbouring one or more oocysts), though this was not statistically significant in the case of field parasites.

³ Data presented in figure 3.1.1A were partially contributed by Dr Mathilde Gendrin and partially by myself. Data presented in figure 3.1.1B and 3.1.2 were wholly contributed by Dr Mathilde Gendrin and are reproduced here, with permission, for the sake of completion. All other data presented in this chapter were generated by myself.

Figure 3.1.2

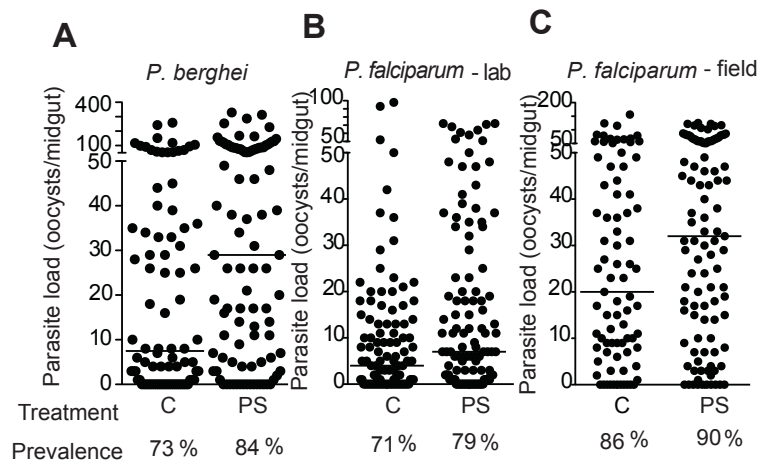


Figure 3.1.2. The effect of therapeutic PS in a human blood meal on *Plasmodium* infection. Taken from Gendrin et al., 2015.

PS in a human bloodmeal results in increased susceptibility to *P. berghei* (A) and laboratory (B) and field (C) *P. falciparum* infection. Each dot represents one midgut, the line shows the median. For full details and methods see Gendrin et al., 2015.

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Therapeutic PS increases mosquito survival

The effect of therapeutic antibiotic feeding on the outcome of *Plasmodium* infection led us to investigate other effects on mosquito physiology, namely survival and reproductive capacity. The presence of therapeutic PS in all three human blood meals was found to significantly increase the survival of mosquitoes relative to those given three control human blood meals (Fig. 3.1.3A). Mosquitoes given therapeutic PS in only the first bloodmeal exhibited an intermediate phenotype. The same effect was found in mosquitoes that fed on a naïve or a *P. berghei* infected mouse either before or after intravenous injection of antibiotics (Fig 3.1.3B).

Figure 3.1.3

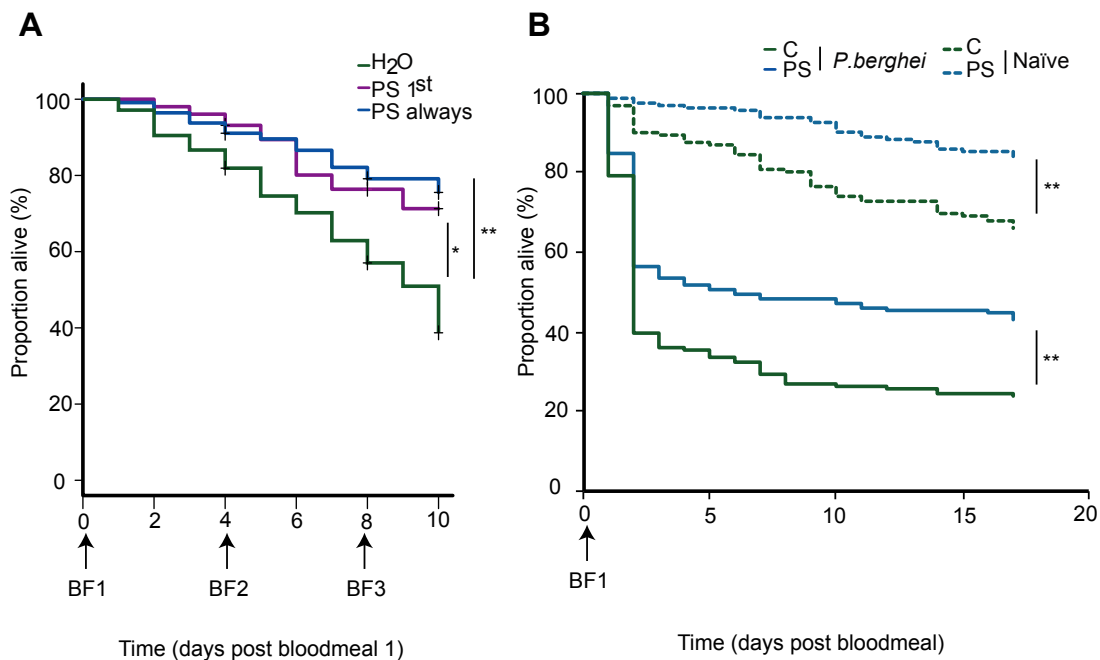


Figure 3.1.3. The presence of therapeutic PS in the blood meal increases mosquito survival

3.1.3A. Mosquito survival over the course of three human blood meals, supplemented with PS in none (H₂O), the 1st (PS 1st) or all three (PS always) blood meals. Data shows a pool of three biological replicates, with at least 50 mosquitoes per condition per replicate. After each bloodmeal, unfed mosquitoes were removed from the experiment and classified as ‘censored’ in the statistical analysis. A log-rank test was used to assess statistical significance.

3.1.3B. Mosquito survival after one control or PS blood meal, taken directly from a *P. berghei* infected or naïve mouse. Data shows a pool of four biological replicates with at least 50 mosquitoes per condition per replicate. A Gehan-Wilcoxon test was used to assess statistical significance.

BF=blood feed; *p<0.05, **p<0.01

Therapeutic PS increases mosquito fecundity

We next investigated effect of therapeutic PS on mosquito fecundity by recording the number of eggs laid per female following a control or PS treated human blood meal. Mosquitoes fed on a human blood meal supplemented with therapeutic PS were both more likely to lay eggs and produced more eggs per oviposition (Fig 3.1.4A). When the eggs were allowed to hatch into larvae to assess their viability, no difference was identified (Fig 3.1.4B).

Figure 3.1.4

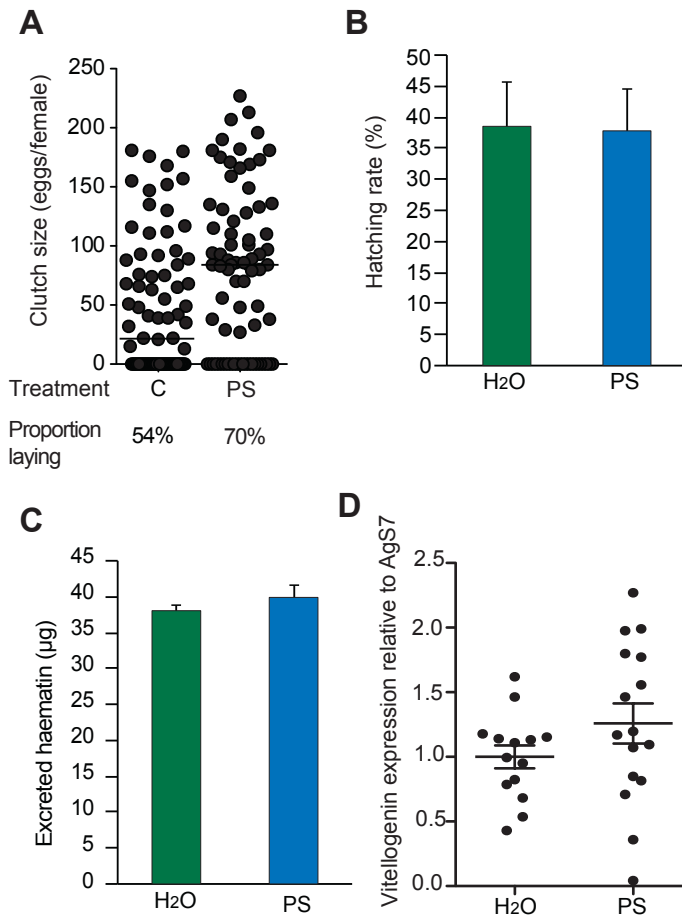


Figure 3.1.4 Therapeutic PS increases mosquito fecundity.

3.1.4A. The addition of PS to a human blood meal increases the proportion of females laying eggs and the number of eggs per female. Data shows the median and data distribution of one representative biological replicate of three. Egg laying (three replicates): $p < 0.01$, ANOVA; number of eggs per female (three replicates): $p < 0.05$, ANOVA.

3.1.4B. The addition of PS to a human blood meal has no effect on the viability of the eggs laid. Data shows the weighted average (weighted on number of eggs) + standard error of three biological replicates, with at least 50 mosquitoes offered a blood meal per condition per replicate. $p > 0.1$, ANOVA.

3.1.4C. The addition of PS to a human blood meal has no effect on the volume of blood imbibed. µg of haematin excreted was quantified by absorbance of the resuspended faecal pellet at 387 nm. Data shows mean + data range for two biological replicates, each consisting of 8-12 mosquitoes per condition. $p > 0.1$, ANOVA.

3.1.4D. The addition of PS to a human blood meal causes a non-significant increase in mean Vg expression. Each dot represents a pool for 5-10 mosquitoes. Line shows mean ± standard error. Data are composed of 4 biological replicates, with each replicate being composed of 2-6 pools. Data are normalised to the mean control (H₂O) value within each biological replicate. $p > 0.1$, ANOVA.

It was hypothesised that mosquitoes offered a PS containing blood meal may imbibe more blood, resulting in elevated fecundity thanks to higher nutritional provision. To test this, the size of the mosquito blood meal was analysed. Blood fed mosquitoes were housed in individual vials until they excreted their blood bolus, approximately 48 h after feeding. The blood boluses were then analysed spectrophotometrically, as previously described (Briegel, 1980), for their haematin content (Fig 3.1.4C). No difference was observed between PS and control mosquitoes in the quantity of haematin excreted, suggesting that the difference in fecundity is not due to a difference in the size of the blood meal.

Previous reports have suggested that NF- κ B signalling acts to inhibit the expression of *Vitellogenin* (*Vg*), which encodes a nutrient transport protein that is essential for egg development (Rono et al., 2010). Given that microbiota proliferation is thought to induce an NF- κ B dependent immune response, it was hypothesised that this may be the cause of the observed increase in fecundity in PS fed mosquitoes. Mean *Vitellogenin* expression in whole mosquitoes exhibited approximately a 25 % increase (Fig 3.1.4D), though this was not statistically significant at this power ($p=0.156$, ANOVA).

Excretion is responsible for the restoration of pre-blood meal microbiota load

Given the observed impact of blood meal dependent microbiota proliferation on mosquito physiology, we sought to further explore the mechanisms underlying the dynamic changes in microbiota load observed over the gonotrophic cycle. We reasoned that this may give insight into why the host permits routine bacterial proliferation that is detrimental to its fitness. In particular, it was asked whether the restoration of pre-feeding bacterial load that is observed by 48 h post blood meal is due to mosquito immune activity or to another factor. Specifically, we hypothesised that the excretion of the blood bolus approximately 48 h after the blood meal may be sufficient to physically remove the microbiota from the gut. If this were the case, it would suggest that the blood meal essentially initiates a bacterial infection, but one that is both predictable and transient; it may be more beneficial to the

mosquito to tolerate the temporary elevation of bacterial load, though this strategy still has its costs, than to induce a costly immune response.

Between 46 and 50 h post blood meal, mosquitoes were separated according to whether or not the blood bolus remained in the gut or had been excreted and their 16S rRNA levels quantified (Fig 3.1.5). cDNA was used instead of DNA here, in an attempt to limit the quantification to live bacteria as much as possible (RNA having a shorter half life than DNA). Indeed, mosquitoes that had not yet excreted their blood bolus harboured significantly more bacteria than those that had ($p < 0.01$, ANOVA). Despite this, the non-excreted mosquitoes harboured less 16S rRNA than the 24 h mosquitoes, with this difference being marginally significant ($p < 0.1$, ANOVA). This could suggest that there is some other mechanism in addition to excretion (such as a mosquito immune response) reducing microbial load. Alternatively, gut bacteria may be less metabolically active at this time point.

Figure 3.1.5

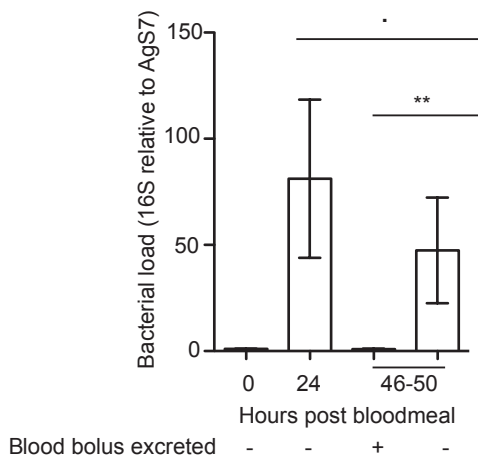


Figure 3.1.5. Blood bolus excretion is largely responsible for the decrease in microbial load at 48 h post bloodmeal. 46-50 h post bloodmeal, mosquitoes were separated according to whether or not they had secreted their blood bolus. Bacterial load in the gut was then assessed by qRT-PCR on cDNA using universal 16S primers. Graph represents two biological replicates, with each replicate consisting of 3-5 pools of 5 guts for each condition. Data are normalised within biological replicates to the mean bacterial load in the sugar fed pools. * $p < 0.1$, ** $p < 0.01$, ANOVA.

Discussion

Here, we show that mosquito survival, reproduction and susceptibility to *Plasmodium* infection are positively affected by the presence of therapeutic PS in the bloodmeal. It was already known that feeding antibiotic-supplemented sugar increases mosquito susceptibility to *Plasmodium* infection, with likely mechanisms including the removal of basal immune induction and the removal of direct anti-parasite effects of the bacteria (Dong et al., 2009; Meister et al., 2009). Here we demonstrate that this effect is maintained with a milder antibiotic treatment, delivered through the blood meal.

Mosquito longevity is amongst the most important mosquito-dependent parameters in determining infectious disease transmission. Mosquito longevity influences the probability of acquiring an infection, of surviving the parasite incubation period and infecting a new individual, as well as the number of gonotrophic cycles per lifetime (and therefore reproductive output), altogether resulting in a non-linear relationship with R_0 . Survival of the host through the parasite extrinsic incubation period is particularly important: here, the daily probability of survival is raised to an exponent, the days taken from gametocyte ingestion to the mosquito being infectious, meaning that mosquito longevity has a very significant bearing on the magnitude of R_0 . Fecundity influences the mosquito:man ratio; whilst this is not as important a parameter as longevity, an increase in mosquito fecundity will still likely translate to a larger mosquito population and an increase in malaria transmission.

Further studies are required to see whether these findings translate to antibiotics that are in common use in malaria endemic regions. Of the antibiotics employed here, penicillin inhibits peptidoglycan synthesis of Gram-positive bacteria (Waxman and Strominger, 1983), whilst streptomycin inhibits protein translation, targeting both Gram types (Griffin et al., 2010). Streptomycin is therefore likely responsible for the reported effects, as the majority of microbiota identified in our colony are Gram-negative. Streptomycin is used in the treatment of tuberculosis and buruli ulcer (WHO Treatment of Tuberculosis Guidelines; WHO Buruli Ulcer Factsheet), though

is not one of the most widely used antibiotics. Rather, ampicillin, amoxicillin and cotrimoxazole are all reported as being highly available in the WHO African region in both the public and private sectors (Lee et al., 2014). Indeed, different antibiotics may affect the microbiota differently depending on their specificity, resulting in the disruption of and subsequent niche colonisation by different bacterial strains and species, each of which are likely to have different effects on mosquito physiology and *Plasmodium* resistance (Bahia et al., 2014). A possible application of these findings would be to recommend the prescription of antibiotics that have minimal or even negative impacts on malaria transmission, or to prioritise bed net distribution to those undergoing antibiotic treatment. What's more, a very simple but potentially highly effective intervention would be to plan the time of day in which antibiotic mass drug administration programmes are administered such that the peak blood antibiotic concentration is in the morning, rather than at night when mosquitoes feed. Depending on the pharmacokinetics of the antibiotics in question, this could ensure that the concentration of antibiotic imbibed by the mosquito is sufficiently low as to have no effect on mosquito longevity, fecundity or *Plasmodium* susceptibility.

In addition to their implications for disease transmission, these findings are highly informative from a basic biology perspective. The fact that the host is permissive to bacterial growth is intriguing, given the detrimental effect that this proliferation has on both survival and fecundity. In the case of the latter at least, it is feasible that it is the immune response rather than the bacteria themselves that cause the reduction in reproductive output. In *Drosophila* there is a well established trade off between immunity and reproduction, with mated females being less able to mount an immune response than unmated females (Short and Lazzaro, 2013; Short et al., 2012). Conversely, infected females have reduced fecundity in a manner that is Imd pathway dependent (Zerofsky et al., 2005), though the affected reproduction pathways have not been identified. A similar phenomenon has been observed in *A. gambiae*, whereby the nutrient transport proteins Vitellogenin and Lipophorin reduce the efficiency of TEP1-dependent parasite killing, and indeed NF- κ B signalling reduces the expression levels of *Vg* after a *P. berghei*-infected blood meal

(Rono et al., 2010). Furthermore, silencing the IMD pathway negative regulator *Caudal* reduces mosquito fecundity, even in the absence of infection (Clayton et al., 2013). Consistent with this, depletion of blood meal-associated microbiota growth here resulted in an increase in mosquito fecundity. The observed increase in mean *Vg* expression, though not statistically significant at this power, would be consistent with a model whereby the increase in fecundity is due to lower NF- κ B signalling in the therapeutic PS fed mosquitoes, causing a derepression of *Vg* expression. Interestingly, this would imply that bacterial proliferation in the gut can induce a systemic immune response in the fat body, where *Vg* is induced for egg production. In *Drosophila*, it has been demonstrated that peptidoglycan fragments derived from a gut infection are able to traverse the genital epithelium and induce a systemic immune response (Gendrin et al., 2009), making this a realistic possibility.

Interestingly, *A. stephensi* mosquitoes that are fully deprived of their microbiota have been shown to have reduced fecundity (Sharma et al., 2013), and indeed *A. aegypti* mosquitoes with a sterile gut have retarded red blood cell lysis and a resultant reduction in egg production (Gaio et al., 2011). It appears that the presence of the microbiota is required to some extent for reproduction, but that the growth that is associated with a bloodmeal tips the balance in the other direction, potentially inducing an immune response that reduces reproductive output.

Blood meal associated bacterial proliferation is also observed to contribute to mosquito mortality. In *Drosophila*, the absence of negative regulators of the *Imd* pathway causes premature death, thought to be due to the derepression of AMP and ROS production that results in increased turnover of gut epithelial cells and a rupture in gut homeostasis (Paredes et al., 2011). A similar phenomenon is seen in ageing flies, whose higher microbiota load causes an increased rate of epithelial renewal and loss of gut integrity (Buchon et al., 2009a). Furthermore, many of the species of bacteria found in the mosquito gut are considered to be pathogenic in other host organisms. *Serratia marcescens*, for example, is a model oral pathogen to *Drosophila* that is

known to cross the epithelial barrier and cause death within six days (Nehme et al., 2007). Therefore, it is not surprising that the unique microbiota proliferation seen in hematophagous insects causes mortality, though to what extent this death is due to classical pathogenesis or to the host response remains unclear.

The finding that excretion is the main cause of the decrease in bloodmeal-associated microbiota load puts these observations in a new perspective. Although it cannot be precluded that many of the bacteria excreted may in fact already be dead (cDNA was analysed instead of DNA in an attempt to detect live bacteria, though the half-life of bacterial 16S rRNA in the mosquito gut is not known), it would suggest that active bacterial killing by the mosquito host may not be necessary for the restoration of homeostasis. Combined with the fact that microbiota proliferation, directly or indirectly, increases host mortality and decreases fecundity, this raises the question of the mechanism used to tolerate the presence of the midgut microbiota, particularly after blood feeding. That is, how the mosquito both prevents and repairs pathogen and immune-derived tissue damage.

3.2 The Mosquito Midgut Transcriptional Response to the Microbiota: A Role for the Peritrophic Matrix in Gut Homeostasis

Abstract

The *Anopheles* gut microbiota is increasingly understood to have wide reaching effects on host physiology in a manner that significantly influences vector competence. The development of interventions that exploit host-microbiota interactions will require a thorough understanding of the mosquito immune- commensal interactions that underlie bacterial persistence and the prevention of microbial overgrowth or dysbiosis. Here, we report a transcriptional analysis of the midgut response to the presence of the gut microbiota across the range of dynamic changes in bacterial load occurring over the blood feeding cycle. As well as characterising the physiological and immune response, we identify elevated expression of several peritrophic matrix (PM)-encoding genes in the presence of the gut microbiota. We show that microbiota depletion by antibiotic treatment results in a disruption of the structure of the PM, and go on to identify a significant role for the PM in gut homeostasis.

Introduction

There is now substantial and growing understanding of immune-microbiota interactions in the model organism *Drosophila*. In this model insect, commensals are controlled largely by the production of ROS by the DUOX system, whilst the other main resistance mechanism in the *Drosophila* gut, the Imd pathway, is under extensive negative regulation to prevent its stimulation by commensals. Mosquitoes are fundamentally different from flies in that they are hematophagous; blood feeding induces both high levels of oxidative stress, potentially precluding further production of ROS for immune control (Oliveira et al., 2011), and triggers substantial microbiota proliferation (Kumar et al., 2010; Pumpuni et al., 1996). In *A. gambiae*, commensals are known to induce the IMD pathway, and knock down of the IMD pathway transcription factor REL2 causes microbiota overgrowth (Dong et al., 2009).

Several immune genes have been identified as being transcriptionally responsive in whole mosquitoes to the presence of the gut microbiota (Dong et al., 2009). However, no studies to date have considered the specific transcriptional response of the midgut epithelium to the presence of the microbiota. Looking specifically at the midgut response is critical, as it facilitates the identification of genes with small changes in expression in the gut that may be masked by expression in the rest of the body.

The major finding from the transcriptional study reported here is the identification of a number of genes encoding putative components of the type I PM as being regulated by the presence of the microbiota. The type I PM is an acellular structure composed of chitin, proteins and glycoproteins that is produced in the gut following a blood meal, where it physically surrounds the blood bolus (Dinglasan et al., 2009). It is distinct from the type II PM, which is produced constitutively in the cardia region of the larval gut (Edwards and Jacobs-Lorena, 2000). The PM has been implicated in playing a role in detoxification of haem and other toxic components of the blood meal, crosslinking them to the PM and targeting them for excretion (Devenport et al., 2006; Lang et al., 2012; Magalhaes, 2014; Pascoa et al., 2002). The PM also modulates the rate of digestion, likely by limiting the rate at which digestive enzymes diffuse into the gut lumen from the epithelium (Villalon et al., 2003). In *A. gambiae*, current understanding is that PM proteins are stored in secretory vesicles in epithelial cells that are then secreted following the blood meal (Devenport et al., 2004, 2005), with the blood meal trigger for this possibly being the physical distension of the midgut (Tellam, 1996).

The PM also acts as a barrier for pathogens, with the disruption of chitinase secretion by *Plasmodium* parasites severely impairing the ability of both *P. falciparum* and *P. berghei* to escape the gut lumen (Dessens et al., 2001; Shahabuddin et al., 1993; Tsai et al., 2001). Depletion of the PM, however, has been shown to have no effect (Shahabuddin et al., 1993, 1995), or indeed to decrease (Kato et al., 2008) the ability of *Plasmodium* to establish infection, indicating that production of chitinase is sufficient to overcome the PM barrier. Indeed, recent data indicates that interaction with a constitutive PM protein,

FREP1, is necessary for *P. falciparum* to infect the epithelium (Zhang et al., 2015).

The role of the PM as a barrier against bacterial infection, however, has not been studied in adult mosquitoes. One protein constituent of the constitutive *Drosophila* type II PM, Drosocrystallin, has been shown to be transcriptionally induced by bacterial infection, to reduce local and systemic Imd pathway stimulation and to reduce susceptibility to bacterial toxins (Kuraishi et al., 2011). More generally, oral infection by *Erwinia carotovora carotovora* (*Ecc15*) has been shown to induce the expression of genes encoding proteins with chitin binding domains, with the induction of a proportion of these genes being Imd pathway dependent (Buchon et al., 2009b). The type II PM of the hematophagous tsetse fly provides infectious *Serratia* bacteria with a protective niche in which they can proliferate without inducing a gut immune response; in this case the disruption of the PM causes increased resistance to bacterial infection, allowing the infection to be swiftly brought under the control of the immune system (Weiss et al., 2014). In the tick *Ixodes scapularis*, the gut microbiota have been shown to induce the PM, in this case via the JAK/STAT pathway (Narasimhan et al., 2014). In this model, the presence of the PM facilitates colonisation by the spirochete bacterium *Borrelia burgdorferi*, possibly by protecting the pathogen from harmful pro-oxidants and cellular components present in the blood meal (Narasimhan et al., 2014). Taken altogether, these data indicate that the precise role of the PM in immune protection is dependent on the dynamics of infection in each particular host-pathogen combination.

Here, we go on to investigate the role of the PM in mosquito microbiota homeostasis. We find that the PM plays a role in resistance to the *Enterobacteriaceae* bacteria present in the gut microbiota, both reducing the extent to which this family of bacteria grows and persists within the gut, and precluding this family of bacteria from seeding a systemic infection.

Results

RNA sequencing of midguts depleted of the native microbiota

We sought to investigate both the impact of the microbiota on the physiology of the midgut, and the mechanisms employed by the midgut to resist and/or tolerate its native bacteria. We therefore undertook a transcriptomic analysis, comparing the midgut transcriptome of control laboratory *A. coluzzii* mosquitoes with laboratory mosquitoes that had been given a robust oral antibiotic treatment (50 µg/ml gentamicin, 60 µg/ml streptomycin, 60 units/ml penicillin) from emergence. Higher antibiotic concentrations have been used previously in *A. gambiae* research with no reported detrimental effects on adult mosquito fitness (Blumberg et al., 2013). qRT-PCR analysis of 16S rRNA indicated that this antibiotic treatment was highly effective in depleting gut bacteria at all time points analysed (Fig 3.2.1). In order to gain insight into the dynamic response of the gut to the different bacterial loads over the blood feeding cycle, guts were sampled at five time points: 3 day old female mosquitoes that had only had access to sugar solution, 5 h post blood meal, 24 h post blood meal, 72 h post blood meal when all mosquitoes had excreted their blood bolus, and 24 h after a second blood meal (i.e. 96 h after the initial bloodmeal). At each time point, 20 midguts were pooled for sequencing. This time course plus/minus antibiotics was repeated with mosquito cohorts from four independent generations, giving four paired biological replicates for each of the five time points.

Figure 3.2.1

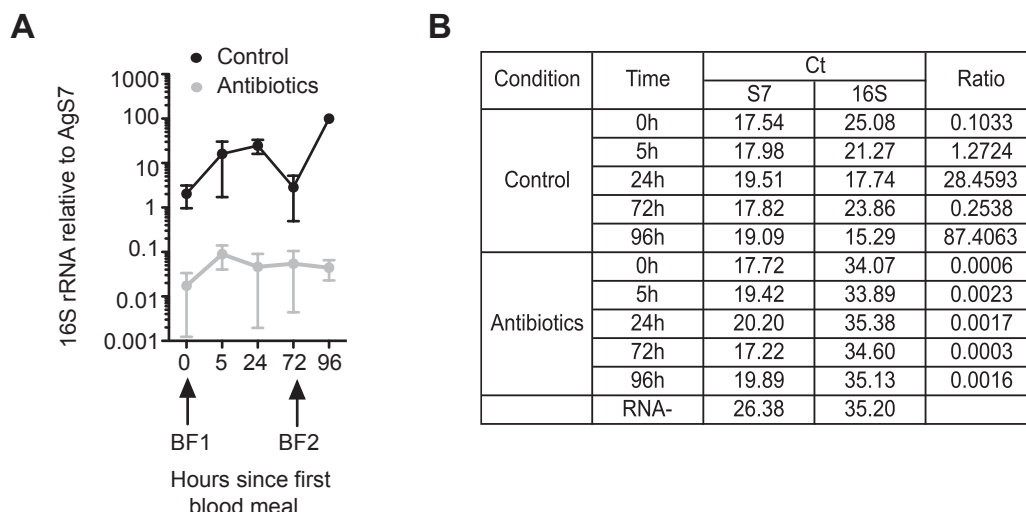


Figure 3.2.1. Bacterial load in control midguts sampled for RNA sequencing and efficacy of antibiotic treatment.

3.2.1A. Bacterial load in control and antibiotics treated gut samples as determined by qRT-PCR on cDNA using universal 16S primers. Data indicates mean and SEM of four biological replicates. Data is normalised to control 96 h samples within each biological replicate. BF=blood feed.

3.2.1B. Efficacy of antibiotic treatment for one representative biological replicate. Table shows threshold cycles (Ct) for the internal control gene (S7) and 16S.

The forty samples of polyA selected RNA were sequenced across four lanes of an Illumina flowcell v3, using an Illumina HiSeq 1500 2x100 bp paired end run. This resulted in a total of 893,247,801 pairs of reads across the ten conditions. Prior to alignment, sequences were filtered for contaminating rRNA and tRNA sequences and Phred quality; an average of 83.8 % of raw reads per sample proceeded to alignment. The splice aware sequence aligner Tophat2 was employed for alignment to the *A. gambiae* PEST genome (AgamP4), with an average of 85.6 % of input sequences per sample aligning uniquely (Fig 3.2.2A). Alignment files were converted to counts using the HTSeq programme; an average of 83.9 % of alignments per sample corresponded to exons in the AgamP4.2 gene annotation (Fig 3.2.2B). Full quality control, alignment and count statistics are available in Table 6.4 in the appendix. A total of 6753 genes (i.e. 49.9% of all annotated genes) had non-zero counts in all forty samples. Principal component analysis (PCA) indicated that the samples cluster according to their blood feeding status, with no obvious outliers (Fig 3.2.2D).

Figure 3.2.2

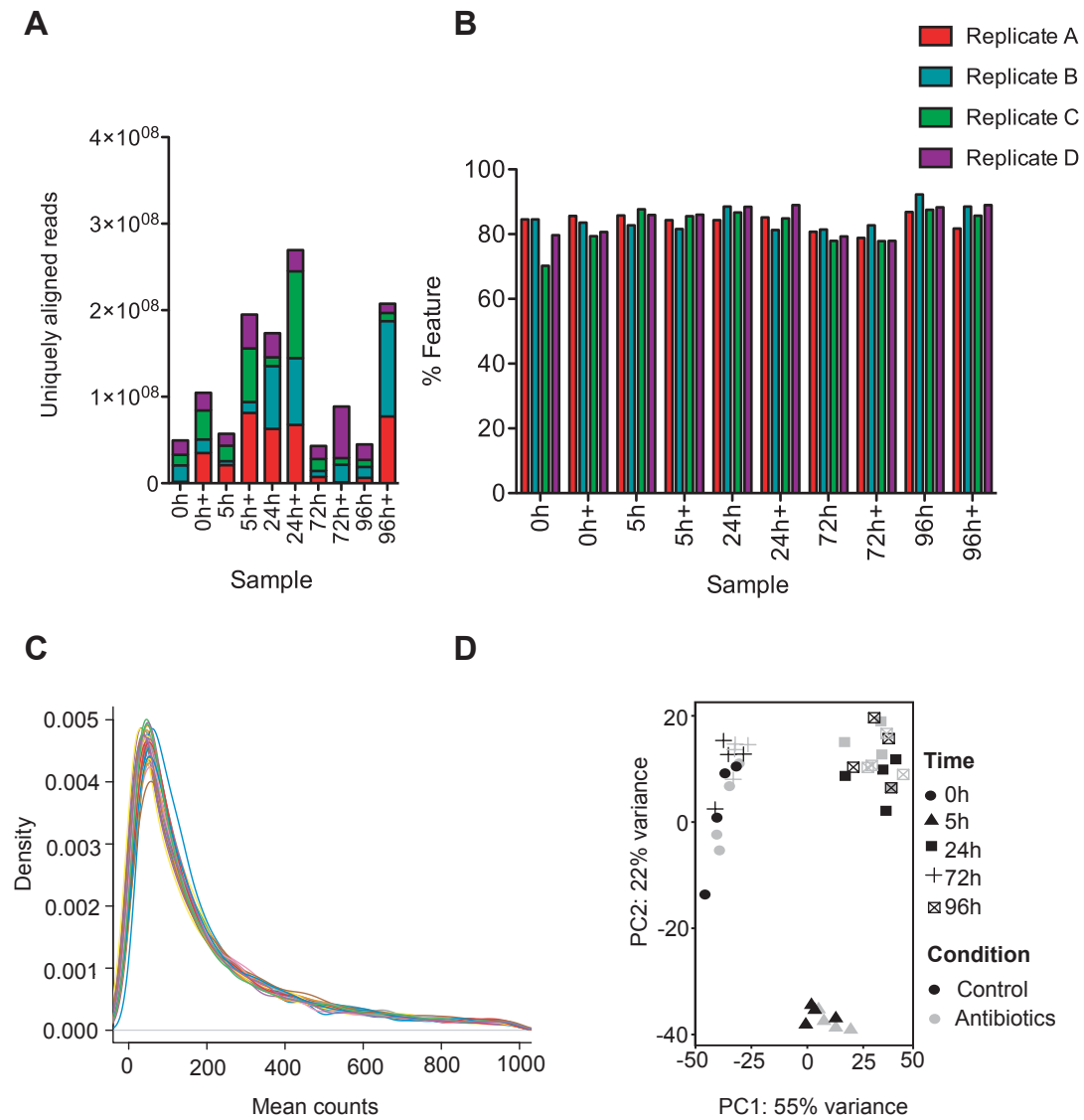


Figure 3.2.2. Post alignment statistics and quality control.

3.2.2A. Number of uniquely aligned paired end reads per sample.

3.2.2B. Percentage of uniquely aligned reads corresponding to a feature after HTSeq counting.

3.2.2C. Distribution of normalised count frequency for features with non-zero counts in all samples. Each line represents one of the forty samples.

3.2.2D. Principal component analysis of all samples after variance stabilising transformation.

Clustering reveals minor overall impact of antibiotic treatment on midgut physiology

We first performed a soft clustering analysis in order to get an initial overview of the impact of antibiotic treatment on the physiology of the midgut over the time course. We identified 7399 genes as being significantly differentially regulated in at least one pairwise comparison between the ten conditions (five time points, plus/minus antibiotics). After variance stabilising transformation of the normalised count values, a median count of the four replicates was calculated for each of the 7399 genes for each condition. Soft clustering of these values was conducted using the Mfuzz package (Fig 3.2.3) (Futschik and Carlisle, 2005). For each of the 12 clusters, significantly enriched KEGG terms were identified with g:Profiler (Reimand et al., 2007) (Table 3.2.1).

Figure 3.2.3

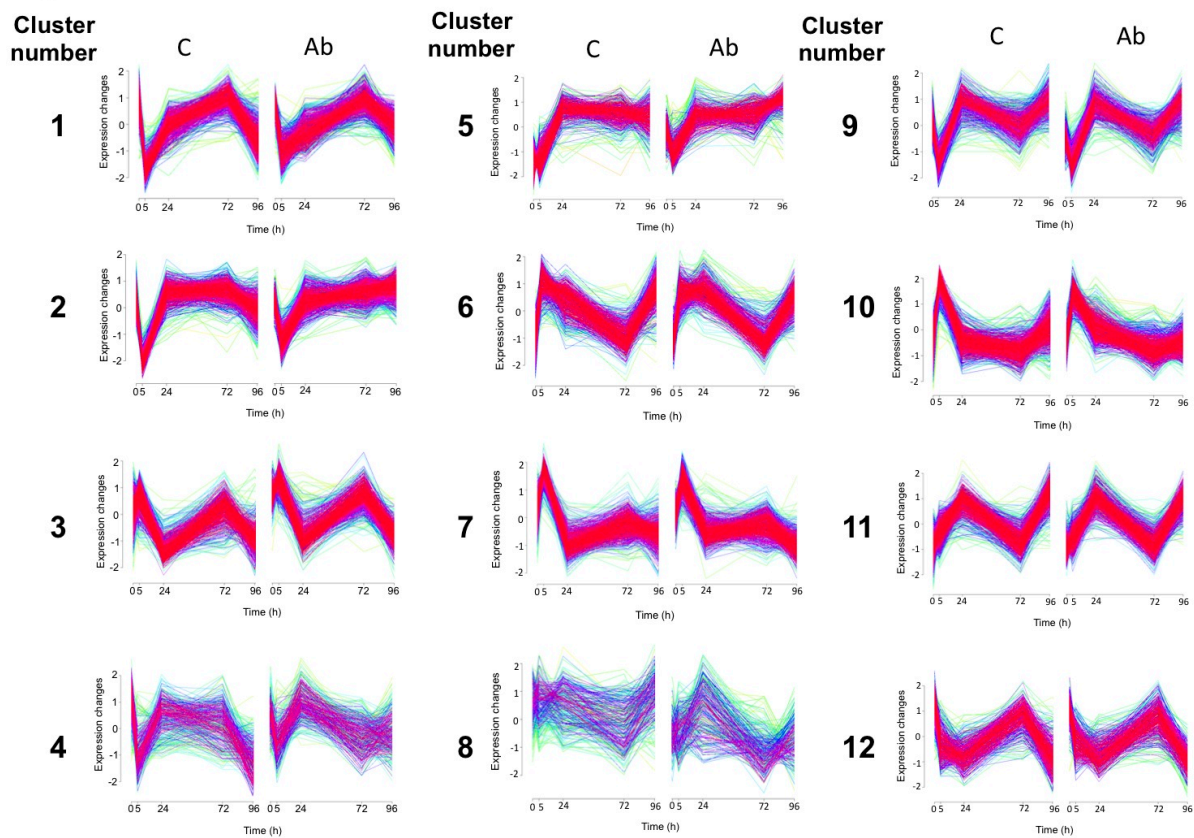


Figure 3.2.3. Soft clustering analysis of midgut expression in control and antibiotic time courses.

Clusters were generated in the Mfuzz package. Each line represents one gene in the cluster, with line colour indicating strength of membership in the cluster (red being the strongest membership and green the weakest). C=control, Ab=antibiotics

Table 3.2.1. Significant KEGG terms associated with the clusters shown in Figure 3.2.3.

Cluster number	Number of Genes	KEGG Term (p value)
1	407	Insulin resistance (0.019)
2	511	mTOR signalling pathway (0.010) Inositol phosphate metabolism (0.0002) Endocytosis (0.0001) Phosphatidylinositol signalling system (0.00001) Ubiquitin mediated proteolysis (0.036)
3	441	Ribosome biogenesis in eukaryotes (6.52E-36) mRNA surveillance pathway (0.014) RNA transport (3.06E-08) RNA polymerase (0.000058) Pyrimidine metabolism (0.0024)
4	153	Glycerolipid metabolism (0.006)
5	355	Lysine degradation (0.045) Endocytosis (0.000057) Phosphatidylinositol signalling system (0.044)
6	408	Fatty acid metabolism (0.003) Nicotinate and nicotinamide metabolism (0.026) Peroxisome (0.001) Glycine, serine and threonine metabolism (0.039) Proteasome (1.69E-22) Valine, leucine and isoleucine degradation (0.000005) Biosynthesis of amino acids (0.030) Metabolic pathways (0.007) Fatty acid degradation (0.0001) Biosynthesis of antibiotics (0.002)
7	598	Ribosome (4.01E-80) DNA replication (0.034) Mismatch repair (0.039) Oxidative phosphorylation (0.0004)
8	80	-
9	321	Lysosome (0.00007)
10	525	Base excision repair (0.034) Metabolic pathways (0.002) Protein processing in endoplasmic reticulum (0.00009) DNA replication (0.000008) Protein export (6.17E-09) Oxidative phosphorylation (6.24E-11)
11	482	Glycosaminoglycan degradation (0.00002) Other glycan degradation (0.049) Sphingolipid metabolism (0.006) Peroxisome (0.000002) Glycosphingolipid biosynthesis- ganglio series (0.003) Lysosome (0.0001)
12	321	Glycolysis/ Gluconeogenesis (0.013) Glycosylphosphatidylinositol (GPI)-anchor biosynthesis (0.025) Metabolic pathways (0.000001) Carbon metabolism (0.005)

Cluster number refers to the number indicated in Figure 3.2.3. Also indicated are the number of genes with membership value in the indicated cluster > 0.7. Significant KEGG terms and associated p values for genes with membership values > 0.7 are indicated.

The first observation, in agreement with the PCA, is that antibiotic treatment has an overall minor effect on the transcriptional changes occurring in the gut in response to blood feeding, with the majority of the 12 clusters appearing

very similar in the control and antibiotic treated time courses. Cluster 8 is an exception to this, with the cluster core being shifted to lower expression at all time points. Although no KEGG terms reach significance in this cluster, the GO term 'defence response to bacterium' is significantly enriched ($p=0.005$). Similarly, cluster 4 appears to be downregulated at 72 h in the antibiotic but not the control time course; in this case 'glycerolipid metabolism' is significantly overrepresented ($p=0.006$).

These data offer insight into the major metabolic processes occurring in the midgut over the gonotrophic cycle: these will be briefly reviewed here before returning to the primary question of this work, the transcriptional response to the microbiota. At 0 and 72 h carbon metabolism and glycolysis are significantly enriched (cluster 12), reflecting the fact that fructose is the main energy source when not digesting blood. By 5 h after the first blood meal, several pathways that are likely involved in blood digestion are upregulated, including fatty acid degradation (cluster 6), two amino acid metabolism pathways (cluster 6) and the peroxisome (clusters 6 and 11), as well as protein processing and export (cluster 10). This is followed at 24 h by an upregulation of endocytosis (cluster 5) and the lysosome (clusters 9 and 11), both associated with nutrient uptake and digestion.

At 5 h, we observe a transient decrease in genes associated with insulin signalling (cluster 1) and mTOR signalling (cluster 2). mTOR signalling is a master regulator of cellular growth, proliferation and metabolism; it is responsive to environmental and cell intrinsic signals, such as extracellular amino acids and insulin signalling (Laplanche and Sabatini, 2009). In *A. aegypti*, mTOR signalling in the midgut is known to synchronise input from elevated amino acid levels and insulin like peptides (ILPs) released from the brain to direct blood meal digestion (Brandon et al., 2008; Gulia-Nuss et al., 2011). In particular, early trypsin expression at 0-12 h post blood meal is regulated at the level of translational initiation in response to elevated amino acid concentration (Brandon et al., 2008; Noriega et al., 1996). The data presented here would be consistent with a model whereby early mTOR signalling also induces translation of components of both the insulin and

mTOR signalling pathways, causing the observed decrease in transcript levels of these components at 5 h post blood meal and initiating a positive feedback loop.

Interestingly, at 5 h post blood meal we additionally observe transient upregulation of DNA replication and mismatch repair pathways (clusters 7 and 10), but no concomitant regulation of cell cycle progression pathways. It has been noted previously in mosquitoes that, following a blood meal, gut epithelial cells undergo endocycling, whereby DNA replicates but cell division does not occur, resulting in polyploidy (Contreras-Garduño et al., 2015). Endoreplication has often been associated with tissues that are involved in nutrient uptake and storage, such as *Drosophila* and *C. elegans* intestinal cells (Hedgecock and White, 1985; Micchelli and Perrimon, 2006) and is proposed to be an adaptation of these cell types to either sustain high levels of protein production, increase cell size or facilitate tissue regeneration after stress (Lee et al., 2009).

Finally, we note extensive regulation of various types of lipid metabolism over the blood feeding cycle, including phosphatidyl inositol signalling (clusters 5 and 2), glycerolipids (cluster 4), sphingolipids and glycosphingolipids (cluster 11) and GPI anchor biosynthesis (cluster 12). These lipids have extensive functions in signal transduction, energy storage and as components of the lipid bilayer. Their regulation likely reflects extensive cellular signalling or membrane remodelling occurring over the blood feeding cycle.

The effect of the microbiota on midgut physiology

Soft clustering analysis revealed that the extensive transcriptional regulation occurring in the gut in response to blood feeding largely masks the more subtle effect of the presence or absence of the microbiota. We therefore performed pairwise differential expression analysis of antibiotic treated and untreated guts at each of the five time points (Fig 3.2.4). In total this analysis identified 890 genes as being significantly differentially regulated by antibiotic treatment at one or more time points.

Figure 3.2.4

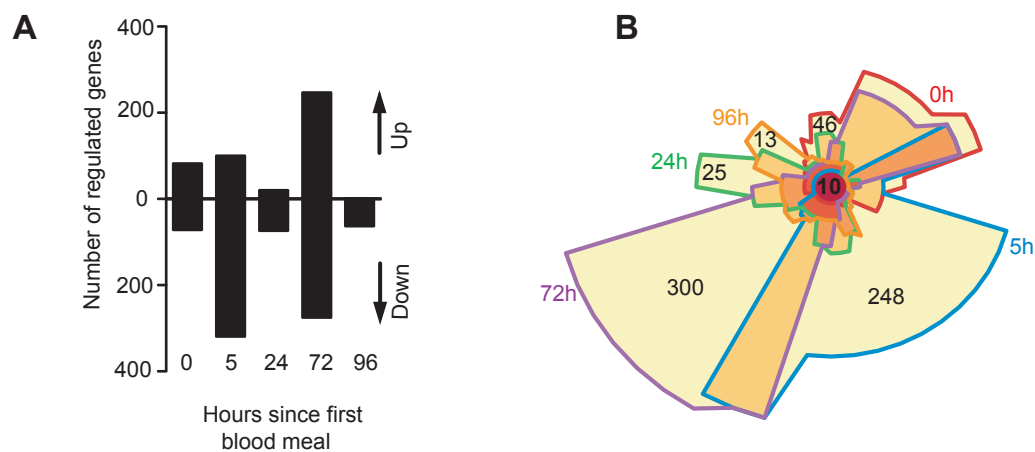


Figure 3.2.4. Pairwise differential expression analysis of the effect of antibiotic treatment at each time point

3.2.4A. Number of genes up/down regulated by antibiotic treatment at each time point. Genes were considered significantly regulated with p value after adjustment for multiple testing below 0.1.

3.2.4B. Venn diagram showing overlap of differentially regulated genes between time points. Colour outlines represent time points as indicated. The number of uniquely regulated genes for each time point is indicated, as well as the ten genes that are differentially regulated at all time points.

Interestingly, we note that 24 h after both the first and second blood meals considerably fewer genes are regulated by the microbiota than at the three other time points, despite the fact that microbiota load in the control guts is 10-100 fold higher at 24 h post blood meal. This suggests either that the gut

bacteria play a more significant role in mosquito physiology at 0, 5, and 72 h than at 24 and 96 h, or that at 24 h after a blood meal highly effective microbiota tolerance responses are employed.

Ten transcripts were identified that are significantly differentially regulated at all time points. Five of these (*CEC1*, *GAM1*, *CEC3*, *PGRPS3* and *PGRPLB*) encode proteins with known roles in immunity- the first three are AMPs whilst the latter two are orthologues of characterised *Drosophila* amidases that regulate the Imd pathway. The remaining five have not previously been associated with the response to the microbiota. Two (AGAP006194 and AGAP006195) encode proteins with a farnesoic acid O-methyl transferase domain (Interpro IPR022041), which is involved in the biosynthesis of juvenile hormone (JH). Another (AGAP011302) is an alkaline phosphatase associated with folate biosynthesis, whilst the remaining two (AGAP009313 and AGAP013543) have signal peptides but no characterised protein domains.

We used g:Profiler (Reimand et al., 2007) to identify enriched up or down regulated KEGG terms and GO Biological Processes at each time point (Table 3.2.2).

Table 3.2.2. KEGG and GO terms (biological processes only) that are significantly enriched after antibiotic treatment.

Time point	Term description	Term ID	Adjusted p value
0h	Defense response to bacterium (n=4)	GO:0042742	0.00000365
	Peptidoglycan catabolic process (n=3)	GO:0009253	0.00448
	Neurotransmitter transport (n=2)	GO:0006836	0.00642
	Glycosaminoglycan degradation (n=2)	KEGG:00531	0.00816
	Oxidation-reduction process (n=16)	GO:0055114	0.00072
	FoxO signaling pathway (n=2)	KEGG:04068	0.05
5h	Proteolysis (n=39)	GO:0006508	0.00834
	Single organism carbohydrate metabolic process (n=64)	GO:0044710	0.0000171
	Signal peptide processing (n=4)	GO:0006465	0.000992
	Organophosphate catabolic process (n=6)	GO:0046434	0.0421
	Histidine metabolism (n=4)	KEGG:00340	0.0216
	beta-Alanine metabolism (n=5)	KEGG:00410	0.0221
	N-Glycan biosynthesis (n=13)	KEGG:00510	0.000000159
	Protein export (n=8)	KEGG:03060	0.000822
	Protein processing in endoplasmic reticulum (n=25)	KEGG:04141	3.03E-09
	Ascorbate and aldarate metabolism (n=5)	KEGG:00053	0.000169
	Ribosome biogenesis (n=9)	GO:0042254	0.000000187
	Ribosome biogenesis in eukaryotes (n=14)	KEGG:03008	2.1E-12
24h	Glutathione metabolism (n=4)	KEGG:00480	0.0147
	Defense response to bacterium (n=4)	GO:0042742	0.0000549
	Peptidoglycan catabolic process (n=3)	GO:0009253	0.0000378
72h	Folate biosynthesis (n=2)	KEGG:00790	0.05
	Sphingolipid metabolic process (n=4)	GO:0006665	0.0337
	Defense response to bacterium (n=4)	GO:0042742	0.00000179
	Protein processing in endoplasmic reticulum (n=14)	KEGG:04141	0.000114
	Ribosome biogenesis (n=7)	GO:0042254	0.0422
	ncRNA processing (n=8)	GO:0034470	0.00752
	Organonitrogen compound biosynthetic process (n=22)	GO:1901566	0.0222
	Oxidation-reduction process (n=26)	GO:0055114	0.0219
	Ubiquinone biosynthetic process (n=3)	GO:0006744	0.0211
	Cofactor biosynthetic process (n=6)	GO:0051188	0.0374
96h	Ribosome biogenesis in eukaryotes (n=16)	KEGG:03008	6.62E-10
	Defense response to bacterium (n=3)	GO:0042742	0.00024
	Peptidoglycan catabolic process (n=3)	GO:0009253	0.000139
	Porphyrin and chlorophyll metabolism (n=2)	KEGG:00860	0.0049
	Starch and sucrose metabolism (n=2)	KEGG:00500	0.00124

Red shaded entries indicate terms that are enriched in genes downregulated by antibiotic treatment. Green shaded entries indicate terms that are enriched in genes upregulated by antibiotic treatment. Only terms with two or more genes are listed. 'n' indicates the number of genes associated with that term that are significantly regulated at the time point indicated.

First, antibiotic treatment results in the downregulation of several pathways involved in nutrient metabolism, both of carbohydrates (GO:0044710, KEGG:00053, KEGG:00500) and proteins (GO:0006508, KEGG:00410, KEGG:00340), particularly at 5 h post bloodmeal. This could suggest that the gut bacteria themselves act as a nutritional resource, providing amino acids and carbohydrates that the mosquito then metabolises, or that the bacteria

contribute to the metabolism of blood and sugar, with the mosquito then absorbing and utilising intermediaries. We also observe a highly significant reduction in protein processing in the endoplasmic reticulum (5 h and 72 h), N-linked glycan biosynthesis (5 h) and protein export (5 h) in the absence of the microbiota. At 5 h after the bloodmeal, the gut epithelium synthesises large quantities of digestive proteins, particularly trypsin-like serine proteases, for blood digestion. Given that we also observe a decrease in amino acid and carbohydrate metabolism, especially at 5 h post bloodmeal, this could reflect a concomitant reduction in the synthesis of digestive enzymes in the absence of the microbiota.

Another apparent contribution of the microbiota to host nutrition is in cofactor and vitamin metabolism, namely folate biosynthesis (24 h) and porphyrin metabolism (96 h). The *A. gambiae* genome does not encode the full complement of enzymes present in the KEGG folate biosynthesis reference pathway, suggesting that the mosquito alone is not able to synthesise folate. The two genes that are significantly upregulated by the presence of the microbiota encode alkaline phosphatases that catalyse the conversion of 7,8-dihydroneopterin 3'-triphosphate to dihydroneopterin (Fig 3.2.5). The *A. gambiae* genome does not encode enzymes that can process dihydroneopterin in the next three conversions. A potential mechanism underlying this observation could be that dihydroneopterin is scavenged by the midgut bacteria for conversion to folate; the mosquito may then reabsorb folate or one of the earlier intermediaries for its own use.

Figure 3.2.5

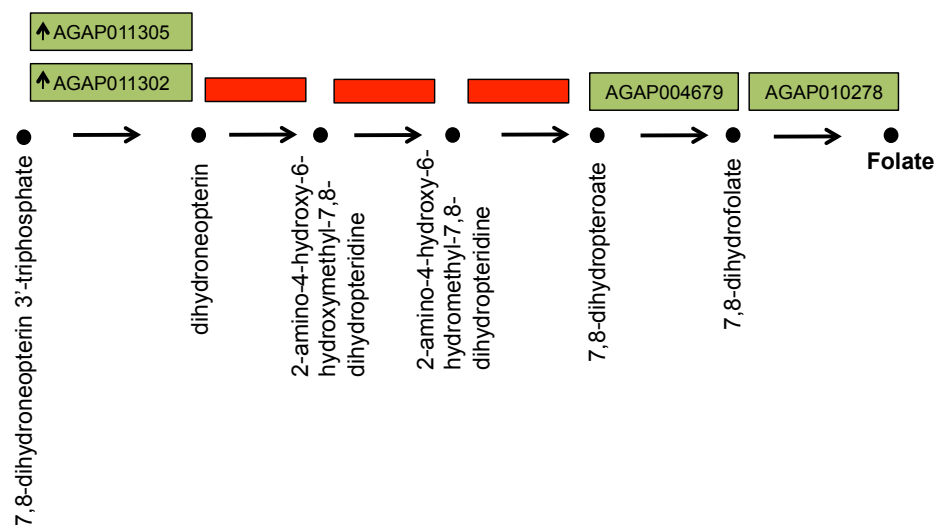


Figure 3.2.5. A section of the KEGG pathway “folate biosynthesis” (KEGG ID: 00790).

Green boxes indicate conversions for which the *A. gambiae* genome encodes the relevant enzyme. Red boxes indicate conversions for which the *A. gambiae* genome does not encode the relevant enzyme. AGAP011305 and AGAP011302 are both upregulated by the presence of the gut microbiota at 24 h post bloodmeal.

At 72 h post bloodmeal, we also observe significant regulation of sphingolipid metabolism. Together with the different profiles observed in cluster 4 (Fig 3.2.3), which is enriched for glycerolipid metabolism, this suggests that the microbiota have a highly significant effect on host lipid metabolism following the excretion of the bloodmeal. Sphingolipids are structural components of the cell membrane as well as acting as signalling molecules that control cell growth, differentiation and apoptosis. In the membrane, sphingolipids are major components of lipid rafts, membrane microdomains that are enriched in cholesterol and signalling proteins. Lipid rafts have been associated with diverse cellular functions such as membrane trafficking, protein sorting and signal transduction. Interestingly, they have also been implicated in microbial pathogenesis; several viruses and bacteria are known to exploit lipid rafts for cellular infection (Mañes et al., 2003), whilst, conversely, lipid rafts are necessary for the internalisation of *P. aeruginosa* by mammalian nasal and tracheal epithelial cells, inducing cellular apoptosis, bacterial death and cytokine release (Grassmé et al., 2003). It would be interesting to explore whether mosquito lipid rafts play a role in controlling or containing the gut microbiota, especially as sphingolipid regulation is observed at 72 h: this time point is preceded by the physical excretion of the blood bolus and the vast majority of the gut microbiota, perhaps suggesting that remaining bacteria must form a close association with the gut epithelium to avoid excretion.

Antibiotic feeding additionally causes the upregulation of ribosome biogenesis (5 and 72 h). Two of the antibiotics used in this experiment, gentamicin and streptomycin, act by targeting the 30S subunit of the bacterial ribosome. It cannot be ruled out that, at the high concentration employed here, the antibiotic may also have had some direct effect on the mosquito eukaryotic ribosome, causing compensatory transcription of ribosomal proteins in the antibiotic treated mosquitoes.

At 72 h we also note upregulation of ubiquinone biosynthesis. Ubiquinone, also known as coenzyme Q10, is a component of the electron transport chain and therefore essential for aerobic respiration, and it also acts as an antioxidant. We further observe upregulation of oxidation-reduction processes

(0 and 72 h) and metabolism of the antioxidant glutathione (5 h). Antibiotic treatment, either directly or because of bacterial depletion, may result in increased energy demands, causing an increase in the rate of cellular respiration and resultant oxidant stress. Alternatively, antibiotic treatment may directly induce oxidative stress, again either in the mosquito or in its bacterial targets. There is growing understanding that bacterial ROS production contributes to cell death after antibiotic treatment regardless of the mode of action of the antibiotic (Dwyer et al., 2014); mosquito antioxidant production may therefore be a direct response to the ROS produced by dying bacteria following antibiotic treatment.

To conclude, these data are suggestive of roles for the mosquito microbiota in diverse physiological functions in the gut, including carbohydrate, protein, vitamin and sphingolipid metabolism. Further complementary studies, for example a metabolomics analysis, would aid in deciphering the precise role of the gut bacteria in these processes.

The immune response to the microbiota

The primary aim of this study was to elucidate the gut epithelial immune response to the microbiota. We examined the transcriptional immune response in a systematic manner, first considering the three main intracellular immune signal transduction pathways (Fig 3.2.6-8), including orthologues of negative and positive regulators that have been identified in *Drosophila*. Next we identified all genes with known roles in immunity or domains that are suggestive of a role in immunity that are regulated at one or more time points by the presence of the microbiota (Fig 3.2.9-3.2.13). The latter were classified into PRRs, regulatory proteins, effectors, ROS-related and neural-associated genes.

The IMD, TOLL and JAK/STAT pathways

Considering first the IMD pathway (Fig 3.2.6), transcripts encoding the putative negative regulators PGRPLB, PGRPS2 and PGRPS3 are all significantly upregulated by the presence of the microbiota at all time points analysed (with the exception of *PGRPS2* at 5 h, which is non-significantly

upregulated), whilst the transcript encoding IAP2, also a negative regulator, is marginally significantly regulated at 5 h. *PGRPS2* and *PGRPS3* in particular are very strongly upregulated following the blood meal in a manner that is completely dependent on the growth of the midgut microbiota, with the antibiotic treated mosquitoes showing no change in expression between 0 and 24 h. These transcripts encode putative amidases; they likely play a role in degrading peptidoglycan fragments to non-immunostimulatory compounds; these data are consistent with the amidase PGRPs playing a key role in the mosquito gut tolerance response to blood meal-associated microbiota proliferation. Interestingly, transcripts encoding the other components of the pathway are regulated in a blood meal dependent, microbiota independent manner: *PGRPLC*, *IMD*, *FADD*, *TAK1*, *CASPAR* and *REL2* are all upregulated 24 h after both the first and second bloodmeal.

Figure 3.2.6

IMD pathway

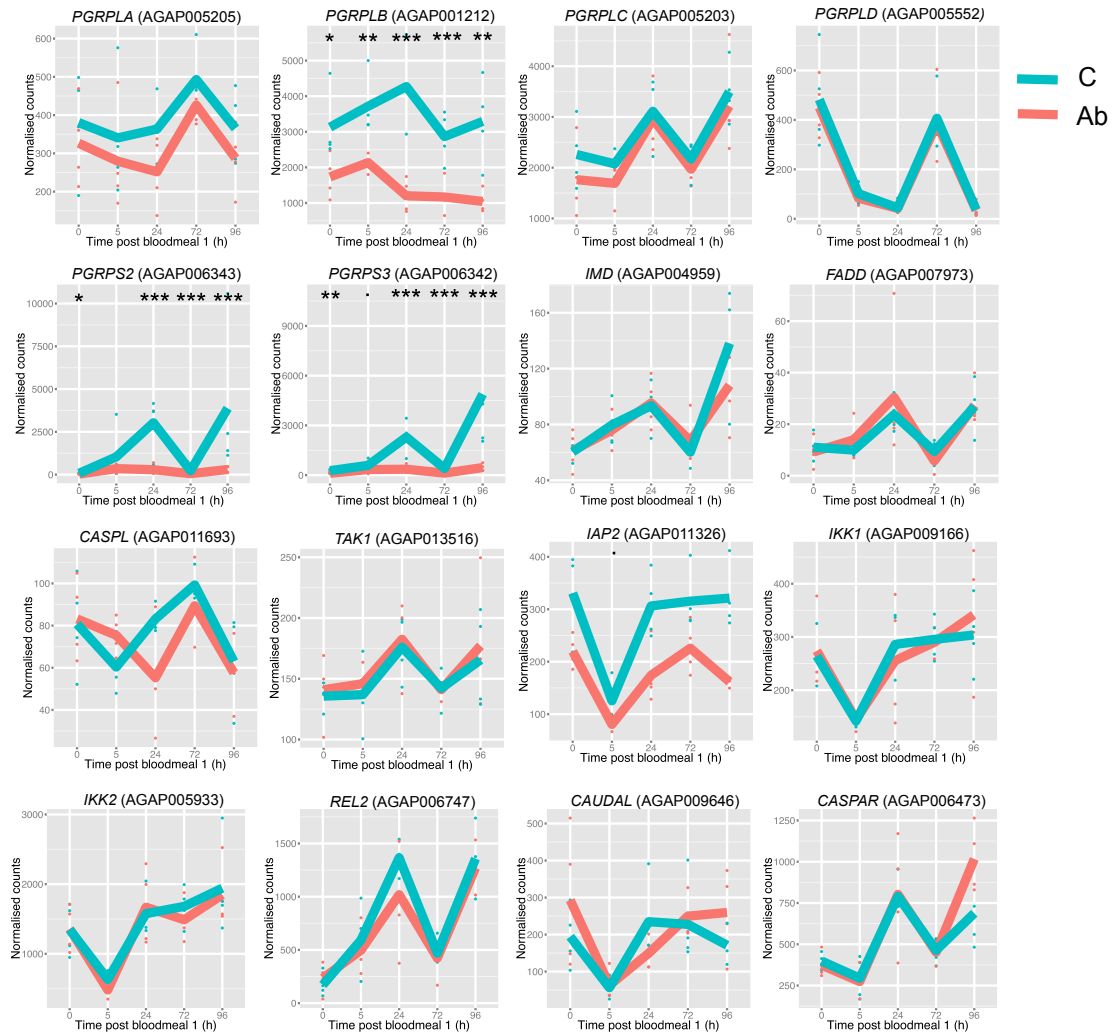


Figure 3.2.6. Normalised counts of genes encoding components of the IMD pathway across control and antibiotic time courses.

See Figure 1.2.1 for a schematic of the IMD pathway. Dots represent one of the four replicates and the line is through the mean. Blood meals were given immediately after the 0 h and 72 h time points. C=control, Ab=Antibiotics. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, . $p < 0.1$, Wald test.

Whilst the IMD pathway is already characterised as playing a role in the midgut response to the microbiota (Dong et al., 2009; Meister et al., 2009), it is not currently clear whether the other major immune pathway, the TOLL pathway, also plays a role. In *A. gambiae*, it is additionally not known which TOLL protein acts as the TOLL pathway receptor in immunity. The *Drosophila* Toll that has been recruited for immunity, Toll-1, does not have a clear *A. gambiae* orthologue, but rather is related to four *A. gambiae* TOLLS: TOLL1A, 1B, 5A and 5B. *Drosophila* Toll-9 has also been linked with immunity when overexpressed (Ooi, 2001) and is the most closely related to vertebrate Toll Like Receptors (TLRs), though so far no immune role has been established in a loss-of-function mutant (Narbonne-Reveau et al., 2011). Here, we see that of TOLL1A, 1B, 5A and 5B, only TOLL5A is consistently detected between samples, though still with low counts (Fig 3.2.7). We do however detect strong and consistent expression of TOLL9, suggesting that this TOLL may play a role in basal TOLL pathway induction in the *A. gambiae* gut.

We also detect low expression of GNBPA2 and three B-type GNBPs, B2, B3 and B4. In *Drosophila*, GGBP1 and GGBP3 signal via the Toll pathway, though it is yet not clear whether this is also true in mosquitoes. The GNBPs have been shown to have activity against bacterial and *Plasmodium* infection and to be induced by bacterial infection, but not by the gut microbiota (Warr et al., 2008). These data corroborate this latter point, suggesting that the GNBPs are unlikely to play a role in the immune response to the microbiota, but likely have a role against exogenous bacterial infection. Finally, the orthologue to *Drosophila* PGRP-SA, PGRPS1, is significantly downregulated by antibiotic treatment 5 h post blood meal. Similar to the GNBPs, PGRPS1 has not formally been demonstrated to act upstream of the TOLL pathway. Nevertheless, its microbiota dependent regulation suggests that PGRPS1 may play a role in the response to the gut microbiota.

Interestingly, transcripts encoding several intracellular components of the TOLL pathway are downregulated at 5 h post blood meal independently of the microbiota, including MYD, TUBE, TRAF6 and REL1. Given the upregulation of translation-associated KEGG terms that is observed at 5 h post blood meal

(Fig 3.2.3 and Table 3.2.1), this could reflect blood meal-dependent translation of a pool of transcripts, allowing a fast anticipatory response, similar to that previously observed for LRIM9 (Upton et al., 2015). It is noteworthy that this downregulation of TOLL pathway components at 5 h coincides with upregulation of the negative regulator *CACTUS*.

Figure 3.2.7

TOLL pathway

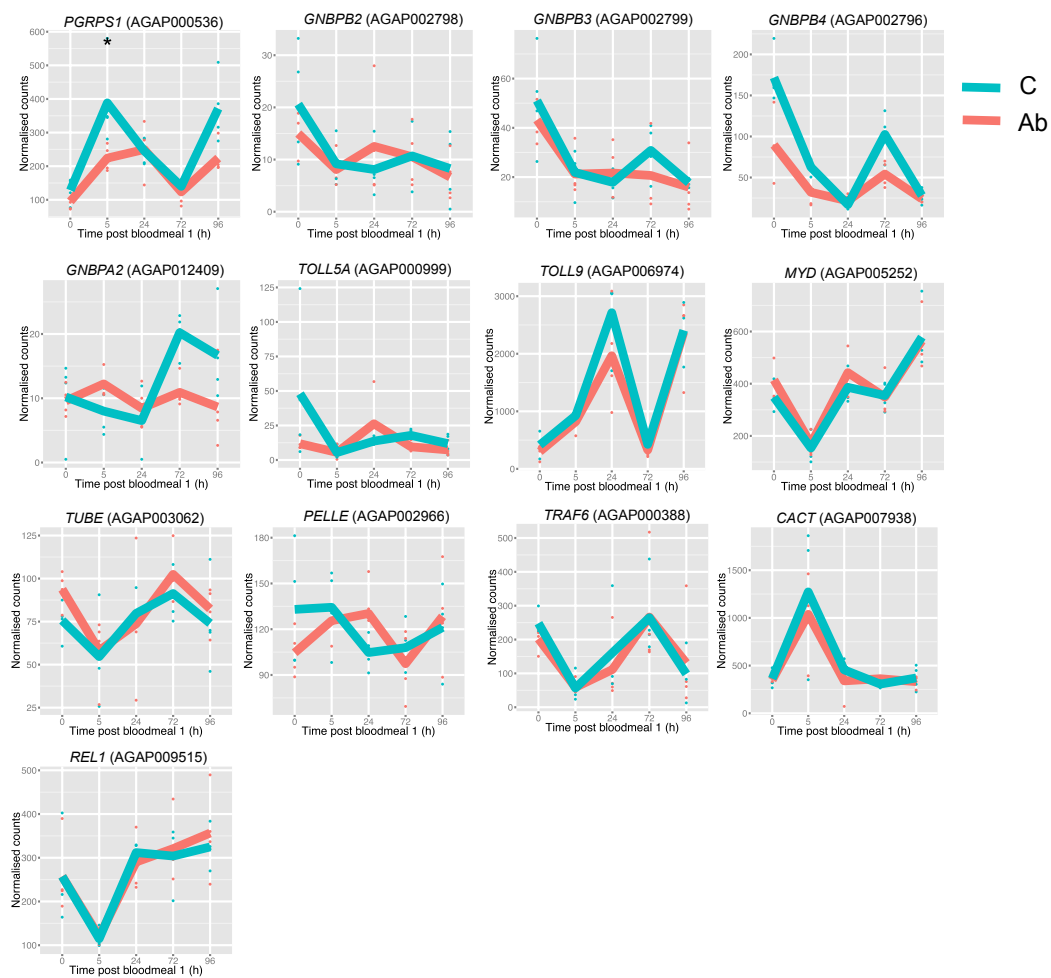


Figure 3.2.7. Normalised counts of genes encoding components of the TOLL pathway across control and antibiotic time courses.

See Figure 1.2.1 for schematic of the TOLL pathway. Dots represent one of the four replicates and the line is through the mean. Blood meals were given immediately after the 0 h and 72 h time points C=Control, Ab=Antibiotics. * $p < 0.05$, Wald test.

Similarly, transcripts encoding two components of the JAK/STAT pathway, the transmembrane receptor DOME and the transcription factor STAT2, are downregulated 5 h post blood meal, again in a microbiota independent manner (Fig 3.2.8). *STAT2* does appear to be somewhat microbiota dependent at 24 h, but this is not statistically significant after adjustment for multiple testing. The other STAT transcription factor, STAT1, does not appear to be expressed in the gut, having very low counts in all samples (data not shown). Again, whether or not the JAK/STAT pathway plays a role in the response to the mosquito microbiota remains unclear.

Figure 3.2.8

JAK/STAT pathway

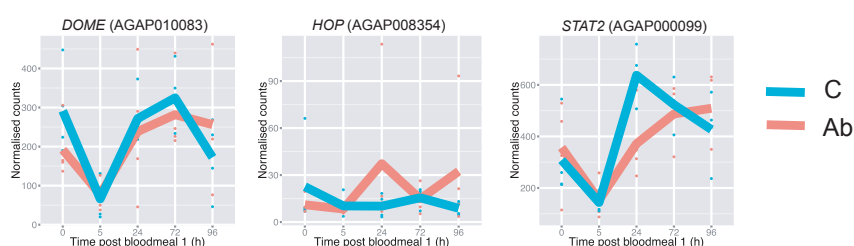


Figure 3.2.8. Normalised counts of genes encoding components of the JAK/STAT pathway across control and antibiotic time courses.

See Figure 1.2.1 for schematic of the JAK/STAT pathway. Dots represent one of the four replicates and the line is through the mean. Blood meals were given immediately after the 0 h and 72 h time points. C=Control, Ab=Antibiotics.

Pattern recognition receptors

Next, considering PRRs not involved in these three major pathways, we identify transcripts encoding two TEPs, two leucine rich immune proteins (LRIMs), three lectins, three fibrinogen domain containing proteins and three MD2-like proteins that are regulated by the microbiota, with the majority of these (11/13) being upregulated by the presence of gut bacteria (Fig 3.2.9).

Figure 3.2.9

Pattern Recognition Receptors

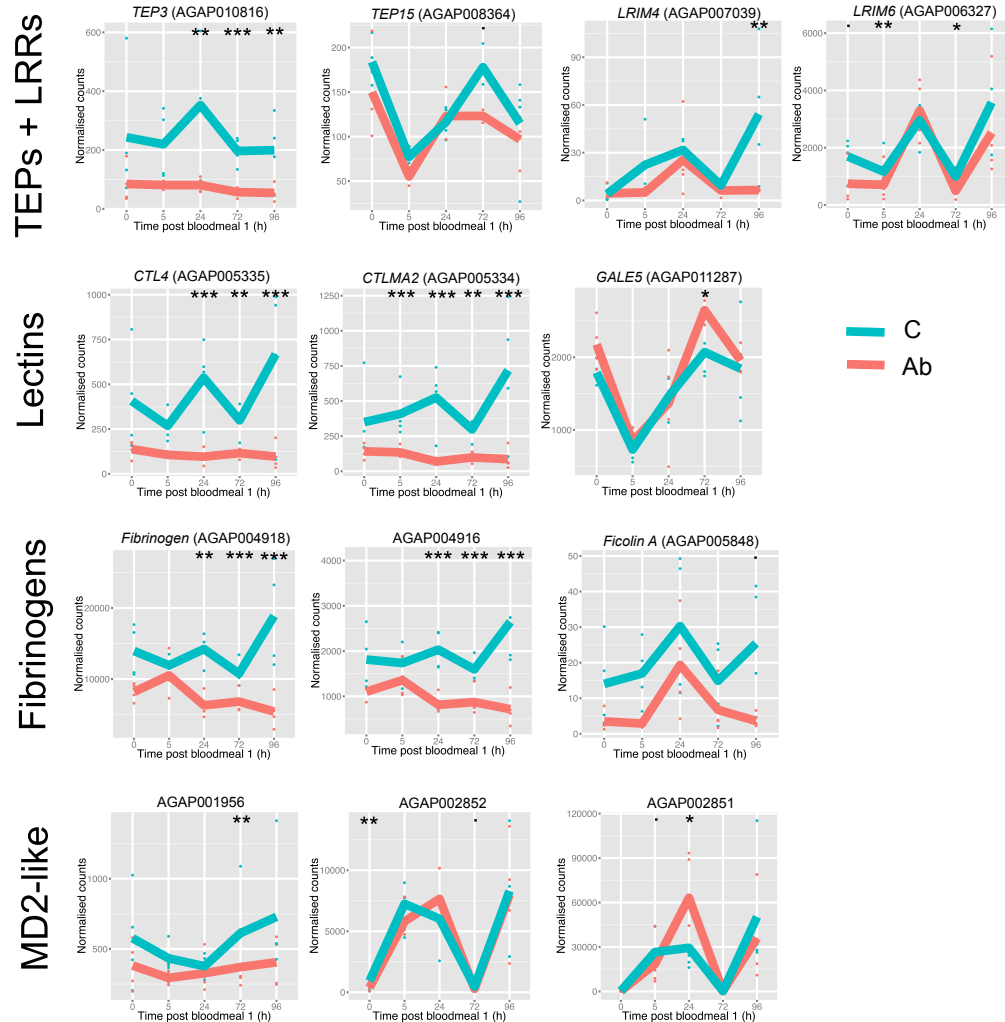


Figure 3.2.9. Normalised counts of genes encoding pattern recognition receptor proteins.

Dots represent one of the four replicates and the line is through the mean. C=Control, Ab=Antibiotics. Blood meals were given immediately after the 0 h and 72 h time points. Genes were selected if they were differentially regulated at one or more time points.

***p<0.001, **p<0.01, *p<0.05, .p<0.1, Wald test.

In vertebrates, the TEPs play a key role in immunity either as components of the complement system or as protease inhibitors (Blandin, 2004). The *A. gambiae* genome encodes 15 TEPs, with TEP1 being the best characterised for its role in opsonising bacteria and parasites for clearance in the haemolymph (Blandin, 2004). Also components of the complement response, two members of the LRIM family, LRIM1 and APL1C, play a role in stabilising circulating TEP1, thereby facilitating its opsonisation of pathogens (Fraiture et al., 2009; Povelones et al., 2009). The LRIMs are a mosquito specific family of proteins containing leucine rich repeat domains, a common domain found in immunity related proteins in diverse phyla. Here, we identified two *TEPs* and two *LRIMs* as being microbiota regulated in the midgut. None of these genes' cognate proteins are well characterised functionally, though TEP3 is known to be a *P. berghei* antagonist (Povelones et al., 2011) and to play a role in the phagocytosis of *E. coli* (Moita et al., 2005). *LRIM4* has been shown to be highly upregulated in the midgut upon *P. falciparum* infection, though its cognate protein has no effect on susceptibility to *P. berghei* infection and does not interact with LRIM1, APL1C or TEP1 (Dong et al., 2006a; Povelones et al., 2011). It is noteworthy that, to date, components of the complement system have been thought of as active largely in the haemolymph. The observation that they are synthesised in the gut in response to the presence of the microbiota in the lumen bears further investigation; as a starting point, it would be key to determine whether these immune proteins are secreted from the luminal or basal side of the epithelial cells, into the gut lumen or the haemolymph respectively.

We also observe the regulation of three immune proteins containing lectin domains, which bind carbohydrate moieties and are often involved in pathogen recognition. The C-type lectins (CTLs), the largest family of animal lectins, bind carbohydrates in a calcium-dependent manner (Schnitger et al., 2009), whilst the galectins are soluble proteins that bind β -galactoside sugars. *GALE5*, a galectin which is found here to be upregulated by antibiotic treatment, is part of a mosquito specific expansion of the galectin family. Other galectins in this group have been associated with the response to virus

infection (Waldock et al., 2012), though *GALE5* itself remains uncharacterised. The fact that it is observed to be upregulated by microbiota depletion could imply that *GALE5* expression is inhibited by the presence of the microbiota. Alternatively, antibiotic treatment may indirectly result in proliferation of the viral microbiota; given the previous association of galectins with antiviral immunity, it would be feasible that this could lead to the observed *GALE5* induction. CTL4 and CTLMA2, whose transcripts are both highly microbiota dependent in their expression, have been shown to circulate in the haemolymph as a disulphide linked heterodimer (Schnitger et al., 2009). Interestingly, CTL4 and CTLMA2 both act as *P. berghei* agonists, reducing the extent to which this parasite is killed by melanisation (Osta et al., 2004), whilst being necessary for the clearance of systemic Gram-negative bacterial infections (Schnitger et al., 2009). The mechanisms underlying this disparity are not yet clear. Similar to the components described above that are associated with a complement response, the role of the CTLs has to date only been studied in the haemolymph. The fact that these genes are transcriptionally responsive to the gut microbiota in the midgut epithelium could suggest that they also play an, as yet unexplored, role in the gut lumen.

We additionally detected the regulation of three genes encoding proteins containing fibrinogen domains. The fibrinogen-related protein (FREP) family is the largest family of PRRs in *A. gambiae*, with 59 members (Dong and Dimopoulos, 2009). In vertebrates, fibrinogen is a glycoprotein that is involved in coagulation; the mosquito FREPs contain a pathogen-interacting fibrinogen-like (FBN) domain at their C terminus, composed of approximately 200 amino acids that are orthologous to the C termini of the fibrinogen β and γ chains, with their N termini likely interacting with other FREPs (Dong and Dimopoulos, 2009). FREPs have long been known to be immune responsive in their expression, and several have been characterised as being involved in anti-bacterial and anti-*Plasmodium* defence (Dong and Dimopoulos, 2009; Dong et al., 2006a). Interestingly one FREP, FREP1, has recently been identified as being integrated into the mosquito PM, where it is directly bound by *Plasmodium* and may facilitate parasite penetration of the PM and the gut epithelium (Zhang et al., 2015). Of the three FREPs identified here as being

microbiota regulated in the gut (AGAP004918, AGAP004916 and AGAP005848), AGAP004918 (FBN25) has been previously shown to be upregulated after systemic *E. coli* infection (Christophides et al., 2002), though none have been functionally characterised. However, two other FREPs, FBN22 and FBN39, have been demonstrated to play a role in immune homeostasis (Dong and Dimopoulos, 2009); this is highly suggestive that a similar role for the three FREPs identified here is likely and merits further investigation.

Finally, we identified three genes belonging to the MD2-like protein family. These genes encode proteins containing a signal peptide and an MD2-like (ML) lipid recognition domain; in the vertebrate innate immune system, the MD2 protein acts as an adaptor to facilitate LPS recognition by TLR4 (Nagai et al., 2002). A binding partner for the mosquito ML proteins has not been identified, and it remains possible that they signal via a TOLL receptor. Of the *A. gambiae* MD2-like proteins, MDL1 has been shown to regulate resistance to *P. falciparum* infection (Dong et al., 2006a), and, interestingly, to form a complex with secreted cytoplasmic actin that mediates phagocytosis of bacteria (Sandiford et al., 2015). However, none of the three genes encoding MD2-like proteins that are characterised here as being microbiota-dependent in their expression have been functionally characterised.

Of all of the PRRs identified here as being microbiota regulated, it is noteworthy that the majority of functional data available concerns their role in the systemic immune response. Further investigation into the role of these PRRs in the local immune response will be critical in understanding the interaction between the midgut epithelium and the gut microbiota. Not least, it will be key to determine whether proteins produced in the cells of the gut epithelium in response to the microbiota are secreted into the lumen or into the haemolymph. The latter scenario could be suggestive of an anticipatory response, pre-empting a potential systemic infection arising from proliferation of microbiota within the gut.

Regulatory proteins

We identified only two differentially expressed genes encoding proteins with putative regulatory roles, the serine protease inhibitor (serpin) SRPN9 and the serine protease CLIPB17 (Fig 3.2.10). The mosquito CLIPs are serine proteases that contain a Clip domain and are involved in protease cascades that lead to melanisation, TOLL pathway activation and complement activation (Yassine et al., 2014). The serpins regulate these proteolytic cascades (Gulley et al., 2013). CLIPB17, identified here as being microbiota dependent in its expression after the second blood meal, is known to promote the melanisation of *P. berghei* ookinetes (Volz et al., 2006), though again a role for this protein in the midgut has not previously been proposed. By contrast, SRPN9 is one of only two serpins previously identified as being enriched in female *A. gambiae* midguts (Warr et al., 2007); the data presented here corroborate this observation, and suggest that this serpin likely plays a key role in regulating the response to the microbiota.

Figure 3.2.10
Regulatory proteins

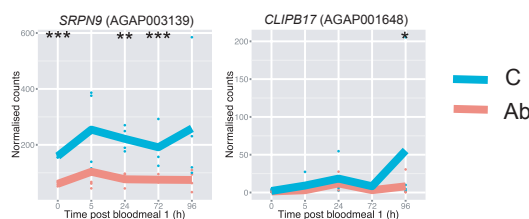


Figure 3.2.10. Normalised counts of genes encoding regulatory proteins.

Dots represent one of the four replicates and the line is through the mean. C=Control, Ab=Antibiotics. Blood meals were given immediately after the 0 h and 72 h time points. Genes were selected if they were differentially regulated at one or more time points. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, Wald test.

Effectors

Of the six AMPs whose transcripts were confidently detected in the midgut (*CEC1-3*, *DEF1-2* and *GAM1*), all were regulated at at least one time point by the microbiota (Fig 3.2.11). Most of these displayed a consistent fold change between the control and antibiotic treated guts across the time course, though interestingly *DEF2* and *CEC2* are only regulated by the microbiota at 0 h and

72 h respectively, whilst being responsive to other, unknown, triggers after blood feeding. Only two C-type lysozymes are microbiota regulated: *LYSC1* appears to be completely dependent on the microbiota for its transcription, whilst *LYSC7*, similar to *CEC2* and *DEF2*, is upregulated by another component of the blood meal at 5 h whilst being microbiota responsive at 72 h. Whilst AMP expression was already known to be microbiota dependent in the gut, these data provide a new level of granularity into the dynamics of the response of each effector across the blood feeding cycle.

Figure 3.2.11
Effectors

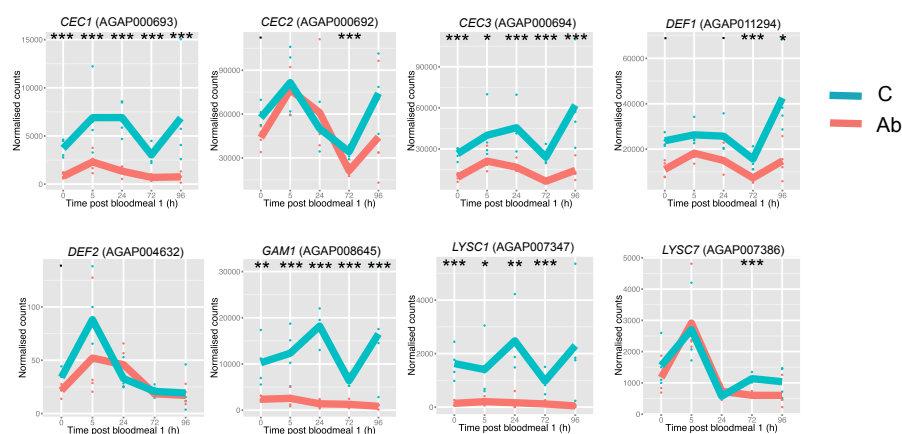


Figure 3.2.11. Normalised counts of genes encoding immune effector proteins. Dots represent one of the four replicates and the line is through the mean. C=Control, Ab=Antibiotics. Blood meals were given immediately after the 0 h and 72 h time points. Genes were selected if they were differentially regulated at one or more time points. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, $p < 0.1$, Wald test.

ROS production and detoxification

In addition to the AMP effectors, we noted the regulation of several enzymes that play a role in producing ROS and in protecting host tissues from their toxic effects. Haem peroxidases (HPXs) catalyse oxidative reactions to produce ROS, whilst reactive oxygen species modulator 1 (ROMO1) induces mitochondrial ROS production and contributes to oxidative stress induced cell death (Shin et al., 2013). Thioredoxin peroxidases (TPX), thioredoxin reductases (TRXR), glutathione peroxidases (GPX), glutathione reductases (GRX) and superoxide dismutases (SOD) are all antioxidants. We observe a range of responses to antibiotic treatment in ROS related genes: antibiotic

treatment leads to lower induction of HPX15 (also known as IMPer) 5 h post bloodmeal, whilst expression of ROMO1 is significantly higher 72 h after feeding. HPX15 has been previously characterised as the immunomodulatory peroxidase that, together with DUOX, cross links proteins in the mucin layer between the PM and the gut epithelium, reducing permeability to immune elicitors (Kumar et al., 2010). Our data corroborates this previous report, which showed HPX15 expression to be microbiota dependent after blood feeding.

With regards to the antioxidants, the expression of three thioredoxins is reduced by antibiotic treatment following blood feeding, whilst the expression of another is significantly increased at 0 h. Three glutathione reductases/ peroxidases are upregulated by antibiotic treatment at 72 h. Altogether, antibiotic treatment seems to cause a decrease in expression of genes associated with ROS production and detoxification at time points when the blood bolus remains in the gut, and an increase of ROS associated gene expression pre-blood feeding and after excretion of the blood bolus. As discussed earlier, however, whether these effects are microbiota dependent or a direct effect of the antibiotic on mosquito tissue cannot be elucidated.

Figure 3.2.12

ROS related

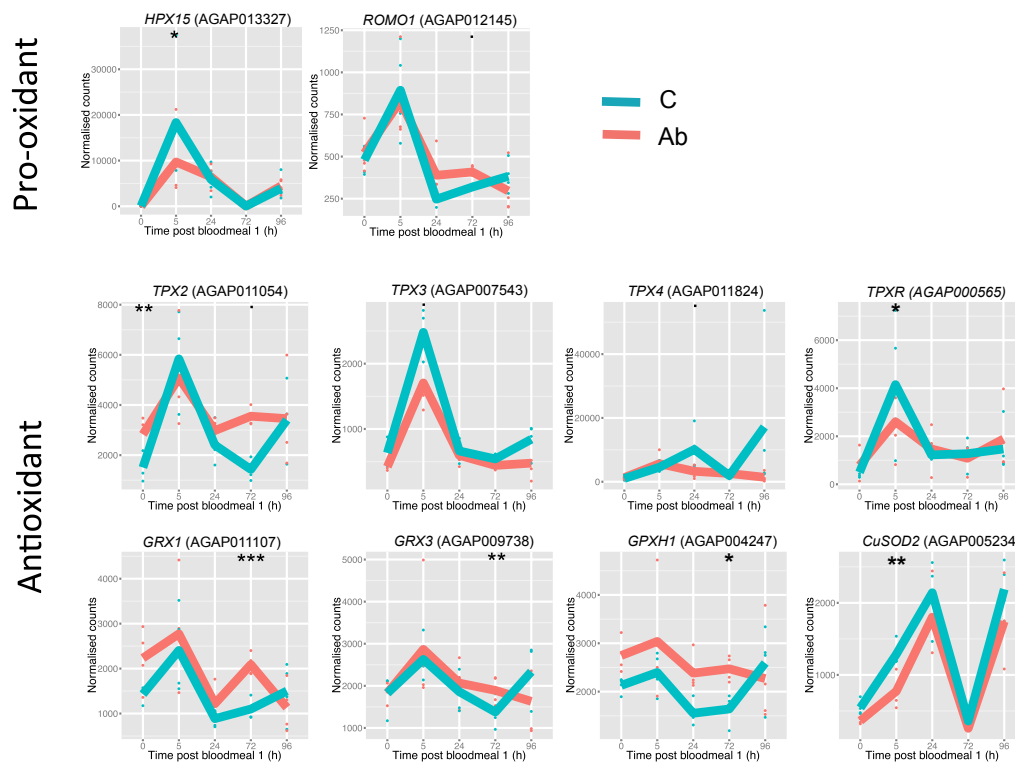


Figure 3.2.12. Normalised counts of genes encoding proteins involved in the production and detoxification of ROS

Dots represent one of the four replicates and the line is through the mean. C=Control, Ab=Antibiotics. Blood meals were given immediately after the 0 h and 72 h time points. Genes were selected if they were differentially regulated at one or more time points. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, . $p < 0.1$, Wald test.

The neural immune response

Finally, we observed the significant regulation of five G-protein coupled receptors (GPCRs), which have potential roles in so-called 'behavioural immunity'. This describes the phenomenon whereby an organism's behaviour is altered upon detection of microbial ligands, most typically as an avoidance response. Indeed, one of the genes that is identified here as being microbiota regulated, *GR9*, has recently been shown to affect the outcome of oral bacterial infection by reducing feeding on sugar baited with *Serratia marcescens* (Stathopoulos et al., 2014). We additionally identify two other gustatory receptor-encoding genes, *GR13* and *GR56*, that are microbiota regulated and may play similar roles. Furthermore, the *Drosophila* orthologue

of Methuselah receptor 6 (GPRMTH6), has been implicated in stress resistance and longevity (Araújo et al., 2013), whilst the *Drosophila* orthologue of the calcitonin receptor GPRCAL3 appears to act as a master network regulator of the circadian clock (Mertens et al., 2005). All of these genes may play a role in influencing mosquito behaviour, either via sugar feeding or host seeking, in a manner that could influence bacterial acquisition. Indeed, it is interesting to note that these GPCRs are differentially regulated at different time points in the blood feeding cycle. This could suggest that different neural pathways are stimulated in different nutritional states (i.e. sugar fed or blood fed), possibly driving distinct mosquito behaviours. This finding represents a potential molecular link between mosquito behaviour and immunity; this is one of the most novel findings presented in this thesis and worthy of further research.

Figure 3.2.13

Neural related

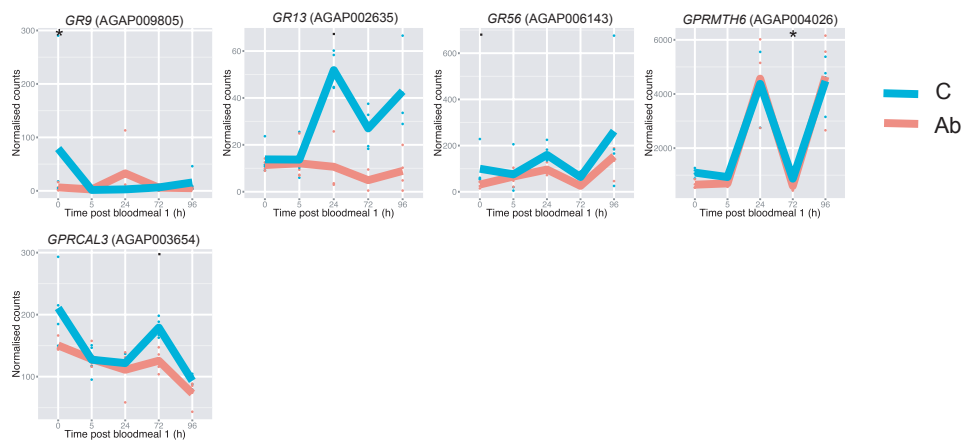


Figure 3.2.13. Normalised counts of genes encoding proteins involved in neuronal systems.

Dots represent one of the four replicates and the line is through the mean. C=Control, Ab=Antibiotics. Blood meals were given immediately after the 0 h and 72 h time points. Genes were selected if they were differentially regulated at one or more time points. * $p < 0.05$, $p < 0.1$, Wald test.

Altogether, by analysing expression specifically in the midgut tissue, at multiple time points over the blood feeding cycle, we have identified a suite of

genes whose expression within the midgut epithelium is responsive to the presence of the gut microbiota. This catalogue of genes provides a strong basis for further investigation into the mosquito response to the presence of bacteria in the gut. Below, we go on to characterise one such response.

Microbiota tolerance following blood feeding: peritrophic matrix-encoding genes are microbiota regulated

Given the dramatic increase in midgut bacterial load 24 h after blood feeding, we were surprised to observe that the expression of most microbiota-dependent immune genes (eg *CEC1*, *CEC3*, *GAM1*, *LYSC1*, *PGRPLB*, *TEP3*, *CTL4*, *CTLMA2*) increased less than two fold or not at all 24 h after a control blood meal. Given that bacteria are actively multiplying following the blood meal and therefore shedding peptidoglycan, with a 10-100 fold increase in bacterial load (Fig 3.2.1), this would suggest that immunogenic ligands in the gut lumen are increased more than 100 fold. Similar fold changes to those observed here have been reported in an *A. gambiae* oral infection model (Stathopoulos et al., 2014); in *Drosophila*, by contrast, oral bacterial infection has been reported to increase AMP expression by up to 77 –fold (Buchon et al., 2009b).

There are several possible mechanisms for the tolerance of the gut to such a dramatic increase in peptidoglycan load, not least the amidase activity of *PGRPS2* and *S3*, two immune genes to be very strongly upregulated upon blood feeding. To investigate this further, we sought to identify genes that are significantly regulated by the microbiota specifically in the blood fed gut, reasoning that such genes may play a role in mediating tolerance to the microbiota specifically after blood feeding. To do this we used DESeq2, specifying a full model with terms ‘time’, ‘condition’ (ie, plus/minus antibiotics) and the ‘time:condition’ interaction, and removing the latter in the reduced model. Only 15 genes were identified as being significantly differentially regulated by antibiotic treatment at different stages of the gonotrophic cycle, after adjusting for multiple testing (Table 3.2.3).

Table 3.2.3. Genes whose regulation by antibiotic treatment varies significantly at different stages of the gonotrophic cycle.

Gene ID	Name	Adjusted p value	Notes
AGAP008731		0.00001	Laccase-like multicopper oxidase 2
AGAP002603		0.00002	Elongation factor 1 alpha-like protein
AGAP006795	Aper1	0.00024	Peritrophin; contains chitin binding domain
AGAP007745		0.00024	
AGAP009225		0.00115	Dimeric dihydrodiol dehydrogenase
AGAP001234		0.00149	Activating signal cointegrator 1 complex subunit 3
AGAP007747		0.00180	
AGAP004935		0.00329	Ankyrin repeat-domain containing protein
AGAP006796		0.00861	Peritrophin; contains chitin binding domain
AGAP011824	TPX4	0.01156	Thioredoxin peroxidase 4
AGAP009993		0.06454	Rio kinase 3
AGAP001032		0.09832	Interferon-related developmental regulator 1
AGAP002643		0.09832	
AGAP005242		0.09832	
AGAP008704		0.09832	Juvenile hormone-inducible protein

Genes with a significant interaction between time and treatment (i.e. plus/minus antibiotics) were identified with a likelihood ratio test followed by adjustment for multiple testing. Genes with an adjusted p value < 0.1 are listed. A brief description of the cognate protein is indicated in the 'notes' column.

Interestingly, two of these genes encode peritrophins. Peritrophins are proteins with one or more chitin binding domains (CBDs) that are structural components of the PM. In *A. gambiae*, genes encoding proteins containing CBDs have been previously noted to be regulated by bacterial infection (Stathopoulos et al., 2014) and by bloodmeal associated microbiota growth (Dong et al., 2009), though a functional role for the PM in modulating the gut response to the microbiota has not yet been demonstrated. After noting that two peritrophins (AGAP006795, or *Aper1*, and AGAP006796) are differentially regulated between the control and antibiotic time courses, we explored whether any other putative PM components are regulated by the microbiota. Although the exact protein constituents of the *A. gambiae* PM are not well established, a proteomic analysis has previously been carried out to shed light on its composition (Dinglasan et al., 2009). In this study, mosquitoes were fed a protein free latex meal, PMs dissected 8 h after feeding and subjected to mass spectrometric analysis. 209 proteins were identified initially and top

candidates prioritised based on their abundance, the presence of a CBD, and if no CBD was present by the presence of a signal peptide, no transmembrane domain or GPI anchor, and no peptidase domain. This left 24 top candidate proteins (Table 3.2.4). Interestingly, we found 12 of these to be significantly regulated by the presence of the microbiota at one or more time points (Table 3.2.4 and Fig 3.2.14A).

Table 3.2.4. Genes identified in (Dinglasan et al., 2009) as top candidates for protein constituents of the PM

Gene ID	Name	Relative abundance (ranking n/209)	Annotation/Comments (taken from Dinglasan et al., 2009)
AGAP006795	AgAper1	7	2 CBD, N- and O-glycosylated; SP
AGAP006796	AgAper9	19	1 CBD; aglycosylated; short sequence; SP
AGAP009830	AgAper14	18	1 CBD; aglycosylated; SP
AGAP010364	AgAper25a	22	2 CBD; O-glycosylated; No SP
AGAP001819	AgAper25b	24	1 CBD (domain 3 of baculovirus spindolin protein); N-glycosylated; SP
AGAP011615	AgAper26	21	3 CBD; N-glycosylated; SP
AGAP010363	AgAper29	25	2 CBD; N- and O-glycosylated; SP
AGAP011616	AgAper30	20	3 CBD; aglycosylated; SP
AGAP006433	AgAper34	26	4 CBD; N- and O-glycosylated; No SP
AGAP006434	AgAper57	23	4 CBD; O-glycosylated and heavily N-glycosylated; SP
AGAP006432	AgICHIT	17	2 CBD; Pro-rich; N-glycosylated, heavily O-glycosylated; mucin domain; SP
AGAP006414	AgChitinase	5	1 CBD; N-glycosylated and heavily O-glycosylated; mucin domain; SP; Chitinase
AGAP006442		31	Conserved hypothetical protein; heavily N-glycosylated; SP, UF
AGAP004883		27	Conserved hypothetical protein; snake toxin-like protein folds/disulfide rich; Thr rich domains; both heavily N- and O-glycosylated; SP, UF
AGAP007860		33	Conserved hypothetical protein; putative protein binding motifs; N-glycosylated; SP, UF
AGAP007612		28	Conserved hypothetical protein; snake toxin-like protein folds/disulfide rich; aglycosylated; SP, UF
AGAP002851		32	Conserved hypothetical protein; MD2-lipid recognition domain; aglycosylated; SP
AGAP001352		30	Conserved hypothetical protein; odorant/hormone binding domain; O-glycosylated; SP
AGAP010132	AgSCRBQ1	34	Conserved hypothetical protein; Croquemort (CD36) scavenger receptor Class B domain; N-glycosylated; SP
AGAP006398		29	Conserved hypothetical protein; galactose-like binding protein/lectin-like domain; N- and O-glycosylated; No SP
AGAP000570		2	Conserved hypothetical protein; aglycosylated; SP, UF
AGAP007745		4	Conserved hypothetical protein; proline rich extension domain, heavily glycosylated, SP, UF
AGAP006194		10	Conserved hypothetical protein; aglycosylated; putative paralogue of AGAP006195; SP, UF
AGAP009313	AgPM9	6	Conserved hypothetical protein; similar to a chemotactic protein; SP, UF

All entries highlighted in grey are identified in this study as being transcriptionally regulated by the presence of the microbiota. Abundance ranking refers to the relative abundance of the protein in the mass spectrometric analysis of Dinglasan et al. CBD= chitin binding domain, SP= signal peptide, UF= unknown function.

Permission was obtained for the republishing of column 4 (see appendix).

Of the 12 genes encoding putative PM components identified in this study, 11 were found to be downregulated by antibiotic treatment (Fig 3.2.14A). The microbiota regulated genes include *AgAper1* (AGAP006795), the first *A. gambiae* PM component encoding gene to be shown to bind chitin (Shen and Jacobs-Lorena, 1998), and the most abundant chitin binding domain (CBD)-containing protein identified by mass spectrometry (Dinglasan et al., 2009). We also note the microbiota-dependent regulation of *ICHIT* (AGAP006432), which has been shown to be transcriptionally induced by both *P. berghei* and bacterial infection and encodes two chitin binding domains and a proline rich domain that may be involved in protein-protein aggregation (Dimopoulos et al., 1998).

In addition to the protein components of the PM, the main structural constituent is chitin, a polymer of N-acetylglucosamine. Insects are able to synthesise chitin (Fig 3.2.14B); fructose-6-phosphate, derived from glucose, is converted to glucosamine-6-phosphate in a rate limiting step catalysed by glucosamine-fructose-6-phosphate aminotransferase (Kato, Dasgupta, Smartt, & Christensen, 2002). Glucosamine-6-phosphate is then metabolised to UDP N-acetylglucosamine, which is subsequently polymerised to chitin by chitin synthase in another rate limiting step. The *A. gambiae* genome encodes two chitin synthase enzymes, *CHS1* (AGAP001741) and *CHS2* (AGAP001205), of which *CHS2* is understood to be expressed in the midgut and responsible for the synthesis of PM-associated chitin (Zhang, Zhang, Park, & Zhu, 2012). In support of this, we found *CHS2*, but not *CHS1*, to be robustly expressed in the gut. Furthermore, we identified the enzymes catalysing both of these rate-limiting steps as being microbiota regulated at the transcript level at one or more of the time points examined (Fig 3.2.14C)

Figure 3.2.14

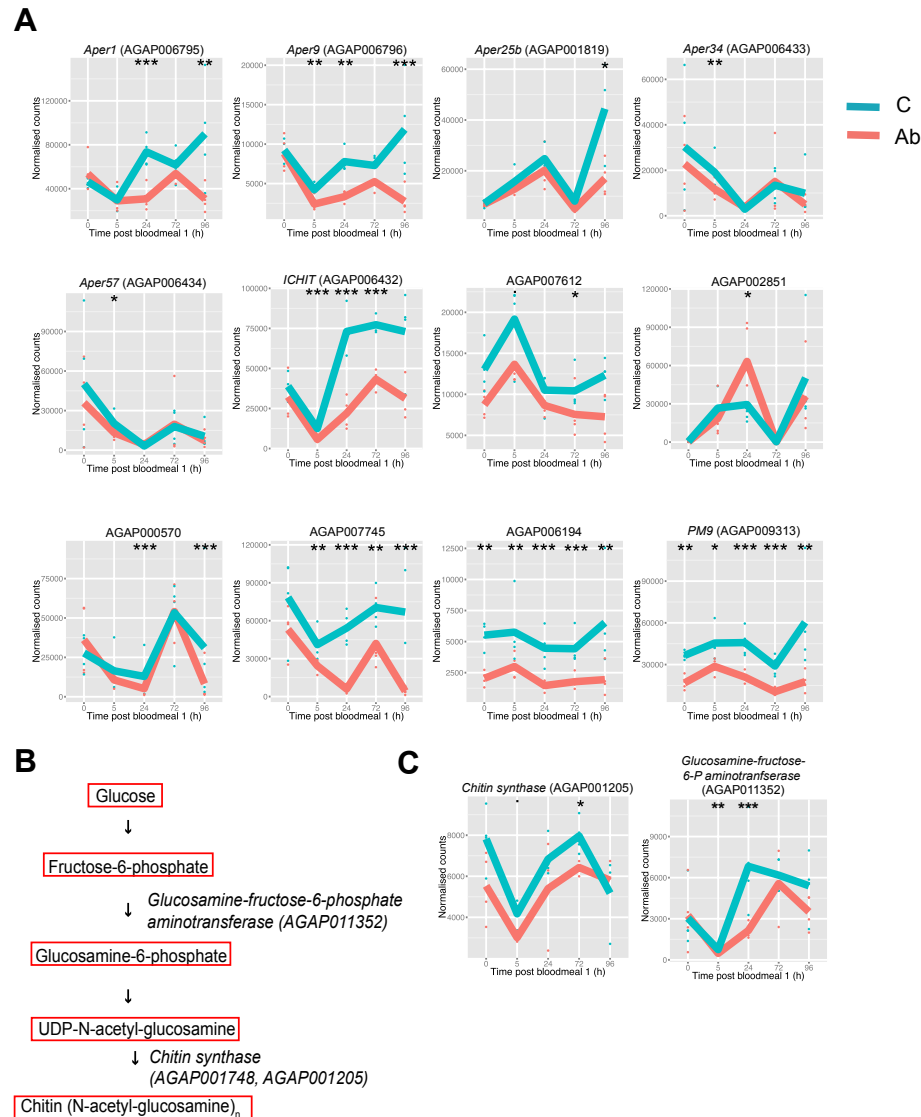


Figure 3.2.14. Protein components of the PM and components of the chitin synthesis pathway are microbiota regulated.

3.2.14A Transcriptional profiles of genes encoding putative components of the PM, as identified in Dinglasan et al, that are microbiota regulated at one or more time points. Blood meals were given immediately after the 0 h and 72 h time points. Dots represent one of the four replicates and the line is through the mean. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, . $p < 0.1$, Wald test.

3.2.14B Schematic of the insect chitin synthesis pathway. Enzymes that catalyse the two rate limiting steps are indicated.

3.2.14C Transcriptional profiles of genes encoding chitin synthase and glucosamine-fructose-6-phosphate aminotransferase. Blood meals were given immediately after the 0 h and 72 h time points. Dots represent one of the four replicates and the line is through the mean. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, . $p < 0.1$, Wald test.

The effect of antibiotic treatment on PM structure

Given that antibiotic treatment significantly reduces the mRNA levels of several components of the PM, we next considered the effect of antibiotic treatment on the structure of the PM (Fig 3.2.15). In particular, we sought to distinguish between a situation where the detection of bacterial elicitors directly induces the PM via a bacteria-stimulated signalling pathway, and one where gut bacteria actively degrade the PM, for example by production of chitinases, resulting in compensatory transcription to maintain a PM of adequate thickness.

In the absence of a standard staining method for insect PMs, whole mosquito abdomens were fixed in Duboscq's Brasil solution 24 h after a human bloodmeal, 5 µm sections cut and stained with hematoxylin and eosin (H&E) to observe the ultrastructure of the tissues. In the control samples, a thick layer of dark pigment was observed surrounding the blood bolus and effectively separating the contents of the lumen from the epithelial cells. We propose that this dark layer consists of haem pigments, bound to the PM; haem is a bi-product of haemoglobin digestion that has been shown to be associated with the PM in *A. aegypti* mosquitoes (Magalhaes, 2014; Pascoa et al., 2002), likely directly via haem binding to CBD-containing proteins (Devenport et al., 2006). In the antibiotic treated mosquitoes, several regions exhibit disruption of this layer; in these cases, it is observed that red blood cells (RBCs) come into direct contact with the microvilli of the epithelial cells (Fig 3.2.15, antibiotics panel). Indeed, RBC contact with the epithelium is observed in significantly more antibiotic fed guts (92 %, n=12) than control guts (6 %, n=18; $p < 0.0001$, Chi-square test). These observations suggest that, indeed, the presence of the microbiota is a prerequisite for the synthesis of a structurally intact PM.

Figure 3.2.15

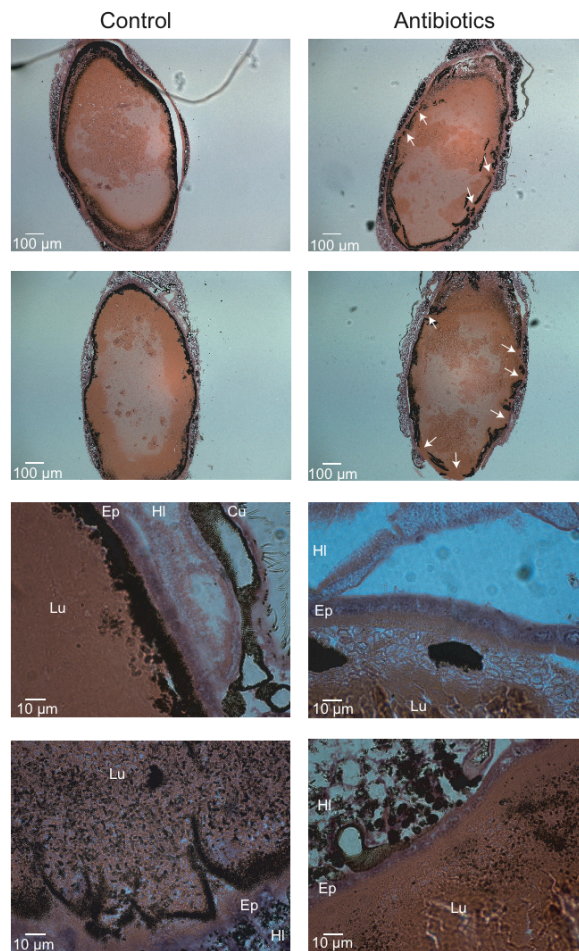


Figure 3.2.15. Thin abdomen sections stained with H&E in control and antibiotic treated mosquito cohorts. Arrows indicate regions where the PM appears to be disrupted, preventing the proper organisation of haem aggregates. Cu= cuticle, Lu= lumen, Ep= epithelium, HI= haemolymph space.

Transcriptional regulation of *Aper1*

The microbiota dependent regulation of one of the microbiota dependent PM genes, *Aper1* (AGAP006795), was verified by qRT-PCR (Fig 3.2.16A). The observation that the microbiota are necessary for the synthesis of a structurally sound PM suggests that gut bacteria directly induce transcription of the PM components. We therefore sought to identify the pathway or pathways that gut bacteria stimulate to induce this response. We based our investigation on the fact that PM components of other insects have previously been shown to be induced by the Imd and JAK/STAT pathways (Buchon et al., 2009b; Narasimhan et al., 2014). Therefore, we first used an *in silico* analysis to identify consensus NF- κ B (GGGRHHYMY) and STAT (TTCNNN(N)GAA) binding motifs in the 500 bp upstream of the start of the protein coding region of the microbiota-regulated PM genes and chitin synthesis pathway enzymes. None of the 14 upstream regions had NF- κ B consensus sites, though five, including *Aper1* and *CHS2*, exhibited STAT binding sites (Fig 6.1, Appendix). As a positive control, 500 bp upstream of the known REL2 responsive gene *CEC1* was also analysed, and found to contain two NF- κ B consensus sequences (Fig 6.1, Appendix). These observations suggest that the microbiota may signal, in part, via the JAK/STAT pathway to induce the PM post blood feeding.

Next, we transiently knocked down the IMD transcription factor, REL2, and the JAK/STAT transcription factor that is expressed in the gut, STAT2, by injection of double stranded RNA (dsRNA), gave mosquitoes a bloodmeal, and quantified *Aper1* transcript levels in the gut by qRT-PCR. Interestingly, REL2 knock down resulted in significantly higher levels of *Aper1* mRNA 24 h post bloodmeal (Fig 3.2.16B), whilst no effect of STAT2 knock down was observed at this time point (Fig 3.2.16C). The positive effect of REL2 knock down on *Aper1* mRNA levels is likely an indirect effect of REL2 knockdown on gut bacterial load, causing elevated induction of *Aper1* via an unknown pathway. These data suggest that, at 24 h post blood meal at least, the JAK/STAT pathway is not responsible for the microbiota dependent induction of *Aper1*, despite the identification of a STAT binding site in its upstream region. Examination of the effect of STAT2 knock down at different time points

post blood meal may well reveal an effect of this pathway and merits further investigation.

Figure 3.2.16

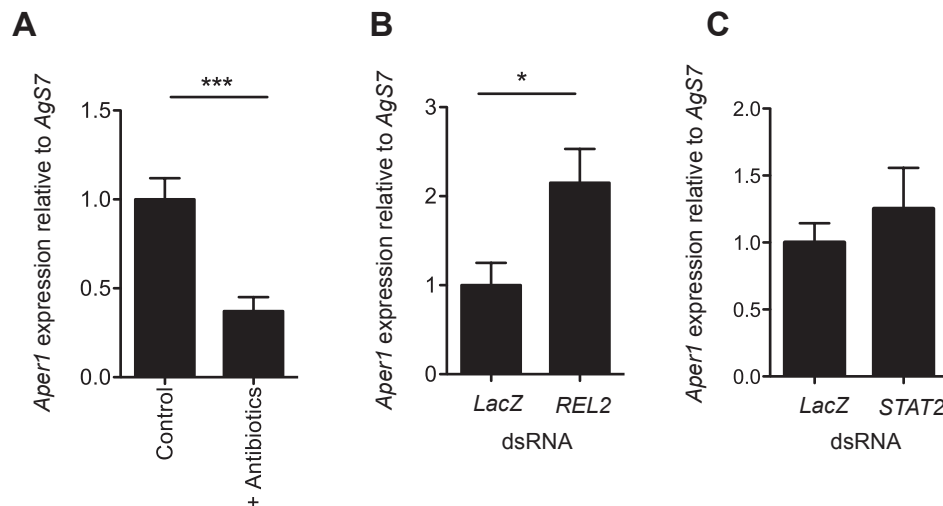


Figure 3.2.16. Transcriptional control of *Aperi1*

3.2.16A. qRT-PCR verification that *Aperi1* transcription is microbiota dependent 24 h post bloodmeal. The same antibiotic treatment was used as that described for the RNA sequencing study described above, using separate batches of mosquito. Data represents 6 pools of 8-10 midguts per condition, derived from two independent biological replicates.

3.2.16B. dsRNA against the IMD pathway transcription factor REL2 increases *Aperi1* expression in the gut 24 h post bloodmeal. Data represents 6-8 pools of 5 midguts per condition, derived from three independent biological replicates.

3.2.16C. dsRNA against the JAK/STAT pathway transcription factor STAT2 has no effect on *Aperi1* expression in the gut 24 h post bloodmeal. Data represents 4-6 pools of 5 midguts per condition, derived from two independent biological replicates.

*** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, . $p < 0.1$, ANOVA.

A-C: Data are normalised within biological replicates to the mean value of the control pool (no antibiotics or dsLacZ). Graphs show the mean and standard error.

The structural integrity of the PM is disrupted by feeding with polyoxin D, a chitin synthase inhibitor

Given that the synthesis of a mature PM is dependent on the gut microbiota, we next sought to investigate whether the PM plays a role in modulating the gut immune response to the microbiota, or in controlling microbiota growth and dissemination. The addition of 100 μ M polyoxin D, a chitin synthase inhibitor, to the bloodmeal has previously been demonstrated to completely abolish synthesis of the *A. gambiae* type I PM 36 h after blood feeding (Shahabuddin et al., 1995). Here, at 24 h post blood meal we observed red blood cell contact with cells of the gut epithelium in H&E stained abdominal sections in significantly more polyoxin D fed mosquitos compared with control mosquitoes (19% of the control mosquitoes, n=32, and 47% of the polyoxin D fed cohort, n=17; $p < 0.05$, Chi-square test) (Fig 3.2.17). Therefore, we decided to use polyoxin D feeding as an experimental disruption of the PM, in order to explore its role in microbiota homeostasis in the gut. To mitigate the fact that we did not observe complete PM disruption in all mosquito gut sections examined, all subsequent experiments are conducted with pools of 8-10 midguts.

Figure 3.2.17

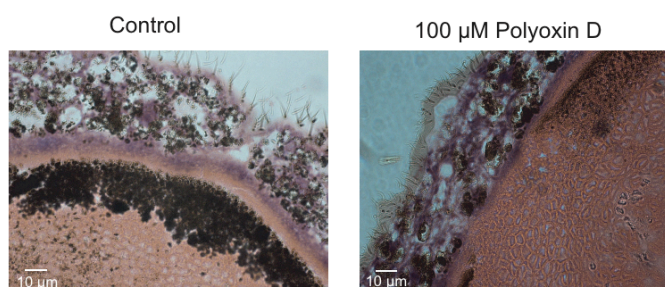


Figure 3.2.17. Feeding with polyoxin D, a chitin synthase inhibitor, disrupts the structural integrity of the PM.

Thin abdominal sections of midguts 24 h after feeding with a control blood meal or a blood meal supplemented with 100 μ M polyoxin D.

The PM contributes to midgut resistance to the microbiota 24 h post blood feeding

We first assessed the effect of polyoxin D feeding on the induction of immune genes in the gut 24 h after blood feeding, the time of peak microbiota load. The expression of three genes, *CEC1*, *GAM1* and *LYSC1*, was analysed; these were chosen as they were all found to be consistently and strongly dependent on the presence of the microbiota for their expression (Fig 3.2.11). After feeding with 100 μ M polyoxin D, we did not find any difference in the expression of *CEC1* or *LYSC1*, though we observed a significant increase in the expression of *GAM1* (Fig 3.2.18A). This difference was dependent on the presence of the gut microbiota, as polyoxin D feeding had no effect on basal *GAM1* expression when mosquitoes were treated with antibiotics from emergence.

We hypothesised that this microbiota-dependent elevated *GAM1* expression could reflect either increased access of bacteria or immune elicitors to the cells of the gut epithelium, or could be due to elevated microbial growth or survival after disruption of the PM. Using qRT-PCR against 16S rRNA, we did not detect a significant increase in total gut microbial load. We also individually assessed the loads of the three main bacterial families that our colony is known to harbour (the *Flavobacteriaceae*, *Acetobacteriaceae* and *Enterobacteriaceae*) (Gendrin et al., 2015). We did not detect a change in the load of bacteria of the *Flavobacteriaceae* and *Acetobacteriaceae* families; however, we did detect a significant increase in the load of the *Enterobacteriaceae* (Fig 3.2.18B).

Figure 3.2.18

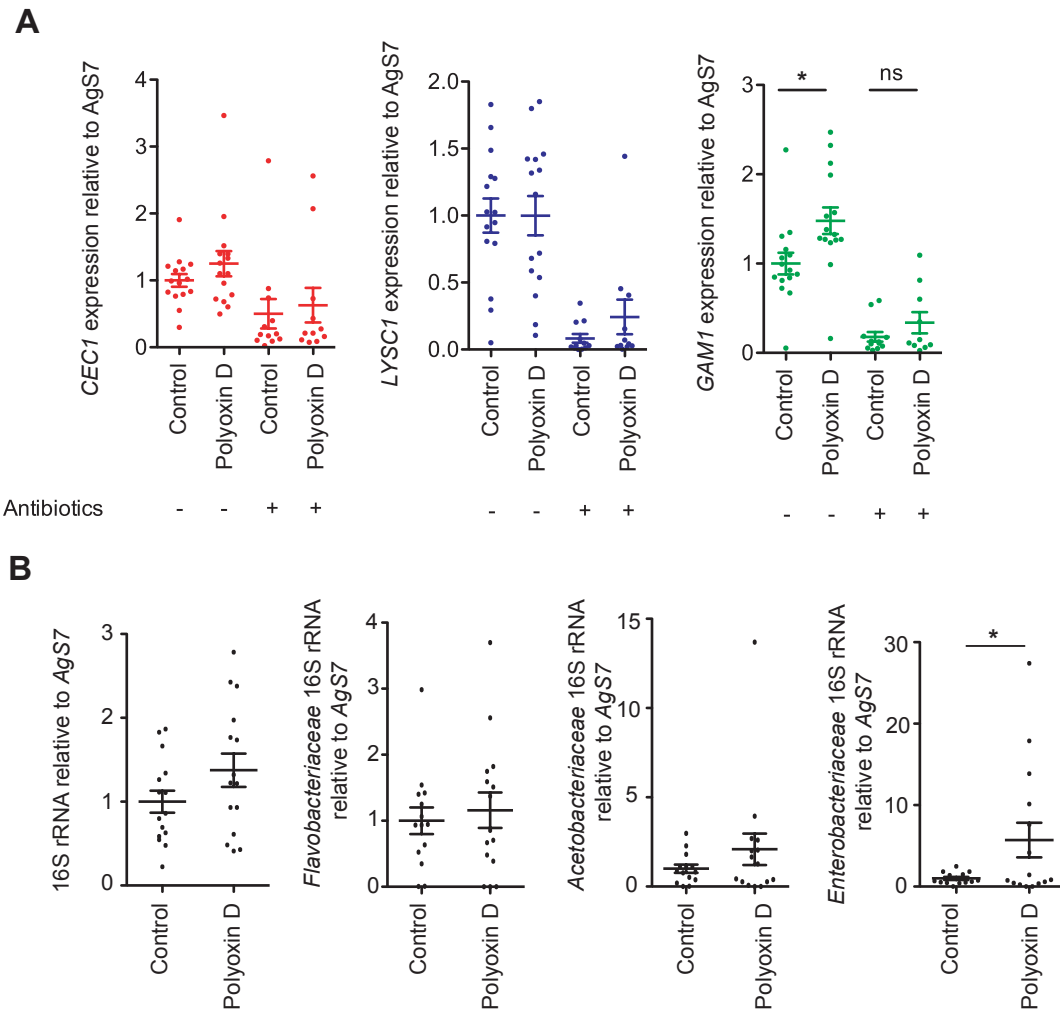


Figure 3.2.18. PM disruption by polyoxin D feeding results in elevated *GAM1* expression and elevated *Enterobacteriaceae* load in the midgut 24 h post blood feeding.

3.2.18A. The effect of the addition of 100 μ M polyoxin D or an equal volume of water (control) to a human bloodmeal on immune induction in the gut. Each dot represents a pool of 8-10 guts, derived from five independent biological replicates. Data are normalised to the average control (water, no antibiotics) value within each biological replicate. Antibiotic treated mosquitoes were fed antibiotics from emergence in both the blood and sugar meals. Line shows the mean $\pm$ standard error.

3.2.18B. The effect of the addition of 100 μ M polyoxin D or an equal volume of water (control) to a human bloodmeal on the total bacterial load or loads of the *Flavobacteriaceae*, *Acetobacteriaceae* and *Enterobacteriaceae* families in the gut, assessed by qRT-PCR on cDNA with universal or family specific 16S primers. Data are normalised to the average control value within each biological replicate. Line shows the mean $\pm$ standard error.

* $p < 0.05$, ANOVA.

To investigate whether it is the same pools of mosquitoes that harbour both increased load of *Enterobacteriaceae* and elevated *GAM1* induction, we explored the relationship between these two variables. Interestingly, in the control cohort of mosquitoes we found a significant positive correlation between relative *Enterobacteriaceae* load and *GAM1* expression, as well as between *Acetobacteriaceae* load and *GAM1* expression, though no such relationship was found between *Flavobacteriaceae* load or total 16S bacterial load and AMP expression (Fig 3.2.19). This could suggest that it is primarily the *Enterobacteriaceae* and *Acetobacteriaceae* families that are responsible for the induction of this AMP in the control guts. In the polyoxin D fed mosquitoes, the positive correlation between *Enterobacteriaceae* load and *GAM1* expression was maintained, though not that between *Acetobacteriaceae* load and *GAM1* expression (Fig 3.2.19). Although this does not prove a causal relationship, these data are consistent with a model whereby the disruption of the PM results in elevated *Enterobacteriaceae* load, which in turn leads to greater induction of *GAM1* in the gut epithelium.

Figure 3.2.19

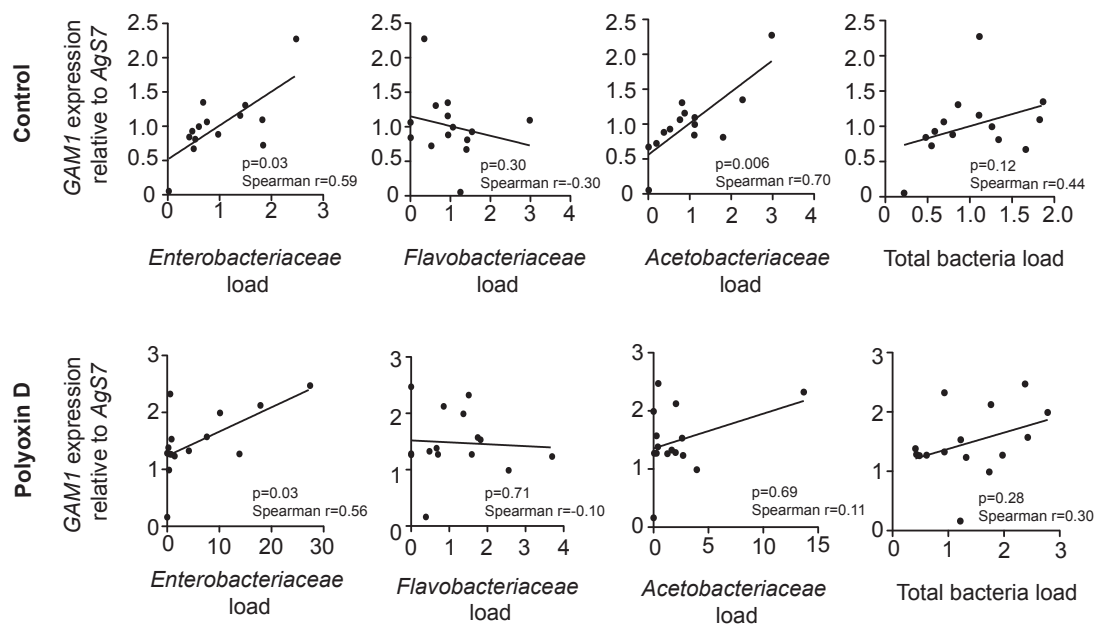


Figure 3.2.19. Relationships between the relative load of specific bacterial families and *GAM1* expression in the midgut of control and polyoxin D fed mosquitoes 24 h post blood feeding.

Scatter plots of the load of specific bacteria families or eubacteria against *GAM1* expression in the midguts of control or polyoxin D fed mosquitoes. Data are normalised to the average control value within each biological replicate. Spearman's rank correlation coefficient and associated p values are indicated.

We next sought to dissect the role of the different components of the PM in microbiota resistance, by exploring whether disruption of the peritrophin component of the PM, as opposed to the chitin component, results in similar effects in the gut system. To do this, we injected mosquitoes with dsRNA against the abundant peritrophin *Aper1*, offered mosquitoes a bloodmeal 24 h after injection, and monitored immune induction and bacterial load in the gut 24 h later. Average *Aper1* transcript levels in the gut, 24 h post blood feeding, were on average 63 % the level of *Aper1* transcript levels in the injection controls.

Interestingly, we found that, similar to polyoxin D feeding, *Aper1* knock down resulted in elevated epithelial immune induction 24 h post blood feeding in a microbiota dependent manner (Fig 3.2.20A). However, in this case *CEC1* was the effector whose expression significantly increased. Unlike after feeding with

polyoxin D, we did not detect a significant change in the load of any family of bacteria (Fig 3.2.20B).

Figure 3.2.20

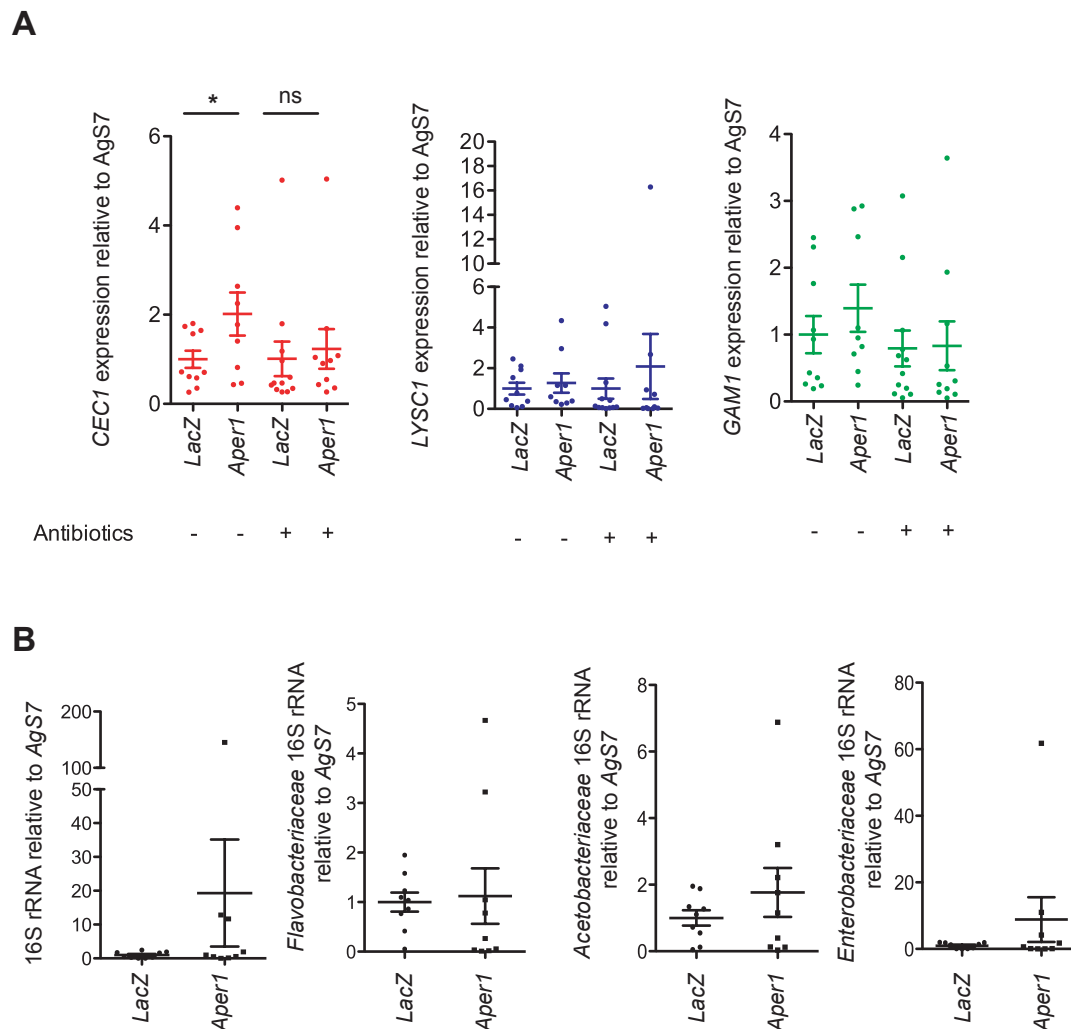


Figure 3.2.20. PM disruption by Aper1 knock down results in elevated *CEC1* expression in the gut 24 h post blood feeding.

3.2.20A. The effect of the injection of dsRNA against Aper or LacZ (control) on immune induction in the gut 24 h after blood feeding. Each dot represents a pool of 3-5 guts, derived from four independent biological replicates. Data are normalised to the average control (LacZ, no antibiotics) value within each biological replicate. Antibiotic treated mosquitoes were fed antibiotics from emergence in both the blood and sugar meals. Line shows the mean $\pm$ standard error.

3.2.20B. The effect of the injection of dsRNA against Aper or LacZ (control) on the total bacterial load or loads of the *Flavobacteriaceae*, *Acetobacteriaceae* and *Enterobacteriaceae* families in the gut. Data are normalised to the average control value within each biological replicate. Line shows the mean $\pm$ standard error.

* $p < 0.05$, ANOVA.

When exploring the relationship between *CEC1* expression and the loads of different bacterial families, we again found that, in the control (dsLacZ) mosquitoes, *Enterobacteriaceae* load correlated significantly with AMP expression (Fig 3.2.21). In this case this was unique to the *Enterobacteriaceae* family. Interestingly, this relationship is not maintained upon *Aper1* knock down. This suggests that *Aper1* knock down results in elevated *CEC1* expression via a mechanism that is independent of an effect on *Enterobacteriaceae* load.

Figure 3.2.21

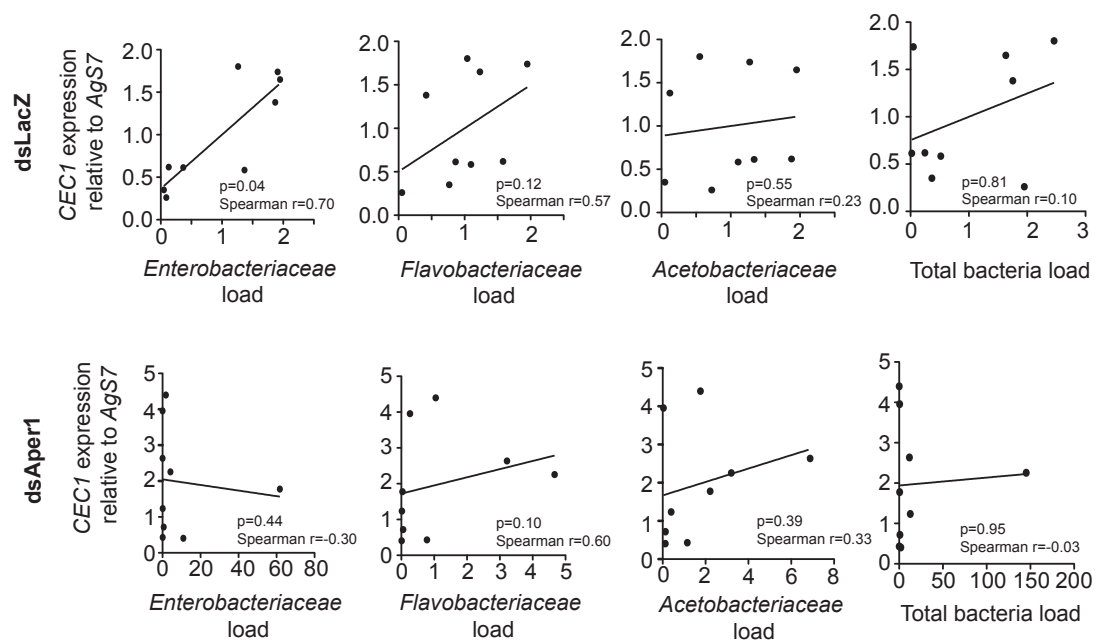


Figure 3.2.21. Relationships between the relative load of specific bacterial families and *CEC1* expression in the midgut of control (dsLacZ) and *Aper1* knock down mosquitoes.

Scatter plots of the load of specific bacteria families or eubacteria against *CEC1* expression in the midguts of mosquitoes after injection of dsRNA against LacZ (control) or *Aper1*. Data are normalised to the average dsLacZ value within each biological replicate. Spearman's rank correlation coefficient and associated p values are indicated.

Taken together, these two distinct methods of disrupting the PM give insight into the interactions between the PM, the gut microbiota and epithelial immunity. First, PM disruption by chitin synthesis inhibition results in elevated *Enterobacteriaceae* load. The observation that *Aper1* knock down does not reproduce this effect could suggest that it is the chitin component of the PM, as opposed to the protein component, that limits *Enterobacteriaceae* growth. Simultaneous targeting of multiple peritrophins would be necessary to verify this, as redundancy between peritrophins, together with weak knock down efficiency, could dampen the magnitude of this effect. Both interventions result in elevated epithelial immune induction in a manner that is dependent on the presence of the microbiota. In the case of polyoxin D feeding, the expression of *GAM1* is significantly increased, whilst in the case of *Aper1* knock down the expression of *CEC1* is significantly increased. The difference in reporter response could reflect distinct mechanisms being at play. After *Aper1* knock down, there is no significant change in bacterial load and no relationship between bacterial load and AMP expression. This suggests that, in this case, elevated *CEC1* expression is independent of any direct effect on bacterial load and composition. One hypothesis would be that this peritrophin plays a role in sequestering bacterial immune elicitors, precluding them from stimulating a response in gut epithelial cells. The elevated *GAM1* expression in polyoxin D fed mosquitoes could be explained by the same mechanism (sequestration of elicitors by the PM), or could be a direct consequence of the increase in bacterial load.

The PM contributes to the restoration of microbiota homeostasis after blood feeding

Upon completion of blood digestion, the PM is believed to be degraded and excreted with the blood bolus (Tellam, 1996). We previously demonstrated that excretion is the main mechanism by which bacteria are removed from the gut after blood feeding (chapter 3.2). To investigate whether the PM plays a role in mediating bacterial excretion, we monitored bacterial load and family composition at 72 h post blood feeding, a time point at which all individuals have excreted their blood bolus, after disruption of the PM by feeding with

polyoxin D⁴. The polyoxin D fed cohort of mosquitoes harboured significantly higher loads of *Enterobacteriaceae* and *Acetobacteriaceae* than the control cohort, as well as non-significantly higher loads of *Flavobacteriaceae* and total 16S rRNA (Fig 3.2.22). These data suggest that the PM prevents bacteria from occupying niches within the gut that cannot be cleared upon excretion of the blood bolus.

Figure 3.2.22

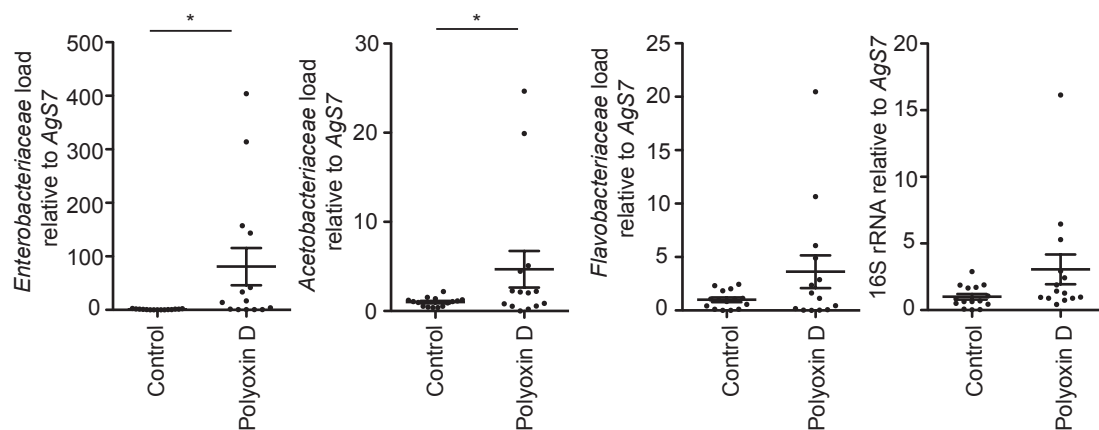


Figure 3.2.22. PM disruption by polyoxin D feeding results in elevated *Enterobacteriaceae* and *Acetobacteriaceae* load in the gut 72 h post blood feeding.

3.2.22. The effect of the addition of 100 μ M polyoxin D or an equal volume of water (control) to a human blood meal on the loads of specific bacterial families and total bacterial load, assessed by qRT-PCR on cDNA with universal or family specific 16S primers. Each dot represents a pool of 8-10 guts, derived from four independent biological replicates. Data are normalised to the average control value within each biological replicate. Line shows the mean $\pm$ standard error.

* $p < 0.05$, ANOVA.

Indeed, in control H&E thin abdominal sections we observed cellular structures that are clearly distinct from blood bolus erythrocytes, occupying what appears to be the ectoperitrophic space (Fig 3.2.23). We hypothesise that these cellular structures are bacteria, and that this population living proximally to the gut epithelium remains in the gut after blood bolus excretion, seeding the microbiota of the next gonotrophic cycle. Disruption of the PM may allow more bacteria to live proximally to the gut epithelium, preventing them from being enclosed within the blood bolus as it is excreted.

⁴ Data is not available on bacterial composition at 72 h for the dsAper1 cohort.

Figure 3.2.23

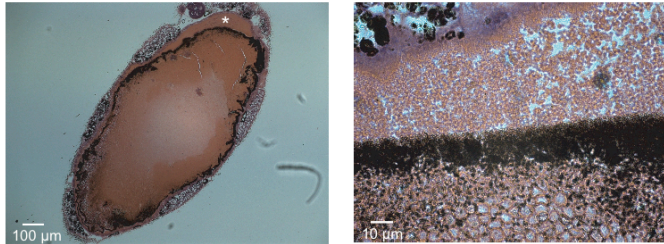


Figure 3.2.23. Observation of cellular structures, hypothesised to be bacteria, occupying the ectoperitrophic space in control guts 24 h post blood feeding.

H&E stained thin sections of control guts. Left hand panel (100X) shows whole abdomen cross section. Right hand panel (1000X) shows the blood bolus (bottom half of the image), enclosed by what we hypothesise to be haem aggregates bound to the PM (dark line). Above this and below the epithelium a distinct cellular structure is observed, which we hypothesise to be bacteria.

The PM prevents dissemination of the midgut microbiota into the body cavity after blood feeding

In *Drosophila* and other insects, one function of a local immune response is to prevent or minimise systemic immune induction arising from an oral infection. As such, we investigated whether the PM plays a role in preventing the induction of a systemic response to microbiota growth within the gut following a blood meal. For each pool of dissected midguts we therefore collected the associated carcass samples, consisting of the abdominal cuticle with the fat body attached, after removal of all other organs (namely the gut, ovaries and malpighian tubules). At 72 h post blood feeding, we observed considerable systemic, microbiota dependent induction of *CEC1* in a subset of the carcasses of the polyoxin D fed mosquitoes (Fig 3.2.24). A similar, but non-significant, trend was observed in the expression of *LYSC1* and *GAM1*.

Figure 3.2.24

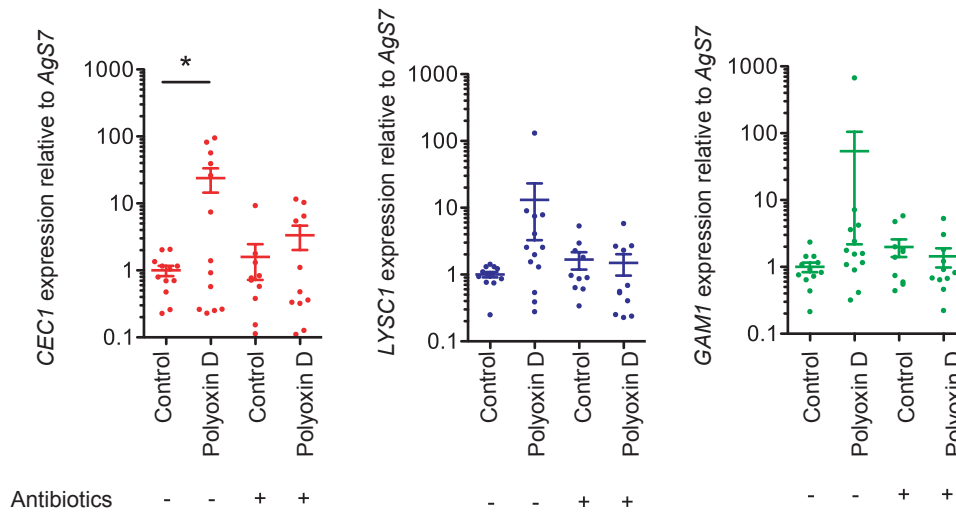


Figure 3.2.24 The effect of the addition of 100 μ M polyoxin D or an equal volume of water to a human blood meal on systemic immune induction 72 h post blood feeding.

Each dot represents a pool of 8-10 carcasses, derived from three independent biological replicates. Data are normalised to the average control (water, no antibiotics) value within each biological replicate. Line shows the mean $\pm$ standard error. * $p < 0.05$, ANOVA.

Mechanistically, we considered that this immune induction could be caused either by translocation of live bacteria from the gut to the haemocoel, or by bacteria or mosquito-derived molecules signalling from the gut to the haemocoel. To investigate the former scenario, we attempted to amplify 16S rRNA from the carcass samples (Fig 3.2.25). In the antibiotic fed mosquitoes, 16S amplification occurred at the level of the qRT-PCR negative controls, whereas 16S rRNA was amplified above this level in a subset of both the control and polyoxin D-fed samples. We observed no significant difference in the relative bacterial load detected in the carcasses of the control or polyoxin D fed mosquito cohorts. At a family specific level we found no significant difference in the incidence or load of the *Flavobacteriaceae* or the *Acetobacteriaceae*. We did, however, observe a significant increase in the detection of *Enterobacteriaceae*; indeed, bacteria of this family were only confidently detected in polyoxin D fed pools of mosquito carcasses, not in the

control pools, in which amplification consistently occurred at the level of the negative PCR controls.

Figure 3.2.25

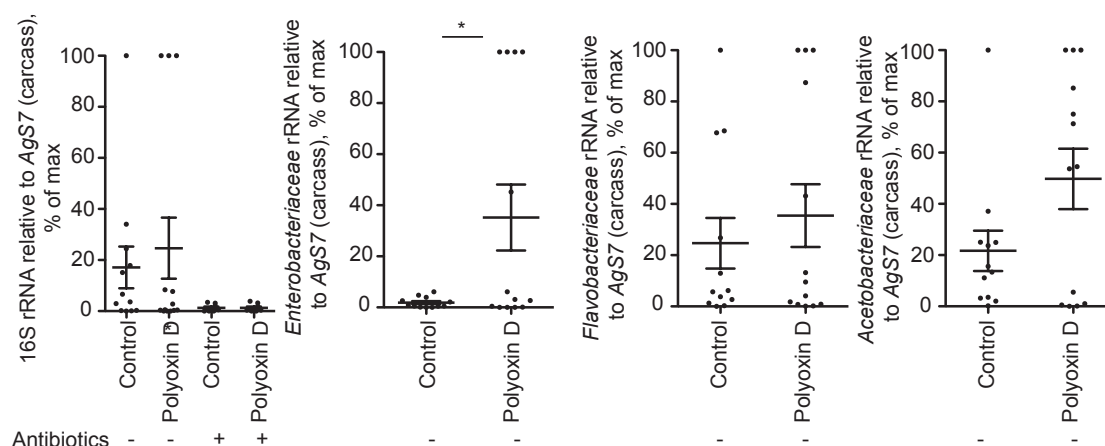


Figure 3.2.25. Detection of eubacteria and specific bacterial families in the carcasses of control and polyoxin D fed mosquitoes 72 h post blood feeding.

Bacterial load was assessed by qRT-PCR on cDNA with universal or family specific 16S primers. Each dot represents a pool of 8-10 carcasses, derived from three independent biological replicates. Data are normalised to the maximum value across all conditions within each biological replicate ('100%'). *p<0.05, ANOVA.

In the polyoxin D fed group, *Enterobacteriaceae* detection in the carcass correlated significantly with *CEC1* induction, with this not being the case in the control group (Fig 3.2.26). *Flavobacteriaceae* and *Acetobacteriaceae* did not have a similar correlation with *CEC1* expression. The clear relationship between the detection of *Enterobacteriaceae* in the carcass and *CEC1* expression, together with the fact that this family of bacteria is detected only in carcasses of polyoxin D fed mosquitoes, strongly suggests that this family of bacteria is able to translocate from the gut to the haemocoel upon disruption of the PM, seeding a systemic infection.

Figure 3.2.26

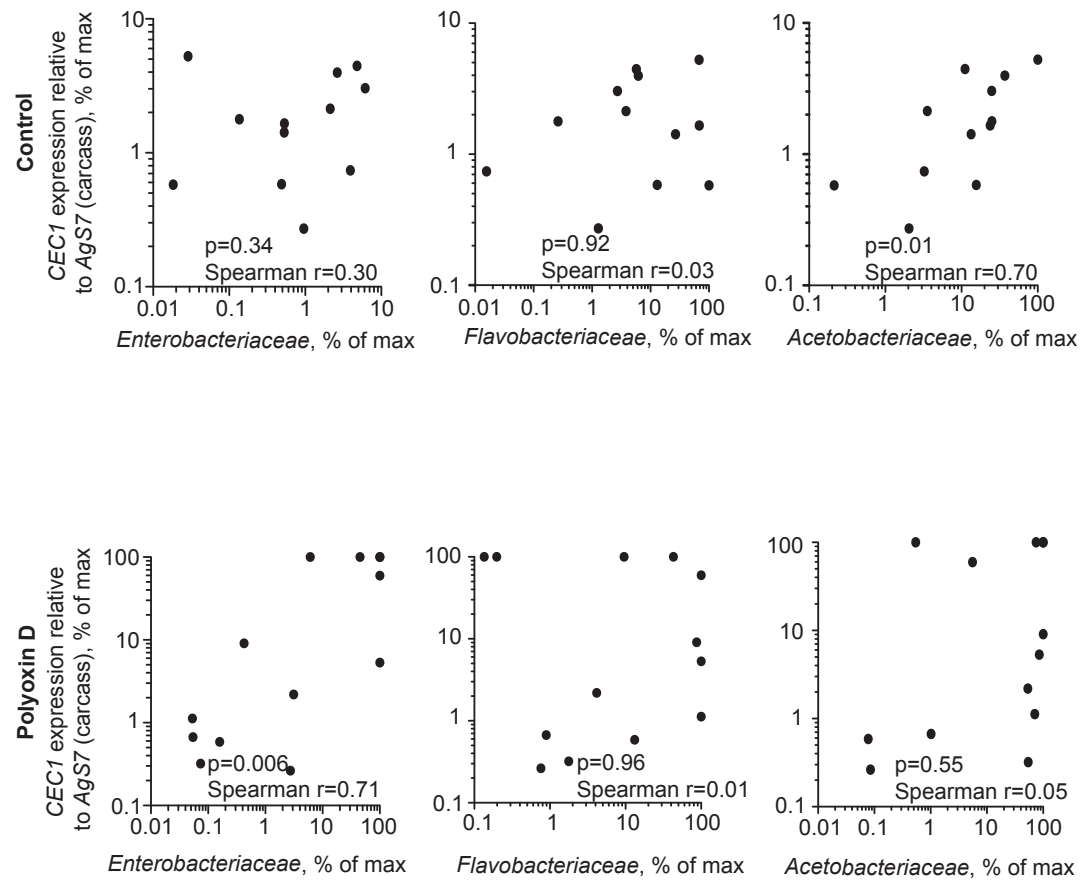


Figure 3.2.26. Relationship between three bacterial families and *CEC1* expression in the carcasses of control and polyoxin D fed mosquitoes 72 h post blood feeding. Scatter plots of the load of specific bacteria families or eubacteria against *CEC1* expression in the midguts of control or polyoxin D fed mosquitoes. Data are normalised to the maximum value across all conditions within each biological replicate ('100%'). Spearman's rank correlation coefficient and associated p values are indicated.

In conclusion, transcriptomic analysis implicated the mosquito gut microbiota in several novel aspects of midgut physiology, including folate biosynthesis and sphingolipid metabolism. We identify many putative components of the type I PM as being microbiota regulated, and go on to show that antibiotic treatment results in a disrupted PM. Using two experimental methods, we show that the PM plays a role in limiting microbiota-dependent immune induction in the gut following a blood meal, and speculate on two potential mechanisms behind this. The PM is also a prerequisite for efficient restoration of microbiota load following blood digestion, possibly by preventing bacteria from occupying niches that are not cleared upon excretion. Finally, we identify a novel role of the PM in preventing gut bacteria from invading the haemolymph and causing a systemic infection.

Discussion

To date, the mosquito microbiota have been identified as facilitating resistance to *Plasmodium* infection (Dong et al., 2009; Meister et al., 2009), being essential to mosquito larval development (Coon et al., 2014), and playing a significant role in adult physiology (Gendrin et al., 2015). The pathways underlying these latter two effects have not yet been established. Here we have identified several nutritional pathways as being differentially regulated in the absence of the gut microbiota, including carbohydrate and protein metabolism at an early time point post blood feeding (5 h). Looking particularly at folate biosynthesis, similar to humans it seems that *A. gambiae* is not able to fully synthesise folate and must therefore acquire it from the diet or the microbiota. In *Drosophila*, microbiota-derived folate was found to be sufficient to compensate for the absence of folate in the diet (Piper et al., 2014). In the presence of the microbiota, two of the genes preceding missing steps in the synthesis pathway are significantly upregulated 24 h post bloodmeal. Interestingly, this could suggest that gut bacteria are able to scavenge an intermediate metabolite, perhaps implying that the mosquito actively transports dihydroneopterin out of gut epithelial cells to be further processed by the microbiota. In one study of human gut bacteria, B vitamin biosynthesis pathways were found to be highly prevalent in microbiota genomes and the majority (71 %) of *Proteobacteria* were found to encode complete folate biosynthesis pathways (Magnúsdóttir et al., 2015). Complementary studies, for example in metabolomics, will be key to fully understanding the role of the gut microbiota in mosquito nutrition.

This transcriptional analysis is the first report of the specific mosquito gut immune response to the presence of the microbiota. By extending our analysis to multiple time points across the gonotrophic cycle and looking specifically at the gut immune response, we have identified considerably more genes as being differentially regulated than a previous analysis employing microarrays to compare control and antibiotic fed whole mosquitoes (Dong et al., 2009). In particular, we have identified several immune genes with characterised roles in the systemic immune response whose role in the gut has not been studied. The putative positive and negative regulator genes

identified here are therefore good candidates for genes with a role in controlling the strength of the immune response and/or microbiota load. Such genes are potential candidates for transmission blocking targets.

Despite using dissected midguts in this experiment, it must be acknowledged that it is not possible to rule out that RNA derived from haemocytes adhering to the gut basal lamina was additionally sequenced. Furthermore, it cannot be ruled out that the feeding of high concentrations of antibiotics could cause a direct transcriptional response in the mosquito; this is particularly the case in the induction of ROS-related genes, making it difficult to draw conclusions on how such genes are regulated by the microbiota. In the future, this experiment should be repeated with an axenic colony of mosquitoes, abrogating the need for antibiotic feeding. Whilst the existence of such a colony has been reported (Lyke et al., 2010), the composition of the larval diet that is required for aseptic development is not yet publically available.

Here, we identify the PM as a key factor in controlling microbiota homeostasis. Several components of the PM and the chitin synthesis pathway were found to be transcriptionally induced by the microbiota. It is not clear which pathways are responsible for this. REL2 knock down was found to cause an increase in *Aper1* expression; this observation could be due to the increase in microbiota load that is expected to be associated with REL2 knock down. Alternatively, REL2 could be a genuine negative regulator of *Aper1* expression. In *Drosophila*, the NF- κ B transcription factor Relish is known to target TAK1, which is upstream of JNK, to the proteasome, thereby negatively regulating JNK signalling (Park et al., 2004). JNK induces several immune responsive genes such as AMPs (Delaney et al., 2006), making it a potential candidate for a regulator of *Aper1* expression. Despite the identification of a consensus STAT binding site in the 500 bp 5' to the *Aper1* translation start site, knock down of STAT2 had no effect on *Aper1* expression 24 h post blood feeding. It is possible that an effect would be observed at a different time point post blood feeding or by looking at different PM reporters with the STAT binding site; indeed, in *I. scapularis* it was found that the expression of only a subset of the PM component encoding genes with the STAT binding site was

STAT dependent at the single time point examined.

The functional data presented here reveal a complex and dynamic relationship between the mosquito microbiota, the PM and local and systemic mosquito immunity. In humans and mice, the inner and outer mucus layers of the gastrointestinal tract form a physical and biochemical barrier between the flora of the gut lumen and the epithelial cells, composed of mucin glycoproteins (Faderl et al., 2015). The mucus layer is at its thickest in the distal colon, the region of highest bacterial colonisation, where it functions as a scaffold for AMPs and immunoglobulin A, and acts to protect against microbiota contact with the epithelium (Johansson et al., 2013, 2014). The outer mucus layer is known to interact with intestinal microbes (Johansson et al., 2008), providing a habitat for O-glycan foraging taxa (Koropatkin et al., 2012). Thus, the defensive nature of mucus is dependent on the entrapment of microbes in the outer layer, but this equally facilitates microbial colonisation in acting as a source of nutrition.

The mosquito PM is analogous to the vertebrate mucus layer, containing heavily glycosylated proteins, though in this case cross-linking chitin. There is some indication that peritrophins derived from flies and crabs are able to interact directly with bacteria, possibly having anti-bacterial action (Huang et al., 2015; Korayem et al., 2004). Furthermore, proteomic analysis of the tsetse type II PM identified 27 proteins derived from the secondary endosymbiont *Sodalis glossinidius*, suggesting that this bacterium lives in close association with the PM (Rose et al., 2014). Here, we observe that the inhibition of chitin synthase results in elevated *Enterobacteriaceae* load; this is consistent with bacteria of this family living in close association with the *A. coluzzii* PM. The PM could limit *Enterobacteriaceae* growth directly, by inducing bacterial death or preventing growth of bacteria bound to the PM, or indirectly, via scaffolding of AMPs and other immune factors (Faderl et al., 2015; Vaishnava et al., 2011). The observation that this effect is limited to the *Enterobacteriaceae* is intriguing. This may reflect different niches within the gut being occupied by different bacterial taxa: for instance, the *Flavobacteriaceae* may live in suspension within the blood bolus whereas the *Enterobacteriaceae* may live

in association with the PM, perhaps in a biofilm-like structure. Indeed, in support of this notion, in mice the *Enterobacteriaceae* family is associated with the induction of colitis, or inflammation of the colon (Garrett et al., 2010).

It is not clear whether the observed increase in immune induction is due to greater bacterial load or altered species composition, translating to a higher concentration of bacterial elicitors, or to increased accessibility of these elicitors to the innate immune receptors in the epithelial membrane. Considering permeability to bacterial elicitors, the PM pore size has been estimated at between 4 and 10 nm in a variety of insects; in *Glossina palpalis* this translates to permeability to a 17kDa protein and impermeability to a 68 kDa protein (Tellam, 1996). TCT, the minimal elicitor of the *Drosophila* Imd pathway, has a molecular mass of approximately 1 kDa, suggesting that it is free to traverse the PM. However larger peptidoglycan fragments and bacteria themselves may be unable to cross the PM.

The second identified role of the PM is in reducing bacterial load at 72 h post bloodmeal. A role in encapsulating the gut lumen contents for excretion would be consistent with this observation. As shown in chapter 3.1, this is the primary means by which bacterial load is brought back to pre bloodmeal levels following blood digestion. Although no obvious defect was observed in the polyoxin D fed mosquitoes' ability to excrete their blood bolus (data not shown), the data presented here would suggest that to some extent the PM is necessary to fully empty the gut microbial contents. Interestingly, we also observed a microbiota-dependent increase in sphingolipid metabolism at this time point; this could suggest that the bacteria that remain in the midgut following the excretion process must form a close association with the epithelial membrane, and, by extension, be able to cross the PM barrier.

In addition to local effects in the gut, upon disruption of the PM we observe the induction of a systemic immune response; our data supports this being a response to a break down in the integrity of the gut barrier and translocation of bacteria of the *Enterobacteriaceae* family into the body cavity. In humans, bacterial translocation is thought to be a common occurrence in healthy

individuals and can present as a complication in the critically ill (Wiest and Rath, 2003). It is understood to be a consequence of bacterial overgrowth (Steffen and Berg, 1983), disruption of the mucosal barrier (Slocum et al., 1992) and impaired immune defence (Owens and Berg, 1980). Our data suggest that the PM is a key barrier to prevent or limit *Enterobacteriaceae* translocation in the mosquito.

In our *A. coluzzii* colony, abundant genera of the *Enterobacteriaceae* family include *Cedecea*, *Enterobacter*, *Ewingella* and *Serratia* (Gendrin et al., 2015). Species of *Enterobacter* have been demonstrated to invade epithelial cells (Krzymińska et al., 2010) and *Serratia marcescens* is a model pathogen in *Drosophila* that, when introduced orally, is able to traverse the gut epithelium (Nehme et al., 2007). Such a phenomenon has not been formally demonstrated in the mosquito, but several lines of evidence point to translocation occurring. First, bacteria are necessary for the induction of immune priming in the haemolymph upon *Plasmodium* infection, suggesting that in the context of parasite-mediated PM degradation and epithelium invasion they are able to traverse the gut barrier (Rodrigues et al., 2010). Second, knock down of immune effectors can result in bacterial proliferation in the haemolymph even in the absence of infection (though in both of these cases the cuticle was damaged by dsRNA injection, providing a possible route of entry for exogenous bacteria) (Dong et al., 2006b; Warr et al., 2008). Third, a GFP *Asaia* strain is able to colonise both the reproductive organs and salivary glands when fed to adults in a blood or sugar meal (Favia et al., 2007). Interestingly, and in further support of this, in the control mosquitoes it was observed that carcass *CEC1* expression correlates only with *Acetobacteriaceae* load. This may imply that, even in the presence of the PM, the *Acetobacteriaceae* are able to translocate from the gut. The data presented here suggest that the PM plays a key role in limiting the ability of other bacterial families, particularly the *Enterobacteriaceae*, from doing the same. Not only is this strongly supportive of the development of paratransgenesis strategies that exploit *Asaia* sp., it additionally suggests a strategy that could significantly increase the ability of genetically-modified *Enterobacteriaceae* to invade the haemolymph.

3.3 PGRPLC dependent activation of the IMD pathway in the mosquito midgut

Abstract

Peptidoglycan recognition proteins (PGRPs) constitute the primary means of bacterial recognition in insects. Recent work in the model organism *Drosophila* has revealed the mechanisms by which the complement of PGRPs refine the sensitivity of different tissues to bacterial elicitors, permitting the persistence of commensal bacteria in the gut whilst maintaining vigilance against bacterial infection. Here, we use *in vivo* knock downs and *in vitro* binding assays to investigate the role of the transmembrane receptor of the IMD pathway, PGRPLC, in inducing basal immunity in the mosquito gut.

Introduction

In *Drosophila*, bacterial infection is perceived primarily by the detection of the bacterial cell wall component peptidoglycan (Leulier et al., 2003). Peptidoglycan is a polymer of alternating residues of N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc) that are cross-linked by a peptide chain of three to five amino acids; peptidoglycan of Gram-negative bacteria and *Bacillus* have a DAP residue in the third amino acid position of the cross-linking peptide bridge, whilst Gram-positive bacteria have a lysine in this position (Schleifer and Kandler, 1972). During the infection process, bacteria shed peptidoglycan as they grow, and these peptidoglycan polymers are likely broken down by host and bacteria enzyme activity into smaller fragments of varying immunostimulatory activity (Stenbak et al., 2004). TCT is an GlcNAc-MurNAc disaccharide with a DAP-containing peptide chain; this peptidoglycan monomer is a potent inducer of the Imd pathway in *Drosophila* (Stenbak et al., 2004).

The *Drosophila* transmembrane protein PGRP-LC was the first identified receptor of the Imd pathway (Choe et al., 2002; Gottar et al., 2002; R  met et al., 2002). *PGRP-LC* encodes three isoforms by alternative splicing that differ only in their extracellular PGRP domains. Intracellularly, the only region of the protein that displays homology with any known protein domains is the Rip

Homotypic Interaction Motif (RHIM) domain (Kaneko et al., 2006), which is essential for signalling. The current working model holds that PGRP-LC recognises a bacterial elicitor, inducing receptor multimerisation, which activates downstream signalling (Choe et al., 2005). PGRP-LC has been shown to interact with Imd; this interaction is not mediated by the RHIM domain and, interestingly, is not essential for activation of the pathway (Choe et al., 2005; Kaneko et al., 2006).

Two of the three PGRP-LC isoforms, LCx and LCa, are understood to function to diversify the recognition capacity of the receptor. LCx is able to bind both polymeric and monomeric peptidoglycan whilst LCa has no peptidoglycan binding capacity (Chang et al., 2005; Mellroth et al., 2005). The binding of TCT induces LCx to heterodimerise with LCa (Chang et al., 2005, 2006), leading to a model whereby the pathway can be stimulated both by polymeric peptidoglycan inducing LCx-LCx homodimerisation and monomeric peptidoglycan inducing LCx-LCa heterodimerisation. Functionally, this model is supported by isoform specific knockdowns in cell culture (Kaneko et al., 2004) and single isoform expression in a PGRP-LC knock out background *in vivo* (Neyen et al., 2012). The third isoform, PGRP-LCy, remains elusive, with several studies failing to attribute it a role (Kaneko et al., 2004; Werner et al., 2003), though there has been a suggestion that it has a mild antagonistic effect (Neyen et al., 2012).

Drosophila additionally has a second Imd pathway receptor, PGRP-LE (Takehana et al., 2002), which acts both in concert with and independently of PGRP-LC. Full length PGRP-LE, with both a RHIM domain and a PGRP domain, is localised intracellularly where it binds TCT, inducing receptor oligomerisation and Imd activity (Kaneko et al., 2006; Lim et al., 2006). The PGRP domain alone is also secreted, binding TCT extracellularly and then interacting with transmembrane PGRP-LCx to induce the Imd pathway (Kaneko et al., 2006).

The third potential Imd pathway receptor, PGRP-LA, is encoded in the same genomic locus as PGRP-LC. It has a RHIM domain, though structural

predictions suggest that the PGRP domain has lost its ability to bind peptidoglycan (Gendrin et al., 2013). Functional studies suggest that PGRP-LA is dispensable for the response to systemic infection but may play a role as a positive regulator of the Imd pathway in barrier epithelia (Gendrin et al., 2013). Whether PGRP-LA dimerises with itself, or indeed with other PGRPs, to induce the Imd pathway, is not currently known. Figure 3.3.1 summarises the known means by which the Imd pathway may be stimulated in *Drosophila*.

Figure 3.3.1

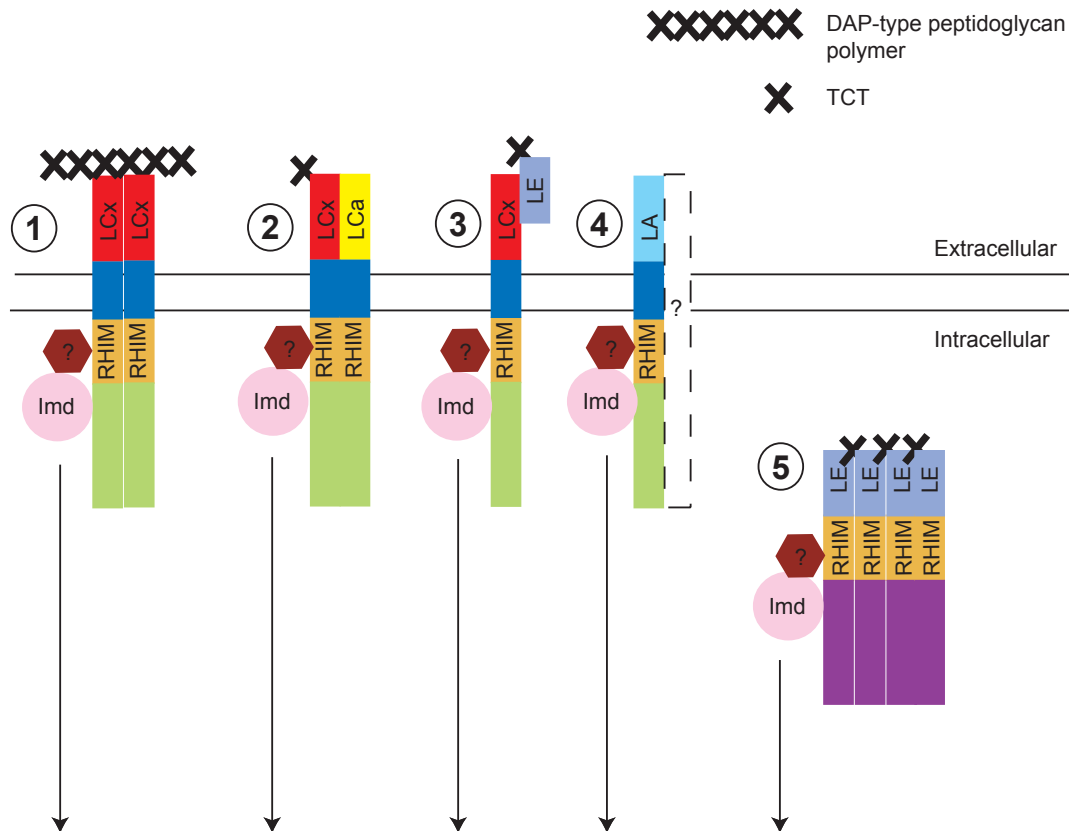


Figure 3.3.1. Ligand induced oligomerisation of peptidoglycan recognition proteins activates the Imd pathway in *Drosophila*. (1) DAP-type polymeric peptidoglycan induces PGRP-LCx homodimerisation. (2) TCT induces LCx/LCa heterodimerisation. (3) Circulating PGRP-LE binds TCT and dimerises with LCx. (4) PGRP-LA is a positive regulator in barrier epithelia, though it is likely not able to bind peptidoglycan and its dimerisation partner is not known. (5) Full length intracellular PGRP-LE binds TCT and oligomerises.

More recently, the relative importance of PGRP-LC and PGRP-LE to bacterial sensing in the gut and the body cavity is becoming apparent. PGRP-LCx is necessary and sufficient for resistance to systemic infection by the Gram negative bacteria *Erwinia carotovora carotovora* 15, indicating that polymeric peptidoglycan is the primary bacterial immune elicitor during systemic infection in the haemolymph (Neyen et al., 2012). This tissue is additionally responsive to TCT both when injected into the haemolymph and when fed orally followed by TCT translocation from the gut, with the former scenario depending solely on LCx/LCa heterodimers and the latter also involving LCx/LE dimers (Neyen et al., 2012).

Both PGRP-LC and PGRP-LE additionally play a role in bacterial detection in the gut. PGRP-LC appears to have its main role in the anterior region of the midgut and the cardia, whilst PGRP-LE is strongly enriched in the midgut (not including the cardia) and plays a significant role here in detecting fed live bacteria and fed TCT, with this Imd stimulation likely mediated by the intracellular form of PGRP-LE (Bosco-Drayon et al., 2012; Neyen et al., 2012). This PRR compartmentalisation reflects a fine-tuning of the responsiveness of different tissues to their different levels of bacterial exposure. The haemolymph, being a nominally sterile environment, uses extracellular receptors to be highly responsive to both polymeric peptidoglycan and TCT. The posterior midgut, meanwhile, relies largely on an intracellular TCT receptor; here the Imd pathway will be stimulated only when bacterial growth reaches such a threshold that TCT is transported intracellularly. This is appropriate for a tissue that is in contact with the gut microbiota.

Hematophagous mosquitoes face the additional challenge of hosting a highly variable microbiota load over the course of their life cycle. IMD pathway stimulation in mosquitoes is considerably less well understood. Experimentally, it has been demonstrated that PGRP-LC augments *A. gambiae* and *A. aegypti* survival to systemic infection with *E. coli*, a Gram negative bacteria, as well as Gram positive bacteria (*S. aureus* and *M. luteus* for each mosquito genus respectively) (Meister et al., 2009; Wang & Beerntsen, 2015). In both mosquitoes PGRP-LE has no detected effect on resistance to systemic *E. coli* infection, and a mildly antagonistic effect on resistance to Gram positive infection (Meister et al., 2009; Wang & Beerntsen, 2015). Intriguingly, whilst *A. aegypti* and *Culex quinquefasciatus* both encode orthologues of *D. melanogaster* PGRP-LE, there is no orthologue in the *A. gambiae* genome. This raises the question of how *A. gambiae* refines its receptiveness to peptidoglycan in tissues such as the midgut, which are exposed to the microbiota. Indeed, it is known that *A. gambiae* PGRP-LC plays a role in controlling microbiota load both in the sugar fed and blood fed gut, with PGRP-LC dependent IMD pathway induction by the microbiota in part

explaining the bacteria dependent reduction in parasite load (Meister et al., 2009).

A. gambiae *PGRPLC* has a similar architecture to its *D. melanogaster* orthologue (Fig. 3.3.2). Three main isoforms (LC1, LC2 and LC3) vary in their extracellular PGRP domains, though unlike *DmPGRP-LC* a common exon contributes to all three isoforms such that the 5' most region of the PGRP domain is the same in each isoform. Interestingly, *AgLC1*, *LC2* and *LC3* share more homology with each other than they do with *DmLCx*, *LCa* and *LCy*; this suggests either that the PGRP domain triplication event occurred twice independently after the divergence of *D. melanogaster* and *A. gambiae*, or that the common ancestor had three domains that have since been subject to concerted evolution in each species (Christophides et al., 2002). Intracellularly, there is a recognisable RHIM domain though there is little other homology with *DmPGRP-LC*. In addition to alternative splicing of the PGRP domain encoding exons, *AgPGRPLC* has also been reported to encode an optional cassette immediately downstream of the transmembrane domain and upstream of the PGRP domain (Meister et al., 2009).

Figure 3.3.2

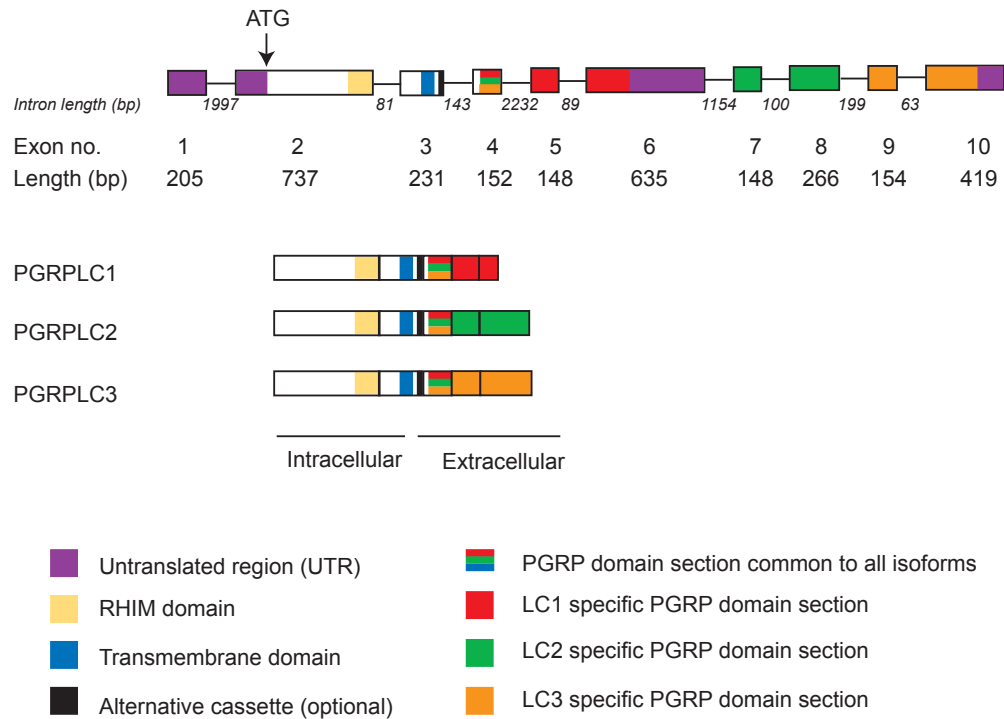


Figure 3.3.2. *A. gambiae* PGRPLC (AGAP005203) gene model and schematic of the three main protein isoforms that it encodes. The intracellular region has a RHIM domain, which is essential for signalling in *Drosophila*. Extracellularly there is an optional alternative cassette followed by the isoform specific PGRP domain.

Functionally, it has been reported that all isoforms contribute to resistance to systemic Gram negative infection, whilst LC1 and LC3, but not LC2, are necessary for resistance to Gram positive infection (Meister et al., 2009). LC3 makes the major contribution to resistance to *P. berghei* infection, with a minor role for LC2 in parasite melanisation (Meister et al., 2009). Additionally, in two mosquito derived cell lines it was found that LC1 is the only isoform that, upon single isoform knock down, is necessary for *CEC1* induction following bacterial challenge (Lin et al., 2007). Reciprocally, overexpression of LC1 was found to be sufficient for *CEC1* induction even in the absence of bacterial challenge, with LC3 overexpression having a similar but milder effect (Lin et al., 2007). Despite these functional data, a model has not so far emerged regarding the functions of the different PGRP ectodomains in the *A. gambiae* immune response.

Given the emerging picture in *Drosophila* of receptor compartmentalisation in the gut, and the fact that the *A. gambiae* genome is lacking in several of the characterised regulators in *Drosophila* (Pirk, PGRP-LF, PGRP-LE), we investigated the role of PGRP-LC in basal immune induction in the gut. We show that the *A. gambiae* midgut is highly compartmentalised in its expression of immune effectors. The splicing of the PGRP-LC isoforms additionally varies both spatially within the gut and temporally, following a blood meal. Using isoform specific knock down, we show that LC2 is necessary for basal immune activation in the sugar fed gut, and that this requirement switches to LC3 24 h after a bloodmeal. Functional analyses of recombinantly produced LC PGRP domains indicate that LC1 and LC3, but not LC2, are able to bind polymeric DAP-type peptidoglycan, whilst in the presence of TCT, LC2 and LC3 form the strongest dimer pair. Altogether, these data lead us to propose a model for the roles of the different PGRP-LC isoforms in the gut, suggesting a mechanism in which one variable receptor is able to sensitise the epithelium to the different levels of midgut bacteria to which it is exposed over the blood feeding cycle.

Results

Spatial and temporal heterogeneity of the gut in expression of immune effectors and PGRPLC isoforms

In the light of recent insights in the *Drosophila* system, it was hypothesised that the gut tissue would be spatially heterogeneous in its immune activity, perhaps in a manner that is dependent on the regions colonised by the gut microbiota. Non blood fed midguts were micro-dissected into four regions as previously described (Warr et al., 2007), and blood fed midguts were micro-dissected into three regions (the posterior region could not be further divided as the blood bolus would burst). Expression of three microbiota dependent AMPs (*CEC1*, *GAM1* and *LYSC1*) and 16S rRNA was analysed in each by qRT-PCR (Fig 3.3.3A-C).

In the sugar fed gut, the posterior region was found to have the highest average bacterial load (Fig 3.3.3B). Upon blood feeding bacterial load increases by approximately 10 fold in the cardia region and 100 fold in the posterior region, with no apparent change in the anterior. Strikingly, all three AMPs analysed are highly overexpressed in the cardia region in both blood fed and sugar fed guts (Fig 3.3.3C). The fact that the posterior region of the gut hosts a higher bacterial load than the more anterior regions and yet induces microbiota-responsive AMPs at a 100-1000 fold lower level suggests profound spatial differences in the sensitivity of the gut epithelium to bacterial elicitors. Indeed, when AMP expression is examined in these two regions of the gut following oral antibiotic treatment of non-blood fed mosquitoes, expression is microbiota-dependent in the cardia but not in the most posterior section of the gut (Fig 3.3.3D). This is not the case after blood feeding, where AMP expression, although still lower in the posterior region, is microbiota dependent (Fig 3.3.3D). This discrepancy is unlikely to be due to a difference in the antibiotic efficacy in different regions of the gut or at different time points, as qRT-PCR indicated that this was greater than 95 % in all tissues examined.

Figure 3.3.3

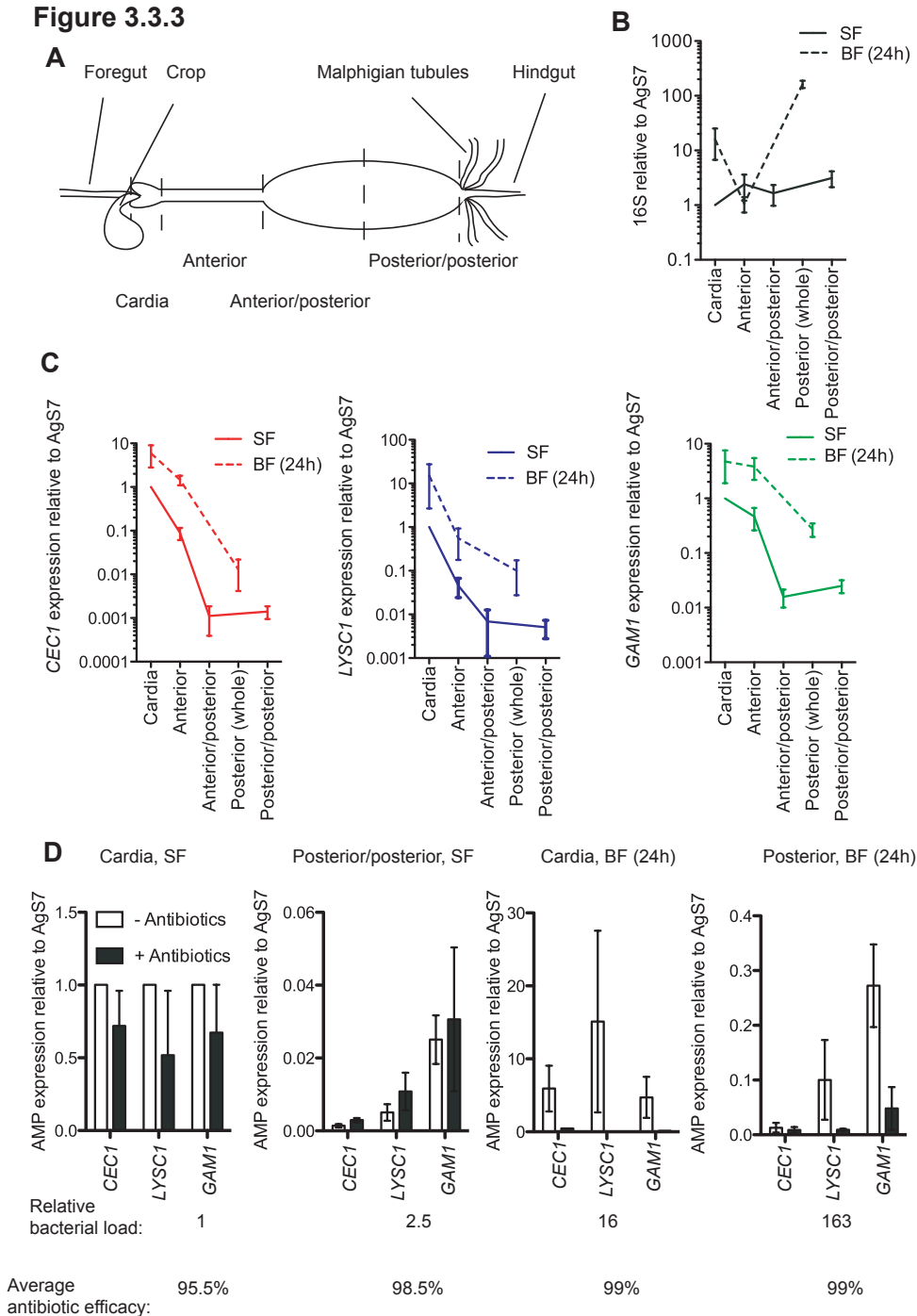


Figure 3.3.3 Spatial heterogeneity of microbiota distribution and AMP expression.

3.3.3A. Schematic of the mosquito gut, with dissected midgut regions indicated.

3.3.3B. 16S rRNA distribution throughout the gut of sugar fed and blood fed (24 h) females, determined by qRT-PCR on cDNA using universal 16S primers. SF: n=5 pools of tissue, of 15-30 individuals per pool, derived from 5 biological replicates. BF: n=2 pools of tissue, approx. 10 individuals per pool, derived from 2 biological replicates.

3.3.3C. Expression of the AMPs *CEC1*, *LYSC1* and *GAM1* throughout the gut of sugar fed and blood fed (24 h) females. SF: n=3 pools of tissue, of 15 individuals per pool, derived from 3 biological replicates. BF: n=2 pools of tissue, approx. 10 individuals per pool, derived from 2 biological replicates.

3.3.3D. Microbiota dependence of AMP expression in the cardia and posterior regions of sugar fed and blood fed (24 h) guts. n=3 pools of tissue, of 15 individuals per pool, derived from 3 biological replicates. BF: n=2 pools of tissue, approx. 10 individuals per pool, derived from 2 biological replicates. Bacterial load relative to the sugar fed cardia and antibiotic efficacy is indicated

All graphs represent the mean and standard error. Data are normalised to the average SF cardia expression level within each biological replicate.

We next examined the spatial distribution of the PGRPLC ectodomain-encoding transcripts across the gut. Given that previous reports have shown evidence of a pool of unspliced *PGRPLC* transcript (Meister et al., 2009), qRT-PCR primers were designed such that one primer spans an exon-exon junction within each isoform to ensure that only spliced mRNA was amplified (Fig 3.3.4). In the case of LC3, the primers span the junction between the common PGRP domain encoding exon and the first LC3 specific exon in order to avoid amplifying the 3' UTR of LC2 (which has been observed to extend into the LC3 region) as well as LC3 mRNA. Melt curve analysis confirmed that a single product was amplified in each case.

Figure 3.3.4

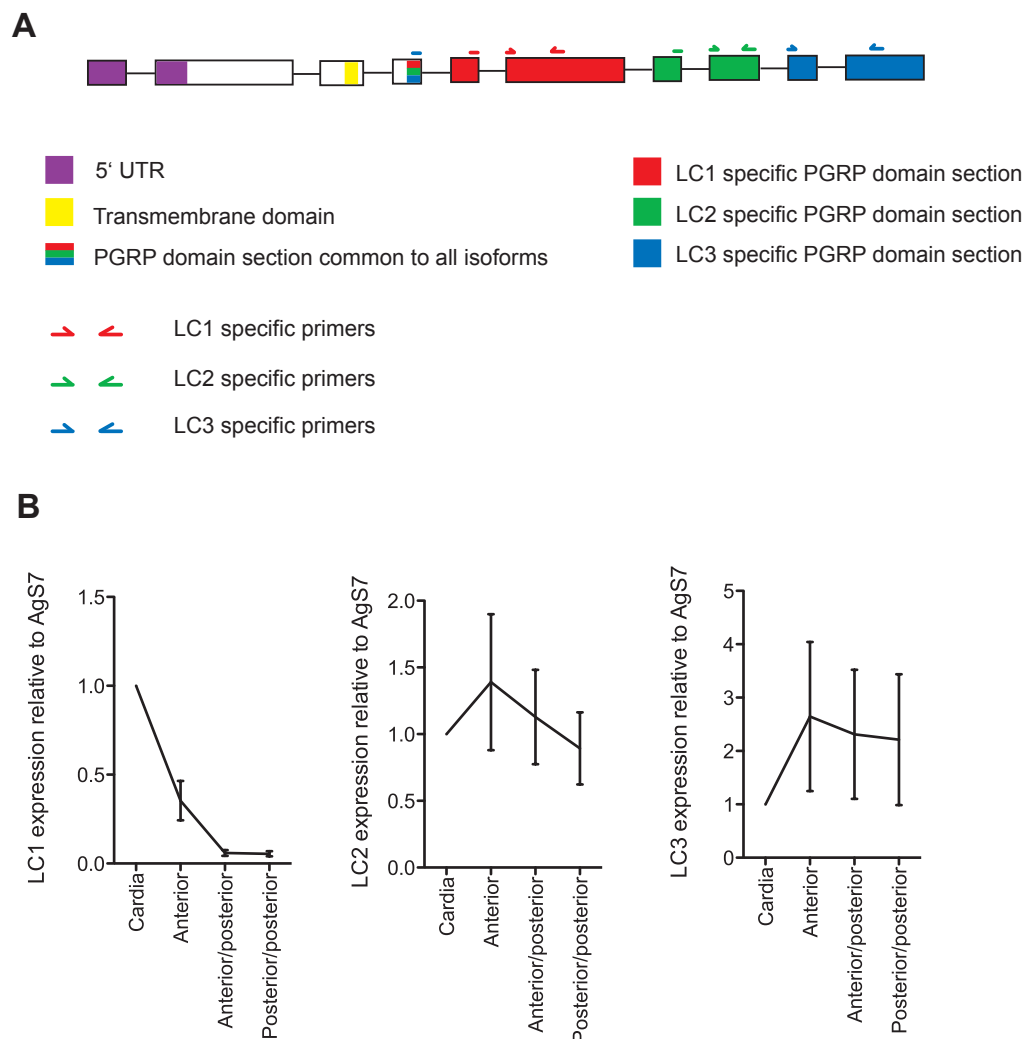


Figure 3.3.4. Expression of *PGRPLC1*, but not *PGRPLC2* and *PGRPLC3*, is spatially heterogeneous within the gut.

3.3.4A. Schematic indicating primer positioning on the *PGRPLC* transcript.

3.3.4B. Expression of the *PGRPLC* isoforms throughout the gut of sugar fed females. $n=5$ pools of tissue, of 15-30 individuals per pool, derived from 5 biological replicates. Graphs show the mean and standard error and data is normalised to the SF cardia expression level within each biological replicate.

PGRPLC1 transcript was found to be heterogeneously localised throughout the gut, exhibiting a similar expression pattern to the AMPs; that is, highly expressed in the cardia. *LC2* and *LC3* show similar patterns of expression to one another, with neither varying significantly along the gut. We additionally observed that *LC2* and *LC3*, but not *LC1*, undergo bloodmeal dependent upregulation specifically in the midgut (Fig 3.3.5).

Figure 3.3.5

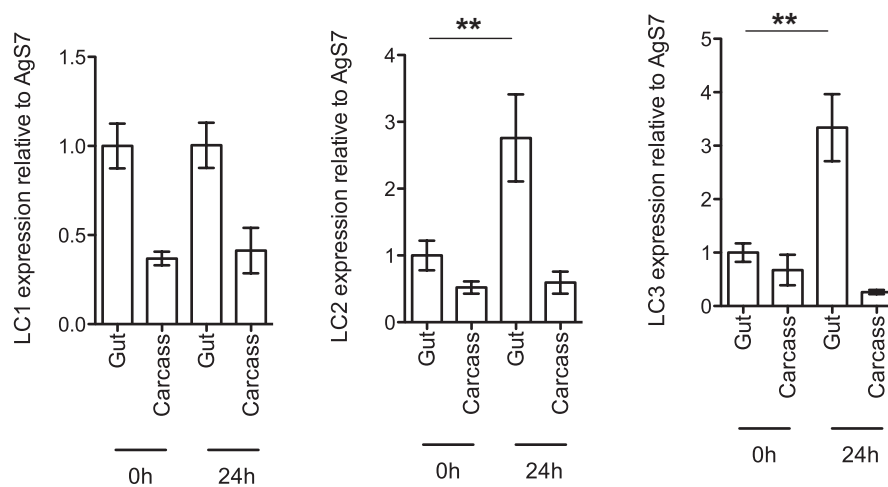


Figure 3.3.5. *PGRPLC2* and *PGRPLC3*, but not *PGRPLC1*, undergo gut specific upregulation upon blood feeding.

n= 9-10 pools of tissue, of 5-10 individuals per pool, derived from 2 biological replicates. ** p<0.01, ANOVA. Graphs show the mean and standard error and data is normalised to the SF gut expression level within each biological replicate.

As all three mature mRNAs are derived from one pre-mRNA, these data could suggest that this variable ratio is due to *LC2* and *LC3* favouring splicing factors being upregulated following the bloodmeal, concomitant with an increase in *PGRPLC* pre-mRNA transcription. Alternatively, this could reflect a bloodmeal dependent change in the half life of the mature *LC2* and *LC3* mRNAs; that is, either a stall in translation or in mRNA degradation could result in the observed increase in mRNA levels. Although antibodies are available, they are not specific enough to assess *in vivo* expression at the protein level (data not shown).

REL2 signalling induces feedback loops to control *PGRPLC* expression

Given the ubiquity of negative feedback loops whereby one outcome of a pathway is to inhibit its own stimulation, we hypothesised that IMD signalling may influence expression of the PGRPLC receptors. The IMD pathway NF- κ B transcription factor REL2 was knocked down by dsRNA injection and expression of the *LC* isoforms analysed by qRT-PCR (Fig 3.3.6).

Figure 3.3.6

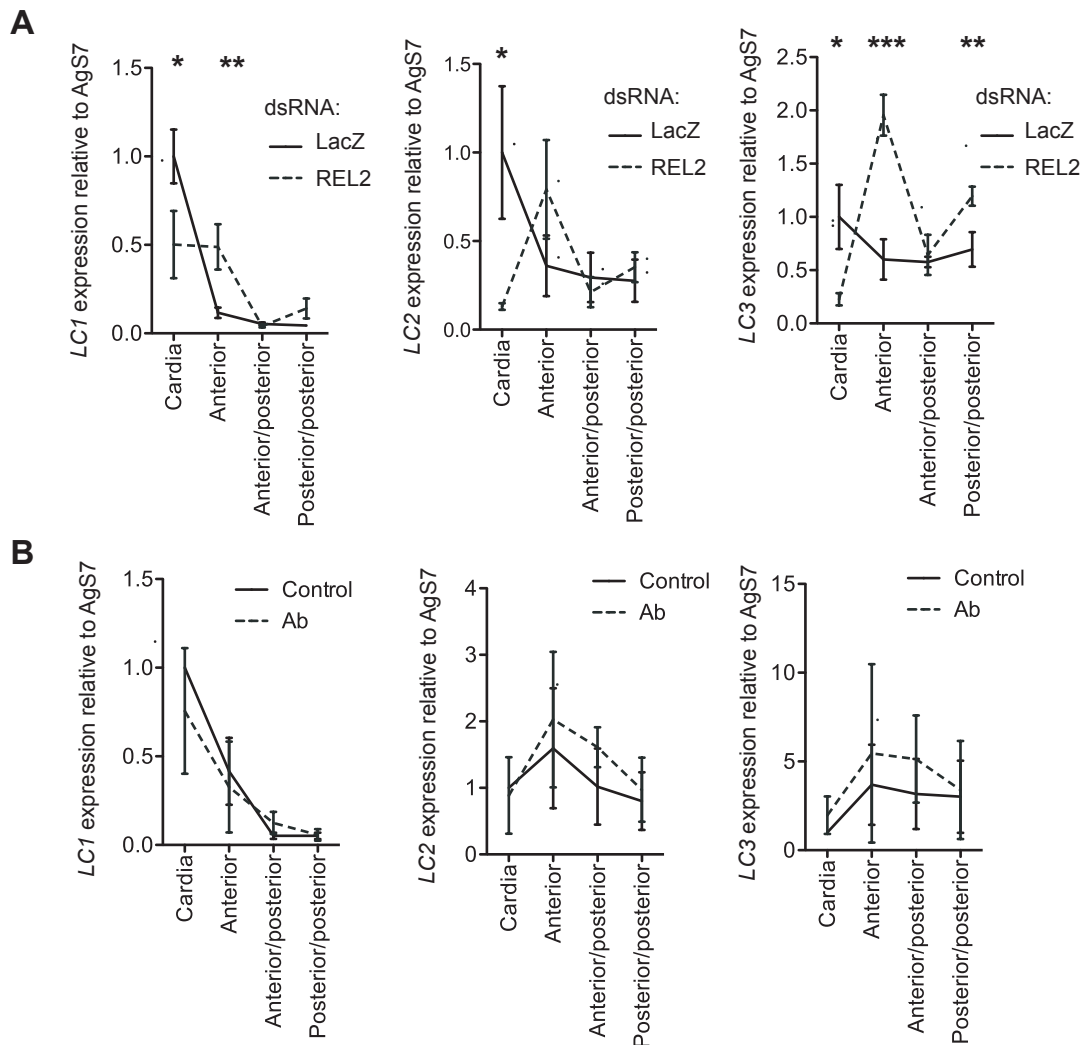


Figure 3.3.6. REL2 signalling induces feedback loops to control the expression of its pathway receptor, *PGRPLC*.

3.3.6A. Expression of *PGRPLC* isoforms throughout the gut 24 h after intrathoracic injection of dsRNA against *LacZ* (control) or *REL2*. $n = 4-5$ pools of tissue, each of 10-20 individuals per pool, derived from 3 (LC1) or 2 (LC2, LC3) biological replicates. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, ANOVA. See Figure 3.3.9 for REL2 knock down efficiency.

3.3.6B. Expression of *PGRPLC* isoforms throughout the non-blood fed, non-injected gut in control or antibiotic treated mosquitoes. $n = 3$ pools of tissue, each of 15 individuals per pool, derived from 3 biological replicates.

Graphs show the mean and standard error and data is normalised to the control/*LacZ* cardia expression level within each biological replicate.

Interestingly, REL2 knockdown led to a significant decrease in the expression of all isoforms in the cardia region and, conversely, a significant increase in the expression of *LC1* and *LC3* in the anterior region, as well as a significant increase in *LC3* in the posterior-most region. However, antibiotic treatment of non-injected mosquitoes did not fully recapitulate this effect. This suggests that the basal level of REL2 signalling induced by the microbiota is not sufficient to have an effect on *PGRPLC* expression; it is possible that the injection process itself induced NF- κ B signalling in the LacZ control. This suggests that a REL2 dependent change in *PGRPLC* isoform ratios could be relevant in an infection model (where NF- κ B signalling is robustly induced), but is unlikely to play a key role in this model of basal immune induction.

All in all, these expression data reveal the functional heterogeneity of the midgut tissue. At the junction between the midgut and the foregut, the cardia exhibits very high expression of AMPs, and in this tissue REL2 signalling induces a positive feedback loop that induces expression of IMD pathway receptors. Together, these data would suggest that the cardia is a highly immunogenic tissue, effectively imposing high pathogen resistance at the junction between the midgut and the external environment. The anterior region, which connects the cardia to the posterior end of the gut, is intermediate in its AMP expression. In this section REL2 signalling appears to induce a negative feedback loop, repressing the expression of its pathway receptors. This is also true of the posterior region, which hosts the highest bacterial load. Here, AMPs are not inducible by the microbiota in the sugar fed gut. The posterior gut therefore appears to be well adapted to tolerate the presence of bacteria. The significance of the different *PGRPLC* isoforms, which are differentially regulated both spatially (*LC1*, Fig 3.3.4) and temporally (*LC2* and *LC3*, Fig 3.3.5), remains to be seen.

Functional analysis of *PGRPLC* isoforms in the gut

It has already been seen that the IMD pathway plays a role in controlling the mosquito gut microbiota (Dong et al., 2011; Meister et al., 2009). We aimed to decipher which *PGRPLC* isoforms are responsible for stimulating IMD pathway basal activity in the different sections of the gut in both non-blood fed

mosquitoes (Fig 3.3.7) and following a blood meal (Fig 3.3.8). To this end, dsRNA against each specific *PGRPLC* ectodomain was injected and the basal expression of three microbiota dependent AMPs analysed 24 h later, or 24 h after a human blood meal (with the blood meal being given 24 h post injections). As a positive control, REL2 knock down mosquitoes were also analysed for the expression of the three microbiota responsive AMPs, in order to establish which of these, if any, is IMD dependent in the midgut. Knock down efficiency and off target effects of each dsRNA were additionally in each gut compartment analysed (Fig 3.3.9).

Figure 3.3.7

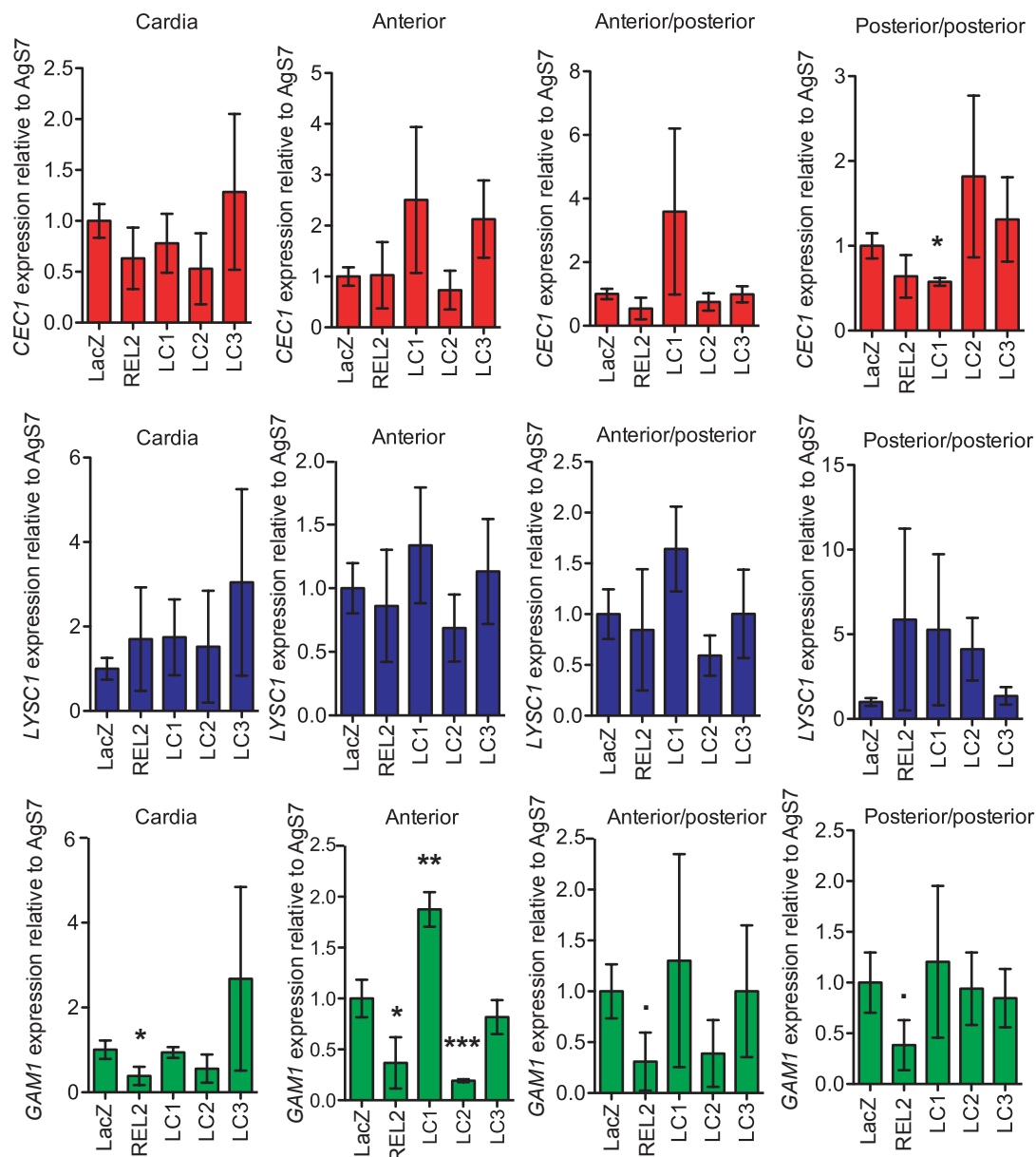


Figure 3.3.7. *CEC1*, *LYSC1* and *GAM1* expression in different sections of the sugar fed midgut following knock down of specific PGRPLC isoforms or REL2.

n=5 pools of tissue, each of 10-20 individuals per pool, derived from 3 biological replicates.

*** p<0.001; ** p<0.01; * p<0.05; . p<0.1, ANOVA.

Graphs show the mean and standard error and data is normalised to the LacZ expression level for each tissue within each biological replicate.

Figure 3.3.8

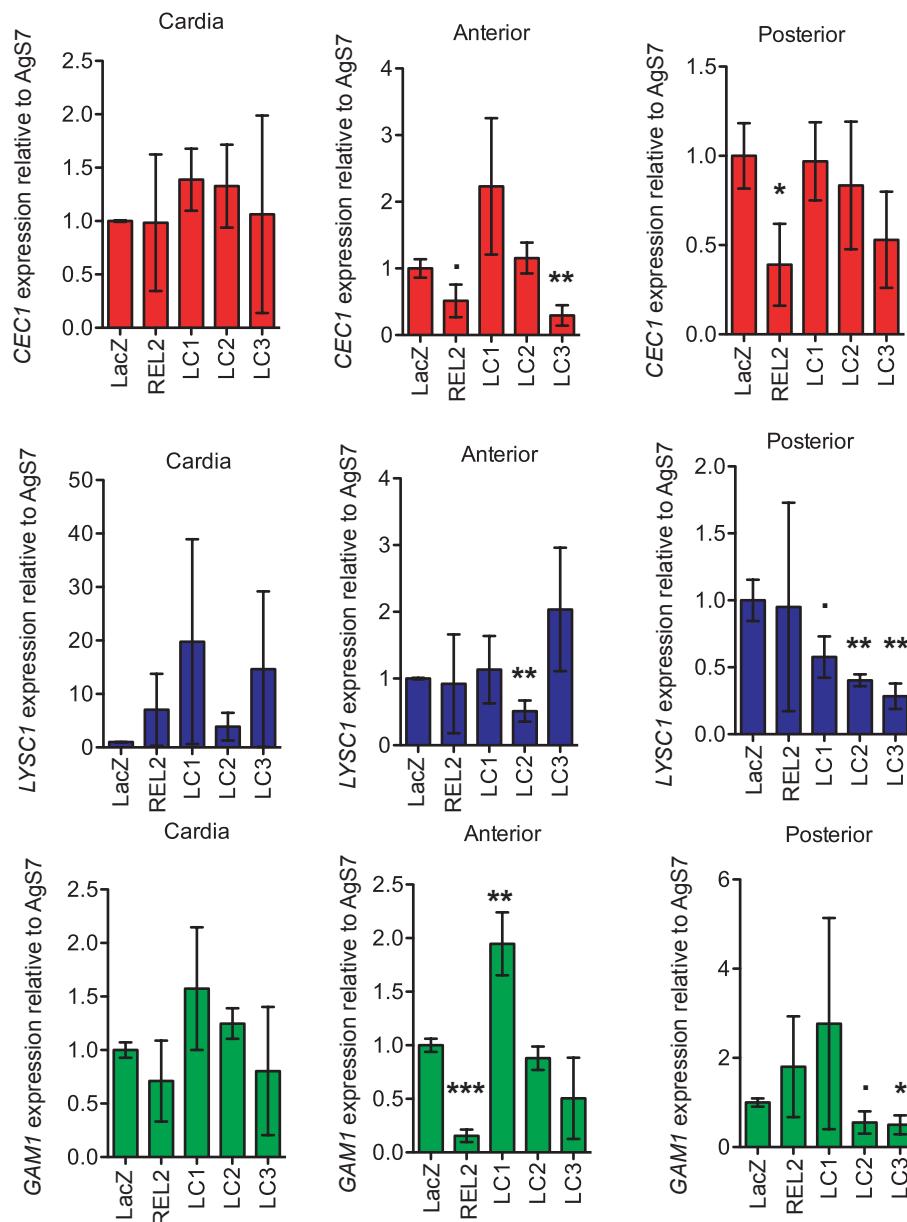


Figure 3.3.8. *CEC1*, *LYSC1* and *GAM1* expression in different sections of the non blood fed gut following knock down of specific PGRPLC isoforms or REL2.

n=4-5 pools of tissue, each of 5-10 individuals per pool, derived from 3-4 independent generations of mosquito. *** p<0.001; ** p<0.01; * p<0.05; . p<0.1, ANOVA. Graphs show the mean and standard error and data is normalised to the LacZ expression level for each tissue within each biological replicate.

A

SF

BF, 24h

Expression as proportion of LacZ control

REL2- LC1- LC2- LC3-

Cardia Anterior Anterior/posterior Posterior/posterior

REL2- LC1- LC2- LC3-

Cardia Anterior Anterior/posterior Posterior (whole)

REL2- LC1- LC2- LC3-

Cardia Anterior Anterior/posterior Posterior (whole)

B

LC1 expression relative to AgS7

LC2 expression relative to AgS7

LC3 expression relative to AgS7

Cardia Anterior Anterior/posterior Posterior/posterior

Cardia Anterior Anterior/posterior Posterior/posterior

Cardia Anterior Anterior/posterior Posterior/posterior

C

LC1 expression relative to AgS7

LC2 expression relative to AgS7

LC3 expression relative to AgS7

Cardia Anterior Anterior/posterior Posterior (whole)

Cardia Anterior Anterior/posterior Posterior (whole)

Cardia Anterior Anterior/posterior Posterior (whole)

dsRNA: LacZ LC1 LC2 LC3

3.3.9A. Knock down efficiencies in each section of the gut in blood and sugar fed females. n=4-5 pools of tissue, each of 10-20 (SF) or 5-10 (BF) individuals per pool, derived from 3-4 independent generations of mosquito. Graphs show the mean and standard error and data is normalised to the average expression level in the LacZ samples for each tissue within each biological replicate.

3.3.9C. Off target effects in each section of the gut in blood fed females. n=3-4 pools of tissue, each of 5-10 individuals per pool, derived from 3 independent generations of mosquito.

159

Considering first which genes are REL2 responsive, *GAM1* is the clearest read out in the sugar fed gut, being significantly or marginally significantly regulated by REL2 in all compartments analysed. After blood feeding, *GAM1* remains strongly REL2 dependent in the anterior region, non-significantly so in the cardia and independent of REL2 in the posterior region. *CEC1*, whose transcription has been shown to be IMD pathway dependent in cell culture (Meister et al., 2005), is not significantly regulated by REL2 in the sugar fed gut, in agreement with previous work (Meister et al., 2009). It is, however, REL2 dependent after blood feeding in the anterior and posterior regions. Finally, *LYSC1* is a microbiota-responsive gene (chapter 3.2) that is completely independent of REL2 in its transcription, showing that the gut microbiota induce immune pathways independent of the IMD pathway.

LC2 knock down mirrors most closely the effect of REL2 knock down in the sugar fed gut, causing significant reduction in *GAM1* expression in the anterior region and non-significant reduction of *GAM1* expression in the cardia and anterior/posterior regions. LC2 dsRNA has no discernible effect on *GAM1* expression in the posterior/posterior region, though this is concomitant with knock down being essentially undetectable in this region (Fig. 3.3.9A). The fact that LC2 is the only isoform whose knock down has a significant effect on REL2-dependent AMP expression would suggest that this is the only essential IMD receptor in the sugar fed gut, implying that LC2 is an essential component of all PGRPLC signalling dimers in these conditions. Whether these signalling dimers consist of LC2 homodimers or LC1/LC2 and/or LC3/LC2 heterodimers, with LC1 and LC3 acting redundantly, remains to be seen.

Following a blood meal, *GAM1* expression remains under the transcriptional control of REL2 in the anterior region of the gut, though its expression is no longer LC2 dependent (Fig 3.3.8), despite improved LC2 knock down efficiency at this time point (Fig 3.3.9A). As LC2 dsRNA additionally exhibits no transitive effects in this region (Fig 3.3.9B,C), this would be consistent with the anterior gut undergoing a functional switch after blood feeding whereby it no longer employs LC2 as an essential IMD pathway receptor.

Surprisingly, no significant effect in the cardia was found upon knocking down LC1, despite this isoform being expressed highly here (Fig 3.3.4) and achieving relatively good knock down efficiency (Fig 3.3.9A). The role of LC1 in the gut therefore remains unclear; one hypothesis is that rather than regulating the IMD dependent response to the microbiota, it may play a role in the response to exogenous bacteria as they enter the gut. Interestingly, we note a consistent antagonistic effect of LC1 on *GAM1* expression in the anterior gut (Figs 3.3.7,3.3.8). This is concurrent with LC1 dsRNA consistently inducing an increase in *LC3* expression (Fig 3.3.9B), a phenomenon that has been noted previously in the midgut (S. Meister, PhD thesis), suggesting that elevated levels of *LC3* could be responsible for the induction of the pathway. However, a role for LC1 as a negative regulator cannot be excluded.

LC3 knock down has no significant effect on AMP expression in any sections of the sugar fed gut. In the blood fed gut, however, *LC3* knock down appears to recapitulate the effect of *REL2* knockdown on *CEC1* expression, causing a significant decrease in expression in the anterior region and a non-significant decrease in the posterior region. The same trend is true of *GAM1* expression in the cardia and anterior regions. Together with the fact the *LC3* knock down efficiency is higher in the sugar fed gut, these data, tentatively, imply that *LC3* is the main isoform required for IMD activation following blood feeding.

Finally, it is interesting to note that the posterior region of the blood fed gut, which has a very high bacterial load, does not rely on *REL2* for the induction of either *GAM1* or *LYSC1*, though both of these AMPs are *LC2* and *LC3* dependent in this region. This would suggest that, at least in this region, *PGRPLC* signalling feeds in to immune signalling pathways other than the IMD pathway. The conclusions that can be drawn from the expression and functional data presented so far are summarised in Figure 3.3.10. To briefly summarise, these data suggest that *LC2* is an essential IMD receptor in the sugar fed midgut (possibly limited to the anterior region), but not in the blood fed midgut. *LC3* is the most likely candidate to take on this role after blood feeding.

Figure 3.3.10

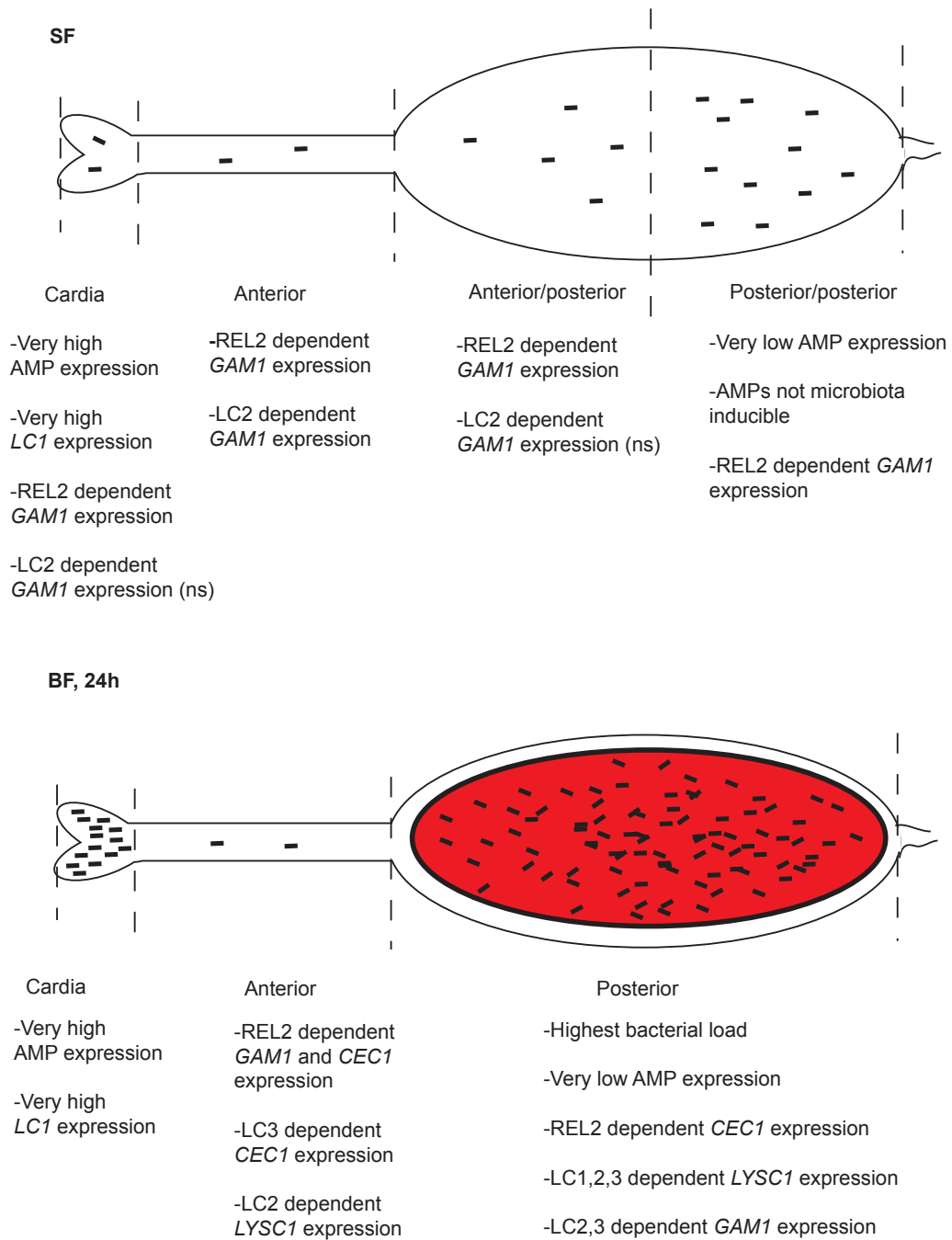


Figure 3.3.10. Summary of expression and functional data on the roles of the PGRPLC isoforms in basal immune induction in the sugar fed and blood fed gut.

Production of recombinant PGRPLC ectodomains

In order to understand further the differing functions of the PGRPLC isoforms, as set out above, the PGRP domains of the three isoforms were recombinantly produced in Sf9 lepidopteran cells, purified and subjected to *in vitro* functional analysis.

A common forward primer was used in combination with isoform specific reverse primers to amplify the PGRP domain of each isoform from an *A. coluzzii* cDNA library (Fig 3.3.11A). In each case, the appropriately sized product was purified by gel extraction to ensure that only spliced cDNA was selected. Ligation independent cloning was then used to clone the inserts into the pLEX-10 vector for expression in Sf9 lepidopteran cells. This vector was selected because it introduces a mouse IgM signal sequence at the N terminus of the recombinant protein; by passing the proteins through the insect cell secretory pathway it was hoped that any post translational modifications that may be essential for the proteins' *in vivo* function would be recapitulated *in vitro*. In addition to the secretion sequence, the vector introduces an N-terminal Strep tag II followed by an enterokinase cleavage site and a C-terminal His tag (10X His). A Tobacco Etch Virus (TEV) cleavage site was introduced in the reverse primers used for insert amplification, such that the His tag could also be cleaved (Fig 3.3.11B). This was deemed necessary because previous work on PGRPs has indicated that His tags may themselves interact with peptidoglycan (Basbous et al., 2011; Persson et al., 2007).

Each construct was transfected into Sf9 cells along with the pLE1-neo helper plasmid to facilitate the selection of stable transformants. Expression and secretion of the protein constructs were confirmed by Western blot (Fig 3.3.11C) and the C-terminal His tag was used to purify the proteins from conditioned media (Fig 3.3.11D).

Figure 3.3.11

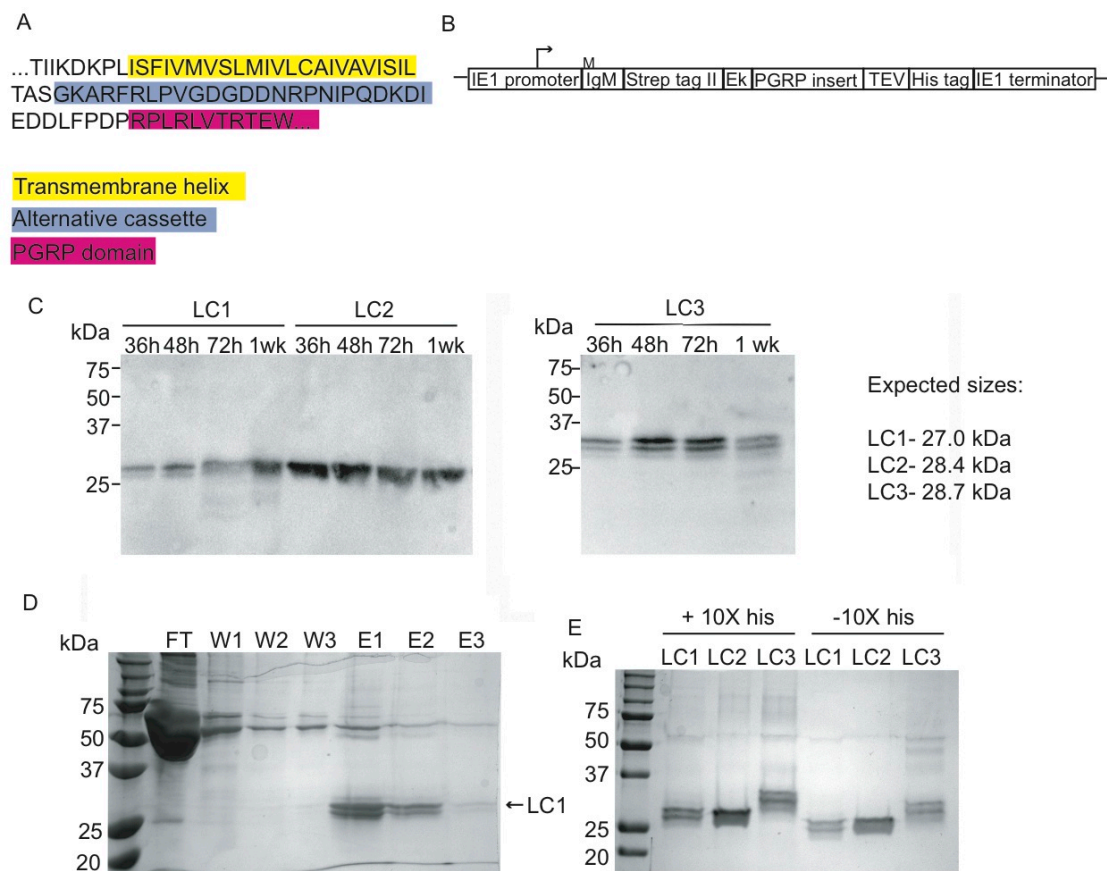


Figure 3.3.11. Production of recombinant PGRP domain proteins.

3.3.11A. Recombinant proteins are produced from the first residue of the PGRP domain (RPLR..), downstream of the transmembrane domain and alternative cassette.

3.3.11B. Schematic of the expression region in the pLEX-10 vector.

3.3.11C. Detection of LC1, LC2 and LC3 PGRP domains in conditioned media. Western blots were probed with anti his antibody (1:2000 in 3% BSA).

3.3.11D. Coomassie stained gel of PGRP LC1 purification by his tag affinity. FT=flow through, W=wash, E=elution.

3.3.11E. Coomassie stained gel of purified proteins before and after his tag cleavage by treatment with TEV protease.

It was observed that LC1 and LC3 migrate as a double and triple band, respectively, whilst LC2 migrates as a single band. We hypothesised that this may be due to glycosylation, a common posttranslational modification in secreted and membrane bound proteins. All of the isoforms have predicted O-glycosylation sites, whilst LC1 and LC3 additionally have two and three predicted N-glycosylation sites respectively (Fig 3.3.12A). Treatment with PNGase F, an N-glycosidase, indeed resulted in a shift in the migration size LC1 and LC3 with no effect on LC2 (Fig. 3.3.12B), suggesting that LC1 and LC3 undergo N-glycosylation.

Figure 3.3.12

A

LC1:

RPLRLVTRTEWLAQPPREELTDLKL PVNNVIAHTA**TEGCTT**QQACKALVQSIQRYHV**N**HTNYGDIGYNFLIG
 GDEFVYEGRGWLKVG**AH**T**K**NYNTISYGIAFIGNYETINRPTEQQMEQLVLLR**NG**TDGGWLAKEYKLCGAS
 QLKGTISPGKYLMEQLRLLPHF**S**ERL

LC2:

RPLRLVTRTEWLAQPPREELTDLKL PVNNVIAHTA**TEGCTT**QAACRLRVRLIQEFHMDGKNYDDITYNFLIG
 GDGHIYEGRNWHKIGAT**I**PGYNSRS**I**TVAFVGEYNYGGKPTKKQIELLK³YLLHFGAKERHLKEGYRIYASEQ
 LDPTVGTGKWLEALHSLPQFVDKEQNKDERPDHE

LC3:

PLRLVTRTEWLAQPPREELTDLKL PVNNVIAHTA**TEGCTT**QTKCMYQVKLIQEFHSSPDSR**N**FSDIAYQFL
 VGGDGNAYEGRGWTKQGAHTKGFNVDSICIAFIG**T**FIADPPPIAQLSAAQQLILLGMKENYLAS**N**YSLYGH
 RQLAPFESPGKALFDIIKT**W**PHWSNKLGSNHWWEPAN**D**TTNRR

Predicted N-linked glycosylation
 Predicted O-linked glycosylation
 Predicted C-linked glycosylation

B

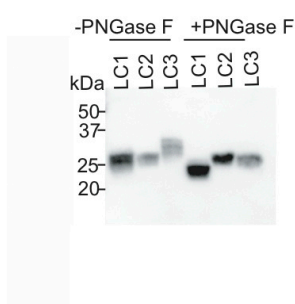


Figure 3.3.12. PGRPLC1 and PGRPLC3 are N-glycosylated.

3.3.12A. Glycosylation sites predicted using the GlycoEP server (Chauhan et al., 2013) using Binary Profile of Patterns (BPP) prediction with SVM threshold set to 0.0.

3.3.12B. PNGase F treatment of recombinant PGRPs. Western blot probed with anti His antibody (1:2000 in 3% BSA).

PGRPLC1 and PGRPLC3, but not PGRPLC2, bind polymeric DAP-type peptidoglycan

We first looked at the ability of the three PGRPLC isoforms to interact with peptidoglycan. As the vast majority of bacteria present in the gut microbiota are Gram negative, we used DAP-type peptidoglycan purified from *E. coli* for this purpose. Each isoform, following His tag cleavage, was incubated with insoluble peptidoglycan, and the supernatant and washed pellet analysed by Western blot. Under the conditions used, LC1 and LC3 are both pulled down with the peptidoglycan, whilst LC2 remains in the supernatant fraction (Fig 3.3.13A). Even when combined with other isoforms, LC2 remains in the supernatant, indicating that it is not able to dimerise with LC1 or LC3 in the presence of polymeric peptidoglycan (Fig 3.3.13B). What's more, we note the appearance of a dimer, likely disulphide bonded, in the LC1/LC2 and LC1/LC3 isoform combinations.

Figure 3.3.13

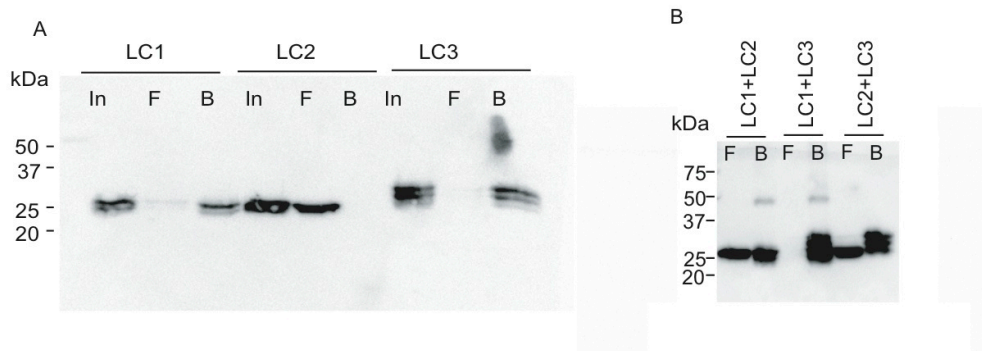


Figure 3.3.13. LC1 and LC3, but not LC2, bind polymeric DAP-type peptidoglycan.

3.3.13A. Pull down assays using insoluble, polymeric DAP-type peptidoglycan and single PGRPLC isoforms. Blot shows one of three independent replicates, each giving the same result.

3.3.12B. Pull down assays using insoluble, polymeric DAP-type peptidoglycan and combinations of PGRPLC isoforms, as indicated. Blot shows one of two independent replicates, both giving the same result.

Western blots are non-reducing and were probed with anti strep tag II antibody (1:1000 in 3% BSA). In=input; F=free; B=bound.

PGRPLC2 and PGRPLC3 form the strongest interaction in the presence of TCT

Next, we considered which of the LC PGRP domains are able to dimerise with one another in the presence of the potent stimulant of the *Drosophila* Imd pathway, TCT. To do this, we combined one isoform with its His tag with another isoform whose His tag has been cleaved, in the presence of the DAP-type peptidoglycan monomer, TCT. Purifying by His tag affinity, we could then pull down the His tagged protein and observe whether the other, non-His tagged, isoform pulled down with it or remained in the flow through (Fig 3.3.14).

Figure 3.3.14

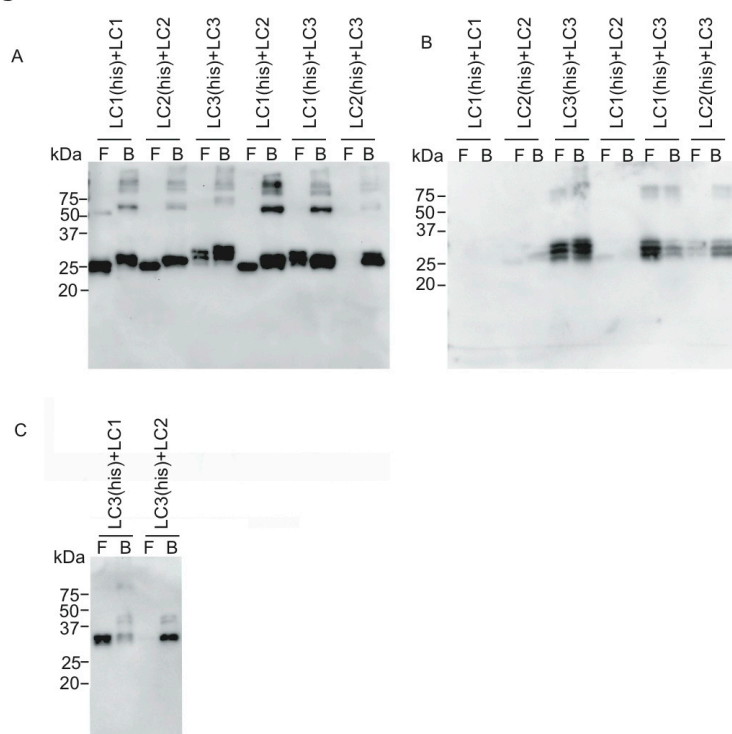


Figure 3.3.14. PGRPLC2 and PGRPLC3 form the strongest interaction in the presence of TCT.

3.3.14A. A His tagged isoform was incubated with a non-His tagged isoform in the combinations indicated, pulled down by His tag affinity and the free and bound fractions analysed. Non-reducing Western blot probed with anti strep-tag II antibody (1:1000 in 3% BSA). Blot shows one of two independent replicates, both giving the same results.

3.3.14B. Same samples as (A), probed with anti-PGRPLC3 antibody (1:500 in 3% BSA).

3.3.14C. LC2 also interacts with His tagged LC3. Western blot probed with anti strep-tag II antibody (1:1000 in 3% BSA).

When probing initially with anti-strep tag II, we found that the only isoform combination that led to near complete pull down was LC2/LC3 (Fig 3.3.14A). This was not due to residual His tag being retained on LC3, as in combination with both LC3(His) and LC1(His), the majority of LC3 is clearly found in the flow through fraction. As LC2(His) and LC3 run at approximately the same size and are difficult to distinguish on a gel, the same samples were separated again and probed with an anti LC3 antibody (Fig. 3.3.14B). Indeed, in combination with LC2(His), LC3 appears mainly in the bound fraction, whereas with LC1(his) and LC3(his), LC3 appears as a prominent band in the free fraction (though with a small proportion pulling down with LC1/3(his)). To validate this finding we performed the reciprocal experiment, pulling down LC1 and LC2 with LC3(His) (Fig 3.3.14C). As expected, LC2 appears almost entirely in the bound fraction whilst LC1 appears mostly in the free fraction, again with a small proportion in the bound fraction.

In addition to the LC2/LC3 interaction, we also observe the appearance of bands at sizes that correspond to dimers and higher oligomers (Fig 3.3.13A). It must be noted that these bands are largely present only in the bound fractions, that is, in the presence of the His tag. Indeed, for example, in the LC2(His)/LC2 combination, multimers are present in the bound fraction but not the free fraction, despite the presence of free LC2. This raises the question of whether these dimers are an artefact caused by the presence of the His tag, a proposition that has some precedence (Wu and Filutowicz, 1999). The exception to this is the LC1/LC1 dimers, which additionally appear in the free fraction. Indeed, aberrant PGRP-His tag interaction could explain why some amount of interaction occurs in all PGRPLC combinations, possibly suggesting that these are artefactual. Despite this, the LC2/LC3 interaction is quantitatively stronger than all other combinations, such that the existence of this interaction can be concluded with confidence.

Discussion

Here, we demonstrate that the mosquito midgut is highly heterogeneous in its immunogenicity. In particular, we observe very high expression of AMPs in the cardia region of sugar fed and blood fed midguts. This high AMP expression correlates with highly enriched *LC1* expression at the mRNA level. The *Drosophila* midgut has long been understood to be compartmentalised in its immune and digestive function (Buchon and Osman, 2015), in particular with enrichment of Imd dependent immune genes in the cardia region (Buchon et al., 2013). This likely both ensures that exogenous bacteria entering the gut encounter a robust initial immune response, and allows the microbiota, that are mainly concentrated in the posterior region, to persist. A very interesting question arising from this work is the mechanism by which the gut epithelium mediates this heterogeneity, given that it is exposed to bacterial ligand along its length. Indeed, the data presented here suggest that AMP expression is not responsive to the presence of the gut microbiota in the posterior-most region of the sugar fed gut, but is microbiota responsive in the sugar fed cardia and in both the cardia and the posterior region after blood feeding. In *Drosophila*, the negative regulator of AMP transcription, Caudal, is specifically expressed in the posterior most region (Buchon et al., 2013) where it represses transcription of AMPs (Ryu et al., 2008). *A. gambiae* Caudal appears to play a similar role (Clayton et al., 2013), though its expression has not been localised to the posterior midgut. Similarly, the putative IMD negative regulators PGRPS1 and PGRPS2 may be specifically expressed in the posterior most region.

One of the other mechanisms underlying this heterogeneity in the *Drosophila* model is the regional use of peptidoglycan receptors, with intracellular PGRP-LE being employed in the posterior most region of the gut. In the absence of an *A. gambiae* *PGRP-LE* orthologue, we asked whether the PGRP-LE isoforms may be employed differentially in the midgut regions to serve a similar purpose. The data presented here do not support this being the case. In the sugar fed gut, LC2 appears to be largely responsible for *GAM1* induction. This is not the case in the posterior/posterior region, though we cannot distinguish whether this is because LC2 truly does not induce *GAM1*

expression there, or because the knock down is not sufficient in this region. Similarly, LC3 appears to play the most significant role after blood feeding, throughout the midgut.

Interestingly, we also observed the LC2 and LC3 dependent induction of *LYSC1* and *GAM1* in the posterior region of the blood fed midgut. In this region, expression of these AMPs is not dependent on REL2. This suggests that PGRPLC signals to pathways other than that IMD pathway in the blood fed posterior midgut. In *Drosophila*, the Imd pathway is known to diverge after TAK1, signalling via either Relish or JNK (Park et al., 2004), and is also known to induce the transcription of *DUOX* via p38 (Ha et al., 2009c). Neither of these interactions has been demonstrated in *Anopheles*, though given the conservation of other intracellular signalling pathways they are likely to be conserved. Indeed, although *GAM1* and *CEC1* transcription is REL2 dependent in other regions of the gut, it must be acknowledged that PGRPLC may also be feeding into other pathways in these regions too.

A significant limitation of the experiments described here is the limited efficiency of knock down, despite employing double the 'standard' concentration of dsRNA and sampling at an early time point post injection. This is likely due to the fact that dsRNA had to access the midgut tissue from the haemolymph, and is consistent with previous reports of detected knock down efficiency in the mosquito midgut (Meister et al., 2009). What's more, in the case of the PGRPLC isoforms, the length of unique sequence available for each isoform limited dsRNA design. In the cardia, significant transitive effects were observed; that is, RNA against one isoform caused a reduction in the expression of the other two isoforms. This may be related to the observation that all dsRNAs were more efficient in the cardia region, possibly simply because the cardia is physically closest to the site of injection. What's more, interestingly, we note that dsLC1 causes an increase in *LC3* expression in all regions of the gut. This could be due to the fact that these two isoforms are common predicted targets for two miRNAs, aga-miR-14 and dme-miR-11, whilst *LC2* does not share common miRNAs with either of the two other isoforms (Griffiths-Jones et al., 2008). The fact that dsLC1 causes an increase

in *LC3* expression could suggest that *LC1* dsRNA interacts with the miRNAs in question, preventing them from targeting the *LC3* transcript. In support of this model, this effect is not observed in the cardia, where *LC1* is highly expressed and is likely the primary target for ds*LC1*, and similarly, ds*LC3* does not have the same effect on *LC1*, likely because *LC3* is highly expressed throughout the gut. To mitigate these challenges associated with the use of RNAi, an isoform specific knock out strategy could be employed In the future: the recent demonstration of CRISPR-Cas based gene targeting in mosquitoes shows that this is now a feasible prospect (Hammond et al., 2016).

What's more, it was observed that REL2 knock down has opposing effects on *LC* expression in the cardia region compared to the other sections of the gut, with REL2 signalling apparently inducing *LC1*, 2 and 3 expression in the cardia and doing the opposite to *LC1* and *LC3* expression in the anterior and posterior regions. The cardia region is derived from both endoderm and ectoderm, whilst the rest of the midgut is derived from the endoderm (Nation, 2015). It is not uncommon for signalling pathways to direct different responses in different cell types (Mullen et al., 2011), though the mechanisms behind this are complex and not well understood. They may depend on active cis-regulatory elements in different cell types (Heinz et al., 2015), co-expression of other transcription factors (Mullen et al., 2011), chromatin context (Arvey et al., 2012) and miRNA networks (Sood et al., 2006). This could also explain why knock down efficiency is higher in the cardia: here, PGRPLC knock down may cause a positive feedback loop, with less REL2 signalling resulting in lower *LC* expression, whilst in the anterior region lower REL2 signalling can cause a negative feedback loop, inducing *LC* expression.

In addition to knock down data, we additionally use recombinant protein expression to explore the binding capacity of the three PGRPLC isoforms. Previous computational modelling predicted that dimerisation of all isoform-isoform combinations was feasible, with the exception of *LC3* homodimers. To our knowledge, the experiments presented here are the first to be performed *in vitro* on the binding capacity of the PGRPLC isoforms. We found that *LC1*

and LC3, but not LC2, are found to be able to bind polymeric DAP-type peptidoglycan. In the presence of the monomeric peptidoglycan unit, TCT, LC2 and LC3 form the strongest interaction of all isoform-isoform combinations. These data are consistent with a model whereby LC2 plays a similar role to *Drosophila* LCa: Dm PGRP-LCa is unable to bind peptidoglycan but acts as an adaptor to facilitate dimerisation with LCx in the presence of TCT. According to the data presented here, LC3 is most likely to act as the LCx equivalent.

This model is strongly supported by previously published data on the functionality of the PGRP-LC isoforms. For example, it was found that all isoforms contribute to resistance to systemic Gram-negative infection, whilst only LC1 and LC3 are necessary for resistance to Gram-positive infection (Meister et al., 2009). Given that only Gram-negative bacteria shed TCT, this is consistent with LC2 only playing a role in the presence of Gram-negatives. In cell line experiments it was found that overexpression of either LC1 or LC3, but not LC2, is sufficient to induce *CEC1* expression in the absence of infection (Lin et al., 2007). Again, this is consistent with LC2 acting as an adapter and alone being unable to dimerise with itself.

Furthermore, our observation that LC2 appears to be the most likely IMD receptor in the sugar fed gut whilst LC3 is the most likely receptor in the blood fed gut is consistent with the previous observation that LC3 is the main isoform to mediate resistance to *P. berghei* (Meister et al., 2009), given that the parasite must be delivered as a component of the bloodmeal. If the model proposed is correct, this would imply that the primary IMD stimulating ligand is TCT in the sugar fed gut (due to the LC2 requirement) and polymeric peptidoglycan in the blood fed gut (as LC2 is no longer required). This could be suggestive of a novel adaptation of the midgut. That is, after blood feeding, when bacteria are replicating substantially and shedding significant quantities of TCT, the IMD pathway may effectively only be responsive to polymeric peptidoglycan. This model should therefore be explored further as a potential novel tolerance mechanism in the mosquito midgut.

Chapter 4: General Discussion

4.1 Discussion

There is growing interest in the potential of manipulating the mosquito microbiota for malaria transmission blocking applications. Several strategies are proposed. A first strategy is paratransgenesis, whereby a bacterial strain expressing anti-*Plasmodium* effector molecules is introduced to the mosquito, rendering it resistant to infection. A second strategy would be to introduce a non-modified bacterial strain that may either naturally provide protection, and/or acts as an entomopathogen to shorten the mosquito life span. Finally, an understanding of the immune control of the microbiota could identify epithelial immune genes that, when targeted by drugs or by knock out, can cause microbiota dysbiosis or overgrowth, again resulting in a shortened mosquito life span or resistance to *Plasmodium*. The converse could also be effective- targeting a negative regulator of the immune response could both increase resistance to *Plasmodium* infection and shorten lifespan, this time via immune-mediated damage to host tissue.

The success of any of these strategies depends on a detailed, systems-based understanding of the mosquito microbiota and its relationship with the mosquito host. It is currently now known what is the physiological or dietary contribution of the gut bacteria to the adult mosquito. Further, the bacterial characteristics that permit persistence within the gut, and indeed dominance within the gut ecosystem in different nutritional states, are not well understood. Equally, the mechanisms employed by the gut epithelium to prevent overstimulation of the gut immune response by the microbiota are not known. This thesis aimed to provide insights into some of these important questions, by examining the mosquito gut and its microbiota as a highly integrated system.

The data presented in this thesis show that reduction of blood meal-associated microbiota growth by antibiotic feeding causes an increase in mosquito life span. *A priori*, this could be explained by reduced tissue damage caused by lower levels of bacterial toxins, or by reduced immune induced tissue damage, or a combination of the two. A previous study showed that overactivation of the IMD pathway leads to both reduced microbial load and

increased life span (Clayton et al., 2013), which would suggest that it is largely the bacteria themselves that cause death, not the immune response. By extension, increasing microbiota growth through targeted disruption of the gut immune response may be expected to reduce the mosquito life span; this would imply that positive immune regulators are potential targets for intervention.

However, this is only the case if the immune system does indeed play a significant role in limiting microbiota growth. Several studies have demonstrated that IMD pathway overactivation can considerably limit microbiota growth following the blood meal, though, reciprocally, the reported microbiota overgrowth associated with PGRPLC knock down at 24 h post blood meal is only approximately 1.5 fold greater than the control (Meister et al., 2009). Furthermore, the data presented here indicate that the primary response after blood feeding is tolerance rather than resistance. For example, at 24 h post blood meal, AMPs are only induced approximately 2 fold as opposed to a 2000 fold increase of the putative IMD pathway negative regulators PGRPS1 and S2. Equally, AMP expression is very low in the blood fed posterior region that houses the majority of the gut microbiota compared with the more anterior regions of the gut that have lower microbial load. What's more, it is demonstrated that the reduction of microbiota load at approximately 48 h post blood meal is caused largely by excretion together with the entire blood bolus. Although this does not necessarily preclude that the excreted bacteria have been killed by an immune response, it does provide a potential explanation as to why a robust immune response does not need to be induced in response to blood meal associated microbiota proliferation.

One factor identified here as playing a role in modulating the gut response at 24 h post bloodmeal is the induction of the PM. Disruption of this structure causes a significant increase in the induction of AMPs at this time point. It is interesting to note that, although statistically significant, the magnitude of the increase in AMP expression following PM disruption is relatively small (only approximately 1.5 fold greater than in the control samples). This observation

might be better understood in the light of the finding that AMP expression is very low in the posterior midgut following blood feeding, such that even a 100 fold increase in expression in the posterior region due to PM disruption would translate to a minor increase in the midgut tissue overall. Furthermore, given that the PM is expected to be permeable to TCT and potentially longer peptidoglycan fragments as well, it is possible that the AMP induction in the case of polyoxin D treatment is due to bacteria not being confined to the posterior midgut but growing into the anterior region as well, as opposed to increased AMP induction in the posterior region. In support of this hypothesis, it is interesting to note that only *GAM1* overexpression reaches statistical significance; in chapter 3.3, it is shown that the expression profile of this AMP shows elevated expression in the anterior region, compared with *CEC1* and *LYSC1*. This could suggest that, indeed, in the case of polyoxin D treatment at least, PM disruption causes bacterial growth into the anterior region of the midgut.

In addition to the PM, the posterior midgut likely employs additional tolerance mechanisms to prevent overstimulation of gut immunity following blood feeding. The data presented here suggest that LC2 is necessary for IMD pathway activation in the sugar fed midgut by sensing TCT as the primary IMD- activating ligand. LC2 binding with LC3 is strongly supported by the data presented here, though LC2 may additionally bind with LC1, or indeed PGRPLA, which has recently been characterised as a positive regulator of the IMD pathway in the gut (Gendrin, et al., under review).

By contrast, LC2 is dispensable after blood feeding, with polymeric peptidoglycan likely being the major stimulator of the pathway at this time point. Further experiments must be performed to verify this model, though it appears to be consistent with previously published data. A question that requires further investigation is whether this is simply due to ligand availability, or is in fact a response intended to minimise stimulation after blood feeding when there is likely significant TCT being shed in the gut. That is, rather than being responsive to TCT, which is likely readily able to diffuse through the PM, IMD pathway stimulation requires interaction with polymeric

peptidoglycan or indeed whole bacteria. This would allow bacteria to replicate and live freely in the endoperitrophic space, only inducing an immune response in the event of escape from within the PM sac. Mechanistically, however, it is not clear how this would work, with both *LC2* and *LC3* being transcriptionally upregulated after blood feeding. It is noteworthy that *LC1* and *LC3* are predicted to be regulated by a common miRNA that does not target *LC2*, suggesting that post transcriptional regulation may play a significant role.

In addition to its role in limiting the gut immune response, the PM also plays a role in restricting the microbiota to the gut compartment. Indeed, this work raises the question of whether and how mosquito tissues other than the gut are colonised by bacteria. It is known that *Asaia* spp. are able to colonise both the salivary glands and the reproductive system, and indeed a GFP strain is able to colonise the both of these organs when fed to adults in a blood or sugar meal (Favia et al., 2007). Although not formally demonstrated, this would imply that this bacterial species is able to escape the midgut, presumably via the haemolymph. The presence of bacteria in the haemolymph could be useful for a paratransgenesis approach to malaria control, as *Plasmodium* is bathed in the haemolymph for the majority of its development. The data presented here regarding the role of the PM in preventing bacterial translocation to the haemolymph could be used to strengthen the likelihood of such an approach succeeding; for example, a paratransgenic candidate could be transformed with a chitinase to overcome the barrier of the PM and increase dissemination through the body.

The paratransgenesis strategy would be further strengthened if some means could be found to ensure that the modified bacterial strain both persists in the microbiota for the lifespan of the mosquito and is a dominant strain within the ecosystem. We observed here that the majority of the gut microbiota are excreted approximately 48 h after the bloodmeal. We also observed that at 24 h post bloodmeal the gut ecosystem is composed of approximately 50 % *Enterobacteriaceae* and 50 % *Flavobacteriaceae*, whilst at 72 h it is almost 100 % *Flavobacteriaceae*, though again the small *Enterobacteriaceae* population expands significantly following the second bloodmeal. This may

imply that the *Flavobacteriaceae* are preferentially able to remain in the gut following excretion, or indeed that between 48 and 72 h gut immunity is preferentially active against the *Enterobacteriaceae*. The potential mechanisms behind the latter scenario are not clear, though some immune modules, containing type III fibronectin domains, have been shown to specifically target this family of bacteria (Stathopoulos et al., 2014). The former scenario could be determined, for instance, by the extent to which different species interact with the PM, or which species are able to cross the PM and interact directly with the gut epithelium. Distinguishing between these two models will be important in determining the characteristics that allow certain species to persist in the gut throughout the gonotrophic cycle.

With regards to which bacterial strains or species are dominant in the midgut, this appears to be dependent to an extent on the nutritional status of the gut, with the *Enterobacteriaceae* preferentially expanding after a bloodmeal. This may be to a large extent dependent on the adaptation of different bacteria to an environment in which blood is being digested; again, an understanding of which genes determine this would be useful for optimising a paratransgenesis strategy. The genome of *Elizabethkingia anophelis* has recently been sequenced and was identified as having well developed iron scavenging, stress response and erythrocyte digestion systems (Kukutla et al., 2014; Li et al., 2015). Similarly, acetic acid bacteria such as *Asaia* are suggested to persist in the insect gut thanks to their ability to thrive in low oxygen concentrations (Chouaia et al., 2014). The genome sequences of several other mosquito isolated strains have also been released recently (Alvarez et al., 2012; Jiang et al., 2012; Pei et al., 2015; Shane et al., 2014), though so far no systematic analysis has emerged of common genes or properties that may be associated with persistence in the mosquito gut.

4.2 Future work

Future work should first seek to validate the principle findings and mechanistic models described here. An important finding of this work is that in the absence of the PM, the microbiota can escape the midgut and cause a systemic infection in the haemolymph. This should be confirmed by a PCR

independent method, for example by microscopically observing bacteria in transit through the gut epithelium. It would also be interesting to see if any gut microbiota species are able to invade even in the presence of the PM; it may be the case that invasion occurs at lower frequency in the presence of the PM, but that this can be immediately brought under control by the systemic immune response. Another essential future experiment will be to determine at greater taxonomic depth which bacterial genera, species or strains are invasive; the fact that bacteria were only detected in a subset of the polyoxin D treated samples would suggest either that it is a specific bacterium that is invasive, or that those individuals that go on to develop an infection are genetically polymorphic in a key immune gene. Our colony has been shown to maintain a high level of genetic variation that makes this a feasible prospect (Stathopoulos et al., 2014).

This work also raises several interesting questions regarding the dynamics of the microbiota, with particular relevance to paratransgenesis, that merit further investigation. For example, it is not known how the gut is recolonised after blood feeding- some bacteria remain in the gut following excretion, so perhaps it is these that seed the community after the first blood meal. If this is the case it would be interesting to understand how these bacteria avoid excretion- whether they must form a close association with the epithelial cells, and whether this does indeed induce the production of lipid rafts as hypothesised based on the RNA-seq data presented here. The H&E stained thin abdominal sections presented here do suggest that bacteria are able to inhabit the endoperitrophic space. Further work should not only confirm that the cellular structures are bacteria, but go on to characterise these at the taxonomic level, perhaps via an *in situ* hybridisation approach. Alternatively, mosquitoes may recolonise their guts by feeding on environmental bacteria. This would have important implications for a paratransgenesis strategy, suggesting that a modified strain may have to be reintroduced to the gut following each bloodmeal in the mosquito life span.

The work described here additionally raises further questions regarding the mechanisms that the gut employs to tolerate the presence of the microbiota.

In particular, it would be key to decipher how AMP expression is reduced specifically in the posterior most region of the gut, despite this region being exposed to high levels of bacterial ligand. This could be achieved by region specific expression of negative or positive regulators at the level of ligand degradation, immune receptor localisation, transcription or translation. To investigate this, an RNA-seq analysis could be performed, looking for genes that are differentially expressed between the gut compartments. This is particularly interesting as it has long been observed that *Plasmodium* oocysts preferentially establish at the posterior region of the midgut (Cociancich et al., 1999), though the reasons for this unclear. One potential explanation, supported by the data presented here, is that the midgut preferentially employs tolerance mechanisms at the posterior end, to allow the persistence of the microbiota.

Here, we have proposed a model for the role of the different PGRPLC isoforms that should be directly tested in future work. This would be best achieved in a cell culture system. For example, a mosquito cell line knocked down for one or more of the LC isoforms could be stimulated by TCT or polymeric peptidoglycan to question directly which isoform is required for IMD activation by each ligand.

Another question that remains to be addressed is whether different bacterial genera are differentially susceptible to the mosquito immune response, and, conversely, whether they are able to stimulate the IMD pathway to different extents. Interestingly, one study found that *Asaia* and *E. coli* both induce AMP expression to the same extent in mosquito haemocytes *in vitro*, though the growth of an *Asaia* culture is considerably less affected by the presence of AMPs than is that of *E. coli*, and, similarly, *Asaia* is considerably less susceptible to phagocytosis by mosquito haemocytes than is *E. coli* (Capone et al., 2013). It would be interesting to observe whether the same is true for other bacterial strains that are isolated from the midgut, perhaps with comparison to environmentally isolated strains of the same species. This would shed light on the extent to which the gut microbiota co-evolved with

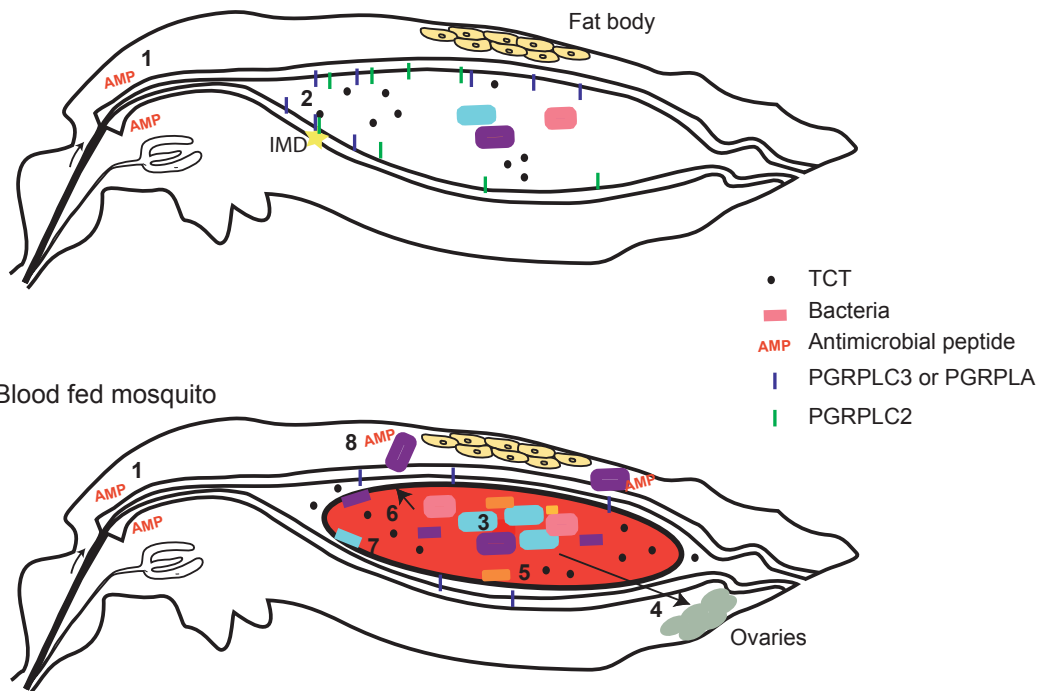
their mosquito host, and equally the extent to which the microbiota are heritable or environmentally acquired.

4.3 Conclusions

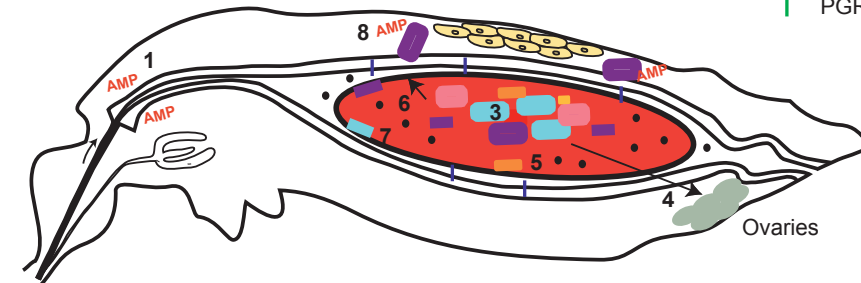
The data presented herein further our understanding of host-microbiota interactions in the malaria vector mosquito *A. coluzzii*. The main conclusions of this work, including proposed functional models, are represented in Figure 4.3.1.

Figure 4.3.1

Sugar fed mosquito



Blood fed mosquito



1. In both the sugar fed and blood fed midguts, the anterior region is highly immunogenic. The posterior tolerates the presence of the microbiota.
2. In the sugar fed gut, TCT is the main immune elicitor. PGRPLC2 plays an essential role as an adaptor, pairing with other PGRPs to stimulate the IMD pathway.
3. Blood feeding stimulates microbiota growth.
4. Blood meal-associated microbiota growth causes a reduction in mosquito fecundity and survival.
5. In the blood fed gut, polymeric peptidoglycan is the main immune elicitor. PGRPLC3, and possibly PGRPLA, are likely the main IMD pathway receptors
6. The gut microbiota contribute to the induction of the peritrophic matrix
7. The peritrophic matrix reduces the growth of the *Enterobacteriaceae* family of bacteria within the gut and facilitates their excretion, possibly by directly binding bacteria.
8. The peritrophic matrix prevents the *Enterobacteriaceae* family of bacteria from traversing the gut epithelium and invading the haemocoel.

Figure 4.3.1. Summary of the main conclusions and models proposed in this thesis.

First, it is demonstrated that the addition of antibiotics to the human blood meal is sufficient to significantly reduce blood meal associated bacterial proliferation, and that this reduction in microbiota growth both increases mosquito survivability and fecundity. Not only does this have potential implications for malaria transmission in regions where antibiotic use is high, it additionally sheds light on the host-microbiota relationship, reframing the microbiota less as symbionts and more as pathobionts- or bacteria that have the potential to become pathogenic to their host. Further, we identify excretion as the primary means by which the excessive microbiota associated with the blood meal are removed from the gut. The mosquito gut transcriptional response to the microbiota reveals that several physiological pathways are perturbed by the absence of the microbiota, highlighting these for further analysis of the nutritional contribution of the bacteria to the mosquito host. Furthermore, we characterise the gut immune response to the presence of the gut bacteria, shedding light on the pathways and effectors likely employed for the control of the microbiota, as well as raising further questions as to how the gut specifically upregulates negative regulators to a greater extent than effectors. Importantly, we identify the PM as playing a key role in gut homeostasis, both reducing immune induction in the gut after a blood meal, ensuring efficient bacterial secretion and preventing bacterial passage from the gut to the body cavity. Finally, we show that the mosquito gut, similar to the *Drosophila* gut, is highly compartmentalised, with AMP expression being localised primarily to the anterior region and the gut microbiota to the posterior. Despite heterogeneity of PGRPLC isoform expression across the length of the gut, we do not find evidence for spatial heterogeneity of function, at least in basal IMD pathway induction. Rather, our data is consistent with PGRPLC2 being the essential isoform in the sugar fed gut and PGRPLC3 in the blood fed midgut. Together with recombinant protein binding assays, these data lead to the proposition of a tentative model of PGRPLC-dependent induction in the midgut, whereby in the sugar fed gut TCT is the primary bacterial elicitor, requiring LC2 for IMD stimulation, while in the blood fed gut this function is fulfilled by polymeric peptidoglycan, which appears to be detected by PGRPLC3. Altogether, this thesis brings together both molecular

and physiological data to unveil new insight into the relationship between the malaria vector mosquito *A. coluzzii* and its gut microbiota.

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Chapter 6: Appendix

Table 6.1. Sequences and amplification efficiencies of primers used in qRT-PCR

Transcript name	AGAP number	Sequence (F)	Sequence (R)	Primer efficiency
S7	AGAP010592	GTGCGGAGTTGGAGAAGA	ATCGGTTGGCGAGAATGC	2.009
16S (eubacteria)	-	TCCTACGGGAGGCAGCAGT	GGACTACCAAGGGTATCTAATCCTG TT	1.783
LYSC1	AGAP007347	AATGTGAAGTGGCAAAGG	AGTCGTTGAACCGTAGC	2.043
CEC1	AGAP000693	ACCAACCAACCACCAAAC	CTTCTCTGCTGCCCTTGAACACT	2.029
GAM1	AGAP008645	CTATCTCAACCGGAAGGGCG	GCCAAACTTTCGCTTGCACT	2.021
Vitellogenin	AGAP004203	CTGGTCTATCCGTTCCGCTC	GACGTCGCTGTTGCCGAATAC	2.006
PGRPLC1 (spliced)	AGAP005203 (-RA&RB)	CACACCAAAAATTACAAACACGATCA	GGCAAAAGCCTCAGCTGTTC	2.051
PGRPLC2 (spliced)	AGAP005203 (-RF&RE)	TTCGCGTTACAATTCCCGC	GCCTCGTCCCTTATTTTGT	1.987
PGRPLC3 (spliced)	AGAP005203 (-RC&RD)	TGCACTACTCAGACGAAATG	GGCTGTAGTTGGAGGCAAGAT	2.093
Aper1	AGAP006795	CCTGATCTGCAACCCACGGAA	GCACTGCTTCTGGGAGTCAT	2.046
REL2	AGAP006747	ACCGATACGGAAAGTGTGCT	GTATCGTTGCGTCGGATTG	2.100
LC1 knock down efficiency	AGAP005203 (-RA&RB)	AGGTATCACGTAAACCAACCA	ACACAAATTCGTCAACACCG	1.912
LC2 knock down efficiency	AGAP005203 (-RF&RE)	GGCAAGTGGCTAATCGAAGC	GGCGCTCGTCCCTTATTTTGC	1.724
LC3 knock down efficiency	AGAP005203 (-RC, RD, RH, RJ)	CCACCGATCGCTCAGCTC	CAGGCTGTAGTTGGAGGCAA	2.052
Flavobacteriaceae 16S	-	TAAGGTTGAAGTGGCTGGAATAA	GTCCATCAGCGTCAGTTAAGACT	1.986
Enterobacteriaceae 16S	-	CGTGCTACAATGGCATATACAAAGAG AAG	AGCATTTCTGATCTACGATTACTAG CGATT	1.970
Acetobacteriaceae 16S	-	GTGCCGATCTCTAAAAGCCGTCTCA	TTCGCTCACCGGCTTCGGGT	1.982

Table 6.2. Sequences of primers used for dsRNA synthesis

Target name	AGAP number	Sequence (F)	Sequence (R)
<i>REL2</i>	AGAP006747	taatcgactcactatagggCGGAGAAAGTCGAAAGA AAACG	taatcgactcactatagggCACAGGCACACCTGA TTGAG
<i>STAT2</i>	AGAP000099	taatcgactcactatagggCCGGAGAGCAACTTC ACGAT	taatcgactcactatagggGATGAACGTGTTGGT AATGAGC
<i>Aper1</i>	AGAP006795	taatcgactcactatagggagaATGGTGATACATTC CGCACGA	taatcgactcactatagggagaCACAGTGGCACA CTTCAAGC
<i>PGRPLC1</i>	AGAP005203 (-RA&RB)	taatcgactcactatagggCGGGATGGCTTAAAG TTGG	taatcgactcactatagggCGCACAGCTTGTACT CCTTG
<i>PGRPLC2</i>	AGAP005203 (-RF&RE)	taatcgactcactatagggGTTACAAATTCGCCGAG CATAA	taatcgactcactatagggCGAGGCATATATTCGG TAACCT
<i>PGRPLC3</i>	AGAP005203 (-RC&RD)	taatcgactcactatagggTTGATCCAGGAGTTTC ATTGG	taatcgactcactatagggAATGTTCCAAATGAACG CGA

Table 6.3. Sequences of primers used for production of recombinant proteins

Target name	Sequence (F)	Sequence (R)
PGRPLC1 PGRP domain	GACGACGACAAGATGAGACCGTTACGGTT AGTGACG	GAGGAGAAGCCCGGTTT GCTCTGGAAGTACAGGTTCTC CAAACGTTTCACTAAATGT
PGRPLC2 PGRP domain	GACGACGACAAGATGAGACCGTTACGGTT AGTGACG	GAGGAGAAGCCCGGTTT GCTCTGGAAGTACAGGTTCTC CTCGTGATCGGGCGCTCG
PGRPLC3 PGRP domain	GACGACGACAAGATGAGACCGTTACGGTT AGTGACG	GAGGAGAAGCCCGGTTT GCTCTGGAAGTACAGGTTCTC TCGTCGGTTTGTGTTCTCG

Table 6.4. Alignment and count statistics of RNAseq data

Time point	Sample	Raw reads (n pairs)	Input to Tophat2 (n pairs)	% to pass pre alignment filtering	Unique alignmen ts (n reads)	Unique alignmen % aligning uniquely	Unique alignmen ts:s:ingleton alignments	Total n pairs processe d by HT- SEQ	Number aligning to feature	Number not aligning to feature	Number ambiguo us	% Feature	% No feature	% Ambiguous			
0h	1	1161446	984291	84.7	1733655	88.1	106885	1626770	920270	778167	136512	5591	84.6	14.8	0.6		
	1+	25459034	19915813	78.2	35069801	88.0	2089753	32980048	18579777	15909589	2548508	121680	85.6	13.7	0.7		
	3	12492104	10750828	86.1	18949339	88.1	1121653	17827686	10035496	8593900	1368840	72756	85.6	13.6	0.7		
	3+	9839640	8782135	89.3	15420405	87.8	951841	14468564	8186123	6840263	1287639	58221	83.6	15.7	0.7		
	4	57409201	8836763	15.4	12259074	69.4	805042	11454032	6532058	4588346	1908710	35002	70.2	29.2	0.5		
	4+	30738104	19741325	64.2	33750416	85.5	2152696	31597720	17951556	14254694	3588543	108319	79.4	20.0	0.6		
5h	6	10846096	9653984	89.0	16610126	86.0	1209950	15400176	8910038	7099318	1745800	64920	79.7	19.6	0.7		
	6+	13177661	11843509	89.9	20404565	86.1	1437349	18967216	10920957	8808364	2031547	81046	80.7	18.6	0.7		
	1	13883501	11930193	85.9	20913285	87.6	1157647	19755638	11035466	9466215	1491988	77263	85.8	13.5	0.7		
	1+	54690605	46440532	84.9	81406644	87.6	4495514	76911130	42951079	36232348	6394611	324120	84.4	14.9	0.8		
	3	3124786	2656117	85.0	4546219	85.6	316669	4229550	2431444	2011938	402662	16844	82.7	16.6	0.7		
	3+	8628455	7277799	84.3	12485911	85.8	888917	11596994	6687414	5454813	1182437	50164	81.6	17.7	0.8		
24h	4	12576113	10244312	81.5	17823628	87.0	1017866	16805762	9420747	8258093	1101143	61511	87.7	11.7	0.7		
	4+	42473645	35489551	83.6	61935527	87.3	3617761	58317766	32776644	28041822	4512610	222212	85.6	13.8	0.7		
	6	9571880	8227294	86.0	13981783	85.0	1001169	1298061	7491476	6439656	997395	54425	86.0	13.3	0.7		
	6+	28302632	23026407	81.4	39255124	85.2	2718252	36536872	20986688	18046374	2763668	176646	86.0	13.2	0.8		
	1	42718406	36536414	85.5	62807227	86.0	3697937	49271764	33252582	28040780	4904676	307126	84.3	14.7	0.9		
	1+	47089435	39479891	83.8	67589973	85.6	3996867	63593106	35793420	30477239	4953174	363007	85.1	13.8	1.0		
72h	3	46280732	42231363	91.3	72488323	85.8	4746263	67742060	38617293	34195226	4060411	361656	88.5	10.5	0.9		
	3+	56429829	47475339	84.1	76986146	81.1	6115808	70870338	41550977	33788212	7468615	294150	81.3	18.0	0.7		
	4	7395030	6086430	82.3	10337420	84.9	719782	9617638	5528601	4792339	684222	52040	86.7	12.4	0.9		
	4+	72809204	58984464	81.0	10028585	85.0	6954463	93331396	53620161	45514712	7656390	449059	84.9	14.3	0.8		
	6	18218679	16498299	90.6	27840046	84.4	1989314	25850732	14914680	13188733	1584936	141011	88.4	10.6	0.9		
	6+	16132127	14540436	90.1	24600048	84.6	1743660	22856388	13171854	11720405	1343972	107477	89.0	10.2	0.8		
96h	1	4799854	4173578	87.0	7277639	87.2	440077	6837562	3858858	3115257	717954	25647	80.7	18.6	0.7		
	1+	827405	708029	85.6	1232030	87.0	80568	1151462	656299	517615	134710	3974	78.9	20.5	0.6		
	3	4405907	4032192	91.5	7031655	87.2	489159	6542496	3760407	3063506	675085	21816	81.5	18.0	0.6		
	3+	13218532	11603971	87.8	20231439	87.2	1299915	18931524	10765677	8907110	1791440	67127	82.7	16.6	0.6		
	4	9705364	8021834	82.7	13794103	86.0	1019727	12774376	7406915	5771381	1590989	44545	77.9	21.5	0.6		
	4+	5354753	4379139	81.8	7548096	86.2	558414	6989682	4053255	3155396	872214	25645	77.8	21.5	0.6		
Average (mean)	6	9586364	8697191	90.7	14988556	86.2	1091116	13897440	8039836	6378412	1608353	53071	79.3	20.0	0.7		
	6+	38284833	34761030	90.8	59770301	86.0	4413929	55356372	32092115	25005664	6870205	216246	77.9	21.4	0.7		
	1	3958175	3588476	90.7	6257029	87.2	388397	5868632	3322713	2886702	405624	30387	86.9	12.2	0.9		
	1+	49104662	44873807	91.4	77169696	86.0	5010382	72159314	41090039	33603043	7152837	334159	81.8	17.4	0.8		
	3	9818896	7324200	74.6	12534809	85.6	762457	11772352	6648633	6134816	462698	51119	92.3	7.0	0.8		
	3+	71224760	64162182	90.1	11004455	85.8	6984661	103059892	58514607	51800805	6288678	425124	88.5	10.7	0.7		
Average (mean)	4	5761965	4841714	84.0	8268072	85.4	529490	7738582	4398781	3850150	504989	43642	87.5	11.5	1.0		
	4+	6625008	5545067	83.7	9459254	85.3	623572	8835682	5041413	4319673	671223	50517	85.7	13.3	1.0		
	6	11949309	10718763	89.7	17881550	83.4	1277286	16604264	9579418	8456982	1033832	88604	88.3	10.8	0.9		
	6+	7173669	6482166	90.4	10854667	83.7	758195	10096472	5806431	5168729	575740	61962	89.0	9.9	1.1		
				83.8		85.6						83.9		15.4		0.8	

Figure 6.1

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GTCTTCGGTTGTACGCGCCCGCGGAAAGATCGGCTCTATCTGCGCGCTCCTCTGCTCGTG
TGAGAAGAAAAAGAAACGGTTCCACGGTACGGCACCAACCACCACTCTCCCTCACTC
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>AGAP000693 (CEC1)
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TCCCGTAGGGCCACGGAAGGGGACTTTTTGAGGTTGAAACGGTTCGAAAGAAAGAAAG
AAAAAAACATTTCATCGAACGCGCGCGTATTATTCCTTTGATCGATTGTACGGGGATCCC
CAGGAATTCCGGGGTAACGCTTTTGGTATATAAAACGGAGCTAATCAATGCAAGGTAT
CAACAGTCGGTTCAAAGCGTTCTGCAGCAAAACATCTTATCAACCCAGAGACCAACCAAC
CACCAACAACCCCAACAACA

```

Figure 6.1. 500 bp 5' of the translation start site of putative PM component encoding genes identified as being regulated by the midgut microbiota. Consensus STAT binding sites are highlighted in red; NF- κ B sites, found only on *CEC1* are highlighted in green.

Permission to reproduce data from (Dinglasan et al., 2009) in Table 3.2.4

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