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2022

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CHARACTERISATION OF IMMUNE RESPONSES TO BACTERIAL STIMULATION IN THE *Anopheles gambiae* COMPLEX

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A dissertation submitted to the Faculty of Health Sciences, University of the Witwatersrand, in fulfilment of the requirements for the degree of Master of Science

Johannesburg, 2021

Declaration

I (Nashrin Fazlerehman Patel) declare that this dissertation is my own unaided work. It is being submitted for the degree of Master of Science at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other university.



(Signature of candidate)

---------24---------- day of ----------December--------20---21------in ---------Johannesburg---

Presentations arising from this study

- **Patel N. F.**, Letinić B. D., Lobb L. N., Zawada J., Dlamini D. M., Mabaso N., Munhenga G., & Oliver S. V. "Characterisation of the relative immune response in zoophilic members of the *Anopheles gambiae* complex". 1st Women in Malaria Virtual Conference. 22-24 March 2021. (Poster Presentation).

- **Patel N. F.**, Jeanrenaud A. C. S. N., Brooke B. D., & Oliver S. V. "Characterising the epigenetic landscape of the *Anopheles gambiae* complex". XXVI International Congress of Entomology, Helsinki, postponed to 17-22 July 2022 (Long oral presentation)

Publications arising from this study

- Manuscript submitted to the journal *Insect Biochemistry and Molecular Biology*: **Patel N. F**., Letinić B. D., Lobb L. N., Zawada J., Dlamini D. M., Mabaso N., Munhenga G., Oliver S. V. Comparison of the effect of bacterial stimulation on the global epigenetic landscape and transcription of immune genes in zoophilic members of the *Anopheles gambiae* complex (Diptera: Culicidae). (Proof in Appendix 2).

- Manuscript in preparation for the journal *Acta Tropica*: **Patel N. F**., Oliver S. V. Characterisation of immune memory in the major malaria vector *Anopheles arabiensis* (Diptera: Culicidae).

Abstract

Anopheles arabiensis, An. merus and *An. quadriannalatus* are three zoophilic members of the *An. gambiae* complex that occur in southern Africa that differ in vector competence. They are considered as major, minor, and non-vector, respectively. This study aimed to characterise the molecular underpinnings of the immune responses in these species in response to bacterial challenges. Furthermore, it assessed whether the insecticide resistant phenotype affects these responses. This was achieved by assessing the global epigenetic landscape and the expression levels of transcripts for *defensin-1* and *gambicin* antimicrobial peptide genes in response to *Streptococcus pyogenes* and *Escherichia coli* challenges. The epigenetic markers 5mC, 5hmC and m6A methylation levels were assessed by ELISA. Histone Acetyl Transferase activity was assessed calorimetrically. The transcript levels of *defensin-1* and *gambicin* were assessed by qRT-PCR. Immune priming was examined in *An. arabiensis*. Species-specific differences in 5mC and m6A levels, as well as *defensin-1* and *gambicin* transcript levels were observed, constitutively and post-immune stimulation. The minor/non-vectors had similar patterns that were different from major vectors. At the constitutive level, there were no differences in the epigenetic landscape and expression of antimicrobial peptide transcripts in insecticide resistant and susceptible *An. arabiensis*. Blood-borne immune priming was an efficient means of inducing immunological memory. Larval immune priming induced immunological memory in a species-specific manner, with a fitness cost in insecticide resistant *An. arabiensis*. Although larval immune priming effects induced transgenerational immunological memory, insecticide susceptible *An. arabiensis* maintained the immunological memory to a greater degree. This study highlights variation in immune responses in the members of the *An. gambiae* complex and the presence of immunological memory.

Acknowledgements

The journey of this research project has been an amazing experience. I have received great support and contributions from many people that I would like to acknowledge.

- I am very thankful to my supervisor, Dr Shüné Oliver, for her constant support, assistance, and guidance on the smallest of the things throughout my journey on this project. From the first discussion until the last, she has been patient and always available.
- I thank my parents for their immense support and prayers. I love you both.
- Thanks to the team in Jozini, for identifying the wild-caught mosquitoes to species complex and sending them live to Johannesburg.
- I thank Dr Blaženka Letinić and Jacek Zawada for the identification of wild-caught mosquitoes, and Leanne Lobb for administering this process.
- Thanks to Dr Blaženka Letinić for her guidance and training on the gene expression experiments.
- Thanks to Ashmika Singh for being an amazing friend and training me on mosquito midgut dissection.
- I thank Arshad Ismail and Senzo Mtshali at The Sequencing Core Facility for assisting me with TapeStation analysis.
- Finally, I would like to thank the staff and students of the Vector Control Unit for providing a friendly environment.

This study was funded by the following organisation:

- The National Research Foundation for the Competitive Support for Unrated Researcher's grant. They are thanked for their financial support.

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List of Abbreviations and Acronyms

- **5hmC**: 5-hydroxymethylcytosine
- **5mC**: 5-methylcytosine
- **A**: Alanine
- **AChE**: Acetylcholinesterase
- **ALKBH-5**: Alkylation repair homolog protein 5
- **AMP**: Antimicrobial peptide
- **ANOVA**: Analysis of Variance
- **BCR**: B-cell receptor
- **cDNA**: Complementary deoxyribonucleic acid
- **CoA**: Coenzyme A
- **CpG**: Cytosine-guanine
- **CTL-4**: C-type lectin 4
- **CTLMA**: C-type lectin mannose binding protein
- **DAP**: Diaminopimelic acid
- **DDT**: Dichlorodiphenyltrichloroethane
- **DEF**: Defensin
- **DEPC**: Diethyl pyrocarbonate
- **DIN**: Deoxyribonucleic acid Integrity Number
- **DNA**: Deoxyribonucleic acid
- **DNMT**: Deoxyribonucleic acid methyltransferase
- **dNTPs:** Deoxynucleotides
- **Dscam**: Downs Syndrome cell adhesion molecule
- **DTT**: Dithiothreitol
- **EDTA**: Ethylenediaminetetraacetic acid
- **ELISA**: Enzyme-Linked Immunosorbent Assay
- **F**: Phenylalanine
- **F1**: Wild-caught *Anopheles arabiensis* first-generation progeny
- **FTO**: Fat mass and obesity-associated protein
- **G**: Glycine
- **GABA**: Gamma (γ)-aminobutyric acid

- **GAMB**: Gambicin
- **GNBP**: Gram-negative binding protein
- **H1**: Histone 1
- **H2A**: Histone 2-A
- **H2B**: Histone 2-B
- **H3**: Histone 3
- **H4**: Histone 4
- **HAT**: Histone acetyl transferase
- **HDAC**: Histone deacetylase
- **HDF**: Haemocyte differentiation factor
- **HDM**: Histone demethylase
- **HMT**: Histone methyltransferase
- **HRP**: Horseradish peroxidase
- **Ig**: Immunoglobulin
- **IGS-2**: Intergenic spacer 2
- **IMD**: Immune deficiency
- **IRS**: Indoor residual spraying
- **ITN**: Insecticide-treated net
- **JAK/STAT**: Janus kinase/signal transducer and activator of transcription
- **K**: Lysine
- **Kdr**: Knockdown resistance
- **L**: Leucine
- **LB**: Lysogeny Broth
- **LPS**: Lipopolysaccharide
- **LRIM**: Leucine-rich repeat immune protein
- **m6A**: N6-methyladenosine
- **METL**: Methyltransferase-like
- **$MgCl_2$**: Magnesium chloride
- **miRNA**: Micro ribonucleic acid
- **mRNA**: Messenger ribonucleic acid
- **NADH**: Nicotinamide adenine dinucleotide
- **ncRNA**: Non-coding ribonucleic acid

- **NF-κB**: Nuclear factor kappa B
- **NOS**: Nitric oxide synthase
- **PAMP**: Pathogen-associated molecular pattern
- **PBS**: Phosphate-buffered saline
- **PCR**: Polymerase Chain Reaction
- **PGRP**: Peptidoglycan recognition protein
- **PMSF**: Phenylmethylsulphonyl fluoride
- **PO**: Phenoloxidase
- **Poly-A**: Poly-adenosine
- **PPO**: Prophenoloxidase
- **PRR**: Pathogen recognition receptor
- **qRT–PCR**: Quantitative real-time Polymerase Chain Reaction
- **R**: Arginine
- **RAG**: Recombination-activation genes
- **RBC**: Red blood cell
- ***Rdl***: Resistance to dieldrin gene
- **Rel**: Relish
- **RINe**: Ribonucleic acid Integrity Number
- **RNA**: Ribonucleic acid
- **ROS**: Reactive oxygen species
- **rRNA**: Ribosomal ribonucleic acid
- **RT**– **PCR**: Reverse transcription Polymerase Chain Reaction
- **RT**: Reverse transcriptase
- **S**: Serine
- **SAM**: S-adenosine methionine
- **siRNA**: Small interfering ribonucleic acid
- **SNP**: Single nucleotide polymorphism
- **TBE**: Tris-Borate-EDTA (EDTA: Ethylenediaminetetraacetic acid)
- **TCR**: T-cell receptor
- **TEP-1**: Thioester-containing protein 1
- **TET**: Ten-eleven translocation
- **tRNA**: Transfer ribonucleic acid

- **Tukey-HSD**: Tukey’s honestly significant difference
- **Upd**: Unpaired
- **UTR**: Untranslated region
- **WHO**: World Health Organisation

CHAPTER ONE: Introduction and literature review

1.1. VECTOR-BORNE DISEASES

Diseases transmitted by animals, generally by the hematophagous (blood-feeding) arthropods, are referred to as vector-borne diseases. These diseases are caused by infectious pathogens such as viruses, bacteria, and parasites. The arthropod vectors ingest the infectious pathogen while feeding on an infected host and thereafter transmit the pathogen into a new uninfected host while taking their subsequent meal (reviewed by Gage *et al.*, 2008). According to the World Health Organisation (**WHO**), "Vector-borne diseases account for more than 17% of all infectious diseases, causing more than 700 000 deaths annually". Ticks, fleas, aquatic snails, blackflies, lice, sandflies, tsetse flies, triatome bugs and mosquitoes are all vectors that contribute to a variety of vector-borne diseases (World Health Organisation, 2020a). Mosquitoes can transmit a variety of diseases and parasites. Mosquito species from the genus *Aedes* transmit viruses such as Dengue, Yellow Fever, Rift Valley fever, Zika and Chikungunya, as well the lymphatic filariasis parasite. Mosquitoes from the *Culex* genus transmit Japanese encephalitis virus, West Nile virus and the lymphatic filariasis parasite. Mosquitoes from the *Anopheles* genus primarily transmit malaria and lymphatic filariasis parasites (World Health Organisation, 2020a).

1.1.1. Malaria

Malaria is the most significant vector-borne disease affecting human populations. In 2019, 229 million malaria cases were reported worldwide, of which 94% of cases were reported from the African continent. An estimated 409 000 malaria deaths were reported worldwide in 2019 (World Health Organisation, 2019). Malaria is caused by protozoan parasites of the genus *Plasmodium* and is transmitted by *Anopheles* mosquitoes which are referred to as malaria vectors (Ross, 1898). Approximately 250 species of the *Plasmodium* parasite have been discovered, of which 48 species infect mammalian hosts (Faust & Dobson, 2015). Five species of the *Plasmodium* parasite have been reported to cause malaria in humans viz: *P. vivax*, *P. falciparum*, *P. ovale*, *P. malariae* and *P. knowlesi*. The *P. falciparum* and *P. vivax* parasites pose a major threat to human populations in Africa and America respectively (World Health Organisation, 2020b).

Malaria is a preventable disease; this can be achieved by preventing the infection (malaria prophylaxis) or restricting the human-vector contact (vector control). Drug chemoprophylaxis

is an effective preventative strategy, often used by tourists travelling to malaria-endemic areas (reviewed by Castelli *et al.*, 2007). Indoor residual spraying (**IRS**) and insecticide-treated nets (**ITNs**) are two types of vector control strategies recommended by the WHO. IRS targets the endophilic (indoor resting) and endophagic (indoor biting) mosquitoes. ITNs provide a protective physical barrier and, the insecticide on the nets repels and kills the endophilic/endophagic mosquitoes (World Health Organisation, 2020b). The emerging resistance to insecticides in *Anopheles* mosquitoes threatens vector control programs, potentially resulting in an increase in malaria cases. According to the World malaria report, mosquitoes in 73 countries have been reported to be resistant to one of the four classes of insecticides (World Health Organisation, 2019).

1.1.2. Malaria in South Africa

Malaria is widespread in 14 countries of southern Africa. The Democratic Republic of Congo and Mozambique were amongst the six countries that accounted for 50% of malaria cases in 2018. In South Africa, malaria is mainly transmitted along the country's borders due to frequent migration (Elimination 8, 2015). Currently, Limpopo, KwaZulu-Natal and Mpumalanga are the provinces where malaria is endemic in South Africa. Subsequently, that puts 10% of the population at risk of developing malaria. Approximately 9500 cases were reported in South Africa in 2018, which is a 50% reduction in comparison to malaria cases in 2017. (World Health Organisation, 2020c). Malaria control and prevention with pyrethrum was first implemented in South Africa in KwaZulu-Natal (de Meillon, 1936). The use of IRS ever since then has led to reduced malaria transmission in South Africa but due to insecticide resistance, the number of infections has started to rise again (Hargreaves *et al.*, 2000).

In 2019, the goal set by the malaria elimination program was to "achieve zero local malaria transmission in South Africa by the year 2023" (National Department of Health, 2019). Malaria elimination is "the interruption of indigenous transmission of a specified malaria parasite species in a defined geographic area" (World Health Organisation, 2018). South Africa and four other countries in southern Africa are working to eliminate malaria (Brooke *et al.*, 2020). However, frequent migration of both parasite reservoirs and vector mosquitoes poses a challenge. Successful malaria elimination in South Africa depends on the economy of the country and new approaches.

1.2. MOSQUITOES AS MALARIA VECTORS

Mosquitoes belong to the order Diptera from the family Culicidae, which consists of multiple genera. Approximately 3500 mosquito species have been recorded worldwide that are grouped into 41 genera (Edwards, 1932; Clements, 1999a). Only the mosquitoes from the *Anopheles* genus transmit malaria to humans (reviewed by Collins & Paskewitz, 1996).

Females require proteins from the blood to produce eggs. Thus, only female mosquitoes are capable of transmitting pathogens. Once the eggs have developed, the female mosquito lays approximately 50–200 eggs on the surface of the preferred aquatic habitat. The eggs hatch within two–three days and larvae emerge. In the presence of adequate nutrients and the appropriate climate, the larvae moult four times before pupating. The adult mosquito then emerges from the non-feeding pupal stage (Figure 1.1; Clements, 1999b).

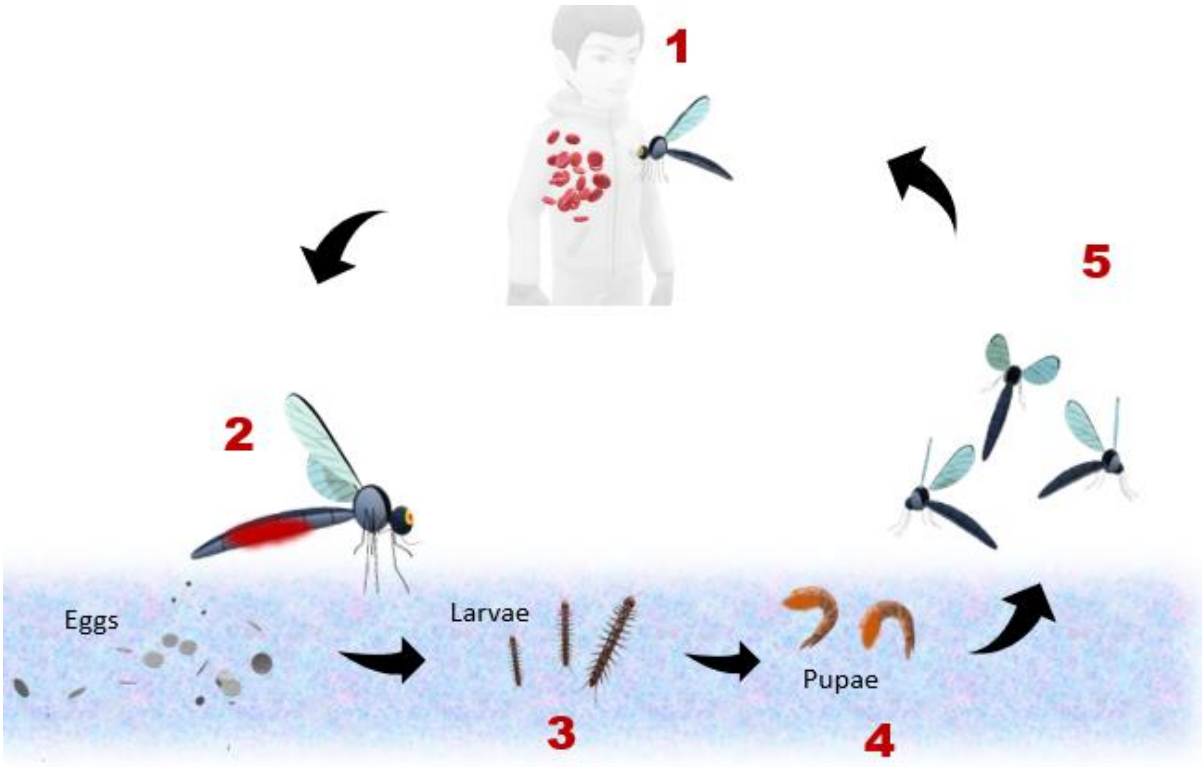


Figure 1. 1: The lifecycle of a mosquito. (1) Female mosquitoes feed on blood to produce eggs. (2) Females then lay eggs on a water body. (3) The eggs hatch and larvae emerge. (4) The larvae feed on plant material and micro-organisms in the water and develop into pupae. (5) The pupae undergo metamorphosis and adult mosquitoes emerge (adapted from Clements, 1999b).

1.2.1. The *Anopheles* genus

The *Anopheles* genus was named and described by a German entomologist (Meigen, 1818). There are currently approximately 460 described *Anopheles* species, of which only 70–80 are

natural vectors of malaria (Harbach, 2013). The remaining species either cannot sustain the development of the *Plasmodium* parasite or are zoophilic (animal-biting). Different *Anopheles* species transmit malaria in different geographic regions, as certain geographic environments support specific species depending on the climate (Centres for Disease Control and Prevention, 2020).

In Africa, approximately 140 *Anopheles* species have been discovered, of which 20 are natural malaria vectors (reviewed by Hay *et al.*, 2000). *Anopheles gambiae*, *An. arabiensis* and *An. funestus* are referred to as the dominant vector species of Africa. *Anopheles merus*, *An. melas*, *An. moucheti* and *An. nili. s.l* are the secondary vectors (minor-vectors) of malaria in Africa. (Sinka *et al.*, 2012). Most *Anopheles* species are found in a complex consisting of sibling species. The sibling species in the *An. gambiae* complex includes *An. gambiae*, *An. arabiensis*, *An. merus* and *An. melas* (Coetzee *et al.*, 2000).

1.2.2. The *Anopheles gambiae* complex

Anopheles gambiae was considered as a biologically mutable species in the 1960s. However, differential larval habitats, feeding and resting behaviours in *An. gambiae* were reported. Advanced molecular techniques led to the discovery of sibling species of *An. gambiae* that led to the description of the *Anopheles gambiae* complex. In 1962, when the West African and East African saltwater species were crossed, they did not mate, nor did they mate with the freshwater strains of *An. gambiae* (Davidson, 1962; Paterson, 1962). The saltwater species from West Africa was named *An. melas* and the one from East Africa was named *An. merus* (Mattingly, 1977).

In 1964, three more sibling species were discovered and named *An. gambiae*, *An. arabiensis* and *An. quadriannulatus* with the observation that they do not interbreed despite all breeding in freshwater (Paterson, 1964; Mattingly, 1977). A few years later, a sixth species of the complex, *An. bwambae* was discovered in Uganda, which is the third saltwater species (Davidson & Hunt, 1973). Studies revealed that *An. gambiae* exists in two molecular forms, the Savannah "S form" and the Mopti "M form" (Gentile *et al.*, 2002). In 2013, these two molecular forms were reclassified as sibling species to the *An. gambiae* complex based on the bionomical and molecular evidence. The S form retained the name *An. gambiae* and the M form of *An. gambiae* was re-named to *An. coluzzii*. The Ethiopian species discovered earlier as *An. quadriannulatus* B (Hunt *et al.*, 1998) was re-named as *An. amharicus* (Coetzee *et al.*, 2013). In 2019, one more species was added to the *An. gambiae* complex. This species was

discovered in Central Africa, being initially identified morphologically as *An. gambiae*. Molecular evaluations identified it as a novel species which was thereafter named *An. fontenillei* (Barrón *et al.*, 2019).

The nine members of the *An. gambiae* complex are morphologically identical but differ genetically. The member species differ in habitats and behaviour, and this contributes to their ability to transmit malaria (Sinka *et al.*, 2010) (Figure 1.2). Certain members behave similarly, with similar habitats yet differ in their ability to transmit malaria (vector competence). Therefore, precise identification of these species is important to assess a malaria threat in a region. Assessment of the difference in the ribosomal ribonucleic acid (**rRNA)** sequence of the intergenic spacer 2 (**IGS-2**) region amongst the members of the *An. gambiae* complex using a conventional Polymerase Chain Reaction (**PCR**) allows identification of individual members of the complex (Scott *et al.*, 1993). In addition to behavioural factors, biological and molecular factors could also be determining the difference in vector competence of the endophilic/exophilic (indoor resting/outdoor resting) and endophagic/exophagic (indoor biting/outdoor biting) members of the *An. gambiae* complex.

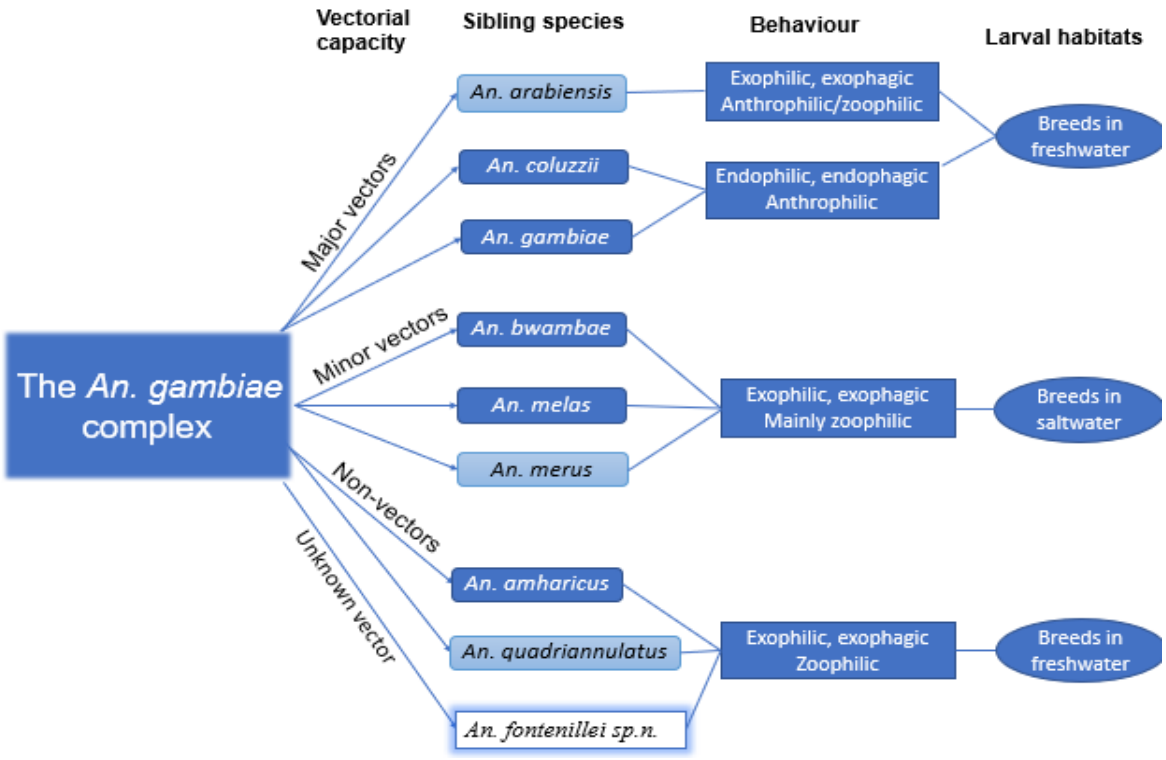


Figure 1. 2: Members of the *Anopheles gambiae* complex. Endophilic species rest indoors whereas exophilic species rest outdoors. Endophagic species bite indoors whereas exophagic species bite outdoors. Zoophilic species bite animals and anthropophilic species bite humans. Species labelled in the shade of a lighter blue are dominant in South Africa (Sinka *et al.*, 2010; Coetzee *et al.*, 2013; Barrón *et al.*, 2019).

1.3. VECTORIAL CAPACITY AND VECTOR COMPETENCE

Vectorial capacity was first defined as "the daily rate at which future inoculations arise from a currently infective case, provided that all female flies biting that case become infected" (Garrett-Jones *et al.*, 1964). Vectorial capacity is a quantitative measure, influenced by a number of factors. This includes variables like the longevity of the insect, parasite incubation time, density, biting rate, as well as vector competence. Vector competence is a term that was used interchangeably with vectorial capacity in the past. However, in modern literature, it refers to the susceptibility of mosquitoes to infection up to the infectious stage. It is the intrinsic capacity of a vector to transmit a pathogen, and it is largely dependent on genetics (reviewed by Beerntsen *et al.*, 2000). Vector competence is one of the factors that can affect vectorial capacity. Vector competence varies between and within species, thus directly creating an effect on the epidemiology of vector-borne diseases (reviewed by Severson *et al.*, 2001).

Vectorial capacity is affected by several intrinsic and extrinsic factors (Figure 1.3) (reviewed by Beerntsen *et al.*, 2000). In this study, the component of vectorial capacity under examination is vector competence. This competence can be affected by factors such as the host feeding preference and genetics. Of the various genetic factors driving vector competence, the immune system of the arthropod vector is a key factor influencing whether the *Plasmodium* infection becomes established and is able to persist within the *Anopheles* mosquito (Dong *et al.*, 2006).

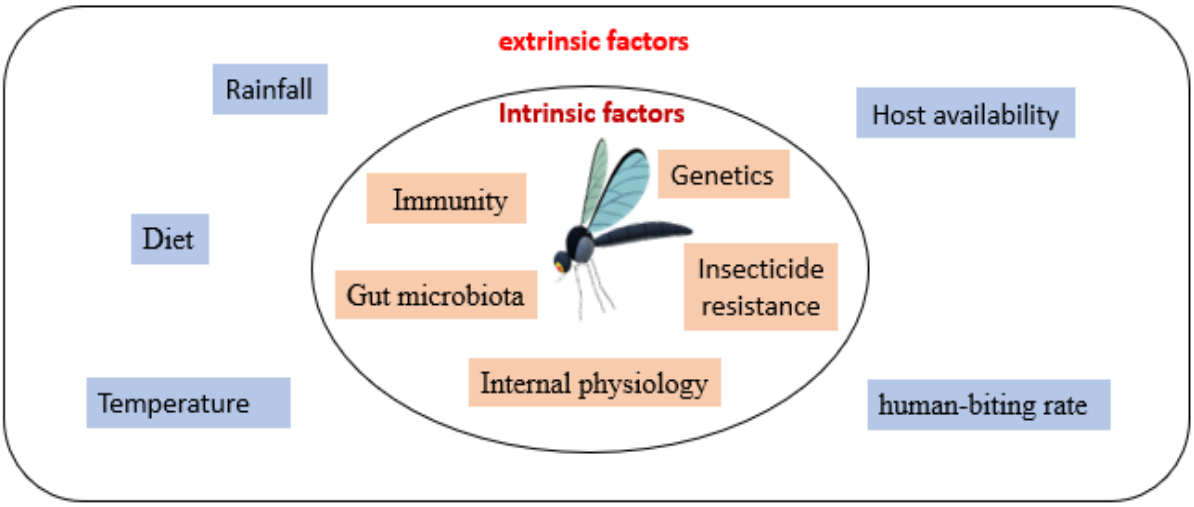


Figure 1. 3: Biological and molecular factors affecting vectorial capacity. Intrinsic factors are traits naturally present within a mosquito species, whereas extrinsic factors act on the mosquito from the outside, affecting the vectorial capacity. Vectorial capacity takes into account all the intrinsic and extrinsic factors that influence the interaction between a pathogen and its host (reviewed by Beerntsen *et al.*, 2000; Habtewold *et al.*, 2017).

1.3.1. The role of the immune system in vector competence

The immune system of an organism is triggered upon detection of non-self, particularly pathogens. This is followed by a series of immune signalling cascades to eliminate that pathogen. The parasites that infect a mosquito also generates a substantial immune response (Dong *et al.*, 2006). As such, the survival and reproduction of the parasite depend on the efficacy of the mosquito's immune response (reviewed by Barillas-Muray *et al.*, 2000). To be a competent vector, the mosquito species needs to be genetically and biochemically compatible to support the development of the parasite. The ingested pathogens also need to survive within the host and evade the host's immune system in order to be transmitted by the vector species (reviewed by Lefèvre *et al.*, 2013). To resist an infection, the mosquitoes generate immune responses in different parts of the body that leads to parasite elimination via lysis, haemocyte-mediated phagocytosis and melanisation (Söderhäll, 2010) (Figure 1.4).

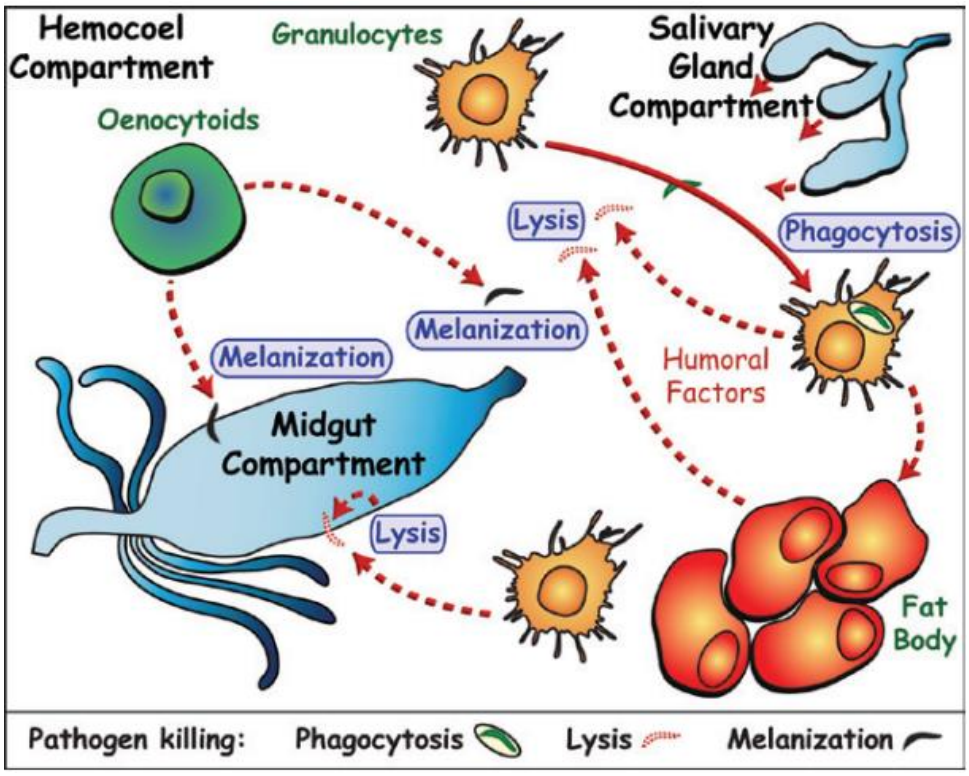


Figure 1. 4: The cellular and humoral immune responses generated by a mosquito upon pathogen invasion. The cellular immune response involves phagocytosis and engulfment of the pathogen by the haemocytes (granulocytes) and eliminating them via lysosomal degradation. The humoral response involves the production of antimicrobial peptides and the phenol-oxidase cascade of melanisation by the fat body that causes pathogen lysis and melanisation, respectively (adapted from Söderhäll, 2010).

Rapid advances have been made in understanding mosquito immunity. The most important barrier that the parasite must traverse is the midgut of the mosquito. Mosquitoes significantly

limit the parasite development in the midgut. The environment in the midgut is hostile to the pathogens, as they need to survive the digestive enzymes in the midgut lumen and successfully penetrate the midgut epithelia (Chege *et al.*, 1996). The immune response begins in the midgut of the mosquito (Osta *et al.*, 2004). The epithelial lining on the gut acts as a physical barrier that activates a systemic immune response upon pathogen invasion (reviewed by Abraham & Jacobs-Lorena, 2004). Following midgut epithelia penetration, the epithelial cells also produce antimicrobial peptides (**AMPs**) and other immune factors that eliminate the pathogen via lysis or melanisation (Figure 1.4) (Dong *et al.*, 2006). The permissiveness of the midgut for parasite penetration into the midgut epithelia and the rate of penetration could be important determinants of vector competence.

The midgut microbiota also plays a role in generating an immune response. The midgut bacteria have been shown to play a protective role against *Plasmodium* infection in *Anopheles* mosquitoes (Pumpuni *et al.*, 1993; Gonzalez-Ceron *et al.*, 2003). Although the exact mechanism(s) are not described, direct microbiota-*Plasmodium* interaction and microbiota induced immune responses have been hypothesised (Dong *et al.*, 2009). As such, an alteration in the midgut microbiota can lead to a difference in immune response, thus it can affect vector competence (reviewed by Weiss & Aksoy, 2011).

Parasite development is energetically costly for the host, and it affects the host's fitness (reviewed in Sheldon & Verhulst, 1996). Therefore, a parasite infection may reduce the host's survival. However, a mosquito vector will tolerate the infection if the cost to eliminate the parasite is greater than the cost to survive. Hence, resource allocation to defence mechanisms is important to generate a strong immune response to eliminate the parasite. Resource allocation may be affected by the degree of parasite virulence, environmental conditions, and reproductive costs (reviewed by Sheldon & Verhulst, 1996; Moret & Schmid-Hempel, 2000). To be a competent vector, the mosquito needs to tolerate the infection, and divert resources to survive and reproduce. To resist an infection is as costly as tolerating it (Hurd *et al.*, 2005), therefore, the ability of the mosquito to allocate resources may affect vector competence.

Despite the array of defence mechanisms, certain mosquitoes are competent vector species for various pathogens (Lowenberger, 1996). Certain parasites have the ability to evade the immune response. One of the ways *P. falciparum* evades the immune system of *Anopheles* mosquitoes is by expressing the Pfs47 protein on its surface, which makes them undetectable to the mosquito immune system (Molina-Cruz *et al.*, 2013). Pfs47 is a protein with various

haplotypes. *Plasmodium falciparum* expresses the haplotypes compatible with its vector, as an incompatible haplotype results in its elimination (Molina-Cruz *et al.*, 2015).

In South Africa several members of the *An. gambiae* complex are present: *An. arabiensis*, *An. merus* and *An. quadriannulatus*. These three species have similar behavioural patterns (exophilic, exophagic and zoophilic) (Sinka *et al.*, 2010). Yet they markedly differ in vector competence for the malaria parasite (Figure 1.2). An increased understanding of the immune responses of these mosquitoes may shed light on their differences in vector competence.

1.3.2. The role of insecticide resistance in vector competence

Insecticides are the most commonly used mechanism for vector control. As such, insecticide resistance is widely prevalent. One of the first mechanisms of resistance to develop was behavioural resistance. Indoor resting mosquitoes started to rest outdoor due to IRS (Elliott, 1972). The constant selection pressure imposed by insecticides led to the development of metabolic resistance and resistance due to changes in the target-site.

Metabolic resistance is the increased detoxification of insecticides due to the overexpression of metabolic enzymes. Metabolic enzymes from the cytochrome P450, general esterase and glutathione S-transferase families degrade and eliminate different forms of insecticides before it reaches its site of action. Target-site resistance occurs due to non-synonymous mutations, usually single nucleotide polymorphisms (**SNPs)** in genes coding for insecticide target proteins. This leads to an altered target-site that prevents the insecticide from binding to its site of action. Therefore, the insecticide has no effect on the insect (reviewed by Hemingway *et al.*, 2004).

Many insecticide-related SNPs have been reported in members of the *An. gambiae* complex. These occur in a variety of proteins. Acetylcholinesterase (**AChE**), which is a target of organophosphates and carbamate insecticides, plays a crucial role in the nervous system. A glycine (**G**) to a serine (**S**) substitution (G119S) in the AChE-coding gene (***Ace-1***) confers carbamate insensitivity (Weill *et al.*, 2003). The neurotransmitter γ-aminobutyric acid (**GABA**) receptor is a ligand-gated chloride channel responsible for chemical signalling in the nervous system and is a target-site for cyclodiene insecticides. A replacement of alanine (**A**) for either serine (**S**) or glycine (**G**) at position 296 in one of the five subunits of the GABA receptor encoded by the *resistance to dieldrin* (***rdl***) gene leads to cyclodiene resistance (Ffrench-Constant *et al.*, 1993). Voltage-gated sodium channels are required for the transmission of action potentials. These channels are the target-sites for the pyrethroid insecticides and

dichlorodiphenyltrichloroethane (**DDT**). The replacement of a leucine (**L**) for either a phenylalanine (**F**) or a serine (**S**) at position 1014 in the *voltage-gated sodium channel* gene leads to knockdown resistance (**kdr**) (Martinez-Torres *et al.*, 1998).

Insecticide resistance often comes with a fitness cost (Rowland, 1991). These costs may have an impact on the fecundity, fertility, longevity, mating success, (Martins *et al.*, 2012; Brito *et al.*, 2013) and possibly vector competence of the mosquitoes. Metabolic resistance plays a role in determining the infectivity of *An. funestus* to the rodent parasite *P. berghei* (Lo & Coetzee, 2013). Metabolic resistance also influences blood-feeding in *An. funestus* that may affect its vector competence (Nouage *et al.*, 2020). Metabolic resistance in *An. gambiae* comes with a lower fitness cost than target-site resistance (Platt *et al.*, 2015), as the primary function of the enzyme is not affected. Pyrethroid resistant *An. gambiae* with the kdr target-site mutation in the voltage-gated sodium channel gene from Tanzania are more competent for *P. falciparum* than susceptible *An. gambiae* (Kabula *et al.*, 2016). The insecticide resistance phenotype has also been reported to enhance West Nile virus vector competence in *Culex quinquefasciatus* (Atyame *et al.*, 2019). Hence there is a possibility that the resistant phenotype could also affect vector competence of the members of the *An. gambiae* complex.

1.3.3. The role of other genetic factors on vector competence

The genetics of both the mosquito-vector and the parasite are essential in determining the vector competence (Collins *et al.*, 1986). The natural refractiveness of a vector may be linked to certain genetic loci (Severson, 1994). With recent advances in genomics, genetic linkage maps have been constructed for vector species to identify genes involved in susceptible and refractory phenotypes (reviewed by Severson *et al.*, 2001). The genetic linkage map of *Ae. aegypti* has allowed researchers to identify multiple genes related to refractoriness (Munstermann & Craig, 1979). This has served as a model organism to study vector biology. Later, the genetic linkage map of *An. gambiae* was constructed and the genome of *An. gambiae* was sequenced (Zheng *et al.*, 1996; Holt *et al.*, 2002). This provided an additional tool for studying the genetic basis of vector competence.

Many genes that support parasite development and the genes that promote parasite elimination have been identified in *An. gambiae.* The leucine-rich repeat immune (***LRIM-1***) gene product plays a role in innate immunity and promotes parasite killing. By contrast, C-type lectin 4 (***CTL-4***) and C-type lectin mannose binding (***CTLMA***) gene products prevent parasite melanisation and protect the parasite from the mosquito's immune system (Osta *et al.*, 2004).

As such, parasite hostile and protector genes in the mosquito genome that may affect its vector competence.

The presence of a particular gene in the mosquito genome may not have an effect on vector competence. This is because there can be variations at gene expression levels. Eukaryotic gene expression is mainly regulated at the level of transcription (reviewed by Phillips, 2008). Environmental and cellular signals control gene transcription by deoxyribonucleic acid (**DNA**) modifications. Inherited modifications to the DNA, without alterations to the DNA sequence are referred to as epigenetics (Russo *et al.*, 1996). The role of epigenetics on vector competence is a new field of research with very little information on mosquito epigenetics. Understanding the epigenetic regulation of gene expression in the members of the *An. gambiae* complex can provide insight of the role of epigenetics on vector competence.

1.4. AIMS AND OBJECTIVES

This study aimed to characterise the molecular underpinnings of the immune response in the three zoophilic members of the *An. gambiae* complex. This was achieved through the completion of the following objectives:

- The characterisation of the global epigenetic signatures upon immune stimulation with Gram-positive and Gram-negative bacteria in *An. arabiensis*, *An. merus* and *An. quadriannalatus*.
- The assessment of the effects of an insecticide resistant phenotype on the global epigenetic signatures of *An. arabiensis* upon immune stimulation.
- The assessment of antimicrobial peptide transcript levels pre and post immune stimulation in *An. arabiensis*, *An. merus* and *An. quadriannalatus*.
- The characterisation of immune priming in insecticide susceptible and resistant strains of *An. arabiensis* by:
    - Comparison of the efficacy of immune priming strategies.
    - Assessment of the effect of immune priming on fertility and fecundity.
    - Assessment of whether immune priming effects are transgenerational.

CHAPTER TWO: Common methods

2.1. STUDY AREA AND MOSQUITO STRAINS

All the experimental procedures were performed in the Vector Control Reference Laboratory located at the National Institute for Communicable Diseases of the National Health Laboratory Service, Sandringham, Johannesburg, South Africa. Four laboratory strains as well as the progeny of wild-caught mosquitoes from the *An. gambiae* complex were used in this study:

1. **SENN**: an insecticide susceptible *An. arabiensis* strain, colonised in 1980 from Sudan (Sennar).

2. **SENN DDT:** an insecticide resistant *An. arabiensis*. The strain was established in 2004 by DDT selection of the SENN strain. The strain has been under continuous DDT selection, which continues to the present day. The strain displays resistance to DDT, λ-cyhalothrin, deltamethrin, permethrin and malathion (Oliver & Brooke, 2017). Resistance is mediated by increased expression of cytochrome P450, Glutathione S-transferase and general esterase detoxification enzymes. The strain is also fixed for the L1014F kdr target-site mutation in the *para-sodium channel* gene (Oliver & Brooke, 2013).

3. **F1**: the first-generation progeny of wild-caught *An. arabiensis.* Wild mosquitoes were collected from Mamfene, KwaZulu-Natal, South Africa. The progeny of mothers identified as *An. arabiensis* were pooled to generate the specimens. The wild specimens were collected between October 2019 and February 2020. This strain displays low level permethrin and bendiocarb resistance.

4. **MAFUS:** an insecticide tolerant *An. merus* strain, colonized in 2012 from Mafayeni, Mpumalanga, South Africa. This strain is reared in saltwater at a concentration of 16g of sodium chloride/litre which is approximately 50% seawater.

5. **SANGWE**: an insecticide susceptible *An. quadriannulatus* strain, collected in 1998 from Sangwe, Zimbabwe.

Anopheles arabiensis, *An. merus* and *An. quadriannulatus* are representative of major, minor, and non-vector species, respectively, from the *An. gambiae* complex (Coetzee *et al.*, 2000; Sinka *et al.*, 2012).

2.1.1. Mosquito rearing protocol

The four laboratory strains were reared in the Botha de Meillon insectary. The temperature of the insectary was maintained at 25°C (±2°C) with a relative humidity of 80% (±5%), a 12:12 hour photoperiod and a 30-minute dusking cycle. The larvae were fed with powdered dog biscuits and brewer's yeast in a 3:1 ratio. The adult mosquitoes were sustained with *ad libitum* access to 10% sucrose (Hunt *et al.*, 2005).

2.1.2. Generation of the F1 progeny of wild-caught *An. arabiensis*

Wild mosquitoes were collected from clay pot traps (Burke *et al.*, 2017) cleared daily as part of the surveillance programme in Mamfene, KwaZulu-Natal (S 27°23′50.5″; E 032° 12′20.1″). Collections took place from November 2019 to March 2020. The females were individually tubed and allowed to lay eggs without any additional feeding to create isofemale lines. Once a female laid her eggs, she was collected for identification using the conventional PCR method of Scott *et al.*, 1993. Once the female was identified as *An. arabiensis*, her F1 progeny were pooled and reared according to Hunt *et al.*, 2005. The F1 progeny were used to represent wild *An. arabiensis*. This was to ensure the age and physiological condition of the mosquitoes, which could not be fully characterised with wild-caught mosquitoes. These mosquitoes were used in the epigenetic experiments (Chapter 3) to compare the effect of immune stimulation on the epigenetic landscape to that of the laboratory *An. arabiensis*.

2.2. IMMUNE STIMULATION WITH BLOOD-BORNE BACTERIA

The immune system of the mosquito is a key factor determining vector competence (reviewed by Barillas-Muray *et al.*, 2000 & Beerntsen *et al.*, 2000). To examine the immune responses of the three zoophilic members of the *An. gambiae* complex, immune stimulation was performed with non-commensal bacterial species. The mosquito strains described in **Section 2.1** were exposed to Gram-positive or Gram-negative bacteria for immune stimulation. The effect of immune stimulation on the epigenetic landscape (Chapter 3) and AMP expression (Chapter 4) was assessed. The effect of a pre-existing insecticide resistance phenotype was considered by the inclusion of two genetically related *An. arabiensis* strains (SENN & SENN DDT) differing in insecticide resistance capacity.

2.2.1. Bacterial culture and growth

Streptococcus pyogenes (ATCC 19615) was used to represent Gram-positive bacteria, while *Escherichia coli* (ATCC 25922) was used to represent Gram-negative bacteria. These strains were chosen as they were not detected in the SENN and SENN-DDT strains (Singh, 2019a)

and subsequently not detected in neither the SANGWE nor MAFUS strains (Singh, 2019b). The bacterial stock was grown to mid-log phase in Lysogeny Broth (**LB –** medium: Davies diagnostics: 610084) overnight at 37°C (Labcon FSIM 16 incubator) (Barnard *et al.*, 2019). The bacterial stock was rotated at 150 rpm to promote aerobic bacterial growth.

2.2.2. Ingestion of an infected blood-meal

Three-day-old female adult mosquitoes from each strain described in **Section 2.1.** were divided into four equal groups (n=30 mosquitoes), with each group receiving a different meal (Figure 2.1).

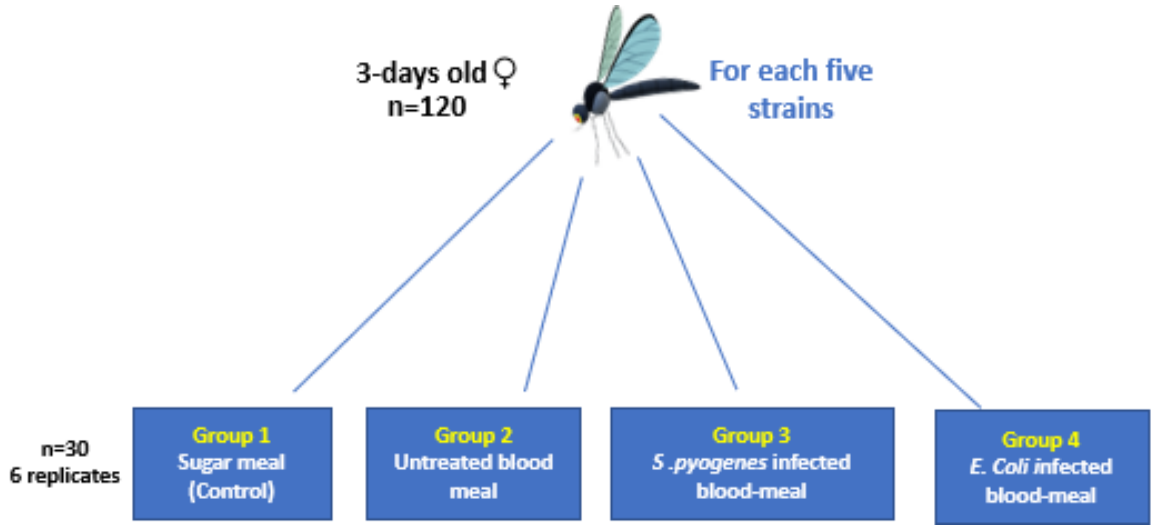


Figure 2. 1: Immune stimulation by blood-borne bacterial supplementation. A total of 120 female mosquitoes from each of the five strains (SENN, SENN DDT, F1 *An. arabiensis*, MAFUS and SANGWE) were divided into four equal groups (30 mosquitoes/group). Each group was provided with a different meal. For nucleic acid extractions, samples were divided into six replicates. Each replicate consisted of five mosquitoes. *Streptococcus pyogenes* represented Gram-positive and *E. coli* represented Gram-negative bacterial challenge.

Group one received *ad libitum* access to 10% sucrose only and constituted the untreated control. Group two received a single untreated blood-meal. This was to control for any effects due to untreated blood treatment alone. Group three received *S. pyogenes* infected blood. Group four received *E. coli* infected blood. Bacteria were supplemented in blood to a final concentration of 0.3% in 15 ml (Barnard *et al.*, 2019). The blood source was defibrinated cow blood that had been filtered through a 0.2µM filter and provided via an artificial membrane feeder (Hemotek™) for four hours. The females were allowed 72 hours to digest the blood and for necessary immune responses to take place before being killed. Once cold-killed, the samples were stored at -70°C until usage.

2.3. EXTRACTION OF RIBONUCLEIC ACID (RNA)

RNA was used in two objectives. In the epigenetic analysis, RNA was used to assess the effect of immune stimulation on messenger RNA (**mRNA**) methylation (Chapter 3). In the analysis of the effect of immune stimulation on AMPs expression (Chapter 4), RNA was used to synthesise the complementary DNA (**cDNA**) template. The cDNA was subsequently used for quantitative real-time PCR (**qRT–PCR**). The RNA was extracted using the Quick-RNA™ MiniPrep kit (Zymo Research: R1054) from each group of each strain according to the manufacturer's instructions (Figure 2.1). The kit is based on a spin-column procedure.

2.3.1. Sample homogenisation

The samples were homogenised by mechanical disruption with steel beads. Prior to homogenisation, the beads were rinsed with a phosphate-free, anionic detergent, thereafter with diethyl pyrocarbonate (**DEPC**) treated water (DEPC: Sigma Aldrich: 159220). The beads were subsequently soaked in RNase AWAY™ (Thermo Scientific: 7003PK) for 15 minutes. The RNase AWAY™ was rinsed off with DEPC-treated water and 70% ethanol, thereafter, autoclaved for 20 minutes. Mosquitoes were homogenised in the kit-provided RNA lysis buffer on a Vortex-Genie® 2 (Scientific Industries) with a bead tube-holder adapter (Machery-Nagel: 740469) at full speed for 20 minutes, according to the manufacturer's instructions.

2.3.2. RNA purification

The mosquito homogenate was centrifuged at 11 000 ×g for one minute (4°C) to pellet the cell debris and other cellular components (HERMLE 2400K: Lasec®). To remove large proteins and genomic DNA (**gDNA**), the supernatant was transferred into a Spin-Away™ Filter (in a collection tube) that binds DNA, and centrifuged (Figure 2.2).

For each sample, absolute ethanol was added to the flow-through to precipitate the RNA, which was subsequently transferred into a Zymo-Spin™ IIICG column to allow RNA binding and, centrifuged for 30 seconds. To remove any traces of DNA, the Zymo-Spin™ IIICG column containing RNA was treated with DNase I, incubated at room temperature for 15 minutes then centrifuged for 30 seconds. The Zymo-Spin™ IIICG columns were subsequently washed once with RNA prep buffer (centrifuged for 30 seconds) and twice with RNA wash buffer (centrifuged for 30 seconds and two minutes, respectively). Thereafter the column was transferred into an RNase-free tube. The RNA on the Zymo-Spin™ IIICG Column was eluted with RNase-free water and stored at -70°C until usage (Figure 2.2). The entire extraction procedure was performed on ice, unless otherwise stated. All centrifugation steps were

performed at 11 000 ×g at 4℃ in a HERMLE 2400K centrifuge (Lasec®) for one minute, unless otherwise stated.

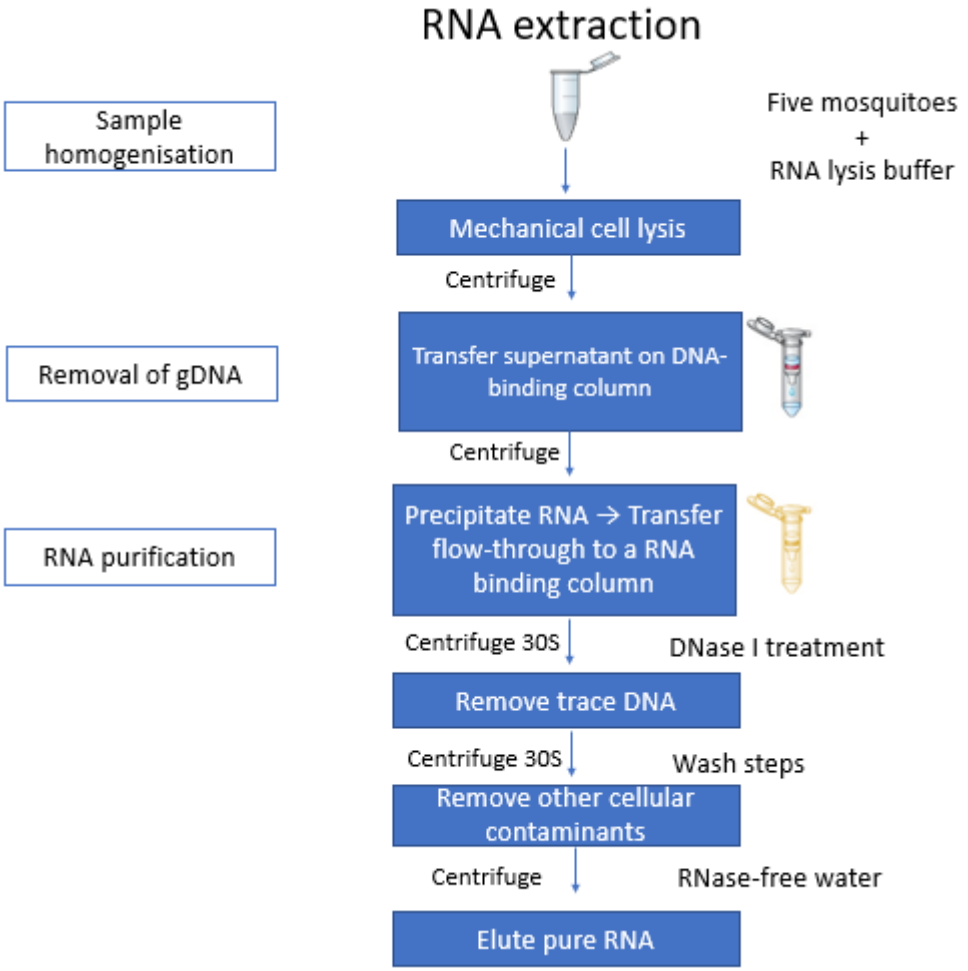


Figure 2. 2: Summary of the RNA extraction protocol. All centrifugation steps were performed at 11 000 ×g for one minute unless stated. Image obtained from Zymo Research: R1054.

2.3.3. Assessment of the RNA quality and integrity

The extracted RNA was quantified and assessed for purity using a Nanodrop™ 2000C spectrophotometer (Thermo Scientific™: Inqaba Biotech™). Pure RNA samples with A260/A280 >1.8, A260/A230 >1.8 and a peak at 260 nm were further assessed for quality and integrity by TapeStation analysis with RNA ScreenTape® (Agilent Technologies: 5067- 5576). The manufacturer's instructions are summarised in Figure 2.3. RNA ScreenTape analysis uses electrophoresis separation of RNA and provides precise information on RNA quantity and integrity. RNA Integrity Number (**RIN^{e}**) provides the overall quality of the RNA sample.

RNA integrity was also assessed using a native 1% 0.5×Tris-Borate-EDTA (**TBE; EDTA:** Ethylenediaminetetraacetic acid) agarose gel electrophoresis at 70V/90mA for 30 minutes. The

preparation of the agarose gel is described in Appendix 3. For sample preparation, 3µl of the extracted RNA was added into 2µl of DEPC-treated water (Invitrogen™: AM9915G) and 5µl of RNA loading dye (Thermo Scientific™: SM1821). The samples were sized using a RiboRuler™ High Range RNA Ladder (Thermo Scientific™: SM1821). Both the samples and the ladder were heated at 70°C for ten minutes in a thermocycler (T100™ Thermal Cycler, Bio-Rad™) to denature the RNA molecules and separate the 26S and 18S subunits. The electrophoretogram was viewed on a ChemiDoc™ XRS+ Molecular Imager® with Image Lab™ software from Bio-Rad™.

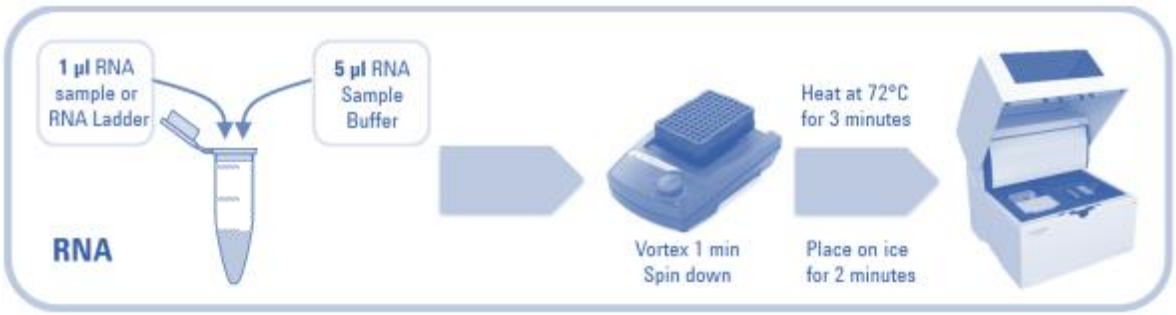


Figure 2. 3: Summary of the RNA TapeStation analysis procedure. A volume of 1µl of the RNA sample was mixed with 5µl of buffer, vortexed and incubated at 72°C for 3 minutes to unfold any secondary RNA structures. Subsequently, the samples were placed on ice for 2 minutes to maintain their unfolded structure and loaded in a TapeStation 4200 instrument (Agilent Technologies).

CHAPTER THREE: Characterisation of the global epigenetic landscape of zoophilic members of the *Anopheles gambiae* complex in response to bacterial challenge

3.1. INTRODUCTION

The word "epigenetics" was derived from the word "epigenesis", which describes the interaction between the genes and their products (Waddington, 1942). Waddington proposed the theory of the "epigenetic landscape", suggesting that there are events beyond the genes that affect the phenotype (Waddington, 1957). Epigenetics is defined as "the study of changes in gene function that are mitotically and/or meiotically heritable and that do not entail a change in DNA sequence" (Riggs *et al.*, 1996). The different cell types of a multicellular organism contain the same genetic material, yet the cells appear different and perform different functions. This is due to the differential expression of the genetic material (Alberts *et al.*, 2002). Epigenetic modifications alter the physical structure of DNA, which provides a cell with a blueprint to "read" only specific genes. Epigenetics is therefore a key determinant of gene expression in a cell, playing a crucial role in driving the specificity of the cell (reviewed by Goldberg *et al.*, 2007).

The three major epigenetic mechanisms include DNA methylation, histone modifications and non-coding RNA (**ncRNA**) (reviewed by Goldberg *et al.*, 2007). Recently, the role of N6-methyladenosine (**m6A**) RNA methylation has also been discovered in regulating gene expression (Meyer *et al.*, 2015). These epigenetic mechanisms regulate gene expression and silencing. Extrinsic factors such as age, diet and environmental stress can induce modifications in the epigenetic landscape of an organism (reviewed by Alegría-Torres *et al.*, 2011).

3.1.1. DNA methylation and demethylation

DNA methylation is the most common DNA modification. This involves the addition of a methyl group onto specific areas of a DNA molecule. DNA methyltransferase (**DNMT**) enzymes catalyse the DNA methylation reaction by adding a methyl group on the 5th carbon of cytosine nucleotides. This results in the formation of 5-methylcytosine (**5mC**) (reviewed by Goll & Bestor, 2005). Therefore, 5mC can be used as a marker to assess DNA methylation. The dietary micronutrient, S-adenosine methionine (**SAM**) is often considered as a universal donor of a methyl group for DNA methylation (reviewed by Zeisel, 2017). The DNMTs are divided into two classes based on their function: the *de novo* and maintenance DNMTs. In

mammals, the DNMT-3 family is responsible for the *de novo* methylation of DNA, establishing a new DNA methylation pattern in the genome during development (Okano *et al.*, 1999). The DNMT-1 family is responsible for the maintenance of previously established DNA methylation patterns during cell division (reviewed by Bestor, 2000).

It has long been known that 5mC methylation exists in the fruit fly *Drosophila melanogaster* (Achwal *et al.*, 1983). However, the presence of *de novo* and maintenance DNMTs have been recently discovered in insects, and these differ from those of mammals (Wang *et al.*, 2006). Almost all DNA methylation occurs on the cytosine nucleotides of the cytosine-guanine (**CpG**) islands in mammals. These are the regions of the genome that have a high density of cytosine and guanine dinucleotides. Approximately 70% of the CpG islands are located in the gene promoters (Saxonov *et al.*, 2006). DNA methylation on a promoter sequence of a gene recruits gene repressor proteins and promotes the formation of heterochromatin (tightly packed DNA). This results in a reduced interaction between the transcriptional factors and DNA, as such, it is associated with gene silencing (reviewed by Goll & Bestor, 2005). Another major function of 5mC DNA methylation in mammals is the X chromosome inactivation in females and genomic imprinting (reviewed by Bird, 2002).

Unlike mammals, DNA methylation in insects is largely present in gene bodies (coding DNA) and rare in intergenic regions such as promoters (Simmen *et al.*, 1999). Methylated gene bodies affect the transcriptional splicing events and can lead to alternative splicing of the mRNA (Foret *et al.*, 2009; Lyko *et al.*, 2010). They may also play a role in maintaining transcript integrity and regulation of mRNA synthesis (Maunakea *et al.*, 2010). Several key components involved in DNA methylation that are present in mammals are yet to be discovered in insects. Whole genome methylation studies in insects like the *Apis mellifera* (honeybee) reveals that DNA methylation levels are low (0.7%) in comparison to mammals (70%) (Strichman-Almashanu *et al.*, 2002; Lyko *et al.*, 2010).

DNA demethylation is the removal of a methyl group, which is catalysed by the ten-eleven translocation (**TET**) dioxygenase enzyme family. In mammals, TET-1 enzymes oxidise the 5mC nucleotides to 5-hydroxymethylcytosine (**5hmC**) (Tahiliani *et al.*, 2009; Williams *et al.*, 2011). The 5hmC molecule serves as an intermediate in the DNA demethylation reaction, which is further modified via deamination and decarboxylation reactions. Therefore, 5hmC can be used as a marker for assessing DNA demethylation. The DNA repair mechanism is initiated,

which replaces the methylated cytosine with an unmethylated cytosine by base excision repair (Tahiliani *et al.*, 2009; Pidugu *et al.*, 2016).

In insects, TET transcripts have been detected in honeybees and ants (Wojciechowski *et al.*, 2014; reviewed by Li-Byarlay, 2016). Recently, an orthologue of TET has been detected in the aphid genome, which upon wheat feeding showed an increase in expression (du Preez *et al.*, 2020). The presence of 5hmC has been discovered in multiple other insect species (reviewed by du Preez *et al.*, 2020). Although the relationship between the TET homologues and 5hmC is unknown in insects, recent literature suggests that the DNA demethylation mechanism could be similar between insects and mammals.

The role of DNA demethylation generally seems to be opposite to that of DNA methylation. As such, DNA demethylation generally corresponds to gene expression, as the active removal of a methyl group from a gene promoter allows access to the transcriptional machinery. This promotes the formation of euchromatin (lightly packed DNA), which activates gene expression in mammals (reviewed by Kangaspeska *et al.*, 2008). A global DNA demethylation during early embryonic development in mammals allows reprogramming of the genome for complete development (Mayer *et al.*, 2000). The increased expression of TET in aphids post feeding (du Preez *et al.*, 2020) suggests an increase in DNA demethylation. Therefore, these enzymes may play a role in gene transcription. Whether or not DNA demethylation has similar functions in insects is unknown.

3.1.2. Histone modifications

Histones are positively charged proteins that wrap the negatively charged DNA forming the basic unit of a chromatin structure referred to as the nucleosome (reviewed in Delange & Smith, 1971). This allows the packaging of the eukaryotic genome in a cell, in the form of chromosomes. The core of a nucleosome particle is composed of two copies of histone 2-A (**H2A**), histone 2-B (**H2B**), histone 3 (**H3**) and histone 4 (**H4**) (Luger *et al.*, 1997). The nucleosome cores are connected with a linker DNA associated with histone 1 (**H1**) – a linker histone. The N-terminal domain of the histone core is rich in positively charged amino acids, which extends outside the nucleosome through the DNA coils. These are referred to as the histone tails (Luger *et al.*, 1997). The histone tails are subject to post-translational modifications. As such, the core histones play a structural role in nucleosome formation and DNA packaging, while the core histone tails play a role in regulating the DNA-histone

interaction. The type of histone modification defines the state of the chromatin (Zheng & Hayes, 2003).

The interaction between the DNA and histones affects gene expression by either forming heterochromatin or euchromatin. Heterochromatin is the transcriptionally inactive form as the tight interaction of the DNA with histones does not allow access to the transcriptional factors. As such, it promotes gene silencing. Euchromatin is the transcriptionally active form as the weak interaction of the DNA with histones allows access to the transcriptional factors, thereby promoting gene expression (Clark & Felsenfeld, 1971; reviewed by Babu & Verma, 1987). The strength of DNA and histone interaction is not only dependent on the DNA modification, but also on the modification of histone proteins.

Histone modification is an epigenetic mechanism that has been initially studied in *D. melanogaster* (Hannah, 1951). Histones are subject to multiple post-translational modifications on the N-terminal tail domain, including acetylation, methylation, sumoylation, glycosylation phosphorylation, crotonylation, butyrylation, and citrullination (reviewed by Herceg & Murr, 2017). These covalent modifications either alter the electrostatic interactions between the DNA and histones or create a steric hindrance for the transcriptional factors. The most common modification is the acetylation and, methylation of the lysine (**K**) residue of H3 and H4 (Yan & Boyd, 2006). Histone modifications and other processes that involve non-histone proteins associated with chromatin structure leads to chromatin remodelling (reviewed by Suganuma & Workman, 2011).

Histone acetylation often involves the reversible addition of an acetyl group to a lysine (**K**) residue of the histone tail. The histone acetyl transferase (**HAT**) enzyme catalyses the transfer of the acetyl group by utilising acetyl coenzyme A (**CoA**) as a donor. Lysine is a positively charged amino acid under physiological conditions, which allows it to bind tightly to the negatively charged DNA. Acetylation of the lysine residue neutralises its positive charge, weakening the interaction between DNA and histones. This promotes the formation of euchromatin. Theoretically, the loosening of chromatin allows access to the transcriptional factors, thus facilitating gene expression (reviewed by Bannister & Kouzarides, 2011). The HAT enzymes are divided into two classes: type A and type B. The type A HAT enzymes acetylate the histone residue N-terminals in the chromatin. The type B HAT enzymes acetylate the free histones in the cytoplasm (reviewed by Parthun, 2007). The level of histone acetylation can be measured by assessing the HAT activity.

In contrast to histone acetylation, histone deacetylation is the removal of the acetyl group from lysine residues of the histone tail. The histone deacetylase (**HDAC**) enzymes catalyse the removal of acetyl groups that restores the positive charge on the lysine residue, thereby restoring the heterochromatin and facilitating gene silencing (reviewed by Bannister & Kouzarides, 2011).

Histone methylation is a complex process. It involves the transfer of 1–3 methyl groups onto a lysine (**K**) residue or 1–2 methyl groups onto an arginine (**R**) residue of the histone tail. Histone methyltransferase (**HMT**) enzymes catalyse the transfer of a methyl group, mainly from SAM, as in DNA methylation (Rea *et al.*, 2000; reviewed by Bedford & Clarke, 2009). Histone methylation does not alter the electrostatic interaction between DNA and histones; instead, it creates steric hindrance for the transcriptional factors and regulatory proteins. Histone methylation promotes gene silencing without altering the chromatin structure (reviewed by Bannister & Kouzarides, 2011). Histone methylation is reversed by a set of enzymes called histone demethylases (**HDMs**). The removal of methyl groups eliminates the steric hindrance and promotes gene expression (Cuthbert *et al.*, 2004).

The combination of a particular set of histone modifications is referred to as the "histone code" for the cellular machinery, with different combinations yielding a distinct functional consequence. The code dictates the same biological function in eukaryotic cells, although there are exceptions. For example, acetylation of H3K9 and H3K27 and trimethylation of H3K4 provides the code for transcriptional activation. The absence of these codes leads to transcriptional repression (Santos-Rosa *et al.*, 2000). By contrast, methylation of H3K9 and H3K27 can also result in transcriptional repression (Lachner *et al.*, 2001; Litt *et al.*, 2001). However, H3K9 methylation is not always associated with gene repression, this modification has also been shown to code for transcriptional activation (Wiencke *et al.*, 2008). The role of histone modification in gene regulation is therefore complex, with the histone code interpreted as being dependent on several cellular factors (Peterson & Laniel, 2004).

Genetic studies in *D. melanogaster* revealed that genes that encode for important elements of heterochromatin are conserved between winged insects and humans (reviewed by Weiler & Wakimoto, 1995). Many studies revealed that the role of histone modification in insects is similar to that of mammals. For example, the trimethylation of H3K9 codes for transcriptional inactivation on the X chromosome of a shield bug *Carpocoris fuscispinus* during meiosis (Viera *et al.*, 2017). The H3 acetylation has been shown to regulate gene expression during

oogenesis and metamorphosis in *D. melanogaster* (Carre *et al.*, 2005). A reduction in histone methylation in the mosquito *An. gambiae* has been shown to negatively affect its growth and development (Sharma *et al.*, 2015). This indicates that histone methylation may affect the lifespan of the mosquitoes.

3.1.3. RNA modifications

Modifications on the RNA molecules were discovered decades ago, however, their functions were not known until recently. Methylation on the eukaryotic mRNA is the most abundant RNA modification (Desrosiers *et al.*, 1974). Of the many different types of RNA modifications, the mRNA methylation on the 6th carbon of the adenosine nucleotide (**m6A**) is of the most importance. The methyltransferase-like (**METL**) enzyme catalyses the transfer of a methyl group from a donor (often SAM) to the adenosine nucleotide on the mRNA. This reaction results in the formation of m6A (Liu *et al.*, 2014). Therefore, m6A can be used as a marker to assess mRNA methylation. The addition of the methyl group is reversible. The fat mass and obesity-associated protein (**FTO**) and the alkylation repair homolog protein (**ALKBH-5**) are responsible for removing the methyl groups (Jia *et al.*, 2011; Zheng *et al.*, 2013).

The m6A mRNA methylation promotes cap-dependent protein translation. The m6A moiety on the 5′ untranslated region (**5′UTR**) recruits eukaryotic initiation factors thus promoting mRNA translation. The absence of m6A methylation leads to reduced translation of the mRNA (Meyer *et al.*, 2015). In mammalian embryonic stem cells, m6A plays a role in determining cell fate (Batista *et al.*, 2014; Aguilo *et al.*, 2015) and therefore plays a role in gene regulation. Although the knowledge of m6A mRNA methylation is limited to mammals, other types of RNA methylation have been reported in certain insects. Low levels of 5mC RNA methylation have been reported to play a role in immune priming in *Tenebrio molitor* (mealworm beetle) (Castro-Vargas *et al.*, 2017).

The role of ncRNAs in gene regulation was discovered recently. Various ncRNAs have been identified to play a role in the epigenetic regulation of gene expression. The small interfering RNA (**siRNA**) alters DNA methylation and histone modifications to cause gene silencing (Morris *et al.*, 2004; Kawasaki & Taira, 2004). The micro-RNA (**miRNA**) is complementary to the target mRNA that promotes the degradation of the transcript thus preventing the mRNA from being translated (Bartel, 2009). Like siRNA, miRNA can also regulate histone modifications (Yuan *et al.*, 2011). Studies in *D. melanogaster* reveal that multiple ncRNAs play a role in the epigenetic regulation of gene expression (reviewed by Li *et al.*, 2019).

3.1.4. The effects of nutrition and environment on insect epigenetics

The external environment can affect the levels of different epigenetic markers and change the epigenetic landscape of an organism, which eventually influences the way an organism develops and functions. In this way, the epigenome links the environment to the organism and its phenotype. Some of these environmental factors include the diet, chemical stress, temperature, and pathogens. For example, the dietary intake of methionine, folate, choline, betaine, and vitamin B affects the production of SAM in the methionine cycle. SAM is the key donor of a methyl group in most epigenetic modifications (reviewed by McKay & Mathers, 2011). Therefore, a deficiency in such nutrients leads to a decrease in the SAM pool, which in turn negatively affects the nucleic acid and histone methylation processes. This can subsequently affect the gene expression and phenotype of a cell/organism. The effect of diet on the phenotype has been demonstrated in honeybees. Feeding the larvae with royal jelly, which contains phenylbutyrate, results in the development of a queen bee. By contrast, feeding with pollen results in worker bees. Phenylbutyrate affects the expression of genes, as it inhibits the activity of HDAC (Chittka & Chittka, 2010).

Larval metal pollutant exposure resulted in epigenetic alteration in the mosquito *An. arabiensis* (Jeanrenaud, 2018). Although the precise mechanism is not known, chemical stress affects the mosquito epigenetics. Alteration in the global DNA methylation in *Aedes albopictus* in response to a variety of epigenetic modulators has been described (Oppold *et al.*, 2015). Large scale deviations from the optimal temperature alters protein activity, including the enzymes that catalyse the epigenetic mechanisms. In *D. melanogaster*, larval exposure to heat stress results in the loss of heterochromatin (Seong *et al.*, 2011).

3.1.5. The impact of an infection on insect epigenetics and immune system

In addition to chemicals and temperature, the role of pathogens on the host's epigenetics is a growing field of research. When a pathogen enters the insect host, the host's immune system is rapidly activated to eliminate the pathogen. As such, the infection results in the activation of gene expression, particularly of those involved in immunity and stress. Eukaryotic gene expression is regulated at many levels. The epigenetic mechanisms, among others, regulate gene expression at the transcriptional level (Jaenisch & Bird, 2003). The toxins released by the pathogen can also affect the epigenetics of the host and hijack the host's innate immune responses (Bierne *et al.*, 2012). Epigenetic mechanisms regulate the immunity against Gram-negative bacteria in the wax moth *Galleria mellonella* (Heitmueller *et al.*, 2017).

There is growing evidence that epigenetic mechanisms regulate immune responses and alters the expression of immune-related genes in insects. Larval immune stimulation with *Serratia entomophila* bacterial challenge resulted in transcriptional reprogramming and increased expression of HDACs in the midgut of *G. mellonella* (Mukherjee *et al.,* 2012). DNA methylation in insects regulates antifungal immunity. An infection with the parasitic fungi *Metarhizium robertsii* altered the DNA methylation patterns in *G. mellonella*. This was observed by the fluctuating expression of DNMTs during the infection (reviewed by Vilcinskas, 2016). A recent study demonstrated that the percentage of 5mC methylation decreases in *T. molitor* post *Micrococcus lysodeikticus* bacterium infection (Castro-Vargas *et al.,* 2017).

The contribution of miRNA in regulating immune response has been demonstrated in *Ae. albopictus* mosquitoes. In response to the West Nile virus, *Ae. albopictus* inhibits the expression of Aae-miR-2940-5p miRNA. This miRNA promotes the expression of a metalloprotease required for viral replication (Slonchak *et al.,* 2014). Therefore, reduced expression of Aae-miR-2940-5p miRNA can lead to reduced viral replication. Similar observations were made in the dengue virus vector *A. aegypti* (Campbell *et al.,* 2014). The miRNA also affects the anti-Plasmodial response in *An. gambiae*. Inhibition of ga-miR-305 miRNA synthesis in *An. gambiae* resulted in increased resistance to the *Plasmodium* infection (Dennison *et al.,* 2015). Although advances are being made in the understanding of insect epigenetics, the role of epigenetics in the invertebrate immune response is poorly understood. This is particularly true for members of the *An. gambiae* complex.

3.1.6. The interplay between epigenetics and vector competence

Vector competence is the intrinsic capacity of a vector to transmit a pathogen. This capacity has a large genetic component (reviewed by Beerntsen *et al.,* 2000). As the immune system of the vector species plays a key role in determining pathogen survival, it is a crucial element of vector competence (Dong *et al.,* 2006). The functioning of the immune system is in turn affected by the epigenetic regulation of immune-related genes (Heitmueller *et al.,* 2017). Therefore, understanding the role of epigenetics on vector competence can provide a new insight on vector-borne disease and vector control. The impact of immune stimulation on insect epigenetics as well as the importance of epigenetics on the regulation of insect immune responses is poorly understood.

The three zoophilic members of the *An. gambiae* complex; *An. arabiensis*, *An. merus* and *An. quadriannalatus* vastly differ in their ability to transmit malaria. This is despite having similar behaviour and genetics (reviewed by Sinka *et al.*, 2012). Studies performed on ticks suggest that epigenetics may affect the vectorial capacity of ticks, as the tick-borne pathogens alter the epigenetic landscape of ticks (De *et al.*, 2021). There is little information available on the epigenetic regulation of immune responses and vector competence in *An. gambia*e and no information on the members of the *An. gambiae* complex. Assessment of the epigenetic landscape of the members of the *An. gambiae* complex can provide a better understanding of their differences in vector competence.

3.1.7. The effect of insecticide resistance on epigenetics

Insecticide resistance is 'a heritable change in the sensitivity of a pest population. It is reflected in the repeated failure of a product to achieve the expected level of control when used according to the label recommendation for that pest species' (Insecticide Resistance Action Committee, 2021). The constant selection pressure of insecticides not only resulted in behavioural resistance but also resulted in changes in DNA sequence that creates the resistance phenotype (Ffrench-Constant *et al.*, 1993; Martinez-Torres *et al.*, 1998; Weill *et al.*, 2003). Recent literature suggests that epigenetic alterations decrease insecticide sensitivity in *Ae. albopictus* (Oppold *et al.*, 2015).

Insecticide resistance can alter vector competence, which is evident in the pyrethroid resistant *An. gambiae*. *Anopheles gambiae* from Tanzania with the kdr target-site mutation had a higher percentage of *P. falciparum* sporozoites than the susceptible *An. gambiae* (Kabula, *et al.*, 2014; Kabula *et al.*, 2016). Understanding the interplay of epigenetics and the insecticide resistance phenotype would advance the understanding of the role of insecticide resistance on vector competence.

The aim of this chapter is to characterise the global epigenetic landscape of *An. arabiensis*, *An. merus* and *An. quadriannalatus* in response to Gram-positive and Gram-negative bacterial challenges, through the following objectives.

- Assessment of the 5mC and 5hmC DNA methylation markers using an Enzyme-Linked Immunosorbent Assay (**ELISA**).
- Assessment of the m6A mRNA methylation marker using ELISA.
- Assessment of histone acetylation by evaluating the HAT activity calorimetrically.

- Assessment of the effect of insecticide resistance phenotype on the epigenetic markers in *An. arabiensis*.
- Compare the epigenetic markers of the laboratory-reared *An. arabiensis* and the F1 progeny of wild-caught *An. arabiensis*.

3.2. MATERIALS AND METHODS

The epigenetic landscape of SENN, SENN DDT, MAFUS, SANGWE and the F1 progeny of wild-caught *An. arabiensis* was characterised. This was done by quantifying the global DNA methylation (5mC and 5hmC) and m6A mRNA methylation levels using ELISA, and the HAT activity calorimetrically. DNA and RNA were extracted from the four groups (control, untreated blood, Gram-positive and Gram-negative bacterial challenged) of each of the five strains (SENN, SENN DDT, MAFUS, SANGWE and F1). The nuclear proteins were extracted from the four groups of each of four laboratory strains. The bacterial stimulation set-up was described in **Section 2.2.2** and illustrated in Figure 2.1. Bacterial supplementation was generally provided by an infected blood-meal. The exception was for the nuclear protein extraction, where the bacteria were provided in a sugar-meal, rather than a blood-meal. This was to avoid any potential interaction between blood proteins and HAT activity. For all analysis, biological replication was established by using strains that originated from different egg batches (cohorts) and replicated using two separate kits (triplicate replicates were limited by cost). Within each biological replicate six technical replicates were conducted, as described in the Appendix 5.

3.2.1. DNA extraction and assessment of the global 5mC and 5hmC DNA methylation

DNA was extracted using the NucleoSpin® DNA insect kit (Macherey-Nagel: PT40101) according to the manufacturer's instructions (Figure 3.1). The kit uses a silica membrane spin-column technology.

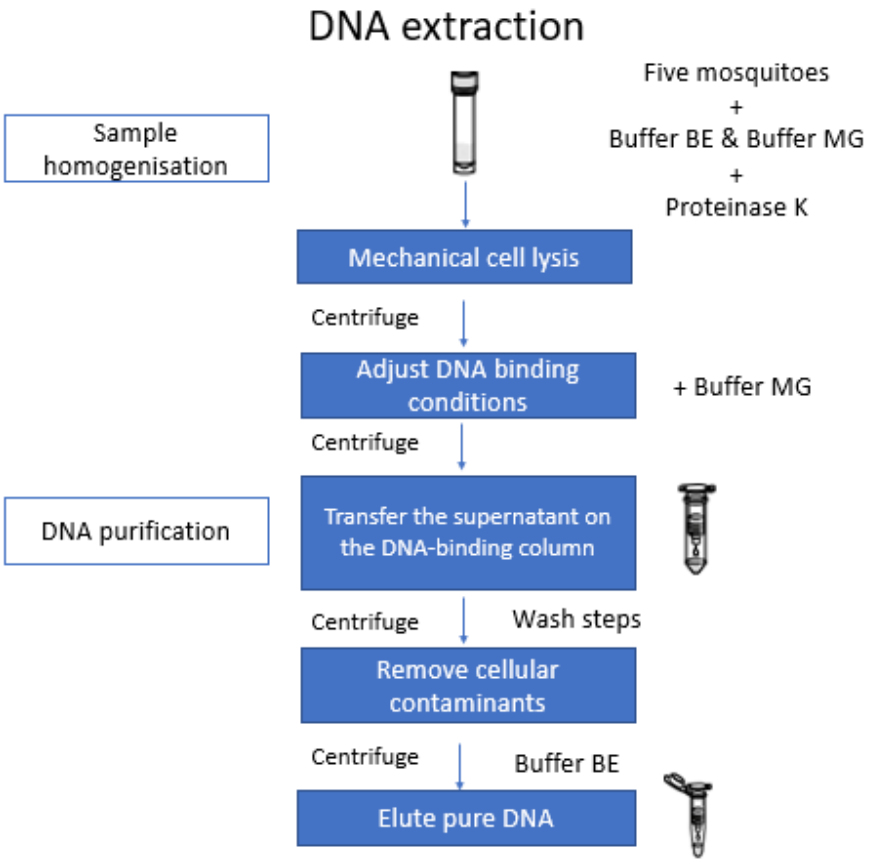


Figure 3. 1: Summary of the DNA extraction protocol. All centrifugation steps were performed at 11 000 ×g (4℃) for 30 seconds. The entire procedure was performed on ice. Image obtained from Macherey-Nagel: PT40101.

Each sample was mechanically homogenised in the kit-provided NucleoSpin® bead tubes with proteinase K, lysis buffer MG and elution buffer BE on a Vortex-Genie®2 (Scientific Industries) with a bead tube-holder adapter (Machery-Nagel: 740469). The chaotropic salts in the lysis buffer removed the hydrate shell around the DNA. Proteinase K digested the contaminating proteins as well as the nucleases that could degrade DNA. To allow efficient DNA binding onto the silica-membrane, high salt concentration buffer MG was added to the homogenate. The tubes were subsequently centrifuged to pellet the cell debris from cellular components. The supernatant was transferred into the NucleoSpin® DNA Insect column to allow DNA binding. Thereafter, the columns were centrifuged to remove contaminants. The columns were thereafter washed with buffer BW and buffer B5 respectively and centrifuged to eliminate any remaining contaminants. The columns were subsequently transferred into the nuclease-free tubes and incubated with a low salt concentration buffer BE for one minute at room temperature then centrifuged. The entire extraction procedure was performed on ice and all the centrifugation steps were performed at 11 000 ×g (4°C) in a HERMLE 2400K centrifuge (Lasec®) for 30 seconds, unless stated otherwise.

The extracted DNA was quantified and assessed for purity using a Nanodrop™ 2000C spectrophotometer (Thermo Scientific, Inqaba Biotech). Pure DNA samples with A260/A280 ≈ 2, A260/A230 >1.8 and a peak at 260 nm were further assessed for quality and integrity by TapeStation analysis with DNA ScreenTape® (Agilent Technologies: 5067- 5365) according to the manufacturer's instructions (Figure 3.2). DNA ScreenTape analysis uses electrophoretic separation of DNA and provides precise information on DNA integrity. DNA Integrity Number (**DIN**) provides the overall quality of the DNA sample. The quantified DNA was utilised in the subsequent DNA methylation analysis.

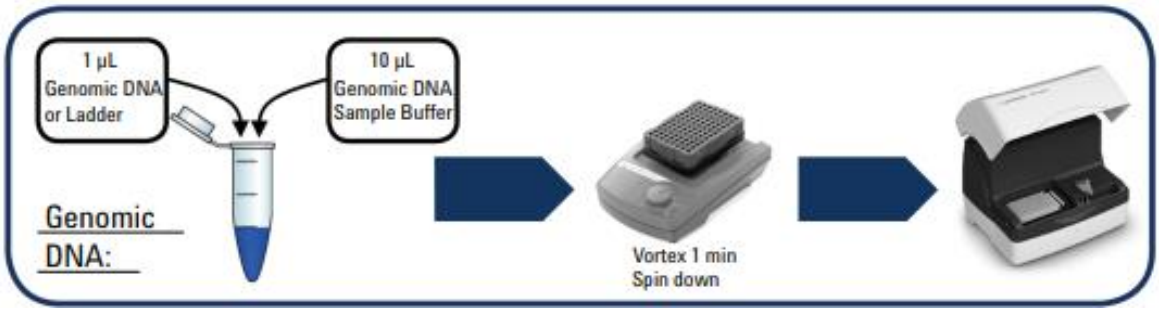


Figure 3. 2: Summary of the DNA TapeStation analysis procedure. One µl of the DNA sample was mixed with 10µl of buffer, vortexed and centrifuged at 2000 ×g to sediment the sample in the tube. Thereafter, the samples were loaded in a TapeStation 4200 instrument (Agilent Technologies).

The assessment of the global 5mC DNA methylation levels was performed using the Imprint® Methylated DNA Quantification Kit (Sigma-Aldrich: MDQ1) according to the manufacturer's instructions. The kit provides a high-throughput measure of 5mC using an indirect ELISA. In which the capture antibody targets the 5mC residues and the detection antibody coupled with an enzyme catalyses the colour change reaction upon substrate addition.

The extracted DNA (200 ng) was added into the individual wells of a 96-well plate, which was incubated for 60 minutes at 37°C in a My Temp™ incubator (Digital Mini-Incubators). The kit-provided positive control (200 ng) and a blank (DNA binding solution) were included as controls. Subsequently, the blocking solution was added into each well to prevent the antibody from binding non-specifically to the wells and incubated for 30 minutes at 37°C. The contents of the wells were removed, and the wells were washed with 1× wash buffer, followed by the addition of 1× capture antibody (anti-5mC) and incubation for 60 minutes at room temperature. The wells were subsequently washed to remove any unbound capture antibody. This was followed by the addition of 1× detection antibody and incubation at room temperature for 30 minutes. Unbound detection antibody was removed by a washing step. The developing

solution, which is the substrate for the enzyme coupled to the detection antibody, was added into each well and the plate was incubated at room temperature in a dark area. After a ten-minute incubation, the reaction was terminated by the addition of a stop solution. The absorbance was then measured at 450nm using a plate reader (SpectraMax® ABS Plus: Molecular Devices). The global methylation levels were calculated relative to the positive control, with the positive control representing 100% methylation, as per the manufacturer's instructions (Appendix 4). The plate layout is illustrated in Appendix 5.

The assessment of global 5hmC DNA methylation was performed using the Quest 5-hmC™ DNA ELISA kit (Zymo Research: D5425) according to the manufacturer's instructions. The kit provides a high-throughput screening to detect 5hmC residues using a sandwich ELISA format, as the DNA is "sandwiched" between the two antibodies. The capture antibody coats the wells, followed by the addition of DNA and thereafter the detection antibody coupled with horseradish peroxidase (**HRP**). HRP catalyses the colour change reaction upon addition of a substrate, which is detected by the plate reader.

The wells were coated with the 1× capture antibody (anti-5hmC) and incubated for 60 minutes at 37℃ (My Temp™ incubator: Digital Mini-Incubators). Thereafter, the wells were washed with 1× ELISA buffer and incubated with the ELISA buffer for 30 minutes at 37℃. The extracted DNA was denatured at 98℃ for 5 minutes in a T100™ Thermal Cycler (Bio-Rad) and placed on ice thereafter to maintain it as single-stranded. After the removal of buffer from the wells, 100ng of the single-stranded DNA was added into the antibody-coated wells and incubated for 60 minutes at 37℃. The kit-provided set of positive controls were used to generate a standard curve, and a blank well with no DNA was used as a negative control. The well contents were removed, the wells were washed with the ELISA buffer, followed by the addition of 1× detection antibody (anti-DNA HRP) and incubation for 30 minutes at 37℃. The wells were subsequently washed with the ELISA buffer, followed by the addition of the HRP substrate, and the HRP developer solution. The plate was incubated at room temperature for approximately 40 minutes until a colour change was observed. The absorbance was measured at 405nm using a 96-well plate reader (SpectraMax® ABS Plus: Molecular Devices) and the global 5hmC levels were calculated using the standard curve generated with the positive controls, where the positive control represented 0.55% hydroxymethylation (Appendix 4). The plate layout is illustrated in Appendix 5.

3.2.2. Assessment of the global m6A mRNA methylation

RNA was extracted using the Quick-RNA™ MiniPrep kit (Zymo Research: R1054) as described in **Section 2.3**. The assessment of global m6A mRNA methylation was performed using the m6A RNA Methylation Quantification Kit (Abcam®, ab185912), according to the manufacturer's instructions. The kit uses the indirect ELISA format.

To each well of a 96-well plate, 200ng of RNA diluted in the binding solution was added and incubated for 90 minutes at 37℃ (My Temp™ incubator: Digital Mini-Incubators). The kit-provided m6A positive control (200ng) and the binding solution (used as the negative control) were added to their respective wells. The wells were washed with 1× wash buffer and 1× anti-m6A capture antibody was added. This was followed by an incubation for 60 minutes at room temperature. Unbound capture antibody was removed by a washing step, then the 0.5× detection antibody was added and incubated for 30 minutes at room temperature. The wash steps were repeated then the enhancer solution was added to each well, followed by incubation for 30 minutes at room temperature. The enhancer solution was removed, the wells were washed and subsequently, a developer solution was added into each well. Once a colour change was observed after 10 minutes, a stop solution was added. The absorbance was measured at 450nm using a 96-well plate reader (SpectraMax® ABS Plus: Molecular Devices). The global m6A mRNA methylation was calculated relative to the positive control (Appendix 4). The plate layout is illustrated in Appendix 5.

3.2.3. Nuclear protein extraction and the assessment of HAT activity

The nuclear protein extraction was performed using the NucBuster™ Protein Extraction Kit (Novagen: 71183). The kit is based on the principle of nuclear membrane lysis, preserving the proteins with protease inhibitors and subsequent precipitation of nuclear proteins with reducing agents (Figure 3.3).

The mosquitoes were mechanically homogenised in 0.1M phosphate-buffered saline (**PBS**) pH 7.0 with protease inhibitors with a Tissue Lyser II (Qiagen®) for four minutes at 25hz. The PBS was supplemented with protease inhibitors to a final concentration of 2mM leupeptin, 4mM phenylmethylsulfonyl fluoride (**PMSF**), 0.5M EDTA and 1× PBS (Appendix 6). The homogenate was subsequently centrifuged at 400 ×g (4℃) for 2 minutes. Thereafter, the supernatant containing non-chitin proteins was transferred into a new tube and centrifuged at 13000 ×g (4℃) for 10 minutes to separate the nucleus from the cytoplasm. The supernatant containing the cytoplasm was discarded, and the pellet was resuspended in the kit-provided

NucBuster Reagent 1 (150 µl per 50 µl of pellet volume). Thereafter, the resuspended pellet was centrifuged at 16000 ×g at 4℃ for 20 minutes to separate the cytoplasmic proteins from the nucleus. The supernatant containing cytoplasmic proteins was discarded and the pellet was resuspended in the kit-provided reagents to disrupt the nuclear membrane and precipitate nuclear proteins. These reagents included 100× Protease Inhibitor Cocktail, 1mM Dithiothreitol (**DTT**), and NucBuster Extraction Reagent 2 (75µl per 50µl of pellet volume). The resuspended pellet was centrifuged at 16000 ×g (4℃) for 20 minutes to collect the nuclear proteins. The supernatant containing nuclear proteins was subsequently stored at -70℃ for later use. The centrifugation steps were performed in a HERMLE 2400K centrifuge (Lasec®).

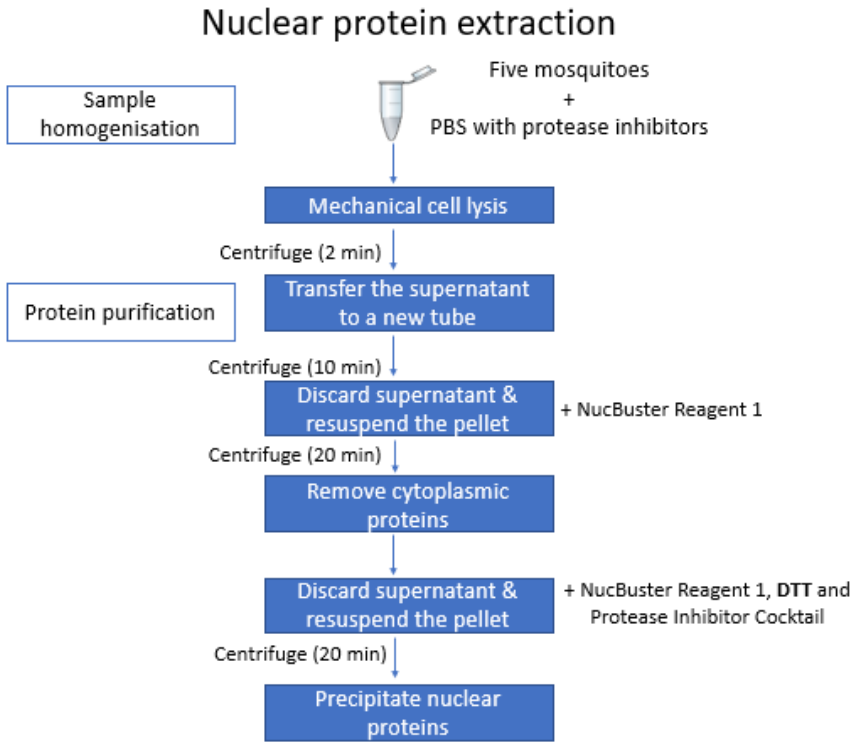


Figure 3. 3: Summary of the nuclear protein extraction protocol. All centrifugation steps were performed at 4℃ and the other steps were performed at room temperature. The extracted proteins were stored at -70℃. PBS= phosphate-buffered saline (pH7.0), DTT= Dithiothreitol (1mM). Image obtained from Novagen: 71183.

The extracted proteins were quantified using a Bradford assay (Bradford, 1976). This protocol is illustrated in Appendix 7. The nuclear proteins were used to assess the HAT activity with the HAT Activity Colorimetric Assay kit (Sigma-Aldrich®, EPI001). The acetylation process releases CoA as a by-product, which is used by the oxidised nicotinamide adenine dinucleotide (**NAD^+**) to produce NADH. The kit measures the production of NADH spectrophotometrically with tetrazolium dye. The extracted proteins were added to the individual wells in the 96-well plate at a final concentration of 50µg/µl. The kit-provided positive control and water as

negative control were used. Into each well, the kit-provided 2× HAT buffer, HAT substrate I and II, and NADH generating enzyme were added according to the manufacturer's instructions. The plate was incubated at 37°C (My Temp™ incubator: Digital Mini-Incubators) for two hours and the absorbance was read at 440nm using a 96-well plate reader (SpectraMax® ABS Plus: Molecular Devices). The background reading was subtracted from each sample and the HAT activity was reported relative to the absorbance. The plate layout is illustrated in Appendix 5.

3.2.4. Statistical analysis

The statistical packages Statistix 8 (Analytical Software, Tallahassee, Florida) and SPSS v22 (IBM Corp. Released 2013. IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp.) were used for analysis. The Shapiro-Wilk test was performed to determine normality (Shapiro & Wilk, 1965). Non-parametric data were assessed using a Kruskal-Wallis Analysis of Variance (**ANOVA**) (Kruskal & Wallis, 1952). Parametric data were assessed using One-Way ANOVA (Gamage & Weerahandi, 1998). Tukey's honestly significant difference (**HSD**) test was used post One-way ANOVA (Tukey, 1949) and Kruskal-Wallis All-Pairwise Comparisons was used post Kruskal-Wallis ANOVA (Kruskal & Wallis, 1952).

3.3. RESULTS

Several global epigenetic marker levels were assessed. The m6A mRNA methylation, 5mC and 5hmC DNA methylation levels were assessed by ELISA. The HAT activity was assessed calorimetrically.

3.3.1. The effect of bacterial stimulation on 5mC DNA methylation levels

The major vector strains (SENN and SENN DDT) had significantly higher constitutive levels of 5mC DNA methylation than the minor/non-vector (MAFUS and SANGWE) (**One-Way ANOVA: $p < 0.001$, $F_{(4, 25)} = 6.89$**). The constitutive 5mC levels between SENN and SENN DDT were not significantly different. (**$p = 0.073$, $F_{(1, 11)} = 4.00$**). The constitutive 5mC levels in SANGWE were significantly lower than that of MAFUS (**$p < 0.001$, $F_{(4, 25)} = 6.89$**) (Figure 3.4).

The untreated blood treatment resulted in a significant reduction in 5mC DNA methylation levels in SENN (**One-Way ANOVA: $p = 0.004$, $F_{(3, 20)} = 6.26$**), SENN DDT (**$p = 0.003$, $F_{(3, 20)} = 6.62$**) and MAFUS (**$p = 0.015$, $F_{(3, 20)} = 4.44$**) compared to their respective untreated counterparts (controls) (Figure 3.4). By contrast, a significant increase in 5mC levels was observed in SANGWE in comparison to its untreated counterpart (**Kruskal-Wallis ANOVA:**

p = 0.011, $F_{(3, 23)} = 4.84$, $\chi^2 = 9.64$). There was a significant increase in 5mC levels (**p = 0.002, $F_{(4, 25)} = 5.55$, $\chi^2 = 13.6$**) in SANGWE post untreated blood treatment in comparison to SENN, SENN DDT and MAFUS (Figure 3.4).

Stimulation with Gram-positive bacteria resulted in a significant reduction in 5mC DNA methylation levels in SENN (**One-Way ANOVA: p = 0.004, $F_{(3, 20)} = 6.26$**) and MAFUS (**p = 0.015, $F_{(3, 20)} = 4.44$**) compared to the 5mC levels of their untreated counterparts (Figure 3.4). The 5mC levels in SENN DDT (**p = 0.668, $F_{(3, 20)} = 0.20$**) and SANGWE (**p = 0.225, $F_{(3, 20)} = 1.58$**) were not significantly different from their untreated counterparts.

There was a significant decrease in 5mC DNA methylation levels in MAFUS and SANGWE compared to SENN and SENN DDT (**Kruskal-Wallis ANOVA: p < 0.001, $F_{(4, 25)} = 12.3$, $\chi^2 = 19.2$**) post Gram-positive bacterial stimulation (Figure 3.4). There was no significant difference in 5mC levels between SENN and SENN DDT (**One-Way ANOVA: p = 0.727, $F_{(1,11)} = 0.13$**) nor between MAFUS and SANGWE (**p = 0.203, $F_{(1,11)} = 1.86$**) post Gram-negative bacterial stimulation.

There was a significant reduction in 5mC DNA methylation levels in SENN (**One-Way ANOVA: p = 0.004, $F_{(3, 20)} = 6.26$**) and SENN DDT (**p = 0.002, $F_{(3, 20)} = 6.62$**) post Gram-negative bacterial stimulation, in comparison to their untreated counterparts (Figure 3.4). No significant differences in 5mC levels were observed in MAFUS and SANGWE post Gram-negative bacterial stimulation compared to their untreated counterparts (**p = 0.177, $F_{(3, 23)} = 1.81$**). There was no significant difference in 5mC levels between the four laboratory strains post Gram-negative bacterial stimulation (**p = 0.867, $F_{(4, 25)} = 0.310$**).

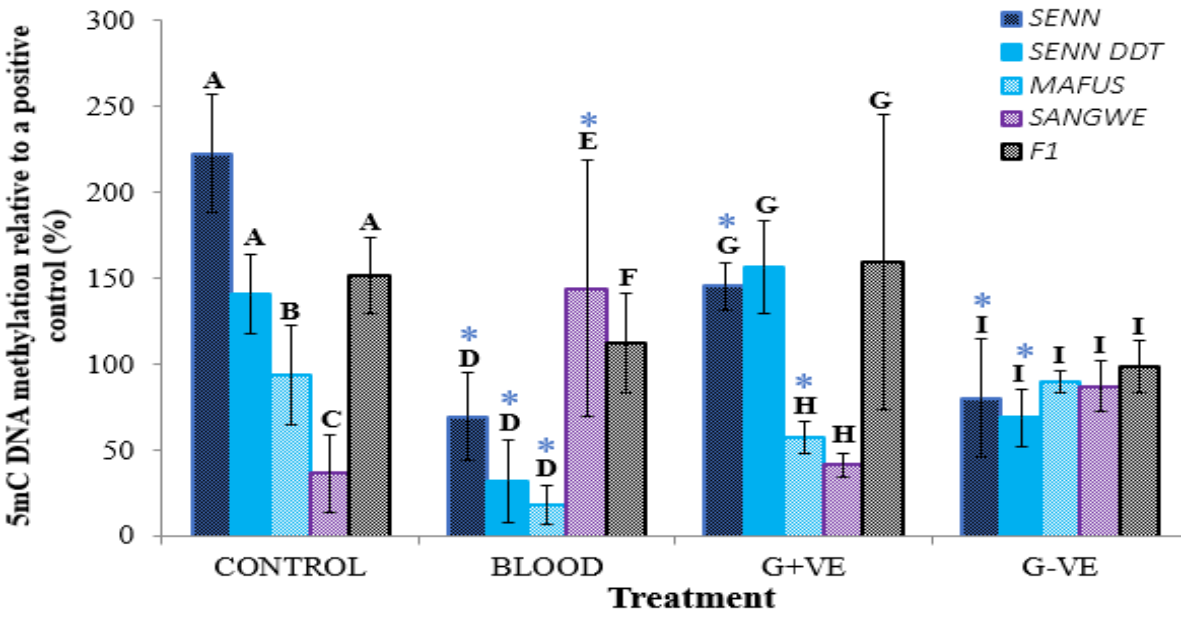


Figure 3. 4: Relative global changes in 5-methylcytosine (5mC) DNA methylation levels in the laboratory strains and F1 *An. arabiensis* in response to immune stimulation induced via a blood-meal. Data presented are relative to a methylated DNA control. SENN–insecticide susceptible *An. arabiensis*, SENN DDT–insecticide resistant *An. arabiensis*, MAFUS–*An. merus*, SANGWE–*An. quadriannalatus* and F1–progeny of wild-caught *An. arabiensis*. CONTROL treatment contains untreated sugar, BLOOD treatment contains untreated blood, G+VE treatment contains *S. pyogenes* bacteria infected blood and G-VE treatment contains *E. coli* infected blood. Asterisks (*) indicate a significant difference from the control of the same strain. Significant differences between strains within the same treatment are indicated by capital letters.

3.3.2. The effect of bacterial stimulation on 5hmC DNA methylation levels

There was no significant difference in 5hmC DNA methylation levels between the four laboratory strains constitutively (**One-Way ANOVA: $p = 0.089$, $F_{(3, 16)} = 2.59$**) (Figure 3.5). The untreated blood treatment did not result in a significant difference in 5hmC levels in the four laboratory strains when compared to their respective untreated counterparts. (**$p = 0.030$, $F_{(7, 39)} = 2.61$; Tukey post-hoc test: Q = 4.58, critical value = 0.321**). However, there was a significant increase in 5hmC levels in SANGWE post untreated blood treatment in comparison to SENN, SENN DDT and MAFUS (**$p < 0.001$, $F_{(4, 21)} = 99.5$**).

Stimulation with Gram-positive bacteria resulted in an increase in 5hmC DNA methylation levels in SENN compared to its untreated counterpart (**One-Way ANOVA: $p = 0.002$, $F_{(3, 18)} = 7.83$**) (Figure 3.5). No significant differences in 5hmC levels were observed in SENN DDT, MAFUS and SANGWE in comparison to their untreated counterparts (**$p = 0.035$, $F_{(3, 32)} = 2.83$; Tukey post-hoc test: Q = 4.33, critical value = 0.322**).

Gram-positive bacterial stimulation resulted in a significant decrease in 5hmC DNA methylation levels in SENN DDT, MAFUS and SANGWE in comparison to SENN (**One-Way ANOVA: $p < 0.001$, $F_{(4, 24)} = 13.5$**) (Figure 3.5). There was no significant difference in 5hmC levels between SENN DDT, MAFUS and SANGWE post Gram-positive bacterial stimulation (**$p < 0.001$, $F_{(4, 24)} = 13.5$; Tukey post-hoc test: Q = 4.17, critical value = 0.280**).

There was no significant difference in 5hmC DNA methylation levels between the four untreated laboratory strains and the strains exposed to Gram-negative bacteria (**One-Way ANOVA: $p = 0.024$, $F_{(7, 43)} = 2.65$; Tukey post-hoc test: Q = 4.57, critical value = 0.281**) (Figure 3.5). There was a significant increase in 5hmC levels in SANGWE compared to SENN, SENN DDT and MAFUS (**$p < 0.001$, $F_{(4, 25)} = 19.8$**) (Figure 3.5). The 5hmC levels in SENN DDT were significantly higher than that of SENN (**$p < 0.001$, $F_{(4, 25)} = 19.8$**). However, there was no significant difference between the 5hmC levels of SENN DDT and MAFUS **($p < 0.001$; Q = 4.15, critical value = 0.281**).

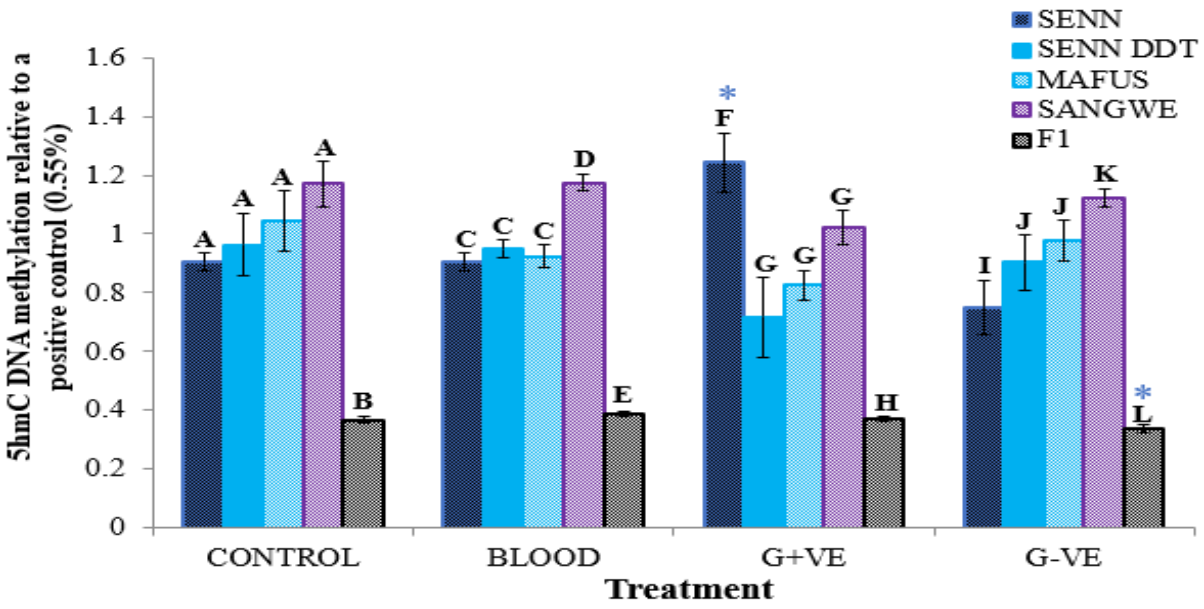


Figure 3. 5: Relative global changes in 5-hydroxymethylcytosine (5hmC) DNA methylation levels in the laboratory strains and F1 *An. arabiensis* in response to immune stimulation induced via a blood-meal. Data presented are relative to a 0.55% methylated DNA control. SENN–insecticide susceptible *An. arabiensis*, SENN DDT–insecticide resistant *An. arabiensis*, MAFUS–*An. merus*, SANGWE–*An. quadriannalatus* and F1–progeny of wild-caught *An. arabiensis*. CONTROL treatment contains untreated sugar, BLOOD treatment contains untreated blood, G+VE treatment contains *S. pyogenes* bacteria infected blood and G-VE treatment contains *E. coli* infected blood. Asterisks (*) indicate a significant difference from the control of the same strain. Significant differences between strains within the same treatment are indicated by capital letters.

3.3.3. The effect of bacterial stimulation on m6A mRNA methylation levels

The constitutive m6A mRNA methylation levels of major vectors (SENN and SENN DDT) were significantly higher than that of minor/non-vectors (MAFUS and SANGWE) (**One-Way ANOVA: $p = 0.004$, $F_{(4, 25)} = 5.12$**) (Figure 3.6). The constitutive m6A levels of SENN and SENN DDT were not significantly different (**$p = 0.629$, $F_{(1, 11)} = 0.17$**). This was also true for MAFUS and SANGWE (**$p = 0.004$, $F_{(4, 25)} = 5.12$; Tukey post-hoc test: $Q = 4.15$, critical value = 0.091**).

No significant differences in the m6A mRNA methylation levels were observed upon untreated blood treatment in SENN, SENN DDT and MAFUS compared to their untreated counterparts (**One-Way ANOVA: $p = 0.553$, $F_{(5, 35)} = 0.810$**) (Figure 3.6). However, increased m6A levels were observed in SANGWE compared to its untreated counterpart (**$p = 0.008$, $F_{(3, 20)} = 5.23$**). A significant increase in m6A levels was also observed in SANGWE in comparison to SENN, SENN DDT and MAFUS post untreated blood treatment (**$p < 0.001$, $F_{(4, 25)} = 9.42$**).

An increase in m6A mRNA methylation levels was observed in SANGWE post Gram-positive bacterial stimulation compared to its untreated counterpart (**One-Way ANOVA: $p = 0.008$, $F_{(3, 20)} = 5.23$**) (Figure 3.6). The m6A levels in SENN, SENN DDT and MAFUS were not significantly different from the m6A levels of their respective untreated counterparts (**$p = 0.343$, $F_{(5, 35)} = 1.18$**). There was no significant difference in m6A levels between the four laboratory strains post Gram-positive bacterial stimulation (**$p = 0.328$, $F_{(4, 25)} = 1.22$**).

There was a significant increase in m6A mRNA methylation levels in SANGWE post Gram-negative bacterial stimulation compared to its untreated counterpart (**One-Way ANOVA: $p = 0.008$, $F_{(3, 20)} = 5.23$**) (Figure 3.6). There was no significant difference between the m6A levels of SENN, SENN DDT and, MAFUS stimulated with Gram-negative bacteria and the m6A levels of their respective untreated counterparts (**$p = 0.367$, $F_{(5, 35)} = 1.13$**). There was no significant difference in m6A levels between the four laboratory strains post stimulation with Gram-negative bacteria (**$p = 0.316$, $F_{(4, 25)} = 1.25$**).

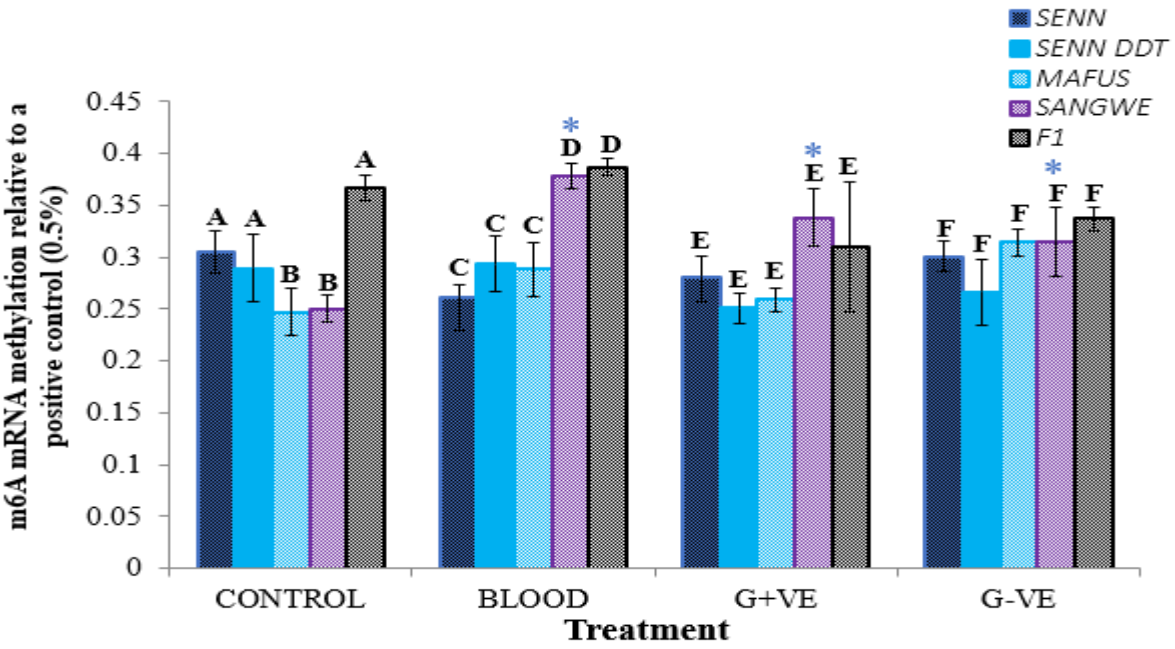


Figure 3. 6: Relative global changes in N6-methyladenosine (m6A) mRNA methylation levels in the laboratory strains and F1 *An. arabiensis* in response to immune stimulation induced via a blood-meal. Data presented are relative to a 0.5% methylated RNA control. SENN–insecticide susceptible *An. arabiensis*, SENN DDT–insecticide resistant *An. arabiensis*, MAFUS–*An. merus*, SANGWE–*An. quadriannalatus* and F1–progeny of wild-caught *An. arabiensis*. CONTROL treatment contains untreated sugar, BLOOD treatment contains untreated blood, G+VE treatment contains *S. pyogenes* bacteria infected blood and G-VE treatment contains *E. coli* infected blood. Asterisks (*) indicate a significant difference from the control of the same strain. Significant differences between strains within the same treatment are indicated by capital letters.

3.3.4 The effect of bacterial stimulation on HAT activity levels

There was no significant difference in the HAT activity between the four laboratory strains when untreated (**One-Way ANOVA: $p = 0.092$, $F_{(3, 20)} = 2.47$**) (Figure 3.7). The untreated blood treatment resulted in a significant reduction in HAT activity in SENN (**$p < 0.001$, $F_{(3, 20)} = 21.8$**), SENN DDT (**$p < 0.001$, $F_{(3, 20)} = 12.4$**) and SANGWE (**$p = 0.030$, $F_{(3, 20)} = 3.64$**) compared their untreated counterparts (Figure 3.7). In contrast, there was no significant difference in the HAT activity between the untreated and blood treated MAFUS strains (**$p = 0.365$, $F_{(3, 20)} = 1.12$**).

The untreated blood treatment resulted in a significant decrease in HAT activity in SENN compared to SENN DDT, MAFUS and SANGWE (**One-Way ANOVA: $p = 0.002$, $F_{(3, 20)} = 6.88$**) (Figure 3.7). There was no significant difference in HAT activity between SENN DDT, MAFUS and SANGWE post untreated blood treatment (**$p = 0.069$, $F_{(2,17)} = 3.20$**).

A significant increase in HAT activity was observed in SENN DDT post Gram-positive bacterial stimulation (**One-Way ANOVA: p = 0.001, $F_{(3, 20)} = 12.4$**) (Figure 3.7). The HAT activity in SENN, MAFUS and SANGWE was not significantly different from that of their untreated counterparts (**p = 0.061, $F_{(5, 35)} = 2.38$**).

Gram-positive bacterial stimulation significantly increased HAT activity in SENN-DDT in comparison to SENN, MAFUS and SANGWE (**One-Way ANOVA: p < 0.001, $F_{(3, 20)} = 8.72$**) (Figure 3.7). HAT activity in MAFUS and SANGWE was significantly lower than that of SENN and SENN-DDT post Gram-positive bacterial challenge (**p = 0.033, $F_{(3,43)} = 3.212$**).

There was no significant difference in HAT activity between the untreated strains and strains exposed to Gram-negative bacteria, in SENN (**One-Way ANOVA: p = 0.567, $F_{(1, 23)} = 0.341$**), SENN DDT (**p = 0.135, $F_{(1,23)} = 2.41$**), MAFUS (**p = 0.723, $F_{(1,23)} = 0.13$**) and SANGWE (**p = 0.748, $F_{(1,23)} = 0.11$**) (Figure 3.7). There was no significant difference in HAT activity between the four strains post exposure to Gram-negative bacteria (**p = 0.047, $F_{(3, 20)} = 6.26$**).

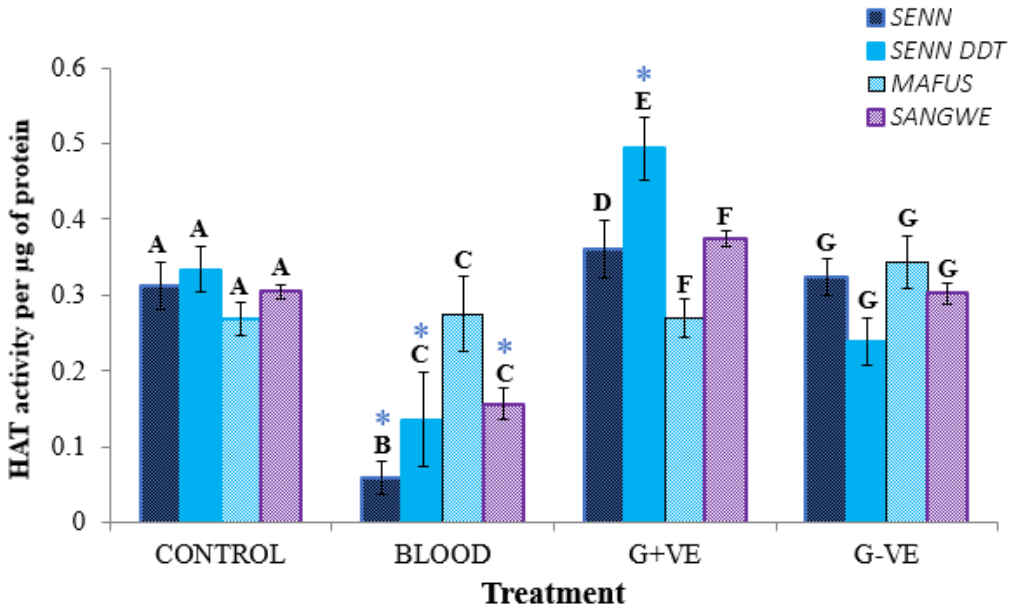


Figure 3. 7: Relative global changes in Histone Acetyl Transferase (HAT) activity in the laboratory strains in response to immune stimulation induced via a sugar-meal. HAT activity is reported as the relative optical density per µg of protein. SENN–insecticide susceptible *An. arabiensis*, SENN DDT–insecticide resistant *An. arabiensis*, MAFUS–*An. merus*, SANGWE–*An. quadriannalatus*. CONTROL treatment contains untreated sugar, BLOOD treatment contains untreated blood, G+VE treatment contains *S. pyogenes* bacteria infected sugar and G-VE treatment contains *E. coli* infected sugar. Asterisks (*) indicate a significant difference from the control of the same strain. Significant differences between strains within the same treatment are indicated by capital letters.

3.3.5. Comparison of the epigenetic markers of laboratory *An. arabiensis* and the F1 progeny of wild-caught *An. arabiensis*.

The levels of methylation markers (5mC, 5hmC and m6A) produced constitutively and post immune stimulation of the two laboratory strains of *An. arabiensis* (SENN and SENN DDT) were compared to that of F1 progeny of wild-caught *An. arabiensis*. The constitutive 5mC DNA methylation levels did not differ between the three strains (**One-Way ANOVA: $p = 0.096$, $F_{(2,17)} = 2.75$**). This was also true for m6A mRNA methylation levels (**$p = 0.076$, $F_{(2, 17)} = 3.08$**) (Figure 3.4 & 3.6). By contrast, the constitutive 5hmC levels in F1 were significantly lower than the constitutive 5hmC DNA methylation levels in SENN and SENN DDT (**$p < 0.001$, $F_{(4, 21)} = 16.4$**) (Figure 3.5).

Untreated blood ingestion resulted in a significantly higher levels of 5mC DNA methylation (**Kruskal-Wallis ANOVA: $p = 0.002$, $F_{(4, 25)} = 5.55$, $\chi^2 = 13.6$**) and m6A mRNA methylation (**One-Way ANOVA: $p < 0.001$, $F_{(4, 25)} = 9.42$**) in the F1 mosquitoes in comparison to SENN and SENN DDT (Figure 3.4 & 3.6). By contrast, 5hmC DNA methylation levels were significantly lower in the F1 mosquitoes compared to SENN and SENN DDT post untreated blood treatment (**$p < 0.001$, $F_{(4, 21)} = 99.5$**) (Figure 3.5).

Stimulation with Gram-positive bacteria did not result in any significant difference in 5mC DNA methylation levels between SENN, SENN DDT and F1 (**Kruskal-Wallis ANOVA: $p < 0.001$, $F_{(4, 25)} = 12.3$, $\chi^2 = 19.2$; Kruskal-Wallis post-hoc test, Z = 2.81, critical value = 14.3**). This was also true for m6A mRNA methylation levels (**One-Way ANOVA: $p = 0.328$, $F_{(4, 25)} = 1.22$**) (Figure 3.4 & 3.6). However, similar to the untreated blood treatment, 5hmC DNA methylation levels in F1 were significantly lower than the 5hmC levels in SENN and SENN DDT post Gram-positive bacterial stimulation (**$p < 0.001$, $F_{(4, 24)} = 13.5$**) (Figure 3.5).

Similar to Gram-positive bacterial stumulation, there was no significant difference in 5mC DNA methylation levels (**One-Way ANOVA: $p = 0.867$, $F_{(4, 25)} = 0.310$**) and m6A mRNA methylation levels (**$p = 0.316$, $F_{(4, 25)} = 1.25$**) between SENN, SENN DDT and F1 post Gram-negative bacterial stimulation (Figure 3.4 & 3.6). Similar to untreated blood and Gram-positive bacteria, Gram-negative bacterial stimulation also resulted in a significantly lower levels of 5hmC DNA methylation in F1 mosquitoes compared to SENN and SENN DDT (**$p < 0.001$, $F_{(4, 25)} = 19.8$**) (Figure 3.5).

3.3.6. The effects of immune stimulation on the epigenetic markers in F1 progeny of wild-caught *An. arabiensis*

Neither untreated blood nor the bacterial stimulation resulted in a significant change in 5mC DNA methylation levels in the F1 progeny in comparison to its untreated counterparts (**One-Way ANOVA: $p = 0.757$, $F_{(3, 20)} = 0.40$**) (Figure 3.4). The 5hmC DNA methylation levels in F1 progeny post untreated blood treatment and exposure to Gram-positive bacteria were not significantly different from that of their untreated counterparts (**$p = 0.019$, $F_{(3,19)} = 4.24$; Tukey post-hoc test: Q = 3.96, critical value = 0.378**) (Figure 3.5). By contrast, there was a significant reduction in 5hmC levels post Gram-negative bacterial stimulation when compared to its untreated counterpart (**$p = 0.019$, $F_{(3, 19)} = 4.24$**). Like 5mC, the m6A mRNA methylation levels of blood treated and immune stimulated F1 progeny were not significantly different from the untreated F1 controls (**$p = 0.376$, $F_{(3.20)} = 1.09$**) (Figure 3.6).

3.4. DISCUSSION

Epigenetic modifications play a vital role in maintaining the housekeeping functions of an organism. This includes roles in development, environmental adaptation, and defence (reviewed by Weiner & Toth, 2012). Epigenetic mechanisms have been shown to be key regulators of innate immune responses to an infection (reviewed by Zhang & Cao, 2019). Only three out of the nine species from the *An. gambiae* complex are natural vectors for malaria (Sinka *et al.*, 2012). As such, there could be a number of complex factors that contributes to their vector competence. As the epigenetics of the insect host changes due to the presence of a pathogen, it could potentially impact the transmission of the disease. It could also affect the vectorial capacity of the insect host and the host-parasite co-evolution (Vilcinskas, 2017). Therefore, the epigenetic landscape of the three zoophilic members of the *An. gambiae* complex with different levels of vector competence was assessed in response to bacterial challenges.

The constitutive levels of 5mC DNA methylation and m6A mRNA methylation in the major vectors (SENN & SENN DDT) and the minor/non-vectors (MAFUS & SANGWE) differed significantly. Both 5mC and m6A markers were higher in the major vector strains at the constitutive level, suggesting a constitutive difference in the epigenetic landscape of these closely related sibling species. Furthermore, the 5mC levels decreased with a decreasing vector competence, with SANGWE having significantly lower 5mC levels than MAFUS. Although 5mC DNA methylation can either cause gene expression or gene silencing depending on the location, it generally corresponds to gene silencing (reviewed by Goll & Bestor, 2005). In the

dipteran model *D. melanogaster*, 5mC results in gene silencing (Phalke *et al.*, 2009). The observed 5mC patterns suggest a lower degree of gene silencing in the minor and non-vector species at the constitutive level. This may suggest a greater level of gene expression in the minor and non-vector species in response to bacterial challenge.

The constitutive 5hmC DNA methylation levels and HAT activity appear to be conserved between the four strains; both are markers of gene expression (Eberharter & Becker, 2002; reviewed by Kangaspeska *et al.*, 2008). This may indicate the conservation of these epigenetic mechanisms in these sibling species in maintaining the expression of genes required for basal metabolism. This finding suggests that the regulatory mechanisms may be based on decreased silencing rather than active increase in gene expression.

The effect of m6A mRNA methylation is more difficult to interpret as its physiological relevance is poorly understood in insects. The association with gene silencing or gene expression is dependent on the location of methylation (reviewed by Zhao *et al.*, 2018). The poly-adenosine (**poly-A**) tail maintains the integrity of the mRNA in many eukaryotes (Guhaniyogi & Brewer, 2001). The addition of a methyl group on the poly-A tail makes it more stable, thus reducing its half-life and promoting increased gene expression. By contrast, adenosine methylation on the protein-coding region of mRNA can prevent the translation process by preventing the access of a transfer RNA (**tRNA**) to its codon. The effect of m6A mRNA methylation on gene regulation cannot be determined by the current experimental methodology. Further investigations of the location of m6A methylation can provide a better understanding of the role of m6A methylation in regulating gene expression.

Blood is a source of proteins required by the female mosquitoes for their eggs and reproduction (Clements, 1999b). Blood-feeding induces widespread changes in gene expression in mosquitoes (Bonizzoni *et al.*, 2011). In general, both 5mC DNA methylation and HAT activity decreased post blood-feeding, these changes correspond to decreased gene silencing and decreased gene expression, respectively (Peterson & Laniel, 2004; Bhutani *et al.*, 2011). This may suggest that each of these markers could be related to a different response. The decrease in 5mC DNA methylation may correspond to the observed overexpression of genes involved in defence against reactive oxygen species (**ROS**), blood-digesting proteins and detoxification enzymes. The decreased HAT activity may correspond to a decreased expression of genes involved in locomotion and response to environmental stimuli (reviewed by Villagra & Frías-Lasserre, 2020). In general, the 5hmC and m6A methylation levels appear to be unaltered post

blood-feeding. This suggests that these two mechanisms may not be involved in the response to blood digestion.

Blood-feeding induced changes in the epigenetic markers between the four laboratory strains. Upon untreated blood treatment, the vector species (SENN, SENN DDT & MAFUS) had similar levels of 5mC, 5hmC and m6A. These were typically lower than that of the non-vector SANGWE. The increased 5hmC and m6A levels in SANGWE suggest a possible increase in gene expression with the assumption that m6A is located on the poly-A tail of the mRNAs. By contrast, increased 5mC levels in SANGWE corresponds to increased gene silencing. This suggests that blood-feeding induces a larger suite of changes, both overexpression and under expression of genes in the non-vector species. These results suggest an underlying genetic difference in this non-vector species compared to its related species in the *An. gambiae* complex that differs in vector competence despite having similar behaviour.

The HAT activity patterns are different from the nucleic acid methylation patterns after blood-feeding. The HAT activity in SENN DDT, MAFUS and SANGWE was significantly higher than that of SENN, suggesting a decreased gene expression in the latter strain. Although these results are incongruent with the nucleic acid methylation results, it still suggests an interspecies difference in the epigenetic response to blood-feeding.

Upon infection, multiple signalling pathways are stimulated and the expression of the immune-related genes such as AMPs and peptidoglycan recognition proteins (**PGRPs**) are upregulated (reviewed by Baxter *et al.*, 2017). The Gram-positive bacteria are recognised by Gram-negative binding protein 1 (**GNBP-1**) and PGRP-SA, which are located on the epithelial cells of midgut. They initiate the Toll-pathway to synthesise specific AMPs against the invaded Gram-positive bacteria (Michel *et al.*, 2001; Ligoxygakis *et al.*, 2002). The Gram-positive bacterial challenge resulted in decreased 5mC DNA methylation and increased 5hmC DNA methylation in the major vector SENN in comparison to its untreated counterpart. This combination of epigenetic modifications suggests that the Gram-positive bacterial challenge could result in increased gene expression in SENN. Although HAT activity was not altered in SENN, increased HAT activity was observed in the insecticide resistant major vector strain SENN DDT. This suggests a differential epigenetic regulation in response to Gram-positive bacterial challenge in a single species that differ in insecticide resistant phenotype.

A decrease in 5mC DNA methylation was also observed in SANGWE post Gram-positive bacterial challenge, which corresponds to a decrease in gene silencing (reviewed by Goll &

Bestor, 2005). The observed increase in m6A mRNA methylation in SANGWE further supports the activation of the immune system. These findings suggest that this non-vector species has a greater response to Gram-positive challenge than other species. Although no direct suggestions can be made about anti-parasite immunity, these responses suggesting increased gene activity in response to bacterial challenge is notable. It is worth investigating whether these altered signatures are related to the increased response of the species to immune challenge (Habtewold *et al.*, 2008)

The Gram-positive bacterial challenge resulted in interspecies differences in 5mC and 5hmC DNA methylation, as well as HAT activity in the laboratory strains. By contrast, no changes were observed in m6A mRNA methylation levels between the laboratory strains. This suggests that unless *S. pyogenes* exposure alters m6A methylation levels in other forms of RNA, the epigenetic response to Gram-positive bacteria may be limited to DNA epigenetic modifications. This also suggests that the mechanism involved in mRNA methylation may be conserved between the members of the *An. gambiae* complex due to their genotypic and phenotypic similarities (Scott *et al.*, 1993; Barrón *et al.*, 2019).

Upon Gram-positive bacterial challenge, the observed low levels of 5mC DNA methylation in minor/non-vectors than major vectors suggest decreased gene silencing in MAFUS and SANGWE. In contrast to 5mC DNA methylation, both HAT activity and 5hmC DNA methylation were higher in the major vectors, which suggest an increased transcriptional activity in SENN and SENN DDT. This suggests that the major and the minor/non-vectors regulate the epigenetic response to Gram-positive bacteria in a different manner. The minor vectors respond by reducing gene silencing and the major vectors respond by increased gene activation. Although the net results are an increase in the expression of relevant genes, the vectors and non-vectors appear to have different biological strategies to achieve this. Although these findings speak to yet unexplored levels of complexity in the regulatory mechanisms, it does suggest a pattern of differential responses to immune stimuli in the major and minor/non-vectors.

The exposure to Gram-negative bacteria resulted in less of a marked difference in the epigenetic landscape than Gram-positive bacteria. Gram-negative bacterial invasion triggers a separate immune signalling pathway from that of Gram-positive bacteria. The transmembrane receptor PGRP-LC stimulates the immune-deficiency (**IMD**) pathway to synthesise immune proteins and AMPS, this receptor is also defensive against *Plasmodium* (Warr *et al.*, 2008). After the

Gram-negative bacterial challenge, decreased 5mC DNA methylation was observed in both the *An. arabiensis* strains SENN and SENN DDT, suggesting an increase in transcriptional activity. The Gram-negative bacterial challenge triggered an increase in m6A mRNA methylation in the non-vector SANGWE, suggesting a possible increase in transcriptional activity. There is evidence from previous studies that the prophenoloxidase (**PPO**) cascade in *An. quadriannulatus* is triggered at a lower level (Habtewold *et al.*, 2008). Although these epigenetic changes correspond to increased transcription after immune stimulation, it is worth noting that *An. arabiensis* and *An. quadriannulatus* may be regulating their responses on different levels. If the non-vector is regulating the epigenetic response at the RNA level, rather than the DNA level, the expression changes could possibly be more rapid accounting for the lower threshold observed in the non-vector.

The interspecies response to Gram-negative bacteria was largely conserved, with no interspecies differences at the 5mC DNA and m6A mRNA methylation, as well as HAT activity levels, post *E. coli* challenge. This lack of epigenetic responsiveness to Gram-negative bacteria may be related to the nature of commensal bacteria. The *Anopheles* mosquitoes generally have gut microbiota dominated by Gram-negative bacteria (Wang *et al.*, 2011; Dada *et al.*, 2018). An epigenetic landscape that is not highly inducible by Gram-negative bacteria may be required to maintain the commensal bacterial population.

The Gram-negative bacterial challenge, however, altered the levels of 5hmC DNA methylation between the strains, while the other epigenetic markers remain conserved. The insecticide susceptible major vector SENN displayed lowest levels of 5hmC DNA methylation while the non-vector SANGWE displayed the highest. As 5hmC corresponds to gene expression (reviewed by Goll & Bestor, 2005), the higher degree of gene expression in the non-vector SANGWE may indicate an increased expression of immune-related genes. The non-vector *An. quadriannulatus* was shown to have an increased tolerance to bacterial challenge and increased immune resistance to *P. berghei*, and the opposite findings were observed in the major vector *An. coluzzii* (Habtewold *et al.*, 2017). These findings are congruent with patterns of 5hmC methylation levels and the potential subsequent gene expression.

Another interesting observation was that the 5hmC DNA methylation levels post Gram-negative bacterial challenge were not different between the insecticide resistant major vector SENN DDT and the minor vector MAFUS. This was not observed in any other epigenetic marker. The reason for this observation could be insecticide tolerance as both SENN DDT and

MAFUS display insecticide tolerance. This suggests that the insecticide resistant phenotype can influence the 5hmC epigenetic landscape. The Gram-negative bacterial challenge generally did not induce interspecies epigenetic changes, where it did occur, it appears to be regulated by 5hmC DNA methylation alterations.

An insecticide is an environmental stressor that causes heritable changes not only in the genotype (Williamson *et al.*, 1996) but also in the epigenetic landscape of an insect (Brevik *et al.*, 2021). The inclusion of insecticide susceptible (SENN) and resistant (SENN DDT) *An. arabiensis* strains from the same genetic background allowed examination of the role of insecticide resistance on the dynamics of immune stimulation on the global epigenetic landscape. The epigenetic markers at the constitutive level between SENN and SENN DDT were not different. This was congruent with the previous study on the effect of metal pollutants on the epigenetic architecture in SENN and SENN DDT. The study demonstrated that the epigenetic marker levels were not statistically different under control conditions (Jeanrenaud, 2018). Blood-feeding only resulted in an epigenetic alteration at the histone level. The insecticide resistant SENN DDT strain had an increased level of HAT activity. Blood-feeding triggers defensive responses that increase insecticide tolerance specifically, in the insecticide resistant strains (Spillings *et al.*, 2008; Oliver & Brooke 2014). The potential increased gene expression induced by blood meals in the SENN DDT strain may be related to this response.

Gram-positive and Gram-negative bacterial challenges did not result in any 5mC DNA and m6A mRNA methylation differences between SENN and SENN DDT, nor in HAT activity post Gram-negative bacterial challenge. The marked differences were observed in the 5hmC DNA methylation. The 5hmC levels in SENN were significantly higher than that of SENN DDT post Gram-positive bacterial challenge and significantly lower than SENN DDT post Gram-negative bacterial challenge. The increased 5hmC levels post Gram-negative bacterial challenge and the increased HAT activity post Gram-positive bacterial challenge in SENN DDT would correspond to increased gene expression. This demonstrates that insecticide resistance does not affect the immune response in a uniform manner. Instead, the presence of the resistance phenotype results in a variable regulation of the induction of immune system rather than basal differences in the immune response. This will be expanded upon in the following chapter.

The basal epigenetic markers were conserved between the laboratory strains and F1 progeny of wild-caught *An. arabiensis* at the 5mC DNA and m6A mRNA methylation levels. Similarly,

the 5mC and m6A methylation levels also did not vary between the laboratory strains and the F1 progeny post immune stimulation with both the bacteria. This suggests that these patterns of epigenetic markers observed in the laboratory strains are not due to a colonisation effect and are representative of the wild population.

These findings also suggest a conservation of the key epigenetic signatures in the laboratory *An. arabiensis* strains. Although this requires experimental validation, it is possible that this may be true for the *An. merus* and *An. quadriannulatus* strains as well. However, the marked deviation from the general pattern was observed at the 5hmC DNA methylation level.

At the constitutive level, 5hmC DNA methylation was significantly lower in the F1 progeny of wild-caught *An. arabiensis* than the laboratory strains. This pattern was maintained after blood-feeding and immune stimulation. Furthermore, Gram-negative bacterial challenge significantly reduced 5hmC levels, which would suggest a reduced level of gene expression. The low 5hmC DNA methylation levels in the F1 progeny may speak to a previous observation in immunological studies. Previous research has demonstrated a marked exaggeration in the immune response and transcriptome divergence of laboratory-reared mosquitoes compared to their wild counterparts (Aguilar *et al.*, 2010; Cornet *et al.*, 2013). It has been suggested that this may be due to the relatively unchallenging environment of the laboratory, which would allow for greater resource allocation to immunity that would not occur in the wild (Tripet, 2009). In the wild, these resources may be allocated to other physiological functions, such as reproduction. As 5hmC DNA methylation corresponds to gene expression (Pastor *et al.*, 2013), the low 5hmC levels seen in F1 progeny may be related to this hypothesis that F1 has not yet shifted their resource allocation to immunity. As such, regulation in these mosquitoes may be more related to changes in gene silencing rather than changes in gene activation.

One limitation for these experiments would be the fact that each strain is different from the other strain because they are from different geographies. SENN and SENN DDT are from Sudan, MAFUS is from South Africa and SANGWE is from Zimbabwe. The observed epigenetic differences may be due to their different geographic origin. They could also be due to their genetic diversity and adaption to the laboratory conditions. However, the expression pattern of majority of the epigenetic markers show a distinction between the major and minor/non-vectors.

Another limitation is the founder effect. The insecticide resistant strain of *An. arabiensis* was selected from the susceptible strain, which may have reduced the genetic diversity of the

insecticide resistant strain SENN DDT. However, all the epigenetic signatures were conserved between the two strains under control conditions. The differences were only observed after immune stimulation. Furthermore, the large-scale conservation in the epigenetic signatures between the *An. arabiensis* from South Africa and Sudan suggests a conservation.

The final limitation is that the results must be interpreted with caution, as the data was analysed by a comparison of means. In order to assess between and within group variation, a generalised linear model (GLM) or generalised linear mixed model (GLMM) would be a better analysis. This would determine the strength of interactions and therefore give a better understanding of the interplay between the factors. At present, the results as analysed represent responses to the challenge only and does not shed appropriate light on interspecies variation.

In conclusion, this study suggests an epigenetic basis for the differences in the immune response of the major, minor, and non-vectors. There are differences in constitutive production levels between the major and minor vectors at the 5mC DNA and m6A mRNA methylation levels with Gram-positive and Gram-negative bacterial challenges resulting in a substantial change in the epigenetic landscape. Furthermore, a comparison of the epigenetic landscape and its responsiveness in the F1 *An. arabiensis* progeny suggests that the findings observed in this study may be largely representative of the findings in the wild. Although observed on a global scale, this may underlie a difference in immunological responses in the members of the *An. gambiae* complex. These findings may therefore potentially be related to vector competence.

CHAPTER FOUR: Assessment of the transcript levels of immune-related genes in zoophilic members of the *Anopheles gambiae* complex in response to bacterial challenge

4.1. INTRODUCTION

The immune system is a complex network of organs, cells, and proteins that defends the organism from foreign bodies and pathogenic microorganisms in the environment. The immune system actively targets the removal of the foreign body; hence, a functional immune system is crucial for the survival of an organism. One of the critical tasks of the immune system is to distinguish self from non-self (Schultz *et al.*, 1987). A weaker immune system can prove fatal for the organism. The immune system consists of two distinct branches, which engage in crosstalk and generate a robust immune response (Bona & Bonilla, 1996).

4.1.1. The innate and the adaptive immune system

The immune system consists of two parts. The first is a non-specific, general defence mechanism referred to as the innate immune system. The second is a specific, specialised defence mechanism referred to as the adaptive immune system (Brandes *et al.*, 2019). The major differences between the innate and the adaptive immune systems are illustrated in Table 4.1.

Table 4. 1: The principle differences between the innate and the adaptive immune system (adapted from Janeway & Medzhitov, 2002; Murphy *et al.*, 2010).

Factor	**Innate immunity**	**Adaptive immunity**
Occurrence	All eukaryotic organisms	Chordates, *Agnatha* onwards
Specificity	Generates a non-specific general response	Generates a specific pathogen targeted response
Action time	Immediate, maximal response	Delayed activation of effector molecules
Distribution	Non-clonal	Clonal
Receptors	Germline encoded and rearrangement not required	Encoded in segments and rearrangement is required
Recognition	Via pathogen-associated molecular pattern recognised by pathogen recognition receptor	Detailed molecular structure recognised by leukocyte antigens
Self-discrimination	Perfect discrimination	Imperfect discrimination
Memory	No immunological memory	Presence of immunological memory

Nonetheless, both branches of the immune system results in a humoral and cellular immune response. The humoral immune response is mediated by AMPs in innate immunity and by antibodies in adaptive immunity. The cellular immune response is mediated by the leukocytes (reviewed in Parkin & Cohen, 2001). The innate immune system provides the first-line defence for all multicellular organisms. The main function of the innate immune system is to eliminate the infection before it becomes systematic (Fearon, 1997). The components of the innate immune system are illustrated in Table 4.2.

Table 4. 2: The components of the mammalian innate immune system (adapted from Alberts *et al.*, 2002).

Component	**Function**
Physical and anatomical barriers such as the skin and mucosal lining of the organs	Acts as a physical barrier to prevent the pathogen entry
The leukocytes: dendritic cells, granulocytes, and macrophages/monocytes	Performs phagocytosis, cytokine production, antigen presentation and destroys the pathogen
Endothelial and epithelial cells	Produce antimicrobial peptides that induce pathogen elimination
Cellular receptors/pathogen recognition receptors	Recognise the pathogenic component and initiate the immune signalling cascades

Innate immunity is a hallmark of most eukaryotic life. In this chapter, the core concepts of innate immunity will be addressed that cover organisms from invertebrates to primates. The generation of the innate immune response occurs within a few minutes to hours of a pathogen invasion. The cellular receptors on the innate immune cells recognise the patterns associated with a group of pathogens. These conserved patterns are known as the pathogen-associated molecular patterns (**PAMPs**). Examples of PAMPs include the components of bacterial cell wall, *Plasmodium* glycosylphosphatidylinositol, viral DNA/RNA, fungal glucans, mannose on the yeast surface (Schofield & Hackett, 1993; reviewed in Brubaker *et al.*, 2015). Cellular receptors that recognise PAMPs are referred to as the pathogen recognition receptors (**PRRs**). The PRRs are germline coded and evolutionarily conserved receptors that recognise clusters of microorganisms and not a specific microorganism (reviewed in Medzhitov & Janeway, 2000). The innate immune system is activated once a PAMP is recognised by its PRR, leading to humoral and cellular innate immune responses that facilitate pathogen elimination (Figure 4.1).

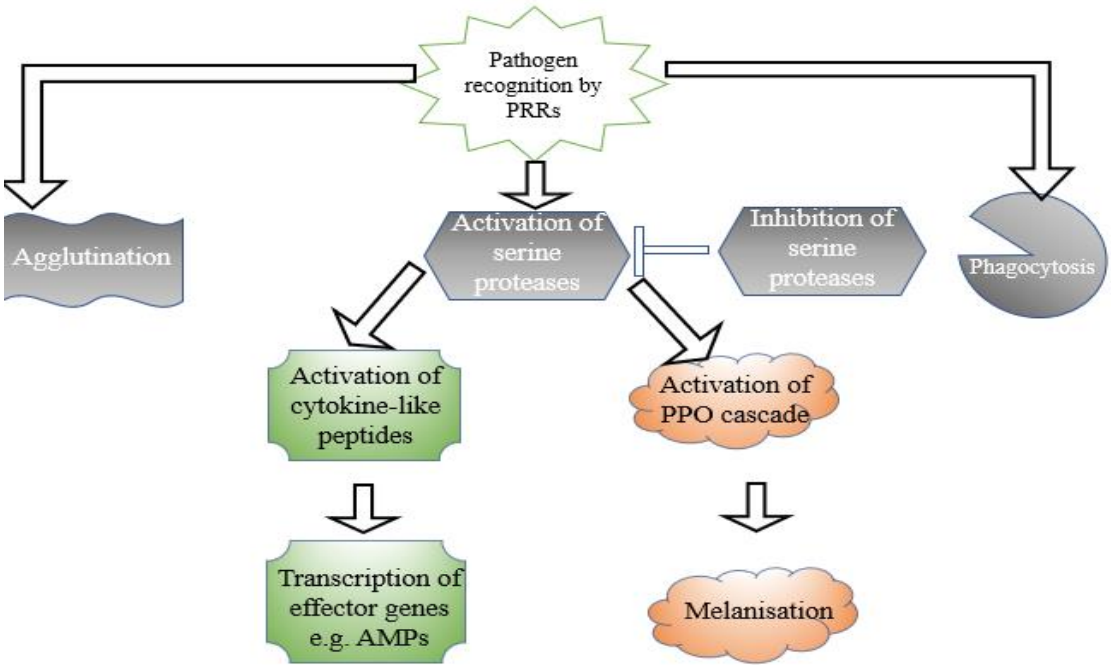


Figure 4. 1: Generalised schematic of the insect immune response to a pathogen challenge. Recognition of the conserved pathogen patterns (such as viral RNA or bacterial peptidoglycan) results in a perfect recognition of self from non-self. This recognition triggers either phagocytosis, agglutination, or serine protease cascades. The latter can result in either melanisation via the prophenoloxidase (PPO) cascade (an early-stage response) or the production of effector molecules (late-stage response). Effector molecules include the antimicrobial peptides (AMPs) and reactive oxygen/nitrogen species (adapted from Barillas-Mury *et al.*, 2000).

The humoral innate immune responses include melanisation, and serine protease immune signalling cascades that produce AMPs and other effector proteins. The cellular immune response is mediated by the phagocytotic cells (reviewed in Rettig *et al.*, 2015).

4.1.2. The immune system of mosquitoes

The adaptive immune system only occurs in chordates from jawless fishes onwards. Mosquitoes and other invertebrate animals use the evolutionary older innate immune system. This is due to the typically shorter lifespan and shorter generation time of these organisms (reviewed by Janeway & Medzhitov, 2002).

The innate immune system of insects consists of physical barriers that prevent pathogen entry, the haemocytes that perform phagocytosis and the fat body, which is the main site of AMP production (reviewed in Boman, 1998). The physical barriers are the initial barrier of the cuticle and several other epithelial barriers, which include the epithelial lining of midgut and the salivary glands. Pathogens that traverse the cuticle into the haemocoel are exposed to both humoral and cellular elements of the immune system. The midgut provides another physical

barrier with the peritrophic matrix that forms after a meal. The salivary lobes are encircled by epithelial cells that form an immunological barrier in salivary glands (reviewed in Kumar *et al.*, 2018).

The pathogens that escape the epithelial barriers could be subject to lysis, phagocytosis, or melanisation. Phagocytosis is a process in which an immune cell engulfs and digests a pathogen. Several cells of the innate immune system perform phagocytosis such as macrophages and dendritic cells. Lysis involves the physical rupture of the cellular membrane of a pathogen. This process is mediated by the AMPs in the innate immune system. (Alberts *et al.,* 2002). Melanisation involves the activation of serine protease cascades that results in cleavage of PPO to Phenoloxidase (**PO**). Quinones are subsequently oxidised by PO and the release of ROS from these reactions contribute to pathogen elimination (reviewed in Kumar *et al.*, 2018).

Upon infection, the arthropod specific PRRs detect PAMPs and initiate immune signalling cascades. This results in the production of effector proteins and AMPs, as well as signal amplification cascades that cause pathogen encapsulation (Kumar *et al.*, 2018).

4.1.3. Pathogen recognition in mosquitoes

The *An. gambiae* genome consists of more than 150 germ-line encoded PRRs that contain either single or multiple pathogen recognition sites. Several PRRs has been linked to the anti-Plasmodial defence. The *thioester-containing protein 1* (***TEP-1***) expressed in the haemocytes and fat body, is one of the first PRR discovered that recognises the *Plasmodium* parasite. However, the exact PAMP recognised by TEP-1 remains unclear (Levashina *et al.*, 2001). The expression of *TEP-1* is regulated by the Janus kinase/signal transducer and activator of transcription (**JAK/STAT**) pathway (Garver *et al.*, 2013). TEP-1 has a strong interaction with the *P. falciparum* ookinete surface and a weak interaction with oocytes, but does not recognise sporozoite (Blandin *et al.*, 2004; reviewed by Dong *et al.*, 2006, Povelones *et al.*, 2016). TEP-1 also promotes bacterial phagocytosis by interacting with the bacterial surface (Levashina *et al.*, 2001). Two alleles of the *TEP-1* gene have been discovered so far. The *TEP-1r* is associated with increased susceptibility to *P. berghei* supporting its development, which was observed by the increased number of oocytes in mosquitoes with this allele. The *TEP-1s* allele is associated with increased resistance to *P. berghei,* promoting the parasite melanisation (Blandin *et al.*, 2009). All strains used in this study are homozygous for the *TEP-1s* allele (Patel, 2019). This

indicates that TEP-1 alone does not contribute to resistance to *Plasmodium* infection, many other immune factors may contribute to the differences in vectorial capacity.

The LRIM-1 protein is another PRR that functions like TEP-1. However, LRIM-1 initiates parasite elimination independent of melanisation. *LRIM-1* expression is induced upon *Plasmodium* invasion, specifically in the midgut of the mosquito during ookinete invasion of the midgut epithelium (Osta *et al.*, 2004; Dong *et al.*, 2006). LRIM also regulates the circulation of the soluble TEP-1 in the haemolymph (Fraiture *et al.*, 2009) that further promotes parasite elimination. Multiple fibrinogen domain immunolectins has been identified as PRRs that recognise and limits *P. falciparum* infection. Fibrinogen-related protein 30 binds to the *P. falciparum* and prevent its development in *An. gambiae* (Niu *et al.*, 2017).

The PGRPs are PRRs that recognises peptidoglycan, which is a key component of the bacterial cell wall. Although insect PGRPs have diverse functions, the four mammalian PGRPs are largely antibacterial (Dziarski *et al.*, 2012). The expression of these receptors is often increased after bacterial challenges (Charroux *et al.*, 2009). PGRPs have a major contribution towards the activation of the Toll and IMD pathways. Seven PGRPs have been identified in the *Anopheles* mosquitoes, of which three are soluble and four are transmembrane receptors (Christophides *et al.*, 2002). The soluble PGRP-SA and PGRP-SD are required for the activation of the Toll pathway. By contrast, PGRP-LC and PGRP-LE are transmembrane receptors, which initiates the IMD pathway (reviewed by Meister *et al.*, 2009).

Although these receptors regulate antibacterial immunity, multiple studies have shown their indirect role in anti-Plasmodial immunity in mosquitoes. PGRP-LC affects the intensity of *P. falciparum* infection in mosquitoes (Meister *et al.*, 2009). PGRP-LA and PGRP-LB have been identified as negative regulators of anti-parasitic defence in *Anopheles* mosquitoes (Gendrin *et al.*, 2017). PGRP-LD regulates the homeostasis of gut microbiota in *An. stephensi* (Song *et al.*, 2018). The mosquito midgut microbiota protects mosquitoes from infections (Barletta *et al.*, 2017), thereby contributing to the defence against *Plasmodium* infection. The exact mechanism of how PGRPs regulates immunity against parasites is unclear.

In addition to the PRRs that specifically recognise the malaria parasites, there are other PRRs that also recognise other types of pathogens. The GNBPs are PRRs with multiple variants, which recognises PAMPs from: fungi, parasites, Gram-positive and Gram-negative bacteria. *GNBPs* are primarily expressed in the midgut, salivary glands and by the haemocytes. Their expression is upregulated after an infection (Dimopoulos *et al.*, 1997). A total of six variants

has been identified in *An. gambiae* that interact with lipopolysaccharide (**LPS**) and glucan molecules from the surface of a pathogen (reviewed by Salgueiro *et al.*, 2016).

The GNBP-B1 is the original form of GNBP identified in *An. gambiae*, initially named AgGBNP (Dimopoulos *et al.*, 1997). *GNBP-B1* expression is induced upon *E. coli* challenge as well as ookinete invasion (Rosinski-Chupin *et al.*, 2007; Warr *et al.*, 2008). GNBP-4 interacts with *E. coli* and *P. berghei* on the midgut epithelial cells of *An. gambiae* and regulates the IMD signalling pathway. However, it is not responsive to *P. falciparum*. GNBP-A2 interacts with *P. falciparum* as well as *E. coli* and promotes their elimination, it also has a weaker interaction with *P. berghei*. GNBP-B2 also interacts with *E. coli* and has been shown to be upregulated after *E. coli* infection in *An. gambiae* (reviewed by Warr *et al.*, 2008).

4.1.4. The immune signalling pathways in mosquito immunity

A series of serine protease cascades activates immune signalling pathways, which are typically initiated by transmembrane receptors. These signalling pathways lead to the production of AMPs and effector proteins that facilitate pathogen elimination. There are three major immune signalling pathways in insects, namely the Toll, IMD and JAK/STAT pathways, which are activated upon pathogen detection. These pathways regulate the humoral immune response (reviewed in Kingsolver & Hardy, 2012). These signalling pathways are well studied and characterised in the fruit fly, *Drosophila melanogaster* (reviewed in Lemaitre & Hoffmann, 2007). External factors such as diet, temperature and chemical stress can affect these signalling pathways by altering the expression of proteins that feed into the system (Horst *et al.*, 2016).

The Toll pathway was initially discovered as a developmental pathway in *D. melanogaster* (Nüsslein-Volhard *et al.*, 1987), with its role in immunity discovered later (Lemaitre *et al.*, 1996). It is activated in response to PAMPs from Gram-positive bacteria and fungi. The lysine-containing peptidoglycans and LPS from the cell wall of Gram-positive bacteria, and glucans from fungi are detected by several PRRs of the Toll Pathway. The GNBP-1, GNBP-3, PGRP-SA and PGRP-SD are a few examples PRRs that activate Toll Pathway (Lemaitre *et al.*, 1996; Gottar *et al.*, 2006). The recognition of a PAMP by its PRR initiates serine protease cascades that leads to the production of the Toll ligand, Spätzle. The binding of Spätzle to the extracellular receptor of the Toll protein initiates a signalling cascade. This eventually leads to the translocation of nuclear factor kappa B (**NF-κB**) transcription factor into the nucleus. Relish-1 (**Rel**) NF-κB promotes the transcription of AMPs in mosquitoes and Dif/Dorsal NF-κB promotes effector proteins expression in *D. melanogaster* (Belvin & Anderson, 1996; Frolet

et al., 2006). Infection with the parasite *Trypanosoma rangeli* causes a decreased activation of the Toll pathway in the kissing bug *Rhodnius prolixus* (Rolandelli *et al.*, 2021), which suppresses the immune response allowing the parasite to replicate.

The Toll pathway is effective in the defence against *P. berghei* and *P. gallinaceum*, rodent and avian malaria parasites, respectively. Silencing cactus, a Rel-1 inhibitor, results in an increased resistance to *P. berghei* in *Anopheles* mosquitoes (Frolet *et al.*, 2006). Similar results have been observed in *Aedes* mosquitoes (Bian *et al.*, 2005). Experimental activation of Rel-2 terminates the development of *P. falciparum* ookinetes (Zakovic & Levashina, 2017). In addition to the antibacterial and anti-Plasmodial activity, the Toll pathway also has antiviral activity. In *Aedes aegypti*, the Toll pathway is reported to control the dengue virus replication (Xi *et al.*, 2008).

The IMD pathway was discovered in *D. melanogaster* carrying a recessive mutation for the *imd* gene. This mutation impaired the expression of AMPs upon Gram-negative bacterial challenge and resulted in a decreased survival of the flies (Lemaitre *et al.*, 1995). The IMD pathway is primarily activated in response to PAMPs from Gram-negative bacteria. The diaminopimelic acid (**DAP**) containing peptidoglycans from the cell wall of Gram-negative bacteria are detected by PGRP-LC and PGRP-LE (Georgel *et al.*, 2001; Choe *et al.*, 2002; Salcedo-Porras *et al.*, 2019). Unlike the Toll pathway, the IMD pathway is directly activated upon PAMP recognition. This results in the recruitment of a signalling complex of proteins that includes the IMD protein. This is followed by a series of signalling cascades that leads to the activation of NF-kB Rel transcription factor. The activated Rel translocate into the nucleus (reviewed in Myllymäki *et al.*, 2014). In mosquitoes, both full-length Rel-2 and cleaved Rel-2 initiates the expression of AMPs in the nucleus (Meister *et al.*, 2005). However, in *D. melanogaster*, the full-length Rel-110 is cleaved into Rel-68 and Rel-49, only the Rel-68 fragment translocate into the nucleus to initiate AMP transcription (Stöven *et al.*, 2000). A decreased activation of the IMD pathway has been observed in *R. prolixus* upon *T. rangeli* parasitic infection. This was observed by the decreased expression of the Rp-Rel after the infection (Rolandelli *et al.*, 2021).

The IMD pathway is more effective in defence against *P. falciparum*. Increasing the expression of Rel-2 and knockdown of its negative regulator Casper creates resistance to *P. falciparum* infection in *An. stephensi*, *An. albimanus* and *An. gambiae* (Garver *et al.*, 2009; Dong *et al.*, 2011; Garver *et al.*, 2012). The transcription factor Caudal also inhibits Rel-2, thus it negatively regulates the IMD pathway. Silencing Caudal in the midgut of *An. gambiae* resulted in an

increased resistance to bacteria and *P. falciparum* infections but not *P. berghei* (Clayton *et al.*, 2013).

The JAK/STAT pathway is least studied in insects. Very little is known about this pathway in mosquitoes even though it is extensively studied in mammals. This pathway is activated by the cytokines released from leukocytes during bacterial and viral infections. In *D. melanogaster*, three cytokine-like proteins: unpaired (**upd**), upd-2, and upd-3 activates the JAK/STAT pathway (Wright *et al.*, 2011). The binding of cytokines to the dimerised receptor Domeless causes the receptor-associated JAK tyrosine kinase Hopscotch 11 (Hop-11) to phosphorylate itself and the cytoplasmic tail of the Dome receptor (Binari & Perrimon 1994; Brown *et al.*, 2001). The phosphorylated sites serve as a docking site for the STAT transcription factor Stat92E (Yan *et al.*, 1996). The phosphorylated STAT travels to the nucleus and initiates gene expression of immune-related and stress-induced genes (reviewed by Kiu & Nicholson, 2012). In addition to gene expression, the JAK/STAT pathway also plays a role in haemocyte differentiation and proliferation (Sorrentino *et al.*, 2004).

The immunological role of the JAK/STAT pathway in *An. gambiae* was first described as antibacterial (Barillas-Mury *et al.*, 1999). The role of JAK/STAT pathway in anti-Plasmodial defence has been recently discovered (Gupta *et al.*, 2009). AgSTAT-A and AgSTAT-B are the two transcriptional factors identified in *Anopheles* mosquitoes (reviewed by Christophides *et al.*, 2004). In response to *Plasmodium* infection, AgSTAT-A mediates the transcriptional activation of nitric oxide synthase (**NOS**) in *An. gambiae*, which diminishes parasite development. The knockdown of AgSTAT-A results in an increase in oocyte number of *P. berghei* and *P. falciparum* in infected mosquitoes (Gupta *et al.*, 2009). NOS catalyses the production of nitric oxide from L-arginine. Nitric oxide has antimicrobial activity and plays a role in innate immune system (Clark *et al.*, 1996). In *An. aquasalis*, the JAK/STAT pathway controls *P. vivax* development in the early stage of infection (Bahia *et al.*, 2011).

Although these immune signalling pathways are known to contribute to anti-Plasmodial defence, the immune evasion strategies of the parasite make the mosquito-borne diseases a burden on the human population. Each of the pathways are initiated by different PAMPs and as such induces different subsets of AMPs (Salcedo-Porras *et al.*, 2019).

4.1.5. Antimicrobial peptides (AMPs): crucial effector molecules of the innate immune system

The AMPs are encoded in the genome and are expressed due to the activation of transcriptional factors and signal transduction pathways upon pathogen recognition (Boman, 2003). AMPs are the main effector molecules of the Toll, IMD and JAK/STAT pathways (Salcedo-Porras et al., 2019). Although AMPs are strongly expressed in the fat body, the expression in the gut and reproductive tracts has also been reported (Tzou *et al.*, 2000). Insect AMPs consist of less than 100 amino acids and are often positively charged. These positively charged AMPs target the negatively charged membrane of pathogens for disruption (reviewed in Brogden, 2005). The hydrophobic portion of the AMP embeds in the cell membrane causing pore formation, which leads to leakage of the cell content and eventually death. AMPs can also alter the pathogen metabolism by targeting the cytoplasmic components (reviewed in Bechinger & Gorr, 2017). There are four types of AMPs reported in mosquitoes to date: Defensins, Cecropins, Attacin and Gambicin (Boman, 2003).

The first family of AMPs identified in mosquitoes was the Defensin family (Chalk *et al.*, 1994). These are small (38–45 amino acids), positively charged AMPs consisting of six cysteine residues that form three disulphide bonds. Their positive charge creates an electrostatic attraction towards the negatively charged cell membrane of the pathogen. The structure of Defensin contains an α-helix connected to two antiparallel β-sheets (Cornet *et al.*, 1995). The α-helix allows Defensins to embed into the cell membrane and cause pore formation (Fujii *et al.*, 1993). In mosquitoes, these peptides are strongly active against Gram-positive bacteria and less effective against Gram-negative bacteria (Lowenberger *et al.*, 1995).

There are four types of Defensin peptides identified in *An. gambiae*: Defensin-1, 2, 3 and 4 (Christophides *et al.*, 2002). Following a bacterial challenge, increased expression of *defensin* was observed in *An. gambiae* larvae and adults (Richman *et al.*, 1996). *Defensin* mRNA has been detected in the salivary glands, fat body cells and midgut of *An. gambiae* after *P. berghei* infection (Dimopoulos *et al.*, 1998). The anti-Plasmodial function of Defensin is not fully understood in the *Anopheles* mosquito. However, it affects the oocytes of *P. gallinaceum* (Shahabuddin *et al.*, 1998). This suggests that Defensin may suppress *Plasmodium* development. Dengue virus inhibits the expression of *defensin-A* in *Ae. aegypti* (Méndez *et al.*, 2021). Defensin has also been implicated in disease progression in the same species. This peptide promotes the Japanese encephalitis virus infection by downregulating the C6/36 cell-surface antiviral protein HSC70B (Liu *et al.*, 2021).

Mosquito Cecropins are also short (35–39 amino acids) peptides consisting of two α-helices linked by a short hinge. In contrast to Defensin, Cecropins lacks cysteine residues thus lack disulphide bonds (Steiner, 1982). The C-terminals of the α-helices is hydrophobic, and N-terminal is amphipathic. This allows Cecropins to cause pathogen death by pore formation in the cell membrane (Moore *et al.*, 1996). Cecropins also have anti-inflammatory properties (Wang *et al.*, 2018). Although Cecropins predominantly target Gram-negative bacteria, it targets Gram-positive bacteria as well. Four types of Cecropin peptides have been identified in *An. gambiae*: Cecropin-1, 2, 3 and 4. A typical feature of all insect Cecropins is a tryptophan residue at either position 1 or 2. However, the mosquito Cecropin lacks this feature (Christophides *et al.*, 2002). Using transgenic technology, *cecropin-A* expression was induced in *An. gambiae*. This resulted in a 60% reduction in *P. berghei* oocytes in comparison to the non-transgenic mosquitoes. (Kim *et al.*, 2004). *Cecropin-3* expression has also been observed in *An. albimanus* during *P. berghei* infection (Pavón *et al.*, 2014). Cecropin has also been demonstrated to have a protective function against a virus. The dengue virus inhibits the expression of *cecropin-A* in *Ae. aegypti* (Méndez *et al.*, 2021).

At 61 amino acids, Gambicin is by far the largest insect AMP. It was first discovered in *An. gambiae* and only occurs in mosquitoes. Like Defensin, Gambicin contains eight cysteine residues that allows the formation of four disulphide bonds. Like the other AMPs, the expression of *gambicin* is induced upon an infection in the fat-body cells, midgut, and haemocytes. Although its mode of action is unknown, it targets both Gram-positive and Gram-negative bacteria, filamentous fungi, as well as *P. berghei* for destruction (Vizioli *et al.*, 2001). Attacin is structurally similar to Cecropin. However, Attacin takes up a form of a random coil in an aqueous solution and helical form in a hydrophobic solution. Its strongly active against Gram-negative bacteria (reviewed in Yi *et al.*, 2014). Attacin inhibits *E. coli* growth by preventing the production of bacterial porins (Carlsson *et al.*,1991).

Despite the efficacy of the immune system, various pathogenic microorganisms evade the immune system. Immune evasion is a strategy used by a pathogen to hide from the host's immune system and continue to reproduce. Pathogens have developed multiple ways to evade the immune system, such as masking the PAMP and enzymatic cleavage of PRRs (reviewed in Rosbjerg *et al.*, 2017).

It has been demonstrated that both IMD and Toll pathways initiate the expression of *defensin-1*, *cecropin-1,* and *gambicin* in *An. stephensi* and *An. gambiae* upon *Plasmodium* infection

(Luna *et al.*, 2006). The immune response and AMP expression in *An. gambiae* have been extensively studied. However, there is a knowledge gap in understanding the immune responses in other members of the *An. gambiae* complex.

The aim of this chapter is to assess the expression of immune-related genes (AMPs and PRR) in *An. arabiensis*, *An. merus* and *An. quadriannlatus* in response to Gram-positive and Gram-negative bacterial challenges. This was achieved by the assessment of transcript levels using a qRT–PCR. The effect of insecticide resistant phenotype was also assessed.

4.2. MATERIALS AND METHODS

4.2.1. RNA extraction and cDNA synthesis

The RNA was extracted from the four control and immune stimulated laboratory strains: SENN, SENN DDT, MAFUS and SANGWE (Chapter 2, Figure 2.1). The Quick-RNA™ MiniPrep kit (Zymo Research: R1054) was used to extract RNA according to the manufacturer's instructions. The RNA extraction protocol is illustrated in Figure 2.2 and described in **Section 2.3**. The extracted RNA was utilised to generate 1000nM of cDNA in replicates of three, using the iScript™ cDNA Synthesis Kit (Bio-Rad: 1708891) according to the manufacturer's instructions. The kit uses a reverse transcriptase (**RT**) enzyme that catalyses the addition of nucleotides using random hexamers that are complementary to the RNA template. The reaction tubes were incubated in a thermal cycler (T100™ Thermal Cycler, Bio-Rad™) using the manufacturer recommended cycling conditions: priming at 25℃ (5min), followed by reverse transcription at 46℃ (20 min) and lastly at 95℃ (1 min) for RT inactivation.

4.2.2. Assessment of the mRNA transcript levels of immune-related genes

To examine the effects of immune stimulation on the expression of immune-related genes, the transcript levels of three genes were assessed. Of the three genes, two were AMPs*: defensin-1* (GenBank accession number: DQ212042; Eggleston *et al.*, 2000) and *gambicin* (GenBank accession number: FJ653776; Vizioli *et al.*, 2001), and one PRR gene *GNBP-B1* (GenBank accession number: XM_308984; Warr *et al.*, 2008). The three genes were amplified using the primer sequences detailed in Table 4.3 in a qRT–PCR at a concentration of 3µM. The 26S and 18S genes were used as reference genes, with the primer sequences detailed in Table 4.3. From the cDNA synthesised in **Section 4.2.1**, 100nM was used as a template for the qRT-PCR reaction in SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad: 1725270) according to the manufacturer's instructions. The supermix contains a Sso7d fusion polymerase that

catalyses the polymerisation, deoxynucleotides (**dNTPs**) to generate the qRT–PCR product, and magnesium chloride (**$MgCl_2$**) to reduce the repulsion between the primers. Detection was based on the SYBR® Green I dye in SsoAdvanced™ Universal SYBR® Green Supermix, which binds to the double stranded DNA. The reactions were performed in a CFX96™ Real-Time system (C1000 Touch™ Thermal Cycler: Bio-Rad) with the following cycling conditions recommended by the manufacturer: DNA denaturation at 98°C (3 min) followed by 40 cycles at 95°C (10 sec) with a final extension at 60°C (30 sec), as instructed by the manufacturer.

Table 4. 3: Primer sequences of the target and reference genes

Primer name	**Primer sequence**
***defensin-1* forward primer**	5′ GGA CAA CTA GGA AGG ACA AAC A 3′
***defensin-1* reverse primer**	5′ ACG GTA GAG TCC TGA GGT AAA 3′
***gambicin* forward primer**	5′ TTT GCT GCT CGG TTG AGT 3′
***gambicin* reverse primer**	5′ TGC AGT CCT CAC AGC TAT TG 3′
***GNBP-B1* forward primer**	5′ GGA CCA GTT TGG GCT AGA TT 3′
***GNBP -B1* reverse primer**	5′ATT CCC GAT TCG AAG GTT ATC A 3′
***18S* forward primer (Reference gene)**	5′ TAC CTG GGC GTT CTA CTC 3′
***18S* reverse primer (Reference gene)**	5′ CTT TGA GCA CTC TAA TTT GTT C 3′
***26S* forward primer (Reference gene)**	5′ GAT AAG GCA ATC AAG AAG TTC G 3′
***26S* reverse primer (Reference gene)**	5′ TAC GGA CAA CCT TCG AGT GG 3′

Note: primers were used at a concentration of 3µM

4.2.3. Statistical analysis

Data generated from qRT–PCR were analysed using the Pfaffl method (Pfaffl *et al.*, 2002). Differences in transcript levels were assessed by One-Way ANOVA using Bio-Rad CFX maestro™ software**.**

4.3. RESULTS

4.3.1. Constitutive levels of AMP transcripts

Constitutive *defensin-1* transcript levels differed between the strains. *Defensin-1* transcript levels were significantly higher in MAFUS and SANGWE relative to SENN, by 11.6 and 4.2-fold respectively (**One-Way ANOVA: $p < 0.001$, $F_{(3, 31)} = 10.4$**) (Figure 4.2A).

There was no significant difference in the basal *defensin-1* expression between SENN and SENN DDT (**One-Way ANOVA: p = 0.995, $F_{(1, 17)}$ = 1.46**). Similarly, *gambicin* transcript levels in SENN DDT were not significantly different from that of SENN (**p = 0.995, $F_{(1, 17)}$ = 1.46**). Like *defensin-1*, constitutive *gambicin* transcript levels were also significantly higher in MAFUS and SANGWE relative to SENN, by 7.4 and 2.4-fold respectively (**One-Way ANOVA: P < 0.001, $F_{(3, 56)}$ = 40.3**) (Figure 4.2B). There was no difference in constitutive levels of *GNBP-B1* transcripts between SENN, SENN DDT, MAFUS and SANGWE (**p = 0.173, $F_{(3, 32)}$ = 1.78**) (Figure 4.5A).

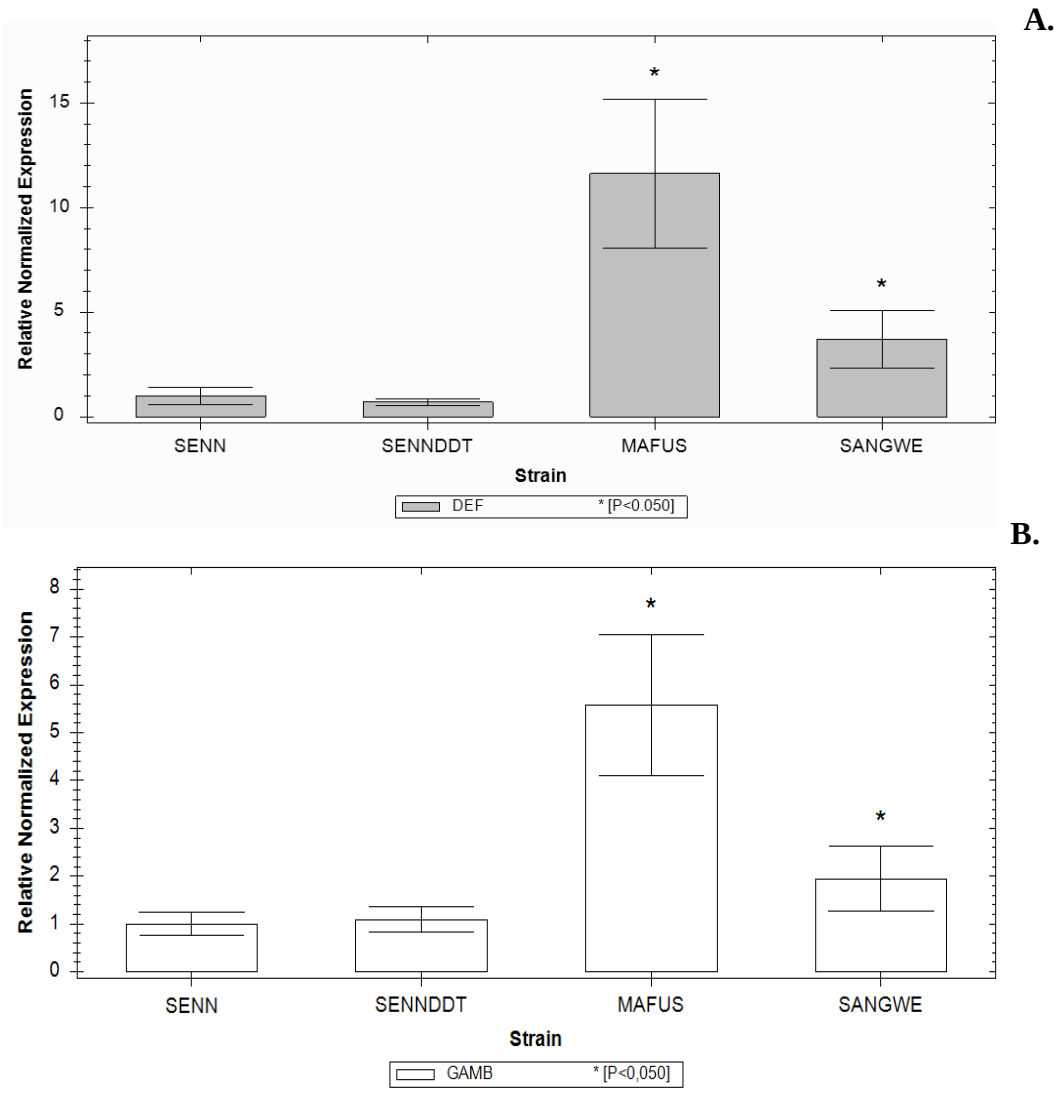


Figure 4. 2: Relative normalised constitutive transcript levels of *defensin-1* and *gambicin*. (A) Relative *defensin-1* (DEF) expression levels. (B) Relative *gambicin* (GAMB) expression levels. SENN – insecticide susceptible *An. arabiensis*, SENN DDT – insecticide resistant *An. arabiensis*, MAFUS – *An. merus*, SANGWE – *An. quadriannalatus.* Significant differences in expression levels relative to SENN are indicated by asterisks (*).

4.3.2. The effect of Gram-negative bacterial challenge on *defensin-1* and *gambicin* transcript levels

Upon stimulation with Gram-negative bacteria, *defensin-1* transcript levels in MAFUS and SANGWE were significantly increased by 43.9 and 10.2-fold respectively, relative to SENN (**One-Way ANOVA: $p < 0.001$, $F_{(3, 43)} = 30.8$**) (Figure 4.3A).

By contrast, *defensin-1* transcript levels in SENN DDT were significantly reduced by 3.1-fold relative to SENN (**One-Way ANOVA: $p = 0.028$, $F_{(1,26)} = 30.8$**). *Gambicin* transcript levels in MAFUS remains significantly higher than SENN, by 4.3-fold post Gram-negative bacterial challenge (**$p = 0.001$, $F_{(3, 28)} = 10.0$**) (Figure 4.3B).

The *gambicin* transcript levels in SANGWE were not significantly different from that of SENN (**One-Way ANOVA: $p = 0.629$, $F_{(1,16)} = 2.1$**). In contrast to *defensin-1*, *gambicin* transcript levels in SENN DDT were increased by 2.1-fold relative to SENN (**$p = 0.008$, $F_{(1, 13)} = 30.8$**). *GNBP-B1* transcript levels were not significantly different between the four strains post Gram-negative bacterial challenge (**$p = 0.426$ F, $_{(3, 30)} = 0.956$**) (Figure 4.5B).

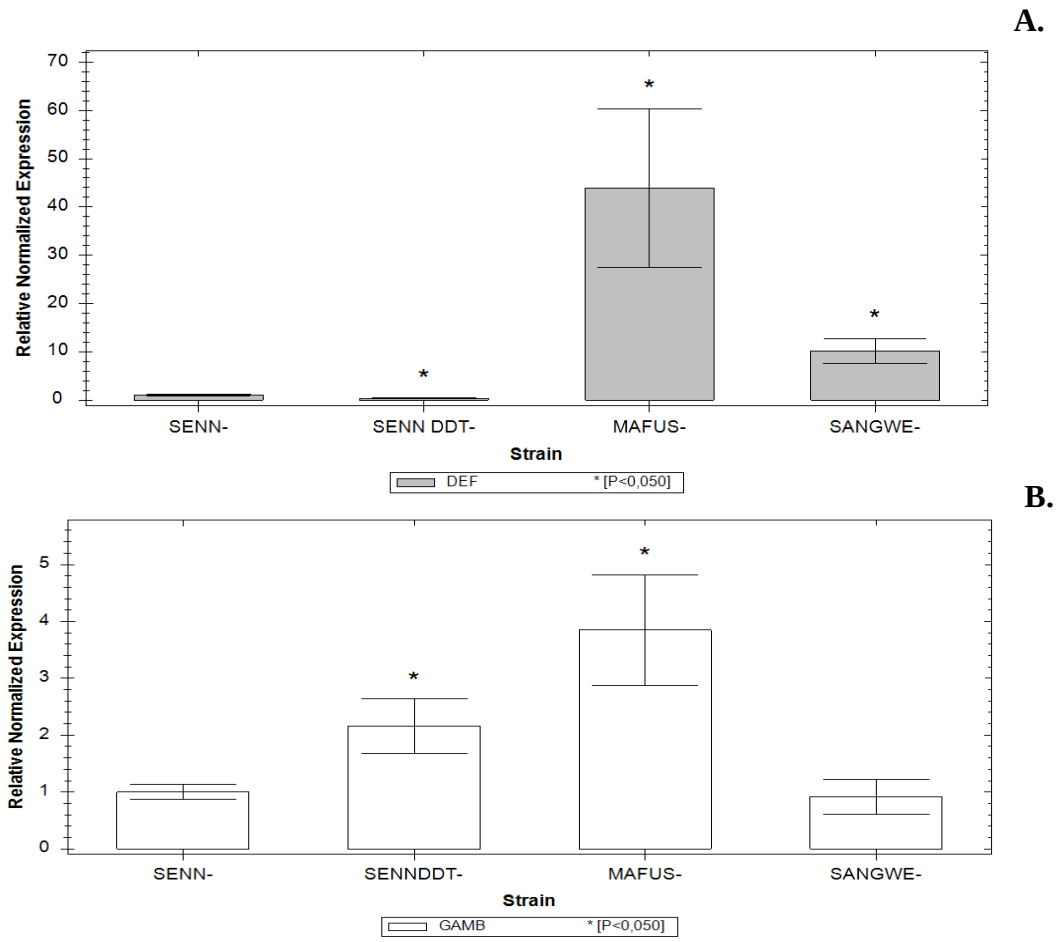


Figure 4. 3: Relative normalised transcripts levels of *defensin-1* and *gambicin* post Gram-negative bacterial challenge**.** (A) Relative *defensin-1* (DEF) expression levels. (B) Relative *gambicin* (GAMB) expression levels. Gram-negative bacteria – *E. coli*. SENN – insecticide susceptible *An. arabiensis*, SENN DDT – insecticide resistant *An. arabiensis*, MAFUS – *An. merus*, SANGWE – *An. quadriannalatus.* Significant differences in expression levels relative to SENN are indicated by asterisks (*).

4.3.3. The effects of Gram-positive bacterial stimulation on *defensin-1* and *gambicin* transcript levels

The Gram-positive bacterial challenge significantly increased *defensin-1* transcript levels in MAFUS and SANGWE relative to SENN, by 14.2 and 6.7-fold respectively (**One-Way ANOVA: $p = 0.002$, $F_{(3, 21)} = 7.30$**) (Figure 4.4A). Similar to Gram-negative bacteria, *defensin-1* transcript levels in SENN DDT were significantly reduced by 9.3-fold relative to SENN (**$p = 0.004$, $F_{(1, 9)} = 1.23$**).

Gambicin transcript levels in MAFUS were significantly increased by 3-fold post Gram-positive bacterial challengerelative to SENN (**One-Way ANOVA: $p < 0.001$, $F_{(3, 32)} = 14.6$**) (Figure 4.4.B). Like *defensin-1*, *gambicin* transcript levels in SENN DDT were significantly reduced by 1.7-fold relative to SENN (**$p = 0.034$, $F_{(1, 19)} = 8.9$**). Unlike *defensin-1*, *gambicin*

transcript levels in SANGWE was reduced by 2.1-fold relative to SENN (**p = 0.006, F $_{(1, 15)}$ = 9.83**). In contrast to Gram-negative bacterial challenge, *GNBP-B1* transcript levels were significantly reduced in SENN DDT, MAFUS and SANGWE by 3.3-fold, 2.3-fold and 12.6-fold respectively, relative to SENN post Gram-positive bacterial challenge (**p < 0.001, F $_{(3, 32)}$ = 56.73**) (Figure 4.5C).

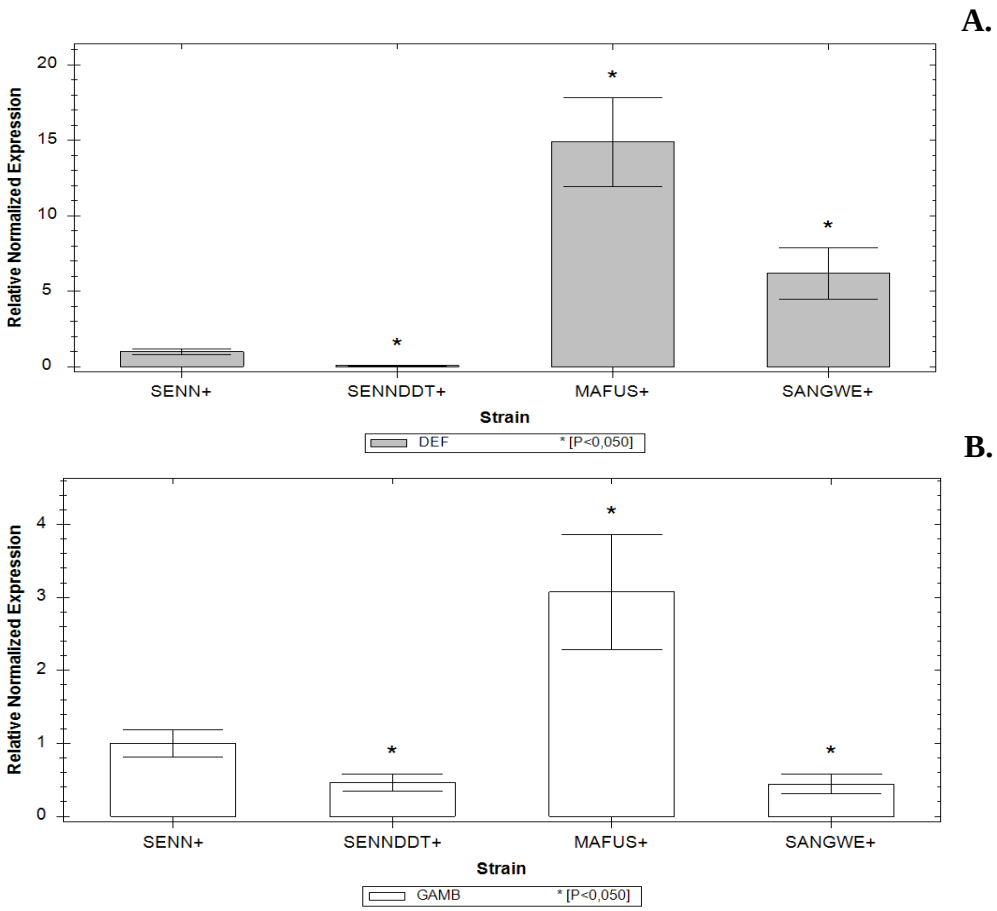


Figure 4. 4: Relative normalised transcript levels of *defensin-1* and *gambicin* post Gram-positive bacterial challenge**.** (A) Relative *defensin-1* (DEF) expression levels. (B) Relative *gambicin* (GAMB) expression levels. Gram-positive bacteria – *S. pyogenes*. SENN – insecticide susceptible *An. arabiensis*, SENN DDT – insecticide resistant *An. arabiensis*, MAFUS – *An. merus*, SANGWE – *An. quadriannalatus.* Significant differences in expression levels relative to SENN are indicated by asterisks (*).

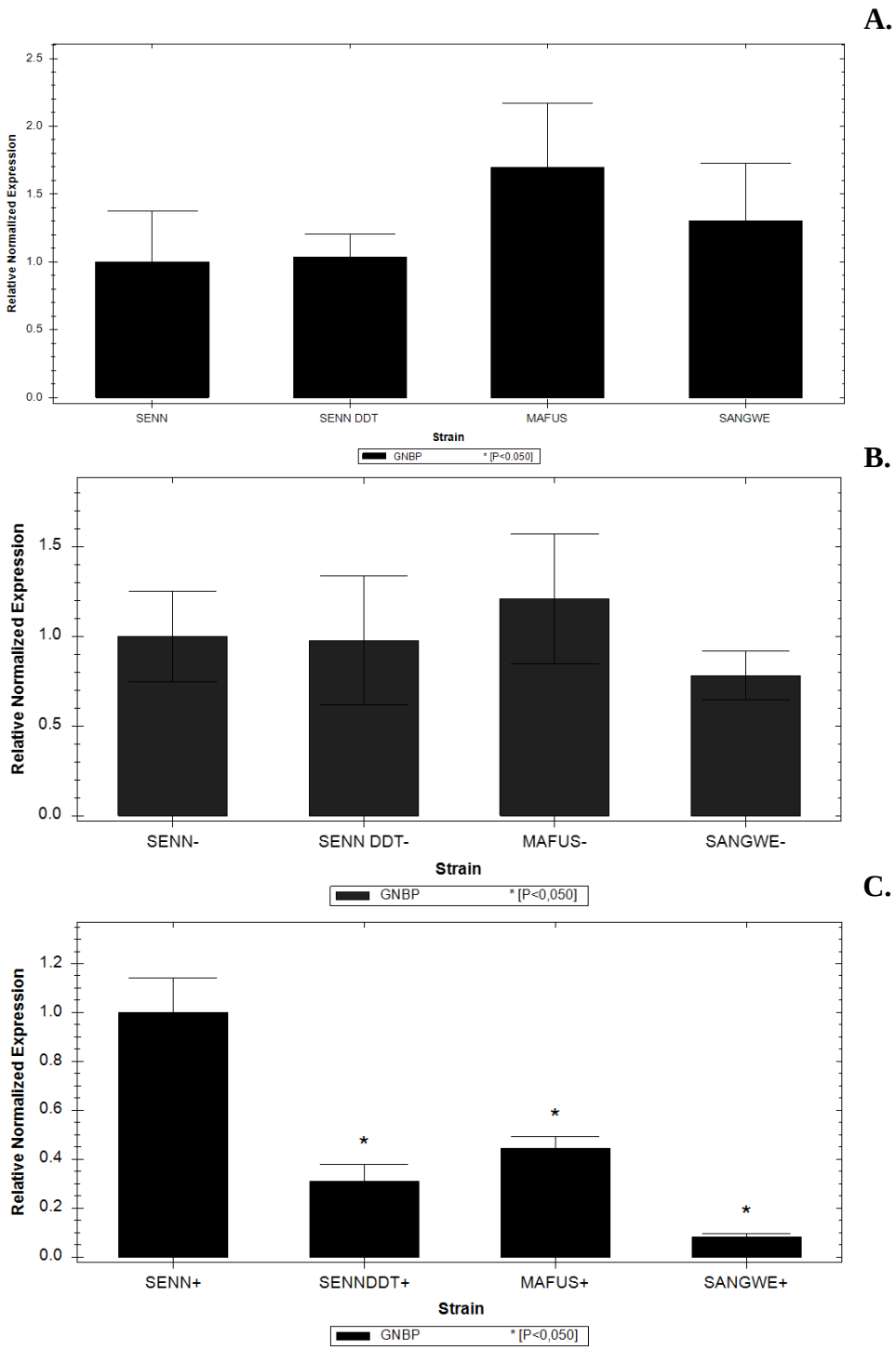


Figure 4. 5: Relative normalised transcript levels of basal *Gram-negative Binding Protein-B1* and post immune stimulation. (A) Relative basal expression levels. (B) Relative expression levels post Gram-negative (*E. coli*) bacterial challenge. (C) Relative expression levels post Gram-positive (*S. pyogenes*) bacterial challenge. SENN – insecticide susceptible *An. arabiensis*, SENN DDT – insecticide resistant *An. arabiensis*, MAFUS – *An. merus*, SANGWE – *An. quadriannalatus.* Significant difference in expression levels relative to SENN are indicated by asterisks (*).

4.4. DISCUSSION

In the previous chapter, the effect of bacterial challenge on the epigenetic landscape of the members of the *Anopheles gambiae* complex was assessed. These findings, however, did not give direct information on the expression of immune-related genes. This chapter focused on assessing the effect of bacterial challenge on the transcription of immune-related genes. These experiments examined the effects of Gram-positive and Gram-negative bacterial challenges on the upstream molecules of immune signaling pathways by assessing the transcript levels of *GNBP-B1*, which is a PRR. These experiments also examined the transcript levels of two effector molecules downstream of the immune signaling pathways, the AMPs *defensin-1* and *gambicin*.

The PRR GNBP-B1 is associated with defence against *E. coli* and *S. aureus* in *An. gambiae* (Warr *et al.*, 2008). The *GNBP-B1* gene is expressed in the midgut at ookinete invasion and in the salivary glands upon sporozoite invasion (Richman *et al.*, 1997; Rosinski-Chupin *et al.*, 2007). As such, the expression of this PRR would be informative about the putative immune response to the parasite. There was no constitutive difference in *GNBP-B1* expression, nor does the Gram-negative bacterial challenge result in a change in expression between the four strains. The Gram-positive bacterial challenge, however, resulted in a change in *GNBP-B1* expression. Relative to the insecticide susceptible *An. arabiensis* (SENN), all three strains had reduced *GNBP-B1* expression. There was a difference in expression in the insecticide susceptible *An. arabiensis* strain SENN and the resistant counterpart SENN DDT with the latter having a significant decrease in expression.

Furthermore, it is worth noting that *GNBP-B1* expression was higher after both Gram-positive and Gram-negative bacterial challenges in comparison to constitutive expression levels. In SENN and SENN DDT, *GNBP-B1* expression was decreased after the Gram-negative bacterial challenge and increased after the Gram-positive bacterial challenge compared to constitutive expression levels. In contrast, both bacterial challenges reduced *GNBP-B1* expression in SANGWE compared to constitutive expression levels. GNBP-B1 appears to be active against both Gram-positive and Gram-negative bacteria. Therefore, the species-specific differences in changes in *GNBP-B1* expression are noteworthy. However, there seems to be no congruency between the expression of this gene and the potential immune response to a parasite. As such, this PRR does not appear to be related to species-specific immune responses that would be relevant to vector competence. An infectious sugar-meal with either Gram-positive (*S. aureus*) or Gram-negative (*E. coli*) bacteria results in an increased expression of *cecropin* in the malaria

vector *An. coluzzii* (Bevivino *et al.*, 2021). Therefore, it is worth examining the responsiveness of downstream effector molecules such as AMPs to bacterial challenge in the other members of the *An. gambiae* complex.

Defensins are induced by both the Toll and IMD pathways (Tanji *et al.*, 2007). They also have anti-Plasmodial activity against oocysts and sporozoites (Shahabuddin *et al.*, 1998). This study highlights several significant findings of the relative expression of *defensin-1*, as well as *gambicin*, between the four strains.

There is a concern about whether insecticide resistance increases the capacity to transmit malaria parasites. This is a pertinent question with the increased burden of insecticide resistance (Minetti *et al.*, 2020). However, this data is relatively limited. The few studies available on the effect of insecticide resistance on parasite development have demonstrated contrasting effects in parasite prevalence. Studies have also demonstrated the effect of insecticide resistance on the proportion of treated mosquitoes successfully infected, parasite intensity, the number of oocysts and sporozoites in infected mosquitoes. In *An. gambiae*, kdr and *ace-1* heterozygotes had increased prevalence, but reduced intensity of the parasites (Collins *et al.*, 2019). In contrast, the L119F-GSTe2 mutation, which confers metabolic resistance was linked to reduced oocyst prevalence but higher intensity in laboratory-reared *An. funestus* (reviewed by Minetti *et al.*, 2020). Although this suggests that insecticide resistance may be associated with increased transmission, it is worth noting the findings of Lo & Coetzee, 2013. Their study of a *P. berghei* model showed that the insecticide susceptible *An. funestus* strain had some of the highest sporozoite levels in the field of research for this model (Lo & Coetzee, 2013). This highlights the complexity of the interaction of the resistance phenotype, which could have a number of genetic underpinnings, and the immune response to the parasite.

The findings on the expression of both *defensin-1* and *gambicin* AMPs genes indicate a lack of difference in expression between SENN and SENN DDT at the constitutive level. However, changes in expression occurred after the bacterial challenge. This suggests that differences in the immune response in these strains are due to differences in the capacity to induce the immune system rather than differences in the basal immune capacity. SENN DDT had significantly lower *defensin-1* expression after both Gram-positive and Gram-negative bacterial challenges. This is congruent with previous studies, as SENN DDT mediates the resistance phenotype by both target-site resistance (L1014F mutation) as well as metabolic resistance. (Oliver &

Brooke, 2013). Therefore, the reduced *defensin-1* expression in SENN DDT could be expected. The increased parasite burden previously observed in insecticide resistant strains may be a result of reduced *defensin-1* expression in those strains (Minetti *et al.*, 2020). It is worth noting that laboratory-reared insecticide resistant *An. gambiae* expresses *defensin* and *cecropin* genes at a higher level than its insecticide susceptible counterpart (Vontas *et al.*, 2005). Unlike this study, however, the strains compared were not genetically related. Similarly, a laboratory-reared insecticide resistant *Culex pipiens* strain showed an increase in expression of AMPs (*cecropin-B* and *gambicin*) post immune stimulation with *E. coli* (Vézilier *et al.*, 2013).

Like Defensin, the mosquito specific Gambicin also has anti-Plasmodial activity. It has lethal effects on ookinetes (Vizioli *et al.*, 2001). Gambicin may have evolved to cope with either the malaria parasite or the mosquito's microbial flora. It also has antimicrobial activity in addition to the anti-Plasmodial activity (Dimopoulos, 2003). Like with *defensin-1*, Gram-positive bacterial challenge resulted in a reduced expression of *gambicin* in the SENN DDT strain. The Gram-negative bacterial challenge, however, resulted in an increased expression of *gambicin* in the SENN DDT relative to SENN. Despite this result, the generalised finding is that immune stimulation resulted in the reduced expression of immune-related genes in SENN DDT. Although these findings add evidence to the previous findings of increased parasite burden of insecticide resistant anophelines (Minetti *et al.*, 2020). It should be noted that decreased AMP expression in the resistant strain is not always guaranteed. What is clear, however, is that the insecticide resistance phenotype results in an altered immune response in the two *An. arabiensis* strains of the same genetic background.

When examining the AMP expression in the minor and non-vector strains, another pattern emerges. Both the minor vector MAFUS and non-vector SANGWE typically have higher levels of AMPs expression, even at the constitutive level. This pattern continues with *defensin-1* expression after both Gram-positive and Gram-negative bacterial challenges. However, this pattern only holds true for *gambicin* expression in MAFUS, the pattern breaks down for *gambicin* expression in SANGWE. Although SANGWE had significantly higher *gambicin* expression relative to SENN at the constitutive level, there was no difference after the Gram-negative bacterial challenge. After the Gram-positive bacterial challenge, SANGWE had significantly reduced *gambicin* expression relative to SENN. It has previously been demonstrated that interspecies nucleotide variation in *defensin* tends to be synonymous. Furthermore, the functionality of the peptide did not vary in members of the *An. gambiae* complex that differed in vector competence (Simard *et al.*, 2007). This finding, coupled with

the results of this study, suggests that regulation at the mRNA level may be one of the regulatory mechanisms that results in immunological variation that may underpin differences in vector competence.

Taken together, there is a marked difference in the expression of immune-related genes in these closely related species of the *An. gambiae* complex. *Defensin-1* expression is remarkably congruent with the capacity of the strains to transmit the parasite. The high levels of AMP expression in the minor vector MAFUS possess challenges to the hypothesis that AMP expression, *defensin-1* in particular, is related to vector competence. If the hypothesis was consistent, SANGWE would have the highest AMPs expression. This may be an artefact due to the rearing of the *An. merus* strain in the laboratory, as the strain is reared at a salt concentration of 50% seawater. As several AMPs, including the Defensins CADEF1 and PDF1.2, have been demonstrated to be involved in osmo-tolerance (Kido *et al.*, 2010). These findings raise the question of larval salinity exposure and the immune response in *An. merus*.

This study suggests a difference in immune responsiveness in both insecticide susceptible and resistant *An. arabiensis*, as well as between *An. merus* and *An. quadriannulatus*. More research, however, is required to confirm these findings. The first is that the experiment must be replicated with material obtained from the wild. This would confirm that the findings are not an artefact of colonisation. Furthermore, transcript levels do not guarantee that changes will be observed at the protein level. Numerous post-translation modifications, changes in protein stability and trafficking, could cause changes in protein expression levels. All these factors could result in differences in AMP activity levels that cannot be detected at the transcription level. These findings therefore need to be confirmed by protein expression studies.

In conclusion, there is a marked difference in the expression of AMPs after immune stimulation in all four strains. Relative to SENN, SENN DDT typically had lower expression of AMPs, while MAFUS and SANGWE had higher expression. The differences between the two *An. arabiensis* strains are not constitutive, while the differences with MAFUS and SANGWE are. The differences do not appear to be mediated upstream of the initiators of the Toll and IMD pathways, but the most marked changes are downstream. The expression of *defensin-1* is most congruent with vector competence of the strains. The particularly high levels of AMPs expression in the *An. merus* strain may be related to its rearing in a high salt concentration. This study suggests a difference in AMP expression in the zoophilic members of the *An.*

gambiae complex. These differences have the potential to be related to the vector competence of these species.

CHAPTER 5: Characterisation of immunological memory in insecticide susceptible and resistant strains of *Anopheles arabiensis*

5.1. INTRODUCTION

The immune system functions to defend an organism from pathogenic microorganisms and foreign molecules (Cohen, 2000). All living organisms possess an innate immune system, but the adaptive immune system only appears in chordates, from jawless fishes and onward (reviewed in Rimer *et al.*, 2014). The innate immune system exists from birth as it is genetically coded. However, the adaptive immune system, which is also known as the acquired immune system, is refined throughout the lifespan of an organism due to the exposure to various pathogens. In contrast to the innate immune system, the acquired immune system is highly specific for a particular pathogen (reviewed in Parkin & Cohen, 2001).

5.1.1. The features of the adaptive immune system

Immunological memory is one of the key features of the adaptive immune system. It allows the immune system to respond rapidly and effectively to a pathogen that it has previously encountered. The information about the pathogen is stored, and upon re-exposure, it is accessed to generate a faster immune response (reviewed in Zinkernagel *et al.*, 1996). This capacity is mediated by clonal expansion of the specific T-cell and B-cell lymphocytes upon pathogen recognition, chromatin rearrangement and further epigenetic reprogramming (reviewed in Netea *et al.*, 2019).

Chromatin rearrangement is the process of dynamic remodelling of the DNA sequence, breaking, joining, or copying segments of the chromosome. This process leads to novel combinations of alleles, which are also inherited by the daughter cells (reviewed in Clancy, 2008). Gene recombination processes in the gene family of immunoglobulins (**Igs**) and T-cell receptors (**TCRs**) contributes greatly to the formation of immunological memory. Three types of gene segments: variable (**V**), diversity (**D**), and joining (**J**), are assembled into one V-D-J exon by breaking and joining a gene. The process of gene recombination is catalysed by recombination-activation gene (**RAG**) enzymes (reviewed in Roth, 2014). The lymphocytes with the novel combination of genes undergoes clonal expansion through the recognition of their specific antigen (reviewed in Campos & Godson, 2003). The clonal expansion also includes the memory cells, which makes the immune response more effective and faster during re-exposure.

Epigenetic reprogramming allows for the differential functioning of a cell without changing the genomic sequence. Several epigenetic mechanisms are crucial for regulating gene expression, as discussed in **Chapter 3**. The changes induced by the epigenetic mechanisms upon an infection are heritable, and as such, they are passed on to the daughter cells during cell division (reviewed in D'Urso & Brickner, 2014). Therefore, the lymphocytes pass on their genetic/epigenetic alterations in response to an infection to their daughter cells, thus creating memory. Epigenetic reprogramming also controls the clonal expansion of the lymphocytes, which is a crucial process for memory generation (Abdelsamed *et al.*, 2017).

Another hallmark of the adaptive immune system is specificity. This is the capacity of the immune system to target specific pathogenic antigens. Specificity is important for memory generation, as the adaptive immune system generates memory for specific antigens (Janeway *et al.*, 2001). The V-D-J recombination not only generate memory but also results in millions of B-cell receptors (**BCR** – transmembrane Ig) and TCR variants that can recognise and neutralise millions of various antigens (reviewed by Campos & Godson, 2003).

5.1.2. The generation of immunological memory

Mosquitoes, like most invertebrates, only have the innate immune system. This is due to their shorter lifespan and short generation time. As such, they circumvent the metabolic demands of the adaptive immune system (reviewed in Janeway & Medzhitov, 2002; Ganeshan & Chawla, 2014). As the innate immune system is genetically encoded, it is limited to genomic mutations and allelic variations for diversity (Janeway *et al.*, 2001). Genomic mutations might occur over a long period, because of the continual immune stimuli with the same pathogen (Loewe, 2008). Therefore, upon a new immune stimulus, the invertebrate species might be eliminated before the genomic change occurs. This raises the question, how do invertebrates cope with repeated immunological challenges? This would be important for scatophagous insects, which are at constant risk of infection from consuming dung, as well as hematophagous insects, which may pick up pathogens in their blood-meal (Rimer *et al.*, 2014). Although invertebrates lack the components associated with the adaptive immunity, there must exist a capacity of acquired immunity that allows the species to adapt and evolve to survive.

Until recently, it was believed that immunological memory was limited to organisms with an adaptive immune system. However, there is ongoing evidence of immunological specificity and memory in multiple invertebrate species that is equivalent to the acquired immune system of vertebrates (Little *et al.*, 2005).

Although the exact mechanism of innate immunological memory generation is unknown, there are a few proposed mechanisms. The Toll and IMD pathways may be "trained" to respond faster by long-lasting upregulation of the pathways (Boutros *et al.*, 2002). This could be achieved by the changes in the phenotype of innate immune cells, which may be induced by the pathogen, or increased expression of PRRs after the first exposure. The increased expression of PRRs upon an infection may be followed by the PRR molecules remaining stable after the infection is cleared (Steiner, 2004; Rodrigues *et al.*, 2010). There is also evidence that the Downs Syndrome cell adhesion molecule (**Dscam**) can be alternatively spliced to generate a broad range of PRRs. As such, this molecule is analogous to the vertebrate immunoglobulins, and it contributes to the generation of innate immunological memory. Dscam also functions to provide specificity to the innate immune system, which is an attribute of the adaptive immune system. The capacity to diversify initially invariant PRRs is an advantage when coping with parasites like *Plasmodium* that rapidly alters their surface antigens to avoid immune detection (Kurtz, 2005). The fact that innate immunological memory can potentially be long-term and can be passed on to the next generation (Little *et al.*, 2003) suggests an epigenetic aspect to the generation of this memory. This is particularly true for histone modifications, where methylation and acetylation have been associated with the generation of immunological memory (Boraschi & Italiani, 2018). There are, however, still substantial gaps in the understanding of how innate immunological memory is generated, as well as on the process as a whole.

The term immune priming is often used interchangeably with immunological memory to describe increased immune response/protection to a particular pathogen upon re-exposure. Immune priming, however, refers to the induction of immunological memory, which occurs as a result of the activated innate immune system (Boraschi & Italiani, 2018). The term priming comes from the initial observations in vertebrates, where the leukocytes primed by an initial stimulus displayed higher reactivity to unrelated stimulus. In this chapter, the terminology proposed by Boraschi & Italiani, 2018 is used. Innate immune memory is referred to as immunological memory. The induction of this memory is referred to as immune priming. Memory-induced decreased responsiveness to a challenge is referred to as tolerance. Memory-induced increased responsiveness to a challenge is referred to as potentiation (Boraschi & Italiani, 2018).

From the terminology, it is clear that immune priming can result in either an increased or decreased responsiveness to the pathogen. Increased tolerance would ensure the survival of the

host organism upon chronic exposure to the pathogen, by preventing tissue damage. Potentiation mechanisms would ensure pathogen elimination (reviewed by Cavaillon & Adib-Conquy, 2007).

5.1.3. Immunological memory in insects

The evidence for the acquired immunity in invertebrates largely comes from the immune priming experiments. Short-term memory specific to the pathogen encountered previously has been reported in many invertebrate organisms such as sponges, cnidarians, and insects (Hildemann *et al.*, 1980; Hartman & Karp, 1989; Salter-Cid & Bigger, 1991). The largest frame of evidence on immunological memory and specificity in invertebrates comes from the arthropod insects. Initial evidence of immunological memory in insects was characterised in an American cockroach (*Periplaneta americana*) using Gram-negative bacteria. After the first few days of priming, the cockroach showed increased tolerance against the bacteria it was primed with, as well as two other Gram-negative bacterial species. After a few days of immune priming, it showed improved protection against the bacteria used to prime than the other bacteria (Faulhaber & Karp, 1992). Similar results of immune tolerance were also observed in bumblebees (*Bombus terrestris*) post immune priming with bacteria (Sadd & Schmid-Hempel, 2006). Several other studies in lepidopterans, dipterans, hymenopterans, coleopterans, and many other insect orders have demonstrated this phenomenon (reviewed by Milutinović *et al.*, 2016).

The mosquito-*Plasmodium* system of *An. gambiae* is most extensively studied amongst other mosquitoes and dipteran insects. *Anopheles gambiae* exposed to *P. berghei* showed an increased tolerance to the parasite upon re-exposure. Immune priming is triggered by the *P. berghei* ookinete midgut invasion, which establishes a close contact between the gut microbiota and the epithelial cells of the midgut (Rodrigues *et al.*, 2010). This releases the haemocyte differentiation factor (**HDF**) by the epithelial cells. HDF consist of a Lipoxin/Lipocalin complex that increases the number of circulating haemocytes (Ramirez *et al.*, 2015). A recent study has also demonstrated another molecule that contributes to immune tolerance in *An. gambiae* mosquitoes. Prostaglandin E2 released by the epithelial cells of the gut attracts the circulating haemocytes to the surface of the midgut (Barletta *et al.*, 2019).

Apart from *An. gambiae,* this phenomenon has been demonstrated in *An. albimanus.* Mosquitoes of this species primed with *P. berghei* displayed an increased expression of AMPs (Contreras-Garduño, 2015). However, to date, there is no current literature on the induction of

immunological memory in the members of the *An. gambiae* complex apart from the nominal member of the group. As the transmission of the *Plasmodium* parasite to its human host is dependent on the successful development of the parasite in the mosquito, the existence of innate immunological memory may provide implications for combating malaria.

The aim of this chapter is to characterise the effect of immune priming in the major vector *An. arabiensis* of the *An. gambiae* complex, with reference to the insecticide resistance. This was achieved by the following objectives:

- To assess the efficacy of immunological memory induction by comparison of the delivery mechanism of immune stimulation:
    - Assessment of immune tolerance by measuring the longevity of young mosquitoes primed with bacteria-infected sugar.
    - Assessment of immune tolerance by measuring the longevity of young mosquitoes primed with bacteria-infected blood.
- To characterise the transgenerational effects of immune priming by assessing:
    - The effect of larval immune priming on the fertility and fecundity of mosquitoes
    - The transgenerational capacity of the mosquitoes to eliminate Gram-negative bacteria by determining the number of viable bacteria in the gut.
    - The effect of larval immune priming on bacterial tolerance, measured as longevity of the second-generation mosquitoes continuously exposed to bacteria.

5.2. MATERIALS AND METHODS

The two strains of *An. arabiensis*, insecticide susceptible (SENN) and insecticide resistant (SENN DDT) were reared in the Botha de Meillon insectary as described in **Section 2.1.1** (Chapter 2). Immune priming was performed using the same bacterial species used in the previous chapters. *Streptococcus pyogenes* (ATCC 19615) was used to represent Gram-positive bacteria and *Escherichia coli* (ATCC 25922) was used to represent Gram-negative bacteria. The bacteria were cultured as described in **Section 2.2.1** (Chapter 2). The bacteria were primarily supplemented in 10% sucrose meals at a concentration of 0.3% in 15 ml (Barnard *et al.*, 2019) and supplemented in blood where stated. All the exposures were performed in triplicate (50 female mosquitoes per replicate) with the three cohorts, each arising from three separate egg batches.

5.2.1. Assessment of the delivery mechanism of immune stimulation in adult mosquitoes

To assess whether non-commensal bacteria ingested via a sugar-meal early in life affect the tolerance to the bacteria later in life, the following experiment was performed (Figure 5.1). Adult female mosquitoes from the SENN and SENN DDT strains were collected upon emergence and were divided into two groups. The first group of mosquitoes (n=50) were provided with Gram-positive bacteria-infected sugar water and the second group (n=50) was provided with Gram-negative bacteria-infected sugar water. The mosquitoes were exposed to the bacteria for three days from emergence, thereafter, maintained on untreated sugar water until day 11. This constituted the sugar priming group. Adult mosquitoes provided with untreated sugar water from emergence constituted the unprimed (control) group (n=50). On day 11, the unprimed and the primed groups were exposed to the bacteria used for the initial priming. A group of females from both groups were exposed to sugar water only from day 11 and this served as an untreated control. For each strain, a group of females were only fed sugar throughout the experiment, and this served as an environmental control (n=50 for each group). Mortality was recorded daily, with cadavers removed daily until all the mosquitoes were dead. The experiment was performed in triplicate.

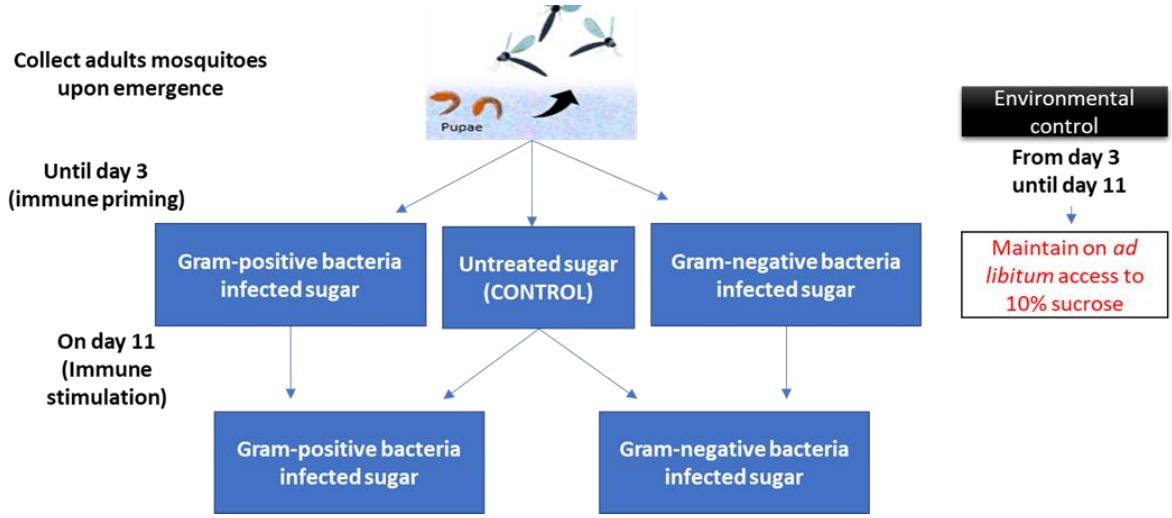


Figure 5. 1: Experimental setup to test the effect of an early life exposure to bacteria on tolerance to re-exposure later in life. Mosquitoes were exposed to bacteria upon emergence, with either Gram-positive bacteria (*S. pyogenes*) or Gram-negative bacteria (*E. coli*) supplemented in sugar. At day 11, the mosquitoes were exposed to the same bacteria used for priming. The unprimed control group of mosquitoes was split into two, with one being exposed to *S. pyogenes* and other being exposed to *E. coli*.

To assess whether non-commensal bacteria ingested via a blood-meal early in life affect the tolerance to the same bacteria later in life, the following experiment was performed (Figure

5.2). Adult male and female mosquitoes from SENN and SENN DDT were collected upon emergence. The males and females were kept together for normal mating. The mosquitoes were maintained on *ad libitum* access to 10% sucrose for three days. Prior to feeding with the bacteria-infected blood, the 3-day old mosquitoes were deprived of sucrose for three hours. This was to encourage feeding on the membrane. The females were either allowed to feed on Gram-positive bacteria-infected blood, Gram-negative bacteria-infected blood, or untreated blood. The blood was treated with 5ml bacteria grown to mid-log phase in 50ml of blood. Mosquitoes were fed for four hours, after which the fully fed females were removed from the cage and split into groups of 50. Each bacterial treatment was split into three groups, while the group fed untreated blood was split into four groups. After the meal, the females were provided with *ad libitum* access to 10% sucrose only until day 11. At day 11, another blood feed was set up in the same manner.

From each of the bacteria-treated groups, the first group was provided with a second infectious blood-meal, and this constituted the exposed primed group. The second group from the primed treatments was fed untreated blood to control for the effect of blood treatment. The third primed group was the group that received no further blood meals, termed the sugar control. The group that was initially only provided an untreated blood-meal constituted the unprimed group. From this group, the first two groups were provided with either a Gram-positive or Gram-negative bacteria-infected blood-meal. This constituted the unprimed exposed groups. The third unprimed group was provided with untreated blood to control for the effect of blood feeding. The final unprimed group was not provided with any further blood and constituted the unprimed sugar control. Where blood feeding occurred, the mosquitoes were fed to repletion. After the second feed on day 11, longevity was monitored until all females were dead. Cadavers were removed daily, with damp filter paper provided to allow egg laying twice a week for the duration of the experiment. The females were provided with *ad libitum* access to 10% sucrose for the duration of the experiment. All meals were provided with an artificial membrane feeder (Hemotek™) for four hours (Figure 5.2).

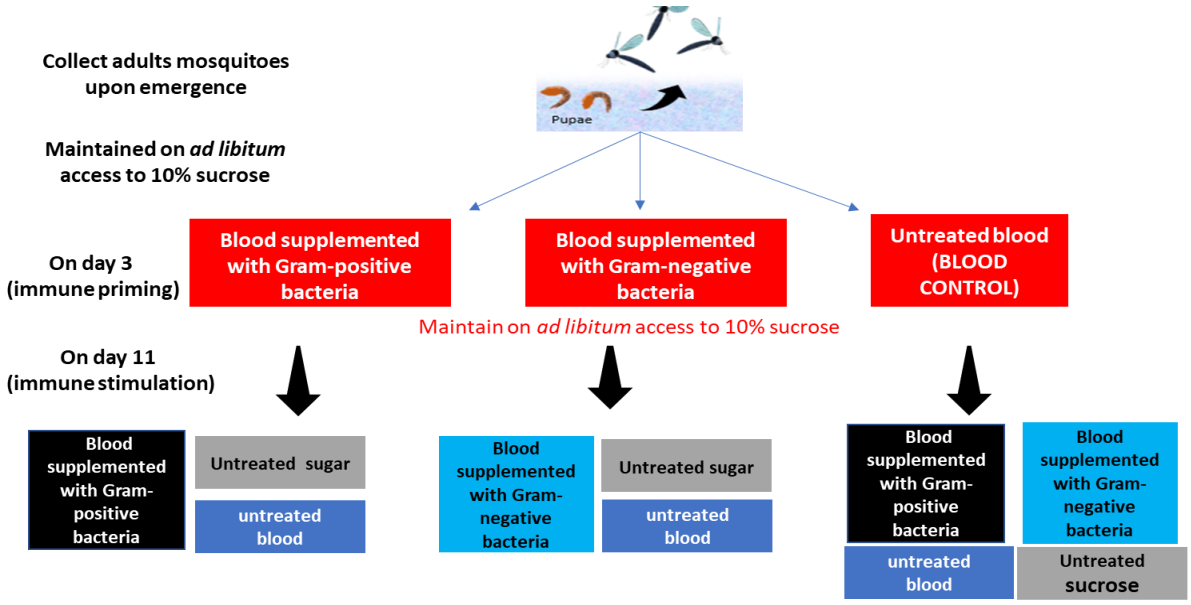


Figure 5. 2: Experimental setup to determine the effect of an infected blood-meal early in life on longevity after the second infected blood-meal later in life. The 3-day old mosquitoes were primed with either Gram-positive (*S. pyogenes*) or Gram-negative (*E. coli*) bacteria in defibrinated cow blood via Hemotek™. Untreated blood group served as the unprimed blood control. On day 11, the immune primed and unprimed mosquitoes were exposed to their respective bacteria (black and cyan blocks). The unprimed group control was separate for both bacteria. The 2nd (blue blocks) and 3rd (grey blocks) sub-groups were provided with untreated blood and untreated sugar respectively.

5.2.2. Evaluation of the transgenerational priming effects in SENN and SENN DDT

To assess whether the effect of immune priming is transgenerational and if it is affected by the insecticide resistance phenotype, the following set of experiments were performed. Firstly, the effect of larval immune priming on the fertility (percentage of eggs that hatch into larvae) and fecundity (total number of eggs produced) of the first-generation (**F1**) and second-generation (**F2**) of SENN and SENN DDT was assessed (Figure 5.3). Secondly, the Gram-negative bacteria used for larval immune priming the F1 SENN and SENN DDT were cultured from the midguts of F2 adult females, and the number of viable bacteria that could be recovered was determined. Finally, the bacterial tolerance of first and second generations of primed SENN and SENN DDT was assessed by measuring the longevity when exposed to bacteria-infected sugar.

To assess the effect of larval immune priming on the fertility and fecundity of SENN and SENN DDT, the F1 larvae were reared in bacteria-infected water (Figure 5.3). A total of 500 first instar larvae per treatment from both strains were reared in an initial dose of 10^6 colony forming units of the relevant bacteria. One group of the F1 larvae were reared in water infected with

Gram-positive bacteria and the other group was reared in water infected with Gram-negative bacteria. These two groups represented the primed groups. A group of F1 larvae were reared in untreated water to represent the unprimed control group. The F1 adult mosquitoes were collected upon emergence and each group was split into two sub-groups. One sub-group was used to obtain F2 mosquitoes, and the other sub-group was used to set up the experiments. To obtain the F2 mosquitoes, F1 adult mosquitoes were allowed to mate and thereafter provided with a Guinea pig-derived blood-meal on days three and seven. On day 11, the eggs of F1 mosquitoes were collected and reared in untreated reverse osmosis water. To set up the fertility and fecundity experiment, 40 males and 20 females of both F1 and F2 adult mosquitoes respectively, were transferred into separate experimental cages upon emergence (Samuel *et al*., 2020). The female mosquitoes were provided with a single Guinea pig-derived blood-meal on day three for egg production, thereafter, maintained on *ad libitum* access to 10% sucrose. On day seven, the egg plates were provided to lay eggs for 24 hours. A total of three days were provided for eggs to hatch, thus on day 11 the egg plates were frozen, the number of eggs and the number of larvae were counted. The experiment was performed in triplicate.

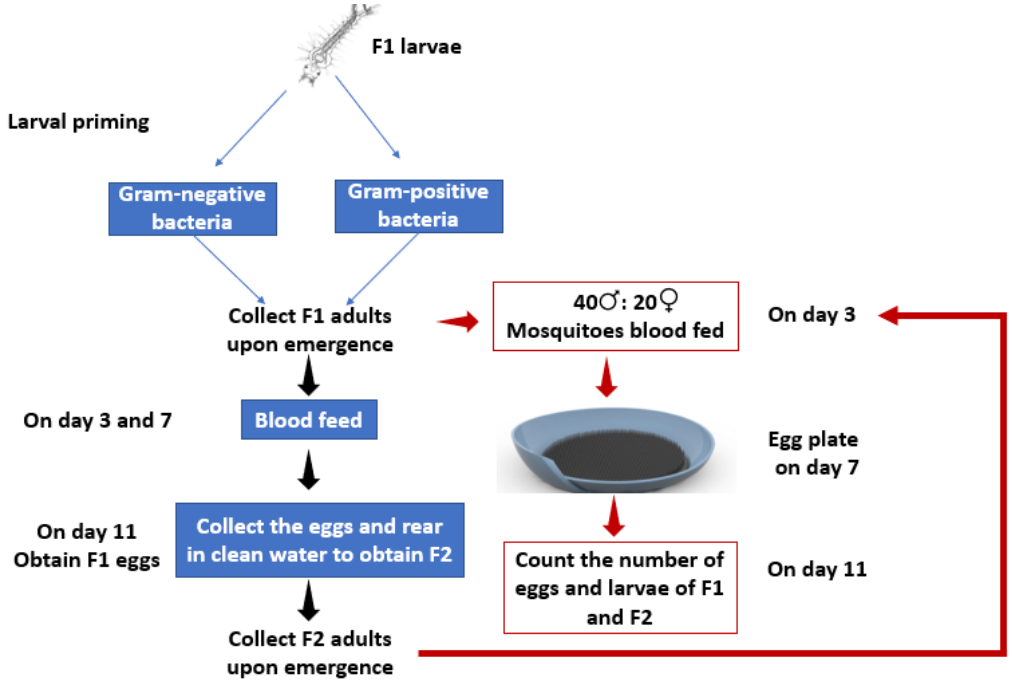


Figure 5. 3: Experimental setup to assess the effects of larval immune priming on the fertility and fecundity of the first and second generations of SENN and SENN DDT. The first-generation larvae (F1) were primed by rearing in water infected with either Gram-positive (*S. pyogenes*) or Gram-negative (*E.* coli) bacteria. The F1 adults collected upon emergence were used to obtain second-generation (F2) mosquitoes, with F1 eggs reared in clean water. Both the F1 and F2 adults were blood-fed, allowed to mate, and lay eggs. After three days, the eggs frozen, and the number of eggs and larvae were counted.

To measure the transgenerational capacity of SENN and SENN DDT to eliminate bacteria in the midgut, the following experiment was performed. The F1 larvae were reared in water infected with Gram-negative bacteria (*E. coli*), representing the larval immune priming event as described in the previous objective. A group of F1 larvae were reared in untreated water constituted the unprimed control group. The F2 mosquitoes were generated as in the previous experiment. The F2 mosquitoes obtained from both the primed and unprimed F1 larvae were further split into two sub-groups (n=30). One sub-group was provided with *E. coli* infected sugar and the other sub-group was provided with untreated sugar for three days upon emergence (Figure 5.4).

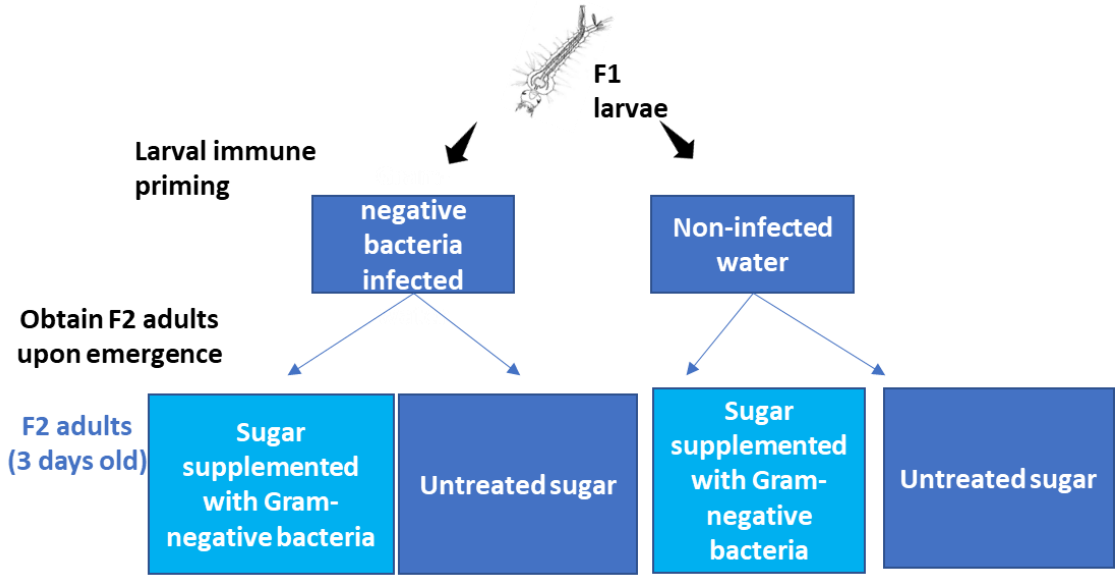


Figure 5. 4: Experimental setup to assess of the effects of larval immune priming on the transgenerational capacity to eliminate *E. coli* from the gut of SENN and SENN DDT. The first-generation (F1) larvae were reared in water infected with Gram-negative bacteria (*E. coli*). F1 larvae reared in untreated water constituted the unprimed control. The F1 adults were used to obtain second-generation (F2) mosquitoes and were reared in untreated water. The primed and unprimed F2 adult females were either provided with *E. coli* infected sugar or untreated sugar. Their guts were sterilely resected, and the presence of viable *E. coli* was assessed on a differential agar medium.

To assess the capacity of F2 mosquitoes to eliminate *E. coli* from the gut, the amount of viable *E. coli* in the midgut were determined and compared to the amount of viable *E. coli* in the F2 unprimed mosquitoes. Prior to gut resection, the female's surface was sterilised using 70% ethanol. The midguts were extracted using sterilised forceps under a stereomicroscope (Olympus SZ61: Wirsam Scientific) in replicates of six with five midguts per replicate. After extraction, the guts were stored in 500µl of 0.1M phosphate-buffered saline (**PBS**) pH 7.4. Subsequently 2ml of lysogeny broth was added to each tube and incubated overnight at 37°C

(Labcon FSIM 16 incubator) to allow optimal bacterial growth. The bacterial culture was diluted 10^6 times in PBS (Franklin *et al.*, 2000). Thereafter, 500µl of the diluted bacterial culture was plated on chromogenic UTI agar plates (Diagnostic media products, South Africa) in duplicate. These plates provide a distinction between the *E. coli* colonies and other bacterial colonies, as the *E. coli* colonies appear pink. *Enteroccoci* would appear as blue colonies, *Coliforms* as purple, *Proteus* as brown and *Staphylococci* as unpigmented. The number of *E. coli* colonies from the primed groups were counted and compared to the number of colonies from unprimed groups.

To assess the effects of larval immune priming on the bacterial tolerance of adult SENN and SENN DDT mosquitoes, the following experiment was performed. A group of F1 larvae were reared in water infected with Gram-positive bacteria and the other group was reared in Gram-negative bacteria, representing the primed groups. F1 larvae reared in non-infected water constituted the unprimed control group. The F2 mosquitoes were generated as described previously. Upon emergence, the adult F1 and F2 mosquitoes of each group were exposed to the bacteria they were primed with, supplemented in sugar (n=50) (Figure 5.5).

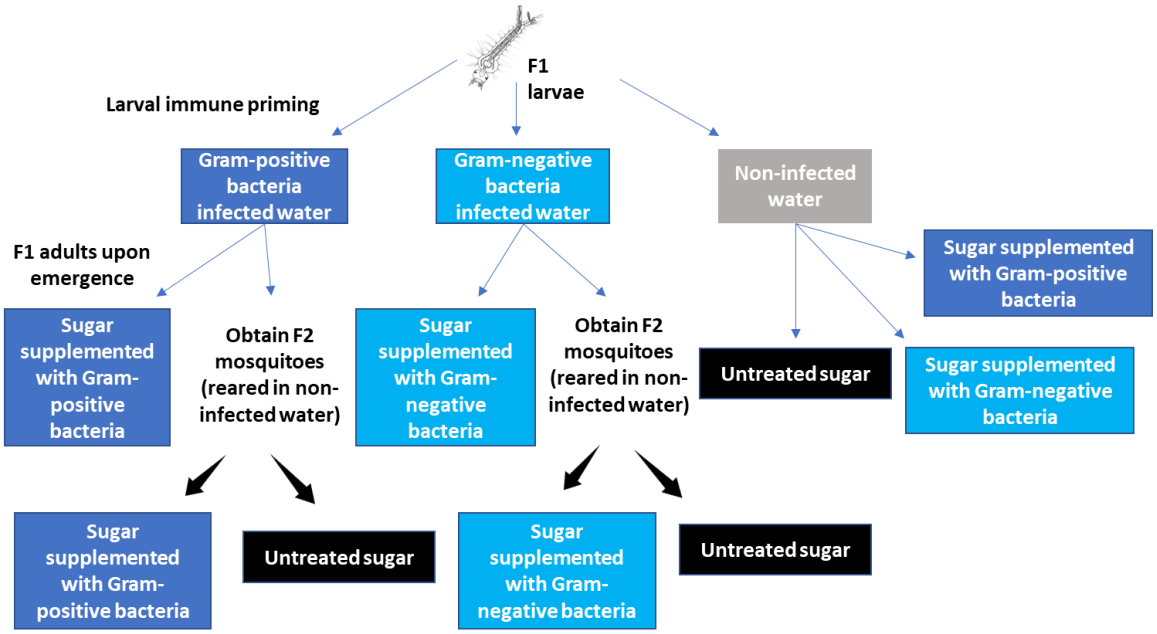


Figure 5. 5: Experimental setup to assess the effects of larval immune priming on bacterial tolerance of SENN and SENN DDT. The first-generation (F1) larvae were primed by rearing in water infected with either Gram-positive (*S. pyogenes*) or Gram-negative bacteria (*E. coli*). F1 larvae reared in non-infected water constituted the control. The second-generation (F2) mosquitoes were generated by rearing the offspring of F1 mosquitoes in untreated water. Upon emergence, both the F1 and F2 adult mosquitoes were exposed to their respective bacteria. A group of F1 unprimed mosquitoes and a group of F2 mosquitoes were maintained on sucrose (control).

A group of F2 adult mosquitoes that were maintained with *ad libitum* access to 10% sucrose constituted the unprimed group for F2 (n=50). The F1 unprimed group (n=150) was split into three sub-groups (n=50): the first sub-group was exposed to Gram-positive bacteria (unprimed control for Gram-positive bacteria). The second sub-group was exposed to Gram-negative bacteria (unprimed control for Gram-negative bacteria) and the third sub-group was maintained on *ad libitum* access to 10% sucrose (untreated control). The mortality was recorded with the number of cadavers observed and removed daily until all the mosquitoes were dead.

5.2.3. Statistical analysis

The statistical packages Statistix 8 (Analytical Software, Tallahassee, Florida) and SPSS v22 (IBM Corp. Released 2013. IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp.) were used for analysis. A Kaplan-Meier estimator was used to assess longevity (Kaplan & Meier, 1958). A Log-rank test was used to test for significance (Mantel 1966). The Shapiro-Wilk test was performed to determine normality (Shapiro & Wilk, 1965). Changes in fertility and fecundity were assessed using One-Way Analysis of Variance (ANOVA) (Gamage & Weerahandi, 1998). Changes in viable *E. coli* in the mosquito gut were assessed using a Kruskal-Wallis ANOVA (Kruskal & Wallis, 1952). To assess significance between groups, a Tukey's honestly significant difference (HSD) test was used post One-Way ANOVA (Tukey, 1949) and Kruskal-Wallis All-Pairwise Comparisons test was used post Kruskal-Wallis ANOVA (Kruskal & Wallis, 1952).

5.3. RESULTS

5.3.1. The effect of priming by an infected sugar meal early in life

In order to understand immune memory, it would be useful to understand the dynamics of immune priming. There are a range of mechanisms that can be used to prime the mosquito, and this experiment aimed to determine whether sugar or blood-priming induced the strongest immune memory. Priming with a sugar meal early in life had a limited effect on immune memory. Immune priming with Gram-positive bacteria (*S. pyogenes*) infected sugar in the early life of insecticide susceptible *An. arabiensis* (SENN) did not affect the tolerance to the bacteria. (Figure 5.6A). There was no significant difference between the longevity of primed and unprimed SENN after being exposed to Gram-positive bacteria (**Log-rank test: $p = 0.637$, $\chi^2 = 0.22$**). Immune priming with Gram-positive bacteria-infected sugar in the early life of insecticide resistant *An. arabiensis* (SENN DDT) however, resulted in an increased longevity after the initial exposure to the Gram-positive bacteria. There was a significant increase in the

longevity of primed SENN DDT in comparison to the unprimed SENN DDT after being exposed to Gram-positive bacteria (**$p < 0.001$, $\chi^2 = 27.8$**) (Figure 5.6B).

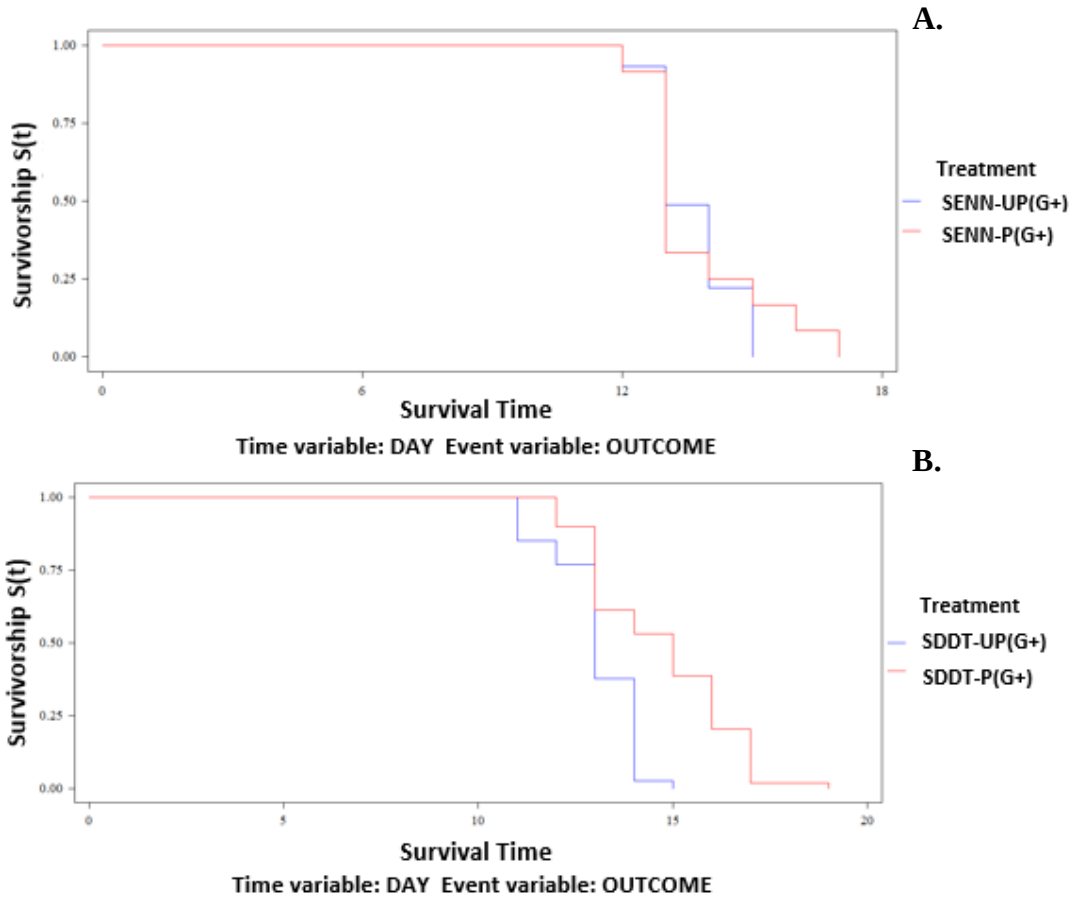


Figure 5. 6: The effects of sugar-delivered Gram-positive bacterial immune priming in early life on bacterial tolerance of SENN and SENN DDT measured as longevity when exposed to *S. pyogenes* infected sugar. SENN-P(G+): SENN primed and challenged with Gram-positive bacteria-infected sugar. SDDT-P(G+): SENN DDT primed and challenged with Gram-positive bacteria-infected sugar. SENN-UP(G+): SENN unprimed challenged with Gram-positive bacteria. SDDT-UP(G+): SENN DDT unprimed challenged with Gram-positive bacteria. A: There was no significant difference between primed and unprimed SENN. B: Primed SENN DDT lived significantly longer

Immune priming with Gram-negative bacteria (*E. coli*) untreated sugar in early life resulted in a significantly reduced longevity in the SENN strain (Figure 5.7A). There was a significant reduction in the longevity of primed SENN in comparison to unprimed SENN after being exposed to the Gram-negative bacteria (**Log-rank test: $p < 0.001$, $\chi^2 = 17.5$**). Unlike Gram-positive bacterial immune priming, Gram-negative bacterial immune priming did not affect the tolerance to the bacteria in SENN DDT. There was no significant difference between the longevity of primed and unprimed SENN DDT (**$p = 0.947$, $\chi^2 = 0.00$**) (Figure 5.7B).

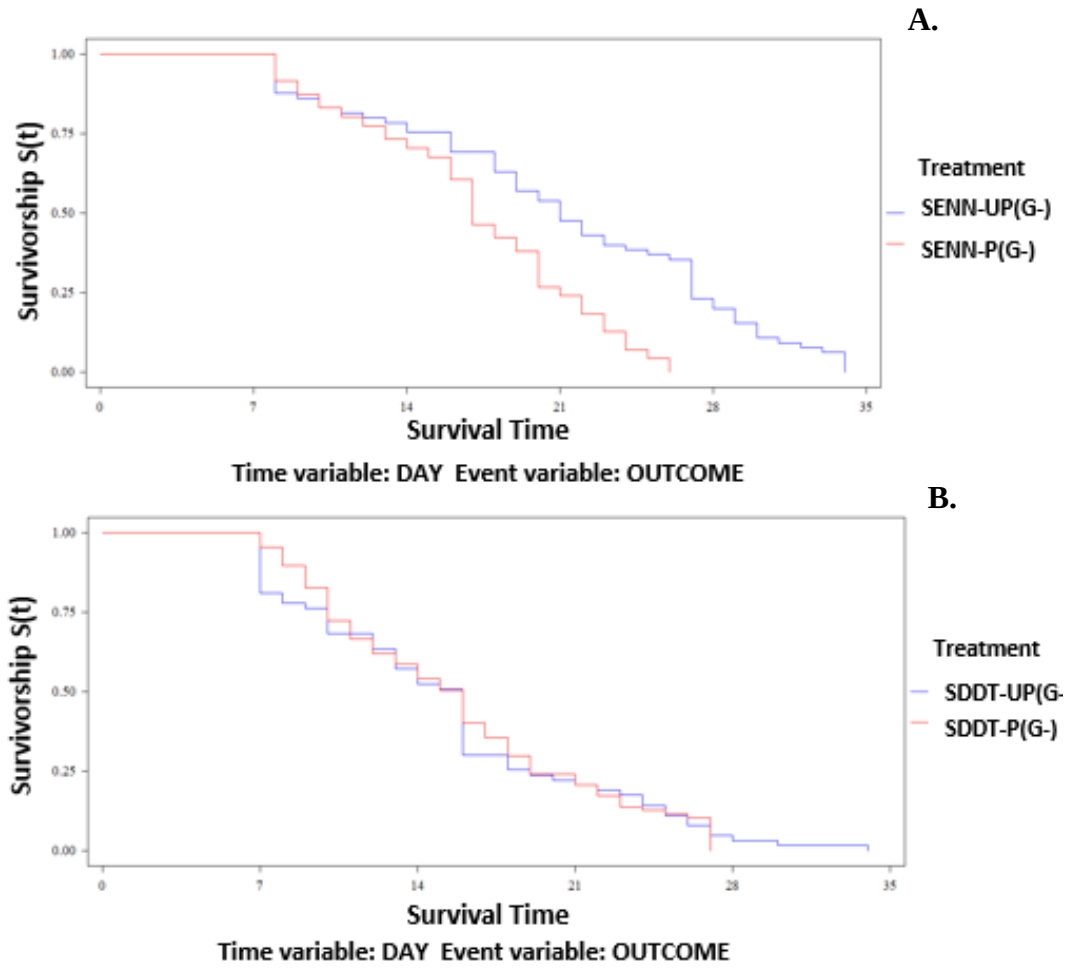


Figure 5. 7: The effects of sugar-delivered Gram-negative bacterial immune priming in early life on bacterial tolerance of SENN and SENN DDT measured as longevity when exposed to *E. coli* infected sugar. SENN-P(G-): SENN primed and challenged with Gram-negative bacteria-infected sugar. SDDT-P(G-): SENN DDT primed and challenged with Gram-negative bacteria-infected sugar. SENN-UP(G-): SENN unprimed challenged with Gram-negative. SDDT-UP(G-): SENN DDT unprimed challenged with Gram-negative. A: Primed SENN lived significantly shorter. B: There was no significant difference in the longevity of primed and unprimed SENN DDT.

The effect of immune priming with bacteria-infected sugar in early life was similar between SENN and SENN DDT after being exposed to the bacteria. There was no significant difference in the longevity between Gram-positive bacterial immune primed SENN and SENN DDT after being exposed to Gram-positive bacteria (**Log-rank test: $p = 0.156$, $\chi^2 = 2.01$**). Similarly, there was no significant difference in the longevity between Gram-negative bacterial immune primed SENN and SENN DDT after being exposed to Gram-negative bacteria (**$p = 0.797$, $\chi^2 = 0.07$**).

In summary, sugar delivery of an initial immune stimuli with Gram-positive bacteria-infected sugar did not affect the tolerance to the bacteria in SENN. However, this delivery mechanism increased tolerance in SENN DDT. An initial sugar delivery of Gram-negative bacteria was

detrimental to SENN, resulting in a subsequent reduction of longevity. Gram-negative bacteria-infected sugar supplementation did not result in any changes of tolerance in the SENN DDT strain.

5.3.2. The effect of priming by an infected blood-meal early in life

Priming the immune system with infected blood resulted in a more marked response in tolerance than the infected sugar. In SENN, Gram-positive bacterial immune priming with blood resulted in an increased tolerance to the bacteria (Figure 5.8A). There was a significant increase in the longevity of Gram-positive bacterial immune primed SENN in comparison to unprimed SENN after being exposed to Gram-positive bacteria supplemented in blood (**Log-rank test: p = 0.034, χ^2 = 4.47**).

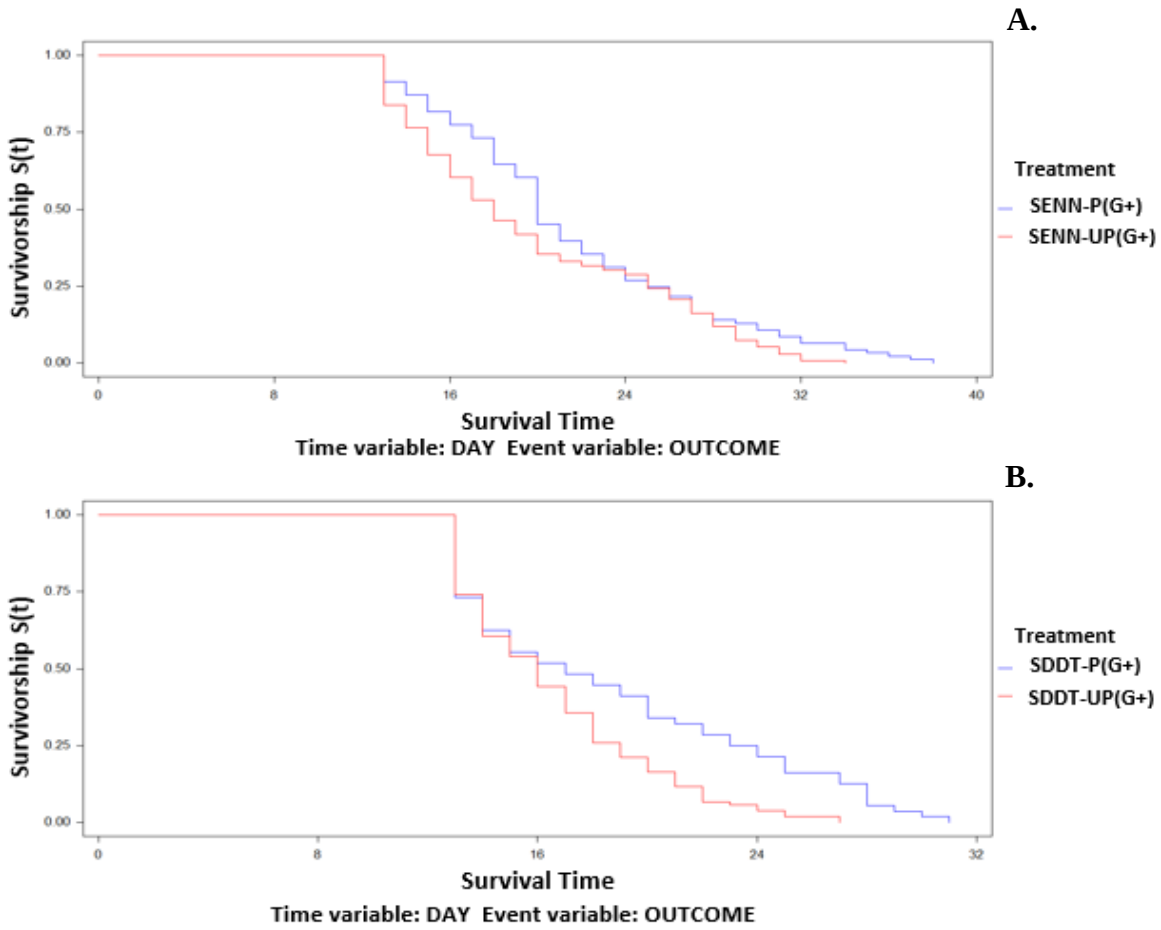


Figure 5. 8: The effect of an initial Gram-positive bacteria-infected blood-meal on subsequent longevity after the second infected blood-meal. SENN-P(G+): SENN primed and challenged with Gram-positive bacteria-infected blood. SDDT-P(G+): SENN DDT primed and challenged with Gram-positive bacteria-infected blood. SENN-UP(G+): SENN unprimed challenged with Gram-positive bacteria-infected blood. SDDT-UP(G+): SENN DDT unprimed challenged with Gram-positive bacteria-infected blood. A: Primed SENN lived significantly longer. B: Primed SENN DDT lived significantly longer.

Like the Gram-positive bacterial immune priming with sugar, blood-borne immune priming also increased tolerance of SENN DDT to the bacteria. (Figure 5.8B). There was a significant increase in the longevity of Gram-positive bacterial immune primed SENN DDT in comparison to unprimed SENN DDT after the exposure to Gram-positive bacteria-infected blood (**Log-Rank test: $p = 0.001$, $\chi^2 = 10.6$**). Blood feeding has previously been demonstrated to increase longevity (Oliver & Brooke, 2014). The increased longevity of the primed mosquitoes was not due to the effects of blood feeding. There was no significant difference between the longevity of primed and unprimed SENN provided with untreated blood (**$p = 0.564$, $\chi^2 = 0.33$**). Similarly, there was no significant difference between the longevity of primed and unprimed SENN DDT provided with untreated blood (**$p = 0.452$, $\chi^2 = 0.57$**)

Immune priming with Gram-negative bacteria-infected blood resulted in an increased tolerance to the bacteria in both SENN and SENN DDT (Figure 5.9), unlike immune priming with Gram-negative bacteria-infected sugar.

There was a significant increase in the longevity of Gram-negative bacterial immune primed SENN in comparison to unprimed SENN after being exposed to Gram-negative bacteria-infected blood (**Log-rank test: $p = 0.019$, $\chi^2 = 5.51$**). Gram-negative bacterial immune priming with blood resulted in a significant increase in the longevity of primed SENN DDT in comparison to unprimed SENN DDT (**$p < 0.001$, $\chi^2 = 16.2$**). The observed results were due to the priming treatment rather than just the blood alone as there was no significant difference between the longevity of primed and unprimed SENN provided with untreated blood (**$p = 0.169$, $\chi^2 = 5.04$**). Similarly, there was no significant difference between the longevity of unprimed SENN DDT provided with untreated blood and unprimed SENN DDT provided with sugar only (**$p = 0.0497$, $\chi^2 = 3.85$**). There was no significant difference between the longevity of primed SENN DDT provided with untreated blood and primed SENN DDT provided with untreated sugar (**$p = 0.0701$, $\chi^2 = 3.28$**).

Initial priming with Gram-positive bacteria-infected blood did not affect the generalised longevity of SENN. There was no significant difference in the longevity between primed SENN and unprimed controls (**Log-rank test: $p = 0.060$, $\chi^2 = 3.53$**). Priming with Gram-negative bacteria-infected blood did not affect the generalised longevity of SENN (**$p = 0.169$, $\chi^2 = 5.04$**). By contrast, the initial immune priming with Gram-positive bacteria-infected blood resulted in a significant reduction in the longevity of primed SENN DDT control in comparison to unprimed SENN DDT control (**$p < 0.001$, $\chi^2 = 20.3$**). Initial immune priming with Gram-

negative bacteria-infected blood had no effect on the longevity of SENN DDT (**p = 0.824**, χ^2 **= 0.05**).

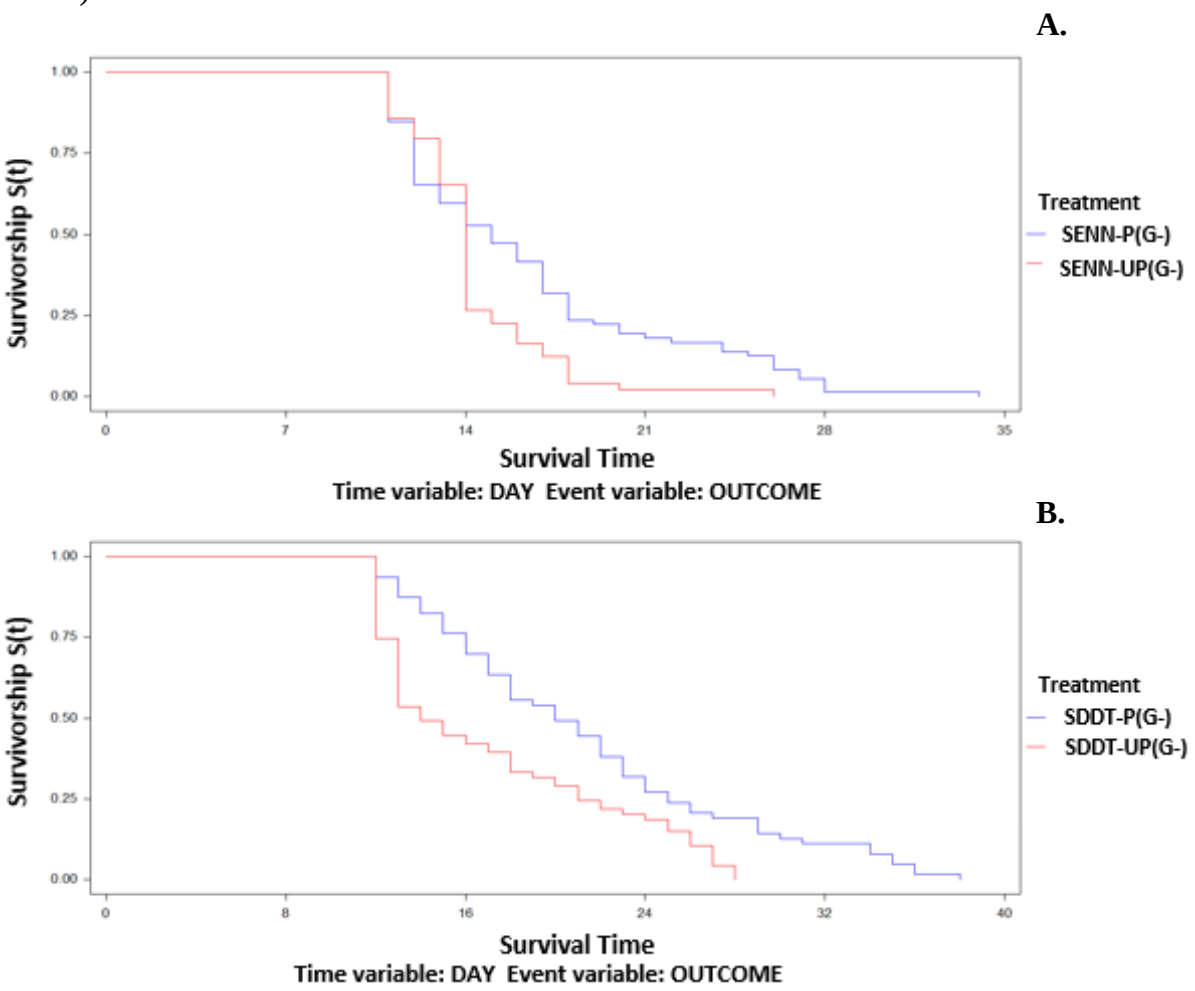


Figure 5. 9: The effect of an initial Gram-negative bacteria-infected blood-meal on subsequent longevity after the second infected blood-meal. SENN-P(G-): SENN primed and challenged with Gram-negative bacteria-infected blood. SDDT-P(G-): SENN DDT primed and challenged with Gram-positive bacteria-infected blood. SENN-UP(G-): SENN unprimed challenged with Gram-negative bacteria. SDDT-UP(G-): SENN DDT unprimed challenged with Gram-negative bacteria. A: Primed SENN lived significantly longer. B: Primed SENN DDT lived significantly longer.

There was a significant increase in the longevity of Gram-negative bacterial immune primed SENN in comparison to unprimed SENN after being exposed to Gram-negative bacteria-infected blood (**Log-rank test: p = 0.019,** χ^2 **= 5.51**). Gram-negative bacterial immune priming with blood resulted in a significant increase in the longevity of primed SENN DDT in comparison to unprimed SENN DDT (**p < 0.001,** χ^2 **= 16.2**). The observed results were due to the priming treatment rather than just the blood alone as there was no significant difference between the longevity of primed and unprimed SENN provided with untreated blood (**p = 0.169,** χ^2 **= 5.04**). Similarly, there was no significant difference between the longevity of

unprimed SENN DDT provided with untreated blood and unprimed SENN DDT provided with sugar only (**$p = 0.0497$, $\chi^2 = 3.85$**). There was no significant difference between the longevity of primed SENN DDT provided with untreated blood and primed SENN DDT provided with untreated sugar (**$p = 0.0701$, $\chi^2 = 3.28$**).

Initial priming with Gram-positive bacteria-infected blood did not affect the generalised longevity of SENN. There was no significant difference in the longevity between primed SENN and unprimed controls (**Log-rank test: $p = 0.060$, $\chi^2 = 3.53$**). Priming with Gram-negative bacteria-infected blood did not affect the generalised longevity of SENN (**$p = 0.169$, $\chi^2 = 5.04$**). By contrast, the initial immune priming with Gram-positive bacteria-infected blood resulted in a significant reduction in the longevity of primed SENN DDT control in comparison to unprimed SENN DDT control (**$p < 0.001$, $\chi^2 = 20.3$**). Initial immune priming with Gram-negative bacteria-infected blood had no effect on the longevity of SENN DDT (**$p = 0.824$**, **$\chi^2 = 0.05$**).

The initial blood-borne immune priming effect differentially affected longevity in SENN and SENN DDT. Under control conditions, SENN had significantly increased longevity compared to SENN DDT (**Log-rank test: $p < 0.001$, $\chi^2 = 32.9$**) (Figure 5.10A). Similarly, after an initial Gram-positive bacterial immune priming, SENN had increased longevity compared to SENN DDT after being exposed to Gram-positive bacteria-infected blood (**$p = 0.012$, $\chi^2 = 5.44$**) (Figure 5.10B). By contrast, after an initial Gram-negative bacterial immune priming, SENN DDT had increased longevity compared to SENN after being exposed to Gram-negative bacteria-infected blood (**$p < 0.001$, $\chi^2 = 52.77$**) (Figure 5.10C).

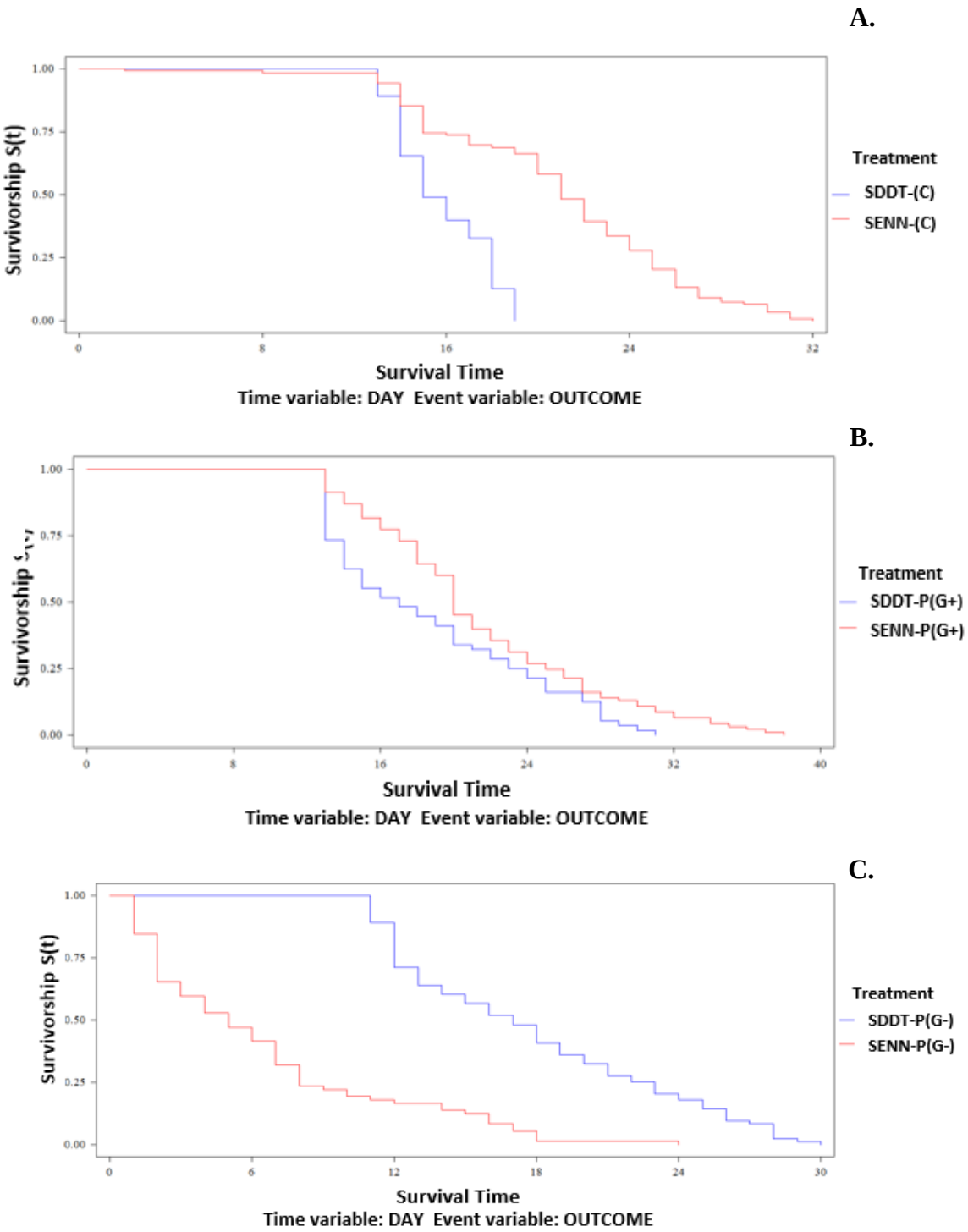


Figure 5. 10: The effect of an initial blood-borne priming event on subsequent longevity of the strains. SENN-(C): SENN provided with initial untreated blood. SDDT-(C): SENN DDT provided with initial untreated blood. SENN-P(G+): SENN primed with Gram-positive bacteria-infected blood. SDDT-P(G+): SENN DDT primed with Gram-positive bacteria-infected blood. SENN-P(G-): SENN primed with Gram-negative bacteria-infected blood. SDDT-P(G-): SENN primed with Gram-negative bacteria-infected blood. A: Under control conditions SENN lived significantly longer. B: After Gram-positive bacterial immune priming, SENN lived significantly longer. C: After Gram-negative bacterial immune priming, SENN DDT lived significantly longer.

In summary, immune priming with bacteria-infected blood resulted in an increased lifespan of both SENN and SENN DDT when challenged with a second infected blood meal. The observed results were not due to blood-feeding. Immune priming did not affect the generalised longevity of SENN. By contrast, immune priming with Gram-positive bacteria decreased generalised longevity of SENN DDT.

5.3.3. Transgenerational effects of larval immune priming: fertility and fecundity

The effects of priming on several traits in the second generation were examined in this study. The first of these was the effect on fertility and fecundity. The Gram-positive bacterial larval immune priming did not affect the fecundity and fertility of both F1 and F2 SENN.

There was no significant difference in the fecundity between the control, and Gram-positive bacterial larval immune primed F1 and F2 SENN (**One-way ANOVA: $p = 0.054$, $F_{(2, 15)} = 3.68$**) (Figure 5.11A). Similarly, there was no significant difference in the fertility between the control, and the larval immune primed F1 and F2 SENN (**$p = 0.157$, $F_{(4, 19)} = 1.87$**) (Figure 5.11B). However, Gram-negative bacterial larval immune priming decreased the fecundity of F1 SENN and increased the fecundity of F2 SENN in comparison to the control (**$p = 0.046$, $F_{(2, 13)} = 3.94$**) (Figure 5.11A). By contrast, there was no significant difference in the fertility between the control, and the larval immune primed F1 and F2 SENN (**$p = 0.157$, $F_{(4, 19)} = 1.87$**) (Figure 5.11B).

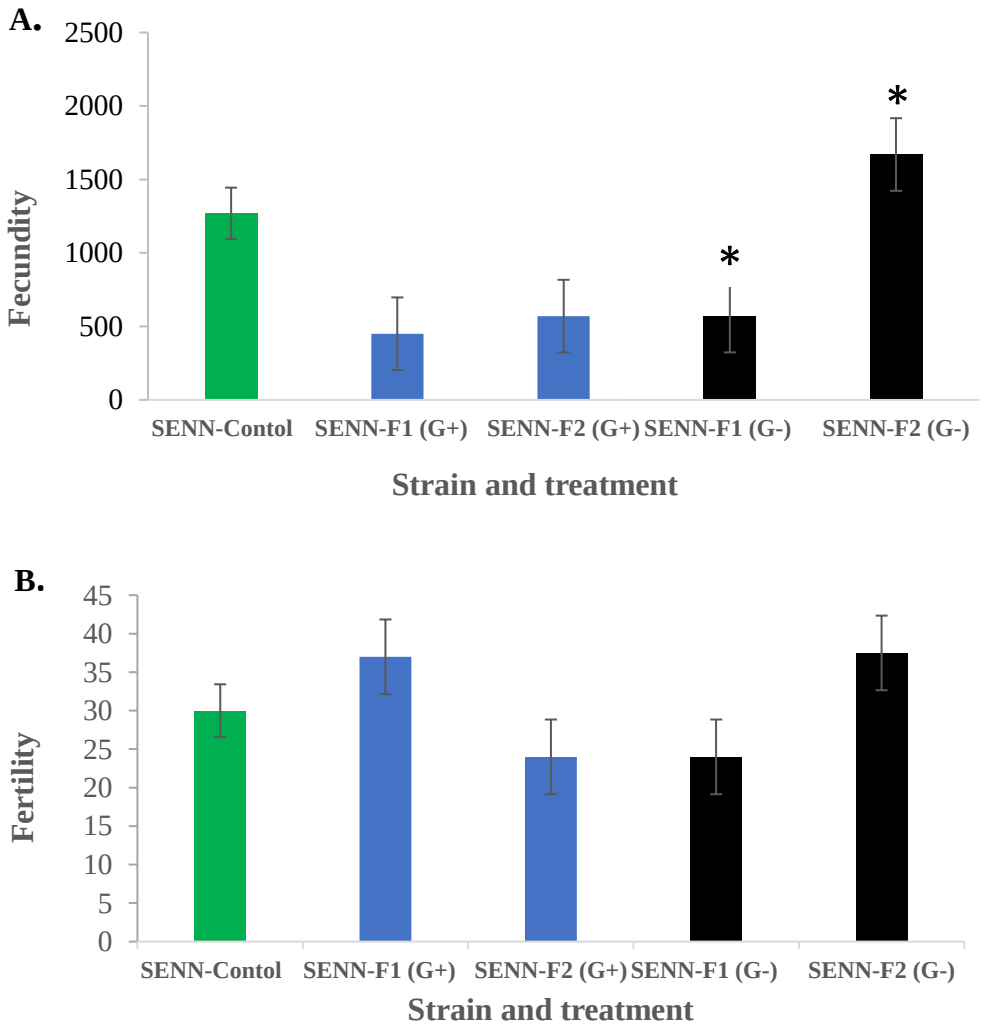


Figure 5. 11: The effect of Gram-positive and Gram-negative bacterial larval immune priming on the fertility and fecundity of SENN. (A) The effect on fecundity – number of eggs produced by the first (F1) and second (F2) generations of insecticide susceptible *An. arabiensis* (SENN). (B). The effect on fertility – percentage of eggs hatched from F1 and F2 SENN. (G+) represents *S. pyogenes* treatment and (G-) represents *E. coli* treatment. Asterisks (*) indicates a significant difference from the untreated SENN control.

The Gram-positive bacterial larval immune priming did not affect the fecundity of SENN DDT, like SENN. There was no significant difference between the control, and the larval immune primed F1 and F2 SENN DDT (**One-way ANOVA: $p = 0.065$, $F_{(2, 9)} = 3.75$**) (Figure 5.12A). By contrast, the Gram-positive bacterial larval immune priming increased the fertility of F1 SENN DDT and decreased the fertility of F2 SENN DDT in comparison to the control (**$p = 0.032$, $F_{(2, 9)} = 5.18$**) (Figure 5.12B). Like SENN, the Gram-negative bacterial larval immune priming resulted in a significant decrease in the fecundity of F1 SENN DDT and a significant increase in the fecundity of F2 SENN DDT in comparison to the control (**$p = 0.016$, $F_{(2, 9)} = 6.75$**) (Figure 5.12A). Unlike Gram-positive bacteria, Gram-negative bacterial larval immune

priming resulted in an increase in the fertility of F2 SENN DDT in comparison to the control (**p = 0.034, $F_{(2, 9)}$ = 5.05**) (Figure 5.12B).

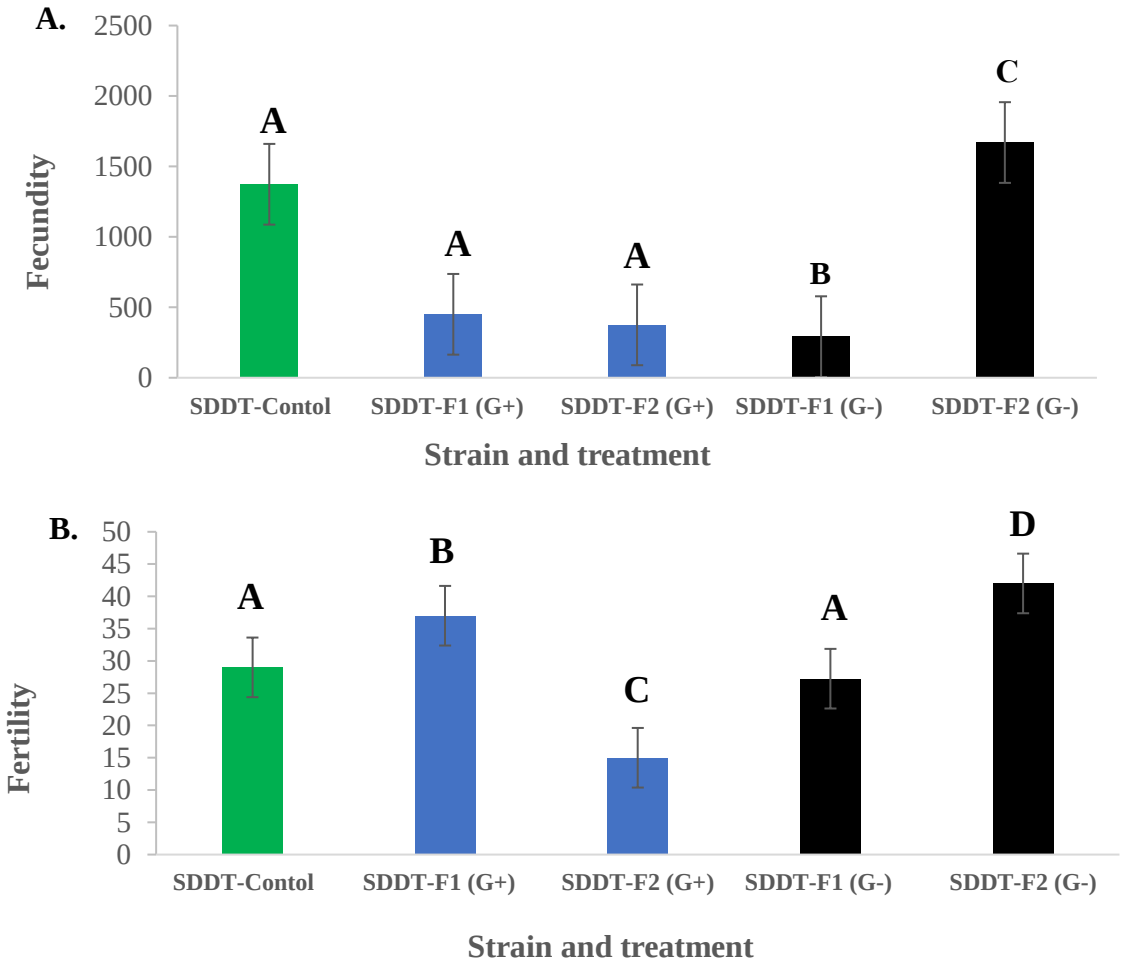


Figure 5. 12: The effect of Gram-positive and Gram-negative bacterial larval immune priming on the fertility and fecundity of SENN DDT. (A). The effect on fecundity – number of eggs produced by the first (F1) and second (F2) generation of insecticide resistant *An. arabiensis* (SENN DDT). (B). The effect on fertility – number of eggs hatched (larvae) from F1 and F2 SENN DDT. (G+) represents *S. pyogenes* treatment and (G-) represents *E. coli* treatment. Significant differences are indicated by different letters.

Larval immune priming did not differentially affect the fecundity and fertility in SENN and SENN DDT. There was no significant difference in the fecundity (**One-way ANOVA: p = 0.834, $F_{(1, 10)}$ = 0.05**) and fertility (**p = 0.842, $F_{(1, 10)}$ = 0.04**) between the SENN and SENN DDT control. The fecundity (**p = 1.00, $F_{(1, 6)}$ = 0.00**) and fertility (**p = 1.00, $F_{(1, 6)}$ = 0.00**) of the Gram-positive bacterial larval immune primed F1 SENN was not significantly different from that of F1 SENN DDT. This was also true for the F2 SENN and SENN DDT (**fecundity: p = 0.390, $F_{(1, 6)}$ = 0.86; fertility: p = 0.409, $F_{(1, 6)}$ = 0.79**). Similarly, there was no significant difference in the fecundity (**p = 0.161, $F_{(1, 6)}$ = 2.56**) and fertility (**p = 0.718, $F_{(1, 6)}$ = 0.14**) of

the Gram-negative bacterial larval immune primed F1 SENN and SENN DDT. This was also true for the F2 SENN and SENN DDT (**fecundity: $p = 1.00$, $F_{(1, 6)} = 0.00$; fertility: $p = 0.183$, $F = 2.27$**).

In summary, Gram-positive bacterial larval immune priming did not affect the fecundity of both F1 and F2 of SENN and SENN DDT. However, Gram-positive bacterial larval immune priming increased the fertility of F1 SENN DDT and decreased the fertility of F2 SENN DDT. By contrast, Gram-negative bacterial larval immune priming decreased the fecundity of F1 SENN and increased the fecundity of F2 SENN. Gram-negative bacterial larval immune priming did not affect the fertility of F1 and F2 SENN. Like SENN, Gram-negative bacterial larval immune priming decreased the fecundity of F1 SENN DDT and increased the fecundity of F2 SENN DDT. Gram-negative bacterial larval immune priming also increased the fertility of F2 SENN DDT, but did not affect the fertility of F1 SENN DDT. Larval immune priming, therefore, had transgenerational effects on fecundity rather than fertility, but there were no large-scale differences between the two strains.

5.3.4. Transgenerational effects of larval immune priming: capacity to eliminate bacteria from the gut after oral challenge

The transgenerational capacity of the mosquito to cope with a bacterial challenge was assessed by determining the number of viable bacteria recovered from the gut after oral challenge. This is a measure of the capacity to eliminate the bacteria. This effect was examined in F2 SENN and SENN DDT strains, where only the parental generation had been primed. The number of *E. coli* colonies cultured from the larval immune primed SENN was significantly lower than that of unprimed SENN after being exposed to *E. coli* (**Kruskal-Wallis One-Way ANOVA: $p = 0.002$, $F_{(1, 18)} = 12.8$, $\chi^2 = 7.91$**) (Figure 5.13). Similarly, the larval immune primed SENN DDT had significantly less viable *E. coli* cultured than the unprimed SENN DDT (**$p < 0.001$, $F_{(1, 18)} = 49.6$, $\chi^2 = 13.9$**). When comparing the viable *E. coli* recovered from SENN and SENN DDT, the unprimed SENN DDT had significantly less viable *E. coli* than unprimed SENN after being exposed to *E. coli* (**$p = 0.019$, $F_{(1, 18)} = 6.69$, $\chi^2 = 5.15$**) (Figure 5.13). By contrast, there was no significant difference in the viable *E. coli* between primed SENN and primed SENN DDT after being exposed to *E. coli* (**$p = 0.905$, $F = 0.01$, $\chi^2 = 0.016$**).

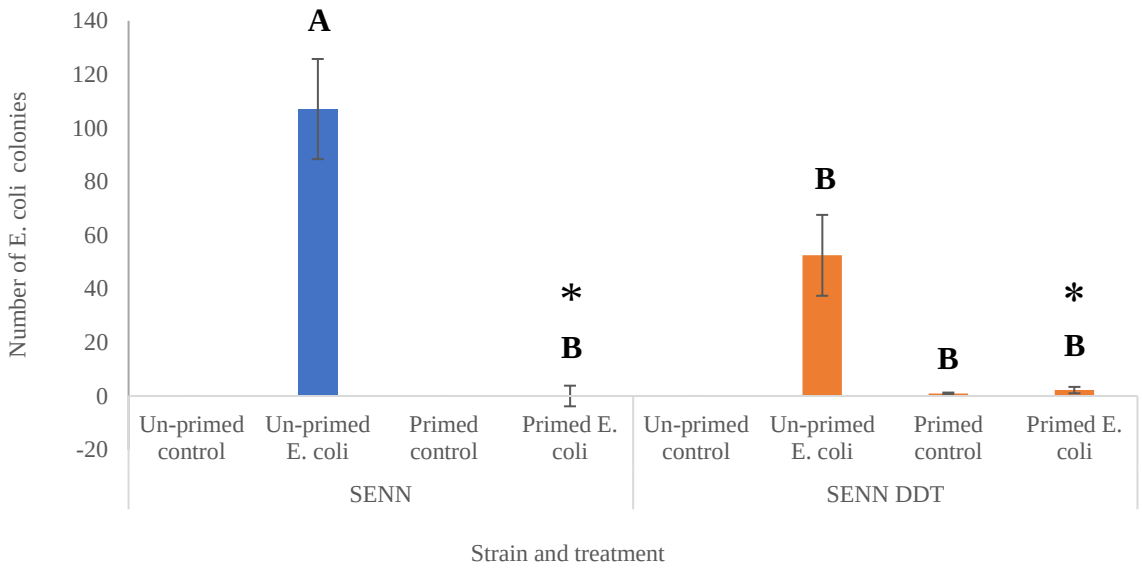


Figure 5. 13: The effect of larval immune priming on the transgenerational capacity to eliminate *E. coli* from the midgut of SENN and SENN DDT. Control: specimen treated with sugar only. *E. coli*: specimen treated with bacterial sugar. Asterisks (*) indicates a significant difference from the control. Differences in groups are indicated by different upper-case letters.

In summary, transgenerational larval immune priming with *E. coli* resulted in a reduced number of viable *E. coli* in the midgut of F2 SENN and SENN DDT, after oral exposure to *E. coli*. Another finding from this experiment was that the unprimed SENN DDT had less viable *E. coli* than unprimed SENN. This effect was lost after priming. The viable *E. coli* collected after priming did not differ between primed SENN and SENN DDT.

5.3.5. Transgenerational effects of larval immune priming: transgenerational tolerance to bacteria

The effect of larval immune priming of the F1 SENN and SENN DDT larvae on the tolerance of F1 and F2 SENN and SENN DDT was assessed by determining the longevity of adults when constantly exposed to bacteria-infected sugar. Gram-positive bacterial larval immune priming SENN resulted in an increased tolerance to the bacteria (Figure 5.14A). Both F1 and F2 Gram-positive bacterial immune primed SENN had increased longevity than the unprimed SENN when continuously exposed to the Gram-positive bacteria (**Log-rank test: $p < 0.001$, $\chi^2 = 48.6$**). There was no significant difference in the longevity between the F1 and F2 SENN (**$p = 0.553$, $\chi^2 = 0.35$**).

Similarly, Gram-positive bacterial larval immune priming in SENN DDT also resulted in increased tolerance to the bacteria (Figure 5.14B). Both F1 and F2 SENN DDT had a significant increase in longevity compared to the unprimed SENN DDT when continuously

exposed Gram-positive bacteria (**$p < 0.001$, $\chi^2 = 146$**). However, the longevity of F2 SENN DDT was reduced compared to that of their F1 counterparts (**$p < 0.001$, $\chi^2 = 11.3$**). The F1 SENN DDT had increased longevity than F1 SENN after being exposed to Gram-positive bacteria (**$p < 0.001$, $\chi^2 = 65.7$**).

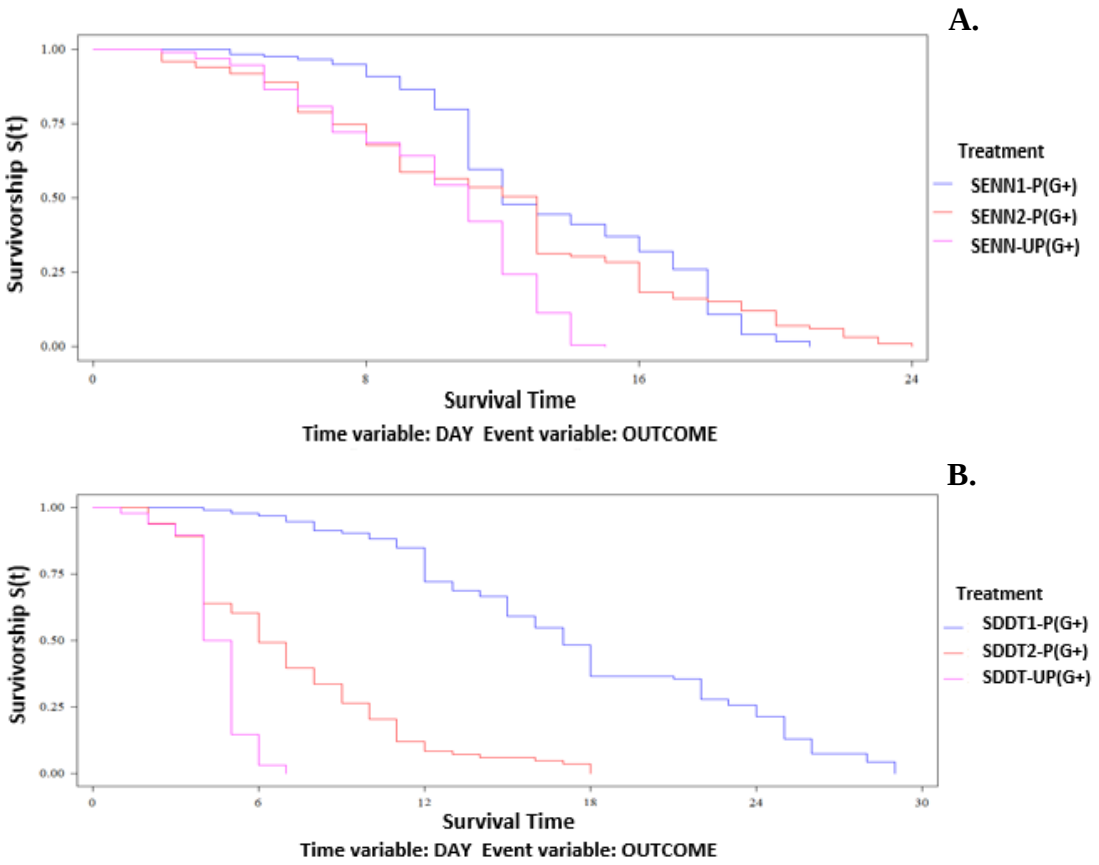


Figure 5. 14: The effect of Gram-positive bacterial larval immune priming on the bacterial tolerance of first and second generations of SENN and SENN DDT. SENN1-(G+): SENN first-generation larval immune primed. SDDT1-(G+): SENN DDT first-generation larval immune primed. SENN2-(G+): unprimed SENN, offspring of primed first-generation. SDDT2-(G+): unprimed SENN DDT, offspring of primed first-generation. SENN-UP(G+): unprimed SENN. SDDT-UP(G+): unprimed SENN DDT. A: For the SENN strain, both generations lived significantly longer than the unprimed strain. There was no significant difference in longevity between the two primed generation. B: For the SENN DDT strain, the primed mosquitoes lived significantly longer than the unprimed, but the F2 generation had a significantly reduced longevity compared to the the F1.

Gram-negative bacterial larval immune priming in SENN did not affect the tolerance to the bacteria (Figure 5.15A). There was no significant difference in the longevity of Gram-positive larval immune primed SENN exposed to Gram-positive bacteria compared to their unprimed counterparts (**Log-rank test: $p = 0.196$, $\chi^2 = 3.26$**). By contrast, Gram-negative bacterial larval immune priming in SENN DDT increased tolerance to the bacteria (Figure 5.15B). Both F1

and F2 primed SENN DDT had a significant increase in longevity compared to the unprimed SENN DDT after being exposed to the Gram-negative bacteria (**$p < 0.001$, $\chi^2 = 101$**). Similar to the results of Gram-positive bacteria, the F1 Gram-negative bacteria immune primed SENN DDT had a significant increase in longevity compared to F2 SENN DDT (**$p < 0.001$, $\chi^2 = 19.9$**).

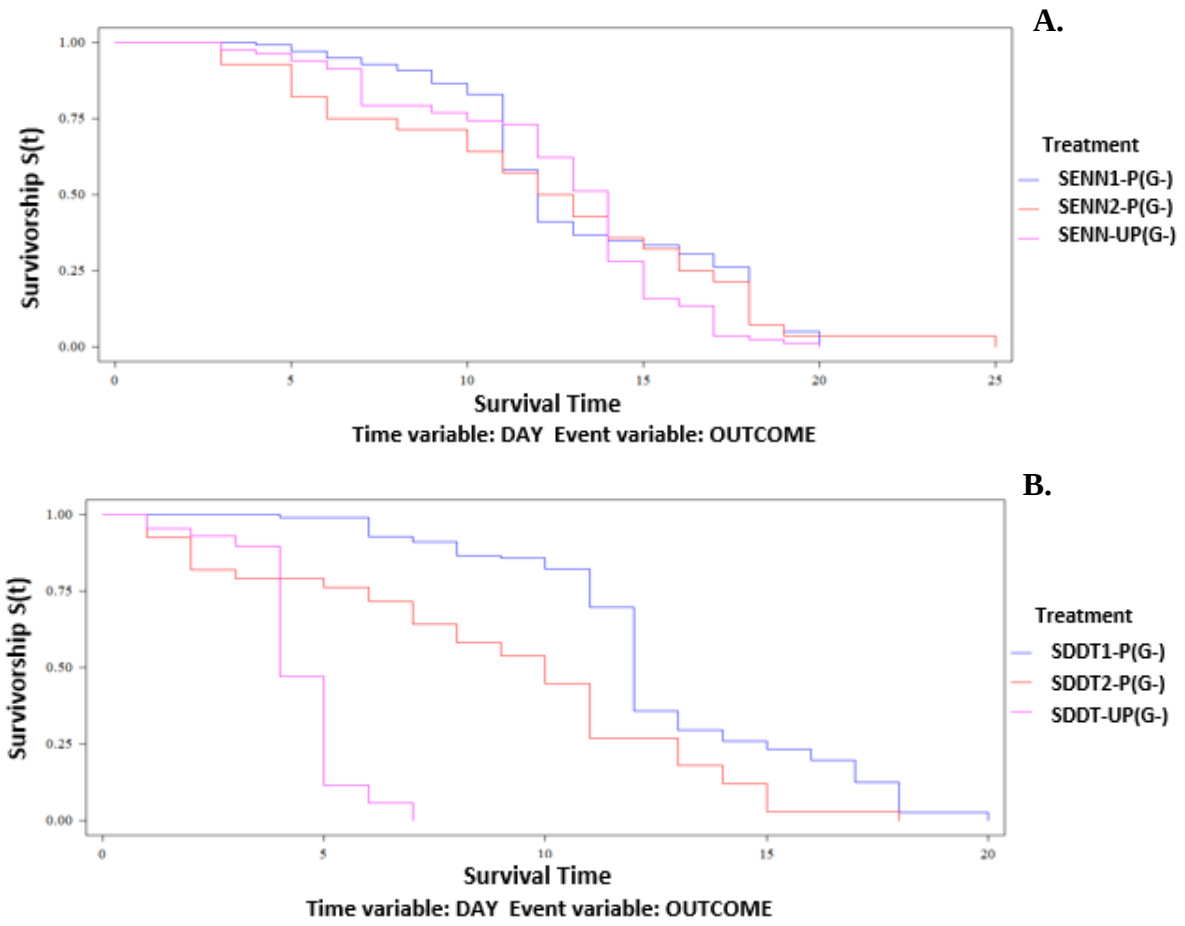


Figure 5. 15: The effect of Gram-negative bacterial larval immune priming on the longevity of first and second generations of SENN and SENN DDT. SENN1-(G-): SENN, first-generation larval immune primed. SDDT1-(G-): SENN DDT first-generation larval immune primed. SENN2-(G-): unprimed SENN, offspring of primed first-generation. SDDT2-(G-): unprimed SENN DDT, offspring of primed first-generation. SENN-UP(G-): unprimed SENN. SDDT-UP(G-): unprimed SENN DDT. A: For the SENN strain, the primed and unprimed mosquitoes did not differ in longevity. B: For the SENN DDT strain, although the primed mosquitoes lived significantly longer than the unprimed, the F1 SENN DDT lived for significantly shorter than the F2 generation.

The effect of larval immune priming on adult tolerance of ingested bacteria differed between SENN and SENN-DDT. Under conditions of continued bacterial exposure F1 Gram-positive bacterial immune primed SENN DDT lived significantly longer than F1 SENN (**Log-rank test: $p < 0.001$, $\chi^2 = 32$**). By contrast, in the F2 generation, Gram-positive bacterial immune

primed SENN lived significantly longer than SENN DDT when continuously exposed to Gram-positive bacteria (**$p < 0.001$, $\chi^2 = 30.3$**) (Figure 5.16).

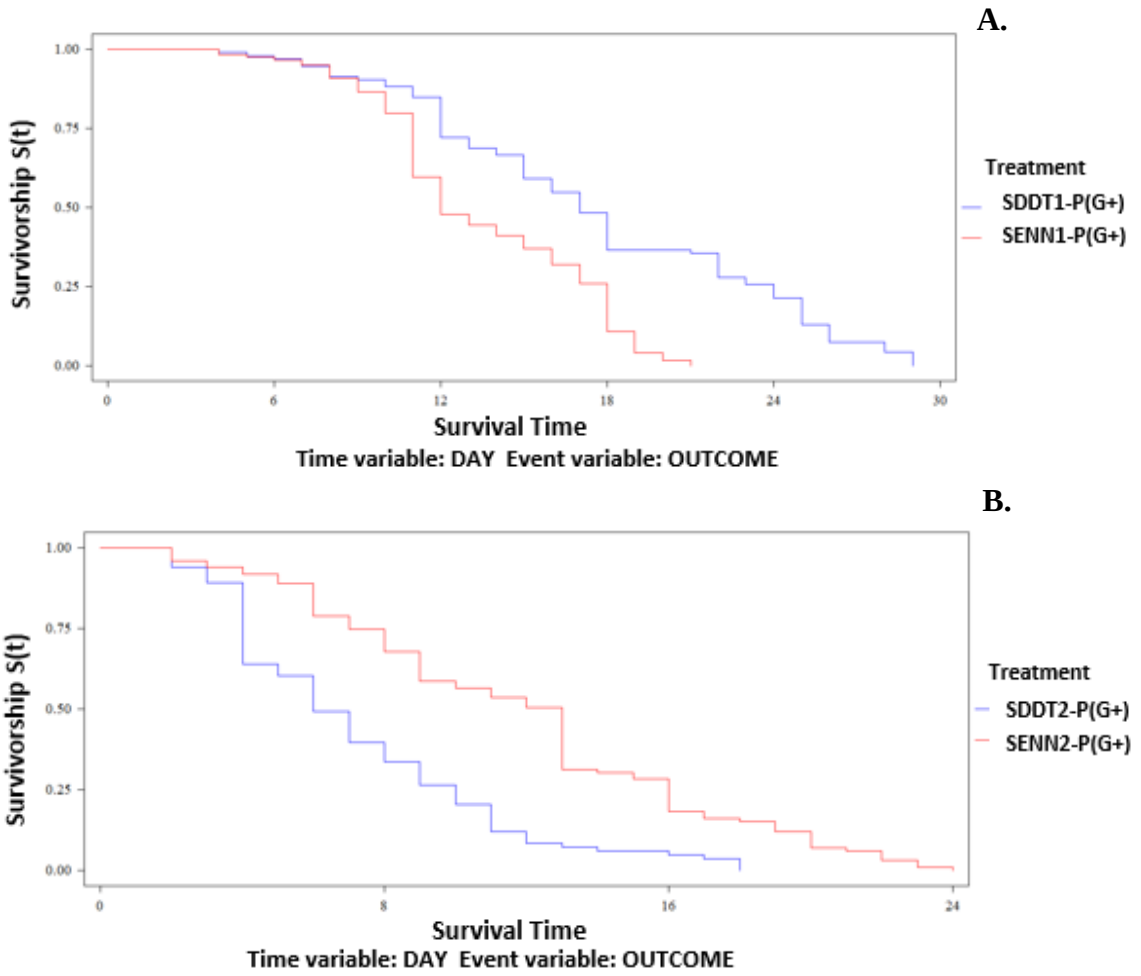


Figure 5. 16: Comparison of the effects of Gram-positive bacterial larval immune priming on the generalised longevity of unchallenged SENN and SENN DDT. SENN1-P(G+): primed SENN first-generation. SDDT1-P(G+): primed SENN DDT first-generation. SENN2-P(G+): unprimed SENN second-generation, offspring of primed first-generation. SDDT2-P(G+): unprimed SENN DDT second-generation, offspring of primed first-generation A: SENN DDT lived significantly longer. B: SENN lived significantly longer.

There was no significant difference between the longevity of F1 Gram-negative bacterial immune primed SENN and SENN DDT (**Log-rank test: $p = 0.154$, $\chi^2 = 2.03$**) (Figure 5.17A). F2 primed SENN lived significantly longer than SENN DDT (**$p = 0.001$, $\chi^2 = 10.4$**) (Figure 5.17B).

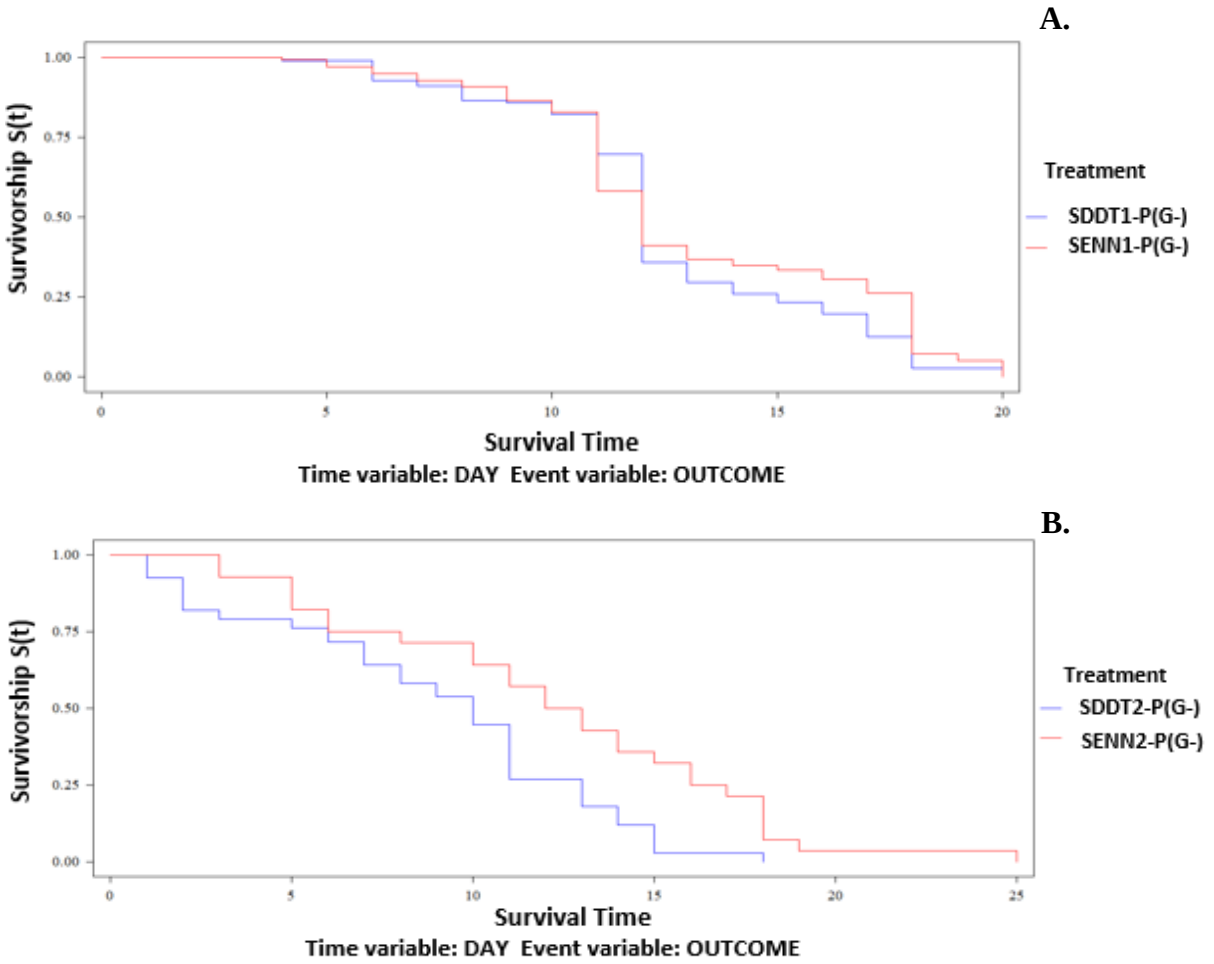


Figure 5. 17: Comparison of the effects of Gram-negative bacterial larval immune priming on the generalised longevity of unchallenged SENN and SENN DDT. SENN1-P(G-): primed SENN first-generation. SDDT1-P(G-): primed SENN DDT first-generation. SENN2-P(G-): unprimed SENN second-generation, offspring of primed first-generation. SDDT2-P(G-): unprimed SENN DDT second-generation, offspring of primed first-generation.. A: There was no significant differences in longevity. B: SENN lived significantly longer.

5.4. DISCUSSION

The subject of immunological memory in mosquitoes is one of the least studied aspects of mosquito biology. There are various reasons why an understanding of this process would be important. However, in the relatively early days of this research, still some key factors that need to be established, particularly on the design of the experimental procedures. When examining two of the latest publications on this subject, Brown *et al.*, 2019 and Powers *et al.*, 2020, it can be noted that this present study addresses several basic issues not covered by the aforementioned studies.

Firstly, this study examined *An. arabiensis*, which is generally less studied than *An. gambiae*, particularly in terms of immunology. Both studies (Brown *et al.*, 2019 & Powers *et al.*, 2020) examined the *An. gambiae* strain G3, which is susceptible to *Plasmodium* and insecticide.

Insecticide resistance is on the increase, with the data suggesting that insecticide resistant populations are exceeding the fully susceptible ones (Knox *et al*., 2014). Furthermore, insecticide resistance is increasingly linked to increased susceptibility to the *Plasmodium* infection (Alout *et al*., 2014). As such, the use of insecticide resistant strains in immunological studies must be considered. This study used two genetically related *An. arabiensis* strains to examine immunological memory, which addresses the issue of the less examined vector, as well as dealing with the questions of the role of insecticide resistance.

Larval infection by contaminated water is a simple mechanism of infection, and has been applied before (e.g., Brown *et al*., 2019). Adult infection, however, is usually performed by blood-feeding (e.g., Contreras-Garduño *et al*., 2014; Contreras-Garduño *et al*., 2015) or injection (e.g., Powers *et al*., 2020; Brown *et al*., 2019). The injection as a delivery mechanism requires skilled personnel, as well as nano-injection equipment, which could be challenging in resource-limited settings. Therefore, if sugar could be used to induce immunological memory, it would simplify the experimental protocol.

Unfortunately, this study demonstrated that sugar-delivered infection was a poor immune priming mechanism. Over and above this, it was particularly unsuccessful for *E. coli* (Gram-negative) immune priming. Although *S. pyogenes* (Gram-positive) sugar-delivered immune priming increased tolerance in the insecticide resistant SENN DDT strain, *E. coli* infected sugar did not increase tolerance. In the insecticide susceptible SENN strain, *E. coli* sugar-delivered immune priming significantly decreased tolerance, which was the only time this was observed in this study. Therefore, not only was sugar-delivered immune priming with *E. coli* not successful in inducing immunological memory, but there were also differences in the response between the strains. This differential response between the strains was also observed with *S. pyogenes* immune priming. When delivered by sugar, this bacterium induced increased tolerance in SENN DDT but not SENN. Therefore, early exposure to infected sugar was not an effective immune priming mechanism. This may be due to the digestive process of sugar. Carbohydrates such as sugar are not digested immediately by mosquitoes. Unlike blood, which is immediately directed to the gut and rapidly digested, sugar is initially stored in the diverticulum (crop) before being released into the gut for digestion. As such, the priming source is not introduced to the gut as rapidly as something provided in a blood-meal (Day, 1954). Thus, sugar delivered as the infection delivery mechanism may reduce the efficacy of the immune priming response.

Immune priming by an initial infected blood-meal was more effective, substantiating previous studies demonstrating that a blood-meal infected with either bacteria or parasites could generate immunological memory (Contreras-Garduño *et al*., 2015). What is notable about this study is that although blood-feeding was an effective immune priming system, there was a difference in response to the two different bacterial strains used. This is a notable comparison to the lack of responsiveness to *E. coli* in the sugar-delivered mechanism. This does highlight the choice of immune priming bacterium. A potential improvement of this study would be the use of an intracellular bacterium for immune priming. Although, only facultatively intracellular bacteria such as *Salmonella typhi* or *Listeria monocytogenes* (McClure *et al*., 2017) could be used, it may be more informative, as the *Plasmodium* parasite is intracellular (reviewed by Beck & Ho, 2021). As such, bacterial immune priming with an intracellular bacterium may not only have a slightly different immune priming effect. However, it may also be a more effective bacterial immune priming tool in understanding the effect of bacterial immune priming on parasite transmission. Both strains used in this study have the capacity for intracellular invasion. The *E. coli* strain is a serotype O6 biotype 1 strain, which is associated with intracellular urinary tract infections (Blum *et al.*,1995). *Streptococcus pyogenes* has recently been recognised as having the capacity for intracellular invasion, albeit typically in white blood cells (Medina *et al*., 2003; Hertzén *et al*., 2012). This may play a role in the efficacy of the immune priming effect.

Regardless of the choice of bacteria, it is clear that there is a difference in the immune priming effects of blood-feeding in SENN and SENN DDT. For the blood-feeding study, both primed SENN and SENN DDT strains had an increased tolerance. When comparing the longevity of Gram-positive bacterial immune primed SENN and SENN DDT, SENN lived significantly longer. By contrast, for the Gram-negative bacterial immune priming study, SENN DDT lived significantly longer than SENN. Although this study does reinforce the efficacy of blood-feeding as an immune priming delivery system, it does highlight the importance of considering the use of insecticide resistant strains in immunological studies. Furthermore, this study demonstrated that the effects observed were due to bacterial supplementation and not simply due to the supplementation of blood. The SENN DDT strain has previously been demonstrated to live longer after multiple blood meals (Oliver & Brooke, 2014). The additional efficacy of blood-feeding induced immune priming may likely be due to the induction of the hormone 20-hydroxyecdysonemay that plays a role in inducing a number of immune functions, including haemocyte differentiation (Ramirez *et al.*, 2015). However, blood-feeding alone, in this case,

did not increase longevity compared to sugar-fed mosquitoes, for both SENN and SENN DDT. This indicates that the observed effect is due to bacterial supplementation rather than the simple act of feeding.

The transgenerational studies have also supported previous studies demonstrating that larval exposure to bacteria was an excellent mechanism of inducing trans-stadial immune activation (Brown *et al*., 2019). Immunological memory has been demonstrated to be relatively long-lasting, often lifelong (Powers *et al*., 2020), as well as transmissible to offspring (Contreras-Garduño *et al*., 2014). The current study examined various aspects of transgenerational immune priming over and above tolerance. Immune priming had a more marked effect on SENN DDT than SENN. In the SENN strain, fertility (hatch percentage) was unaffected, while fecundity (numbers of eggs produced) was affected in most cases. Gram-negative bacterial immune priming had the same effect on fecundity in both strains. The first-generation had a significantly reduced fecundity after immune priming, but this was recovered in the unprimed second-generation, where fecundity exceeded the fertility of the control naïve mosquitoes. Although previous studies have demonstrated a reduction in egg production (a reproductive cost) due to immune priming (Contreras-Garduño *et al*., 2014), this effect does not appear to be transgenerational.

The previous objectives measured the success of immune priming as tolerance to the bacterial challenge, measured as longevity when exposed to bacteria-infected sugar. This is not the only mechanism of measuring the success of immune priming. The transgenerational capacity to eliminate *E. coli* from the gut was also determined. This experiment was the first to highlight key differences in the immune responses between the two strains. Unprimed SENN DDT exposed to *E. coli* infected sugar had less viable *E. coli* recovered from the midgut than SENN. This suggests that SENN DDT had a better capacity to cope with the bacterial challenge. This supports previous studies that demonstrated that SENN DDT had a better phenotypic capacity to cope with the bacterial challenge. These studies showed that SENN DDT lived longer when exposed to *S. pyogenes* and *E. coli*, both at the control and elevated temperatures (Barnard *et al*., 2019). SENN DDT crude protein lysate also resulted in a significantly greater clearing of both bacterial species using the Kirby-Bauer disc diffusion test (Gotte, 2020; Nonjola, 2020). However, this advantage was lost in the second-generation, where the amount of viable *E. coli* recovered from second generations of primed SENN and SENN DDT exposed to *E. coli* did not differ significantly. This suggests that immunological memory wanes in the SENN DDT strain. This finding is substantiated by the transgenerational tolerance studies.

Immune priming mosquitoes by exposure to bacteria as larvae were an effective immune priming mechanism in this study. Both Gram-positive and Gram-negative bacterial challenges increased bacterial tolerance in SENN DDT. This is not unprecedented, as this has been demonstrated before (Brown *et al*., 2019). Interestingly, *E. coli* immune priming was not successful in the SENN strain. This is a repeating theme, as sugar-delivered immune priming with *E. coli* was detrimental. What is of interest is the success of the transgenerational immune priming effects between the two strains. For Gram-positive bacterial immune priming, the first-generation of primed SENN DDT lived significantly longer than SENN. In the second-generation, unprimed SENN lived significantly longer. Generally, there was no significant difference in the longevity of the Gram-negative bacterial immune primed mosquitoes, with the exception of second-generation unprimed SENN, which lived significantly longer. When comparing the longevity of the first and second generations of primed mosquitoes, there was no significant difference in SENN. By contrast, the longevity of the second-generation of primed SENN DDT was significantly reduced after both the Gram-positive and Gram-negative bacterial challenges. This supports the previous findings that immunological memory attenuates more in the SENN DDT strain than the SENN strain.

This attenuation of the immunological memory in SENN DDT may be a result of the fitness cost associated with insecticide resistance. Fitness costs are common in insecticide resistant strains (Kliot & Ghanim, 2012). One of the key fitness costs is a decrease in longevity (Otali *et al.*, 2014). Gram-positive bacterial larval immune priming induced a fitness cost in SENN DDT, as immune priming reduced the generalised longevity. This was also observed after blood-borne immune priming with Gram-positive bacteria. SENN DDT mediates the resistance phenotype by both metabolic and target-site resistance (Oliver & Brooke, 2017). As such, it is likely due to this metabolic toll that the immune tolerance in SENN DDT attenuates. This again demonstrates that it is crucial to include insecticide resistant strains in immunological studies.

Immunological memory is potentially an important aspect in transmission dynamics. Previous studies on trans-stadial immune priming suggested that larval immune priming increased the response to bacteria, although this was not true for the anti-parasite response. The immune priming increased the susceptibility of *An. albimanus* to *P. yoelii* (Brown *et al*., 2019). If this is found to be true for *P. falciparum*, larval bacterial immune priming may increase the parasite transmission.

Although, this study provides valuable considerations for the studies of immune priming, it has several limitations. The biggest limitation is the use of laboratory strains. It has been previously demonstrated that the laboratory strains have a more exaggerated immune response than their wild counterparts (Cornet *et al*., 2013). Although more challenging, this experiment must be replicated in F1 wild mosquitoes. A second limitation is that although tolerance to bacterial challenge is a measure of the success of immune priming, further measures of the success of immune priming is required. An examination of changes in haemocyte populations would also provide more mechanistic explanations of the findings. Finally, the expansion of the study to include more members of the *An. gambiae* complex would be informative. A limitation of the study is that a confirmation of the bacterial load in the tolerance experiments would have improved the study by confirming whether the bacterial load differed in the two strains. This was performed in the transgenerational study by examining viable bacterial load, but this would not have accounted for non-viable bacteria that had been neutralised by the immune system. This could have been performed by quantifying 16s transcripts by qRT-PCR.

In conclusion, blood-borne immune priming was more effective in inducing immunological memory than sugar-delivered immune priming. *S. pyogenes* differed in the efficacy of immune priming than *E. coli*. The SENN and SENN DDT strains differed in the capacity to induce immunological memory, as the immune priming comes at a fitness cost in SENN DDT. The effects were transgenerational, with SENN maintaining immunological memory better than SENN DDT.

CHAPTER SIX: General discussion and conclusions

Mosquito immunity is a critical component of the biology of the mosquito. It is however, challenging to study, particularly because immunity is a life-history trait that is not as well conserved in the laboratory strains as other traits. There are still numerous questions about the immune system of the mosquito, and it is particularly fascinating that the epigenetic regulation of mosquito immunity is poorly examined. Mosquito epigenetics, in general, are poorly examined. This is highlighted by the fact that the term '*Plasmodium* epigenetics' returns 277 results compared to the 24 results for '*Anopheles* epigenetics' on Pubmed.

It is also worth noting that the studies on anopheline epigenetics of the *An. gambiae* complex are typically focused on the nominal member of the complex. Therefore, the opportunity to compare the epigenetic patterns of other members of the *An. gambiae* complex is useful basic information. In resource limited-environments, epigenetic characterisation using ELISA kits is a more cost-effective, if less accurate, method of baseline study than next-generation sequencing options. The epigenetic experiments in this study represents a validation of the use of commercial kits for mosquito research, which were originally optimised for mammals. This study demonstrated that the kits can also be used to quantify nucleic acid methylation in *Anopheles* mosquitoes. Due to the size, and as such the subsequent amount of nucleic acid of the mosquito, they cannot be used to characterise a single mosquito. However, several useful findings have emerged from this study.

Another limitation of the epigenetic study was that the 5mC and 5hmC DNA methylation kits were from two separate companies. This was based on the superior sensitivity of the Sigma-Aldrich® DNA methylation kit, which did not have a matching 5hmC DNA methylation kit. This was based on a previous study (Jeanrenaud, 2019). The Zymo® 5hmC kit was more sensitive than the 5mC kit, and, as such, was used to quantify this methylation. Unfortunately, these kits calculated methylation relative to their positive controls differently. As such, a direct comparison between the 5mC and 5hmC levels could not be made. Instead, the relative methylation patterns were compared. Typically, there was a pattern of low 5mC with high 5hmC DNA methylation and vice versa. These patterns generally correspond to increased gene expression and decreased gene expression, respectively. It is possible that the 5hmC kit was less sensitive in comparison to the 5mC kit. As such, the lack of change at the 5hmC level may be an artefact of the kits' sensitivity and may be an underrepresentation of the effect on 5hmC

methylation. Furthermore, examination of epigenetic patterns of specific genes in a specific tissue, rather than the whole body, may reveal differences that are masked in this study. The epigenetic patterns in the midgut, reproductive organs and salivary glands are of potential interest following an infection.

A key finding of the epigenetic study was that both *An. arabiensis* strains SENN and SENN DDT had similar methylation patterns at the constitutive level. This agrees with the previous examination of the effects of metal exposure on the epigenetic architecture of these two strains. In the latter study, the control 5mC, 5hmC and m6A methylation levels, as well as HAT activity, did not differ between the two strains, and changes were observed only after the exposure metal stress (Jeanrenaud, 2019). As with the aforementioned study, the difference in epigenetics became apparent in response to a stressor, in this case, an immune stimulation. This is particularly true for the Gram-positive bacterial challenge. This is expected, as critical organs like the midgut are dominated by Gram-negative bacteria (Dada *et al*., 2018). As such, Gram-positive bacteria are likely to represent non-self and therefore may result in a greater immunological response. It is worth noting that Gram-positive bacterial challenge resulted in greater changes in the insecticide resistant SENN DDT strain, which is congruent with the different gut microbiota of SENN and SENN DDT (Barnard *et al*., 2019). This finding emphasises the relatively greater effect of *S. pyogenes* on the epigenetic landscape of SENN and SENN DDT strains in general.

There was a general conservation of 5mC DNA and m6A mRNA methylation markers between the F1 progeny of wild-caught and the laboratory strains of *An. arabiensis*. This suggests that the findings of these methylation markers in laboratory-reared *An. arabiensis* are representative of wild mosquitoes and are not due to the colonisation effects. However, the 5hmC DNA methylation levels of F1 mosquitoes were typically lower than that of the laboratory strains. It has been previously demonstrated that laboratory-reared mosquitoes have a greater phenoloxidase activity than their wild counterparts, particularly for the insecticide resistant strains (Cornet *et al*., 2013). As 5hmC is associated with gene expression (Jaenisch & Bird, 2003), these low levels observed in wild mosquitoes may be related to the reduced phenoloxidase activity. This may be due to the less challenging laboratory environment, which results in greater resource allocation to factors such as immunity due to the lack of predation and other environmental challenges. Therefore, the findings of particularly low 5hmC levels do not invalidate the 5mC and m6A methylation results.

Generally, there were few differences in the m6A mRNA methylation levels and HAT activity. This again, may not necessarily suggest that there are no differences in RNA methylation patterns or histone modification. This is because m6A methylation is not limited to mRNA. Examining the methylation in other types of RNA may require a more specialised RNA extraction than the generalised extraction procedure of the Quick-RNA™ MiniPrep kit. Furthermore, the HAT activity is not the only determinant of histone modification, as there are several other histone-modifying enzymes that could alter the epigenetic regulation (Lodish *et al.*, 2000; Zheng & Hayes, 2003). Histone ubiquitination could also play a role. Alternate histone ubiquitination can activate or repress gene expression (reviewed by Meas & Mao, 2015). As such, the interaction between histone modification and immunity represents an independent study.

This study not only examined epigenetics as a regulatory mechanism. Changes in the transcript levels were also examined to assess the effects of immune stimulation on specific immune related genes. A PRR transmembrane immune receptor upstream, as well as AMPs downstream of the immune signalling pathways were examined in this study. The regulation of the upstream PRR, GNBP-B1 was affected by the Gram-positive bacterial challenge. Species-specific changes in the expression of *GNBP-B1* were observed. Although there were no differences in the constitutive levels of expression of this PRR, the response to an immune stimulation varied. This already indicated that there was a potential for the expression of downstream effector molecules to differ, which was indeed observed. There was a marked variation in the AMPs expression level of transcripts between the species, even at the constitutive level. It is interesting to note the congruency of the 5mC DNA methylation patterns (gene silencing) at the constitutive level and after the Gram-positive bacterial challenge, and the AMPs transcript levels. The *An. arabiensis* strains had higher levels of 5mC in those cases, as well as lower transcript levels. This pattern was not observed after Gram-negative bacterial challenge, but it does suggest a further validation of the findings from the commercial methylation quantification kits.

Similar to the methylation patterns, there was a clear distinction in the transcript expression levels of AMPs between the *An. arabiensis* strains and the *An. merus* and *An. quadriannulatus* strains. Further congruence with the epigenetic studies can be seen when examining the differences in the AMPs transcript expression levels in the SENN and SENN DDT strains. Like with all the epigenetic markers, there were no differences at the constitutive level, but differences in AMP transcript levels were observed after the bacterial challenges. These

findings suggest that these strains do not have a baseline difference in immunity, but rather differs in generating a response to an immune stimulation.

There has been a substantial amount of research on the effect of AMPs on anti-Plasmodial defence. However, the role of AMPs is not fully understood, with many studies examining their role using non-*P. falciparum* models (as reviewed in Vale *et al*., 2014). Although these molecules represent attractive control targets, it is not clear whether they would be useful against the most severe type of malaria parasites. However, the fact that there are basal differences in the AMPs transcript expression levels in the major, minor, and non-vectors, which is only increased after immune stimulation, suggests that it may play a role in species-specific differences in immunity. It is worth noting that the changes in AMP transcript levels may not be specific, as observed by the high levels of expression in the *An. merus* strain, which is reared in saltwater. In that case, the expression may at least be in part attributable to osmotic defence (Kido *et al.*, 2010). This does not, however, invalidate the marked differences observed between the major vector, the minor and the non-vector.

Most of the current literature suggests that insecticide resistant anophelines are more susceptible to the parasitic infection than their susceptible counterparts (reviewed in McCarroll & Hemingway, 2002). This is regardless of whether the resistance phenotype is mediated by target-site or metabolic resistance. The insecticide resistant strain in this study, SENN DDT, mediates resistance by both mechanisms (Oliver & Brooke, 2017). It is interesting to note that even with a bacterial challenge, the expression levels of transcripts for both AMPs was typically lower in SENN DDT than in SENN. With the exception for *gambicin* expression after Gram-negative challenge, this is particularly noteworthy considering the phenotypic tolerance studies, where SENN DDT showed an excellent tolerance to bacterial challenges.

As it was clear from the previously mentioned studies, the insecticide resistance phenotype does have a distinct effect on the immune responsiveness of the mosquito. For this reason, the studies on immunological memory focussed on the SENN and SENN DDT strain. Future studies will replicate and expand these experiments to include the MAFUS and SANGWE strains. From these phenotypic studies, a difference not only in the immune response of SENN and SENN DDT was noted, but a difference in the immune response to *S. pyogenes* and *E. coli* bacteria as well.

One of the aims of the immune priming studies was to assess the differences in the efficacy of the initial immune stimulation on the generation of immunological memory. The provision of

an infected sugar meal was the least efficient mechanism of inducing immunological memory. It was so inefficient, that only the Gram-positive bacterial challenge in SENN DDT resulted in an increased tolerance upon a second challenge. This inefficiency may be due to the digestion process. Sugar is not usually digested immediately, but rather stored initially in the diverticulum until required. This is in contrast to blood, which is rapidly digested (Clements, 1999b). As such, the system would be exposed to a blood-borne challenge more rapidly than a sugar-borne challenge. This may influence the success of the immune priming response. It is also worth noting that SENN DDT generated immunological memory. This again highlights the good phenotypic responsiveness of the SENN DDT strain to bacteria, contrasting with the lower relative AMP transcript levels after bacterial challenges.

The blood-borne immune priming experiments were performed slightly differently from other immune priming experiments. The longevity was measured after one or two infected blood meals, rather than measuring tolerance to a continuous supply of infected sugar. This is partly to accommodate the delayed effects that would be observed from an infected sugar-meal. It also removed extraneous variables from the blood-borne study. The initial infected blood-meal resulted in an increased longevity after the exposure to a second infected blood-meal. This effect was independent of the effect of blood feeding on longevity (Oliver and Brooke, 2014). These findings suggests that a bacteria-infected blood-meal early in life could heighten the immune response later in life. The blood feeding study also demonstrated that there were differences in the responses elicited by the Gram-positive and Gram-negative bacteria. The latter resulted in a greater change in longevity.

It is worth noting that although the primed SENN DDT strain showed increased longevity after the second infected meal, this immune priming effect came with a cost. The initial immune priming resulted in a generalised decrease in longevity in the SENN DDT strain, which was not observed in the SENN strain. This suggests that the generation of immunological memory in SENN DDT incurs a fitness cost. Additional fitness costs were observed when examining the transgenic effects of immune priming.

The fitness costs in SENN DDT were observed when examining transgenerational larval immune priming. When initially primed as larvae, regardless of whether *S. pyogenes* or *E. coli* was administered, there was a strong increase in tolerance to the same bacteria. However, this tolerance was markedly reduced in the second-generation, although the second-generation still had a greater tolerance than the naïve (control) population. This pattern was not observed in

SENN. This supports the observations of a fitness cost in the SENN DDT strain as seen in the blood-borne immune priming study. Furthermore, it also suggests that SENN maintains immunological memory better than its insecticide resistant counterpart.

The larval immune stimulation can increase adult tolerance to the same bacteria and these effects are transgenerational. Blood feeding with an infected meal at an early age results in a greater tolerance when challenged the second time. It is therefore clear that immunological memory can be generated by both short-term and long-term immune stimulation. As these effects are transgenerational, they may be mediated by the epigenetic changes, as it was observed that bacterial challenges can alter the epigenetic landscape.

It has already been demonstrated that SENN DDT had a more marked initial phenotypic tolerance to bacterial challenge. However, a recent study demonstrated that increased bacterial tolerance by larval immune priming resulted in an increased susceptibility to *Plasmodium* infection (Brown *et al*, 2019). This finding, as well as the decreased expression of AMPs that have anti-Plasmodial activity, may underlie the increased prevalence and intensity of parasitic infection in insecticide resistant anophelines.

These findings raise the question of the significance of immunological memory in SENN and SENN DDT. It is not clear for how many generations the findings of Brown *et al*., 2019 are valid. As such, the only way to fully understand the effect of bacterial immune priming on the interaction between the mosquito and parasite is by exposing the primed females to parasite using membrane-feeding assays. That, however, is beyond the remit of this study, which was largely focussed on using a bacterial model system. Nonetheless, this study does demonstrate that differences exist in inducing immunological memory and maintenance in insecticide resistant *An. arabiensis*.

In conclusion, this study demonstrates that there are species-specific differences in the epigenetic landscape as well as the expression levels of transcripts for AMPs between the three zoophilic members of the *An. gambiae* complex. Insecticide resistance alters the responsiveness of *An. arabiensis* to immune stimulation. Immunological memory is best generated in *An. arabiensis* by an infected blood-meal or larval immune priming with bacteria. The effects of larval immune priming are transgenerational, and this response is affected by the insecticide resistance phenotype. These findings provide baseline information on the epigenetic landscape of the two poorly studied members of the *An. gambiae* complex. It also

provides insight into the role of insecticide resistance in immune responsiveness, which may ultimately inform immune-based interventions for vector control.

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APPENDICES

APPENDIX 1: Ethics waiver

ANIMAL RESEARCH ETHICS COMMITTEE
Registration number: AREC-101210-002

Date: 09/06/2020

Certificate reference: Waiver 09-06-2020-O
Category: O
Applicant: Nashrin Fazlerehman Patel (1434246)
Department: National Institute for Communicable Diseases/Wits Research Institute for Malaria (University of the Witwatersrand)
Tel: 011 386 6485; **Email**: 1434246@student.wits.ac.za

Re: Waiver from the Animal Ethics Research Committee of the University of the Witwatersrand

This letter is to confirm that Nashrin Fazlerehman Patel (1434246), National Institute for Communicable Diseases/Wits Research Institute for Malaria (WITS), does not require full Animal Ethics Research Committee clearance to undertake the work titled **'Characterization of the immune responses of the *Anopheles gambiae* complex to bacterial stimulation'**.

Reason for waiver
This study will utilize invertebrate animals only and will not make use of any vertebrate material.

Details of the study
The aim of this study, therefore, is to characterize the immune response of *An. arabiensis*, *An. merus* and *An. quadriannulatus* from the *An. gambiae* complex in response to *Streptococcus pyogenes* (Gram-positive bacteria) and *Escherichia coli* (Gram-negative bacteria) infection by assessing the expression of anti-microbial peptides. Furthermore, it will be determined if these immune responses are heritable, affected by changes in epigenetics or affected by insecticide-resistance.

The individuals covered by the waiver are Nashrin Fazlerehman Patel and Dr Shüné Oliver.

Please contact me should you require further information.

Yours sincerely

Prof Frederic Michel
Chair: Animal Research Ethics Committee
University of the Witwatersrand

APPENDIX 2: Manuscript arising from this study (proof of submission)

This will be submitted upon approval from all co-authors

NATIONAL INSTITUTE FOR COMMUNICABLE DISEASES

of the ***NATIONAL HEALTH LABORATORY SERVICE***

NICD



Vector Control Reference Laboratory

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To the editor, Insect Biochemistry Molecular Biology X/05/2021

Our manuscript entitled "**Comparison of the effect of bacterial stimulation on the global epigenetic landscape and transcription of immune genes in zoophilic members of the *Anopheles gambiae* complex (Diptera: Culicidae)"** is submitted to you as an original manuscript.

In this study, we examined the global epigenetic landscape (5mC, 5hmC, m6A-methylation as Histone Acetyl Transferase activity) as well the transcript levels of immune related genes (GNBP-1, Defensin-1 and Gambicin) in response to bacterial stimulation (*Streptococcus pyogenes*, *Escherichia coli*). The factors were examined in four laboratory strains, covering 3 species, *Anopheles arabiensis*, *An. merus* and *An. quadriannulatus*, representing a major, minor, and non-vector species respectively.

We found species-specific variation in 5mC and m6A methylation between the strains, even at the constitutive level. The markers tended to differ between the major vectors and the minor/non-vectors. As an insecticide susceptible and resistant strain of *An. arabiensis* were used, it was observed that basal levels of the markers did not differ but differed upon response to immune challenge. The markers observed in laboratory *An. arabiensis* was largely conserved in F1 females. The species-specific patterns were also reflected in the mRNA transcript analysis. The minor and non-vectors had higher levels of antimicrobial peptide expression than both *An. arabiensis* strains. These findings have potential implications for transmission dynamics.

[All authors have read and approve the manuscript]. The authors declare no competing interests. This original manuscript has not been published before or submitted for publication elsewhere. We hope that you will consider our manuscript for publication.

Yours sincerely,

Dr. Shüné Oliver
Senior Medical Scientist, Senior Researcher
Vector Control Reference Laboratory
Centre for Emerging Zoonotic and Parasitic Diseases
National Institute for Communicable Diseases
Johannesburg, South Africa.

APPENDIX 3: Protocol of native 1% Tris-borate-EDTA (TBE; EDTA - Ethylenediaminetetraacetic acid) agarose gel preparation

The detailed step by step protocol is as follows:

1. Mix 0.5 g of agarose powder into 50 ml of 0.5× TBE buffer in a beaker and boil.

    1.1. To prepare 0.5× TBE buffer, add 20 ml of 5× TBE buffer into 180 ml of Diethyl pyrocarbonate (**DEPC**) treated water.

    1.2. To prepare DEPC-treated water, add 1 ml of commercially available DEPC solution (Invitrogen™: AM9915G) into 999 ml of distilled water.

    1.3. To prepare 5×TBE buffer, add 54 g Tris base, 27.5 g boric acid and 20 ml of 0.5M EDTA into 800 ml of DEPC-treated water.

2. Once the mixture is cooled, add 1 µl of ethidium bromide dye (Sigma-Aldrich: E1510) before pouring the mixture into the casting tray. Sterilise all the equipment using 3% hydrogen peroxide.

APPENDIX 4: Formulae for the calculation of relative epigenetic markers

1. **5-methylcytosine (5mC) calculation (100%)**

1.1. Average the absorbance at 450 nm (**A450**) for the blank, samples and the positive methylated control.

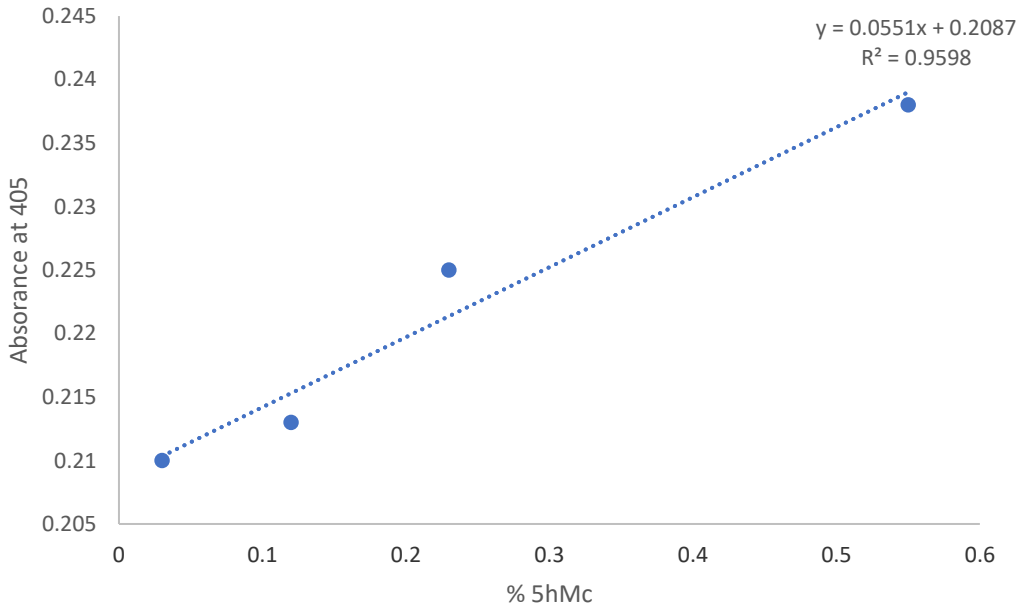
1.2. Thereafter use the following formula to calculate % 5mC

$$\frac{A450\ \text{sample} - \text{A450 blank}}{\text{A450 positve control} - \text{A450 blank}} \times 100$$

2. **5-hydroxymethylcytosine (5hmC) calculation (55%)**

2.1. Generate a standard curve from the kit-provided control DNA set

The standard curve



2.2. Therefore % 5hmC = (A_{sample} – y-intercept) / slope

- Where A_{sample} is the absorbance of the sample, measured using a plate reader
- Y- intercept = 0.2087 (from the standard curve)
- Slope = 0.05551 (from the standard curve)

3. <u>N6-methyladenosine (m6A) calculation (100%)</u>

3.1. Average the absorbance at 450 nm (**A_{450}**) for samples, the negative control, and the positive methylated control.

3.2. Thereafter calculate the m6A % using the following formula

$$\frac{(\text{A450 sample} - \text{A450 negative control}) \div \text{S}}{(\text{A450 positive control} - \text{A450 negative control}) \div \text{P}} \times 100$$

- Where S is the amount of RNA added, which is 200 ng
- P is the amount of positive control added, which is 1 ng

APPENDIX 5: The 96-well ELISA plate layout for the epigenetic study

SENN control	**SENN** control	**SENN** control	**SENN** control	**SENN** control	**SENN** control	**SENN** Gram (+)	**SENN** Gram (+)	**SENN** Gram (+)	**SENN** Gram (+)	**SENN** Gram (+)	**SENN** Gram (+)
S DDT control	**S DDT** control	**S DDT** control	**S DDT** control	**S DDT** control	**S DDT** control	**S DDT** Gram (+)	**S DDT** Gram (+)	**S DDT** Gram (+)	**S DDT** Gram (+)	**S DDT** Gram (+)	**S DDT** Gram (+)
MAFUS control	**MAFUS** control	**MAFUS** control	**MAFUS** control	**MAFUS** control	**MAFUS** Control	**MAFUS** Gram (+)	**MAFUS** Gram (+)	**MAFUS** Gram (+)	**MAFUS** Gram (+)	**MAFUS** Gram (+)	**MAFUS** Gram (+)
SANGWE control	**SANGWE** control	**SANGWE** control	**SANGWE** control	**SANGWE** contol	**SANGWE** Control	**SANGWE** Gram (+)	**SANGWE** Gram (+)	**SANGWE** Gram (+)	**SANGWE** Gram (+)	**SANGWE** Gram (+)	**SANGWE** Gram (+)
SENN Blood control	**SENN** Blood control	**SENN** Blood control	**SENN** Blood control	**SENN** Blood control	**SENN** Blood control	**SENN** Gram (-)	**SENN** Gram (-)	**SENN** Gram (-)	**SENN** Gram (-)	**SENN** Gram (-)	**SENN** Gram (-)
S DDT Blood control	**S DDT** Blood control	**S DDT** Blood control	**S DDT** Blood control	**S DDT** Blood control	**S DDT** Blood control	**S DDT** Gram (-)	**S DDT** Gram (-)	**S DDT** Gram (-)	**S DDT** Gram (-)	**S DDT** Gram (-)	**S DDT** Gram (-)
MAFUS Blood control	**MAFUS** Blood control	**MAFUS** Blood control	**MAFUS** Blood control	**MAFUS** Blood control	**MAFUS** Blood control	**MAFUS** Gram (-)	**MAFUS** Gram (-)	**MAFUS** Gram (-)	**MAFUS** Gram (-)	**MAFUS** Gram (-)	**MAFUS** Gram (-)
SANGWE Blood control	**SANGWE** Blood control	**SANGWE** Blood control	**SANGWE** Blood control	**SANGWE** Blood control	**SANGWE** Blood control	**SANGWE** Gram (-)	**SANGWE** Gram (-)	**SANGWE** Gram (-)	**SANGWE** Gram (-)	**SANGWE** Gram (-)	**SANGWE** Gram (-)

SENN is the insecticide susceptible *An. arabiensis*. S DDT is **SENN DDT**, insecticide resistant *An. arabiensis*. **MAFUS** is *An. merus*. **SANGWE** is *An. quadriannulatus*. Gram (+) is the Gram-positive bacteria *Streptococcus pyogenes* and Gram (-) is the Gram-negative bacteria *Escherichia coli*

APPENDIX 6: Preparation of Phosphate-buffered saline (PBS) with protease inhibitors.

The detailed step by step protocol is as follows:

1. To prepare 1× PBS (pH = 7.2), combine 137 mM Sodium chloride (**NaCl**), 43 mM Sodium phosphate dihydrate (**Na_2HPO_4**), 27 mM potassium chloride (**KCL**) and 15 mM monopotassium phosphate (**KH_2PO_4**).

2. To prepare 10 mM leupeptin stock solution, add 5 mg of leupeptin hemi sulphate to 1.05 ml of distilled water. Thereafter, dilute the stock to 2 mM in distilled water.

3. To prepare 1M phenylmethylsulphonyl fluoride (**PMSF**) stock solution, add 17.42 g of PMSF (MW=174.19 g/mol) in 100% dimethyl sulfoxide in a final volume of 100 ml. Thereafter, dilute the 1M PMSF to 4 mM in distilled water.

4. To prepare 0.5M Ethylenediaminetetraacetic acid (**EDTA**), add 18.31 g of EDTA disodium salt dihydrate (MW=372.24 g/mol) to 80 ml of distilled water

5. Combine 1× PBS, 2 mM of leupeptin, 4 mM of PMSF and 0.5 M EDTA (final pH = 8.0)

APPENDIX 7: Bradford assay

1. Prepare standard concentration of bovine protein by series of dilution: 00, 10, 20, 30, 40, 50, 100, 200, 300, 400, 500 and 1000 µg/ µl.

2. Add the proteins into individual wells of a plate.

3. Add in 1.5 ml Bradford reagent (Bio-Rad: 5000006) and incubate for 5 minutes at room temperature.

4. After color development, measure the absorbance at 495 nm.

5. Generate a standard curve using the absorbance and standard concentrations.

6. Add in 1 µl of the extracted nuclear proteins into a separate plate, add in Bradford reagent and measure the absorbance.

7. Use the standard curve to determine the concentration of nuclear protein

Standard curve

