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Malaria parasite metabolite and mosquito vector dynamics

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Malaria parasite metabolite and mosquito vector dynamics

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

Johannesburg, 2021

Declaration

I declare that this Dissertation is my own, unaided work. It is being submitted for the Degree of Master of Science (MSc Med) at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other University.

EERLANK
(Signature of candidate)

15th day of September 2021

Publications arising from this research project

*****Due to the skills I have obtained during my MSc project. I was involved in other publications that arised from infection studies I participated in. Appendix A

Reader J, van der Watt M, Taylor D, Le Manach C, Mittal N, Ottilie S, Theron A, Moyo P, **Erlank E**, Nardini L, Venter N, Lauterbach S, Bezuidenhout B, Horatscheck A, van Heerden A, Spillman NJ, Cowell AN, Connacher J, Opperman D, Orchard LM, Llinas M, Istvan ES, Goldberg DE, Boyle GA, Calvo D, Mancama D, Coetzer TL, Winzeler EA, Duffy J, Koekemoer LL, Basarab G, Chibale K, Birkholtz LM. 2021. Multistage and transmission-blocking targeted antimalarials discovered from the open-source MMV Pandemic Response Box. Nature Communications. 12:269.

Role in the publication:
I was responsible for coordinating infection feedings as well as conducting the standard membrane feeding assays, maintaining infected mosquitoes, midgut dissections of mosquitoes, data collection and analysis.

Presentations arising from this research project

Presentation was accepted for the H3D Symposium that was due to take place in March 2021 but it has been postponed till 2022 due to COVID-19 travel restrictions.

Abstract

Plasmodium falciparum makes its human host more attractive for its vector companion, the *Anopheles* mosquito. Once in the host, the malaria parasite synthesizes isoprenoids via the 2-C-methyl-D-erythritol 4-phosphate pathway. The precursor for isoprenoids in this pathway is known as (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMBPP). HMBPP activates red blood cells to release volatile organic compounds that acts as an attractant to the mosquito to stimulate blood feeding. Only a few anopheline mosquito species are known to transmit the human malaria parasite. These mosquito can be classified as either major or minor vectors, depending on their impact on malaria transmission. The main African malaria vectors are responsible for 95% of malaria cases while minor vectors contribute to the remaining 5%.

During this study the aim was to evaluate if HMBPP influences *P. falciparum* susceptibility, feeding rate and attraction in major, minor and non-vector mosquito species. The standard membrane feeding assay was used to artificially infect mosquitoes and evaluate feeding rate, whereas attraction assays was conducted with a dual choice chamber.

Results revealed that both the major vector, *An. gambiae s.s.*, and the minor vector, *An. merus,* had an increase in both prevalence and intensity of oocysts. An increase in feeding rate and attraction was also observed for some vectors, in the presence of HMBPP. These findings could play an important role in understanding the role of parasite volatiles in malaria transmission.

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The shared data and assistance with field work undertaken in this project would not have been possible without the dedication by the SIT team and Dr. Givemore Munhenga.

The generosity and assistance of the team at the University of Pretoria, who supplied the infected blood for many of the assays conducted in this study, is greatly appreciated and a special thanks to Prof. Lyn-Marie Birkholtz and Dr. Janette Reader for their continued support.

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ABBREVIATIONS

SFMA: Standard Membrane Feeding Assay

ELISA: Enzyme-linked Immunosorbent Assay

PCR: Polymerase Chain Reaction

HMBPP: (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate

CHAPTER ONE: Introduction

1.1. Malaria Overview

Malaria continues to be one of the most destructive diseases and despite efforts to fight it, an estimate of 229 million cases were reported in 2019 – of which approximately 409,000 were fatal (World malaria report, 2020).
This infectious disease has shown to follow a seasonal trend with climate factors also contributing (Caminade et al. 2013). Furthermore, malaria endemic areas are more affected, since malaria transmission tends to present itself in short seasons or as epidemics that can result in high mortalities (Caminade et al. 2013). In an effort to eliminate malaria, the WHO introduced the global malaria eradication program in the 1950's, during which 79 countries achieved elimination status. The most successful countries were however the ones with highly seasonal malaria transmission, including northern Africa and America as well as Australia, and *Plasmodium vivax* was mainly responsible for infections (Feachem et al. 2010). Limitations in public health services and the extend of the *Plasmodium* infections in the endemic areas of Africa resulted in the replacement of malaria elimination prospects with control policies by the WHO in the early 1970's (WHO, 1970). To date the African region still accounts for approximately 94% of cases (World malaria report, 2020) and several countries are still affected by residual transmission. South Africa is among these countries where residual transmission can be attributed to the fact that it borders other African malaria endemic countries (Morris et al. 2013).

Though it is known that malaria is caused by a protozoan parasite and that an *Anopheles* mosquito vector and a vertebrate host are needed (Laveran, 1884), the knowledge regarding malaria transmission still remains inadequate. It is known that about 60, out of more than 500 anopheline mosquito species, are described as malaria vectors (Gillies & De Meillon, 1968; Gillies & Coetzee, 1987; Manguin, 2008; Sinka et al. 2010) and 48 of approximately 250 *Plasmodium* parasite species are responsible for infecting mammal hosts (Faust & Dobson, 2015). Furthermore, the *P. falciparum* parasite remains the greatest contributor of this disease and it was responsible for 99,7% of estimated sub-Saharan Africa malaria cases in 2018 (World

Malaria Report, 2019). Its most efficient mosquito companions include the *An. gambiae* complex and the *An. funestus* group. The combination of the parasite and the latter species group and complex, are responsible for 90% of deaths in sub-Saharan Africa (Fontenille et al. 1997; Fontenille & Simard, 2004; Cohuet et al. 2004; Lwetoijera et al. 2014). Although there's been a reduction of 40% in the prevalence of *P. falciparum,* across Africa, by controlling the mosquito vector with long lasting insecticide nets (LLINs) (Bhatt et al. 2015) malaria transmission still remains a problem. This could be explained by the fact that this control method might only encourage vector species to feed on people when they are not using LLINs (White et al. 1972; Mbogo et al. 1993; Kiszewski et al. 2004; Lyimo and Furguson, 2009; Takken & Verhulst, 2013). This includes people who don't have LLINs as well as those being exposed during early mornings or evenings when not sleeping (Takken, 2002).

Vector control strategies are further complicated by the fact that the *Anopheles* genus consists of species complexes and groups, where the different species within these complexes and groups exhibit different feeding behaviors and roles in malaria transmission (Gillies & Coetzee, 1987; Kyalo et al. 2017; Wiebe et al. 2017). The *An. gambiae* complex consists of nine sibling species which are morphologically indistinguishable and includes vector and non-vector species (Gillies & Coetzee, 1987; Barron et al. 2019; Coetzee 2020). Furthermore this complex comprises freshwater and saltwater breeders (Coetzee et al. 2013). The three major vectors of this complex include *An. gambiae s.s., An. coluzzii* and *An. arabiensis* which exhibit different behaviors. *Anopheles gambiae s.s.* preferentially bite human hosts indoors and *An. coluzzii* exhibit similar behavior, whereas *An. arabiensis* has a more diverse behavior and will feed indoors or outdoors on human and other mammalian hosts (Gillies & Coetzee, 1987; Sinka et al. 2010; Akogbéto et al, 2018). *Anopheles funestus s.s.* is the most anthropophilic and predominant member of the eleven members that form part of the African *An. funestus group* (Gillies & De Meillon, 1968; Gillies & Coetzee, 1987; Harbach, 2013). The latter species group also differs from the *An. gambiae* complex in terms of ecology, biology and genetics (Fontenille & Simard, 2004; Coetzee & Koekemoer, 2013). Consequently, depending on their role in malaria transmission, vectors can be classified as either major or minor (Cohuet 2009). Major malaria vectors are described as vectors that are responsible for more than 95% of the total malaria transmission globally and for sub-Saharan Africa these

species include: *An. gambiae*, *An. arabiensis*, *An. coluzzii* and *An. funestus* (Gillies and De Meillon, 1968; Gillies and Coetzee, 1987; Antonio-Nkondjio et al. 2006; Sinka et al. 2010; Coetzee et al. 2013; Kyalo et al. 2017). Minor vectors are thus responsible for the remaining malaria transmission, and these species include *An. melas*, *An. merus* and *An. bwambae* (from the *An. gambiae* complex) and *An. rivulorum* from the *An. funestus* group (Gillies & Coetzee 1987; Wilkes et al. 1996; Antonio-Nkondjio et al. 2006; Kawada et al. 2012; Kyalo et al. 2017).

The interaction between the *Plasmodium* parasite and its vectors also plays an important role when considering malaria control interventions. These parasites, especially *P. falciparum*, have been found to alter the behavior of its mosquito vectors and consequently enhance malaria transmission (Cator et al. 2012). Recent studies found that the parasite metabolite, (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMBPP), is responsible for increased attraction and infection in a mixed strain mosquito vector of *An. gambiae s.l*. (Emami et al. 2017). This parasite-host manipulation phenomenon has however not been studied extensively in African malaria vectors and it is therefore important not to only concentrate on the mosquito vectors but to follow a holistic approach with regards to the transmission cycle between the parasite and the vectors.

1.2 Aim, Objectives and hypotheses

The aim of this study was to monitor the seasonal changes of vectors in KwaZulu-Natal and determine if mosquito species were affected by the presence of the *P. falciparum* metabolite, HMBPP. Furthermore, this study was conducted in order to gain important information concerning the complex interaction between the malaria parasite and its mosquito companion. As the parasite evolve to manipulate its vector, it is important to remain vigilant as human malaria infections might be affected as a result. Being informed about how different vector species behave in the presence of the parasite, could assist in following a holistic approach that might prevent further infection, for example by adjusting vector control strategies to target species that form part of the cycle.

Specific objectives pertaining to the laboratory study included:

- Evaluating the seasonal change and overall mosquito vector dynamics between the major-, minor- and non-vectors of South Africa
- Determining the effect of HMBPP on the attraction of the major-, minor- and non-vector mosquito species
- Determining the effect of HMBPP on the feeding behavior of the major-, minor- and non-vectors
- Determining the effect of HMBPP on the susceptibility to *P. falciparum* of different vector species

The main hypotheses included:

1. The vector dynamics of the minor- and non-vector species will change in the occurrence of changes in the major vector population
2. The feeding rate and attraction behavior will be altered in the presence of HMBPP
3. Since the blood meal size is suggested to increase in the presence of HMBPP (Emami et al. 2017), the prediction would be that more gametocytes would be ingested and thus a higher number of oocysts would be present in the mosquito midgut.

1.3 Literature Review

1.3.1 Mosquito vector dynamics and seasonal variation of species

The potential for an *Anopheles* mosquito to become a vector depends on several factors, including the ability to support malaria parasite development, environmental conditions, blood feeding behavior and adult longevity/survival (Beier, 1998). More recent studies provides support for the fact that environmental factors such as temperature and rainfall could affect the transmission potential of a vector, due to changes in the biology of the vector (Afrane et al. 2012; Paaijmans et al. 2009; Paaijmans et al 2012; Mordecai et al. 2013). Larval condition has also been found to have an effect on adult traits that are important for vector competence, such as adult longevity and body size (Plaistow et al. 2001; Pulkkinen & Ebert, 2004). Although the mechanisms responsible for regulating malaria parasite-vector interactions are still being evaluated, historically, it was proposed that only certain mosquito species are able to support the parasite cycle (Beier, 1998). According to Beier (1998) only certain mosquito species are able to take up gametocytes from parasite-infected blood, and support the formation of the sporozoite stage of the parasite, which are transmitted to humans. However, recent studies provided support for the hypothesis that most anophelines might be able to support parasite development, but due to host feeding preferences, it might limit their role in malaria transmission (De Meillon et al. 1977; Temu et al. 2007; Burke et al. 2017).

These mosquito vector dynamics also play an important role, when considering control methods to reduce or eliminate certain mosquito species. It brings the question to mind: would these control methods, which focus on eradication or elimination of current major mosquito vector species, not create a niche for minor vector species to fulfill the roles of the current ones? Ecologists proposed the exclusion principle which is described as the existence of two non-interbreeding species in the same geographic area, where one species multiplies faster than the second species, and eventually replacement of the latter species will take place (Elton, 1927; Hardin, 1960). One form of species replacement takes place when introduced species numbers increase due to external interference, such as weather, habitat alteration or man-made intervention which in turn causes resident species numbers to decline (Herbold & Moyle, 1986). Species replacement was suspected to

occur in south west Uganda where the minor vector, *An. rivulorum,* replaced the major vector, *An. funestus,* due to the fact that the latter species was eliminated by house-spraying (Aboul-Nasr, 1970). The same phenomenan was observed in the inland parts of Kenya and Tanzania, where the elimination of *An. funestus* led to a sharp increase in the number of *An. rivulorum* mosquitoes (Gilies & Smith, 1960). It could therefore be suggested that if minor vector numbers are not limited (by low abundance or survival) and thus flourish, they can be considered as main vectors since these minor vectors might be able to maintain malaria transmission (Wilkes et al. 1996; Antonio-Nkondjio et al. 2006). This was also described by Temu et al. (1998) where the minor vector, *An. merus*, was found to be the main vector with the highest infection status. Additional studies by Quakyi et al. (2000) also proposed that high malaria transmission, which is maintained by the major vectors, could aid the minor vectors' performance, by keeping the parasite cycle going.

In addition to different malaria vector species and their role in malaria transmission, both transmission and parasitaemia might present seasonal variation to some extend (Smith et al. 1993). In such regions where low but periodic transmission occurs, epidemics might be the result (Bruce-Chwatt, 1985). Although the same transmission pattern is observed in South Africa (Hargreaves et al. 2000; Maharaj et al. 2013), in general malaria transmission is low and follows a seasonal trend in the malaria endemic provinces (Burke et al. 2019). Low-level transmission is described as transmission by minor vectors, of which members of both the *An. funestus* group and *An. gambiae* complex mosquitoes form part of (Gillies & Coetzee, 1987; Coetzee & Hunt, 1998). Low-level transmission could further be the result of effective malaria control that results in low abundance of main vector species. Furthermore, the current major vector species responsible for malaria transmission in South Africa includes *An. arabiensis* (a member of the *An. gambiae* complex) and historically it also included *An. funestus* (from the *An. funestus* group) (Hargreaves, et al. 2000; Brooke et al. 2013).

Anopheles funestus s.s. is amongst the species that are responsible for malaria epidemics, when present in South Africa, (Fontenille et al. 1990; Hargreaves et al. 2000). *Anopheles arabiensis* is mostly responsible for the continuation of seasonal malaria transmission in the absence of *An. funestus* (Coetzee & Hunt 1998; Coetzee et al. 2013; Dandalo et al. 2017). Some minor vectors that are responsible for low-

level malaria transmission includes *An. merus*, a member of the *An. gambiae* complex, as well as *An. vaneedeni* and *An. parensis* (both members of the *An. funestus* group).

Anopheles merus has been regarded as a minor vector in the country and has been directly involvement in malaria transmission in the southern parts of Mozambique (Cuamba & Mendis 2009). The fact that this species occurs in all the malaria endemic provinces of South Africa, is therefore a cause for concern (Mbokazi et al. 2018; Coetzee et al. 2000). *Anopheles vaneedeni* has been a suspected minor vector in sporadic malaria cases in the historic northern Transvaal (Gillies & Coetzee, 1987) and has recently been incriminated as a minor vector, as it is suspected to be involved in residual transmission in KwaZulu-Natal (KZN), South Africa (Burke et al. 2017). Another species that also recently tested positive for *P. falciparum* in the same province is *An. parensis* (Burke et al. 2019). Although several other members of the *An. funestus* group, including *An. leesoni* and *An. rivulorum*, are perennially present in KZN, their role in malaria transmission needs further investigation (Mouatcho et al. 2007; Burke et al. 2017).

KwaZulu-Natal is amongst the South African malaria endemic provinces where the primary malaria control method is insecticide based indoor residual spraying (IRS). *Anopheles arabiensis* is currently the main vector in this province and relatively abundant in the study site (Dandalo et al. 2017), however, IRS does not target this species as efficiently as *An. funestus* due to the nature of its feeding and resting behaviours (Gillies & Coetzee, 1987; Sinka et al. 2010; Coetzee & Hunt 1998). The latter species was therefore controlled efficiently and could be eliminated, despite its up flare in the year 2000 (Hargreaves et al. 2000).

A number of minor vector species co-exists with *An. arabiensis*, and also needs to be considered since control interventions, combined with seasonal variation, might enhance the transmission potential of minor vectors. Furthermore, as vector dynamics are constantly changing and more minor vector species are being incriminated in malaria transmission, it would be suspected to see an increase in the abundance and parasite transmission capacity of current minor vector species, as major vector numbers are declining, due to external factors such as control interventions. This phenomenon was also visible in the Kruger National Park where

An. arabiensis numbers declined due to possible draught in the area which resulted in an increase in the number of *An. quadriannulatus* (a non-vector species) mosquitoes (Munhenga et al. 2014; Munhenga, unpublished data).

1.3.2 Mosquito blood feeding and attraction behavior

The blood sucking habit of insects is believed to have originated from a close and extended association with vertebrates (Lehane, 2005). Even unspecialized insects adapted to become blood-sucking ones as a result of the high nutritive value and easy digestibility of blood (Lehane, 2005). The estimated number of blood feeding insect species is 14,000 of which only 300 – 400 are of medical and agricultural importance (Adams, 1999; Lehane, 2005). Numerous blood-sucking insects have caused huge destruction as several diseases are caused by the transmission of parasites to both humans and animals (Lehane, 2005). Like for most other blood-sucking insects, mosquitoes are dependent on blood for the vital nutrients and proteins that are needed for egg production and therefore blood feeding is crucial for obtaining reproductive fitness (Takken & Verhulst, 2013). The term blood feeding can be described as a combination of behaviors which includes host detection, probing and piercing, location and ingestion of blood as well as termination of the feed (Friend & Smith, 1977).

It is assumed that the blood source selected by female mosquitoes depends on genetic factors (Kilpatrick et al. 2007; Huang et al. 2009) and Southwood (1977) also proposed that the historic enviro nMent of a species influences its niche. All insects do not feed equally well from the same host and thus exhibit host choice preference. Host preference is a behavioral trait that is believed to have been shaped by native and external factors (Takken & Verhulst, 2013). Mosquitoes can be classified as either specialist feeders, which tend to feed on certain hosts, whereas the generalists are more opportunistic and make use of a variety of hosts (Takken & Verhulst, 2013). Furthermore, it was proposed that niche shifts and adaptation to different resources is possible (Chaves et al. 2010). This is due to the holometabolic trait of mosquitoes, which allows them to exploit different resource i.e. feeding on different hosts (Chaves et al. 2010). Feeding behavior also differs between different mosquito vector species. *Anopheles arabiensis* tends to rest outdoors and in the absence of insecticides, it will

also rest indoors together with exhibiting a diverse feeding habit (both human and cattle), whereas *An. funestus* is a highly anthropophilic and endophilic mosquito and thus mostly feed on human hosts (Coetzee & Fontenille, 2004; Sinka et al. 2010).

In order for a mosquito to feed on a host, the host must be located and although some of the blood feeding mosquitoes are not fastidious when it comes to finding the right host (Takken & Verhulst, 2013), host attraction plays an important role in the location process. Several factors need to be taken into account when considering mosquito-host attraction, including heat, visual cues and olfaction (Takken & Knols, 1999; Lehane, 2005). At close proximity, mosquitoes can sense heat with thermosentitive neurons that are found in the sensilla on the tip of the antennae (Khan et al. 1966; Gingl et al. 2005). (Fig. 1.1). Visual cues, on the other hand, are perceived by the compound eyes of the mosquito, which can detect differences in light intensity (Muir et al. 1992; Moon et al. 2014).

Odorants that are emitted by the host, are received by the olfactory receptors, which are located within sensilla on the palps and antennae of the mosquito (Fig.1.1) (Meijerink et al. 2001; Qiu et al. 2006; Carey et al. 2010). Some of these cues are host blood source, human skin bacteria and carbon dioxide (CO_2) (Dekker et al. 2005; Verhulst et al. 2011). Despite the fact that some colonized *An. gambiae* strains lack effective CO_2 receptors, these mosquitoes are still able to locate a host (McMeniman et al. 2014). Furthermore, the skin bacteria are responsible for producing volatile compounds, such as lactic acid, ammonia and ketones (Davis & Sokolove, 1976; Braks et al. 2001; Verhulst et al. 2010). Body heat and relative humidity form part of external cues that are responsible for the dispersal of semiochemicals by convection currents which are generated by body heat and humidity (De Jong & Knols, 1995; Olanga et al. 2010).

Olfactory cues are received by chemosensory receptors, including odorant receptors, ionotropic receptors and gustatory receptors (Suh et al. 2014). These receptors allow mosquitoes to integrate taste and odor in the host location process (Riabinina et al. 2016). The orco gene that is expressed by the olfactory receptor neurons, connects the suboesophageal zone of the brain to the labella on the proboscis, in order to facilitate this process (Riabinina et al. 2016).

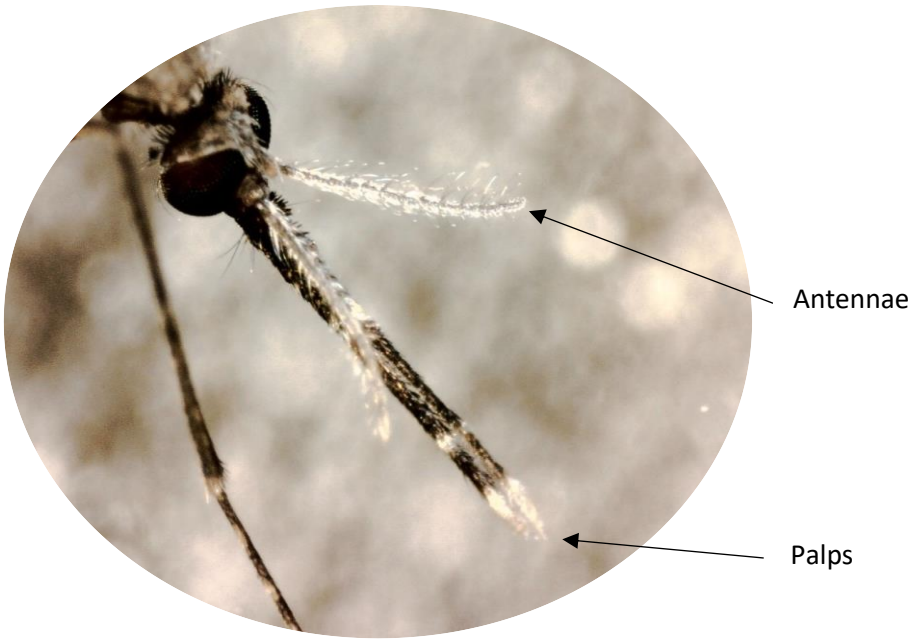


Fig.1.1. Head and mouthparts of a female anopheline mosquito.

1.3.3 Parasite host manipulation

The presence of parasites is another important factor that needs to be considered as studies have shown that mosquito feeding behavior and attraction is affected upon infection of the mosquito with human malaria parasites (Koella & Packer, 1996; Anderson et al. 1999; Emami et al. 2017). Parasites that increase malaria transmission by changing the behaviour of their hosts, is not a scarce phenomenon in nature (Moore, 2002). Though several studies have suggested that parasites alter the feeding behaviour and thus increase the feeding rate of the vector, once the mosquito is already infected (Rossignol et al. 1984; Schwartz & Koella, 2001; Koella et al. 2002), others found that infection status did not determine feeding behaviour (Li et al. 1992; Hogg & Hurd, 1995). In addition to changes in feeding behaviour, the *P. falciparum* parasite has also been found to enhance attraction of its vectors to the infected hosts. The *P. falciparum* metabolite, HMBPP, has been suggested to be responsible for these manipulative changes in the mosquito (Emami et al. 2017).

Mosquitoes' parasite companions (in particular *P. falciparum* and *P. vivax*) are host specific (Takken & Verhulst, 2013) and therefore these parasites would guide the mosquitoes to find a human host. The 'manipulation hypothesis' has been described as the way in which the malaria parasite enhances transmission by adaptive

manipulation of the malaria vector (Cator et al. 2012). Evidence suggested that manipulation by the parasite, by means of increasing the mosquito feeding rate, together with causing changes in the probing behavior of sporozoite infected females, might change the biting frequency of the vector (Cator et al. 2012). This in turn also increases the likelihood of the vector to attempt to feed and thus enhance possible transmission (Cator et al. 2012). This was also supported by a study where *An. gambiae* mosquitoes that were infected with *P. falciparum* sporozoites, showed elongated probing durations and an increase in the number of probes while feeding (Wekesa et al. 1992). As a result, the chance of more sporozoites being transmitted to the human host increases and therefore enhanced transmission of the parasite is facilitated through this probing behavior (Wekesa et al. 1992).

It has also been suggested that the parasite is responsible for possible changes in mosquito behavior, through the manipulation of host odors (De Moraes et al. 2014). This might result in a cycle where the infected human hosts are more attractive to the mosquitoes, resulting in higher anthropophilic feeding behavior, which will dramatically influence malaria transmission (Takken & Verhulst, 2013). A study in Kenya showed that children with *P. falciparum* gametocytes were more attractive to mosquitoes than those without gametocytes (Lacroix et al. 2005; Busula et al. 2017). An explanation for this is that attractive volatile compounds such as terpenes that are produced by malaria parasites result in increased attraction of mosquitoes to the host (Nyasembe et al. 2012; De Moraes et al. 2014; Kelly et al. 2015, de Boer et al. 2017). Studies conducted in human, mouse and avian malaria parasites further suggested that volatile organic compounds (VOCs) found in exhaled breath, are changed by *Plasmodium* infection and this might consequently result in increased recognition by the mosquitoes (Schaber et al. 2018).

Although human odor, which consists of numerous chemicals, play a critical role in indicating human presence (Bernier et al. 2000; Takken et al. 1999), *P. falciparum* terpenes also play a vital role in assisting with mosquito attraction. These parasites produced terpenes, which form part of the attractive volatile compounds, and fall into the isoprenoids class (Emami et al. 2017). Furthermore, these terpenes consist of isopentenyl (IPP) and its isoform dimethylallyl pyrophosphate (DMAPP) (Fig.1.1). The synthesis of IPP and DMAPP occurs through the mevalonate pathway that is used by humans, mosquitoes and other higher eukaryotes (Emami et al. 2017). This crucial

metabolic pathway is involved in several cellular processes by the synthesis of isoprenoids – both sterol (like cholesterol) and non-sterol (Buhaescu & Izzedine, 2007). In contrast to this, apicomplexan parasites, such as *P. falciparum*, and other eubacteria use an alternative pathway, known as the 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway (Fig.1.1), for IPP and DMAPP synthesis (Morita et al. 2007; Emami et al. 2017). The MEP pathway uses (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMBPP) as a precursor for the production of these isoprenoids (IPP and DMAPP) (Emami et al. 2017). HMBPP is not only known to activate human Vɣ9Vδ2 T-cells (which was found to trigger immune response in *An. gambiae s.l.*) (Morita et al. 2007; Lindberg 2013), but has also been found to trigger red blood cells to release VOCs. This is based on the same principle in which *P. falciparum* infected red blood cells act as an attractant to stimulate blood feeding by mosquito vectors (Emami et al. 2017). There is however insufficient knowledge regarding the effect of HMBPP on African mosquito species.

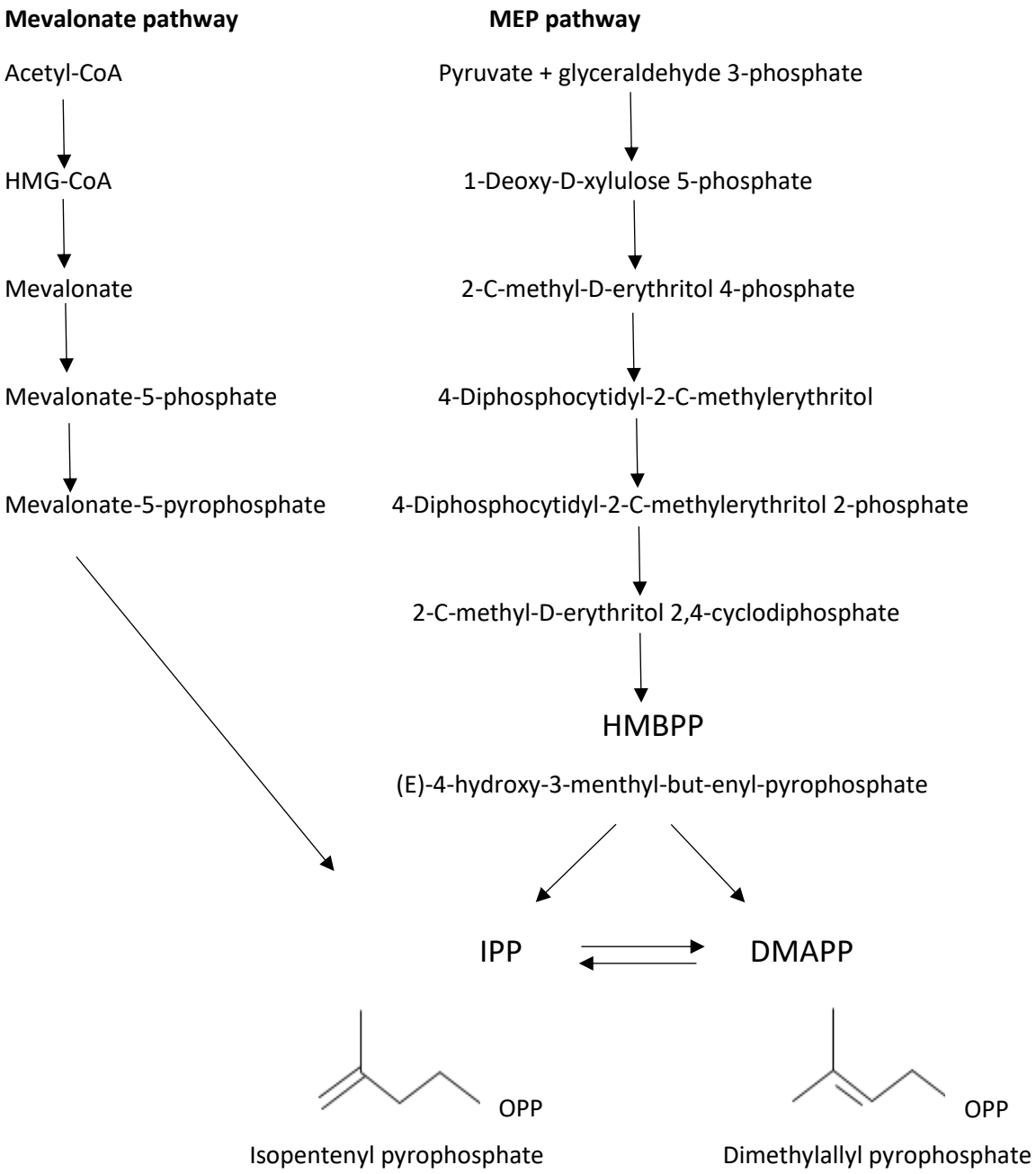


Fig. 1.2. Isoprenoid biosynthesis through the mevalonate and MEP pathways. The mevalonate pathway is found in higher eukaryotes whereas the MEP pathway is used by most eubacteria and apicomplexan protozoa.

1.3.4 Parasite susceptibility of different mosquito vectors

Gametocytes and sporozoites are the big culprits in the transmission cycle between the parasite and the vector (Delves et al. 2013). The female and male gametocytes form part of the sexual stage of the parasite and are ingested by the mosquito when taking a blood meal from an infected human host (Smith et al. 2014). The sexual phase of *P. falciparum* needs to develop *in vivo* and can be separated into gametocytogenesis and gametogenesis (Sinden, 1981). Gametocytogenesis is the phase that takes place within the mosquito vector and is necessary for the mature gametocytes to differentiate into motile male microgametes and a single sessile female macrogamete (Sinden, 1981). Gametogenesis, on the other hand, involves the activation and fertilization preparation of gametocytes (Bousema & Drakeley 2011).

Exflagellation is the term that is used to describe the transformation process of male gametocytes into gametes as well as their emergence from red blood cells (Delves, 2013). Since the exflagellation process takes place within the mosquito, several suggestions have been made to further explain vector susceptibility to the parasite. Species-specific stimulation of exflagellation was earlier considered a possible mechanism for aiding mosquito susceptibility (Micks et al. 1948; Nijhout, 1979). Recent studies found that one of the important transformation processes (gametocytes into gametes), that forms part of the development of the *P. falciparum* parasite, is triggered by a favourable change in enviro nMent within the mosquito gut. Among these favourable conditions is the presence of the mosquito derived gametocyte-activating factor xanthurenic acid (Garcia et al. 1998). The latter is among the contributing factors, which enables gametocytes to become mature and thus enhance the chance that a mosquito becomes infective (Oaks et al. 1991). Other factors include oxygen and carbon dioxide concentration, pH and temperature factor (Oaks et al. 1991).

After exflagellation, the fusion of the male and female gametes takes place as well as the formation of a zygote from which the motile ookinete originates (Sinden, 1981). The ookinete exits the bloodmeal, which was taken up by the mosquito, and it enters the midgut epithelium of the mosquito (Sinden, 1981). The ookinete matures and

forms an oocyst from which motile sporozoites originate (Oaks et al. 1991). These sporozoites travel to the salivary glands of the mosquito, from where they are injected into the host upon being bitten by the mosquito (Oaks et al. 1991). In order for the parasite to become infective, it needs to undergo an incubation period of 14-15 days (MacDonald, 1955; Emami et al. 2017)

Vector susceptibility is an important parameter for parasite transmission (Cohuet et al. 2009). A combination of a vector's susceptibility and parasite infectivity could be described as vector competence and it involves both the host's defence mechanisms and the parasite's ability to overcome those defences (Lefevre, 2013). The susceptibility of certain *Anopheles* species to the malaria parasite is a mysterious phenomenon and the mechanisms involved is not well known (Oaks et al. 1991). Weathersby (1952) suspected that the inability of certain mosquito species to sustain the development of the parasite could be due to a toxin being present that inhibits the development of the parasite. It might also be that the absence of factors, which are essential for parasite development, plays a role (Weathersby, 1952). More recent studies have shown that nitric oxide (NO) is also involved in termination of parasite development. Once the mosquito ingests infected blood from a human host, there is a substantial increase in the levels of nitrates and NO in the mosquito midgut (Luckhart et al. 2003; Peterson et al. 2007). The midgut epithelium could also naturally contain NO and upregulation of NOs has also been detected in *An. gambiae* midguts (Dimopoulos et al. 1998; Luckhart et al. 1998). Oxyhaemoglobin and haeme which are released by the digestion of red blood cells in the mosquito, can react with NO to produce NO metabolites which are toxic and can negatively influence parasite development (Peterson et al. 2007).

Besides vertebrate-derived components of blood that forms part of the modulatory components in preventing parasite development, mosquito immunity also involves defence peptides which can inhibit sporogonic development (Sinden et al. 2004). The development of proteins on the surface of the parasite, once inside the midgut of the mosquito, could also be recognized by the mosquito antibodies and as a result inhibit further development of the parasite (Gozar et al. 1998; Margos et al. 1998). In addition to this, melanisation and lysis of *Plasmodium* parasites also occurs. A strain of *An. gambiae* has been found to melanise the ookinetes of *P. berghei* and allopatric strains of *P. falciparum* whereas another strain of *An. gambiae* lysed *P. gallinaceum*

ookinetes (Collins et al. 1986). Melanisation of these ookinetes occurs as they emerge from the midgut epithelium whereas the lysis of ookinetes takes place while these ookinetes traverse the midgut epithelium (Vernick et al. 1995). Furthermore, for mosquitoes to be classified as successful vectors of the parasite, they need to carry sporozoites to their hosts. If these sporozoites are not able to migrate and successfully penetrate the mosquito salivary glands, it might be another possible factor contributing to the mosquito's resistance to parasites (Collins et al. 1986).

Measurable infection related traits could be evaluated in order to determine the competence of a vector species – these are parasite prevalence and intensity (Lafevre, 2013). Prevalence could be described as the proportion of mosquitoes that have been exposed to the parasite, which harbours at least one oocyst in the midgut, whereas parasite intensity denotes the oocyst number per infected gut (Lafevre, 2013). Both low prevalence and intensity could indicate high anti-infection resistance by the mosquito or high antigrowth resistance to parasite reproduction in the mosquito, respectively. High prevalence and intensity would therefore imply the opposite (Lafevre, 2013). HMBPP was found to be released by *P. falciparum* into the blood and to regulate sporogenesis in the mosquito (Emami et al. 2019). Furthermore, it was shown to increase the size of the blood meal taken by the mosquito and there by increases the number of oocysts in the mosquito midgut (Emami et al 2019). The addition of HMBPP to *P. falciparum* blood would therefore increase the likelihood of higher oocyst prevalence and intensity in African mosquito vectors as well.

CHAPTER TWO: Materials and Methods

2.1 Sampling of wild mosquitoes

The wild mosquitoes in this study were collected at sentinel sites which were established by members of the Sterile Insect Technique (SIT) project. These sites were located in Mamfene, in the Umkhanyakude district of KwaZulu-Natal (KZN). Collections varied between larval collections and adult mosquito collections (with mouth aspirators). Resting adult mosquitoes were mainly collected from clay pots (Fig. 2.1a) or a modified plastic bucket (Dandalo et al. 2017). In addition, some adults were collected from tyres around cattle kraals (Fig.2.1b) as well as CO_2 traps which were placed at sentinel sites *ad hoc* during this time. Collections ranged over an 8-month period (Aug. 2019 – March 2020) as *An. funestus* group species were not available during the months prior to this time and due to COVID 19 restrictions, no fieldwork was conducted after March 2020. Mosquitoes were collected monthly and apart from assisting with fieldwork occasionally, mosquitoes were mostly collected by the SIT team based in KZN. Adult mosquito collections, as well as the larvae and pupae, were brought back to the Vector Control Reference Laboratory and the Wits Research Institute for Malaria in Johannesburg, South Africa, where further rearing and processing of samples took place. Adult female mosquitoes were placed into egg laying vials (Choi et al. 2014), of which each contained a piece of wet filter paper to provide the appropriate substance for egg laying. The F1 generation was further reared through to adults, which were then preserved in silica tubes for further morphological and molecular identification.




Fig. 2.1a &b. Resting adult mosquitoes being collected from a clay pot and the search for resting adult mosquitoes in tyres at a sentinel site in Mamfene, KwaZulu-Natal.

2.2. Wild caught mosquito identification

Mosquito identification needs to be conducted on both morphological and molecular level, in order to ensure accurate species identification (Erlank et al. 2018). Mosquitoes were separated, based on morphological differences, with the use of a dichotomous key by Coetzee (2020). Adult mosquitoes were morphologically separated into *Anopheles gambiae* complex, *An. funestus* group and other anophelines. After the morphological identification, further molecular identification was conducted by the polymerase chain reaction (PCR) assay. As *An. gambiae* complex species data was generated for the SIT project, PCR assay was conducted by the relevant team and did not form part of this study.

2.2.1 PCR species identification and seasonal trend

For identification of *An. funestus* group mosquitoes on a molecular level, DNA was extracted from a mosquito leg or wing of this species group with the use of the prepGEM Insect DNA extraction kit (ZyGEM; Cat no. E3BG6M; New Zealand). An insectary reared *An. funestus* mosquito, from the FUMOZ colony, was used as a positive control and a negative control was included during the DNA extraction assay and PCR assay. Post DNA extraction, specimens were further analysed with the use

of species-specific polymerase chain (PCR) reaction assay according to the PCR protocol for the *An. funestus* group (Koekemoer et al. 2002; Cohuet et al. 2003). A PCR master mix was made up, consisting of 1.25 µl of 10 x reaction buffer (100 mM Tris-HCL, 500 mM KCl), dNTP mixture (which includes 2.5 mM of each dNTP), 25 mM $MgCl_2$ (ThermoFisher Scientific; Cat no. A31021; South Africa). A concentration of 3.3 pmol of each of the following primers: universal, *An. funestus, An. leesoni, An. parensis, An. rivulorum, An. rivulorum-like* and *An. vaneedeni* (primer sequences indicated in Table 2.1) was added. This was followed by the addition of Taq-polymerase (5 units/µl) (ThermoFisher Scientific; Cat no. R001AM; South Africa) and 3.65 µl deionised H_2O. Each volume was multiplied by the number of samples to be analysed, including the positive control and negative controls for DNA extraction and the PCR master mix, and the mixture was vortexed and centrifuged. Aliquots of 12.5 µl was then made into the individual PCR tubes, containing 0.5 µl of the sample DNA template. The PCR tubes were placed into the PCR machine (Biorad T100 Thermal Cycler) which was programmed according to the following cycling conditions: 94℃ for 2 minutes and a 30 x repetition of the following conditions: 94℃ for 30 seconds, 45℃ for 30 seconds, 72℃ for 40 seconds and 72℃ for 5 minutes.

Table 2.1: Species-specific primer sequences for members of the *An. funestus* group (Koekemoer et al. 2002; Cohuet et al. 2003).

Primer name	Sequence (5’ to 3’)
UV	TGT GAA CTG CAG GAC ACA T
FUN	GCA TCG ATG GGT TAA TCA TG
VAN	TGT CGA CTT GGT AGC CGA AC
RIV	CAA GCC GTT CGA CCC TGA TT
RIVLIKE	CCG CCT CCC GTG GAG TGG GGG
PAR	TGC GGT CCC AAG CTA GGT TC
LEES	TAC ACG GGC GCC ATG TAG TT

The PCR product of each assay was electrophoresed on a 2.5% Tris–acetate–ethylenediamine tetraacetic acid (TAE) agarose gel, which was stained with ethidium bromide (0.5 µg/ml). Gels were viewed with a Geldoc system where after amplicon sizes were compared against the corresponding molecular weight marker: *An. vaneedeni* (587 bp), *An. funestus* (505 bp), *An. rivul*orum (411 bp), *An. rivulorum*-like (313 bp), *An*. *parensis* (252 bp) and *An. lee*soni (146 bp).

Species identification results for the *An. gambiae* complex mosquitoes were obtained from the SIT team. This was included in the results section in order to compare the abundance of the current major vector in the area, *An. arabiensis* (which is a member of the *An. gambiae* complex), to the minor and non-vector numbers of the *An. funestus* group.

Monthly rainfall data were also obtained from the weather station in Mamfene in order to establish if there is a correlation between mosquito abundance and rainfall during this period. No temperature or humidity data was however available due to these sensors being damaged by cattle.

2.2.2 Data analysis

The Pearson's correlation analysis was used in order to determine if there was a positive correlation between the abundance of species within the *An. funestus* group and *An. gambiae* complex as well as between the major vector, *An. arabiensis* and minor- and non-vector species of both the *An. gambiae* complex and *An. funestus* groups. Pearson's correlation was also used for rainfall and mosquito abundance. Analysis was conducted in GraphPad Prism (9.0.1) (Motulsky, 1998) and results were displayed as the r-value which is indicative of the coefficient of determination. A two-tailed *P*-value indicates the goodness of fit where a significant *P*-value rejects the idea that any correlation is due to random sampling.

2.3. Wild caught mosquito infection profiling with the Enzyme linked Immunosorbent Assay

The infectivity of wild mosquitoes was detected by the Enzyme linked Immunosorbent Assay or better known as ELISA Wittz et al. 1987). This immunoassay is based on the principle where the enzyme acts as a signal generator in conjunction with a solid phase reactant and thus indicates an anitigen-anitibody reaction which is signalled through an enzyme (Beier et al. 1987). This assay has been widely used in antibody detection across human and animal illnesses and for the detection of the *P. falciparum* parasite, a repetitive antigen on the

circumsporozoite protein of the latter parasite is recognized by a monoclonal antibody as used in the ELISA assay (Beier et al. 1987).

The *P. falciparum* ELISA assay was conducted as per the Malaria Research & Reference Reagent Resource Center (MR4) protocol over a period of two consecutive days. The *P. falciparum* sporozoite ELISA Reagent Kit (BEI Resources: https://www.beiresources.org/, NIAID, NIH: Plasmodium falciparum Sporozoite ELISA Reagent Kit, contributed by Robert A. Wirtz, Cat No. MR4-890; United States) was used.

The following buffer solutions were prepared prior to analysis. A stock solution of phenol red (100 mg/ml) (ThermoFisher Scientific; Cat No. ICN10260405; South Africa) was prepared. A 1 x Phosphate Buffered Saline (PBS) solution was also made by dissolving PBS tablets (ThermoFisher Scientific; Cat No. BP2944; South Africa) in water and maintaining a concentration of 10 mM and pH of 7.4. Blocking buffer consisted of 2.5g casein (Merch; Catalog No. C7078; South Africa), 0.1 N NaOH (ThermoThermoFisher Scientific; Cat No. AC124260010; South Africa) and 10 mM 1x PBS solution. The 0.1 N NaOH was boiled in a flask and casein was slowly added. Once the casein dissolved and cooled down to room temperature, the PBS was added. A 1N concentration of HCL was added in order to reach a pH of 7.4. Phenol red (100 mg/ml) was then added to the blocking buffer (BB) to act as pH indicator. A 1x PBS- Tween solution was prepared by mixing 10mM PBS with 0.05% Tween-20 (ThermoThermoFisher Scientific; Cat No. BP337; South Africa). Lastly the grinding (homogenising) buffer was prepared by adding 125 µl of Octylphenoxy poly(ethyleneoxy) ethanol (IGEPAL CA-630) (ThermoThermoFisher Scientific; Cat No. ICN19859650; South Africa) to 25 ml of BB. A ratio of 1:1 of solution A to solution B (Qiagen; Cat No. 51200032; South Africa) was achieved in order to prepare the 2, 2'-azino-di (3-ethylbenzthiazoline-6-sulfonate) (ABTS) substrate solution. This latter mixture was wrapped in foil due to its photo-sensitivity. All the solutions were vortexed and stored at 4℃ for 1 week.

A 96-well clear, round bottom PVC microtitre plate (Merck (Sigma-Aldrich); CAS number 2797, South Africa) was coated with the *Plasmodium falciparum* monoclonal antibody (mAb) Pf2A10 (BEI Resources, https://www.beiresources.org/) (0.5 mg/ml)) of which the stock solution consisted of mAb and a 1:1 glycerol to water solution. A

volume of 50 µl of the solution was added to each well and the plate was covered and incubate for 24 hours at 4˚C.

Mosquitoes (including 7 unfed female mosquitoes, from the FUMOZ colony, that were used as negative controls) were prepared by dissecting the mosquito and removing the head and thorax from the abdomen, with the use of a scalpel. The same method was used for the wild caught mosquitoes, which were preserved individually in 1.5 ml microcentrifuge tubes with silica gel, after collection. Each sample was removed from the tube and following the removal of the head and thorax, each sample (together with the negative controls) was placed into an individual 1.5 ml microcentrifuge tube with 50 µl grinding buffer. A 3 mm stainless steel bead (BMG Spartan, South Africa) was added, where after tubes were placed in the TissueLyser II (Qiagen) and were shaken at a high speed for 4 minutes at a frequency of 25Hz for 4 minutes. After samples homogenised, each bead was removed and 150µl of blocking buffer was added to each tube, to have a total volume of 200µl. Samples were stored at 4℃ for 24 hours and after usage remaining samples were stored at -20℃ for periods longer than 24 hours.

After the 24 hour incubation period of the *P. falciparum* antibody coated plate, PSB-antibody mixture was aspired from the plate wells. The wells were filled with blocking buffer and left to incubate for 1 hour at room temperature. After the incubation period, the blocking buffer was poured off and samples and controls were added to the plate. The positive control, a purified recombinant protein, was prepared by adding 20 µg of capture mAb stock (Wirtz et al. 1987, BEI Resources, https://www.beiresources.org/) to 5 ml of PBS. The positive control was added to well A1 and 50 µl of each negative control was added to the last seven wells. Samples were added in the same quantity to the remainder of the wells and the sample number was recorded against the corresponding well number. The plate was covered with foil and incubated for two hours at room temperature.

After incubation the wells were aspired and the plate was washed twice with PBS-Tween. A volume of 50 µl PfA10 peroxidase labelled antibody (0.5 mg/ml) diluted in BB (for a final concentration of 5.0 µg/5 ml) was added to each well. Due to the photosensitivity of the peroxidase labelled antibody, the plate was covered with foil

and incubated at room temperature for an hour. After the incubation period, the wells were aspired and the plate was washed four times with PBS-Tween. A volume of 100 µl of ABTS substrate solution was added to each well and the plate was incubated for 30-60 minutes to allow for the colour change to take place.

The absorbency was determined for each well at 405 nM, with the use of a plate reader (Labsystems Multiskan RC) and Ascent software version 2.6. The cut-off value was determined with the following calculation: 2 x mean absorbance values of negative samples. Samples with absorbance values above that of the cut-off value, was regarded as positive and those with absorbance values below the calculated cut-off was regarded as negative. Positive samples had to be repeated by first boiling the sample at 100°C for 10 minutes and repeating the process in order to confirm the presence of the circumsporozoite protein (Durnez et al. 2011).

2.4. Attraction and feeding rate

2.4.1 Biological material

Colonised mosquitoes: Insectary mosquito colonies that were used during this study included: FUMOZ (*An. funestus* from Mozambique, colonized in 2000 by Hunt et al., (2005)), KWAG (*An. arabiensis* from Mamfene, KwaZulu-Natal, SA, colonized in 2005 by Mouatcho et al. (2009), COGS (*An. coluzzii* from the Congo, colonized in 2009, (Koekemoer et al. 2011), MAFUS (*An. merus* from the Kruger National Park, SA, colonized in 2012 (Munhenga et al. 2014)), SANGWE (*An. quadriannulatus* from Zimbabwe, colonized in 1998). Mosquitoes were reared in a biosafety level 2 (BSL2) insectary and conditions were kept at 25℃ and a relative humidity of 80% (Hunt et al. 2005). The lighting cycle was set at 12-h day/night, including dusk/dawn cycles that lasted 45 minutes. Adult mosquitoes were maintained on a 10% sucrose solution.

Wild mosquitoes: Mosquitoes used for the feeding or attraction study, were the progeny of the females (section 2.1) collected and placed in egg laying vials (Fig. 2.2.). Due to the low number of eggs that were produced by wild female mosquitoes, the F1 generation was very limited for this study, therefore on average 15 mosquitoes were used for each of the three technical replicates. All adult mosquitoes

were identified with the use of the dichotomous key of Coetzee (2020) and species identification was conducted by PCR assays according to the standard protocols for the *An. gambiae* complex (Scott et al. 1993) and the *An. funestus* group (Koekemoer et al. 2002) as described in section 2.2.



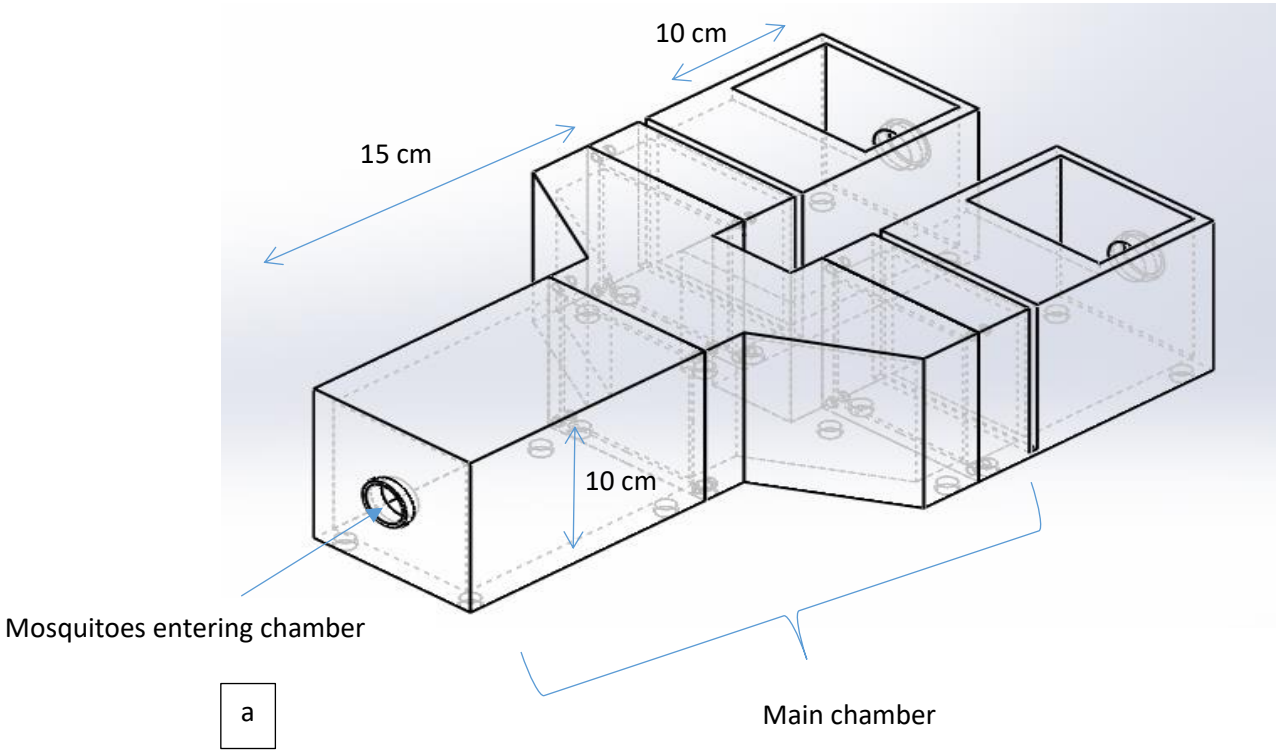
Fig. 2.2. Egg laying vial containing a wild caught female *Anopheles* mosquito

2.4.2. Mosquito attraction and dual choice chamber assays

i. Evaluation of the choice chamber

Prior to conducting attraction assays, as described in the next section, the effectiveness of the choice chamber (Fig. 2.5.a) was evaluated using colonized mosquitoes *An. funestus s.s.* (FUMOZ) and *An. arabiensis* (KWAG). Experiments were conducted separately on each species and included three biological replicates of 20 female mosquitoes per replicate. The mosquitoes were provided with a choice between sugar water and cow blood (collected from the Karan beef abattoir as part of the larger SIT project). Each source was located at a different arm of the chamber. The blood and sugar water sources were also rotated between the two arms, with each biological replicate to ensure that positioning effects do not interfere with the source that was selected by the female (Fig. 2.5.b & c). As the laboratory mosquito

colonies were sustained on sugar water daily, the blood would have been the expected source of preference. Analyses were conducted and described in more detail (section iii) below. An additional control measure, to assess if mosquitoes alternate between the two arms (or between different food sources), was not evaluated at this stage.



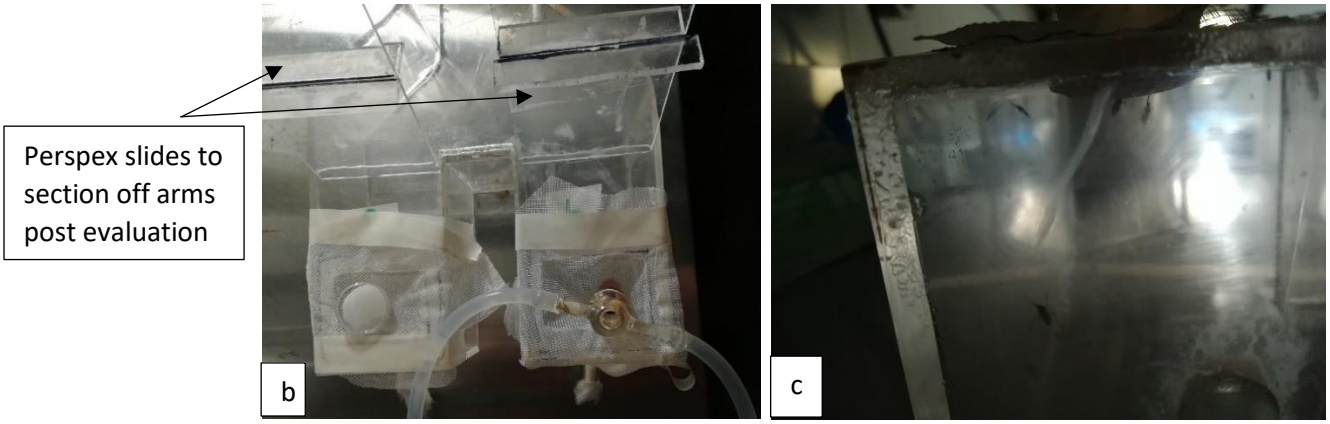


Fig. 2.3.a, b & c. Dual choice chamber with dimensions of the main chamber and two arms (a) Dual choice chamber evaluation with sugar water and cow blood (b) Mosquitoes entering the preferred arm of the choice chamber (c).

ii. Attraction behavior of major-, minor and non-vectors in the presence of HMBPP

The dual choice chamber (Fig.2.3.a) was used to evaluate the attractiveness of mosquitoes towards human blood only (control group) vs. HMBPP-supplemented

human blood (test group). Species included all four major African vectors (*An. gambiae* s.s*., An. coluzzii, An. arabiensis* and *An. funestus s.s*.) as well as the minor vector, *An. merus,* and non-vector, *An. quadriannulatus* obtained from colonies. Wild caught *An. vaneedeni*, member of the *An. funestus* group, was also included in this study. Two glass feeders were set up according to the standard membrane feeding assay (SMFA) protocol as described in section 2.4.3. Blood type O+ was used and was loaded into the feeders - one feeder was used for the test group which consisted of 10 µM HMBPP (Merck (Sigma-Aldrich); CAS number 396726-03-7; South Africa) that was added to 1 ml blood. The feeder, containing HMBPP-supplemented blood, was placed on top of the net that covered the one arm of the choice chamber, whereas a different feeder was placed on the other arm, for the control group (blood only). With each biological replicate, the sources were alternated between the left and right arm of the chamber. Mosquitoes were starved for 24 hours (with distilled water provided), prior to feeding. Females (5 - 7 day old) were transferred into the main arm of the chamber one-by-one. Each mosquito was allowed 1 minute to choose which arm to enter, before the next mosquito was blown into the chamber. Mosquitoes were free to alternate between the two arms as well as to move around in the main section of the chamber. An additional control measure, to assess if mosquitoes alternate between the two arms (or between different food sources), was not evaluated at this stage. After 20 minutes, the arms were isolated from the main chamber by closing the gates (Fig 2.3.b. & c). The total number of mosquitoes in each arm section were recorded. Three biological replicates of 20 mosquitoes per species were conducted. The percentage of mosquitoes selecting the specific blood source was calculated and the preference index was calculated in R-Studio (1.3.1093) with the generalized linear mixed model (GLMM) as described in the next section.

iii. Data analyses

To account for variance between biological datasets, the generalized linear mixed model (GLMM) was used (Crawley, 2013) with the multiple regression model (lme4 package) incorporating replicate as a random factor for both control and treatment groups. The use of the GLMM models with random variables do not restrict statistical evaluation to normal distributed data. Thus, semi-parametric models are used for

evaluating attraction and feeding behaviour during this study. All analyses were done in R-Studio and results were entered into GraphPad Prism (9.0.1) to construct the graphs.

2.4.3. Mosquito feeding rate and HMBPP

In order to determine if HMBPP plays a role in mosquito feeding rate, the SMFA was conducted and the feeding rate was determined between the control (blood) and treatment (HMBPP-supplemented blood) groups for different mosquito species.

The SMFA was conducted by supplying blood through a glass feeder system that was connected to a water bath of which the temperature was maintained at 37°C (Fig. 2.4). One millilitre of blood (O^+ human blood that was donated) (Ethics no. M200692, Appendix B) was added to the glass feeder, which was covered with cow intestine at the bottom to form a membrane, of the control group. The same applied to the test group but in addition to adding 1 ml blood to the feeder, 10 µM of HMBPP was added and mixed with the blood, before adding it to the feeder. Since a concentration of 10 µM/ml is similar to the concentration of undiluted gametocyte-infected red blood cells (Emami et al. 2017), the concentration was kept constant throughout the study. Each blood filled glass feeder was placed horizontally on top of each cup (350 ml). Each cup consisted of 25 unfed female *An. gambiae* complex or 30 *An. funestus* group mosquitoes that were allowed to feed for 40 minutes. Prior to conducting the SMFA, unfed females were starved for 24 hours with distilled water soaked cotton pads. Each replicate consisted of three technical replicates of both the control and treatment groups and three biological replicates were conducted. After feeding, mosquitoes were immobilised by placing them in the freezer for 10 minutes and the number of unfed (Fig. 2.5.a) versus fed (Fig. 2.5.b) mosquitoes was recorded for each treatment.



Fig. 2.4. Set-up for the standard membrane feeding assay with feeding cups placed under the feeding system with water (37℃) circulating from the water bath (A) to the connected feeders (B).



Fig.2.5.a. Microscopic image of an unfed female anopheline mosquito.



Fig.2.5.b. Microscopic image of a fully fed female anopheline mosquito

2.4.4. HMBPP dose-response evaluation

Different dosages of HMBPP (1 µM and 100 µM) were evaluated in addition to the standard 10 µM that was used throughout the study, in order to determine if the

HMBPP-induced feeding rate was dose dependant. Feeding rate experiments were conducted as per the previously stated SMFA protocol (Section 2.4.3), using the two major vectors, *An. funestus s.s*. and *An. gambiae s.s.* Each biological replicate consisted of three technical replicates/HMBPP concentration. A total of three biological replicates was completed. The feeding rate was established by recording the number of fed females per concentration of HMBPP evaluated.

i. Data analysis

A dose-response graph was constructed using GrapPad Prism (9.0.1) and statistical difference between concentrations, for each species, was determined with the paired t-test (Kim, 2015) as both sets of data was normally distributed as confirmed with the Shapiro-Wilk test (Hanusz et al. 2016). In order to determine the statistical difference between the two species, at each concentration, the unpaired t-test was used.

2.5. Artificial infection of different mosquito species with *Plasmodium falciparum*

2.5.1. Infection through SMFA

In order to determine if HMBPP has an effect on the susceptibility of major (including *An. arabiensis, An. gambiae s.s., An. coluzzii* and *An. funestus s.s*), minor (*An. merus*) and non-vector (*An. quadriannulatus*) mosquitoes to *P. falciparum,* female mosquitoes were fed on human blood containing mature gametocyte culture. A gametocyte culture consisted of Stage V gametocytes from the NF54 parasite strain. The gametocytemia of cultures varied between 1.5-2.5%, with a 50% hematocrit in A+ male serum with added fresh red blood cells (blood group was dependent on the donor) (Reader et al. 2021). Prior to setting up the SMFA, the gametocyte culture was assessed to ensure exflagellation of male gametes and the presence of a 3:1 female:male ratio. The gametocyte culture was provided by the Biochemistry Department of the University of Pretoria. The same protocol was followed as per section 2.4.3. to conduct feeding rate assays through SMFA. Each experiment consisted of three technical and biological replicates of which the control group was

supplied with human blood, containing gametocyte culture only, whereas the test group received gametocyte infected blood with added HMBPP (10 µM/1 ml blood) (Merck (Sigma-Aldrich); CAS number 396726-03-7; South Africa). Each cup consisted of 25 *An. gambiae* complex (*An. gambiae s.s.; An. coluzzii; An. arabiensis*; *An. merus* and *An. quadriannulatus*) and 30 *An. funestus* group (*An. funestus s.s.*) females (5-7 days old) (Fig.2.6). Female mosquitoes accessed the blood by piercing through the membrane and were allowed to feed for 40 minutes in dark conditions. Mosquitoes were starved for 2 – 3 hours prior to feeding. All unfed mosquitoes were removed with a mouth aspirator and fully fed mosquitoes were housed in a BSL2 facility of the Maureen Coetzee insectary, at a temperature of 25°C and 80% relative humidity and a 12 hour day/night cycle with a 45 dusk/dawn transition of 45 minutes. Mosquitoes were provided with a 10% sugar water solution, containing 0.05% 4-aminobenzoic acid (PABA) (Merck (Sigma-Aldrich); CAS number 150-13-0; South Africa), until eight days post-infection.

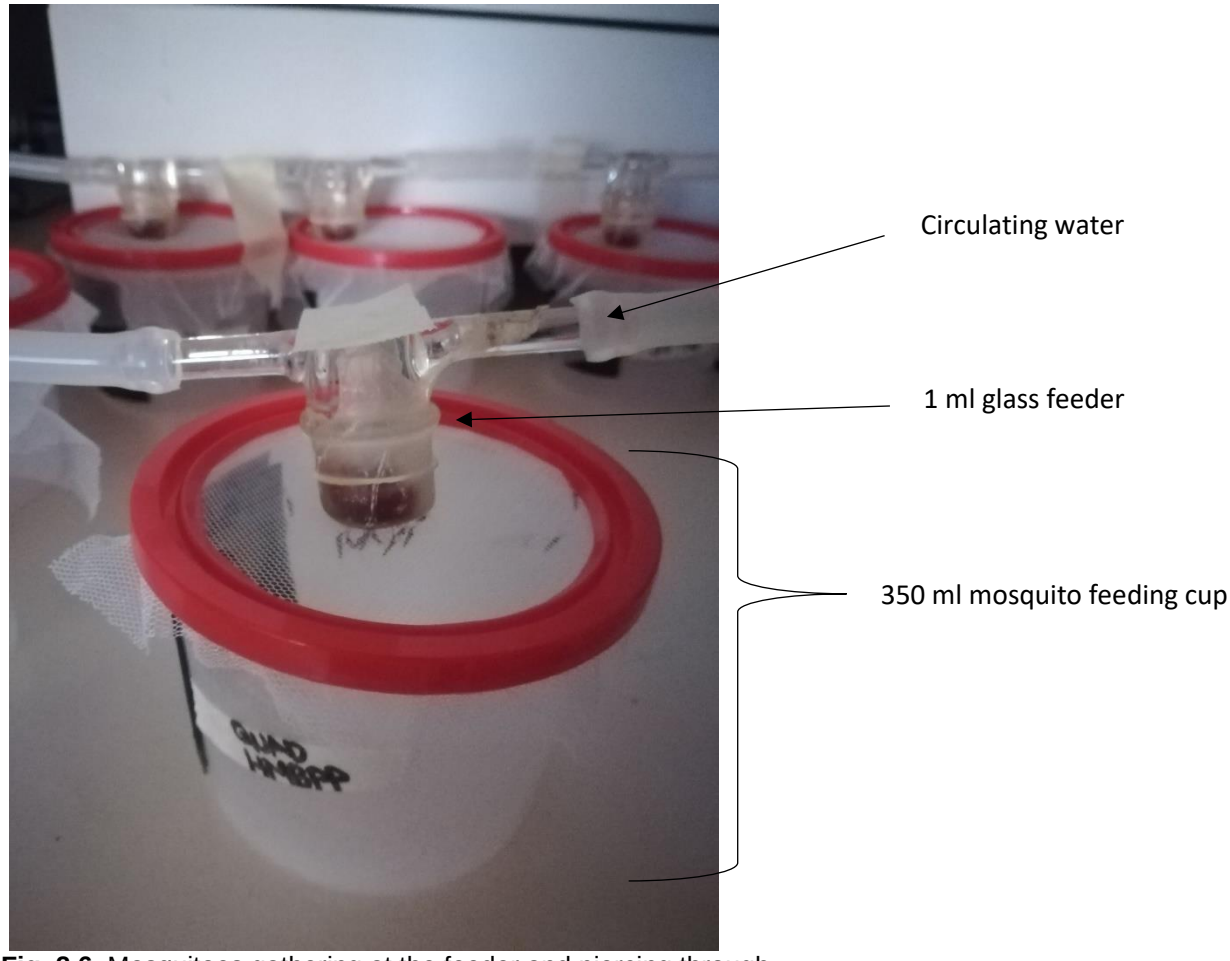


Fig. 2.6. Mosquitoes gathering at the feeder and piercing through the membrane, feeding on HMBPP-supplemented gametocyte culture.

2.5.2 Midgut dissections

On days 8 – 10, post-infection, mosquitoes were immobilized and with the use of dissecting needles, the midguts were removed from infected specimens. Mosquitoes were dissected in PBS and guts were subsequently transferred to a droplet of 0.1% mercurochrome (Merck (Sigma-Aldrich); CAS number 129-16-8; South Africa). For better visibility, guts were stained between 8 to 10 minutes prior to visualisation under the microscope. After staining, the presence of oocysts was easily detected, as oocysts stained a darker shade of red, in comparison to the rest of the midgut.The prevalence (presence of oocysts) (Fig. 2.7a) and intensity (number of oocysts) were determined by viewing the guts under bright field illumination at 10x - 20x magnification (Delves & Sinden, 2010). Mosquitoes that had no oocysts in the midgut therefore had a prevalence of 0 (Fig. 2.7b)

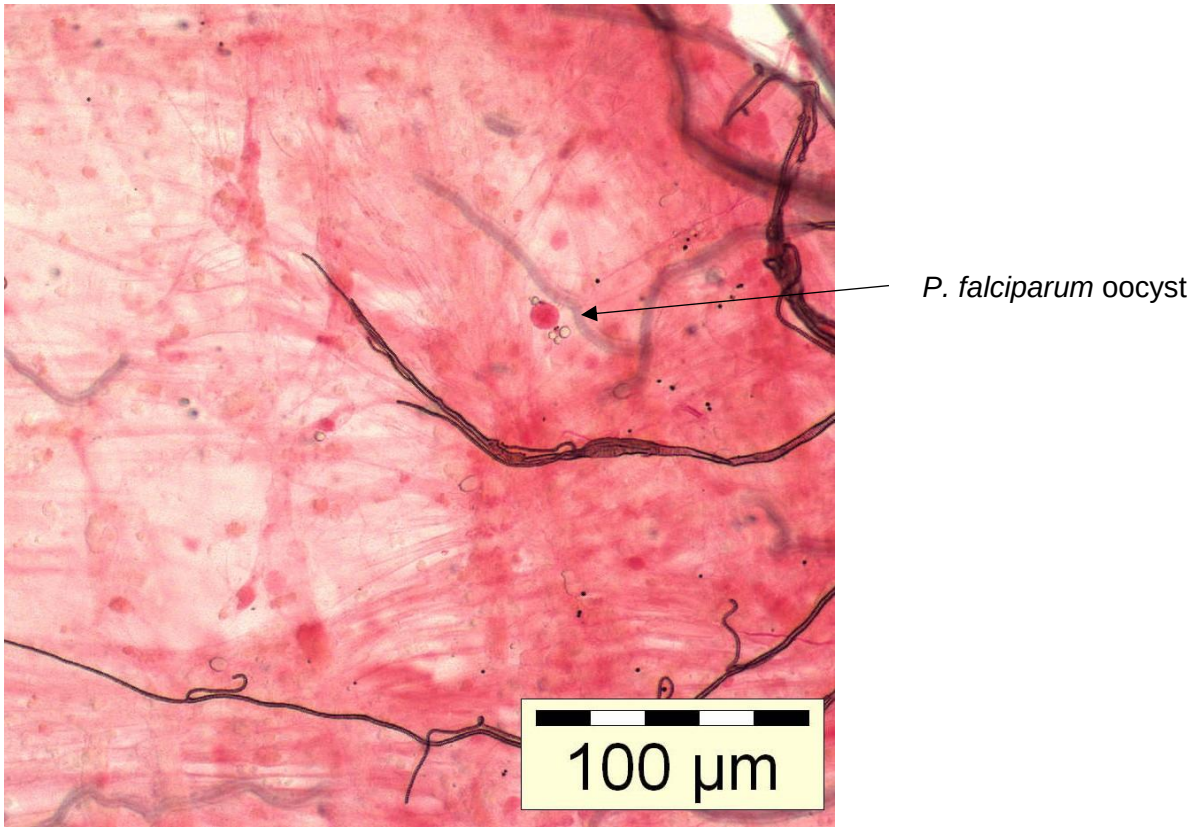


Fig. 2.7.a. Midgut of a major vector, *An. funestus,* containing a *P. falciparum* oocysts.

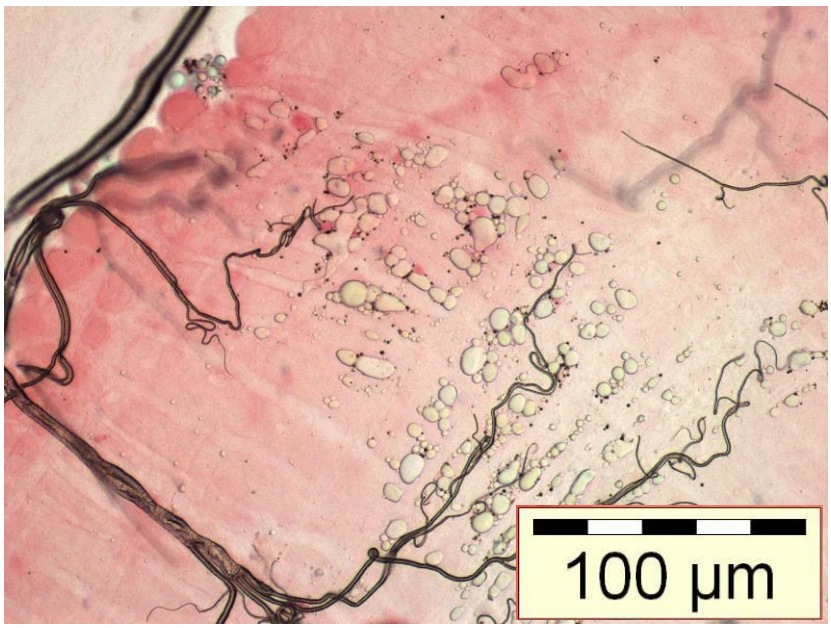


Fig. 2.7.b. Midgut of an uninfected *An. funestus* mosquito with no parasite oocysts.

2.5.3 Oocyst prevalence and intensity data capture and analysis

Oocyst prevalence and intensity data were entered into Excel spreadsheets and the oocyst prevalence and intensity was determined for the control and treatment group according to each species. Data analysis was conducted in R-Studio (1.3.1093) and the Generalised Mixed Linear model was used to analyse oocyst prevalence and intensity data with high variance (Crawley, 2013). This model includes the semi-parametric model to evaluate data that is not normally distributed and therefore the model is not restricted to normally distributed data. Therefore, normality tests were not conducted for these assays. The multiple regression model was used to account for replication as a random factor. Results are reported as beta-estimate-, Chi-square- and *P*-values, with df=1. These values were entered into GraphPad Prism (9.0.1) in order to construct graphs displayed.

CHAPTER THREE- Results

3.1 Mosquito vector dynamics

3.1.1. Species identification and seasonal variation

A total of 182 anopheline mosquitoes that were morphologically identified as belonging to the *An. funestus* group, showed amplification during the *An. funestus* multiplex PCR assay. Molecular species identification revealed that four members of the *An. funestus* group were present, *An. parensis, An. leesoni, An. vaneedeni* and *An. rivulorum* (Fig. 3.1). Twenty seven samples (15%) failed to amplify even after repeating the PCR assay and were not included in the data. This is possibly due to DNA degradation of the specimens in the field or specimens were morphologically misidentified and failed to amplify.



Fig. 3.1. Agarose gel for the PCR identification of members of the *An. funestus* group. Lanes 1 and 28 indicate molecular weight markers whereas lanes 2, 3 and 4 are controls for *An. funestus* and negative controls for DNA extraction and PCR respectively. Lanes 5, 7, 12, 14-16, 19 and 23 are *An. leesoni* (146 bp) whereas lanes 6, 17-18, 21 and 24-25 indicates samples identified as *An. vaneedeni* (587 bp). Lanes 8-11, 13, 20, 22 and 26-27 contained samples which did not amplify during the assay.

Seasonal variation in the relative abundance of members of the *An. funestus* group is represented in figure 3.2 and Table 3.1. *Anopheles parensis* was found to be the most abundant species over the 8-month collection period with a total of 64

mosquitoes identified, which comprises 35% of the total sample size. *Anopheles vaneedeni* was the second most abundant species with a total of 51 specimens (28%) and together with *An. parensis*, these species were present from August 2019-March 2020 (Fig. 3.2).

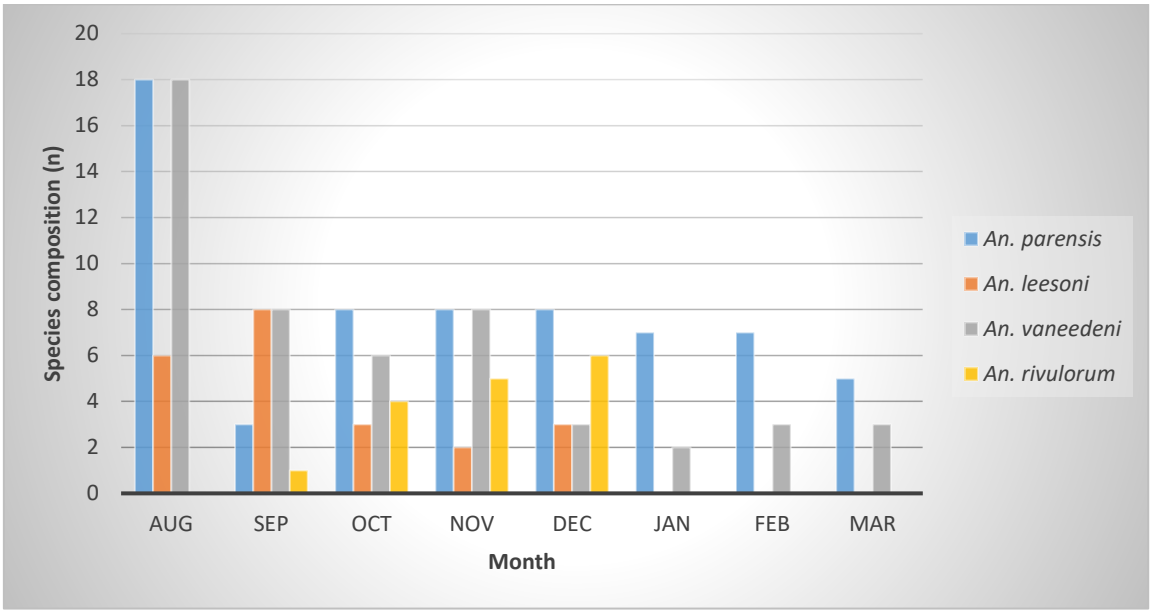


Fig.3.2. Relative species composition (n) of members from the *An. funestus* group (the minor vectors, *An. parensis & An. vaneedeni and non-vectors An. leesoni, & An. rivulorum*) from the Mamfene region in KwaZulu-Natal as collected over a period of 8 months.

Table 3.1: The absolute number of anopheline species from the *An. funestus* group collected over an 8-month period from sites in Mamfene, KwaZulu-Natal. The percentage mosquitoes collected per collection method is indicated.

Variable	*Number of An. funestus group mosquitoes collected*
Month	
Aug	42
Sep	20
Oct	21
Nov	23
Dec	20
Jan	9
Feb	10
Mar	8
Collection method	
Clay pot	60%
Modified plastic bucket	12%
Tyre	19%
Other methods(CO_2 traps, landing catches etc.)	9%

From October to December the abundance for *An. parensis* remained stable but started to decline from January (Fig. 3.2). *Anopheles vaneedeni* numbers also remained constant, after the spike in August, but the abundance started to decline in December (Fig. 3.2). The latter species numbers started to increase again from February. Overall a positive correlation was found between *An. parensis* and *An. vaneedeni* (r= 0.77, *P*=0.024) (Appendix C). *Anopheles leesoni* comprised 12% of the total number of *An. funestus* group collections. Furthermore, *An. leesoni* was only present in collections made from August to December, with the highest abundance during September (Fig. 3.2). *Anopheles rivulorum* was only present in September to December collections and its abundance increased monthly (Fig. 3.2). This species contributed 9% of the total specimens that amplified during the PCR analysis. No correlation was found between other members of the *An. funestus* group (Appendix C).

The highest number of mosquitoes were collected during August (n=42) whereas fewer *An. funestus* group mosquitoes were collected during January (n=9) to March (n=8) (Table 3.1). Most collections came from clay pots (60%) and modified plastic buckets was the individual collection method that collected the least mosquitoes (n=12) (Table 3.1).

Seasonal variation of the relative abundance for members of the *An. gambiae* complex is illustrated in Figure 3.3, Table 3.2. *Anopheles arabiensis* dominated collections with a total of 3,550 (99.4%) mosquitoes collected over the 8-month period while the minor vector, *An. merus*, contributed towards 0.5% of the total number. The non-vector species, *An. quadriannulatus,* only accounted for the remaining 0.1% of collections (data curtesy of the SIT group). The number of *An. arabiensis* decreased from August to September but started to increase again in October and November (Fig 3.3). For December, the *An. arabiensis* number was lower, but a sharp increase was observed for January. Although there was a decline in the number of *An. arabiensis* mosquitoes in February, the numbers increased again in March (Fig. 3.3).

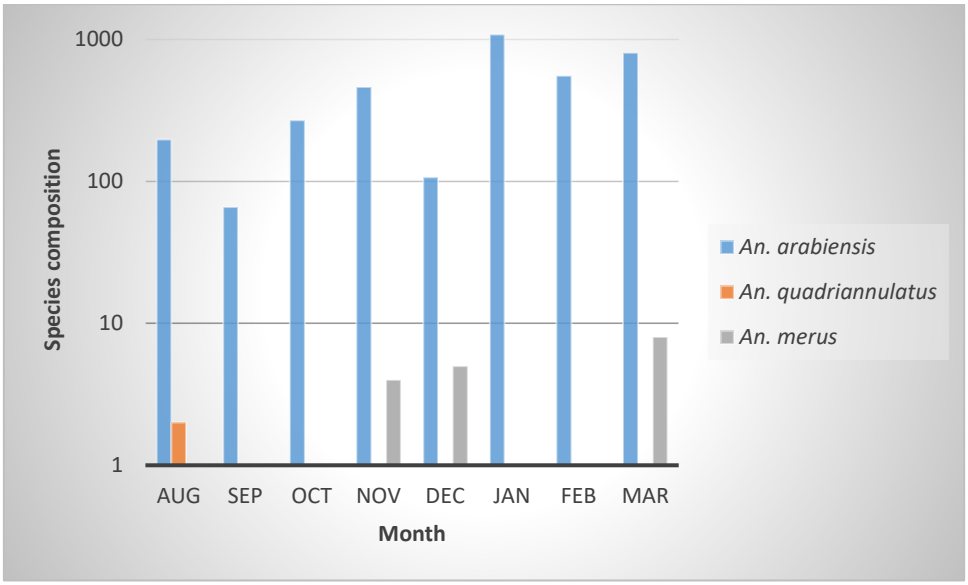


Fig. 3.3. Relative species composition of members from the *An. gambiae* complex (the major vector, *An. arabiensis,* the minor vector, *An.merus* and non-vector*An. quadriannulatus*) from the Mamfene region in KwaZulu-Natal as collected over a period of 8 months.

Table 3.2: The absolute number of anopheline species from the *An. gambiae* complex collected over an 8-month period from sites in Mamfene, KwaZulu-Natal. The percentage of mosquitoes collected per trap is indicated.

Variable	*Number of An. gambiae complex mosquitoes collected*
Month	
Aug	199
Sep	66
Oct	269
Nov	468
Dec	112
Jan	1087
Feb	557
Mar	815
Collection method	
Clay pot	48%
Modified plastic bucket	2,4%
Tyre	0,07%
Other methods (CO_2 traps, human landing catches etc.)	49,5%

The minor vector, *An. merus,* was only present in collections from November, December and March. In comparison to the major vector, the peak time for *An. merus* collections was in March, whereas the highest number of *An. arabiensis* specimens was collected in January (Fig.3.3). The second highest number of *An. merus* mosquitoes collected, was during December, when *An. arabiensis* collections were low in comparison to November and January (Fig.3.3). A total of 50% of *An.*

quadriannulatus collections was made during August, while *An. arabiensis* collections were also lower (Fig.3.3). Collections for *An. quadriannulatus* also coincided with the months during which the collections for *An. arabiensis* were lower, but no correlation was found between the non-vector and the major vector.

Collections for the *An. gambiae* complex mosquitoes were the highest during January (n=1087), with the least number of mosquitoes collected during September (n=66) (Table 3.2). As with the *An. funestus* group collections, the clay pots were responsible for most of the collections (48%), per single collection method, of *An. gambiae* complex mosquitoes (Table 3.2).

A comparison between species of the *An. funestus* group and *An. gambiae* complex is presented in Fig. 3.4. The overall numbers of the non-vector (*An. leesoni*, *An. rivulorum* and *An. quadriannulatus*) and minor vector (*An. parensis, An. vaneedeni* and *An. merus*) populations remain low in comparison to that of the major vector. No correlation was found between the major vector, *An. arabiensis*, and minor- (*An. parensis*: r=-0.21, P=0.60; *An. vaneedeni*: r=-0.50, P=0.20; *An. merus*: r=0.27, P=0.51) or non-vector (*An. leesoni*: r=-0.80, P=0.64; *An. rivulorum*: r=-0.46, P=0.24; *An. quadriannulatus*: r=-0.19, P=0.65) species (Table 3.3).

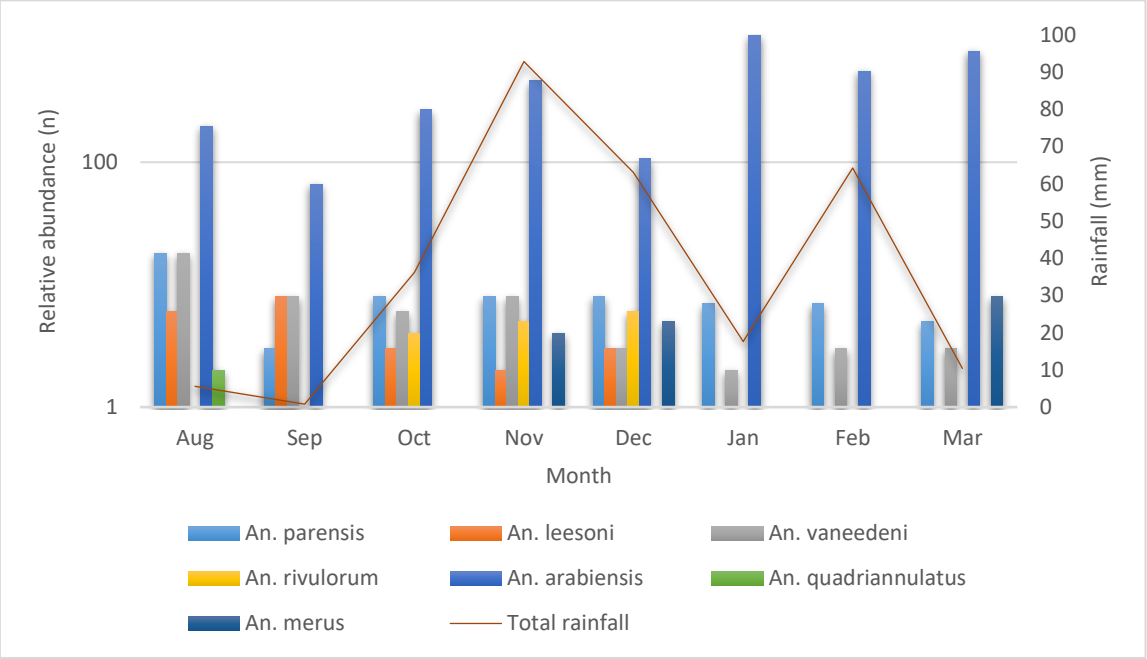


Fig. 3.4. The relative abundance of the major vector, *An. arabiensis* and minor (*An. parensis*, *An. vaneedeni,* and *An. merus*) and non-vector (*An. rivulorum, An. leesoni,* and *An. quadriannulatus*) species from the *An. funestus* group and *An. gambiae* complex over an 8-month period. A logarithmic scale displays relative abundance data. Average monthly rainfall (mm) is also indicated for the period.

Table 3.3: Pearson's correlation between the relative abundance of the major vector, *An. arabiensis* and all other minor- (*An. parensis, An. vaneedeni* and *An. merus*) and non-vector (*An. leesoni, An.* rivulorum and *An. quadriannulatus*) species from the *An. gambiae* complex and *An. funestus* group. Results are displayed as r- and two-tailed *P*-values for each species (*P*>0.05 indicates no significant difference).

	An. parensis	*An. leesoni*	*An. vaneedeni*	*An. rivulorum*	*An. quadriannulatus*	*An. merus*
An. arabiensis	r=-0.21 *P*=0.60	r=-0.80 *P*=0.64	r=-0.50 *P*=0.20	r=-0.46 *P*=0.24	r=-0.19 *P*=0.65	r=0.27 *P*=0.51

In terms of rainfall, *An. arabiensis* numbers were low during the drier month (September) and increased again as rainfall increased in October and November (Fig. 3.4). The relative abundance of *An. arabiensis* was, however, at its peak in January, while the rainfall was much lower. The same was observed for March when *An. arabiensis* numbers were high during a drier period. No correlation was found between *An. arabiensis* abundance and rainfall (r= -0.06, *P*=0.88) (Table 3.4) or any of the other species (Table 3.4).

Table 3.4: Pearson's correlation between rainfall and mosquitoes from the *An. gambiae* complex and *An. funestus* group. Results are displayed as r and two-tailed *P*-values for each species (*P*>0.05 indicates no significant difference).

Rainfall	*An. parensis*	*An. leesoni*	*An. vaneedeni*	*An. rivulorum*	*An. arabiensis*	*An. quadriannulatus*	*An. merus*
Pearson's Correlation	r=-0.05 *P*=0.89	r=-0.40 *P*=0.33	r=-0.27 *P*=0.51	r=0.67 *P*=0.06	r=-0.06 *P*=0.88	r=0.13 *P*=0.72	r=0.23 *P*=0.58

3.1.2. Wild caught mosquito infection profiling

The ELISA assay confirmed the absence of *P. falciparum* in the wild female *An. funestus* group mosquitoes collected, including *An. parensis, An. vaneedeni, An. leesoni* and *An. rivulorum*. None of the samples had an optical density value higher than that of the cut-off value and thus all of the samples were regarded as negative. Representation of the optical density values is displayed in Fig. 3.5 for nine of the samples analysed.

Measurement count: 1 Filter: 405												
	1	2	3	4	5	6	7	8	9	10	11	12
A	0.235	0.088	0.081	0.071	0.061	0.050	0.040	0.047	0.043			
B												
C												
D												
E												
F												
G												
H						0.079	0.069	0.059	0.060	0.049	0.044	0.048

Fig. 3.5. Screening of mosquitoes for *P. falciparum* infection. Optical density values are measured at 405 nM. Well A1 represents the positive control, which consists of purified recombinant protein, and wells A2-9 contain the wild caught samples. Insectary reared unfed female mosquitoes were used as negative controls, which was added to the last 7 wells of the plate, H6-12. Cut off value was calculated to be 0,12.

3.2. Mosquito Blood Feeding Behaviour, Attraction and Parasite Host Manipulation

In order to further investigate mosquito feeding behaviour and infection in the laboratory, an artificial feeding system was developed. Rodhain conducted the first artificial feeding of insects, via a membrane, in 1912 (Rutledge et al. 1964). The feeding system has been redesigned and to date it is one of the most common systems used (Rutledge et al. 1964). During this study the standard membrane feeding assay (SMFA) was used to evaluate if changes in feeding and attraction behaviour occurred in uninfected African mosquito vector species..

3.2.1. Mosquito attraction and HMBPP

3.2.1.1. Choice chamber evaluation

It was vital to establish if the choice chamber was sensitive enough to allow adult female mosquitoes to select between different food sources. Mosquitoes' attraction was therefore evaluated between sugar water vs blood. The proportion of *An. arabiensis* mosquitoes that showed attraction towards the blood source was significantly higher (P=0.018) than those that had selected the sugar water (Table 3.5). The same was observed for *An. funestus* (P=0.032) (Table 3.5).

Table 3.5. Statistical analysis of the choice chamber evaluation. Cow blood and sugar water were the two sources used for attraction evaluation. Results are presented for two major vector species, (n=3).

Species		HMBPP-supplemented blood (Treatment)	Blood only (Control)
An. arabiensis	Sample size	60	60
	Beta-estimate	31.1	30
	Chisq value, Df=1	55	
	P-value	0.018*	
An. funestus	Sample size	60	60
	Beta-estimate	30.6	29.3
	Chisq value, Df=1	4.59	
	P-value	0.032*	

*Denotes significant difference

3.2.1.2. HMBPP and attraction of different mosquitoes

It was evident that the choice chamber design was sufficient, and I evaluated the possibility that female mosquitoes were more attracted to HMBPP-supplemented blood. The major vector, *An. arabiensis*, was more attracted to HMBPP-supplemented blood (*P*=0.0022) (Fig. 3.6, Table 3.6) whereas the opposite was observed for for *An. coluzzii* (*P*=0.0039) and *An. gambiae* (*P*=0.0023) (Fig. 3.7 & 3.8, Table 3.6). *Anopheles funestus* showed a similar response to HMBPP-induced blood as *An. arabiensis*, with increased attraction towards it (*P*=0.011) (Fig. 3.9, Table 3.6). Both minor vectors, *An. merus* and wild caught *An. vaneedeni*, showed higher attraction towards blood containing the metabolite (*P*=0.011, Fig. 3.10, Table 3.6 and *P*<0.001, Fig. 3.11, Table 3.6). The strongest significance was observed for *An. vaneedeni.* The non-vector, *An. quadriannulatus*, was more attracted towards the blood in the control group (*P*=0.012, Fig. 3.12, Table 3.6).

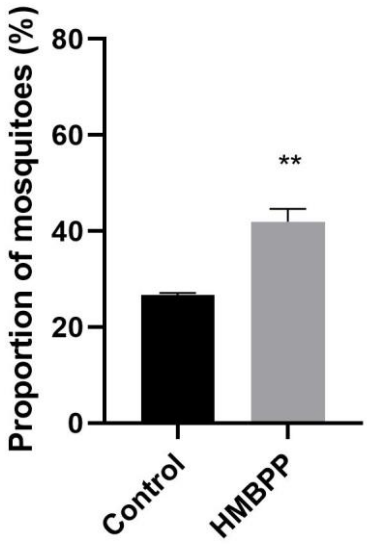


Fig. 3.6. Attraction of the major vector, *An. arabiensis,* females towards the control and treatment (HMBPP-supplemented blood) group. Error bars, ± SE Asterisks indicates significant difference (*P<0.05, **P<0.01, ***P<0.001).

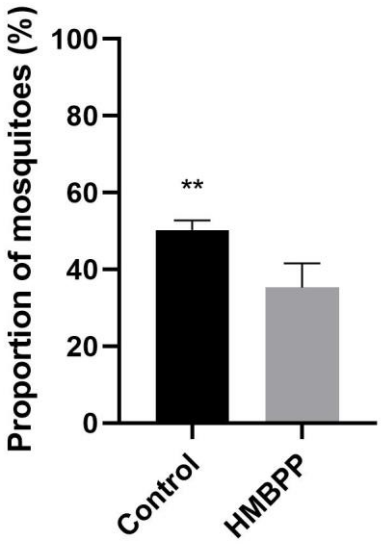


Fig. 3.7. Attraction of the major vector, *An. coluzzii,* females towards the control and treatment (HMBPP-supplemented blood) group. Error bars, ± SE Asterisks denotes significant difference (*P<0.05, **P<0.01, ***P<0.001).

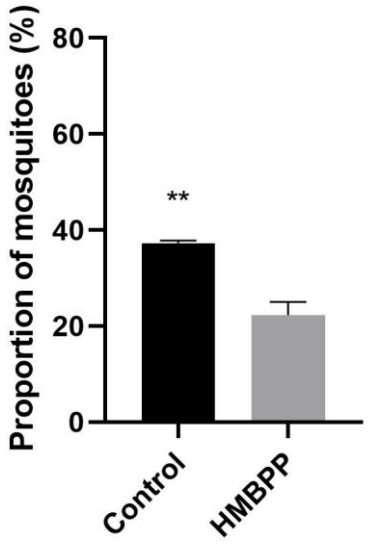


Fig. 3.8. Attraction of the major vector, *An. gambiae,* females towards the control and treatment (HMBPP-supplemented blood) group. Error bars, ± SE are indicated, and asterisks show significant difference (*P<0.05. **P<0.01. ***P<0.001).

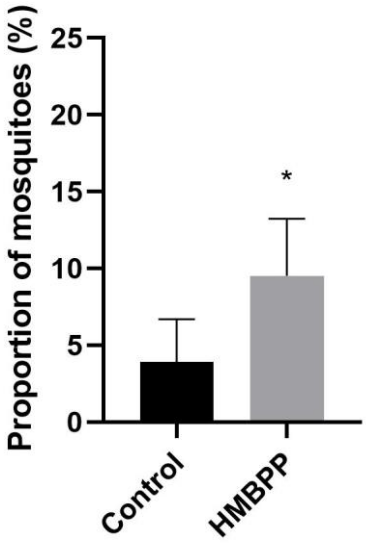


Fig. 3.9. Attraction of the major vector, *An. funestus,* females towards the control and treatment (HMBPP-supplemented blood) group. Error bars, ± SE Asterisks indicates significant difference (*P<0.05, **P<0.01, ***P<0.001).

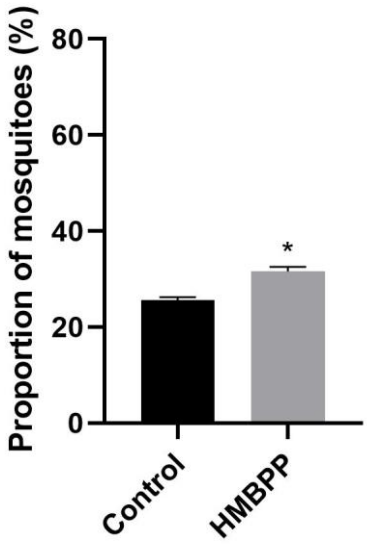


Fig. 3.10. Attraction of the minor vector, *An. merus,* females towards the control and treatment (HMBPP-supplemented blood) group. Error bars, ± SE, are indicated and asterisks show significant difference (**P*<0.05, ***P*<0.01, ****P*<0.001).

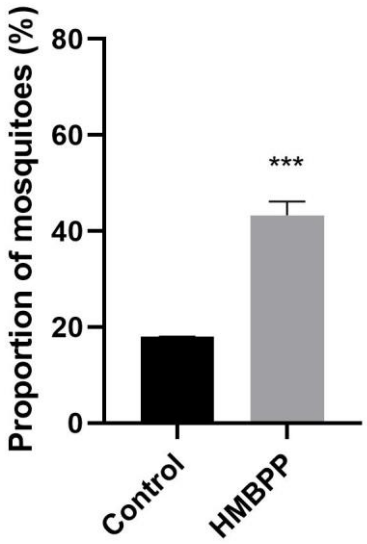


Fig. 3.11. Attraction of the minor vector, *An. vaneedeni,* females towards the control and treatment (HMBPP-supplemented blood) group. Error bars, ± SE, Asterisks denote significant difference (**P*<0.05, ***P*<0.01, ****P*<0.001).

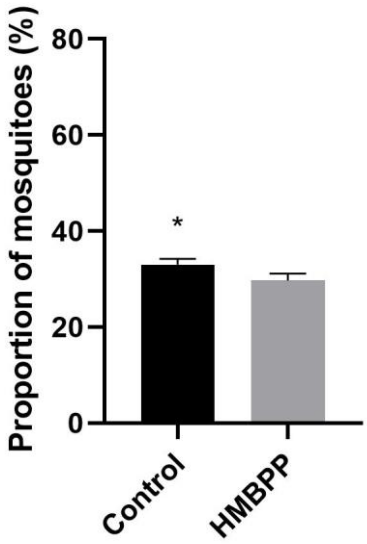


Fig. 3.12. Attraction of the non-vector, *An. quadriannulatus*, females towards the control and treatment (HMBPP-supplemented blood) group. Error bars, ± SE, Asterisks denote significant difference (**P*<0.05, ***P*<0.01, ****P*<0.001).

Table 3.6. Statistical analysis of the attraction towards HMBPP-supplemented blood (treatment group) and blood only (control group). Results are presented for major vectors (*An. arabiensis, An. gambiae*, *An. coluzzii* and *An. funestus*), minor vectors (*An. merus* and *An. vaneedeni*) and the non-vector *An. quadriannulatus* (n=3)

Species		HMBPP-supplemented blood (Treatment)	Blood only (Control)
An. arabiensis	Sample size	60	60
	Beta-estimation	41.9	26.6
	Chisq value, Df=1	9.36	
	P-value	0.0022	
	P value summary	**	
An. gambiae	Sample size	60	60
	Beta-estimation	22.3	37.3
	Chisq value, Df=1	9.26	
	P-value		0.0023
	P value summary		**
An. coluzzii	Sample size	60	60
	Beta-estimation	35.3	50.3
	Chisq value, Df=1	8.28	
	P-value		0.0039
	P value summary		**
An. funestus	Sample size	60	60
	Beta-estimation	5.6	3.9
	Chisq value, Df=1	6.46	
	P-value	0.011	
	P value summary	*	
An. merus	Sample size	60	60
	Beta-estimation	31.6	25.6
	Chisq value, Df=1	6.42	
	P-value	0.011	
	P value summary	*	
An. vaneedeni	Sample size	60	60
	Beta-estimation	43.3	18.0
	Chisq value, Df=1	10.94	
	P-value	0.0009	
	P value summary	***	
An. quadriannu-latus	Sample size	60	60
	Beta-estimation	29.6	33.0
	Chisq value, Df=1	6.18	
	P-value		0.012
	P value summary		*

*$P<0.05$, **$P<0.01$, ***$P<0.001$

3.2.2 Feeding rate

Glass feeders were used to determine if HMBPP spiking of blood increase blood feeding rate in colonized *An. arabiensis*, *An. coluzzii*, *An. gambiae*, *An. funestus*, *An. merus* and *An. quadriannulatus*. The feeding rate for *An. arabiensis* was significantly higher (*P*=0.0015) in the treatment group (HMBPP-supplemented blood) than in the control group (Table 3.7). The significantly higher feeding rate in the treatment group was also observed for *An. coluzzii* (*P*=0.025) and *An. gambiae* (P=0.015) (Table 3.7). In contrast to these findings, the other major vector, *An. funestus,* did not show increased feeding rate in the treatment group (*P*=0.022) (Table 3.7). Furthermore, the minor vector, *An. merus*, had a feeding rate that was significantly higher in the control group (*P*=0.026) (Table 3.7) whereas for the non-vector, *An. quadriannulatus*, a significantly higher feeding rate was observed in the treatment group (*P*=0.0042) (Table 3.7).

The aim of this study was not to compare the effect of HMBPP on the feeding rate between species and therefore results do not reflect such comparisons.

Table 3.7. Statistical analysis of the feeding rate between HMBPP-supplemented blood (treatment group) and blood only (control group). Results are presented for major vectors (*An. arabiensis, An. gambiae*, *An. coluzzii* and *An. funestus*), the minor vector, *An. merus,* and the non-vector, *An. quadriannulatus*.

Species		HMBPP-supplemented blood (Treatment)	Blood only (Control)
An. arabiensis	Sample size	225	225
	Beta-estimate	56.5	41.7
	Chisq value, Df=1	9.98	
	P-value	0.0015**	
An. gambiae	Sample size	225	225
	Beta-estimate	24.7	21.8
	Chisq value, Df=1	5.82	
	P-value	0.015*	
An. coluzzii	Sample size	225	225
	Beta-estimate	45.1	43.7
	Chisq value, Df=1	5.01	
	P-value	0.025*	
An. funestus	Sample size	270	270
	Beta-estimate	13.3	13.8
	Chisq value, Df=1	5.21	
	P-value		0.022*
An. merus	Sample size	225	225
	Beta-estimate	14.9	15.2
	Chisq value, Df=1	4.94	
	P-value		0.026*
An. quadriannu-latus	Sample size	225	225
	Beta-estimate	47.8	37.8
	Chisq value, Df=1	8.16	
	P-value	0.0042**	

*$P<0.05$, **$P<0.01$

3.2.3 HMBPP Dose response

A standard HMBPP concentration of 10 µM was used throughout the study, yet the effect of dosage on different vectors was determined in order to get additional insight and a dose-response evaluation was conducted.

Anopheles funestus showed an upward trend as the HMBPP concentration increased (Fig. 3.13). A steady incline was observed as the HMBPP increased from the dosage

range of 1 µM to 100 µM. No significant difference was however found for comparisons between the standard concentration (10 µM) and the low (1 µM) and high (100 µM) concentrations repsectively (*P*=0.095; *P*=0.11). The same results were not observed for *An. gambiae*, as the opposite effect was detected in this species (Fig. 3.13). *Anopheles gambiae* showed a steady decrease in feeding rate from the lowest concentration (1 µM) to the standard 10 µM. This was followed by a more rapid declination towards the highest concentration of 100 µM. Again no significant difference was detected between the standard and both the low and high concentration *(P*=0.97; *P*=0.24). A significant difference was observed between *An. gambiae* and *An. funestus* at a 1 µM concentration (*P*<0.01**) whereas no significant difference was found between the latter species at 10 µM (*P*=0.093) and 100 µM (*P*=0.71).

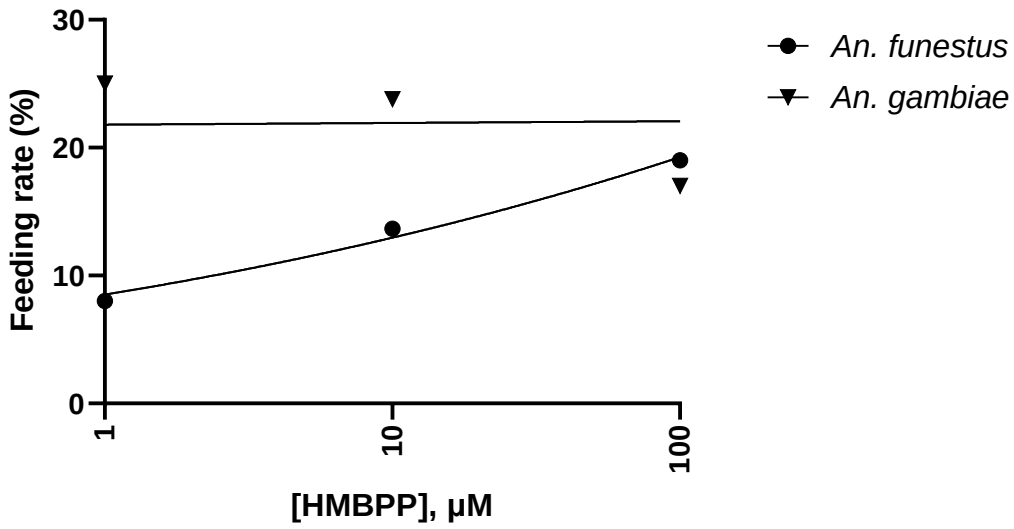


Fig. 3.13. Dose response curve representing different HMBPP concentrations represented as percentage feeding rate. The feeding rates for both major vectors, *An. funestus* and *An. gambiae* are illustrated.

3.3. Susceptibility of mosquito vector species to the *P. falciparum* parasite

The dose response curved showed a significant difference in feeding rate between the standard and lower HMBPP concentration, therefore the standard concentration of 10 µM HMBPP was used to determine the impact of this metabolite on vector susceptibility to *P. falciparum*.

3.3.1 *Plasmodium falciparum* susceptibility of major vectors

Anopheles arabiensis showed no statistical difference for oocyst prevalence between the control and treatment groups (*P*=0.13, Chisq=6.20, Df=1). The overall mean prevalence for both groups was 60% (Fig. 3.14a, Table 3.8). In terms of oocyst intensity, the treatment group had a significantly higher number of oocysts per midgut, reaching 5,8 oocysts per gut (*P*=0.012, Chisq=6.18,Df=1) in comparison to the 3,3 in the control group (Fig.3.14b, Table 3.8).

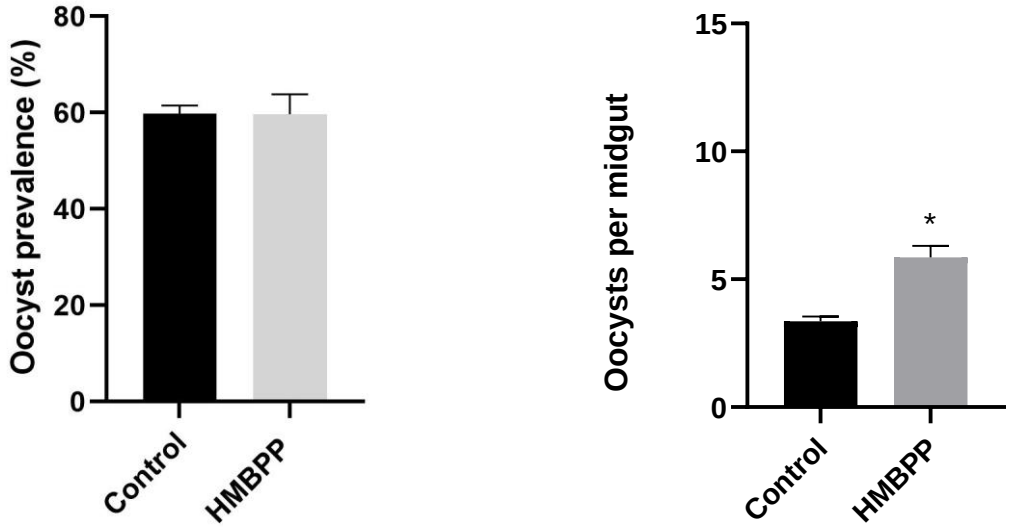


Fig. 3.14a & b. Percentage oocyst prevalence for the control and test group in *An. arabiensis* midguts (a) and average number of oocyst per midgut (intensity) in *An. arabiensis* females (b). Error bars, ± SE, asterisks denotes significant difference (**P*<0.05, ***P*<0.01, ****P*<0.001).

Table 3.8: Statistical significance in oocyst prevalence and intensity between HMBPP-supplemented *P. falciparum* infected blood (treatment) and *P. falciparum* infected blood only (control) of *An. arabiensis* mosquitoes. Results are reported as beta-estimate-, Chi-square- and *P*-values, with df=1. Data are from three biological replicates .

Treatment	Infected blood only (Control)	HMBPP-supplemented infected blood (Treatment)
Sample size	77	62
Prevalence *P*-value	*P*=0,13, ns	
Chisq prevalence value	6,20	
Prevalence beta-estimate value	59,75	59,67
Intensity *P*-value		*P*=0,012, *
Chisq intensity value		6,18
Intensity beta-estimate value	3,36	5,86

The oocyst prevalence in *An. gambiae* females was significantly higher in the presence of HMBPP (78%) (*P*<0.001, Chisq=11.03, Df=1) than in the control group (60%) (Fig. 3.15a, Table 3.9). The females in the treatment group had a mean number of oocysts of 9,2 per midgut, compared to 8,1 in the control group (Fig. 3.15b, Table 3.9). The difference was, however, not statistical significant (*P*=0.057, Chisq=3.61, Df=1).

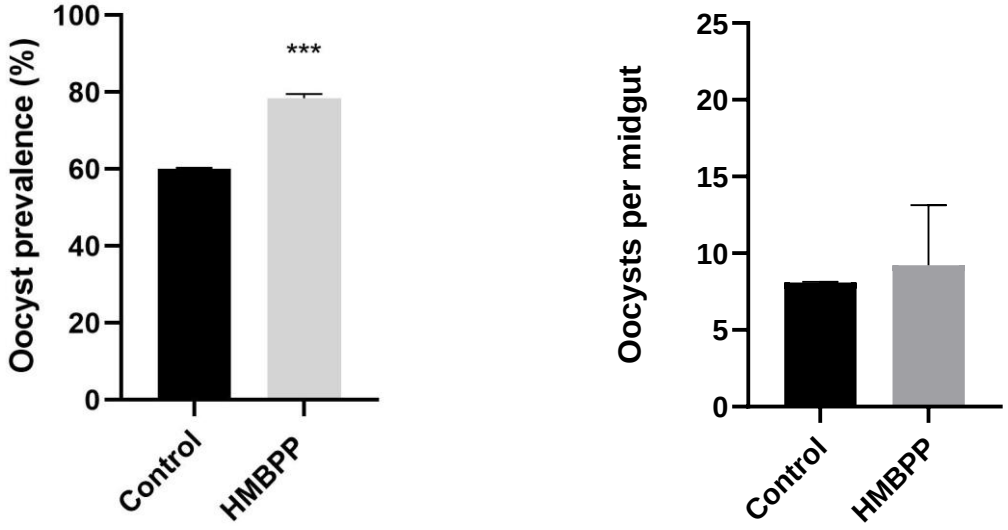


Fig. 3.15 a & b. Percentage oocyst prevalence for the control and test group in *An. gambiae s.s*. midguts (a) and average number of oocyst per midgut (intensity) in *An. gambiae s.s.* females (b). Error bars, ± SE, asterisks denotes significant difference (**P*<0.05, ***P*<0.01, ****P*<0.001).

Table 3.9. Statistical significance in oocyst prevalence and intensity between HMBPP-supplemented *P. falciparum* infected blood and *P. falciparum* infected blood only (control) of *An. gambiae s.s.* mosquitoes. Results are reported as beta-estimate-, Chi-square- and *P*-values, with df=1. Data are from three biological replicates.

Treatment	Infected blood only (Control)	HMBPP-supplemented infected blood (Treatment)
Sample size	68	56
Prevalence *P*-value		*P*<0.001, ***
Chisq value		11,03
Prevalence beta-estimate value	60,06	78,44
Intensity *P*-value		*P*=0,057, ns
Chisq value		3,61
Intensity beta-estimate value	8.10	9,21

Anopheles coluzzii had an oocyst prevalence that was significantly higher in the treatment group (*P*=0.002, Chisq=9.42, Df=1) than in the control group (Fig. 3.16a, Table 3.10). A precentage prevalence of 74% was observed in the treatment group and for the control group it was 60%. In terms of oocyst intensity, no significant difference was observed between the treatment and control groups as the mean number of oocysts per midgut was 4,6 and 4,4 respectively (*P*=0.15, Chisq=2.04, Df=1). (Fig. 3.16b, Table 3.10).

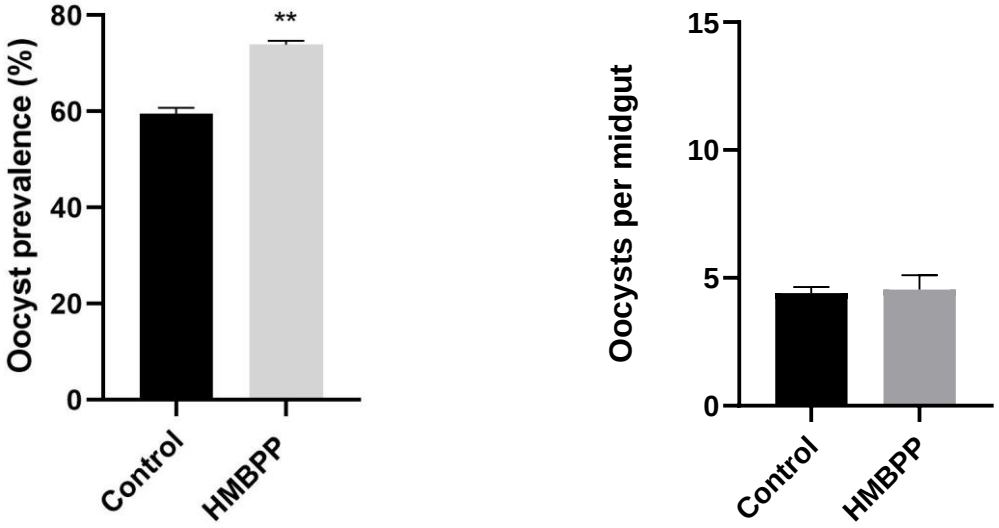


Fig. 3.16a & b. Percentage oocyst prevalence for the control and test group in *An. coluzzii* midguts (a) and average number of oocyst per midgut (intensity) in *An. coluzzii* females (b). Error bars, ± SE, asterisks denotes significant difference (**P*<0.05, ***P*<0.01, ****P*<0.001).

Table 3.10. Statistical significance in oocyst prevalence and intensity between HMBPP-supplemented *P. falciparum* infected blood (treatment) and *P. falciparum* infected blood only (control) of *An. coluzzii* mosquitoes. Results are reported as beta-estimate-, Chi-square- and *P*-values, with df=1. Data are from three biological replicates.

Treatment	Infected blood only (Control)	HMBPP-supplemented infected blood (Treatment)
Sample size	75	72
Prevalence *P*-value		*P*=0,002, **
Chisq value		9.42
Prevalence beta-estimate value	59.53	73.92
Intensity *P*-value		*P*=0,15, ns
Chisq value		2.04
Intensity beta-estimate value	4.41	4.55

The same significant increase in prevalence was observed for *An. funestus* as the oocyst prevalence was higher (70%) in the presence of HMBPP in comparison to the control group (63%) (*P*=0.012, Chisq=6.19,Df=1) (Fig. 3.17a, Table 3.11). A higher oocyst intensity was also observed for the group that was treated with HMBPP with 3,3 versus 2,6 observed for the respective groups. The difference in oocyst intensity was, however, not significant (*P*=0.15, Chisq=2.07, Df=1) (Fig.3.17b, Table 3.11).

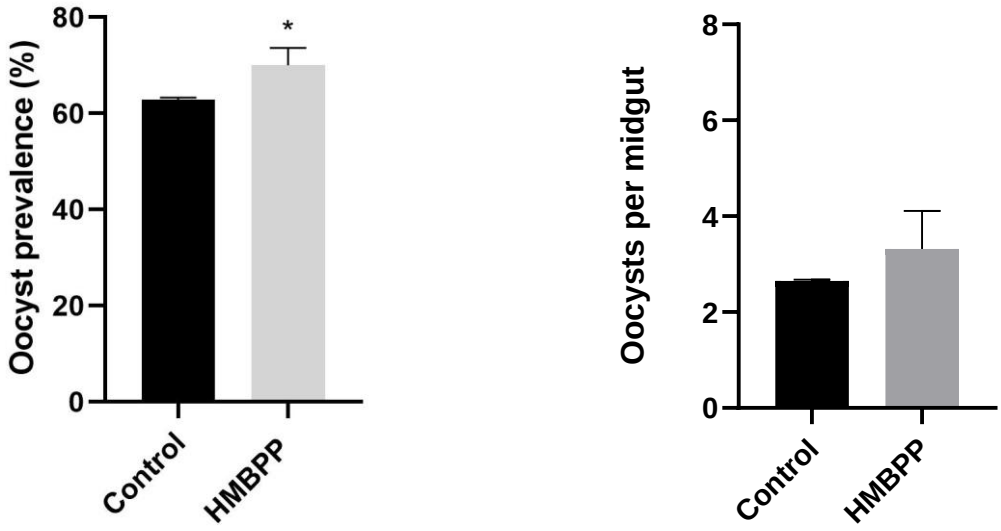


Fig. 3.17 a & b. Percentage oocyst prevalence for the control and test group in *An. funestus s.s*. midguts (a) and average number of oocyst per midgut (intensity) in *An. funestus s.s.* females (b). Error bars, ± SE, asterisks denote significant difference (**P*<0.05, ***P*<0.01, ****P*<0.001).

Table 3.11. Statistical significance in oocyst prevalence and intensity between HMBPP-supplemented *P. falciparum* infected blood and *P. falciparum* infected blood only (control) of *An. funestus s.s* Mosquitoes. Results are reported as beta-estimate-, Chi-square- and *P*-values, with df=1. Data are from three biological replicates.

Treatment	Infected blood only (Control)	HMBPP-supplemented infected blood (Treatment)
Sample size	79	61
Prevalence *P*-value		*P*=0,012, *
Chisq value		6,19
Prevalence beta-estimate value	62.86	70.03
Intensity *P*-value		*P*=0,15, ns
Chisq value		2,07
Intensity beta-estimate value	2.65	3.32

3.3.2. *Plasmodium falciparum* susceptibility of a minor vector

Anopheles merus also had a significantly higher mean oocyst prevalence with the addition of HMBPP (67%) in comparison to the control group, at 44% (*P*<0.001, Chisq=12.50, Df=1) (Fig. 3.18a, Table 3.12). The oocyst intensity did not differ significantly between the control and treatment groups (*P*=0.095, Chisq=2.77, Df=1) (Fig. 3.18b, Table 3.12).

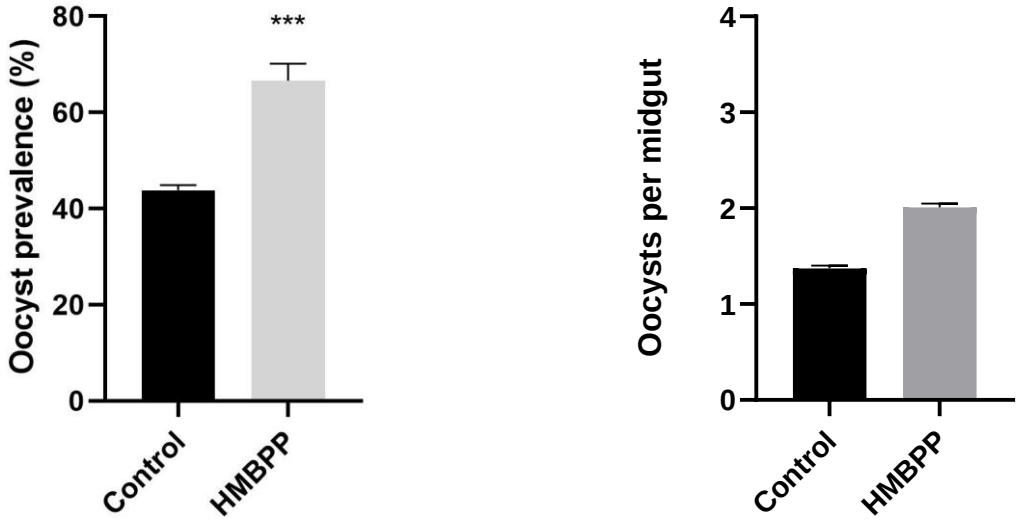


Fig. 3.18a & b. Percentage oocyst prevalence for the control and test group in *An. merus* midguts (a) and average number of oocyst per midgut (intensity) in *An. merus* females (b). Error bars, ± SE, asterisks denotes significant difference (**P*<0.05, ***P*<0.01, ****P*<0.001).

Table 3.12: Statistical significance in oocyst prevalence and intensity between HMBPP-supplemented *P. falciparum* infected blood (treatment) and *P. falciparum* infected blood only (control) of *An. merus* mosquitoes. Results are reported as beta-estimate-, Chi-square- and *P*-values, with df=1. Data are from three biological replicates.

Treatment	Infected blood only (Control)	HMBPP-supplemented infected blood (Treatment)
Sample size	60	73
Prevalence *P*-value		*P*<0.001, ***
Chisq value		12,50
Prevalence beta-estimate value	43.86	66.66
Intensity *P*-value		*P*=0.095, ns
Chisq value		2,77
Intensity beta-estimate value	1.37	2.01

3.3.3 *Plasmodium falciparum* susceptibility of a non-vector

The oocyst prevalence for *An. quadriannulatus* showed a significant increase in the presence of HMBPP (71%) (*P*=0.006, Chisq=7.38, Df=1) in comparison to the control group (62%) (Fig 3.19a, Table 3.13). The mean oocyst intensity was higher in the treatment group (3,1) than in the cotrol group (2,6), but the difference was not significant (*P*=0.18, Chisq=1.75, Df=1) (Fig. 3.19b, Table 3.13).

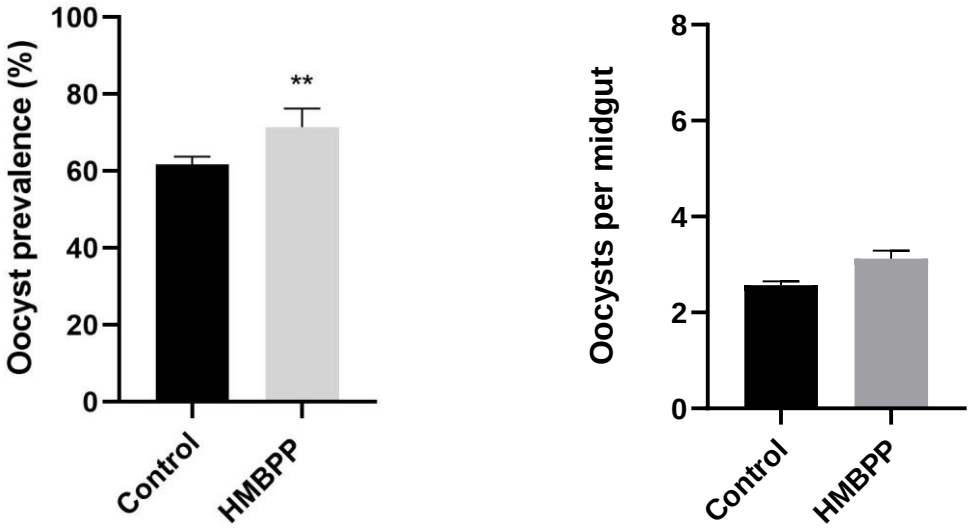


Fig. 3.19 a & b. Percentage oocyst prevalence for the control and test group in *An. quadriannulatus* midguts (a) and average number of oocyst per midgut (intensity) in *An. quadriannulatus* females (b). Error bars, ± SE, asterisks denotes significant difference (**P*<0.05, ***P*<0.01, ****P*<0.001).

Table 3.13. Statistical significance in oocyst prevalence and intensity between HMBPP-supplemented *P. falciparum* infected blood and *P. falciparum* infected blood only (control) of *An. quadriannulatus* mosquitoes. Results are reported as beta-estimate-, Chi-square- and *P*-values, with df=1. Data are from three biological replicates.

Treatment	Infected blood only (Control)	HMBPP-supplemented infected blood (Treatment)
Sample size	47	49
Prevalence *P*-value		*P*=0,006, ***
Chisq value		7,38
Prevalence beta-estimate value	61.70	71.42
Intensity *P*-value		*P*=0,18, ns
Chisq value		1,75
Intensity beta-estimate value	2.57	3.12

A summary of the effect of HMBPP on the feeding and attraction behavior, as well as parasite susceptibility is presented in table 3.14.

Table 3.14. The overall effect of HMBPP on the attraction, feeding rate, oocyst prevalence and intensity of different anopheline mosquitoes. An increase of the relevant behavior is indicated for the control and treated (HMBPP) groups.

	Attraction		**Feeding rate**		**Oocyst prevalence**		**Oocyst intensity**	
	Control	Treated	Control	Treated	Control	Treated	Control	Treated
An. arabiensis		X		X				X
An. gambiae	X			X		X		
An. coluzzii	X			X		X		
An. funestus		X	X			X		
An. merus		X	X			X		
An. quadriannulatus	X			X		X		
An. vaneedeni		X						

CHAPTER FOUR – General Discussion

4.1 Seasonal variation and vector dynamics

4.1.1 Species identification and seasonal variation

Data collections from the same region for the period of January – December 2018 (NICD, 2018) correlates with data from this study. *Anopheles arabiensis* dominated overall collections, with a contribution of 46% towards total samples collected. The latter species also constituted 94% of all data for the *An. gambiae* complex during 2016 but no seasonal data were however presented (NICD, 2016). During the current study, collections for this species was however lower during December; and this could be possibly due to less sampling taking place over the holiday period.

Anopheles parensis was the most abundant species of the *An. funestus* group, with a contribution of 35%. This also correlates with data from 2016 where *An. parensis* contributed to 51% of the *An. funestus* group (NICD, 2016). The latter species also dominated collections (56%) during the late summer months of 2018 (NICD, 2018). Since the relative abundance of *An. arabiensis* was higher in comparison to the other minor- (such as *An. merus* and *An. parensis*) and non-vector (including *An. quadriannulatus*) numbers, it could explain why other species are not flourishing as their numbers are possibly suppressed by competition between the major vector, *An. arabiensis*. Another possible explanation might be that most of the sampling took place in fresh water bodies, where the major vector occurs and since *An. merus* is a salt water species (Sinka et al. 2010), collections of this species might have been limited. Environmental (humidity, rainfall and temperature) and ecological factors (such as breeding site availability, suitable vegetation to provide adequate carbohydrates such as nectar) also determines if species will be present and how abundant they will be at specific sites (Mwangangi et al. 2007; Muturi et al. 2008). The fact that no correlation between the abundance of *An. arabiensis* and rainfall was observed, could possibly be attributed to some of the other except breeding site availability. Run-off water from the Jozini dam which provides drinking water for the village and subsistence farmers, can also provide ample permanent breeding

sources for this species and thereby its abundance could also be sustained during drier months.

Furthermore, this scenario could possibly mimic the one from the Pare district from Tanzania, where the major vector in this area, *An. funestus s.s.*, was abundant during some seasons but as house-spraying started to take place, *An. funestus s.s.* numbers declined drastically and *An. rivulorum* numbers increased dramatically (Gillies & Smith 1960). During the periods in which *An. funestus s.s.* numbers were high, *An. rivulorum* was only present in small numbers (Gillies & Smith 1960). This might lead to the prediction that if *An. arabiensis* numbers are restricted, due to indoor residual spraying (IRS) or environmental factors, species like *An. parensis* or *An. merus* could possibly occupy the niche of the major malaria vector in the area. Data published by Munhenga et al. (2014) showed a rapid increase in the number of *An. quadriannulatus* specimens, after the reduction in *An. arabiensis* numbers during November and December 2012. Although this was observed in one collection site in the Kruger National Park, the change in vector population dynamics is still evident and might possibly be due to environmental factors such as draught or a change in host availability.

Limitations of this study is however that in order to interpret the abundance, the number of mosquitoes collected as well as the collection efforts need to be normalised (Dandalo et al. 2017). This was however not part of the current study as the data belongs to the SIT team and therefore further analysis, using the much larger data set, will be conducted by the team.

4.1.2 Wild caught mosquito infection profiling

Although the circumsporozoite protein (CSP) could not be detected in any of the *An. funestus* group species that were collected during the period of August 2019 to March 2020, Burke et al. (2017) showed high infection rates (2%) in the zoophilic member, *An. vaneedeni* in 2017. This was while the main malaria vector, *An. arabiensis,* showed low infection rates (0.5-0.7%) (Dandalo et al. 2017). Although the sample size of *An. vaneedeni* in this study was low (n=51), their impact on transmission is still unknown and therefore future surveillance and investigation of

this species would be crucial. No comparative ELISA data for the *An. gambiae* complex was however available for this study period.

It could therefore be concluded that mosquito vector dynamics is constantly changing within the *Anopheles* genus and the hypothesis that species would replace each other could thus not be rejected. The once abundant major vector, *An. funestus,* was not collected during this study and is it is therefore a good indication that the malaria control program is effective in preventing the return of this species. As vector dynamics are changing and some species are becoming more abundant, like *An. merus*, it is important to monitor the situation while also taking into consideration that species replacement might be a possibility.

4.2. Parasite metabolite (HMBPP) and mosquito behaviour

4.2.1. Mosquito attraction and HMBPP

The choice chamber evaluation confirmed that the chamber was accessible for the mosquitoes and allowed them to detect different olfactory cues derived from the different sources (blood and sugar water). It also took into account possible positioning effects of the arms and therefore the chamber was eligible to be used for further attraction assays. Future research should investigate if the length of the arms influence the strength of the attraction as well as evaluation of the methodology of introducing the mosquitoes to the chamber.

Increased attraction towards HMBPP-supplemented blood was observed for *An. arabiensis*. This correlates with attraction data from Emami et al. (2017) for *An. gambiae s.l.* Field studies conducted on wild *An. arabiensis* mosquitoes in Ethiopia also showed increased attraction in the presence of HMBPP (Debebe et al. 2020). Results for *An. coluzzii* and *An. gambiae* obtained during the current study, do however not correlate with findings by Emami et al. (2017) since blood without HMBPP was the preferred source in this study. Both minor vectors, *An. merus* and *An. vaneedeni*, showed more attraction towards HMBPP, whereas the non-vector, *An. quadriannulatus* was more attracted towards the control group blood. Comparative data for these species were however not available to date and to my knowledge have not been published. Plasticity in host preference was also found to

exist in mosquitoes, where mosquitoes learn and adapt their preference behaviour in response to experiencing a specific host cue (Takken & Verhulst, 2013).

Attraction to HMBPP-supplemented blood is suggested to be a result of increased emission of volatiles (Emami et al. 2017), including one of the key attractants, CO_2 (Frame et al. 1972; Omondi et al. 2015). An increase in aldehydes C8 to C10 as well as monoterpenes (α- and β-pinene, limonene) has also been found to be a results of the presence of HMBPP, in combination with red blood cells (Emami et al. 2017). The fact that different mosquito species are not attracted to their host in the same way, might be due to different olfactory stimuli. Studies have discovered that more specialist feeders, including *An. gambiae*, had a preference for human-specific volatiles (De Jong & Knols, 1995; Dekker et al. 1998). It was also found that more generalist species, such as the zoophilic *An. quadriannulatus*, is mainly attracted to CO_2 that is present in exhaled human breath (De Jong & Knols, 1995; Dekker et al. 1998). This in turn could explain why HMBPP did not have similar effects on different vector species, since although HMBPP did show an increase in the CO_2 emission, it was only by 16%, compared to an increase of 1.7 to 5.2 factors for aldehydes (Emami et al. 2017).

Future investigations would however be needed to evaluate some contributing factors, such as, the experimental conduction of such attraction assays (choice chambers might be too small), the effect of small sample size on the outcome as well as the evaluation of the colonization effect on species. Colonies that were exposed to selection pressure to adapt to artificial feeding might show different outcomes than natural occurring ones.

4.2.2. Mosquito feeding behaviour in the presence of the parasite metabolite

The significantly higher feeding rate that was observed for all major vectors within the *An. gambiae* complex (including *An. arabiensis, An. coluzzii* and *An. gambiae*), in the presence of HMBPP, correlates with data from Emami et al. (2017). This increase in feeding rate was also observed in the non-vector species, *An. quadriannulatus*. Emami et al. (2017) reported doubled feeding rate in *An. gambiae s.l.* (Keele colony,

which is a mixture of 4 strains) (Ranford-Cartwright et al. 2016) in the presence of HMBPP, in comparison to the control group, thus HMBPP was suggested to be a feeding stimulant. Contrary to this, *An. funestus* and *An. merus* showed a higher feeding rate in the control group. This needs further investigation, however, the different feeding behaviours of these species, low sample size or the impact of colonization might contribute towards these variations observed. Despite the diverse behaviours that were observed for *An. merus* and *An. quadriannulatus* during this study, both these species colonies were reared and maintained through guinea pig feeding. This is in contrast to the other species colonies that have been sustained on cow blood through an artificial feeding system. This might further explain why all members from the *An. gambiae* complex showed an increase in feeding rate, as these colonies were already used to artificial blood feeding and thus possibly more sensitive to detect the presence of HMBPP. This was however not true for *An. funestus*, even though this colony was also maintained on an artificial blood feeding system. As no similar studies were conducted on the major vector, *An. funestus,* and the minor- and non-vector species, *An. merus* and *An. quadriannulatus*, findings in this study could not be compared.

HMBPP thus acted as a feeding stimulant for most of the species evaluated (66%), including major vectors of the *An. gambiae* complex. Contrary to this, *An. funestus* preferred untreated blood.

4.2.3. Dose response evaluation

Findings in the current study for *An. gambiae s.s.* does not correlate with results obtained by Emami et al. (2017) as results in the current study showed a decrease in feeding rate as the HMBPP concentration increased. This is in contrast to their findings, where the feeding rate of *An. gambiae s.l.* increased as the HMBPP concentration increased. This is possibly due to the fact that these are two different strains of *An. gambiae* mosquitoes and most likely have different feeding behaviours due to colonization. The opposite was however true for *An. funestus*, however no comparative data was available for this species. The species specific difference that was found at a concentration of 1 µM could be due to different feeding behaviors of *An. gambiae* and *An. funestus* as their feeding preferences also differed during the feeding rate assays that were conducted at a concentration of 10 µM.

A 10 µM concentration of HMBPP has been found to mimic the concentration of undiluted gametocyte-infected red blood cells, in terms of the amount of volatiles that are released. Therefore observing a higher feeding rate at the lowest concentration, might suggest that the *An. gambiae s.s.* strain that was used for this study, might be stimulated by lower concentrations of HMBPP. As higher dosages of HMBPP is not expected to necessarily increase the amount of CO_2 and other volatiles that are emitted, findings for *An. funestus* is interesting. Further investigations regarding the effect of HMBPP concentration and evaluation of a wider range of concentrations, would be needed for both species as well as others that were not evaluated during this study.

4.3. Mosquito susceptibility

Results obtained from this study, indicates that the major vector, *An. gambiae s.s.,* showed a great significant increase in mean oocyst prevalence, in the presence of HMBPP. The same was true for all major species, except *An. arabienis*. Contrary to this, *An. arabiensis* was the only species that showed a significant increase in the number of oocysts per midgut. Data from Emami et al. (2017) showed an increase in oocyst intensity of *An. gambiae s.l.*, although they did not find a significant difference in oocyst prevalence, with the addition of HMBPP. This is contradictory to findings from the current study as oocyst prevalence was more affected in the presence of HMBPP. This is possibly the result of the difference in the *An. gambiae* strain between the two studies. Since they form part of the same species complex, another possible explanation for this could be attributed to a combination of the up- and down regulation of genes which led to the alteration of gene expression of the modulatory machinery at neural synapses (Emami et al. 2017). It could therefore be that genes might not be regulated in the same way as it is in African *An. gambiae s.s.* mosquitoes - although it was not the objective of this study to determine gene regulation in the presence of HMBPP, it would be worth investigating in future studies. A significant difference was also observed for the other major vector, *An. funestus*, in terms of oocyst prevalence. No comparative data for this species was however available.

Interesting results also arised from this study with regards to the minor vector, *An. merus,* as a great significant increase in *P. falciparum* prevalence was detected, in the presence of HMBPP. Mozambican studies found a steep increase in sporozoite infectivity rate of 4.13% in *An. merus* between 2007 and 2009 (Sharp et al. 2007; Cuamba & Mendis, 2009). In a South African context, data also supports the theory that *An. merus* might become more important as a vector species while the country is aiming to eliminate malaria. It is also possibly indicative that this might be a result of a rise in the relative abundance of this species (Mbokazi et al. 2018) as well as expansion of its geographical range since it has been occupying breeding habitats that was previously occupied by other mosquito species (Govere et al. 2000). *Anopeles quadriannulatus*, the non-vector of the *An. gambiae* complex, has also shown an increase in oocyst prevalence, when HMBPP is involved. Supportive data for this has shown that *An. quadriannulatus* is indeed susceptible to *P. falciparum* (Takken et al. 1999) but the presence of the parasite has never been discovered in wild populations of this species (Coetzee et al. 2000).

Overall form the six species evaluated for P. falciparum susceptibility, 83% (5 out of 6 species) showed an increase in oocyts prevalence when fed on infected blood treated with HMBPP. The major vector, *An. arabiensis,* was the only species that showed an increase in the intensity of infection. These findings will have an impact on malaria transmission if the same is true in a field setting.

CHAPTER FIVE - General Conclusion

This study therefore concludes that feeding- and attraction behaviour, as well as parasite susceptibility of South-African mosquito vector- and non-vector species are affected by the presence of the *P. falciparum* parasite metabolite, HMBPP. The different responses in feeding rate, in the presence of HMBPP, would require further investigation, since even despite the fact that different species have different feeding behaviors, some of them showed similar behaviors in response to HMBPP. This might however be due to the fact that HMBPP alters their behaviour, as it had been reported that plasticity in host preference exists in mosquitoes. Both current major mosquito vectors also showed increased attraction towards the HMBPP metabolite and the same results were observed for the two minor vectors, including the newly incriminated *An. vaneedeni*. It could therefore imply that these vectors might exhibit the same behaviour towards individuals that harbours the *P. falciparum* parasite. In turn this could cause greater concern for vector control strategies. *Plasmodium falciparum* oocyst prevalence were significantly higher in most of the species, which might be an indication that the transmission potential of these different vector species are possibly enhanced by parasite infected blood.

This study would however need to be evaluated using wild caught samples, in order to account for factors such as colonisation effects, the lack of environmental conditions and the loss of genetic traits through rearing mosquitoes under laboratory conditions for long periods. The identification of other attractant compounds that are produced by *Plasmodium* could also be further investigated and evaluated.

CHAPTER SIX – References

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CHAPTER EIGHT – Appendices

Appendix A: Publication

*See supplementary file

Appendix B: Ethics approval

UNIVERSITY OF THE WITWATERSRAND JOHANNESBURG

R14/49 Ms E Erlank

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
CLEARANCE CERTIFICATE NO. M200692

NAME: (Principal Investigator)	Ms E Erlank
DEPARTMENT:	School of Pathology Department of Virology Wits Research Institute for Malaria Medical School University
PROJECT TITLE:	Malaria parasite metabolite and mosquito vector dynamics
DATE CONSIDERED:	Ad hoc
DECISION:	Approved unconditionally
CONDITIONS:	
SUPERVISOR:	Professor L Koekemoer
APPROVED BY:	Dr CB Penny, Chairperson, HREC (Medical)
DATE OF APPROVAL:	2020/07/14

This clearance certificate is valid for 5 years from the date of approval. Extension may be applied for.

DECLARATION OF INVESTIGATORS

To be completed in duplicate and ONE COPY returned to the Research Office Secretary on the 3rd Floor, Phillip Tobias Building, Parktown, University of the Witwatersrand, Johannesburg.
I/we fully understand the conditions under which I am/we are authorized to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated, from the research protocol as approved, I/we undertake to submit details to the Committee. I agree to submit a yearly progress report. When a funder requires annual re-certification, the application date will be one year after the date when the study was initially reviewed. In this case, the study was initially reviewed in June and will therefore reports and re-certification will be due early in the month of June each year. Unreported changes to the application may invalidate the clearance given by the HREC (Medical).

Principal Investigator Signature

15/07/2020
Date

To whom it may concern,

Student : Erica Erlank

Student number : 1785302

Degree : Masters

Study Title : Malaria parasite metabolite and mosquito vector dynamics

Department: Wits Research Institute for Malaria; School of Pathology

Supervisors: Prof Lizette Koekemoer, Dr Noushin Emami

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Full clearance certificate from the AREC of the University of the Witwatersrand is NOT required.

Reason: This study uses mosquitoes (invertebrates).

Comment/Notes : Full ethics application was made – waiver issued.

Appendix C

Table 1: Pearson's correlation values for abundance between different species within the *An. funestus* group.

	An. parensis	*An. leesoni*	*An. vaneedeni*	*An. rivulorum*
An. parensis		r= 0.21 P= 0.60	r= 0.77 P= 0.60	r= -0.06 P= 0.88
An. leesoni	r= 0.21 P= 0.60		r= 0.68 P= 0.06	r= 0.07 P= 0.86
An. vaneedeni	r= 0.77 P= 0.024*	r= 0.68 P= 0.06		r= -0.13 P=0.76
An. rivulorum	r= -0.06 P= 0.88	r= 0.07 P= 0.86	r= 0.68 P= 0.06	