Blood-meal identification and impact of blood feeding on female-specific esterase in the *Anopheles funestus* group

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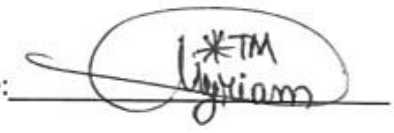
Signature:  Date: 14 June 2022

DEDICATION

I dedicate this work to my family, Mwamba, for their unwavering support!

PUBLICATIONS ARISING FROM THIS DISSERTATION

1) **Mwamba, T.M.**, Dahan-Moss, Y., Munhenga, G., Koekemoer, L.L. 2022. Impact of climatic factors on blood feeding patterns of *Anopheles funestus* group. *Draft in progress.*

   Role: TMM conceived and designed the protocol for the study; TMM conducted all laboratory experiments including optimisation of the blood meal extraction and PCR assays. TMM also analysed and interpreted the data, and wrote the manuscript.

2) Dahan-Moss, Y., Jamesboy, E., Lobb, L., Letinic, B., Zawada, J., Langa, Z., **Mwamba, M.**, Vogel, E., Kaiser, M., Masatola, T. *et al.* 2020. Malaria vector surveillance report, South Africa, January – December 2019. *NICD Public Health Surveillance Bulletin*, 18(1), 62-75. Non-peer reviewed.

   Role: MM conducted laboratory experiments for species of the *An. funestus* group from KwaZulu-Natal, and gathered the data.

3) Dahan-Moss, Y., Jamesboy, E., Gillbert, A., Burke, A., Kaiser, M., **Mwamba, M.**, Ekoka, E., Oliver, S., Braack, L., Munhenga, G. *et al.* 2019. Malaria vector surveillance report, South Africa, January – December 2018. *NICD Public Health Surveillance Bulletin*, 17(1), 47-59. Non-peer reviewed.

   Role: MM conducted laboratory experiments for species of the *An. funestus* group from KwaZulu-Natal, and gathered the data.

PRESENTATIONS ARISING FROM THIS DISSERTATION

1) **Mwamba, T.M**., Dahan-Moss, Y. and Koekemoer, L.L. 15 October 2020. Blood-meal identification of *Anopheles funestus* group (Diptera: Culicidae) from Mamfene, KwaZulu-Natal confirms their zoophilic nature, Wits Research Day.

   Role: TM conceived and designed the protocol for the study; TM conducted all laboratory experiments. TM also analysed and interpreted the data, and wrote the manuscript.

2) **Mwamba, T.M**., Dahan-Moss, Y. and Koekemoer, L.L. 15 October 2019. Blood-meal identification and impact of blood feeding on female-specific esterase in the *Anopheles funestus* group (Diptera: Culicidae), Masters Progress Update, Journal club, NICD.

   Role: TM conducted all laboratory experiments, and complied preliminary results of her masters' project which included species identification, enzyme-linked immunosorbent assay, and optimisation of blood meal PCR.

3) **Mwamba, T.M**. 19 June 2018. Blood feeding biology and behaviour in mosquitoes, VCRL Journal club, NICD.

   Role: TM researched, compiled the presentation, and presented the topic.

ABSTRACT

South Africa is experiencing low-level residual malaria transmission in Limpopo, Mpumalanga, and KwaZulu-Natal. This is partly due to outdoor biting and resting of *Anopheles* species that are not affected by the indoor spraying of insecticides. *Anopheles funestus* of the *An. funestus* group is a major malaria vector in southern Africa, while other species of the group, aside from *An. rivulorum-like*, have been implicated as secondary vectors. It is necessary to investigate the potential role of *An. funestus* group species in malaria transmission in South Africa in order to reduce incidences of malaria. This requires understanding the blood (host) feeding preferences of *Anopheles* species as well as understanding the underlying factors, such as esterase activity, linked with blood feeding biology. Wild caught *An. funestus* group specimens collected from Mamfene area in KwaZulu-Natal were identified to species level using *An. funestus* species-specific polymerase chain reaction (PCR). The presence of *Plasmodium falciparum* in the female specimens was examined using a *P. falciparum* circumsporozoite ELISA. The specific host preference(s) (human, cattle, goat, pig, dog and chicken) of the wild female specimens was studied using blood meal PCR. The impact of climatic factors (temperature, relative humidity and precipitation) on the specimens' host preferences was also analysed. Finally, the link between blood feeding of laboratory colonised *An. funestus s.s.* and its esterases' activity was determined using an isoenzyme electrophoresis assay.

A total of 826 females representing four *An. funestus* group species (*An. leesoni*, *An. parensis*, *An. rivulorum* and *An. vaneedeni*) were identified, with *An. parensis* being the most common species. None of the females were infected with the malaria parasite nor had they fed on humans. These members of the *An. funestus* group showed a clear preference for cattle and climatic parameters did not influence the host feeding preferences. Increase in esterases activity (EST-9, -7, -4, and -1) was observed after blood feeding of laboratory colonised *An. funestus s.s.* females. In conclusion, members of the *An. funestus* group collected from Mamfene, which have previously been implicated as secondary malaria vectors, did not feed on humans and were not infected with *P. falciparum*. This lowers the role of these species in malaria transmission for KwaZulu-Natal. However, entomological surveillance of *Anopheles* species should continue to increase sample size and provide the malaria vector control with scientific evidence to guide future activities. This study also demonstrated that blood feeding influenced specific esterases activity in a major malaria vector *An. funestus.* Further analysis of these esterases' activity and their link with blood feeding is necessary as it could provide essential information regarding the *An. funestus* vectorial capability.

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NOMENCLATURE

ABTS	2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)
ANOVA	Analysis of variance
BB	Blocking buffer
BF	Blood fed
BM	Blood meal
bp	Base pair
CDC	Centres for Disease Control
CI	Confidence interval
CO_2	Carbon dioxide
COVID-19	Coronavirus disease 2019
CSP	Circumsporozoite protein
Cytb	Cytochrome b
DDT	Dichlorodiphenyltrichloroethane
dH_2O	Distilled water
ddH_2O	Double distilled water
DMP	Diagnostic media production
dNTP	Dinucleotide triphosphate
DNA	Deoxyribose Nucleic Acid
EDTA	Ethylenediaminetetraacetic acid
EIR	Entomological inoculation rate
ELISA	Enzyme-linked immunosorbent assay
EST	Esterase
EtOH	Ethanol
G	Gravid
FOR	Forward
FP	Feeding proportion
FUMOZ	*Anopheles funestus* strain from Mozambique
GB	Grinding buffer
Hb	Haemoglobin
HBI	Human blood index
HG	Half gravid
HSD	Honestly significant difference
HT	Head and thorax

IEE	Isoenzyme gel electrophoresis
IRS	Indoor residual spraying
ITS	Internal transcribed spacer region
KZN	KwaZulu-Natal
L	Litre
LOD	Limit of detection
$MgCl_2$	Magnesium chloride
NaOAc	Sodium Acetate anhydrous ($C_2H_3NaO_2$)
NaOH	Sodium hydroxide
NC	Negative control
NEC	Negative extraction control
NF	Non-fed
NICD	National Institute for Communicable Diseases
NIH	National Institute of Health
nm	Nanometre
NTC	No template control
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PBM	Post blood meal
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
Pf	*Plasmodium falciparum*
pH	Potential of hydrogen
QC	Quality control
RBC	Red blood cells
rDNA	ribosomal DNA
REV	Reverse
RNA	Ribonucleic acid
rpm	Revolution per minute
SANDoH	South African National Department of Health
SIT	Sterile insect technique
s.s.	*Sensu stricto*
TAE	Tris(hydroxymethyl) aminomethane-acetate
Ta	Annealing temperature

Taq	*Thermus aquaticus* polymerase
TBE	Tris Boric
TE	Tris-EDTA
Tris-HCl	Tris(hydroxymethyl) aminomethane chloride
µl	Microlitre
µM	Micromolar
UN	Universal
VCRL	Vector Control Reference Laboratory
WHO	World Health Organization
X^2	Chi-square

CHAPTER ONE

General introductory chapter: Literature review

1.1. Global malaria burden

Malaria is a life threatening, vector borne disease caused by five parasite species of the *Plasmodium* genus; *Plasmodium falciparum* and *P. vivax* are the major causative agents (WHO, 2021), with *P. ovale, P. malariae*, and *P. knowlesi* also etiological agents of human malaria (Cox-Singh and Singh, 2008; Singh and Daneshvar, 2013). The parasites are transmitted through the bites of infected mosquito vectors of the *Anopheles* genus (Diptera: Culicidae). Despite malaria being a largely preventable and treatable disease, it remains one of the leading causes of morbidity and mortality in low-income countries (WHO, 2021). Since the turn of the 21st century, the World Health Organization (WHO) has published yearly global estimates of malaria cases and deaths were estimated at 241 million and 627 000 respectively for 2020 (WHO, 2021). Following a two decade reflection on malaria milestones (2000-2020), malaria cases were shown to have decreased by 5.8 % in 2019 compared to the year 2000, and by 7 % compared to 2010 (WHO, 2021), (**fig 1.1A)**. In addition, there was a 37.7 % reduction in malaria deaths in 2019 compared to 2000 (WHO, 2021) (**fig 1.1B)**. However, as evidenced in the 2021 world malaria report for 2020, these advances were reversed in 2020 where there was an increase in both malaria cases and deaths by 5.8 % and 11 % respectively compared to 2019 (WHO, 2021) (**fig 1.1**).

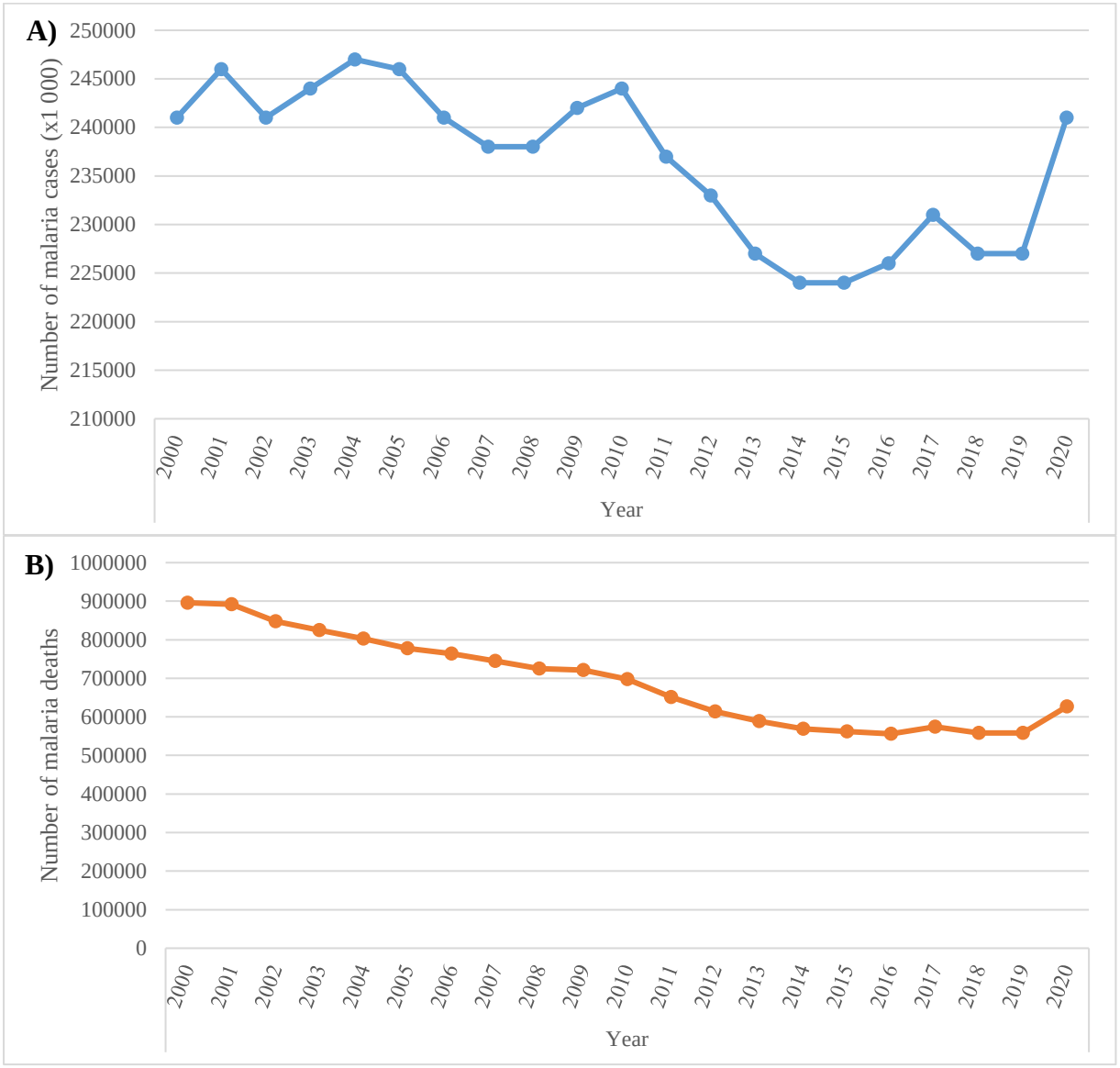

Figure 1.1. Global statistics on malaria 2000-2020. A) Estimated global malaria cases 2000-2020 (x 1 000). **B)** Estimated global malaria deaths. **Citation:** data derived from WHO (2021).

The WHO had already predicted this turn of events, in that an increase in malaria cases would be expected for the 2020 year as a result of the impact of the COVID-19 pandemic on malaria endemic countries (WHO, 2020, 2021). Although it is still early to tell of the full extent of the COVID-19 pandemic impact on malaria cases and deaths, there is some evidence of a negative impact at this stage as seen in the 2021 report. The WHO (2020, 2021) reported that there was a rapid increase of malaria cases globally, at the brink of the COVID-19 pandemic in March 2020, owing to COVID-19 related disruptions of essential malaria services and its associated restrictions. Avenues were successfully explored to mitigate the negative impact of the pandemic in malaria-affected regions (WHO, 2021). These include resolving bottlenecks in the manufacturing of malaria medicines, mitigating disruptions in shipment of these, guiding

responses in countries experiencing disruptions, assisting the completion of malaria prevention campaigns, as well as reducing disruptions of malaria diagnosis and treatment (Brooke *et al.*, 2020; WHO, 2020, 2021).

The African continent continues to bear the brunt of global malaria cases. The estimated number of malaria cases in Africa for the 2020 season was 228 million, accounting for 94.6 % of global malaria cases, and of all malaria deaths that occurred in 2020 (WHO, 2021). Of the malaria cases, 96 % were accounted for by 29 African countries, with Nigeria leading the board at 27 %, followed by the Democratic Republic of Congo (DRC) at 12 % (WHO, 2021). Children under the age of five remain the most vulnerable group, as they accounted for two thirds of all malaria cases (WHO, 2021).

1.2. Malaria epidemiology in South Africa

Based on the 2020 world malaria report (WHO, 2020), South Africa was amongst the few African countries on track to achieve the global technical strategy (GTS) 2020 target, which is defined as a 40 % reduction in malaria morbidity and mortality using a baseline of 2015 (WHO, 2020, 2021). South Africa achieved the GTS 2020 mortality milestone with reduction in mortality rate of 40 % or more, however the country did not meet the GTS 2020 morbidity milestone as a result of increase in the number of malaria cases in 2020 (WHO, 2021). South Africa had achieved 61.6 % reduction in malaria incidence cases in 2019 compared to 2010, however, the country only achieved 37.6 % reduction in malaria cases when compared to 2015 (**fig 1.2)** (WHO, 2021). Malaria in South Africa was also affected by the COVID-19 pandemic, and the continual decrease in malaria cases after the 2017 upsurge was reversed owing to an increase of 1 367 cases (31 %) in 2020 compared to 2019 (**fig 1.2**) (WHO, 2021). To continue encouraging malaria endemic countries towards elimination, the E-2025 initiative was developed on the backdrop of E-2020, and South Africa is amongst 25 E-2025 countries capable of eliminating malaria by 2025 (WHO, 2021). The country also has a nationwide elimination programme to try and meet this target (SANDoH, 2019).

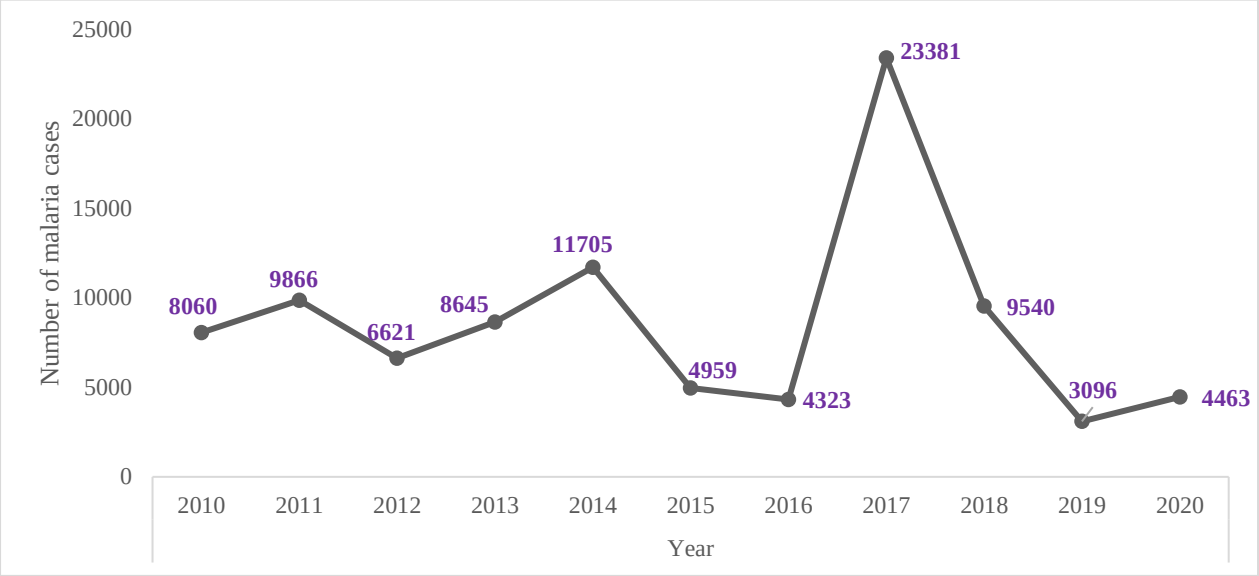

Figure 1.2. Number of indigenous malaria cases in South Africa, 2010-2020. Graph plotted using data from WHO (2021).

Malaria in South Africa is confined to three endemic provinces, Limpopo, Mpumalanga, and KwaZulu-Natal (KZN) (Brooke *et al.*, 2013; SANDoH, 2019; Moonasar *et al.*, 2021). Initially, the country had adopted a malaria elimination agenda in 2012 for its three endemic provinces to eliminate malaria by 2018 (SANDoH, 2012). Since then, the provinces had begun showing a reduction in the number of locally acquired cases. However, the country was unable to reach its elimination target of 2018 and this target was then moved to 2025 (Maharaj *et al.*, 2019; SANDoH, 2019; WHO, 2021). This delay in achieving appropriate targets was due to the national upsurge of malaria cases in 2017 (NICD, 2018; Moonasar *et al.*, 2021; WHO, 2021) (**fig 1.2).**

There is ongoing low-level residual malaria transmission in the three endemic provinces regardless of years of control interventions (Coetzee *et al.*, 2013a; SANDoH, 2019). Two main hypotheses have been devised to explain this phenomenon. The one hypothesis states that the ongoing transmission is sustained by outdoor biting of malaria mosquito vectors that are largely unaffected by indoor vector control interventions (De Meillon *et al.*, 1977; Carnevale and Manguin, 2021). The other hypothesis is that there is an asymptomatic and/or subclinical parasite reservoir in the population, which allows for continual seeding transmission (Young and Hardman, 1948; Greenwood, 1987; Okell *et al.*, 2012). This study focused on the first

hypothesis to determine whether outdoor feeding and resting anophelines, such as members of the *Anopheles funestus* group, contribute to the ongoing transmission in KZN.

The province of KZN, also known as the 'garden province', is the second most populous province of South Africa. It shares borders with three other South African provinces, Eastern Cape, Free State and Mpumalanga. It also shares borders with countries such as Mozambique, Lesotho and Eswatini. The KZN province is typically known for its high humidity, and its coastal regions are subtropical but experiences cooler temperatures towards the inland regions. This province is closest to malaria elimination compared to Limpopo and Mpumalanga, contributing less than 1 % of the national malaria burden since 2005 (Moonasar *et al.*, 2012; Raman *et al.*, 2016, 2020). Kwazulu-Natal is classified as a "very low transmission intensity" zone, with only three of its eight districts endemic for malaria (Raman *et al.*, 2020). Those districts uMkhanyakude, King Cetshwayo, and Zululand, have maintained very low transmission reporting incidence rates of <1 per 1 000 population at risk (Raman *et al.*, 2016; Moonasar *et al.*, 2021), despite the upsurge of malaria cases experienced in 2017. In a more recent study aimed at identifying the factors that are driving and sustaining low-level malaria transmission in KZN, Raman *et al.* (2020) reported a low malaria prevalence of 2 % (67/2 979) amongst asymptomatic individuals, most of which were imported cases. This confirmed that low level of local malaria transmission occurs in the province and is partly contributed by asymptomatic malaria carriers.

Malaria transmission has remained low in KZN as a result of successful vector control methods, mainly indoor residual spraying (IRS), that target indoor resting and feeding mosquitoes such as *An. funestus s.s* (Brooke *et al.*, 2013). This species has been eradicated in South Africa and currently, *An. arabiensis* is the main vector species present in South Africa. A study involving a large and intensive long-term entomological surveillance outdoors at three sentinel sites in KZN, confirmed *An. arabiensis* to be infected with *P. falciparum* and that it plays a role in malaria transmission (Dandalo *et al.*, 2017). This supported the hypothesis that low-level residual malaria transmission occurring in the endemic provinces were caused in part by outdoor feeding and resting anophelines, such as *An. arabiensis* (De Meillon *et al*., 1977; Mouatcho *et al.*, 2009). This hypothesis was further supported when other mainly zoophilic outdoor resting and feeding species such as *An. vaneedeni* and *An. parensis*, members of the *An. funestus* group, were incriminated as potential malaria vectors in KZN (Burke *et al.*, 2017; Burke *et al*., 2019).

1.3. Malaria vector control in South Africa

Anopheles arabiensis, a member of the *An. gambiae* complex, and *An. funestus s.s.* are recognized as major malaria vectors in South Africa (Moonasar *et al.*, 2021). The addition of *An. vaneedeni* and *An. parensis* to the list of secondary vectors requires additional evidence. *Anopheles funestus s.s.*, hereafter referred to as *An. funestus*, was one of the main contributors for the malaria epidemic that occurred in the years 1996 through to 2000 (WHO, 2008; Coetzee *et al.*, 2013a). Vector control is effective at controlling the spread of malaria by reducing the population of mosquito vectors. IRS involves the application of insecticides to the inner walls of houses at certain yearly frequencies, and has been the mainstay vector control method in South Africa for nearly eight decades (Coetzee *et al.*, 2013a; SANDoH, 2019). The programme uses dichloro-diphenyl- trichloroethane (DDT) and pyrethroids (e.g. alpha-cypermethrin and deltamethrin) in a mosaic approach (Brooke *et al.*, 2013; Coetzee *et al.*, 2013a).

Malaria was hyperendemic to large areas of South Africa up until the early 1950's, however, the introduction of DDT for IRS in the mid-1940's, provided an effective vector control strategy which reduced malaria transmission (Coetzee *et al.*, 2013a). Nevertheless, due to concerns surrounding DDT-induced bed bug resistance and persistence of the insecticide in the environment, a policy change was successfully implemented for the substitution of DDT with pyrethroids for IRS in 1995. This, however, led to a sudden exponential increase in malaria cases during the 1999/2000 season, from less than 10 000 cases in 1995, to nearly 70 000 cases in 2000 (Hargreaves *et al.*, 2000; Coetzee *et al.*, 2013a). An entomological investigation was carried out by Hargreaves *et al.* (2000) in northern KZN, along the Mozambican border. The authors reported that 83 % of *An. funestus* collected were resistant to pyrethroids, and 5.4 % of them were infected with *P. falciparum.*

Subsequent to these findings, DDT in combination with pyrethroids, were reintroduced for IRS in 2001 and additional consented efforts were used to eliminate pyrethroid resistant *An. funestus* from South Africa (Coetzee *et al.*, 2013a; Maharaj *et al.*, 2013). IRS remains the focus of vector control in South Africa (Brooke *et al.*, 2020). Evidently, this resulted in the sensible decrease of malaria cases, as well as the effective eradication of *An. funestus* within the borders of South Africa (Coetzee *et al.*, 2013a; Maharaj *et al.*, 2013; Dandalo *et al.*, 2017; Dahan-Moss *et al.*, 2021). The vector control impact has restricted malaria transmission to the most northern-eastern regions of the three endemic provinces and for almost 20 years now, low-level residual malaria

transmission has been a tricky phenomenon in those regions. South Africa sharing borders with malaria endemic countries, such as Mozambique, Zimbabwe and Eswatini, contribute to imported malaria in South Africa, and the continued presence of *An. arabiensis* appear to be a major contributor (Coetzee *et al.*, 2013b; Dandalo *et al.*, 2017; Braack *et al.*, 2020). This until recently, when zoophilic members of the *An. funestus* group, *An. vaneedeni* and *An. parensis*, were newly incriminated as potential malaria vectors (Burke *et al.*, 2017, 2019).

Although vector control strategies have been effective at controlling indoor biting *Anopheles* vectors, this approach is insufficient in low transmission settings where residual transmission is sustained by outdoor biting vectors (Sougoufara *et al*., 2020; Moonasar *et al.*, 2021), such as *An. arabiensis* in South Africa (Dandalo *et al.*, 2017). Hence, there is a need for vector control approaches that target outdoor biting anopheline vectors. Sougoufara *et al.* (2020) provided an in-depth review of a wide range of outdoor vector control tools. Amongst these are larvicides, which have been used selectively as a complementary method to enhance the effect of IRS (Moonasar *et al.*, 2021), seeing that there are no large scale vector control tools for outdoor feeding and resting *Anopheles*. Biolarvicides specifically, utilize toxic proteins naturally produced by soil bacteria, and are therefore safer for the environment (Sougoufara *et al.*, 2020). This tool was not always successful against the malaria epidemic and was shown to be three times more costly than IRS in South Africa (Coetzee *et al.*, 2013a). Significant success in its use has been demonstrated elsewhere such as Tanzania, Kenya and Burkina Faso (Derua *et al.*, 2019).

Other vector control-based approaches include the sterile insect technique (SIT) (Sougoufara *et al*., 2020). This method relies on the mass release of laboratory sterilised male mosquitoes to induce sufficient sterility in wild female mosquitoes which can suppress and/or eliminate a targeted insect population (Klassen and Vreysen, 2021). In South Africa, the SIT programme targets only one particular *Anopheles* vector, that is *An. arabiensis* in KZN (Munhenga *et al.*, 2011, 2016; Kaiser *et al.*, 2021). This programme is still in its development stages. So far, Kaiser *et al*. (2021) have been able to obtain estimates of the population size of *An. arabiensis*, which will be used as a guideline in determining the number of sterilised males of the species that can be used for a SIT pilot trial. However, this approach is lengthy and requires extensive infrastructure that can prove costly (Sougoufara *et al*., 2020; Kaiser *et al.*, 2021). Other approaches have also been proposed. These include the use of attractive toxic sugar baits

(ATSB) and endectocides, which are cost-effective and efficient for large scale implementation (Sougoufara *et al*., 2020). Endectocides involve the use of ivermectin drug treatment, which works to reduce the insect population that feed on humans, and can be used on *Anopheles* to reduce the risk of malaria transmission. Sugar baits are made of attractive toxic compounds, and can be used to mislead mosquitoes by attracting them with fruity sugar solutions that contain toxic compounds to kill them (Müller *et al.*, 2010; Sougoufara *et al*., 2020). Protocols for these two novel methods are under review to determine public health value (Sougoufara *et al*., 2020).

1.4. Biology of haematogenous mosquitoes

1.4.1. Blood sucking insects: classification and biology

Adult mosquitoes feed on plant nectar to obtain carbohydrates necessary for their energy requirement, whilst mosquito larvae feed on yeast, bacteria, protozoa and other plants and microorganisms found in water (Lehane, 2005). The adult females of most mosquito species, including *Anopheles*, require blood meal from which they obtain protein and iron necessary for each egg production (Lehane, 2005; Kim *et al*., 2011). The females can lay between 30-300 eggs at one oviposition depending on the species (Lehane, 2005; Okal *et al.*, 2015; Ngowo *et al.*, 2021), and they ingest more than three times their body mass of blood (Lehane, 2005). Blood feeding, termed haematophagy, is a behaviour exhibited by most arthropod vectors of human pathogens, as it is during blood feeding that pathogens can be transmitted (Lehane, 2005; Kim *et al*., 2011; Hol *et al*., 2020). Blood feeding is divided into three stages, first insertion, where the female inserts the proboscis, their needle-like mouth part, into the skin of the host until it reaches the capillary blood (Lehane, 2005; Hol *et al*., 2020). Then salivation occurs at the site of insertion, with the female mosquitoes injecting anti-coagulants and enzymatic factors, such as anti-thrombin, which numbs that area of the skin. These keep the blood from clotting, resulting in a sub-dermal blood pool with continual probing into the capillaries of the host (Lehane, 2005). This allows for feeding from the liquid blood pool as a final step. As aforementioned, it is during this process that mosquito-borne pathogens are ingested as well as released into the host.

1.4.2. Blood sucking insects: behaviour

It is hypothesized that the blood seeking habit of insects is a result, in part, of a prolonged close association with vertebrates (Lehane, 2005). There are different aspects to blood feeding

behaviour, including host specificity or host choice, feeding periodicity, feeding and resting location, as well as host attraction (Lehane, 2005). Host choice is defined as species of host animal(s) from which blood-sucking insects obtain their blood meals (Lehane, 2005; Chaves *et al.*, 2010). Many mosquitoes are host-specific: anthropophilic mosquitoes feed almost exclusively on humans, whilst zoophilic mosquitoes mainly feed on other animal vertebrates (Lehane, 2005). Some mosquitoes can exhibit a combination of both behaviours. When it comes to feeding and resting locations, insects that feed indoors or outdoors are endophilic and exophilic respectively, whilst resting indoors or outdoors are termed endophilic or exophilic respectively (Lehane, 2005; Chaves *et al.*, 2010). It is this blood feeding requirement that makes certain insects, vectors of several diseases, examples include: Dengue disease caused by dengue virus and transmitted by *Aedes* species, West Nile fever caused by West Nile virus (WNV) transmitted by *Culex* species, and *Anopheles s*pecies that transmit the *Plasmodium* parasites that cause malaria (Jupp, 2005; Lehane, 2005). Species of the *An. funestus* group are traditionally and historically characterized as zoophilic and exophilic (Gillies and Coetzee, 1987), with the exception of the cryptic species *An. funestus*, characterized as anthropophilic and endophilic. However, because species of the *An. funestus* group have behavioural plasticity, they adapt depending on host availability and other environmental factors.

Feeding periodicity refers to certain times of a 24-hour cycle during which blood feeding occurs (Lehane, 2005). *Anopheles* species are nocturnal, feeding between sunset and sunrise (Lehane, 2005). Mosquitoes' ability to find a host and feed is driven by a factor known as host attraction: mosquitoes are able to detect their host using a combination of sensual, olfactory and thermal cues (Lehane, 2005; Batista *et al*., 2014; Emami *et al.*, 2017). Examples include visually spotting a prey, smelling CO_2 and other body attractants such as lactic acid and ammonia compounds enriched in human odour, as well as thermal detection of body heat (Dekker *et al*., 2005; Lehane, 2005; Batista *et al*., 2014; Emami *et al.*, 2017; Wooding *et al.*, 2020). Body odour has been of particular interest, especially in persons already infected with malaria parasites. Emami *et al.* (2017) determined that a precursor called (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMBPP), which parasites require for growth, was the factor that caused changes in odour profiles of malaria infected hosts. This caused increased host attraction to mosquitoes, resulting in subsequent blood feeding by mosquitoes. This *Plasmodium* precursor has also been recorded to stimulate feeding in main mosquito vectors, *An. arabiensis* and *An. gambiae s.s.* (Stromsky *et al.*, 2021).

1.5. The *Anopheles funestus* group

1.5.1. Species composition

In Sub-Saharan Africa, the most important vectors for malaria transmission include: *An. arabiensis* Patton, *An. coluzzii*, *An. gambiae* from the *An. gambiae* complex, as well as *An. funestus* from the *An. funestus* group (Gillies and De Meillon, 1968; Gillies and Coetzee, 1987; Coetzee *et al.*, 2013b; Coetzee, 2020). Although *An. funestus* is one of the most important human malaria vectors in Africa, it is understudied compared to *An. gambiae*.

The *An. funestus* group contains eleven recognized African sibling species: *An. funestus*, *An. leesoni*, *An. rivulorum*, *An. vaneedeni*, *An. parensis*, *An. brucei*, *An. aruni*, *An. fuscivenosus*, *An. confusus* (**fig 1.3. A-C**) (Gillies and De Meillon, 1968; Coetzee *et al.*, 2013b; Derua *et al.*, 2015; Coetzee, 2020), *An. rivulorum-like* first discovered in Cameroon (Cohuet *et al.*, 2003), and *An. funestus-like* discovered in Malawi in 2009 (**fig 1.3. C**) (Spillings *et al.*, 2009). *Anopheles funestus* is the predominant vector species of the group. It is highly anthropophilic and endophilic, and transmits *P. falciparum* more efficiently than other species within this group (Coetzee and Fontenille, 2004; Coetzee and Koekemoer, 2013; Vezenegho *et al.*, 2013; Derua *et al.*, 2015; Ogola *et al.*, 2018; Braack *et al.*, 2020). Those characteristics make it a highly efficient malaria vector. Other species of the group are predominantly zoophilic and prefer to feed on vertebrates other than humans (Coetzee and Fontenille, 2004; Temu *et al.*, 2007; Kawada *et al.*, 2012; Vezenegho *et al.*, 2013).

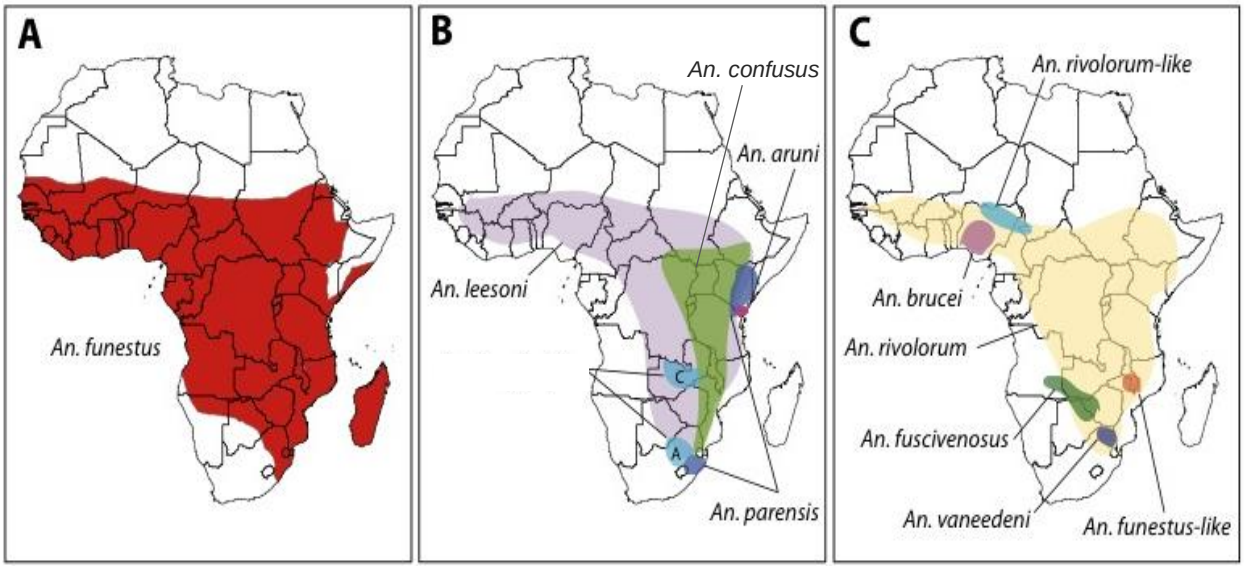

Figure 1.3. African maps with distribution of the 11 sibling species of the *An. funestus* group. **A:** distribution of *An. funestus*; **B:** distribution of *An. leesoni*, *A. parensis*, *An. aruni*, and *An. confusus*; **C:** distribution of *An. brucei*, *An. rivulorum*, *An. fuscivenosus*, *An. vaneedeni*, *An. funestus-like*, and *An. rivulorum-like*. **Citation:** Map adapted from Dia *et al*. (2013).

1.5.2. Biology and distribution of the *An. funestus* group

There is limited knowledge about the biology and ecology of the *An. funestus* group when compared to *An. gambiae c*omplex. Less is also known about the importance of *An. funestus* group's biology in relation to insecticide resistance which can negatively impact the effectiveness of malaria vector control strategies, or challenge the development of new and/or alternative control methods (Dahan-Moss and Koekemoer, 2016; Ngowo *et al.*, 2021). The knowledge gap has been largely attributed to the difficulties in colonizing *An. funestus* strains (Coetzee and Koekemoer, 2013; Ngowo *et al.*, 2021). The behavioural plasticity in resting and feeding patterns of species of the *An. funestus* group, allow them to adapt and survive in a broad range of environments. Members of the *An. funestus* group also vary in their ecology, feeding and resting biology. Focus on the common members of the *An. funestus* group found in South Africa is elaborated on below.

Anopheles funestus has a widespread distribution covering the entire sub-Saharan African region (**fig 1.3A**). As mentioned, it is highly anthropophilic and endophilic (Coetzee and Fontenille, 2004; Vezenegho *et al.*, 2013; Derua *et al.*, 2015; Ogola *et al.*, 2018; Braack *et al.*, 2020; Mburu *et al*., 2021). However, it has also been found to rest outdoors (Mulamba *et al.*, 2014; Debrah *et*

al., 2021). The species breeds in environments with semi-permanent, or natural/man-made permanent water bodies, in rice fields, or emerging vegetation (Gillies and De Meillon, 1968; Dia *et al*., 2013; Debrah *et al.*, 2021). The limiting factors to the development of *An. funestus* include extreme temperatures and heavy rain, the latter is known to wash out breeding sites (Dia *et al*., 2013).

Anopheles leesoni also exhibits a wide distribution across sub-Saharan Africa, but mostly localized in the savannah region of eastern and western Africa (**fig 1.3B**). The species has been incriminated as a secondary vector in Tanzania, east Africa (Temu *et al.*, 2007; Kweka *et al.*, 2008). The species is predominant outdoors (Awolola *et al.*, 2005), but it has also shown to have an endophilic tendency that allows them to feed on humans indoors (Temu *et al.*, 2007; Derua *et al.*, 2015). Temu *et al*. (2007) found that the 29.5 % of this species (26/88) had tested positive for *P. falciparum* by nested PCR, with eight of them having fed on humans indoors. The larvae of *An. leesoni* can be found in association with those of *An. funestus* at the edges of slow flowing streams (Gillies and De Meillon, 1968; Dia *et al*., 2013).

Anopheles parensis' distribution is mainly limited to East Africa (**fig 1.3B**) (Dia *et al*., 2013), and include Tanzania (Temu *et al.*, 2007; Kweka *et al.*, 2008; Derua *et al.*, 2015), South Africa (Gillies and De Meillon, 1968; Mouatcho *et al.*, 2007; Burke *et al.*, 2017, 2019), Kenya (Muturi *et al.*, 2009), Uganda (Mulamba *et al.*, 2014), and more recently in Malawi (Mburu *et al.*, 2021). The species is mainly exophilic and zoophilic (Muturi *et al.*, 2009; Mulamba *et al.*, 2014). They are the most common species of the *An. funestus* group found outdoors in KZN, South Africa (Burke *et al.*, 2017, 2019; Dahan-Moss *et al.*, 2021). Some population of *An. parensis* show a strong inclination to rest indoors (Kamau *et al.*, 2003; Mouatcho *et al.*, 2007; Temu *et al.*, 2007; Muturi *et al.*, 2009; Derua *et al.*, 2015; Mburu *et al.*, 2021). Those studies failed to detect *P. falciparum* in *An. parensis* except Temu *et al.* (2007) in Tanzania, who found one *An. parensis* resting indoors that was *P. falciparum* positive using nested PCR. The species was also found to have an anthropophilic tendency when indoors (Muturi *et al.*, 2009). *Anopheles parensis* in South Africa was recently found infected with *P. falciparum*, and confirmed by sequencing (Burke *et al.*, 2019). This means that it is a potential malaria vector. The larvae of *An. parensis* develop in permanent water bodies such as swamps and ponds between reeds and emergent vegetation (Gillies and De Meillon, 1968; Dia *et al*., 2013).

Anopheles rivulorum exhibits a wide distribution but mostly focalized in east and west Africa (**fig 1.3C**). The species has been incriminated as a potential malaria vector in Tanzania and Kenya (Wilkes *et al*., 1996; Temu *et al.*, 2007; Kawada *et al.*, 2012). It is considered mainly exophilic and zoophilic species (Gillies and De Meillon, 1968; Awolola *et al.*, 2005; Dia *et al*., 2013), however, *An. rivulorum* was shown to strongly feed on humans in other settings when collected indoors (Kweka *et al.*, 2008; Debrah *et al.*, 2021). The larvae of *An. rivulorum* can be found in the same water bodies as those of *An. funestus* (Dia *et al*., 2013; Debrah *et al.*, 2021).

Since its first discovery in Cameroon (**fig 1.3C**), *An. rivulorum-like* has also been reported in other countries including, but not limited to, Zambia (Norris and Norris, 2015), South Africa (Mouatcho *et al.*, 2018; Braack *et al.*, 2020), Cameroon again (Elanga-Ndille *et al.*, 2019), Malawi (Mburu *et al*., 2021), and Mali (Wragge *et al.*, 2021). *Anopheles rivulorum-like* is exophilic in South Africa, and appears to be restricted to the province of Limpopo (Mouatcho *et al.*, 2018; Braack *et al.*, 2020; Dahan-Moss *et al.*, 2021). The species also appears to have a strong endophilic tendency (Mouatcho *et al.*, 2018; Elanga-Ndille *et al.*, 2019). There is little evidence on their host preference. One *An. rivulorum-like* collected indoor in Malawi had fed on goat blood, implying a zoophilic tendency (Mburu *et al.*, 2021). There is currently no information about the breeding site(s) of *An. rivulorum-like*.

Anopheles vaneedeni is an outdoor biting species, mostly recorded from South Africa (Gillies and De Meillon, 1968; De Meillon *et al.*, 1977; Burke *et al.*, 2017, 2019; Dahan-Moss *et al.*, 2021). Additional records of *An. vaneedeni* have only been reported from Kenya (Kweka *et al.*, 2013; Ogola *et al.*, 2018). The species has been caught feeding on humans outdoors (De Meillon *et al.*, 1977). It was newly found naturally infected with *P. falciparum* circumsporozoite protein (CSP) in South Africa (Burke *et al.*, 2017). There is currently no study that have produced evidence of host preferences of this species. Their larval habitats are not extensively studied but do not seem to differ from those of *An. funestus* (Gillies and Coetzee, 1987; Dia *et al*., 2013).

1.6. Factors affecting the ecology of *Anopheles* species

As with any other infectious diseases, malaria transmission is well elucidated when applying the epidemiological triad, involving the human host, the agent *Plasmodium*, and the environment,

with *Anopheles* vectors as vehicles. The environmental factors are largely classified into climatic and non-climatic factors (Craig *et al.*, 2004a, 2004b; Kumar and Reddy, 2014).

1.6.1. Non-climatic factors

Although malaria transmission and its epidemics are mainly driven by climatic determinants, "many non-climatic factors may alter or override the effect of climate" (Craig *et al.*, 2004a; Craig *et al.*, 2004b; Kumar and Reddy, 2014). Non-climatic factors include but are not limited to anti-malarial drug resistance, suppressed host immunity, cross-border migration, especially between malaria endemic and non-malaria endemic regions, as well as insecticide resistance (Craig, *et al.*, 2004b). Whilst the former two factors mainly affect the biology of *Plasmodium* species, the latter two mostly affect the ecology of *Anopheles* vectors (Craig *et al.*, 2004b; Lehane, 2005). Other known non-climatic factors include the malaria vectors and availability of hosts (Kumar and Reddy, 2014). Factors such as cross-border migration, drug resistance, and insecticide resistance were listed as possible causes of the drastic increase of malaria in the years 1999/2000 in South Africa. Resistance to deltamethrin was demonstrated to be the main driver for *An. funestus* to reinvade South Africa after its elimination from the country in the 1950's (Hargreaves *et al.*, 2000; Coetzee *et al.*, 2013a; Maharaj *et al.*, 2013). Imported malaria due to cross-border movement from highly endemic neighbouring countries to South Africa, was identified as one of the contributors towards the upsurge in malaria cases for the 2016/2017 malaria season (Maharaj *et al.*, 2019; Raman *et al.*, 2020; Moonasar *et al.*, 2021). Most of the malaria cases in KZN for the 2016/2017 period were imported (Moonasar *et al.*, 2021). Reduction in IRS was also identified as a contributing factor. Moonasar *et al*. (2021) reported that the number of structures targeted for IRS in Limpopo had decreased from ~ 1.4 million during the 2015/2016 malaria season to <1 million in the 2016/2017 malaria season. Braack *et al.* (2020) had also reported the collection of one *An. funestus* outdoors in Limpopo. The authors suggested that the species may have been imported. However, there may have been more since the success of collections depends on methods employed and on the aim of the surveillance. Hence, non-climatic factors are also of importance in malaria transmission and epidemics.

1.6.2. Climatic factors

Climatic factors are the major limiting environmental factors that play an important role in malaria epidemics, especially considering that malaria transmission occurs during distinct

seasons. Additionally, *Plasmodium spp* and the *Anopheles* that transmit them are strongly affected by climatic determinants. Temperature affects the development of both parasite and vector (Craig *et al.*, 2004a; Kumar and Reddy, 2014). Rainfall allows formation of breeding sites for mosquito vectors, whilst temperature plus humidity play a role in mosquito behaviour, survival and proliferation (Craig *et al.*, 2004a; Dia *et al*., 2013; Kumar and Reddy, 2014). Altogether, these three climatic factors are crucial for various biological processes of both *Plasmodium* parasites and anopheline vectors (Craig *et al.*, 2004a; Kumar and Reddy, 2014). Environmental pressures such as drought and increase in ambient temperature may impact mosquito host-seeking behaviour for blood meal (Gatton *et al.*, 2013; Stone *et al.*, 2016). For instance, *Anopheles* mosquitoes can shift from feeding on cattle outdoors to feeding on humans more readily (Mouatcho *et al.*, 2007; Temu *et al.*, 2007; Kweka *et al.*, 2008; Kawada *et al.*, 2012). This is because drought can result in loss of cattle from which those mosquitoes normally obtain their blood meal (Abbas *et al*., 2019; Ndlovu and Demlie, 2020). This could lead mosquitoes to rather feed on human hosts outdoors, resulting in the persistence of low-level residual malaria transmission.

Blood feeding frequency is directly proportional to temperature. Subsequent to the effect of temperature on mosquito feeding frequency, the time between blood meal digestion, egg development, oviposition and re-feeding gets reduced at high temperatures, thereby allowing more feeding at shorter intervals (Lee *et al*., 2013; Walton and Reisen, 2014; Shapiro *et al*., 2017; Agyekum *et al*., 2021). Examples of this interplay include the study by Christiansen-Jucht *et al.* (2015), in which they found that the number of laid eggs by adult *An. gambiae* decreased with increased temperature, this is because at higher temperature the oviposition time becomes shorter resulting in low number of eggs laid. Additionally, the biting/feeding rates of *An. stephensi* doubled when temperature was increased from 21 °C to 32 °C (Shapiro *et al*., 2017). By this, one can imply that if *Anopheles* species that feed on humans bite and feed more with increasing temperature, malaria transmission increases. This explains why malaria is seasonal, mostly occurring during the warmer seasons of summer than in colder seasons of winter, especially in South Africa (Raman *et al.*, 2016; Ikeda *et al.*, 2017). However, as stated above, temperature interacts with other climatic factors to influence mosquito development and blood feeding behaviour. Studies have shown that the survival of mosquitoes is prolonged at high humidity, preferably 60-70 % (Yamana and Eltahir, 2013; Kumar and Reddy, 2014; Drakou *et al.*, 2020). The right amount of rainfall is required for the formation of breeding sites for mosquitoes (Yamana and Eltahir, 2013; Kumar and Reddy, 2014). The additive effect of these

climatic variables not only increases mosquitoes' life span, but also promotes increase in population size, and increased chances of seeking blood meal to develop more eggs. This can subsequently affect malaria transmission dynamics.

Further study into other branches of biology need to advance as well. Dahan-Moss and Koekemoer (2016) highlighted that a better understanding of the reproductive, behavioural and ecological characteristics of malaria vectors, such as *An. funestus*, will inform better application of vector control strategies. Enzyme expression mediate the underlying physiological mechanisms that govern the afore mentioned characteristics. Examples of biological processes mediated by enzymes include mating, and post-blood meal (PBM) mechanisms. There are two 20E-regulated chymotrypsin-like serine proteases expressed in *An. gambiae* females, which make them more susceptible to mating (Bascuñán *et al.*, 2020).

Generally, during blood feeding, haematogenous insects release a combination of anticoagulating, antiplatelet and vasolidator compounds from their salivary glands into the wound to prevent blood clotting, thereby resulting in a subdermal pool of blood from which they feed on (Lehane, 2005; Rizzo *et al.*, 2011). The salivary gland protein 6 (SG6) aids in effective blood feeding in both *An. gambiae s.s*. and *An. funestus* (Lombardo *et al.*, 2009; Rizzo *et al.*, 2011). An orthologue of this protein has also been detected in *An. stephensi* (Valenzuela *et al.*, 2003). Enzymes can also be found in the gut, such as trypsin and esterases, implying that they may play a digestive role for blood meal and non-blood meals (Lehane, 2005), other roles of esterases have been described.

1.7. Classification and role of esterases in mosquitoes

Seven classes of enzymes exist, including isomerases, translocases, lyases, ligases, transferases, oxidoreductases, and hydrolases (de Souza Vandenberghe *et al.*, 2020). Esterases, which belong to the class of hydrolases, are composed of subclasses including lipases, peptidases, phosphatases, glycosidases, and nucleosidases (Montella *et al*., 2012; de Souza Vandenberghe *et al.*, 2020). Esterases are of special interest in insects as they are linked to several biological processes including reproduction (Freyvogel *et al*., 1968; Lu *et al.*, 2012; Rawal *et al.*, 2016), insecticide resistance (Devonshire, 1991; Montella *et al*., 2012; Weedall *et al.*, 2020), and behaviour such as blood feeding including its digestion (Freyvogel *et al*., 1968; Lehane, 2005;

Marinotti *et al.*, 2006; Cui *et al.*, 2020). Esterases are subsequently grouped as either alpha esterases for hydrolysing alpha-naphthyl acetate, or as beta esterases based on their ability to hydrolyze beta-naphthyl acetate (Williams, 1985; Montella *et al.*, 2012). Based on the inhibition of esterases by certain substrates (some of which are used in insecticides), esterases are further classified as either cholinesterases (as well as acetylcholinesterase), carboxylesterases, arylesterases, acetylesterases (Williams, 1985). Cholinesterase activity is inhibited by organophosphates and serine sulphate; carboxylesterase activity is inhibited by organophosphates; arylesterase activity is inhibited by sulphydryl reagents and acetylesterases are not inhibited by any of those substrates (Freyvogel *et al*., 1968; Montella *et al*., 2012).

Esterases are of particular importance because of their role in mediating resistance to insecticides that are used for vector control programmes. Five common classes of insecticides are used to control malaria vectors; they include organochlorines, pyrethroids, carbamates and organophosphates, and neonicotinoids (Nauen, 2007; WHO, 2021). These insecticides work by targeting the central nervous system of insects, and extensive use of these is widely known to result in insecticide resistance (WHO, 2020). Of the four types of insecticide resistance mechanisms, metabolic resistance is a major mechanism widely shared across all four groups of insecticides (Montella *et al*., 2012), and it is characterized by rapid insecticide detoxification due to change in mosquito's enzyme systems (Devonshire, 1991; WHO, 2020). This occurs as a result of over-expression or mutation that increases the expression of detoxifying enzymes, namely glutathione S-transferases (GSTs), cytochrome P450s (P450s), and esterases such as carboxylesterases (COEs) (Hemingway and Ranson, 2000; Liu, 2015). Metabolic resistance mechanism by esterases is most common because most of the insecticides used, contain ester bonds (e.g.DDT, pyrethroids, malathion) of substituted phosphoric, carbamic or cyclocarboxylic acids (Devonshire, 1991; Montella *et al*., 2012). Those insecticides are consequently subject to degradation by esterase enzymes.

Additionally, studies have shown that certain mosquito behaviour contribute to the insecticide resistance phenotypes of *Anopheles*. An important behaviour is the blood feeding requirement of the females. Spillings *et al*. (2008), as well as Oliver and Brooke (2014) showed that a blood meal enhanced insecticide resistance in already resistant strains of *An. funestus* and *An. arabiensis* respectively. The same was not observed in susceptible strains. In addition, Oliver and Brooke (2014) showed that multiple blood feeding increased the longevity of mated and

unmated pyrethroid-resistant *An. arabiensis* females, as well as that of the unmated susceptible females, thereby giving them a competitive advantage. Against this background, female mosquitoes were shown to be more resistant to insecticides than males (Oliver and Brooke, 2014). It is possible that this phenomenon is mediated by underlying biochemical processes initiated by a blood meal. Hence, there could be some female-specific esterase(s) that contribute to insecticide resistance. Specific roles of esterases and how they are affected by blood feeding, are explored in chapter four.

1.8. Rationale of the study

Following the implementation of enhanced vector surveillance systems in the Mpumalanga and KZN provinces, Burke *et al*. (2017) reported on two *An. vaneedeni* females, one from each of the two provinces (KZN (n=1/51) and Mpumalanga (n=1/41), infected with *P. falciparum* sporozoites. This provided the first evidence of natural populations of *An. vaneedeni* infected with *P. falciparum*. This species is historically recognized as being exophilic and zoophilic and so at the time, it posed a threat to the South African malaria elimination agenda that was initially set for 2018 but now pushed to 2025 (SANDoH, 2019; Moonasar *et al.*, 2021; WHO, 2021). Following that, one *An. parensis* female collected from Mamfene was also infected with *P. falciparum* (n=1/154) (Burke *et al.*, 2019). Both species are considered potential secondary malaria vectors, contributing to the very low transmission intensity status that is KZN. Further investigation would be required to provide additional evidence of the vectorial capacity of both *An. vaneedeni* and *An. parensis*.

For an *Anopheles* species to be considered a malaria vector, it has to meet certain criteria. Amongst others, the detection of *Plasmodium* in the salivary glands of the *Anopheles* species, the intrinsic incubation period of *Plasmodium*, as well as host preferences specifically the isolation of human DNA from its blood meal (Kent and Norris, 2005; Ghosh *et al.*, 2009; Gunathilaka *et al.*, 2016). Hence, this study endeavoured to establish the range of vertebrate hosts of outdoor seeking species of the *An. funestus* group, as well as their *Plasmodium* infectivity in Mamfene, KZN. Furthermore, the study investigated if there was a change in host-choice, and whether the ongoing drought in KZN, in the period 2014-2017, possibly resulted in a change in the mosquito's host preference. Understanding the role played by climatic factors in determining mosquito host preferences could provide information to help develop more efficient

tools and other control measures to better combat malaria, and possibly other mosquito-borne diseases.

To further understand the biology of female *An. funestus*, this study also investigated the effect of blood meal on the activity of EST-2. This esterase was identified as an arylesterase, and a female-specific esterase, by Dahan-Moss and Koekemoer in 2016. As a female-specific esterases, there are a few roles it may play, including aiding blood feeding, in blood meal digestion, in development of eggs, and/or in insecticide resistance. This will provide baseline information for future projects and shedding more knowledge of *An. funestus* female biology.

1.9. Research aim and objectives

The main aim of this study was to determine the range of host preferences of outdoor resting *An. funestus* group species in the period 2014-2019 in the KwaZulu-Natal (KZN) province that is nearing malaria elimination, and determine their vector status. To link the vector-host interaction, the range of vertebrate hosts was determined for these outdoor resting *Anopheles* species. How the different climatic factors, temperature, relative humidity and rainfall affect the feeding proportions was also investigated. Finally, the impact of a blood meal on the activity of *An. funestus* female-specific esterase was investigated. Ethical clearance was obtained for this study from the animal research ethics committee (AREC) (**Appendix 1A & 1B**).

1.9.1. Specific Objectives:

1) Species-specific identification of archived and newly collected wild *An. funestus* group samples from KZN (2014-2019), followed by vector incrimination and determination of their blood meal source(s).

2) Determining the impact of climate data on host feeding preferences of blood fed species of the *An. funestus* group.

3) Investigate the impact of a blood meal on the activity of a female-specific esterase, EST-2, in *An. funestus*.

CHAPTER TWO

Species composition and *Plasmodium falciparum* detection in the *Anopheles funestus group* from Mamfene, KZN

2.1. Introduction

2.1.1. Identification of species of the *An. funestus* group

Identification of *Anopheles* mosquitoes has been historically performed based on morphological keys as it is a tool to triage between the different groups or complexes of mosquito species. However, species within the *An. funestus* group have similar adult morphology and thus, this tool cannot discriminate between the cryptic species of the group (Gillies and De Meillon, 1968; Gillies and Coetzee, 1987; Spillings *et al.*, 2009). As such, novel methods with higher discriminatory power have been explored such as DNA-based molecular methods first published in 1999 (Koekemoer *et al.*, 1999). Additional molecular methods have aided in differentiating between the members of the *An. funestus* group (Hackett *et al.*, 2000; Koekemoer *et al.*, 2002; Cohuet *et al.*, 2003). Identification of *An. funestus* by polymerase chain reaction (PCR) is based on interspecies variations in the internal transcribed region 2 (*ITS2*) region of the ribosomal DNA (rDNA) locus. The rDNA is characterised as being represented in multiple copies and contains regions with highly variable regions (**fig 2.1)** (Paskewitz and Collins, 1997).

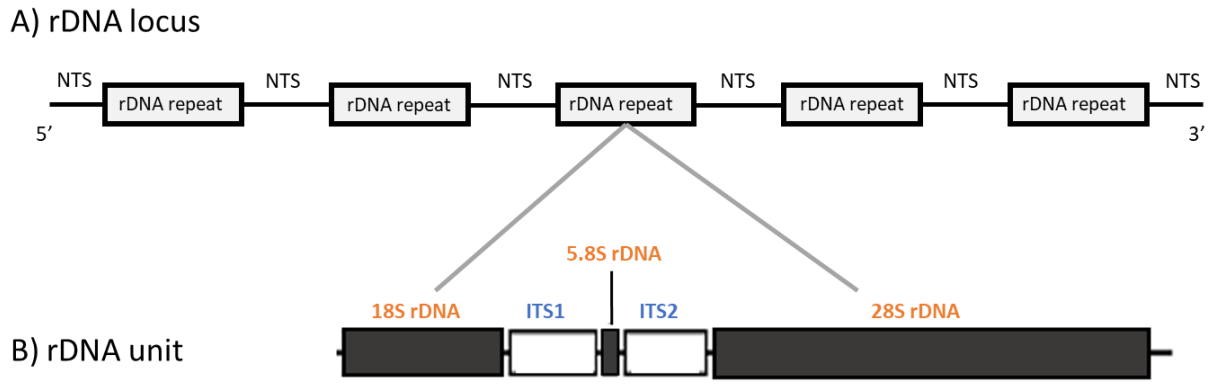

Figure 2.1. Schematic diagram of a transcription unit of the ribosomal DNA. A) Tandems of the rDNA locus separated by non-transcribed spacer region (NTS). **B)** An rDNA unit comprised of 18S rDNA, 5.8S, and 28S rDNA genes, separated by two internal transcribed spacers (*ITS*). Citation: adapted from (Paskewitz and Collins, 1997).

Although the *An. funestus* group comprises 11 African recognized species, the rDNA PCR method initially developed by Koekemoer *et al.* (2002) focused on identifying the five most common species of the *An. funestus* group. These species include, *An. funestus sensu stricto*, *An. vaneedeni*, *An. rivulorum*, *An. leesoni*, and *An. parensis*. However, Hackett *et al.* (2000) noted that "*An. rivulorum"* occurring in Burkina Faso (western Africa) and *An. rivulorum* occurring in Kenya and South Africa (eastern and southern Africa) showed *ITS2* sequence variation of approximately 19 %, implying the existence of a cryptic species. Cohuet *et al.* (2003) discovered a considerable divergence between *An. rivulorum* from Cameroon, compared to the ones from South Africa. "*Anopheles rivulorum*" from Cameroon were confirmed as new cryptic species and provisionally named *An. rivulorum-like*. Cohuet *et al.* (2003) designed *An. rivulorum-like* species-specific primer and integrated it into the multiplex cocktail PCR that was developed by Koekemoer *et al.* (2002). Using this assay, *An. rivulorum-like* has since been reported from other countries including Zambia (Norris and Norris, 2015), South Africa (Mouatcho *et al.*, 2018; Braack *et al.*, 2020), Cameroon (Elanga-Ndille *et al.*, 2019), Malawi (Mburu *et al*., 2021), and Mali (Wragge *et al.*, 2021).

Identification of *Anopheles* to the species level is also a vital initial step when determining *Plasmodium* infectivity in *Anopheles* vectors. This is to avoid incriminating the wrong species as a vector, should it be infected with *Plasmodium* parasites. It is important to start the identification prior to detecting *Plasmodium* circumsporozoite protein (CSP) in the salivary glands of female anophelines. Once *Plasmodium* CSP is detected in a specific *Anopheles* species, *ITS2* PCR followed by sequencing is performed, to confirm the *Anopheles* species identity (Durnez *et al.*, 2011; Burke *et al.*, 2017, 2019; Dahan-Moss *et al.*, 2020a). Dahan-Moss *et al.* (2020a) found that several species of the *An. gambiae* complex, including the major vector *An. arabiensis*, amplified as *An. leesoni* when processed using the *An. funestus* multiplex PCR assay. This raises concern on reports that have identified *An. leesoni* as a vector such as the study by Temu *et al.* (2007) in Tanzania. The authors reported that of the 88 *An. leesoni* that were identified by PCR, 26 of them (29.5 %) were infected with *P. falciparum*. However, the investigators did not follow up with sequencing of *An. leesoni* and the *P. falciparum* they were infected with to confirm their identity. Based on the finding by Dahan-Moss *et al.* (2020a), it is possible that *An. leesoni* identified by Temu *et al*. (2007) could have been misidentified, but without sequencing PCR this remains unclear. Dahan-Moss *et al.* (2020a) emphasized on the importance of using morphological keys as a first step in species identification before progressing to molecular methods. Furthermore, sequencing of the *ITS2* is strongly

recommended to confirm identification of abnormal or unexpected molecular results (Dahan-Moss *et al.*, 2020a), as sequencing provides the highest discriminatory power.

2.1.2. Determining *Plasmodium* infectivity rate

The enzyme-linked immunosorbent assay (ELISA) method is mostly used for determining sporozoite rate, which is the proportion of *Plasmodium*-infected anophelines. It is also used to estimate the entomological inoculation rate (EIR), an important key indicator for estimating malaria transmission (Durnez *et al.*, 2011). The ELISA method is widely used, but numerous studies have noted that it can produce false positive results (Lochouarn and Fontenille, 1999; Sylla *et al*., 2000; Koekemoer *et al.*, 2001; Mouatcho *et al.*, 2007; Abraham *et al*., 2017; Hendershot *et al.*, 2021). One convincing hypothesis devised to explain the false positive ELISA is that, in the case where a female mosquito had taken a blood meal, some antigens in the plasma of those hosts can cross-react with the *Plasmodium*-specific monoclonal antibody (mAb) (Somboon *et al*., 1993; Lochouarn and Fontenille, 1999). These cross-reacting antigens appear to only be present in the head and thorax of mosquitoes that are mainly zoophilic (Durnez *et al.*, 2011). However, those cross-reacting antigen factors are heat unstable, thus can be destroyed by boiling at 100 °C for 10 minutes, whilst *Plasmodium* CSP are heat-stable (Durnez *et al.*, 2011). Hence, Durnez *et al*. (2011) recommended that all positive ELISA homogenates, be re-run by adding the boiling step prior to hybridization. This would result in false positive ELISA results switching to negative ELISA results, whilst true positive ELISA results would remain positive. To further reduce the chances of a false positive ELISA result, all positive ELISA specimens should be subsequently subjected to a *P. falciparum*-specific nested PCR followed by sequencing (Snounou *et al.*, 1993). In summary, confirming *Plasmodium* infectivity should follow a two-step ELISA method followed by a nested PCR specific for detecting *Plasmodium* sporozoites.

2.2. Objective

The overall objective of this study was to determine the species composition of *An. funestus* group and their vectorial capacity to establish their role in the residual malaria transmission occurring in KwaZulu-Natal (KZN), South Africa.

2.2.1. Specific objectives

1. To identify members of the *An. funestus* group that occur outdoors in the Mamfene, KZN.
2. To detect the presence of *P. falciparum* circumsporozoite protein (CSP) in the salivary glands of identified females.

2.3. Materials and methods

2.3.1. Study site and experimental design

Study Site

Wild *An. funestus* species used in this study were collected from the Mamfene locality in the northern part of KwaZulu-Natal province of South Africa (Dandalo *et al.*, 2017). The area forms part of the Jozini local municipality of the uMkhanyakude District in the province. Three of the ten sentinel sites in that area were selected, where an intense sterile insect technique (SIT) project is underway: Section 2 (S 27°24'14.2"; E 032° 12'41.8"), Section 8 (S 27°27'34.3"; E 032°10'43.7") and Section 9 (S 27°23'50.5"; E 032° 12'20.1") (**Fig. 2.2.**).

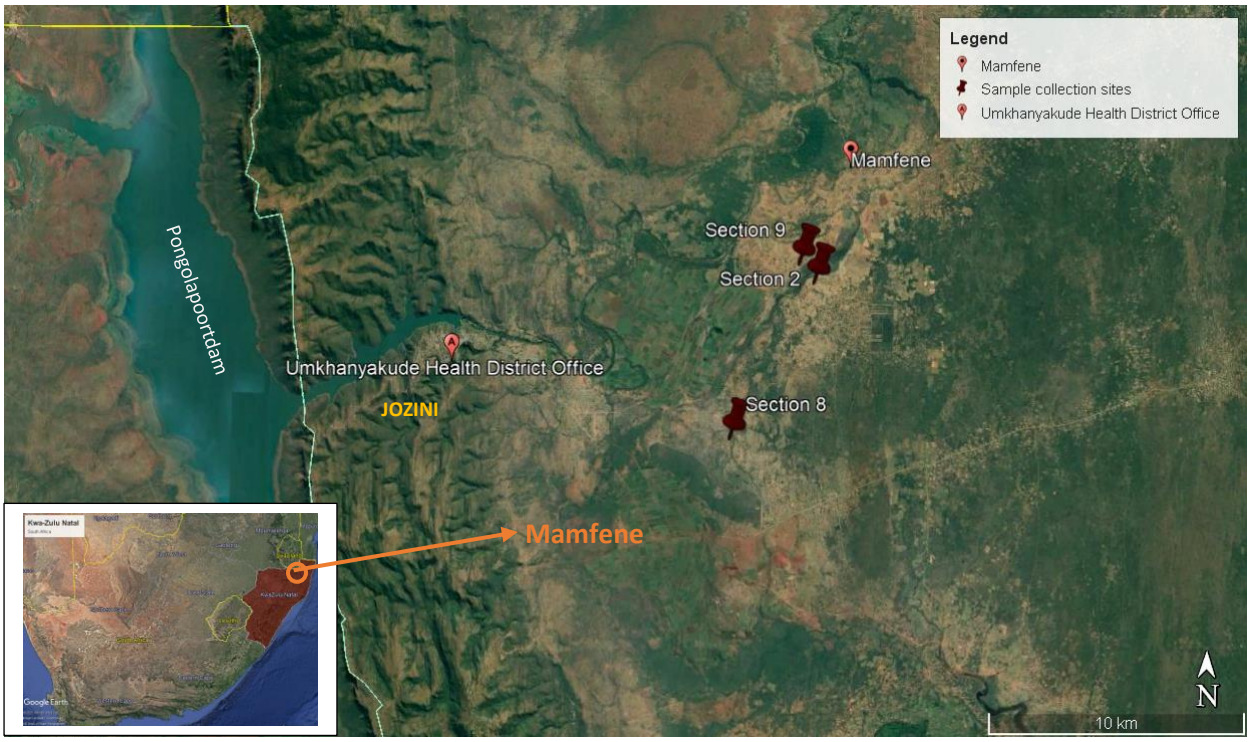

Figure 2.2. Map of Mamfene, area in northern KwaZulu-Natal, South Africa, showing three mosquito sampling sites: Sections 2, 8 and 9. Map designed on Google earth Pro (v7.3.3).

Biological material

Wild caught An. funestus group: Archived specimens and newly collected female *An. funestus* mosquitoes were analysed for vertebrate blood-meal preferences. Wild materials for the period 1 January 2014 – 31 December 2016 were already processed in previous surveillance activities by the SIT team, whilst wild materials from 1 May 2017 – 30 June 2019 were processed in this study. For archived data collection (2014 – 2016), the Vector Control Reference Laboratory (VCRL) museum database was used to recover records on species identification, *P. falciparum* ELISA results and blood meal status. Museum storage locations recorded on the database were used to recover all females to confirm blood meal status and process them accordingly. Additionally, all females in archived collection, with missing species identification or *P. falciparum* ELISA results were also recovered and processed.

Adult *Anopheles* mosquito insects were collected outdoors twice a week every month (i.e. eight times per month) for both collections periods mentioned above. Various collection methods were used, such as clay pots, carbon dioxide traps, outside buckets as well as *Anopheles* found resting near a cattle kraal, and on tyres at the sentinel sites. After collection, mosquitoes were transported to entomological laboratories near the sentinel sites in Mamfene. The mosquitoes were immobilised and euthanised using 70 % chloroform, to allow for morphological identification to genus level, using dichotomous keys (Gillies and Coetzee, 1987; Coetzee, 2020). All mosquitoes identified as *An. funestus* group species were used in this study. They were preserved in tubes containing silica gel, which were transported to VCRL at the National Institute for Communicable Diseases (NICD), for molecular processing.

Colonised An. funestus: Positive controls for the PCR assays were obtained from insectary reared *An. funestus* (FUMOZ) housed in the Botha de Meillon Insectary at the NICD. This colony was established in the year 2000 using wild-caught *An. funestus* material from southern Mozambique (Hunt *et al.*, 2005). The colony is maintained under the following environment: 80 % humidity, a constant temperature of 25 °C $\pm$ 2 °C, 12-hour day-night photoperiod, and 45 minutes' dusk-dawn cycle. Adult females are fed *ad libitum* with 10 % sugar water solution but replaced twice weekly with fresh solution, and blood-fed with guinea pigs twice weekly. *Anopheles leesoni*, *An. parensis*, *An. rivulorum*, and *An. vaneedeni* controls were obtained from previous surveillance activities done.

2.3.2. DNA extraction for species identification

Deoxyribose nucleic acid (DNA) for species identification was extracted from one leg of individual mosquito using the prepGEM® Insect kit (**Appendix 2A**, including supplier details), according to the manufacturer's instructions with slight modification in that a quarter of the recommended volumes for the reagents were used per DNA extraction. Each 10 µl mastermix consisted of 10x extraction buffer, *prep*GEM enzyme, made up to 10 µl with deionized water (**Appendix 2A**). Individual legs were then submerged in the extraction mix and incubated in a thermocycler at 75 °C for 15 minutes and 95 °C for 5 minutes. Control specimens: *An. funestus*, *An. leesoni*, *An. parensis*, *An. rivulorum*, and *An. vaneedeni* were extracted using the same protocol. *An. rivulorum-like* control was not available. One negative extraction control (NEC), i.e. 1 µl deionized water, was added to detect potential contamination during the extraction stage.

2.3.3. Species-specific identification of *An. funestus* group by multiplex PCR

Anopheles funestus-specific multiplex PCR was performed to distinguish between the six target species as described (Koekemoer *et al.*, 2002; Cohuet *et al.*, 2003). These species were amplified using unique reverse primers, and a universal forward primer (UV), to amplify their uniquely sized fragments (**Table 2.1**).

Table 2.1. Primer sequences for *An. funestus*-specific multiplex PCR (Koekemoer *et al.*, 2002; Cohuet *et al.*, 2003)

Primers	Sequence (5' ⟶ 3')	Tm (°C)	Species Identity	Amplicon size (bp)
UV	TGT GAA CTG CAG GAC ACA T	55.3	-	-
FUN	GCA TCG ATG GGT TAA TCA TG	52.4	*An. funestus*	505
VAN	TGT CGA CTT GGT AGC CGA AC	58.0	*An. vaneedeni*	587
RIV	CAA GCC GTT CGA CCC TGA TT	58.8	*An. rivulorum*	411
PAR	TGC GGT CCC AAG CTA GGT TC	60.5	*An. parensis*	252
LEES	TAC ACG GGC GCC ATG TAG TT	60.2	*An. leesoni*	146
RIVLIKE	CCG CCT CCC GTG GAG TGG GGG	60.7	*An. rivulorum-like*	313

Each 12.5 µl PCR mastermix consisted of 10x buffer (100 mM Tris-HCl (pH 8.3), 500 mM KCl), 200 µM of each dNTP, 1.5 mM $MgCl_2$, 0.266 pmol of each primer, and 0.04 units of the

thermostable *Taq* DNA polymerase (**Appendix 2B,** including supplier details). The following cycling conditions were used for the reaction: initial hot start at 94 °C for 2 minutes, followed by 30 cycles of (denaturation at 94 °C for 30 seconds, annealing at 45 °C for 30 seconds, and elongation at 72 °C for 40 seconds), and final extension at 72 °C for 5 minutes. All five positive controls from extraction were included, as well as two negative controls, one was the negative extraction control (NEC) and the second was a no template control for PCR (NTC).

Successful amplification was confirmed by resolving 12.5 µl PCR product mixed with 4 µl O'Range 6x Loading dye (**Appendix 2C**) on 2.5 % ethidium bromide-stained agarose gel (**Appendix 2C,** including supplier details). The products as well as a 100 bp DNA ladder were electrophoresed at 120V, 400 mA for ~100 minutes. The products on the gel were then viewed in the Molecular Imager® ChemiDoc™XRS^{+} and analysed using Image Lab™ Software (Cat No: 1708265, Bio-Rad, South Africa). For a PCR to be considered valid, the following quality control (QC) was fulfilled: both the negative extraction control (NEC) and the PCR no template control (NTC) should not have any amplicon present. At least three of the five positive controls inclusive of the *An. funestus,* should amplify, producing amplicons at their respective fragment sizes (**table 2.1)**.

2.3.4. Enzyme-linked immunosorbent assay for the detection of *P. falciparum* sporozoites

The heads and thoraces of all females from the *An. funestus* group were tested for the presence of *P. falciparum* CSP using an indirect sandwich enzyme-linked immunosorbent assay (ELISA) method as described, with slight modifications (Wirtz *et al.*, 1987; Durnez *et al.*, 2011) (**fig 2.3).**

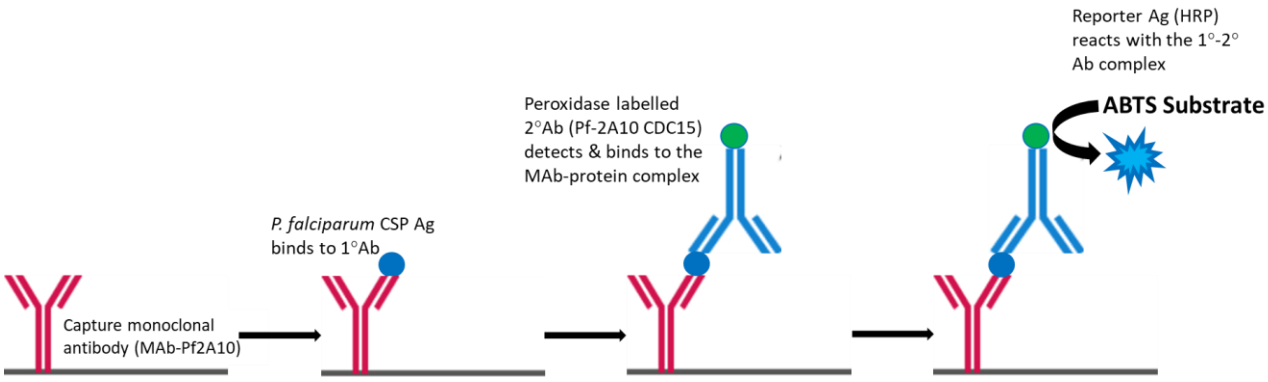

Figure 2.3. Principle and laboratory procedure of sandwich enzyme-linked immunosorbent assay (ELISA) for detection of *P. falciparum* circumsporozoite protein (*Pf*CSP). Adapted from the protocol provided in Methods in *Anopheles* research (Benedict, 2015).

Reagents were prepared a day or two before each experiment. Buffers, including grinding buffer (GB), blocking buffer (BB), phosphate buffered saline (PBS), PBS-Tween, and all necessary reagents used for ELISA were prepared as described in **Appendix 2D** (including supplier details). The following reagents were obtained through BEI resources, NIAID, NIH: *Plasmodium falciparum* Sporozoite ELISA Reagent Kit, MRA-890, contributed by Robert A. Wirtz. The primary antibody was a capture monoclonal antibody prepared in PBS (0.200 µg/50µl/well). This primary antibody mix was then added to each well. The plate was covered with foil (to prevent drying) and stored overnight at 4 °C to allow for coating of the plate. Samples and controls were homogenised in 50 µl grinding buffer (GB) (0.5 % w/v Casein, 0.05 % Igpal/NP-40, 0.1 % w/v phenol red, in 10 mM PBS, pH 7.4) (**Appendix 2D**), in the QIAGEN® TissueLyser II (Cat No: 85300, Qiagen, South Africa) using one 5 mm stainless bead in each sample at 25 Hz for 4 minutes. After homogenisation, 150 µl BB (0.5 % Casein and 0.1N NaOH in 10mM PBS, pH 7.4, plus 0.1 % phenol red was added to each tube to make up the volume to 200 µl. Homogenised samples were also kept in the freezer (-20 °C) until processing the next day.

After 24 hours, the blocking buffer was dispensed over the plate and incubated at room temperature for 1 hr. This was to block the areas of the microwell unbound by the primary antibody, to prevent non-specific binding of the secondary antibody. The blocking buffer was then discarded and excess liquid was blotted off on a paper towel. Then 50 µl of the controls and each sample was added into the wells and incubated at room temperature for 2 hours. The positive control used (2 pg/µl), was a synthetic peptide *P. falciparum* circumsporozoite protein (Pf-PC positive control, MRA-890, BEI resources), whilst seven adult insectary-reared females that fed on sugar-water were used as negative controls (**fig 2.4)**.

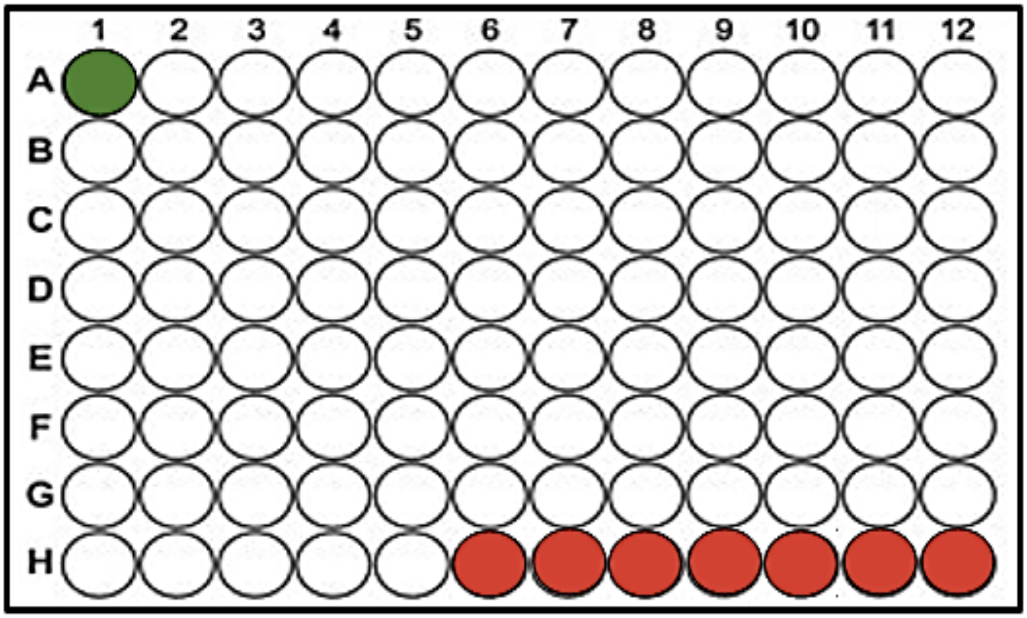

Figure 2.4. A 96-well plate set-up for *P. falciparum* ELISA. Well A1: positive control (synthetic peptide). Wells H6-H12: Negative controls (NCs), 7 uninfected insectary female samples.

The samples and controls were incubated for 2 hours at room temperature. Afterwards, the plate was manually washed twice, using PBS-Tween solution (10 mM PBS, 0.05 % Tween 20) (**Appendix 2D**). Following this, each well of the plate was coated with 50 µl of the secondary antibody mix which included 10 µl peroxidase labelled antibody (Pf-2A10 CDC15, Cat No: 37-00-24-4: KPL) per 5.0 mL blocking buffer for a 96-well plate). After a 1 hr incubation at room temperature, the plate was washed four times with the PBS-Tween solution. Finally, 100 µl of 2,2'-azino-biz-(3-ethylbenzothiazoline-6-sulphonic acid (ABTS 1) peroxidase substrate was added to each well and incubated at room temperature for 30-60 minutes, and used to detect the antibody-antigen complex resulting in a colour change.

The ELISA results were interpreted by determining the optical density (OD) in each well measured at 405 nm absorbance, using a 96-well microtitre plate reader, a SpectraMax® ABS Plus and SoftMax® Pro 7 data acquisition and analysis software (Cat No.: 76266-086, Seperations, South Africa). Analysis from the software were used to interpret significance of the optical density of each sample.

For each ELISA, a critical cut-off value was calculated as follows:

$$\frac{\sum(405 \text{ nm of} -\text{ve controls})}{n} \text{ x } 2,$$

defined as two times the average of optical density of the negative controls (Beier *et al.*, 1990), with n being the number of negative controls used, which is seven. For analysis of ELISA results, any given assay was considered valid if the OD value of the positive control was above the calculated cut-off value. Every tested sample with a value above the calculated critical cut-off value was considered positive for *P. falciparum* CSP.

The ELISA homogenates of all *P. falciparum*-positive samples were then processed a second time as described above with the exception that the homogenates were heated to 100 °C for 10 minutes prior to hybridization to the primary antibody in the second round (Durnez *et al.*, 2011). The heating step was performed to test the stability of the antigen being tested, because the assay is known to bind to the CSP in the salivary glands as well as in other mosquito tissues (Durnez *et al.*, 2011). Samples that were positive a second time were considered true positives and were further processed by a *P. falciparum* nested-PCR.

2.3.5. Phenol-chloroform DNA extraction

Further confirmation of the presence of *P. falciparum* in the *An. funestus* group samples was necessary to exclude false positive results. The phenol-chloroform DNA extraction method was used to extract DNA from ELISA homogenates that were positive for the presence of *P. falciparum* CSP. The protocol by Barker *et al.* (1998) was employed with slight modifications. First the remaining volume of homogenates (100 µl) was made up to 200 µl using 1x TE (elution) buffer (10 mM Tris-HCl, pH 8.5). Then an equal volume of phenol:chloroform:isoamyl (PCI) alcohol solution (25:24:1) (**Appendix 2E**), pH 8.0, was added to the homogenate and centrifuged at 13 000 rpm for 5 minutes resulting in a three-phase formation of upper aqueous phase containing DNA, an interface phase containing protein, and a bottom oily phase containing phenol/chloroform. Approximately 180 µl of the aqueous phase containing DNA was transferred to a second tube.

The phenol/chloroform extraction was repeated in the first tube to retrieve more DNA, by adding 200 µl of 1x TE buffer. The mixture was then vortexed vigorously and centrifuged at 13 000 rpm for 5 minutes. The supernatant was then added to a second tube. In the second tube, an equal volume of chloroform:isoamyl (24:1) (**Appendix 2E**) was added, and the tube was

vortexed for 1 minute and centrifuged for 3 minutes at 13 000 rpm. The mixture was again divided into three phases and the top supernatant/aqueous phase was transferred into a third tube, where 10 µl 3M sodium acetate (NaOAc, pH 5.2) was added for every 50 µl DNA (in the aqueous phase) to precipitate DNA out of solution. The solution was mixed well, and 2.5x volume of absolute/100 % ethanol (EtOH) was added (2.5x EtOH x (10 µl NaOAc x 50µl supernatant)), then incubated at 20 °C overnight.

The following day, the ethanol solution was centrifuged at 13 000 rpm for 20 minutes at 4 °C, in order to collect the precipitated DNA. The alcohol supernatant was decanted without disturbing the pellet of DNA. The pellet was washed by adding 300 µl of 70 % EtOH, vortexed three times, and centrifuged at 13 000 rpm for 15 minutes at 4 °C. The supernatant was decanted and the wash with 70% EtOH was repeated. Residual EtOH was removed gently. The pellet was air dried at room temperature for 10 minutes, and finally 30 µl of 1x TE buffer was added to dissolve and resuspend the pellet of DNA. The extracted DNA was processed immediately or stored at -20 °C.

2.3.6. *Plasmodium falciparum*-nested PCR

A nested PCR was used to confirm the presence of *P. falciparum* parasite in DNA extracted from homogenates with positive ELISA results, using the nested PCR for *P. falciparum* described by Snounou *et al.* (1993). The PCR detection is based on the small subunit ribosomal RNA (ssrRNA) genes of *Plasmodium* parasites. It is a two-stage PCR in which the primary amplification cycle involves detection of the *Plasmodium* genus using genus-primers rPLU5 and rPLU6 (Macrogen, Europe) (**table 2.2)**. A total volume of 12.5 µl PCR mastermix was used, each containing 10x buffer (100 mM Tris-HCl (pH 8.3), 500 mM KCl), 200 µM of each dNTP, 1.5 mM $MgCl_2$, 2 µM of each primer, and 0.04 units of the thermostable *Taq* DNA polymerase (**Appendix 2E**). The cycling conditions were: an initial hot start at 95 °C for 5 minutes, followed by 25 cycles of (58 °C for 2 minutes, 72°C for 2 minutes, and 94 °C for 1 minutes), and a final extension at 72 °C for 5 minutes. An NEC and NTC were included in every PCR assay. An aliquot from the primary PCR (1 µl) was used as a DNA template for the secondary PCR using primers specific for *P. falciparum*; rFAL 1 and rFAL 2 (Macrogen, Europe) (**table 2.2)**. PCR products were resolved on 2.5 % agarose gel and visualized as in section 2.3.3. The *P. falciparum* amplification product has a uniquely sized fragment of 205 bp.

Table 2.2. Nested-PCR primer sequences for *P. falciparum*-specific identification (Snounou *et al.*, 1993)

Organism	Primer name	Oligonucleotide sequence (5'-3')	Target gene	Amplicon size (bp)
Plasmodium genus	rPLU5	CCTGTTGTTGCCTTAAACTTC	ssrRNA	1200
Plasmodium genus	rPLU6	TTAAAATTGTTGCAGTTAAAACG	ssrRNA	
P. falciparum	rFAL 1	CATCGGCACAAATTTAGTCG	ssrRNA	205
P. falciparum	rFAL 2	CCTAATCTTAGTACTTGTACCCTTCCTC	ssrRNA	

ssrRNA: small subunit ribosomal RNA

2.3.7. Data analysis

All analyses were performed in STATISTICA version 13.0.17 (TIBCO®) to obtain summary of descriptive analysis. Species abundance and composition were determined by the number of each positively identified species over the total number of *An. funestus* specimens identified. Sporozoite infectivity rate was determined by the proportion of *P. falciparum* CSP-positive *An. funestus* females, of the total number of tested females.

2.4. Results

2.4.1. Species composition of the *An. funestus* group

Successful amplification of an unknown specimen was classified as either: *An. funestus s.s.* if amplified at 505 bp; *An. leesoni* (146bp); *An. parensis* (252 bp); *An. rivulorum-like* (313 bp); *An. rivulorum* (411 bp); or *An. vaneedeni* (587 bp)**. Figure 2.5** is an illustrated example of PCR amplicons obtained. A total number of 1 137 wild caught females, collected over the study period (2014-2019), were morphologically identified as belonging to *An. funestus* group, and of these, a total of 826 specimens (72.6 %) were successfully identified to species level by PCR (**fig 2.6 & 2.7)**. The remaining 311 (27.4 %) *Anopheles* were most likely misidentified morphologically, and could belong to other *Anopheles* groups/complexes. The unidentified samples may have also been due to incorrect storage, or not stored shortly after death, which may have degraded DNA.

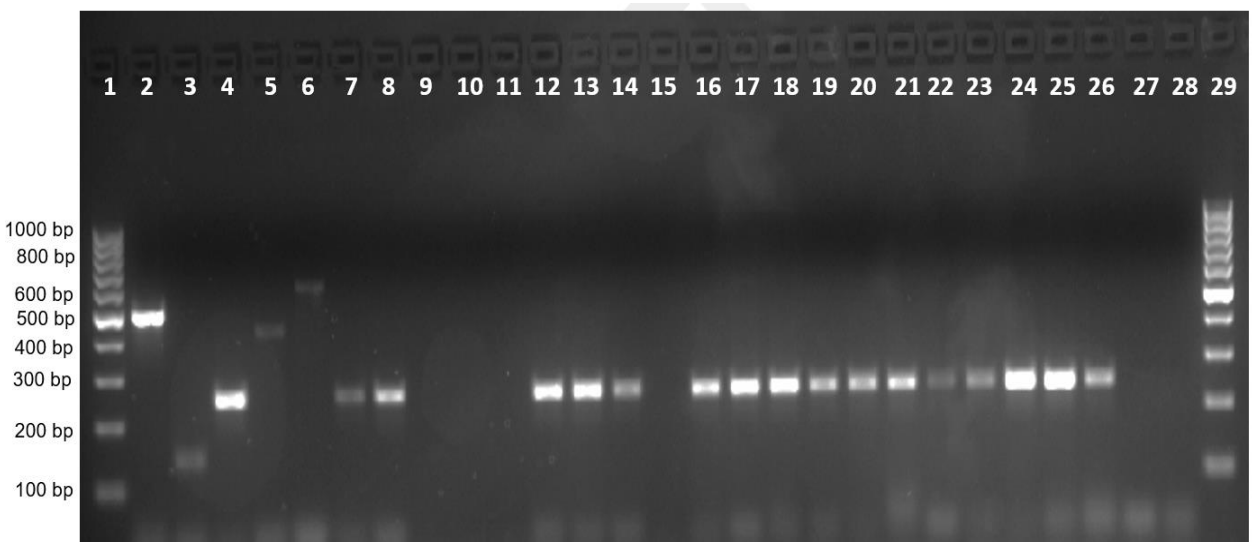

Figure 2.5. *Anopheles funestus* species-specific PCR amplicons, gel electrophoresed on a 2.5 % ethidium bromide-stained agarose gel. Lanes 1 and 29: DNA ladder (O'Gene ruler 100 bp). Lanes 2-6: positive controls for *An. funestus s.s*. = 505 bp, *An. leesoni* = 146 bp, *An. parensis* = 252 bp, *An. rivulorum* = 411 bp, and *An. vaneedeni*= 587 bp. Lanes 7, 8, 12-14, 16-26: specimens identified as *An. parensis*. Lanes 9-11: No amplicon. Lanes 27 & 28: NEC and NTC respectively.

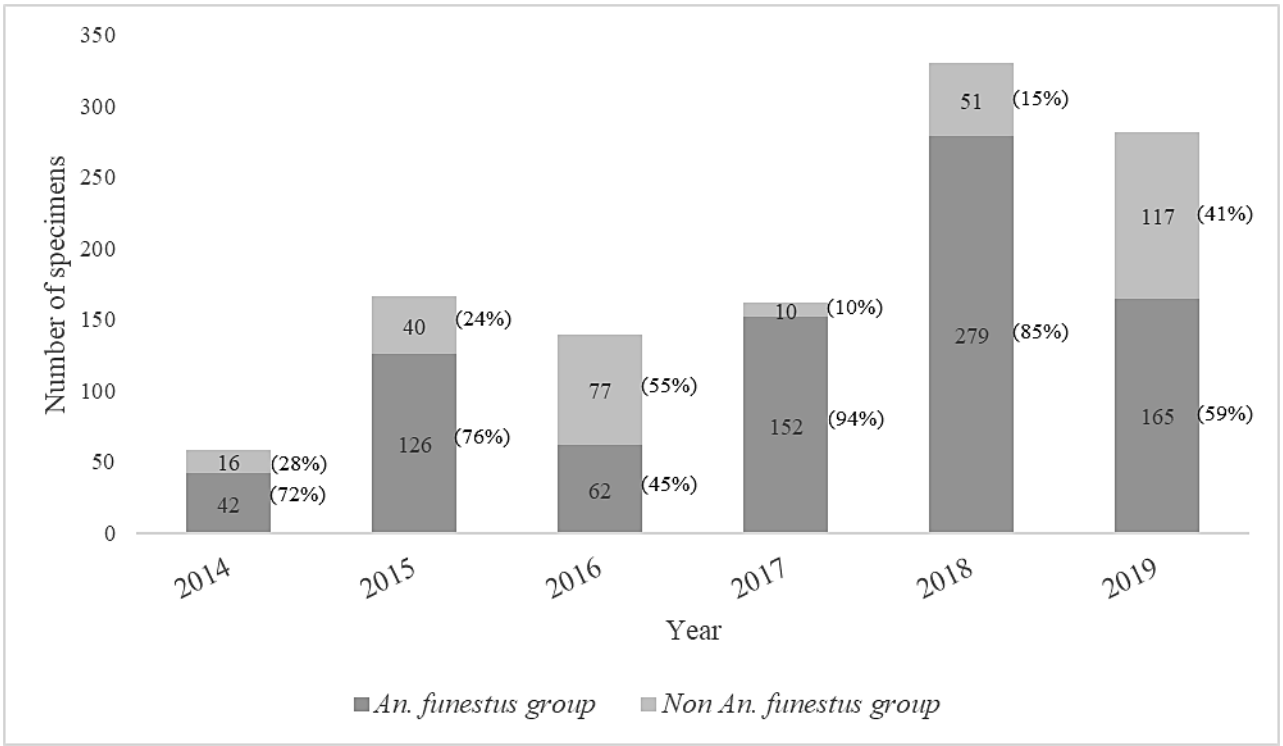

Figure 2.6. Number PCR identified *An. funestus* group specimens (n, %), in the total wild caught population of *An. funestus* group from Mamfene, KZN (2014-2019).

Of the six species targeted in this study, four species of the *An. funestus* group were successfully identified from the outdoor collections **(fig 2.7)**. An overview of the results shows that *An. parensis* is the most abundant of the four species identified in this study (n=447, 54.1 %), followed by *An. vaneedeni* (n=161, 19.5 %), *An. rivulorum* (n=141, 17.1 %), and *An. leesoni* being the least abundant (n=77, 9.3 %). Furthermore, *An. parensis* was the most abundant of the species during time period of 2014-2019, except in 2016, where *An. rivulorum* was the most abundant *An. funestus* group species (n= 31, 50 %), **(fig 2.7)**. Throughout the study period, spanning 2014-2019, *An. funestus* and *An. rivulorum-like* were not detected.

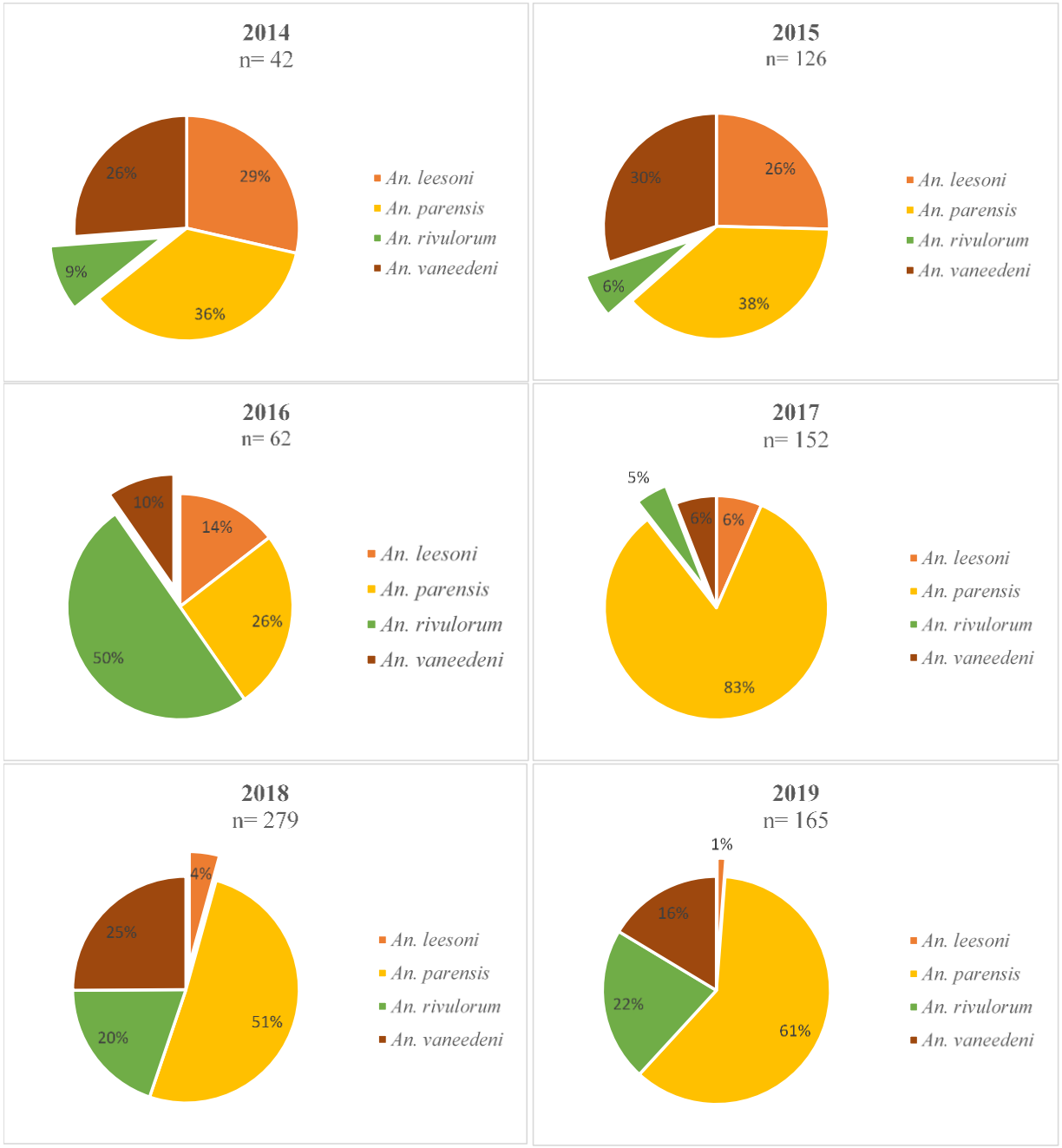

Figure 2.7. Relative proportions of member species of the *An. funestus* group collected outdoors in Mamfene, KZN. These proportions are based on *Anopheles* specimens collected during the periods January-December in 2014, 2015, 2016, & 2018; May-December 2018, January-June 2019.

2.4.2. Sporozoite infectivity of species of the *An. funestus* group

Species identification, detection, and analysis of *P. falciparum* CSP for specimens collected in the years 2014 - 2016 were already performed (Burke *et al.*, 2017). Specimens collected between January 2017 - May 2019 were processed for identification and sporozoite presence

status in this study is listed in **table 2.3.** Infected species of *An. funestus* group were not detected in 2017. In 2018, one female *An. rivulorum* was a confirmed positive for *P. falciparum* CSP by ELISA but not by nested-PCR (**table 2.3)**. In 2019, another *An. rivulorum* and one *An. parensis* were confirmed positive by ELISA. However, DNA extracted from the ELISA homogenates of these specimens to confirm *P. falciparum* infection by PCR did not amplify.

Table 2.3. Results of *P. falciparum* circumsporozoite detection for the *An. funestus* group, in archived and newly collected specimens, and their feeding status

Year	# of Positive ELISA	Species	Blood meal	PCR & sequencing	*P. falciparum* infectivity rate
2014-2016	1*	*An. vaneedeni**	N/A	Confirmed*	1.96 %*
2017	0	N/A	N/A	N/A	N/A
2018	1	*An. rivulorum*	NF	No amplification	0
2019	2	*An. parensis* *An. rivulorum*	NF NF	No amplification No amplification	0

*: Data published by Burke *et al.* (2017)

NA: Not applicable

NF: Non blood fed

2.5. Discussion

2.5.1. Outdoor composition of *An. funestus* group species in Mamfene

For vector-borne diseases such as malaria, species identification is crucial for implicating the correct anopheline species as vector(s) involved in malaria transmission. The wide range and increasing number of available vectors can negatively impact on the epidemiology of malaria by increasing malaria incidence. Four common members of the *An. funestus* group were identified in the outdoor collections, *An. parensis*, *An. leesoni*, *An. rivulorum*, and *An. vaneedeni*. Data from this study contributed to the surveillance reports by Dahan-Moss *et al*. (2019, 2020b). Previous malaria surveillance activities and peer-reviewed research have also reported the presence of these four species (Christian *et al.*, 2017; Dandalo *et al.*, 2017; Burke *et al.*, 2019). Irrespective of the year of collection, *An. parensis* appeared to be the most abundant of the *An.*

funestus group identified in this study. Interestingly, the highest proportion of *An. parensis* was in observed in 2017 (83 %) (**fig 2.7**), which corresponded with the sudden increase in malaria cases for the 2016/2017 season (NICD, 2018; Moonasar *et al.,* 2021), as well as the finding of one *An. parensis* infected with *P. falciparum*, collected in October 2017 in Mamfene (Burke *et al*., 2019). Put together, it provides an overwhelming evidence of interconnected event. This puts the focus on *An. parensis* that may be contributing to the low residual transmission in KZN.

Anopheles rivulorum-like, first discovered in Cameroon (Hackett *et al.*, 2000), is also known to occur in Limpopo Province and Kruger National Park in South Africa (Mouatcho *et al.*, 2018; Braack *et al.*, 2020; Dahan-Moss *et al.*, 2021). Against this background, *An. rivulorum-like* was also targeted for amplification in this study. However, *An. rivulorum-like* was not identified in our collections, and has not yet been implicated as a malaria vector (Mouatcho *et al.*, 2018; Elanga-Ndille *et al.*, 2019; Mburu *et al.*, 2021).

Generally, *An. funestus* is the most common species in the *An. funestus* group, and most efficient at transmitting *P. falciparum* as well as *P. vivax* parasites, which makes it a major malaria vector in sub-Saharan Africa (Gillies and De Meillon, 1968; Coetzee and Fontenille, 2004; Coetzee *et al.*, 2013a). This is owing to its highly endophilic and anthropophilic nature (Gillies and De Meillon, 1968; Coetzee and Fontenille, 2004). However, *An. funestus* was not detected in this study. This species was last recorded from the collection in the Mamfene area in 2005 using indoor collections, and only comprised 0.9 % (n= 2/220) of the collection (Mouatcho *et al.*, 2007). However, in a recently published study, Braack *et al.* (2020) reported 2018 collection comprising one *An. funestus* and one *An. gambiae s.s* sample from Limpopo province. These species are generally regarded as being eliminated from within South Africa owing to the effectiveness of IRS (Hargreaves *et al.*, 2000; Brooke *et al.*, 2013; Coetzee *et al*., 2013a), with additional backing of recent malaria vector surveillance reports for South Africa (Dahan *et al.*, 2019; Dahan-moss *et al.*, 2020b). Unfortunately, the species identities were not confirmed with *ITS2* sequencing by Braack *et al.* (2020). Although *An. funestus* has previously been recorded in the country, this sequence confirmation for the *An. gambiae* sample would have provided vital information for the control program. This would have been the first report of this species in the country and it would have eliminated the possibility that the morphologically misidentified sample were included in the molecular array. Erlank *et al*. (2018) found that several species (not members of the *An. gambiae* complex) can be wrongly speciated by PCR as belonging to the *An.*

gambiae complex or the *An. funestus* group as a result of poor or wrong identification at the morphological level. Therefore, sequencing of the *ITS2* region is important for vector control planning since *An. gambiae* and *An. funestus* are still prevalent in neighbouring Zimbabwe (Masendu *et al.*, 2005; Zengenene *et al.*, 2020) and Mozambique (Casimiro *et al.*, 2006; Riveron *et al.*, 2019).

2.5.2. Vector status of species of the *An. funestus* group

Various requirements must be met to classify a specific *Anopheles* mosquito as a successful malaria vector. One major requirement is the non-refractoriness to *Plasmodium*, which is the ability of *Anopheles* to enable the parasite to complete its life cycle within the *Anopheles* host (Ghosh *et al.*, 2009; Mueller *et al.*, 2010). This means that successful detection of the infectious *Plasmodium* CSP in the salivary glands of female *Anopheles*, is indicative of a completed *Plasmodium* life cycle. This requirement has been met by the newly incriminated *An. vaneedeni* and *An. parensis* (Burke *et al.*, 2017, 2019). However, subsequent surveillance activities did not confirm *Plasmodium* infections in these species. Another major requirement is the host seeking behaviour to obtain a blood meal, and frequency of feeding (Kent and Norris, 2005; Gunathilaka *et al.*, 2016). This means that although a mosquito may be a vector for malaria, it would need to display anthropophilic behaviour to be implicated in human malaria. Detection of *Plasmodium* CSP in the salivary is used to classify an *Anopheles* species as a potential malaria vector, but only with the added evidence of feeding on humans can the species role in human malaria transmission be elucidated.

Unlike primary or "main" malaria vectors, defining and incriminating secondary vectors is not as clear-cut. Nonetheless, a secondary vector is defined as *Anopheles* found to be infective with *Plasmodium* parasites, thus capable of transmitting *Plasmodium*, but is unlikely to be a vector due to its primary zoophilic and exophilic nature (Gillies, 1964; Wilkes *et al*., 1996; Afrane *et al*., 2016). They include anophelines "known to play or suspected of playing a minor part in malaria transmission" (Gillies, 1964).

Data on *P. falciparum* infectivity rate for the archived collection period (January 2014-December 2016) have been reported by Burke *et al.* (2017). In their study, only two *An. vaneedeni* females collected in 2015, one in Mpumalanga and another Mamfene in KZN, were

infected with *P. falciparum* CSP. However, there was no way to confirm if this species had fed on humans, hence it was not possible to directly implicate it in the ongoing residual malaria transmission. Amongst the newly collected specimens (identified in 2018 and 2019, **table 2.3**), two *An. rivulorum* and one *An. parensis* were confirmed positive for *P. falciparum* CSP by ELISA. DNA extracted from the homogenates of positive ELISA samples in this study, *An. parensis* and *An. rivulorum*, did not amplify using the *Plasmodium*-specific nested PCR assay. This means that these specimens might have been false positives. However, other reasons may explain the results, including insufficient *Plasmodium* DNA in the remaining ELISA homogenate that could not be detected by PCR.

Although PCR was negative for the ELISA-positive *An. parensis* and *An. rivulorum* in this study, these species have been identified as malaria vectors in other settings, *Anopheles rivulorum* in Tanzania and Kenya, and *An. parensis* in Tanzania (Wilkes *et al*., 1996; Temu *et al.*, 2007; Kawada *et al.*, 2012). Of these, *An. rivulorum* and *An. parensis* in Tanzania (Temu *et al.*, 2007), and ELISA-positive *An. rivulorum* identified by Koekemoer *et al*. (2001) and Kawada *et al*. (2012), were confirmed positive for *P. falciparum* on PCR. However, neither the *P. falciparum* PCR products nor the *Anopheles* species were sequenced for further confirmation in all three studies. As in my study, these studies also employed the highly sensitive nested PCR assay by Snounou *et al*. (1993), for confirmation of *P. falciparum* infection. However, owing to the nature of the PCR, it is possible that the PCR also amplified parasite DNA found in the thoracic haemocele, rather than parasite DNA found in the salivary glands alone (Temu *et al*., 2007).

Additionally, one *An. parensis* resting outdoors in Mamfene, was infected with *P. falciparum* CSP and both the *Anopheles* and *Plasmodium* species were confirmed by sequencing PCR (Burke *et al.*, 2019). Considering that *An. parensis* is the most abundant of *An. funestus* group identified in the Mamfene area (Dandalo *et al.*, 2017; Burke *et al.*, 2019), as well as this study, the report by Burke *et al.* (2019) is concerning as a standalone finding. Although they are generally considered strongly exophilic and zoophilic (Gillies and De Meillon, 1968), *An. parensis* has also been found to have endophilic tendencies during an intense survey in Mamfene, KZN (Mouatcho *et al.*, 2007), in Kenya (Kamau *et al.*, 2003), and Tanzania (Temu *et al.*, 2007). According to Mouatcho *et al*. (2007), the *P. falciparum* positivity in *An. parensis* was confirmed three times by ELISA, but failed confirmation using the same nested PCR assay, and

none of them had fed on humans whilst indoors (Mouatcho *et al*., 2007). Of the 20 (13 %) *An. parensis* that were *P. falciparum* positive by ELISA, 65 % (13/20) had taken a blood meal of cattle origin only. Since cattle are outdoors, the finding suggest that they fed on cattle outdoors and rested indoors after taking their blood meal. Taken together, the finding by Burke *et al.* (2019) on *An. parensis* being infected with *P. falciparum* warrants intense future investigations of both indoor and outdoor resting *Anopheles* species, including those recognized as primarily zoophilic.

Emphasis is drawn on confirming the identification of *P. falciparum* detected, as well as the species of *Anopheles* infected with it. This is because misidentification of *Anopheles* species can occur, and this could result in implicating the wrong species as a malaria vector if found infected with *P. falciparum* (Erlank *et al*., 2018; Dahan-Moss *et al*., 2020a). Aside from *An. parensis*, Temu *et al.* (2007) also reported that 26/88 (29.5 %) *An. leesoni* were positive for *P. falciparum* on PCR. However, the *Anopheles* species and *P. falciparum* PCR products were not confirmed by sequencing. As mentioned previously, Dahan-Moss *et al*. (2020a) showed that some members of the *An. gambiae* complex can be misidentified as *An. leesoni* on PCR, which could have been the case with *An. leesoni* in Temu *et al*. (2007). This highlights the importance of confirming not only the *Plasmodium* infectivity in an *Anopheles* species, but also the *Anopheles* species itself by PCR and sequencing (Dahan-Moss *et al*., 2020a). *Anopheles leesoni* is also primarily zoophilic and exophilic. Therefore, Burke *et al.*'s (2019) recommendation is emphasized, that there is a rising need for malaria control intervention(s) that target outdoor feeding and resting vector populations.

Anopheles species misidentification was also observed in this study. A number of specimens were not identified using the *An. funestus* PCR. The assay is highly sensitive for specimens that are morphologically identified as members of the *An. funestus* group. On average, the PCR detection method was successful at identifying ~ 72 % of mosquito species during the collection period. The largest proportion of specimens that failed identification were in 2016 (55 %) and in 2019 (41 %). This can be attributed to DNA degradation. Moreover, it could be that some of the specimens were misclassified morphologically as in Erlank *et al*. (2018). There were 49 specimens collected in 2018-2019 that were identified as *An. leesoni* by PCR. This raised a suspicion because confirmed *An. leesoni* numbers were competing with those of *An. parensis*, which are the most common of the *An. funestus* group in Mamfene and KZN as a whole

(Dandalo *et al.*, 2017; Burke *et al.*, 2019; Dahan-Moss *et al.*, 2021). This finding was notified and investigated. An entomologist at VCRL performed morphology on those 49 specimens and classified them as *An. marshallii* complex (**supplementary table 2.1, Appendix 2F**). Root cause analysis revealed that those specimens were not identified morphologically before I received them for PCR identification. Although these samples were excluded from analysis in this study, it is possible that the other specimens that failed identification (**fig 2.6**) may belong to other *Anopheles* species which could not be amplified using the *An. funestus* species-specific PCR. This goes further to show the importance of morphological identification of anophelines, especially if the misidentified species happen to be infected with *Plasmodium* circumsporozoite protein.

As previously mentioned, sporozoite rate is important in calculating the entomological inoculation rate (EIR) as an indicator for estimating malaria transmission, calculated as a product of the sporozoite rate and the human biting rate (Shaukat *et al.*, 2010). The human biting rate is the average number of bites per person per unit of time, measured based on mosquito capture methods which assume that the mosquito fed on a human to allow transmission, these methods include human landing catches (HLC), window exit traps, and CDC light traps (WHO, 1975; Shaukat *et al*., 2010; Abraham *et al*., 2017). The variations in capture methods can result in many errors. Additionally, these capture methods solely depend on direct exposure of mosquitoes to humans. Confirmation of human blood meal in those mosquitoes will require an assay to perform host identification. Moreover, in studies such as mine where a direct approach is not taken, but rather a general interest of host preferences of outdoor feeding *Anopheles* is sought after, blood meal identification is required to confirm human as a host. Transmission to humans can also be confirmed if a *Plasmodium*-infected *Anopheles* has taken a human blood meal. In our study however, calculating the entomological inoculation rate may not be a reliable approach to estimate transmission risk, due to the low residual transmission experienced in Mamfene, KZN, with most malaria cases being imported from neighbouring Mozambique (Raman *et al*., 2020). Surveillance activities in such settings may only be able to establish transmission risk by determining which *Anopheles* species contribute to residual transmission, their abundance, and what their interaction with human is.

CHAPTER THREE

Blood meal source identification of *An. funestus* group and role of climatic parameters

3.1. Introduction

3.1.1. Importance of blood meal identification

The host preferences of haematogenous insects affect their capacity to transmit diseases. Hence, the range of hosts of *Anopheles* vectors affects their capacity to transmit malaria (Garret-Jones, 1964). Determining blood meal sources of malaria vectors in a specific geographic region is a critical step into understanding the epidemiology of the disease and the transmission dynamics of the pathogen (Garret-Jones, 1964; Lehane, 2005; Gatton *et al.*, 2013). Not only does the technique give insight about vector-host-pathogen interactions, in so doing, it provides information on host-specificity and whether *Anopheles* mosquitoes are primarily zoophilic or anthropophilic. Changes in trophic preferences can also be assessed in this manner. Blood meal source affects the capacity of *Anopheles* to transmit malaria by revealing whether humans are at risk of malaria by calculating the human blood index (HBI), an important component of vectorial capacity (Garret-Jones, 1964). This index is calculated as the estimated proportion of the blood meals obtained by a mosquito population. A representative sample of a single species, collected in a specific area and period is required. Garret-Jones (1964) explained that the best estimate of HBI requires factoring in the unweighted mean of samples collected from human dwellings, and the unweighted mean from other resting places. The author elaborated on three formulae depending on the available data collected in the field, and they are performed per individual species collected. Formula 1 calculates the "true human blood index", and takes into account the number of resting places in the collection area, the number of blood fed specimens, and the proportion of blood fed specimens containing human blood. When the number of resting places is not available, formula 2 calculates the ratio of samples containing human blood by the total number of fed specimens. Formula 2 is often used (Bashar *et al.*, 2012; Gunathilaka *et al.*, 2016; Debrah *et al.*, 2021), however, it is deemed erroneous and underestimates the HBI (Garrett-Jones, 1964). An alternative to formula 2, formula 3 calculates the ratio of sum of proportion that fed on human by the total number of collection sites.

3.1.2. Current host preferences of *Anopheles* species in South Africa

The study by Mouatcho *et al.* (2007) provided the first and only evidence, to my knowledge, of blood meal sources identification of *Anopheles* in South Africa. In that study, the blood feeding preference of indoor resting species of the *An. funestus* group, specifically *An. parensis*, from Mamfene, KZN, was evaluated by screening for human, cattle, chicken, goat, and sheep blood by direct ELISA, in both half-gravid and fully fed mosquitoes. Blood meal analysis of 169 *An. parensis* revealed a broad spectrum of hosts as blood meal sources. The results indicated that *An. parensis* had fed on all vertebrate species screened for, with cattle (cattle) blood meal as the most common food source and human blood meal the least common (Mouatcho *et al.*, 2007). Multiple blood meals were also detected in that study. No additional research has looked at the host preferences of the *An. funestus* group or any other *Anopheles* species in South Africa. This study presents the second evidence of host preferences in South Africa, but the first for outdoor feeding and resting *Anopheles*.

3.1.3. Methods used for blood meal identification

Various techniques have been used in the identification of blood meal sources of haematophagous arthropod vectors of different species. A number of serological tests first used for blood meal source identification include fluorescent antibody technique (Gentry *et al*., 1967) and traditionally, the precipitin test (Tempelis and Lofy, 1963) and latex agglutination test (Boorman *et al.*, 1977). However, these methods vary in disadvantages between lack of sensitivity, specificity, and extensive manual preparation. Indirect sandwich ELISA methods are more sensitive and specific, and have previously been used as routine methods for identification of host species in wild-caught *Anopheles* mosquitoes (Service, *et al.*, 1986; Mouatcho *et al.*, 2007), but this method is technically more difficult and cross hybridisation leading to inaccurate results were reported (Hunter and Bayly, 1991; Kent, 2009; Koekemoer, *pers comm*). However, with DNA-based molecular methods, blood meal identification in haematogenous arthropods, such as *Anopheles*, allow for high-throughput screening, and provide enhanced sensitivity and specificity (Irwin *et al.*, 1991; Kirstein and Gray, 1996; Kent and Norris, 2005; Gunathilaka *et al.*, 2016; Mbewe *et al.*, 2021).

In this study, two multiplex polymerase chain reactions (PCR), with *cytochrome b* (*Cytb*) and *12S rRNA* as molecular gene targets, were used to identify and differentiate between hosts' DNA

(**fig 3.1)**. Both molecular loci are found within the mitochondrial genome, which is a well-studied genome and widely used for vertebrate species identification. The mitochondrial genome (mtDNA) has limited variation within the same species while inter-species variation is large, and this is due to the high proportion of evolutionary-caused nucleotide substitution within the genome (Matsuda *et al.*, 2005; Tobe *et al*., 2010). The *12S rRNA* gene is located upstream of the *Cytb* gene, whilst *Cytb* is located downstream of the genome between bases 14 747 and 15 887 (**fig 3.1)**. As a mitochondrial gene, *Cytb* is especially useful for identifying vertebrate DNA in blood fed arthropods due to its high copy number; it also has sufficient genetic variation at the primary sequence level to discriminate between different vertebrate taxa thereby providing reliable identification (Irwin *et al*., 1991; Kirstein and Gray, 1996; Ngo and Kramer, 2003).

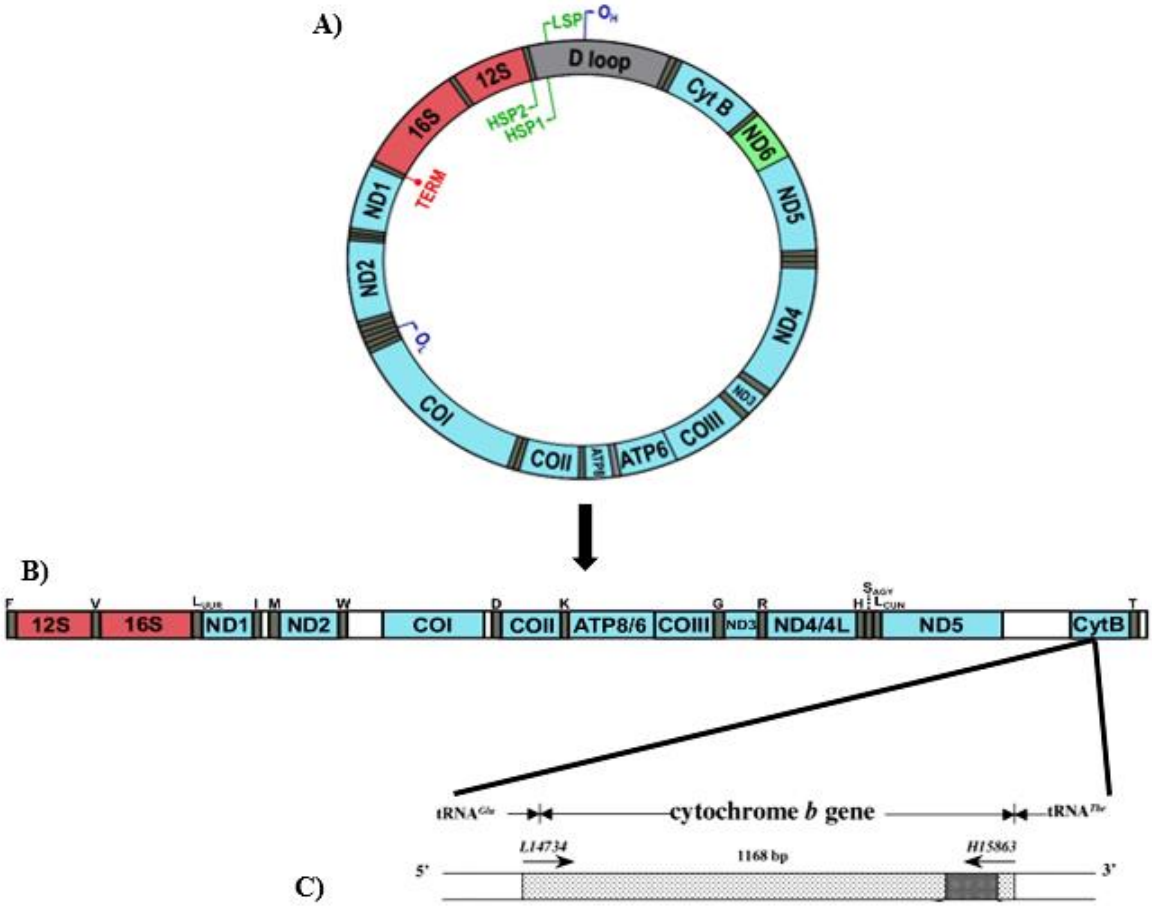

Figure 3.1. Schematic representation of a typical mitochondrial genome. A. Circular mitochondrial DNA (mtDNA). **B.** Gene order of mtDNA in typical vertebrates containing target genes, *12S rRNA* and *Cytb*, used for blood meal PCR assays in this study. **C.** *Cytochrome b* gene. **Citation:** modified from Matsuda *et al.* (2005).

For the *cytochrome b* target, five vertebrate species were identified for which primers had been designed (Kent and Norris, 2005). The species targeted for identification in this study using the

Cytb gene include human (*Homo sapiens*), dog (*Canis familiaris*), cattle (*Bos taurus*), goat (*Capra aegagrus hircus*), and pig (*Sus scrofa*). The rationale for selection of the five vertebrate species in this study was based on their abundance in the study area, Mamfene in KZN (Dr Munhenga, G., *pers comm*). Additionally, since there is an interest in chicken (*Gallus domesticus*) as a potential blood meal source for the outdoor biting *An. funestus* group, a second multiplex PCR assay developed by Cahyadi *et al.* (2018) was included. In this assay, the *12S rRNA* gene was targeted to differentiate between chicken host DNA, and two other species, cattle and pig. This study presents the first evidence of host-preferences of *Anopheles* in South Africa using DNA-based methods. As indicated in the introductory chapter, climatic parameters are the major limiting factors in malaria epidemics because of its seasonality. Temperature, humidity and rainfall influence the development and host-seeking behaviour of mosquitoes (Gatton *et al.*, 2013; Stone *et al.*, 2016). Hence, this study also presents the first evidence on the impact of climatic parameters on host preferences of *Anopheles*.

3.2. Objectives

The aim of this study was to identify host preferences amongst blood fed species of the *An. funestus* group females, to identify if there was a change in feeding proportions by these species, and determine how climatic parameters affect their host choice.

3.2.1. Specific objectives

1. To optimise a multiplex PCR assay to identify blood meal source(s) amongst fed females of the *An. funestus* group.
2. To determine how and which climatic parameters play a role on the host preferences and feeding proportions of *An. funestus* group females in KZN.

3.3. Materials and methods

3.3.1. Selection of blood fed females by light microscopy

Only female species of *An. funestus* group that were successfully identified by PCR were subsequently analysed for blood meal. The blood meal status of a female *Anopheles* mosquitoes was identified based on the abdominal/physiological appearance using a dissecting light

microscope. Selection with the naked eye can be misleading, as it is difficult to distinguish between gravid/half gravid from fed females in that manner because the abdomen would look engorged and dark in both cases, especially with archived specimens that were stored on desiccant.

Blood meal classification described by Beier (2002) was used to identify blood-fed females for DNA extraction (**fig 3.2**). An empty or sugar-fed abdomen is classified as non-blood fed (**NF**): no blood meal. Fed females would have engorged abdomens (**F**): freshly fed females will have a bright red abdomen, but for an archived/silica-preserved specimen, the blood would have dried and darkened the whole abdomen. Half-gravid (**HG**) means the blood is covering 3-4 segments of the upper part of the abdomen, and gravid (**G):** the blood is absent or shown as a small black patch on the ventral surface of abdomen and appears engorged as the ovaries/eggs are covering almost all of abdomen.

Figure 3.2. Classification of blood-feeding stages in *Anopheles* mosquitoes. From left to right: non-blood fed stage, blood fed stage, half-gravid stage, and gravid stage. **Citation:** images captured using an iPhone 6s (12-megapixel back camera: 4032x3024 pixels).

3.3.2. Optimisation of DNA extraction from a blood meal

3.3.2.1. Sample preparation

Female species of *An. funestus* group classified as fed in the previous section (3.3.1), had DNA extracted from their abdomens. The abdomen was separated from the rest of the body and used for DNA extraction (Kent and Norris, 2005). This was to minimise mosquitoes' DNA and maximise isolation of host DNA. Dried field specimens required rehydration to solubilise the blood meal before DNA extraction. To replicate their conditions for optimisation, the FUMOZ colony mosquitoes and colonised *An. gambiae* females, used as positive controls, were collected

after receiving human and cattle blood meals respectively. The controls were kept in tubes containing desiccant (silica gel) at room temperature for a week to allow them to dry. This was done to replicate wild caught specimens, which were also kept on desiccants before processing. Afterwards, individual abdomens were placed in a 1.5 ml microcentrifuge tube containing 50 µl phosphate buffered saline (PBS) (pH 7.2) at room temperature, to rehydrate the blood in their abdomens as described by Ungureanu (1972), with slight modifications. Subsets of females were then incubated at 5-minute intervals from 5-30 minutes at room temperature. The amount of blood released from the abdomen into the PBS solution was visually estimated at each time point. Maximum blood release was observed at 20-30 minutes incubation (**fig 3.3)**. Twenty-minute incubation period was used henceforth. After rehydration, the specimen was ready to be homogenised for DNA extraction.

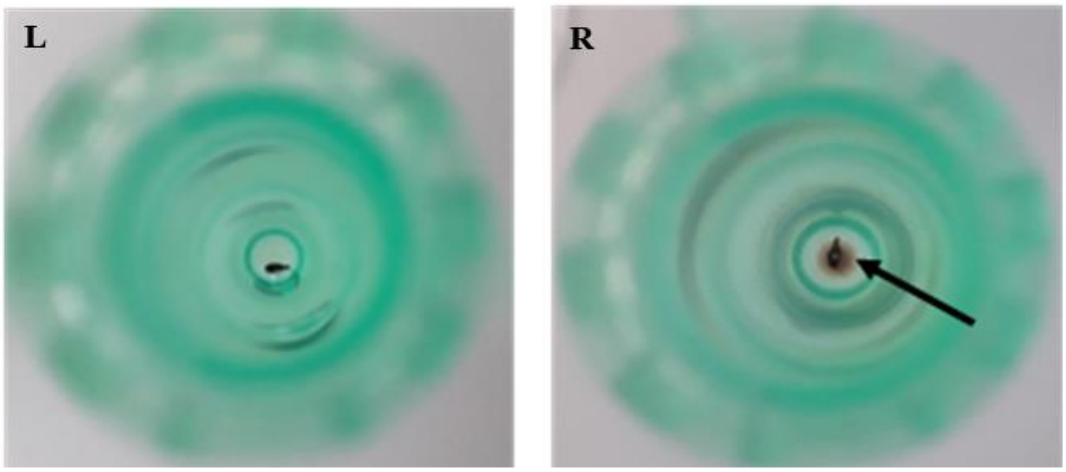

Figure 3.3. Rehydration procedure of a blood fed *Anopheles* in PBS (pH 7.2). L (left): 0-minute post-incubation. **R(right):** 20-minute post-incubation. Solid black arrow: blood released in PBS solution. **Citation:** images captured using a Samsung SM-J260F (2448x3264 pixels back camera).

3.3.2.2. DNA isolation from blood meal

Initially, three different extraction methods which isolate DNA from a blood meal were evaluated. These DNA extraction methods included a manual DNA extraction using Collins' method (Collins *et al.*, 1987) (**Appendix 3A**), and two commercial DNA extraction kits, the ZymoResearch's Quick-DNA™ Microprep Plus kit (**Appendix 3B**), and the Invitrogen's PureLink™ Genomic DNA Mini Kit (Cat No: K1820-02, Thermo Scientific, SA). The Collin's method was used as described (**Appendix 3A**). The kit-based DNA extraction methods were

performed following the manufacturer's instructions. The blood lysate protocol was used with the PureLink™ Genomic DNA Mini kit (described below), and the biological fluids & cells protocol was used with the Quick-DNA™ Microprep Plus kit (**Appendix 3B**). DNA extracted using the different methods was quantified using the NanoDrop™ 2000/2000c Spectrophotometer (Cat No: ND2000C, Inqaba biotec™, South Africa) following the manufacturer's instructions. Subsets of blood meal extracted were eluted at different volumes ranging from 200 µl down to 30 µl to determine which elution volume would provide the optimal DNA yield for PCR.

Genomic DNA extracted using the Invitrogen's PureLink™ Genomic DNA Mini kit with slight modifications was the optimum method as it produced good DNA yield compared to the other two methods. Hence this kit was used for subsequent DNA extraction from our dried field specimens. The dried field specimens were rehydrated using the optimised method described in section 3.3.2.1, and DNA extraction performed using the Invitrogen kit. After rehydration, one 5 mm stainless bead was placed in each sample and homogenised in the TissueLyser II (Cat No: 85300, QIAGEN®, South Africa) at 25 Hz for 4 minutes. This process released the blood meal into the solution. The beads were then rinsed off with ~ 140 µl PBS to bring the total starting volume to ~ 200 µl. The blood lysate protocol was then followed as described by the manufacturer (**fig 3.4**). DNA was eluted in 30 µl genomic elution buffer. Two negative controls were included, a negative extraction control of 200 µl PBS (NEC), and a negative template control (NeTC) containing a non-blood fed female from the FUMOZ colony in 50 µl PBS. The NeTC was included to ensure that non-specific binding of the blood meal primers with mosquito DNA does not occur during the blood meal PCR. The extracted DNA was stored at -20˚C or used immediately. Once analysed using blood meal PCR, DNA was stored at -70 °C for long term storage.

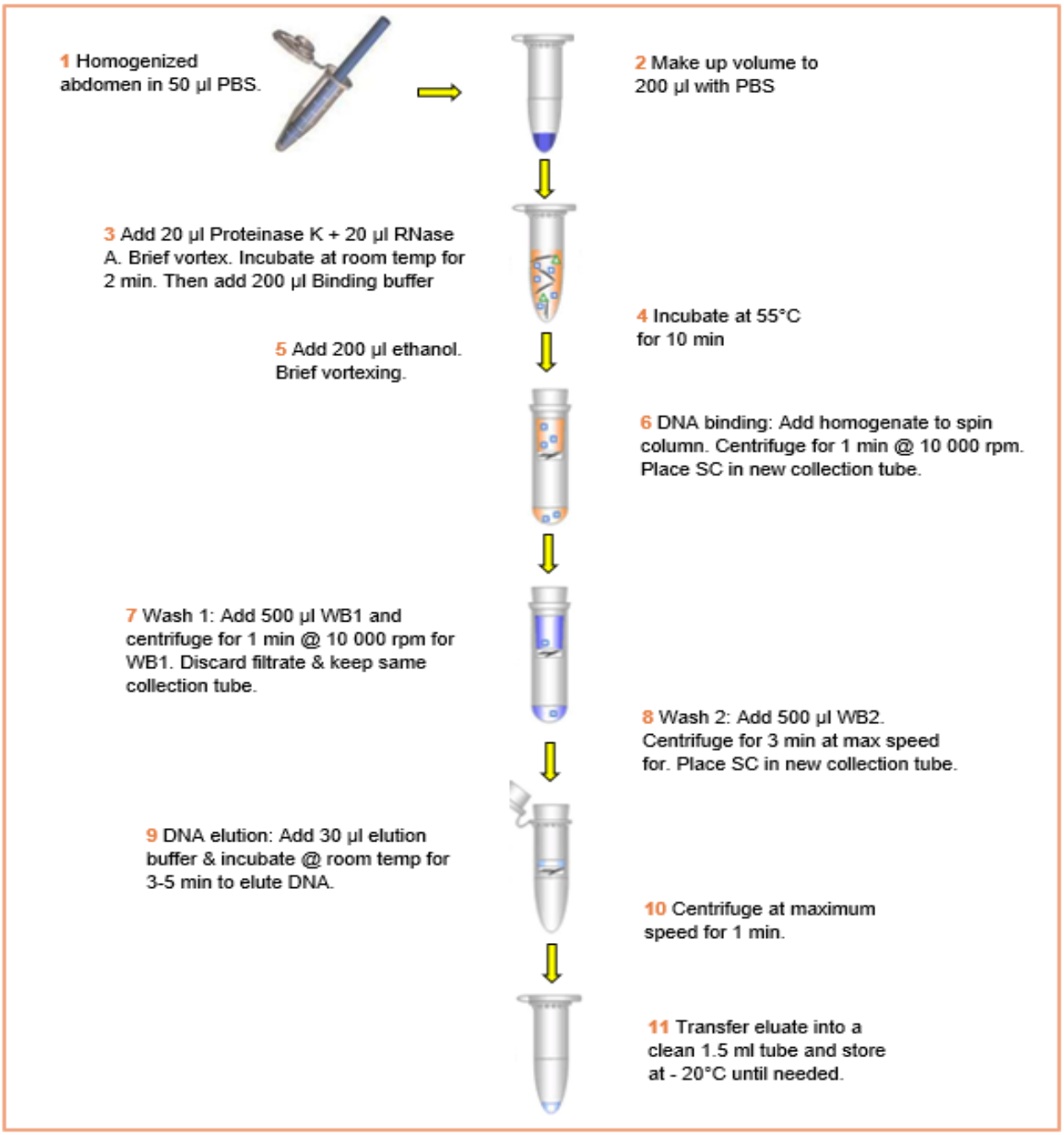

Figure 3.4. Laboratory procedure for extraction of genomic DNA from host blood in *Anopheles* mosquitoes. SC: Spin column; WB: wash buffer.

3.3.3. Optimisation of blood meal primers using temperature gradient protocol

3.3.3.1. Positive controls and PCR primers

Positive control mosquitoes or specific blood meal sources used for optimisation and further processing for the blood meal identification PCR are described (**table 3.1)**.

Table 3.1. Malaria uninfected positive controls for blood meal PCR, their sources and mosquito feeding methods.

Positive control	Source	Feeding method
Human (*Homo sapiens*)[1]	Dead female *An. funestus* specimens that were fed on uninfected control blood at Wits Research institute for malaria (WRIM) insectary (**Appendix 1B**).	Membrane feeding
Cattle (*Bos taurus*)[1,2]	Colonised *An. gambiae* at VCRL insectary fed on cattle blood.	Fresh, cattle whole blood warmed to 27 °C through membrane feeding
Goat (*Capra hircus*)[1]	Stored goat DNA, identified from previous surveillance activities in *Anopheles* mosquitoes.	N/A
Pig (*Sus scrofa*)[1,2]	DNA extracted from purchased meat	N/A
Dog (*Canis familiaris*)[1]	Stored dog DNA, identified from previous surveillance activities in *Anopheles* mosquitoes.	N/A
Chicken (*Gallus domesticus*)[2]	DNA extracted from purchased meat	N/A

[1]Positive controls used in the Kent and Norris (2005) PCR assay

[2]Positive controls used in the Cahyadi *et al.* (2018) PCR assay

N/A: Not applicable

Two conventional multiplex-PCR assays were used in this study using species-specific primers designed by Kent and Norris (2005), and Cahyadi *et al*. (2018) for the detection of human, cattle, goat, pig, dog, and chicken DNA in the blood meal (**table 3.2**). All primers were purchased from Macrogen, Europe. The Kent and Norris' (2005) PCR assay comprised of vertebrate-specific forward primers and a universal reverse primer (UNREV); whilst the Cahyadi *et al*.'s (2018) PCR assay comprised of vertebrate-specific reverse primers, with a universal forward primer (UNFOR) (**table 3.2**).

Table 3.2. Primer sequences for conventional blood meal PCR

Organism	Primer	Oligonucleotide sequence (5'-3')	Target gene	Tm (°C)	Amplicon size (bp)
Human	Human741F	GGCTTACTTCTCTTCATTCTCTCCT	*cyt b*[1]	64.2	334
Dog	Dog368F	GGAATTGTACTATTATTCGCAACCAT	*cyt b*[1]	61.6	680
Cattle	Cow121F	CATCGGCACAAATTTAGTCG	*cyt b*[1]	56.4	561
Goat	Goat894F	CCTAATCTTAGTACTTGTACCCTTCCTC	*cyt b*[1]	67.2	132
Pig	Pig573F	CCTCGCAGCCGTACATCTC	*cyt b*[1]	61.7	453
	UNREV1025	GGTTGTCCTCCAATTCATGTTA	*cyt b*[1]	59.4	-
	UNFOR	ACCGCGGTCATACGATTAAC	12S rRNA[2]		-
Cattle	SP_R	AGTGCGTCGGCTATTGTAGG	12S rRNA[2]		155
Pig	BB_R	GAATTGGCAAGGGTTGGTAA	12S rRNA[2]		357
Chicken	A_R	CGGTATGTACGTGCCTCAGA	12S rRNA[2]		611

[1]Kent and Norris (2005)
[2]Cahyadi *et al.* (2018)
Tm: melting temperature

3.3.3.2. Kent and Norris (2005) PCR assay

This assay targeted the most common vertebrate/host species, using primers designed by Kent and Norris (2005) (table **3.2**). The PCR assay was optimised for our setting, using gradient annealing temperature (Ta) method (**table 3.3)**. A 57.5 °C annealing temperature was optimal for all primers and was used. PCR was carried out in a final volume of 12.5 µl, consisting of 10x buffer (100 mM Tris-HCl (pH 8.3), 500 mM KCl), 200 µM of each dNTP, 1.5 mM $MgCl_2$, 50 µM of each primer, and 5 units of the thermostable *Taq* DNA polymerase (**Appendix 3C**).

Table 3.3. Kent and Norris (2005) blood meal PCR cycling conditions for Ta optimisation

Cycling conditions	Temp (°C)	Time (minutes)
Initial denaturation/Hot Start	95	5
Steps 1-3 repeated through 35 cycles		
Step 1	95	1
Step 2	51.4 - 68 (gradient)	1
Step 3	72	1
Final extension	72	7

3.3.3.3. Cahyadi *et al.* (2018) PCR assay

This second assay was used to investigate whether chicken is a potential blood meal source for outdoor feeding species of *An. funestus* group. Following the protocol described Cahyadi *et al*. (2018), PCR was performed on all samples that did not amplify in the first PCR assay. Additionally, the primers from this assay were also used to amplify cattle and pig DNA (**table 3.2**). No optimisation for the assay was required as the cycling conditions described by the authors proved optimal upon testing (**table 3.4**). Each 12.5 µl PCR mastermix consisted of 10x buffer (100 mM Tris-HCl (pH 8.3), 500 mM KCl), 200 µM of each dNTP, 1.5 mM $MgCl_2$, 10 µM of each primer, and 5 units of the thermostable *Taq* DNA polymerase (**Appendix 3D**).

Table 3.4. Cahyadi *et al.* (2018) blood meal PCR cycling conditions

Cycling conditions	Temp (°C)	Time (sec)
Initial denaturation/Hot Start	95	180
Steps 1-3 repeated through 30 cycles		
Step 1	95	15
Step 2	60	30
Step 3	72	30
Final extension	72	180

3.3.4. Verification of blood meal PCR assay using *Anopheles* mosquitoes from Mozambique

The optimised Kent and Norris (2005) and the Cahyadi *et al.* (2018) PCR assays were applied to wild caught specimens of *An. funestus* group and *An. gambiae* complex, from Mozambique, mostly known to be anthropophilic. They were collected from September to November 2019. This was done to verify that the assay can detect human DNA from blood meal of wild caught *Anopheles* specimens.

3.3.5. Data analysis

3.3.5.1. Blood meal identification of host preferences

PCR products were resolved on a 2.5 % ethidium-stained agarose gel, visualized in the Molecular Imager® ChemiDoc™XRS$^+$ system (Cat No: 170-8071, Bio-Rad) and analysed using Image Lab™ Software (Cat No: 1709690, Bio-Rad). A blood meal PCR assay was considered valid if all negative controls did not amplify. For the Kent and Norris (2005) PCR assay, at least three of the five positive controls should have amplified. Successful amplification of an unknown host DNA was classified as either goat if amplified at 132 bp, human (334 bp), pig (453 bp), cattle (561 bp), or dog (680 bp). For the Cahyadi *et al*. (2018) PCR assay, at least two of the three positive controls should have amplified. Amplification of an unknown host DNA was classified as either cattle if amplified at 155 bp, 357 bp for pig, or 611 bp for chicken. The human blood index (HBI), defined as "the proportion of freshly engorged mosquitoes containing human blood", was calculated based on formula 3 for the ratio of sum of proportion that fed on human by the total number of collection sites, for each species of *An. funestus* group, as described (Garret-Jones, 1964):

$$\text{HBI (formula 3)} = \frac{\Sigma \text{ proportion fed on human}}{\text{Total number of collection sites}}$$

3.3.5.2. Collection of climatic data sets

Meteorological data for 1 January 2014 – 31 December 2019 was obtained from the automatic Mamfene weather station (Jozini, KZN). The mean, minimum and maximum measurements of three climatic parameters were extracted: temperature (in °C), precipitation (in mm), as well as humidity (in %). The study period was filtered for missing values for each month and each year separately. Each month was considered complete if there were three or less missing days per month (less than 10 % of the month), and a full year was considered complete if there was only one month missing. Every month and year under analysis met these conditions. Monthly temperature means, relative humidity means, as well as monthly precipitation totals (in mm) were calculated from the daily data. Weather seasons were defined as follows: summer = 1 December – 28/29 February; autumn = 1 March– 31 May; winter = 1 June – 31 August; and spring = 1 September – 30 November.

3.3.5.3. Statistical analysis

All statistical analysis of the data including descriptive analysis were performed using STATISTICA version 13.0.17, and XLSTAT (by Addinsoft, 23.3.1186.0, 2019). Statistical significance was measured at $p < 0.05$, thus measuring significance level at 95 % confidence interval (CI). The Shapiro-Wilk test was used to determine normality.

Temporal pattern of climatic parameters

The monthly mean temperatures (°C) for the period 2014 – 2019 were normally distributed. To investigate if mean monthly temperatures changed over the study period, the non-parametric Mann-Kendall trend test was applied, performed in XLSTAT. If the null hypothesis, which states that there is no trend in the climatic parameters across the study years, was accepted, then analysis of variance (ANOVA) and Tukey's HSD (honestly significant difference) test for normally distributed climatic parameters, temperature (°C) and relative humidity (%), were performed. Friedman's test for non-parametric climatic parameter, precipitation (mm), was performed to determine differences by month-to-month comparisons. Analysis of variance (ANOVA), Tukey's HSP test, and Friedman's test were performed in STATISTICA.

Calculation of blood feeding proportions (FP)

First data quality control (QC) was performed. Samples for the year 2014 were used in another project and could not be triaged for blood meal analysis. This period was therefore excluded in further analysis with feeding proportions. Feeding data for January 2017-April 2017 was also used in another project, hence this period was also excluded for further analysis with climatic parameters. Finally, specimens were collected after June 2019 for this study were also excluded, hence climatic variables for that period were not used for analysis with feeding proportions. The study therefore considered full 12 month periods of blood feeding data: January-December 2015, January-December 2016, May 2017-April 2018, and May 2018-April 2019. Thus the total number of blood fed specimens successfully identified by blood meal PCR used for analysis was N=218.

Feeding proportion (FP) expressed as a percentage, for each species of *An. funestus* group. Feeding proportions were calculated in two ways:

1. Mean feeding proportions were calculated for two collection periods. Archived collection (January 2015 – December 2016 = 24 months), and newly collected data (May 2017 – April 2019 = 24 months). Data were combined into monthly averages for each collection period, resulting in 12 data points. e.g. average FP for January months in the archived collection period. The mean FPs for each *Anopheles* species, irrespective of vertebrate host they fed on, were then compared for the archived versus the newly collected data, using the non-parametric Mann-Whitney U test. Formula:

   FP = N_x / N_T ,

   where N_x is the total number of an *Anopheles* species that were blood fed, per month

   N_T is total number of all *Anopheles* species that were blood fed, per month

2. Annual feeding proportions were calculated per *Anopheles* species per vertebrate host. for 4 collection years: year 1: 2015 (January-December), year 2: 2016 (January-December), year 3: May 2017-April 2018, and year 4: May 2018-April 2019. Chi square test was used to determine if there was a difference in annual feeding proportions per *Anopheles* species per year.

   FP on a specific host = N_{xh} / N_T,

   where N_{xh} is the number of specimens of an *Anopheles* species that fed on a particular host, per year

   N_T is total number of blood fed specimens of that *Anopheles* species (per year)

Relationship between feeding proportions and climatic parameters

The non-parametric Spearman's correlation was first used to determine association correlation between mean feeding proportions of each *Anopheles* species and weather parameters in archived and newly collected data, irrespective of vertebrate host they fed on. Then time-series analysis was performed for statistically significant correlations to examine the impact of climatic parameters on mean feeding proportions of mosquitoes. The same analyses were run for annual feeding proportions per *Anopheles* species per vertebrate host species, with corresponding annual climatic parameters.

3.4. Results

3.4.1. Optimised DNA extraction from mosquito blood meal

Rehydration of fed female mosquitoes, used as positive controls, was efficient in 50 µl PBS (pH 7.4) for 20-30 minutes at room temperature, which maximised DNA isolation. Initially, DNA eluted in >100 µl elution buffer from extraction kits used, failed to be amplified during blood meal PCR. Subsequently elution volumes ≤ 100 µl elution buffer were evaluated. The DNA concentrations of specimens extracted with the ZymoResearch kit were considerably lower than those extracted with the Invitrogen kit with corresponding elution volumes (**table 3.5).** The negative template controls (non-blood fed female) blood meal PCR was performed on extracted DNA, using cycling conditions by Kent and Norris (2005).

Table 3.5. Nanodrop results comparing DNA yield by two kit-based methods and at different elution volumes.

#	Sample ID	Elution volume (µl)	Nucleic Acid Conc. (ng/µl)	Sample Type
1	ZYMO 1-Bovine blood meal	100	3,5	DNA
2	ZYMO 2-Bovine blood meal	50	5,3	DNA
3	ZYMO 3-Bovine blood meal	25	9,3	DNA
4	ZYMO 4-Human blood meal	100	1,9	DNA
5	ZYMO 5-Human blood meal	50	3,8	DNA
6	ZYMO 6- Negative template control	50	7,7	DNA
7	INVITROGEN 1-Bovine blood meal	100	2,1	DNA
8	INVITROGEN 2-Bovine blood meal	50	13	DNA
9	INVITROGEN 3-Bovine blood meal	25	16,8	DNA
10	INVITROGEN 4-Human blood meal	100	18,1	DNA
11	INVITROGEN 4-Human blood meal	100	18,5	DNA
12	INVITROGEN 5- Human blood meal	50	11	DNA
13	INVITROGEN 6- Human blood meal	25	11,4	DNA
14	INVITROGEN 7- Negative template control	50	8,9	DNA
15	Negative extraction control	50	0	DNA

Blood meal PCR amplification was successful for DNA extracted using the Invitrogen kit only. Samples that were extracted using the Invitrogen kit and eluted in 100 µl elution buffer, showed variable DNA concentrations (**table 3.5**). Regardless, all of these were used for blood meal PCR identification, including cattle DNA with concentration of 2.1 ng/µl (**table 3.5)** amplified a faint fragment successfully (lane 7, **fig 3.5)**. Cattle DNA in lanes 7 and 9 amplified at 561 bp as

expected, but cattle DNA in lane 9 produced higher yield as it was eluted in 25 µl compared to DNA in lane 7 eluted in 100 µl (**fig 3.5**). No analysis for DNA in lane 8 eluted in 50 µl, as the mastermix had evaporated. Human DNA eluted in 100 µl and 25 µl amplified at 334 bp as expected (lanes 10 & 13), but not human DNA eluted in 50 µl (lane 11) (**fig 3.5**). Amplification of blood meal DNA was not observed for the extraction control (lane 12, non-blood fed female) containing the non-blood fed female mosquito, indicating that the PCR is specific for vertebrate host species, and not mosquito DNA.

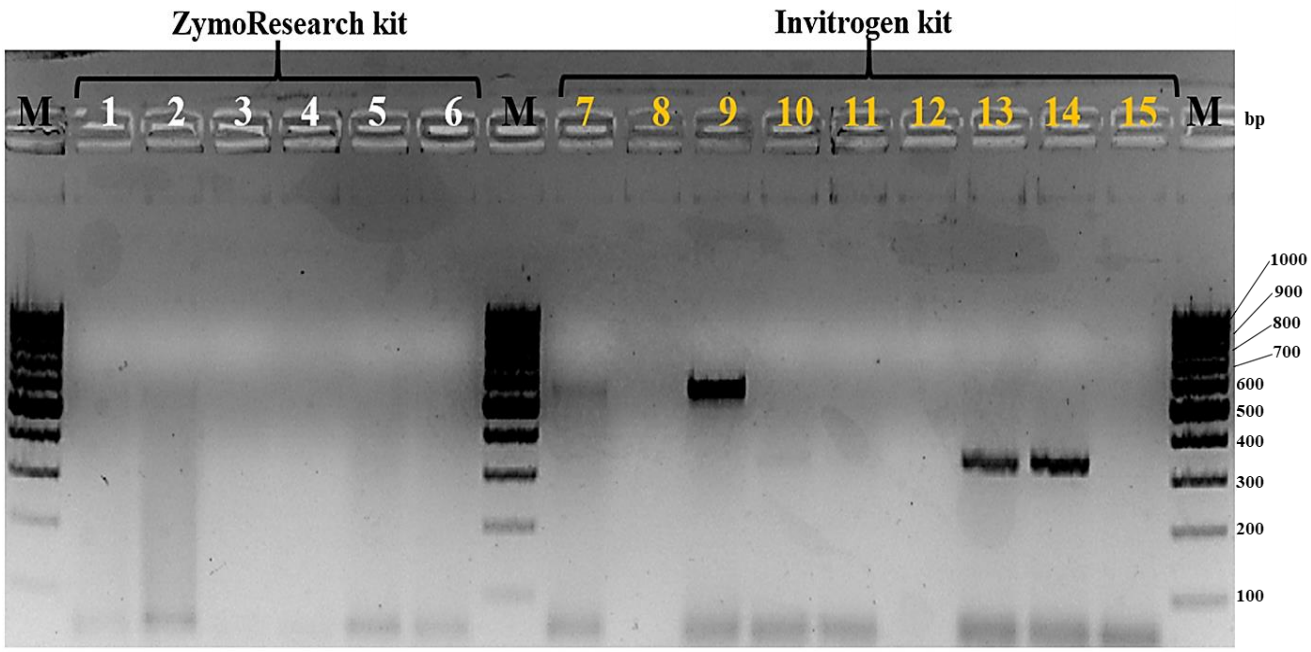

Figure 3.5. PCR products of blood meal extracted using two DNA extraction kits. M: Molecular weight marker: O'Gene ruler 100 bp. Results for DNA extracted using: ZymoResearch kit in white- lanes 1-6; and the Invitrogen kit in yellow- lanes 7-15. Lanes 1-3: DNA eluted in 100 µl, 50µl, and 25 µl elution buffer respectively. Lanes 4 and 5: human DNA eluted in 100 µL, and 50 µL elution buffer respectively. Lanes 7 and 9: cattle DNA eluted in 100 µl and 25 µl elution buffer respectively. Lane 8: empty. Lanes 10 and 11: human DNA eluted in 100 µl, and 50 µl elution buffer respectively. Lanes 13 and 14: human DNA eluted in 25 µl elution buffer. Lanes 6 and 12: Negative extraction controls, and lane 15: PCR no template control.

3.4.2. Optimised blood meal PCR

Only Kent and Norris (2005) PCR was optimised, as described in the methods (section 3.3.3), using *cytochrome b* primers. Product visualization showed that the PCR reaction produced a

single amplicon according to the target size of each specific vertebrate species. Non-specific amplification did not occur. Amplification of the *cytochrome b* gene resulted in PCR amplicons of 132 bp (for goats), 334 bp (for humans), 453 bp (for pigs), 561 bp (for cattles), and 680 bp (for dogs), respectively. Based on the results obtained, the optimum annealing temperature in this study was 57.5 °C, at which all positive controls tested amplified (**table 3.6)**.

Table 3.6. Result of annealing temperature (Ta) optimisation for the Kent and Norris (2005) PCR assay

Vertebrate Species	Scientific Name	Annealing temperatures (Ta) (in °C) tested in gradient steps							
		68	**66.8**	**64.8**	**61.7**	**57.5**	**54.6**	**52.6**	**51.4**
Neg	N/A	-	-	-	-	-	-	-	-
NTC	N/A	-	-	-	-	-	-	-	-
Human	*Homo sapiens*	+	+	+	-	+	+	+	+
Dog	*Canis familiaris*	-	-	-	+	+	-	-	+
Cattle	*Bos taurus*	+	-	+	+	+	+	-	+
Goat	*Capra aegagrus hircus*	-	-	-	-	+	+	+	-
Pig	*Sus scrofa*	+	+	+	+	+	+	+	+

Neg: Negative extraction control

NTC: PCR No template control

+: successful amplification

-: no amplification

3.4.3. Identification of host DNA

An example of results obtained using the Kent and Norris (2005) assay is illustrated in **fig 3.6**. Four of five positive controls successfully amplified, goat at 132 bp, human at 334 bp, pig at 453 bp, and cattle at 561 bp. Dog positive control did not amplify. Two of 27 blood meals amplified at 132 bp as the goat control; 13/27 amplified at 561 bp as the cattle control; whilst the remainder 12/27 did not amplify.

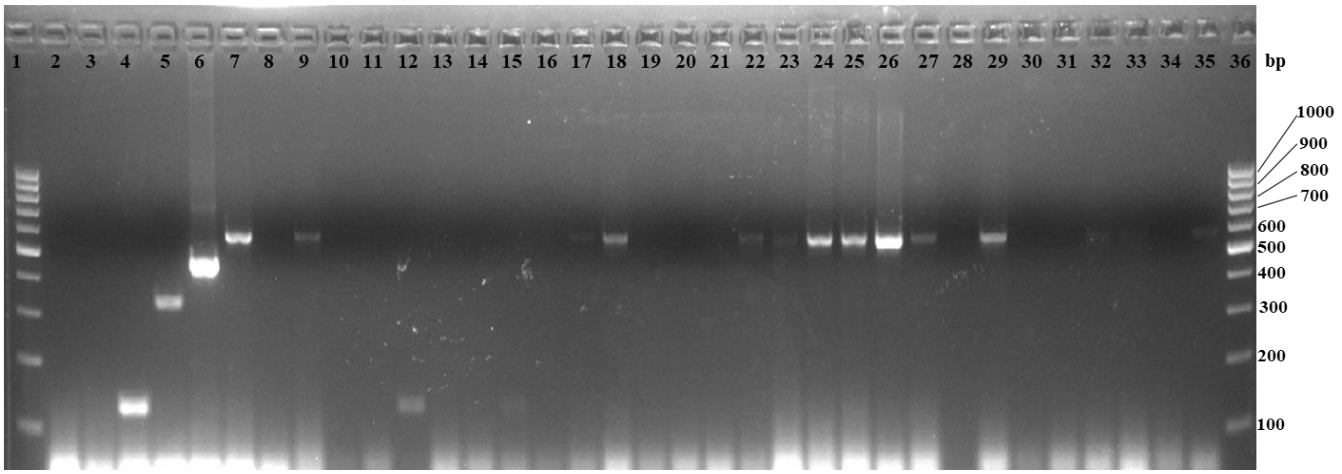

Figure 3.6. Kent and Norris (2005) blood meal PCR gel electrophoresis result. Lanes 1 and 36: DNA ladder (O'Gene ruler 100 bp). Lanes 2 & 3: NEC and NTC respectively= no amplicon. Lanes 4-8*:* positive controls for goat 132 bp; human 334 bp; pig 453 bp; cattle 561 bp; and dog= no amplicon, respectively. Lanes 12 & 15= goat DNA. Lanes 9, 17, 18, 22-27, 29, 32, 33, and 35= cattle DNA. Lanes 10, 11, 13, 14, 16, 19-21, 28, 30, 31 and 34 = no amplification.

An example of results obtained using Cahyadi *et al*. (2018) assay is illustrated in **fig 3.7**. All positive controls amplified, cattle at 155 bp, pig at 357 bp, and chicken at 611 bp. Fourteen of 29 blood meals amplified at 155 bp as the cattle control; 1/29 had mixed blood meal of cattle and pig; 1/29 amplified at 357 bp as the pig control; the remaining 13/29 did not amplify.

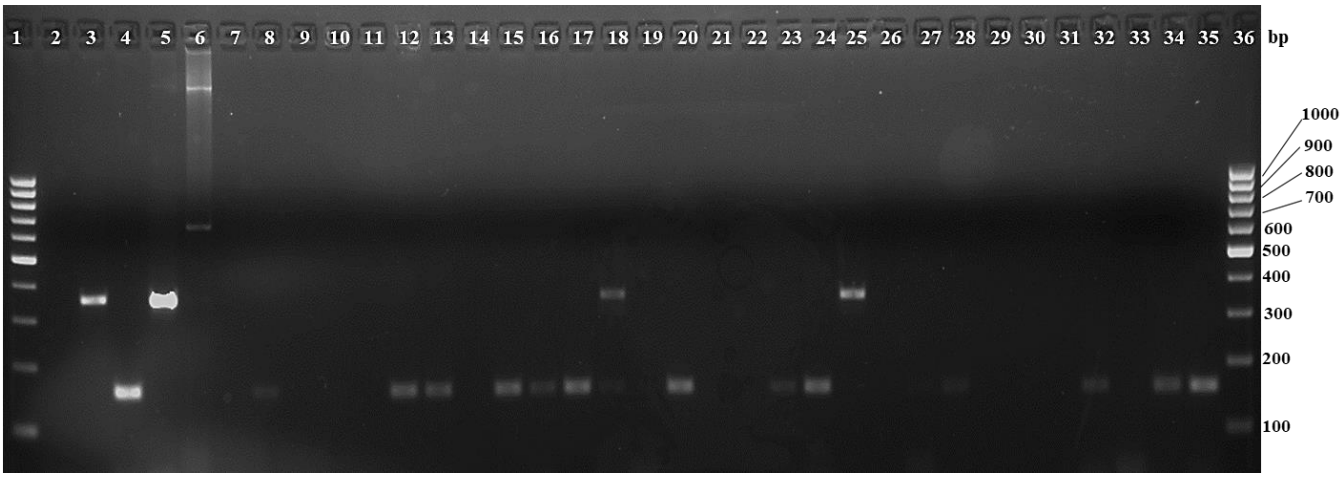

Figure 3.7. Cahyadi *et al*. (2018) blood meal PCR gel electrophoresis result. Lanes 1 and 36: DNA ladder (O'Gene ruler 100 bp). Lanes 2 & 7: NEC and NTC respectively = no amplicon. Lanes 3-6*:* positive controls for pig 357 bp; cattle 155 bp, pig 357 bp, chicken 611 bp. Lanes 8, 12, 13, 15-17; 20, 23, 24, 27, 28, 32, 34 & 35 = cattle DNA. Lane 18: mixed blood meal of cattle & pig; Lane 25: pig host DNA. Lanes 9-11, 14, 19, 21, 22, 26, 29-31, & 33 = no amplicon.

3.4.4. Verification of blood meal PCR using Mozambican samples

A total of 360 wild *Anopheles* female specimens from Mozambique were received for blood meal analysis using Kent and Norris (2005) and Cahyadi *et al.* (2018) PCR assays. They were successfully identified to species level by Bianca E Silva (MSc student at VCRL). Four *An. gambiae* and three *An. arabiensis* of the *An. gambiae* complex, 340 *An. funestus*, two *An. leesoni*, and eight *An. vaneedeni* of the *An. funestus* group were identified. Blood meal status were already determined in the field. Positive blood meal identification was achieved for 40 % of blood fed specimens (n=140). Of these, 95 % (N=136) had fed on human blood (**table 3.7**). *Anopheles funestus* was the most common species of the collection, and mostly fed on humans (N=132), followed by goat (N=2) (**table 3.7**).

Tale 3.7. Composition of vertebrate host species in *Anopheles* species from Mozambique

	PCR result								
Species	Human	Dog	Cattle	Goat	Pig	Human+ Goat	Chicken	BM not identified	**Total**
An. gambiae	3	0	0	0	0	0	0	4	**7**
An. arabiensis	0	0	0	0	0	0	0	3	**3**
An. funestus	132	0	0	2	0	2	0	204	**340**
An. leesoni	0	0	0	0	0	0	0	2	**2**
An. vaneedeni	1	0	1	2	0	0	0	4	**8**
Total	136	0	1	4	0	2	0	217	**360**

The human blood index (HBI), the ratio of sum of proportions that fed on human by the total number of collection sites, was calculated for each species of the *An. funestus* group and of the *An. gambiae* complex, as described (Garret-Jones, 1964). The number of collection sites in Mozambique was N=4, Maganja, Quitupo, Senga and Vila Palma (**table 3.8**). The HBI was 0.005 for *An. gambiae*, 0.31 for *An. funestus*, and 0.003 for *An. vaneedeni* (**table 3.8**). The optimised assay can successfully identify blood meal of human origin and the assay was subsequently used to test archived and newly collected samples from Mamfene, KZN.

Table 3.8. Human blood index (HBI) of *Anopheles* species from Mozambique

	Collection site A: Maganja	Collection site B: Quitupo	Collection site C: Senga	Collection site D: Vila_Palma	HBI
Blood fed specimens collected	125	204	25	6	
No. (and proportion) of *An. gambiae* containing human blood	3 (0.02)	0	0	0	0.005
No. (and proportion) of *An. arabiensis* containing human blood	0	0	0	0	0
No. (and proportion) of *An. funestus* containing human blood	42 (0.34)	83 (0.41)	8 (0.32)	1 (0.17)	0.31
No. (and proportion) of *An. leesoni* containing human blood	0	0	0	0	0
No. (and proportion) of *An. vaneedeni* containing human blood	1 (0.01)	0	0	0	0.003

HBI: human blood index

3.4.5. Blood meal status and source of *An. funestus* group from Mamfene

Blood meal status of 826 *An. funestus* group females in this study was used to classify them as non-fed (39.35 %, n= 325), blood fed (30.39 %, n=251), gravid (22.40 %, n=185) and half-gravid (7.51 %, n=62) (**table 3.9**). The blood meal assays used, successfully identified host DNA in 220 (87.65 %) of 251 blood-fed specimens belonging to all four species of *An. funestus* group identified in this study (**table 3.9**). Using the Kent and Norris (2005) PCR assay, 135 (54 %) of 251 fed females had their host DNA identified on first attempt using 1 µl template. Re-amplification using 1.5 µl template was performed for samples that failed on the first attempt, and obtained an additional 36 (14 %) positive blood meal identifications. Hence, successful amplification using the Kent and Norris (2005) assay was at 68 % (171 of 251). The remaining 80 samples were processed using Cahyadi *et al.* (2018) PCR assay, which positively identified host DNA for 49 additional fed females.

Table 3.9. Physiological status of *Anopheles* species from Mamfene, and blood meal identified using Kent and Norris (2005) and Cahyadi *et al.* (2018) PCR assays.

	Physiological status					
Species	Non Fed	Gravid	Half-gravid	Blood-fed	**BM identified (%)**	**BM not identified (%)**
An. leesoni	30	19	4	24	22 (92)	2 (8)
An. parensis	173	97	39	136	121 (90)	15 (10)
An. rivulorum	64	25	9	43	37 (86)	6 (14)
An. vaneedeni	58	44	10	48	40 (83)	8 (17)
Total	**325**	**185**	**62**	**251**	**220 (88)**	**31 (12)**

BM: Blood meal

DNA of human origin was not detected in any of the four *An. funestus* group species collected outdoors, resulting in an HBI of 0 for all four *Anopheles* species. Chicken DNA was not identified in either one of the fed females collected outdoors. The host preference of *An. funestus* group species from Mamfene was 71.71 % *Bos taurus* (cattle), 9.16 % *Capra aegagrus hircus* (goat), 1.19 % *Sus scrofa* (pig), and 0.40 % *Canis lupus familiaris* (dog) (**fig 3.8**). Only one mosquito, *An. parensis*, was identified positive for dog DNA (**fig 3.8).** Only 12.35 % of the blood fed females had unidentifiable blood meal origins.

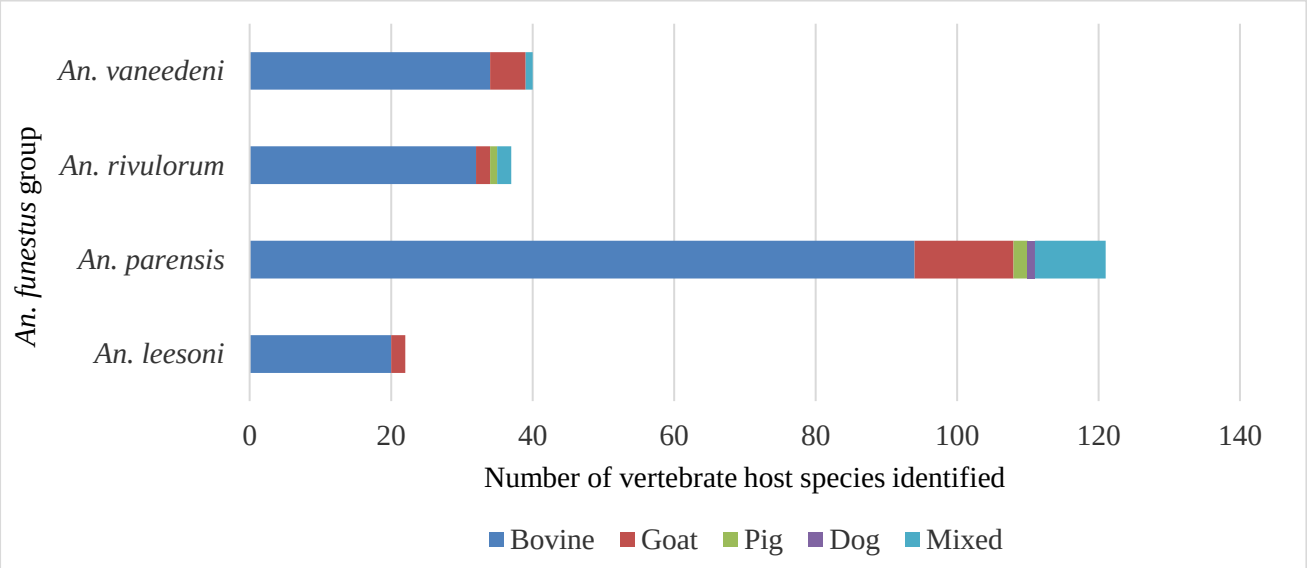

Figure 3.8. Overall composition of vertebrate hosts identified per *An. funestus* group species from KZN.

Three groups of mixed host species were identified, the most common of which was 3.98 % of mixed cattle and goat blood (n=10), then 0.40 % (n=1) of mixed pig and dog blood, and 0.80 % of mixed cattle and pig blood (n=2), (**table 3.10**). Mixed blood meal was detected in *An. parensis*, *An. rivulorum*, and *An. vaneedeni*, but not in *An. leesoni* (**table 3.10**).

Table 3.10. Mixed blood meal of *An. funestus* group species from Mamfene

Species	Cattle+Goat	Cattle+Pig	Pig+Dog
An. leesoni	0	0	0
An. parensis	9	0	1
An. rivulorum	1	1	0
An. vaneedeni	0	1	0
Total	**10**	**2**	**1**

3.4.6. Temporal pattern of climatic parameters

A general trend of all three climatic parameters in Mamfene, KZN, were modelled (**fig 3.9 - fig 3.11).** Mean monthly temperatures, mean monthly relative humidity, and total monthly rainfall were described for the period January 2015-December 2019 for which blood meal data was collected. All three parameters follow a seasonal pattern where the highest values are seen during the summer seasons, December-February, and the lowest values for all three parameters lowest during winter seasons, June-August.

3.4.6.1. Temperature

The highest temperatures, as high as 28.8 °C was experienced in summer (December – February), whilst the lowest temperatures were in winter (June-July) at 17.4 °C (**fig 3.9**). The Mann-Kendall test revealed that there is no trend in the monthly mean temperatures across the study years in Mamfene (Kendall's tau = -0.067, S= -1.000, Var (S) = 28.333 [-0.713, 0.829], *p* = 1.000). A *p* values > 0.05 indicates that the mean monthly temperatures were not the same between 2015-2019.

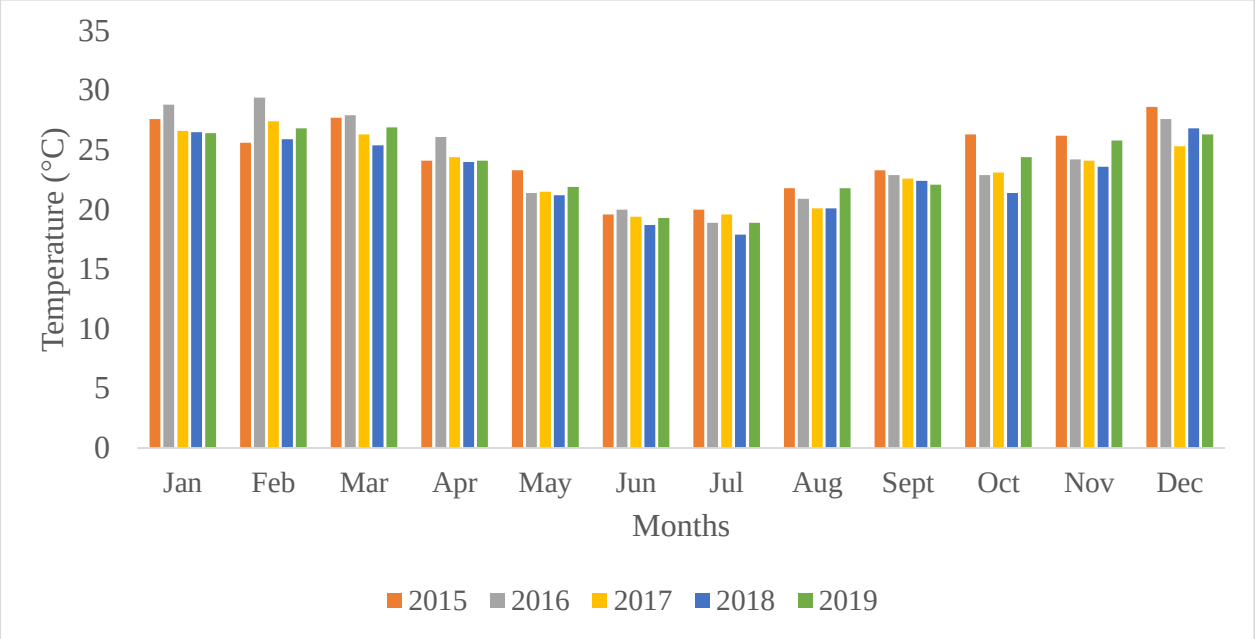

Figure 3.9. Temporal trend of temperatures in Mamfene, KwaZulu-Natal. Average monthly values for January 2015-December 2019.

A one-way ANOVA revealed that there was a statistical difference in mean monthly temperatures between at least two years ($F_{(5, 55)}$=[9.982], $p < 0.001$). Multiple comparisons of mean monthly temperatures for 2015-2019 using Tukey's HSD test revealed that the mean monthly temperatures were statistically different between six pairs of years (**supplemental table 3.1, appendix 3E)**. The years 2015 and 2016 experienced the highest mean monthly temperatures compared to the other years ($p < 0.01$, 95 % CI) (**fig 3.9**).

3.4.6.2. Relative humidity

Relative Humidity (%) were higher during the months of March-May (Autumn) as high as 79.3 % compared to other seasons. Mean monthly relative humidity values start to decrease in winter (June-August) and into spring (September-November) (**fig 3.10**). There was no trend in the monthly mean humidity values across the study years in Mamfene as revealed by the Mann-Kendall test (Kendall's tau = 0.333, S=5.000, Var (S) =28.333 [-1.467, 1.392], $p = 0.348$). A *p*-value > 0.05 implied that there was no trend in the data and that the mean monthly relative humidity values were not the same between 2015-2019.

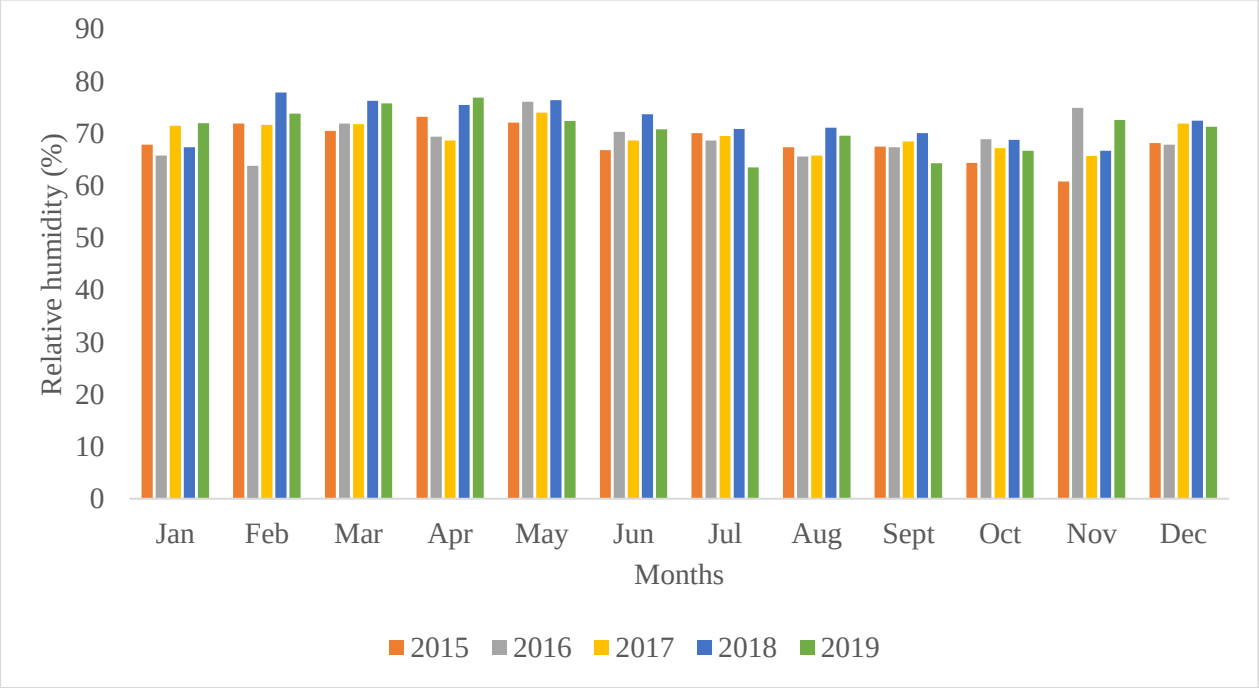

Figure 3.10. Temporal trend of relative humidity in Mamfene, KwaZulu-Natal. Average monthly values for January 2015-December 2019.

There was a statistical difference in mean monthly relative humidity values between at least two years ($F_{(5, 55)}$=[2.893], p = 0.0213) based on a one-way ANOVA. Tukey's HSD test for multiple comparisons found that the mean relative humidity values were statistically different only between 2015 and 2018 (p = 0.017, 95 % CI) (**supplemental table 3.2, appendix 3E)**, with 2015 having the lowest mean relative humidity compared to 2018 (**fig 3.10**).

3.4.6.3. Rainfall

Very little to no rainfall occurred in June, whilst high rainfall is mostly seen in summer (December – February), and beginning of autumn (March) (**fig 3.11**). The total monthly rainfall across the study years in Mamfene were different. The Mann-Kendall test revealed that there was no trend in these values (Kendall's tau = 0.000, S=-9.000, Var (S) = 28.333 [-71.200, 148.667], p = 0.091). The rainfall data (mm) was normally distributed for 2016 and 2017 (p > 0.05), but not for 2015, 2018 and 2019 (p < 0.05). The year 2015 experienced the lowest total rainfall (343.4 mm) in comparison to the other years, 386.6 mm for 2016, 441.6 mm for 2017, 789.4 mm for 2018 with the highest rainfall, and 485.4 mm for 2019 (fig 3.11). However, Friedman's test showed that there was no statistically difference in the mean annual rainfalls (ANOVA Chi Sqr. $X^2_{(12, 5)}$ = 9.196 p = 0.102).

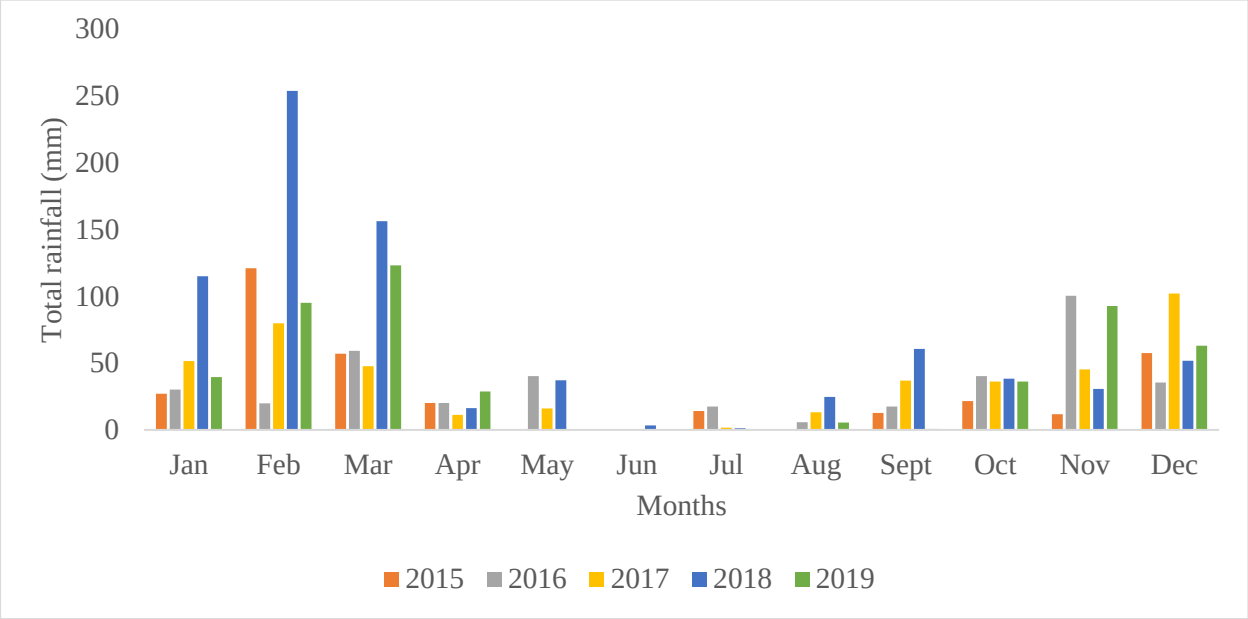

Figure 3.11. Temporal trend of rainfall in Mamfene, KwaZulu-Natal. Total monthly values for January 2015-December 2019.

3.4.7. Association between mean monthly feeding proportions and climatic parameters in archived and newly collected data, irrespective of host

Spearman's correlation and time series analysis were used to show the relationship between mean feeding proportions and climatic parameters for each species of the *An. funestus* group from Mamfene, in archived and newly collected data, irrespective of what vertebrate hosts was fed on. Archived blood feeding data were comprised of data from January 2015 to December 2016 (N=24), whilst newly collected data were comprised on data from May 2017-April 2019 (N=24). In both periods, data were combined into mean monthly feeding proportions expressed as percentages resulting in 12 data points in each period. Feeding proportions were calculated as the ratio of number of blood fed specimens of specific *Anopheles* species by the total number of blood fed specimens of all *Anopheles* species per month. As with feeding proportions in both collection periods, climatic data were also combined into monthly averages resulting in 12 data points in each period for each climatic variable.

3.4.7.1. *Anopheles parensis*

The mean feeding proportions for *An. parensis* were significantly lower for the archived period (Jan 2015 - Dec 2016) (Median = 0 %, n=12), compared to the new period (May 2017 - April

2019) (Median = 50 %, n=12), Mann-Whitney U = 14.000, Z = -3.320, p = 0.001. Spearman's correlation revealed that this difference was attributable to a positive correlation observed between the mean feeding proportions of *An. parensis* and mean relative humidity in the archived data (r = 0.593, p <0.05, 95 % CI) (**table 3.11, fig 3.10A**).

Table 3.11. Spearman's coefficient correlation (r-values) between mean feeding proportions of female sampled and mean monthly temperature, mean monthly relative humidity, and monthly total precipitation, for archived data (January 2015 – December 2016).

Climatic variables	*An. leesoni*	*An. parensis*	*An. rivulorum*	*An. vaneedeni*
	r-value	r-value	r-value	r-value
Monthly temperature (°C)	-0.118	0.232	0.408	0.437
Monthly Average Relative Humidity (%)	0.120	0.593*	0.140	0.416
Monthly Precipitation (mm)	0.100	0.416	0.250	0.621*

* Correlation is significant at the 0.05 level (2-tailed), p < 0.05, 95 % CI, as marked on STATISTICA

In the archived collection, there was a general increase in mean feeding proportions of *An. parensis* as mean relative humidity increased (**fig 3.12**). The highest feeding proportion of *An. parensis* (81 %) corresponded with the second highest mean relative humidity of 71.3 %; this pattern was observed in late summer (February) and in autumn (April) (**fig 3.12**). The lowest mean relative humidity of 66.5 % corresponded with no feeding for *An. parensis* (**fig 3.12**).

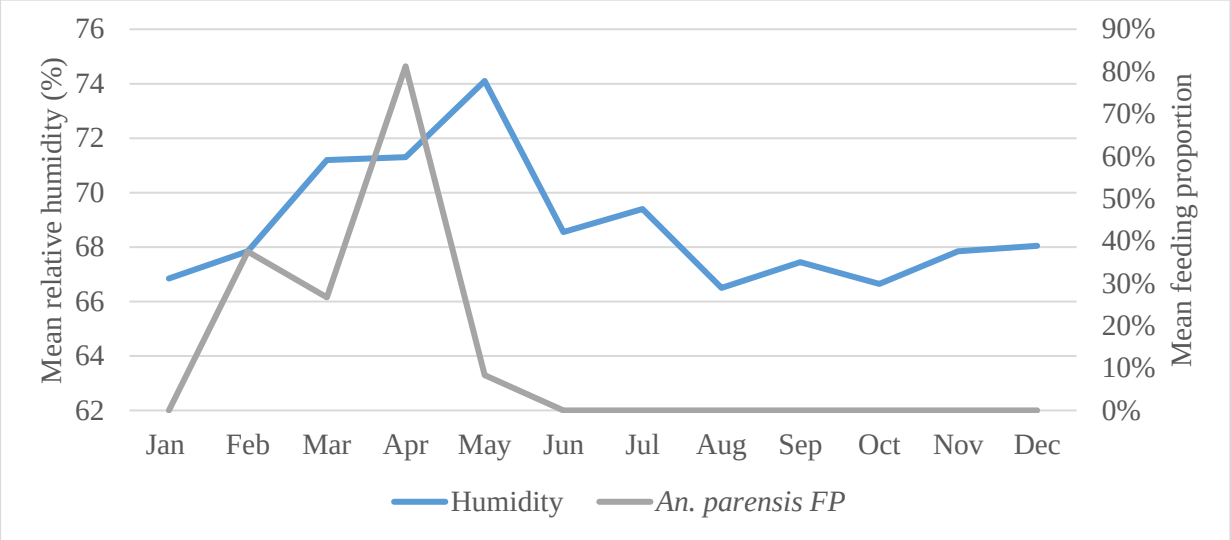

Figure 3.12. Time series correlation between mean monthly feeding proportions of *An. parensis* and mean monthly relative humidity in Mamfene. FP: feeding proportion.

3.4.7.2. *Anopheles vaneedeni*

The Mann-Whitney U test showed that the mean feeding proportions for *An. vaneedeni* were lower in the archived period (Jan 2015 - Dec 2016) (Median = 0 %, n=12) when compared to those in new period (May 2017 - April 2019) (Median = 10.1 %, n=12), $U = 31.000$, $Z = -2.338$, $p = 0.019$. The lower FP of *An. vaneedeni* in the archived data was associated with mean total precipitation, with strong positive correlation ($r = 0.621$, $p < 0.05$, 95 % CI) (**table 3.11, fig 3.13A**). Increasing rainfall of up to 70 mm was favourable for *An. vaneedeni* feeding up to 45 % FP (**fig 3.13A**).

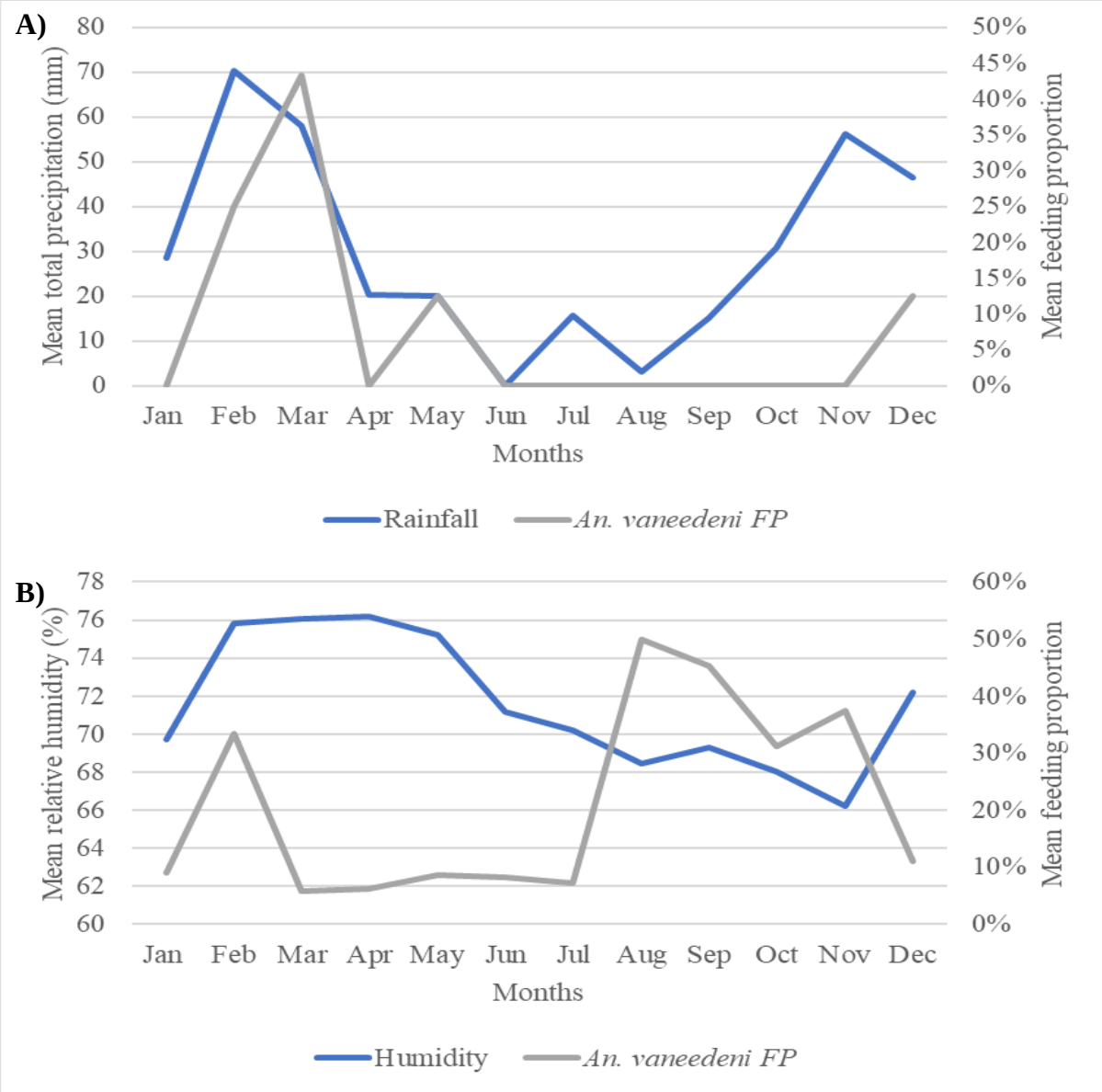

Figure 3.13. Time series correlation between mean monthly feeding proportions of *An. vaneedeni* and climatic parameters in Mamfene. A) correlation between mean monthly feeding proportions of *An. vaneedeni* and mean monthly rainfall in the archived period. **B)** correlation between mean monthly feeding proportions of *An. vaneedeni* and mean monthly relative humidity in the new period. FP: feeding proportion.

The mean feeding proportions of *An. vaneedeni* showed a strong negative correlation with mean monthly relative humidity in the new collection period (**table 3.12**). Their mean feeding proportions decreased with increase mean relative humidity (r = -0.692, $p < 0.05$, 95 % CI) (**fig 3.13B**). The highest mean feeding proportions for *An. vaneedeni* were recorded at the end of winter (August) and during spring (November) with lower relative humidity below 70 % (**fig 3.13B**).

Table 3.12. Spearman's coefficient correlation (r-values) between mean feeding proportions of female sampled and mean monthly temperature, mean monthly relative humidity, and monthly total precipitation, for newly collected data (May 2017 – April 2019).

Climatic variables	*An. leesoni*	*An. parensis*	*An. rivulorum*	*An. vaneedeni*
	r-value	r-value	r-value	r-value
Monthly temperature (°C)	-0.378	-0.252	0.538	-0.028
Monthly Average Relative Humidity (%)	-0.214	-0.088	0.655*	-0.692*
Monthly Precipitation (mm)	-0.513**	-0.368	0.328	0.147

* Correlation is significant at the 0.05 level (2-tailed), $p < 0.05$, 95 %, as marked on STATISTICA

** Correlation is significant at the 0.1 level (2-tailed), $p < 0.1$, 90 %, as marked on STATISTICA

3.4.7.3. *Anopheles rivulorum*

Mann-Whitney U test result indicated that the mean feeding proportions for *An. rivulorum* did not differ between the archived period (Jan 2015 - Dec 2016) (Median = 0 %, n=12) compared to the new period (May 2017 - April 2019) (Median = 5.4 %, n=12), $U = 67.000$, $Z = -0.260$, $p = 0.765$. However, feeding proportions of *An. rivulorum* showed a strong positive correlation with mean monthly relative humidity in the new period (r = 0.655, $p < 0.05$, 95 % CI) (**table 3.12, fig 3.12**). The highest feeding proportions were recorded during autumn, 44 % in March and 37 % in April, which corresponded to the highest relative humidity values, >76 % for both months (**fig 3.12**). Relative humidity < 70 % were not favourable for feeding above 15 %.

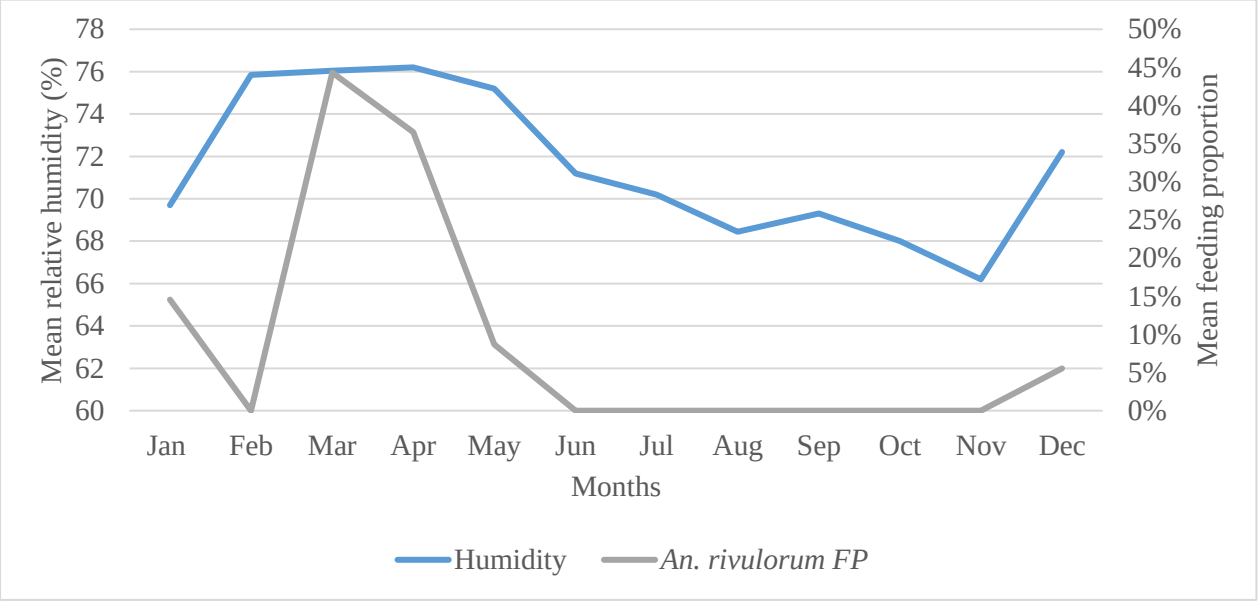

Figure 3.14. Time series correlation between mean monthly feeding proportions of *An. rivulorum* and mean relative humidity in newly collected data (combined May 2017-April 2019), from Mamfene. FP: feeding proportion.

3.4.7.4. *Anopheles leesoni*

The median mean feeding proportion of *An. leesoni* was higher in the archived period (Jan 2015 - Dec 2016) (Median = 14 %, n=12), compared to the new period (May 2017 - April 2019) (Median = 2.8 %, n=12), but not statistically different based on a Mann-Whitney U test, U =40, Z = 1.891, p = 0.059. Only 95 % CI was considered for significant correlations, however, it was noted that at 90 % CI only *An. leesoni* FPs showed strong association with rainfall and that was in the new collection period (**table 3.12).**

3.4.8. Association between annual feeding proportions and climatic parameters

Change in annual feeding proportions per *Anopheles* per vertebrate host species were evaluated and Spearman's correlation was used to show the relationship between those annual feeding proportions and climatic parameters for each species of the *An. funestus* group from Mamfene, KZN. Results are summarized in **table 3.13**. The relationships were studied for four collection years, year 1 represented 2015 (January-December, N=12), year 2 represented 2016 (January-December, N=12), year 3 represent May 2017-April 2018 (N=12), and year 4 represented May 2018-April 2019 (N=12).

Table 3.13. Proportion of host feeding by different species of the *An. funestus* group (n/N, % rounded off to the nearest percentage)

Host	Year	*An. leesoni*	*An. parensis*	*An. rivulorum*	*An. vaneedeni*	Annual mean temperature (°C)	Annual mean relative humidity (%)	Annual total rainfall (mm)
Bovine	Year 1-2015	11/13 (85%)	8/11 (73%)	2/3 (67%)	9/9 (100%)	24.5	68.4	343.4
	Year 2-2016	0	2/3 (67%)	3/3 (100%)	2/2 (100%)	24.3	69.2	386.6
	Year 3-May2017-April2018	5/5 (100%)	52/58 (90%)	15/16 (94%)	5/6 (83%)	23.1	70.7	792.8
	Year 4-May2018-Apr2019	4/4 (100%)	40/58 (69%)	14/17 (82%)	18/23 (78%)	23.0	72.4	534.8
Goat	Year 1-2015	2/13 (15%)	3/11 (27%)	1/3 (33%)	0	24.5	68.4	343.4
	Year 2-2016	0	1/8 (33%)	0	0	24.3	69.2	386.6
	Year 3-May2017-April2018	0	5/58 (9%)	0	1/6 (17%)	23.1	70.7	792.8
	Year 4-May2018-Apr2019	0	14/58 (24%)	2/17 (12%)	4/23 (17%)	23.0	72.4	534.8
Dog	Year 1-2015	0	0	0	0	24.5	68.4	343.4
	Year 2-2016	0	0	0	0	24.3	69.2	386.6
	Year 3-May2017-April2018	0	1/58 (2%)	0	0	23.1	70.7	792.8
	Year 4-May2018-Apr2019	0	1/58 (2%)	0	0	23.0	72.4	534.8
Pig	Year 1-2015	0	0	0	0	24.5	68.4	343.4
	Year 2-2016	0	0	0	0	24.3	69.2	386.6
	Year 3-May2017-April2018	0	0	1/16 (6%)	0	23.1	70.7	792.8
	Year 4-May2018-Apr2019	0	3/58 (5%)	1/17 (6%)	1/23 (4%)	23.0	72.4	534.8
Total		22	130	39	40			

3.4.8.1. Feeding on cattle

Chi-square statistics revealed that feeding proportions of cattle did not change for *An. parensis*, *An. rivulorum*, and *An. vaneedeni* over the four years, (**table 3.13**). For *An. leesoni*, their feeding proportions in year 1 (2015), year 3 (May 2017-April 2018), and year 4 (May 2018- April 2019) were the same. However, since no blood fed *An. leesoni* were collected in 2016 (**table 3.13**), a Chi-square test showed that *An. leesoni* fed less in 2016 compared to the other years, X^2(df=9, N=22) = 11.52, $p < 0.001$. Spearman's correlation between annual feeding proportions of *An. leesoni* on cattle and climatic parameters were not significant (**supplementary table 3.1, Appendix 3F**). Although there was no change in the cattle FP of *An. vaneedeni*, Pearson's correlation showed that its annual cattle FP had a strong positive association with mean annual temperature (r = 0.986, $p < 0.05$) and a strong negative correlation with mean annual relative humidity (r = -0.958, $p < 0.05$) (**supplementary table 3.1, supplementary fig 3.1, Appendix 3F**). Cattle FP of *An. vaneedeni* decreased with decreasing temperature in years 3 and 4 (**supplementary fig 3.1A, Appendix 3F**). Annual temperatures of ≥23 °C corresponded to ≥ 75 % FP on cow for *An. vaneedeni*. In contrast, cattle FP of *An. vaneedeni* decreased with increasing relative humidity also in years 3 and 4, but they still recorded ~80 % FP at 72 % humidity (**supplementary fig 3.1B, Appendix 3F**).

3.4.8.2. Feeding on goat

The feeding proportions on goat for *An. leesoni*, *An. rivulorum*, and *An. vaneedeni* did not change for the four years investigated (**table 3.13**). The goat feeding proportions of *An. parensis* were the same for year 1 (2015), year 2 (2016) and year 4 (May 2018- April 2019). The goat feeding proportion of *An. parensis* was significantly lower in year 3 (May 2017- April 2018) (5/58, 9 %) compared to year 4 (May 2018 – April 2019) (14/58, 24 %), X^2(df=9, N=130) = 10.70, $p < 0.04$. However, there was no indication that it was a result of vertebrate host shift. Additionally, correlating *An. parensis* annual FP with annual climatic parameters did not show an association to explain the lower FP in year 3 (**supplementary table 3.1, Appendix 3F).**

3.4.8.3. Feeding on dog

There was no significant change in feeding on dog blood for all four *Anopheles* species as none of them fed on dog blood as detected by PCR, except for one *An. parensis* female with dog

blood in both year 3 and year 4 (**table 3.13**). Chi-square test result indicated that dog FP of *An. parensis* were the same for all collection years. Although Spearman's correlation indicated that the annual dog FPs of *An. parensis* had significant positive correlation with mean annual temperatures (r = -0.993, $p < 0.05$) (**supplementary table 3.1)**, it is important to note that only one *An. parensis* fed on dog in years 3 and 4 each (**table 3.13).**

3.4.8.4. Feeding on pig

Blood meal PCR did not detect pig blood DNA in fed *An. leesoni* for all the collection years. *Anopheles parensis* and *An. vaneedeni* that fed on pig were only detected in May 2018-April 2019 (**table 3.13**), but not significantly higher than subsequent years X^2(df=9, N=130) = 3.72, p>0.1 and X^2(df=9, N=40) = 0.739, $p > 0.1$. However, there was no significant association for these with climatic parameters. There was no change in annual pig FP for *An. rivulorum*, even though pig blood was only detected in the species in years 3 and 4 (**table 3.13**). There was no significant correlation between pig FP of all *Anopheles* species and the three climatic parameters (**supplementary table 3.1, Appendix 3F**).

3.5. Discussion

3.5.1. Blood meal sources of outdoor *An. funestus* group in Mamfene

The HBI for this study was zero, because none of the fed-female species of the *An. funestus* group had taken a human blood meal. This can be attributed to the wider range of non-human vertebrate hosts that were available outdoors as host preferences. The findings of this study confirmed that all four *Anopheles* species identified from outdoors in Mamfene area, showed no anthropophilic tendencies, instead they were solely zoophilic. All four species of the *An. funestus* group identified in this study, *An. leesoni, An. parensis, An. rivulorum*, and *An. vaneedeni*, showed strong preference for feeding on cattle, followed by feeding on goats. No chicken DNA was detected in any of the blood fed *Anopheles* females in this study, implying that chickens are not the preferred host of the species of the *An. funestus* group. Our conclusion could be explained from the study done in *An. arabiensis*, a malaria vector and a member of the *An. gambiae* complex, where Jaleta *et al.* (2016) discovered that chicken volatiles repel host-seeking *An. arabiensis*. Thus the volatiles exuded by chickens repelled host-seeking mosquitoes (Jaleta *et al.*, 2016), and this could also be the case for the species of *An. funestus* group

identified in this study. Although a previous study had identified chicken DNA in 3.75 % (N=169) amongst their blood fed *An. parensis* females collected from Mamfene (Mouatcho *et al.*, 2007), it is important to note that an ELISA method, rather than PCR, was used by Mouatcho *et al*. (2007) for blood meal identification. This ELISA method is based on detection of host IgG levels, and cross-reactivity may occur and lead to inaccurate results (Hunter and Bayly, 1991; Kent, 2009; Koekemoer, *pers comm*). Recently, a study reported on the artificial feeding of *An. funestus* using chicken blood, and was done under laboratory conditions (Ngowo *et al.*, 2021). This means that *Anopheles* can feed on chicken blood but only under controlled conditions.

Host preference is affected by animal/human ratio, and in areas where there are more animals than humans, mosquitoes tend to be more exophilic and zoophilic. It is evident that *An. leesoni, An. parensis, An. rivulorum,* and *An. vaneedeni* resting outdoors in Mamfene are mainly zoophilic. However, in settings where members of the *An. funestus* group were collected indoors or using human baited traps, human DNA was detected in the blood fed specimens (Mouatcho *et al.*, 2007; Temu *et al.*, 2007; Kweka *et al.*, 2008; Kawada *et al.*, 2012).

The PCR assays used were successful at identifying mixed blood meals in this study, which confirmed the findings of Kent and Norris (2005) that "multiple blood meals from different mammals could also be detected in a single mosquito". More importantly, this study showed that blood meals can also be identified from long term dried-stored female mosquitoes. The blood meal identification assay employed at the extraction step appeared to have contributed to the high success rate of samples stored since 2015. Host DNA failed identification in 12.35 % (31/251) of our fed females. However, this was most probably justified by the low quality of extracted DNA as a result of blood meal digestion within the specimen prior to collection. Alternatively, unidentified host DNA in *An. leesoni*, *An. parensis*, *An. rivulorum*, and *An. vaneedeni* may have fed on vertebrates other than the ones targeted in our assays. Such other possible vertebrate species found in Mamfene area are cats. Cat DNA has been successfully isolated from blood fed *Anopheles* species in Sri Lanka (Gunathilaka *et al*., 2016). Horse and sheep are also found in KZN, but these were not common at the Mamfene households where wild *An. funestus* members were collected from (Dr Givermore Munhenga, pers. comm)."

Various other factors are known to affect successful blood meal identification, including the post-feeding time of mosquitoes before storage during which enzyme degradation of host DNA in the mosquito gut occurs; the various ages of blood fed individual mosquitoes; storage time, DNA extraction method for host DNA, as well as the method of preservation (Reeves *et al.*, 2016). In this study the only method of mosquito preservation used was desiccation on silica gel tubes, a method which Reeves *et al.* (2016) proved to be less effective at maintaining the integrity of host DNA and should be avoided. However, the method developed by Kent and Norris (2005) for mosquito blood meal identification, was successful at amplifying host DNA from mosquitoes preserved on silica. This is because the method targets shorter amplicons, which are well suited for degraded host DNA. The target genes used, the *cytochrome b* gene, as well as the *12S rRNA* gene, are mitochondrial genes that have inter-species variation that help in discriminating between closely related species. They are present in multiple copies, thus increasing the chance of detection even with degraded DNA.

My study was limited in the fact that only outdoor feeding/resting mosquitoes were collected, and blood specimens constituted a low sample size. However, because it was a longitudinal study, it confirmed the principal zoophilic nature of outdoor members of the *An. funestus* group in Mamfene. Nonetheless, it would be beneficial to undertake a longitudinal study with the same objective, for indoor and outdoor feeding/resting *Anopheles* in Mamfene, to fully establish their ecology. Additionally, I could not determine the host feeding index (HFI), which is the ratio of proportion of blood feeds on one host with respect to another by the expected comparative proportion of feeds on those two hosts (Richards *et al*., 2006; Gunathilaka *et al*., 2016), neither the forage ratio (FR), described as preference of a particular vertebrate over other available hosts (Hess *et al*., 1968; Gunathilaka *et al*., 2016). Calculations of these blood indices require knowledge of the total number of available hosts and number of study sites, which were not recorded in this study due to lack of resources and personnel to do household tally of the different hosts. Other limitations included the lack of measured DNA concentration for each fed female processed. This would have helped in determining the limit of detection (LOD) for blood meal PCR. However, DNA concentration could not be measured for every specimen extracted, as the volume of available DNA, of 30 µl, was insufficient to do so. This is because LOD is determined by doing a serial dilution of the positive sample with the lowest concentration. In this study, only fed (F) females were processed for blood meal analysis, whilst one other study successfully identified host DNA from half-gravid, gravid and even physiologically unfed females using an ELISA method (Bashar *et al.*, 2012).

Owing to the disadvantages of low sensitivity and specificity in using ELISA for host blood meal identification, I suggest that future bionomic studies into host preferences can consider expanding blood meal identification to all physiological statuses of female mosquitoes, to see if the same phenomenon by Bashar *et al*. (2012) can be replicated using PCR assays. However, certain aspects would have to be considered, such as the sensitivity of the molecular assays and the size of the mosquito. Species of the *An. funestus* group are relatively smaller in size (average 2.50 mm wing length) compared to *An. gambiae s.s.* (average 2.94 mm wing length) (Mwangangi *et al.*, 2004; Ngowo *et al.*, 2021). Thus, species of the *An. funestus* group take smaller blood meals, which may decrease the success of blood meal identification in this group. Other more sensitive methods for host identification to overcome this limiting factor includes, targeted high-throughput (Illumina) sequencing method (Logue *et al.*, 2016). However, sequencing methods are still time consuming, costly and technically demanding. Niare *et al*. (2016), were first to explore the novel use of MALDI-TOF MS proteomic method for host blood meal identification in freshly engorged fed mosquitoes for up to 24 hours post-blood meal. It proved to be a rapid tool and present a dual advantage in identifying the arthropod species as well as its blood meal origin (Niare *et al*., 2016; Tandina *et al*., 2020). This proteomic method been shown to be successful in detecting multiple blood meals (Tandina *et al*., 2020).

3.5.2. Role of climatic parameters on monthly feeding proportions of individual species of the *An. funestus* group, irrespective of vertebrate host

When comparing the mean feeding proportions (FP) between the archived data (January 2015 – December 2016) and newly collected data (May 2017 – April 2019), the mean FP of *An. leesoni*, and *An. rivulorum* did not change ($p > 0.05$, 95 % CI). This means that irrespective of the fed female sample sizes across the two collection periods, the mean feeding proportion for these two species did not differ. However, the mean feeding proportion of *An. parensis* was significantly lower in the archived collection (January 2015 – December 2016) in comparison to the newly collected data (May 2017 – April 2019).

3.5.2.1. *Anopheles parensis*

Correlation showed that the significantly lower mean monthly FP of *An. parensis* in the archived collection (2015-2016) was associated with mean relative humidity in a positive correlation. The lower feeding proportion of the species in the archived period raised the question whether this

phenomenon could have been a result of extreme drought experienced in the years 2015 – 2016. The most common form of drought in ecological studies, meteorological drought, is defined as the occurrence of less than normal rainfall, mainly characterized by increased atmospheric temperatures and low relative humidity (Wilhite and Glantz, 1985; WMO, 1992). It is widely known that the province of KwaZulu-Natal experienced drought in recent years. Various studies have assessed the drought in the province and its impact on the livelihood of its inhabitants (Abbas *et al*., 2019; Vetter *et al*., 2020; Ndlovu and Demlie, 2020). These evidence-based studies all indicated that KZN experienced the worst drought during 2015/2016, with 2015 being the driest as a result of the negative El-Ninõ southern oscillation (ENSO) which is associated with drought conditions (Ndlovu and Demlie, 2020).

Climatic variables pre-2014 for Mamfene in KZN were not retrieved, and based on our analysis there was no statistical difference in monthly rainfall for 2014-2019. However, in a study conducted by Ndlovu and Demlie (2020), rainfall data for KZN spanning 48 years (1970-2017) indicated that 2015 and 2016 were the driest years. This backs up our findings that 2015 and 2016 recorded the lowest total annual rainfalls of 343.4 mm and 386.6 mm, although statistically insignificant to the other years. These annual rainfalls are below our Mamfene annual average of 476.8 mm/year, and also below the South African and World annual averages of 500 mm/year and 860 mm/year respectively (Ndlovu and Demlie, 2020). To add to the definition of meteorological drought, our analysis showed that 2015 and 2016 had the highest mean monthly temperatures. Additionally, 2015 also had the lowest relative humidity compared to the years in our study, but was only statistically significant from 2018. It can therefore be implied that 2015 met all three criteria for a meteorological drought period.

Against this background, the lower feeding proportion of *An. parensis* in the archived period (2015-2016) was possibly a result of drought conditions. This is based on the sample population with a blood meal. In addition, the study found that the mean feeding proportions of *An. parensis* in the archived data (2015-2016) were strongly affected by mean relative humidity (%). Hence, the low mean relative humidity influenced the low mean feeding proportion. The opposite is also true, this implies that *An. parensis* females are more active and feed more with increasing humidity. This coincides with a report which indicated that relatively high humidity is a favourable condition which allows mosquitoes to survive longer, thus reproducing more (Yamana and Eltahir, 2013; Drakou *et al.*, 2020), which subsequently increases the need for a

blood meal to develop more eggs. Another factor that could explain why *An. parensis* fed less stems from studies which indicated that drought had resulted in loss of cattle and vegetation in KZN (Abbas *et al*., 2019; Vetter *et al*., 2020), but that the impact of herd mortality and decrease in grass biomass were most apparent in 2016 (Abbas *et al*., 2019; Vetter *et al*., 2020).

3.5.2.2. *Anopheles vaneedeni*

The mean feeding proportion of *An. vaneedeni* negatively correlated with mean relative humidity in the new collection period, especially during autumn (March-May) and (June & July). These periods experience higher rainfall. The species' FP of >10 % during those periods were recorded at mean relative humidity between 66 %-70 %. It has been established that favourable humidity levels have a ripple effect on other mosquitoes' behaviours, including increased biting (Yamana and Etlahir, 2013; Kumar and Reddy, 2014; Drakou *et al*., 2020). Rainfall was also a strong determinant for feeding by *An. vaneedeni* in the archived period, with a positive association. Rainfall is required for the formation of breeding sites for mosquitoes (Yamana and Etlahir, 2013; Kumar and Reddy, 2014), and increasing rainfall increases the number of water bodies for breeding. Although not statistically significant, lower annual rainfall (~365 mm) was recorded for the archived period compared to the new period (~664 mm). This seems to have strongly affected feeding proportion of *An. vaneedeni* in the archived period, resulting in lower FP in the archived period.

3.5.2.3. *Anopheles rivulorum*

The mean feeding proportions of *An. rivulorum* was higher in the archived data (January 2015 – December 2016) compared to the newly collected data (May 2017- April 2019), however not statistically significant. Noticeably, their feeding proportions showed positive correlation with temperature, relative humidity and precipitation in both collection periods, but was only strongly positively associated with mean relative humidity in the new collection period. Increasing relative humidity positively influences mosquitoe's survival, and have been reported to be more active at high humidity > 60 % (Kumar and Reddy, 2014). Mean relative humidity was significantly higher in 2018 (new period) compared to 2015 (archived period), and this may have positively influenced the feeding proportions recorded. Relative humidity had a significant impact on the feeding of *An. rivulorum* in the new collection, where feeding was only recorded when relative humidity was > 60 %, and they appear to tolerate relative humidity up to 76 %.

3.5.2.4. *Anopheles leesoni*

There was no significant correlation between the mean FPs of *An. leesoni* and the three climatic parameters in the archived and new collections. This is in contrast with *An. parensis*, and indicates that different species exhibit different ecological behaviours. Although not significant at $p < 0.05$, the mean feeding proportion of *An. leesoni* had a stronger negative correlation with rainfall in the new collection period (May 2017-April 2019) at 90 % CI. Increasing rainfall increases the number of water bodies for breeding (Yamana and Etlahir, 2013; Kumar and Reddy, 2014). However, it appears that increasing rainfall inhibited blood feeding by *An. leesoni* in this study, at 90 % CI. Reasonably so, rain presents an obstacle to host-seeking. Malaria is seasonal and transmission peaks during the warm and rainy seasons whilst it is lower during cool and dry seasons (Raman *et al.*, 2016; Ikeda *et al.*, 2017), and this is a result of increasing biting activity of *Anopheles* at warmer temperatures (Shapiro *et al*., 2017), and subsequently during rainy seasons. However, a study by Vantaux *et al.* (2021) have found that the biting rate/feeding proportion of various *Anopheles* species was higher during the dry and cool season compared to the rainy and warm season, which appears to be the case for *An. leesoni* in our study.

3.5.3. Role of climatic parameters on annual feeding proportions for individual *Anopheles* species, and per vertebrate host

Monthly feeding proportions per *Anopheles* species per vertebrate host in this study was not possible. Due to the small sample size, calculating monthly FP per host in this scenario thinned out the data resulting in many zeros that did not allow performing statistical tests for analysis. Hence, the study reported annual data to establish change in feeding proportions for each *Anopheles* species on each vertebrate host species they fed on, and how they correlated with the annual climatic parameters under investigation. Overall, there was no change in the annual FP on dog and pig by *An. leesoni, An. parensis, An. rivulorum,* and *An. vaneedeni*. There was a change in annual cattle FP of *An. leesoni*, and a change in annual goat FP of *An. parensis*, but none of these changes were influenced by any of the three climatic parameters assessed.

Although there was no change in annual FP of *An. vaneedeni* on cattle, correlation based on temporal pattern of feeding showed that their FP were generally influenced by mean annual temperature and relative humidity. *Anopheles vaneedeni* FP on cattle increased with increased

temperature and vice versa. This correlates with findings that biting rates and feeding frequencies of mosquitoes in general increase at warmer temperatures (Lee *et al*., 2013; Shapiro *et al*., 2017). This is because, at warmer temperatures, cell metabolism is increased causing a mosquito to digest blood meal faster and causing them to feed more often (Lee *et al*., 2013). Additionally, warmer temperatures are known to decrease the gonotrophic cycle of *Anopheles*, so that the time between a blood meal, oviposition and the next blood meal is shortened (Walton and Reisen, 2014; Shapiro *et al*., 2017; Agyekum *et al.*, 2021). In this study, mean annual temperatures were warmer in 2015 and 2016 where *An. vaneedeni* FP on cow were higher. However, *An. vaneedeni* FP on cattle had an inverse relationship with relative humidity in that humidity of >70 % caused a decline in their FP. This confirms findings that relative humidity of 60-70 % is favourable for mosquitoes' survival, giving them prolonged time to feed more frequently (Yamana and Eltahir, 2013; Kumar and Reddy, 2014; Drakou *et al.*, 2020). In summary, it appears that the climatic parameters studied here did not affect the host choice of members of the *An. funestus* group, but rather how much they feed in terms of feeding proportions.

CHAPTER FOUR

Impact of a blood meal on the activity of esterases

4.1. Introduction

4.1.1. Role of esterases in blood fed mosquitoes

The salivary glands and midgut are directly involved in transmission of malaria parasites (Lehane, 2005). Digestion of blood meal and non-blood (sugar) meal in insects is mediated in the midgut. Different digestive enzymes, including invertases, amylases and esterases, play digestive roles in the gut (Gooding, 1972, 1977; Lehane, 2005). The question remains which of these play a role in blood digestion, and which in non-blood digestion. Freyvogel *et al.* (1968) presented one of the first reports of esterase activity. They had identified multiple high levels of non-specific esterases in the stomach epithelium of 14 mosquito species including *An. stephensi*. The authors observed that esterases may play a role in mediating lipids to digest blood meals.

To note, blood meal from different hosts can induce different digestive enzymes in an insect (Lehane, 2005). For example, blood from avian hosts contain nucleated red blood cells (RBCs), which may induce high levels of digestive DNAses (Lehane, 2005). However, humans and other mammalian vertebrates have anucleated blood cells, which may induce different enzymes. The serum of mammalian vertebrates contain proteinase inhibitors, and insects need to overcome such obstacle in order to digest a blood meal (Lehane, 2005). The overcoming mechanism can either be the stimulation of the insect to secrete more trypsin, or to secrete invertase, amylase, and/or esterases (Freyvogel *et al*., 1968; Gooding, 1972). Using genomic and proteomic approaches in *An. gambiae*, Marinotti *et al.* (2006) and Cui *et al.* (2020) showed that blood meal triggered midgut cells to secrete enzymes useful for blood meal digestion. The most common enzyme they identified were trypsin-like peptidases, a subclass of esterases. Other midgut esterases can be involved in reducing the virulence of pathogens to protect the vector. For instance, the lethality of *Pseudomonas*, a gram-negative bacteria present in the midgut of many mosquitoes, was reduced as a result of increased arylesterase activity in the midgut of *Drosophila melanogaster* (Stoltz *et al.*, 2008).

In addition to the esterase role in the midgut, certain proteins were found to be either upregulated or downregulated in the salivary glands of *An. gambiae* and *An. culicifacies* post blood meal (PBM) (Marinotti *et al.*, 2006; Rawal *et al.*, 2016). An example were hydrolytic

enzymes, some of which could be esterases, were downregulated PBM compared to the sugar fed group (Rawal *et al.*, 2016). Female specific esterases, EST-A, and -B, were isolated from the gland cells of *An. stephensi* (Poehling and Meyer, 1980). Poehling and Meyer (1980) showed that these esterases had strong phosphatase activity and their functions include production and transport of secretions, as well as energy metabolism. It is also possible that EST-A and -B play a role in the mechanism of *Plasmodium* parasites transmission based on their localization in the mosquito salivary gland. Moreover, it appears that blood meal launches a cascade of interconnected events. Rawal *et al.* (2016) found that aldehyde dehydrogenase was overexpressed PBM, and this protein regulates Juvenile hormone (JH) which subsequently promotes reproductive maturation in adult female mosquitoes. Additionally, the hydrolysis of JH is catalysed by JH esterase, a carboxylesterase (Edgar *et al.*, 2000).

4.1.2. Isoenzyme gel electrophoresis method for the study of esterases

Biochemical assays are widely used for the study of enzyme activity and expression. Such assays include polyacrylamide electrophoresis (PAGE) method (Micales and Bonde, 1995), which help broaden the study of enzymes, specifically of esterases. The native PAGE method is suitable for esterases, since these are isoenzymes with similar functions but different molecular sizes (Micales and Bonde, 1995). Hence maintaining their native nature aids in separating the different forms of esterases based on size. Dahan-Moss and Koekemoer (2016) used this method to identify nine common esterases in the laboratory colonised *An. funestus* from Mozambique, (FUMOZ) (Hunt *et al.*, 2005), as well as in wild male and female adults of *An. funestus* from Zimbabwe (FUZIM) (Dahan-Moss and Koekemoer, 2016). They denoted them EST-9 to EST-1 using the naming system of Mahon *et al*. (1976).

Amongst the nine esterases, Dahan-Moss and Koekemoer (2016) identified EST-2 as a female-specific esterase. This esterase was further classified as an arylesterase, based on this, its activity may carry physiological and adaptive significance that could influence certain characteristics such as insecticide resistance (Dahan-Moss and Koekemoer, 2016). Another female-specific arylesterase was identified in the salivary gland of female *An. stephensi* Liston (called Esterase-A) (Poehling and Meyer, 1980). If the EST-2 identified by Dahan-Moss and Koekemoer (2016) shares the same localization as EST-A, it could also be present in the salivary gland of *An. funestus* females, and potentially play a role during blood-feeding and consequently in the transmission of *Plasmodium* parasites. Alternatively, or additionally, this esterase could play a potential role in insecticide resistance. The body of evidence presented in the introduction, on

the effect of blood feeding on the underlying physiological mechanisms of *Anopheles* mosquitoes, and a special interest on the female-specific EST-2 with potential role in parasite transmission, provide the basis for studying esterases. Understanding the mechanism of blood meal induced esterases could provide information useful to target mosquitoes for vector control.

Identifying and characterizing these enzymes was achieved using non-denaturing native PAGE (Dahan-Moss and Koekemoer, 2016). No characterization was required in this study as Dahan-Moss and Koekemoer (2016) had already characterized those esterases based on inhibition criteria. In this study, the activities of the 9 esterases were investigated in the different body parts of *An. funestus* females. Freyvogel *et al.* (1968) showed that studying the distribution of esterases helps in elucidating their function(s) in mosquitoes. This study used two soaking solutions to identify the esterases, the first being a solution containing alpha naphthyl acetate since all nine esterases had preferentially hydrolysed the alpha naphthyl acetate substrate (Dahan-Moss and Koekemoer, 2016). The second solution contained alpha naphthyl acetate and Fast Blue RR (4-benzoylamino-2‘,5’-dimethoxy-aniline), a dye used to stain the native PAGE gels. Fast Blue RR binds to the esterase reaction product of alkaline phosphatase and precipitates it as an insoluble compound that appear black on the enzyme position on the gel, resulting in the formation of a visible band (**fig 4.1**).

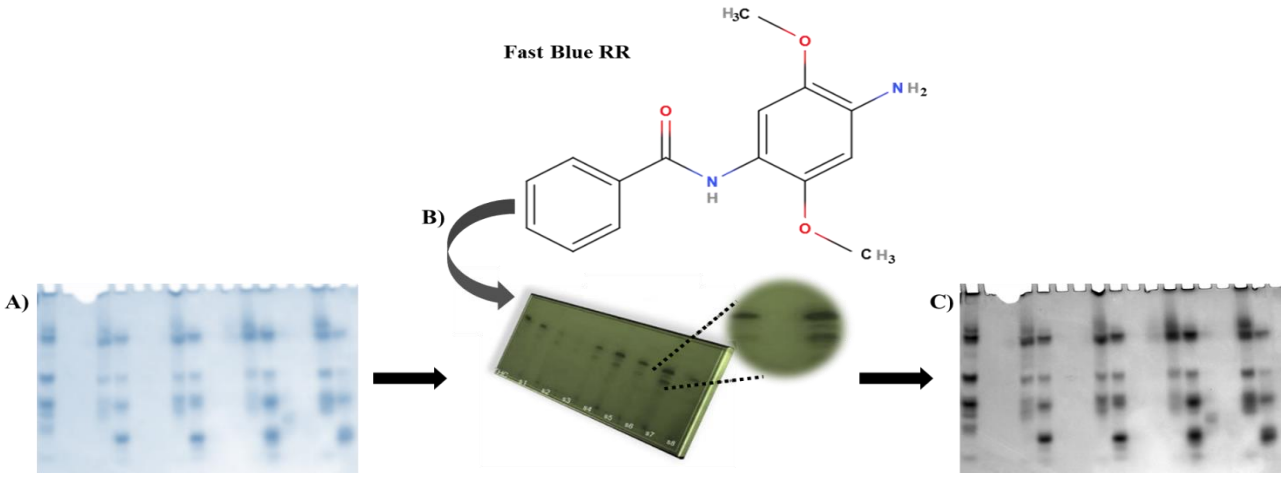

Figure 4.1. Principle of Fast Blue RR dye in isoenzyme gel electrophoresis. A) Zymogram of an unstained gel after incubation in soaking solution in which alpha-naphthyl acetate is hydrolysed to produce an alkaline phosphatase compound called naphthol appearing as blue on the gel. **B)** the gel is soaked in a second soaking solution of alpha-naphthyl acetate plus Fast Blue RR dye, which binds to the naphthol compound and precipitates it into an insoluble compound appearing black at enzyme position on the gel. **C)** Zymogram of the isoenzyme gel after distaining background and enzyme bands become more visible. **Citation:** 2D chemical structure of Fast Blue RR drawn using MolView free online web application (Bergwerf, 2015).

To my knowledge, there has not been any study into the impact of a blood meal on the activity of esterases in *An. funestus*. Gathering knowledge on the different activities of esterases post blood meal in *An. funestus* may contribute to developing malaria blocking strategies. Such knowledge will be achieved by studying the function of esterases, and how their contributory role in development and reproductive maturity directly or indirectly contribute to parasite transmission.

4.2. Objectives

The main aim of this study was to determine whether the activities of the nine esterases, EST-9 to EST-1, are affected by blood feeding in *An. funestus* females.

4.2.1. Specific Objectives

1. To determine the activity levels of the nine esterase, in the different body sections of *An. funestus* females, as function of blood meal, age, and mating.
2. To determine which of the female-specific esterase, EST-2 identified by Dahan and Koekemoer is specific to blood feeding in *An. funestus*.

4.3. Materials and methods

4.3.1. Experimental design

Three experiments were conducted in this study and consisted of a blood meal experiment, mating experiment, and age experiment. All experiments were set up in the Botha de Meillon insectary using female mosquitoes of the FUMOZ colony. Two gels with two biological repeats on each gel were electrophoresed for each experiment to allow analysis of four biological repeats. Sufficient *An. funestus* females, and males where necessary, were collected to account for this. Each biological repeat comprised three tests, test 1 was the upper body (head and thorax), test 2 was the abdomen, and test 3 was the spermatheca.

Blood meal experiment

The blood meal experiment was set up to investigate whether blood meal affected the activities of esterases, by studying blood-fed (B) versus non-blood fed females. Twenty-five 3-day old unmated females, were placed into two cages each. In the first cage, females were supplemented with 10 % sucrose solution until day 10 (**Appendix 4A)**, and in the second cage, the females were also maintained with 10 % sucrose solution, then were fed on a guinea pig at day 10. Live females from both cages were collected when females were 10 days old. Females were then placed in 1.5 mL microcentrifuge tubes and prepared as described section 4.3.2 below.

Mating experiment

This was set up to investigate how the activities of esterases were affected in mated versus non-mated females. Newly-emerged females (n=25) were placed into a cage and fed with 10 % sucrose solution to represent the *non-mated* group. The second cage contained 25 newly-emerged females and 50 newly-emerged males (1 female: 2 males) and were allowed to mate until day 10, and represented the *mated* group. A ratio of 1 female: 2 males supplemented on 10 % sucrose solution allows for ~60 % insemination success (Kalonji, 2021). The mating experiment was done in duplicate to increase the chances of collecting 20 successfully inseminated females altogether for four biological repeats of five females each, as not all females would have been inseminated in the first set-up. Adults in the *mated group* had access to 10 % sucrose solution for the duration of the experiment. At day 10, the females from *non-mated* and *mated* groups were collected and prepared as described below.

Age experiment

The age experiment was set up to investigate the activities of esterases in newly-emerged (8-12 hrs post-eclosion) versus unmated adult females (10 days post-eclosion). The 10-day period was selected so to replicate *An. funestus* longevity in the wild. The longevity of adult female *Anopheles* generally live on average 10-14 days in the wild, even though they can live up to a month when a captivity (Davidson, 1954; Service and Townson, 2002). Newly-emerged females were between 8-12 hours post-eclosion. Twenty-five newly-emerged females placed in one cage and fed with 10 % sucrose solution, were allowed to age until day 10 to represent the *adult* group, whilst another 25 newly-emerged females were immediately collected to represent the *newly-emerged* group, and were prepared as below.

4.3.2. Preparation of specimens

All collected specimens were immediately euthanized in the fridge. All subsequent preparations were done on ice, to prevent loss of enzyme activity.

Blood meal experiment

Freshly fed females were visibly examined for blood in the abdomen, to confirm that they had a blood meal. Examination by light microscopy was not necessary, as the females were freshly fed.

Mating experiment

The spermatheca capsule (where female mosquitoes store sperm) were dissected out of the abdomen as described by Ungureanu (1972). The spermathecae were placed on a slide with PBS, and examined under the Olympus BX50 microscope, connected to a camera with software (Olympus Stream Essentials.Ink). Successfully mated females had evidence of sperm, filamentous-like structures, in their spermatheca, whilst females who had not mated had empty spermathecae (**fig 4.2**).

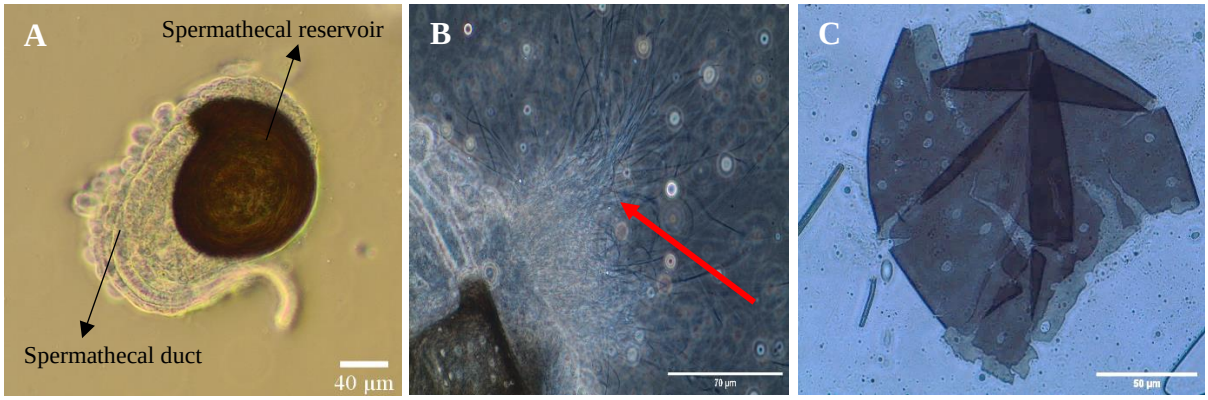

Figure 4.2. Spermatheca of *Anopheles funestus* female. **A.** Spermathecal structure from a mated female, 100x magnification. **B.** Ruptured spermatheca from a mated female, showing filamentous-like sperm structures indicated in red, 400x magnification. **C.** Ruptured spermatheca from a non-mated female, no evidence of sperm as evident from the transparent morphology, 400x magnification.

Pooling of body sections

In order to investigate the expression level of EST-2 in *An. funestus* females, after collection from the insectary, each female specimen was separated into three body sections, the upper body (head including mouth parts, and thorax), the abdomen (including reproductive organs, excluding the spermatheca), and the spermatheca (**fig 4.3.**). The isoenzyme experiment and all reagents used are described (**Appendix 4B**).

For each experiment, separate body sections were pooled from five individual females; this number was estimated to represent a full female body. Each body section from five females was pooled into a 1.5 mL microcentrifuge tubes containing 80 µl grinding buffer (10 % sucrose, 33% Tris-boric acid-ethylenediaminetetraacetic acid (TBE) buffer, pH 8.9). Pooling the same body parts from five samples provided sufficient homogenate to allow for isoenzyme analysis. The wings and legs were excluded. Pooled samples were homogenised manually with a clean sterile pestle. The homogenised samples were kept frozen (-70 °C) until processing, to prevent degradation of enzyme activity before analysis could be performed, but no longer than 12 months (Micales and Bonde, 1995).

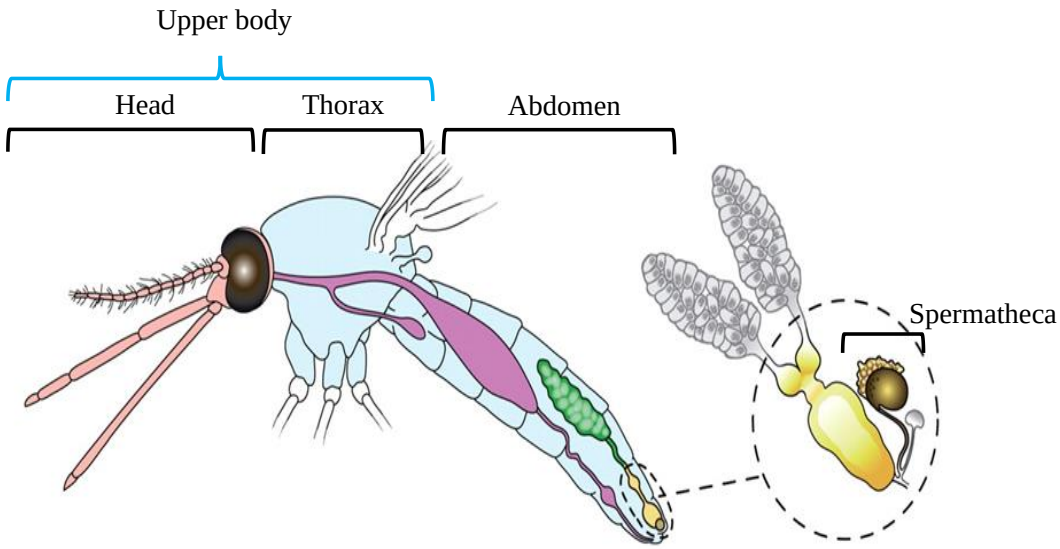

Figure 4.3. Schematic diagram of a whole female *Anopheles* mosquito. Body sections used for the isoenzyme assay are: 1. upper body (head and thorax), 2. abdomen (the gut in purple and ovaries in green), and 3. the spermatheca (in brown) from the lower reproductive tract (in yellow). **Citation:** modified from Rogers *et al.* (2008).

4.3.3. Isozyme gel electrophoresis

Isoenzyme experiment (IEE) set-up and gel electrophoresis

The protocol described by Dahan-Moss and Koekemoer (2016) was used, with slight modification. A 7.5 % polyacrylamide gel was prepared and allowed to set overnight prior to electrophoresis the following day. Homogenates were retrieved from the freezer (-70 °C), defrosted on ice and centrifuged at 13 000 rpm, 4 °C, for 5 minutes to collect tissue debris as a pellet, whilst isolating esterases in the supernatant. Eight microliters of the supernatant mixed with 4 µl of 1 % bromophenol blue (used as a tracking dye) was loaded onto the loading wells of the continuous non-denaturing native 7.5 % polyacrylamide gel. The homogenates were then electrophoresed at a constant amperage of 40 mA, 50V for 3 hours with continuous cooling at 4 °C in the PROTEAN® II xi cell electrophoresis instrument (Cat No: 165-1801, Bio-Rad, South Africa). Eight microliters of haemoglobin (Hb) extracted from cattle blood was used as a marker to track the migration of esterases through the gel. The gel set-up for each experiment is illustrated (**fig 4.4**).

Controls

Three controls were included for each gel (**fig 4.4**)**.** The haemoglobin (Hb) marker was prepared, (**Appendix 4B**), and included in the first well and last well of every gel. One whole female (F), unmated 10-day-old, was also added to use as a reference. A negative control, made up of the grinding buffer alone, was added.

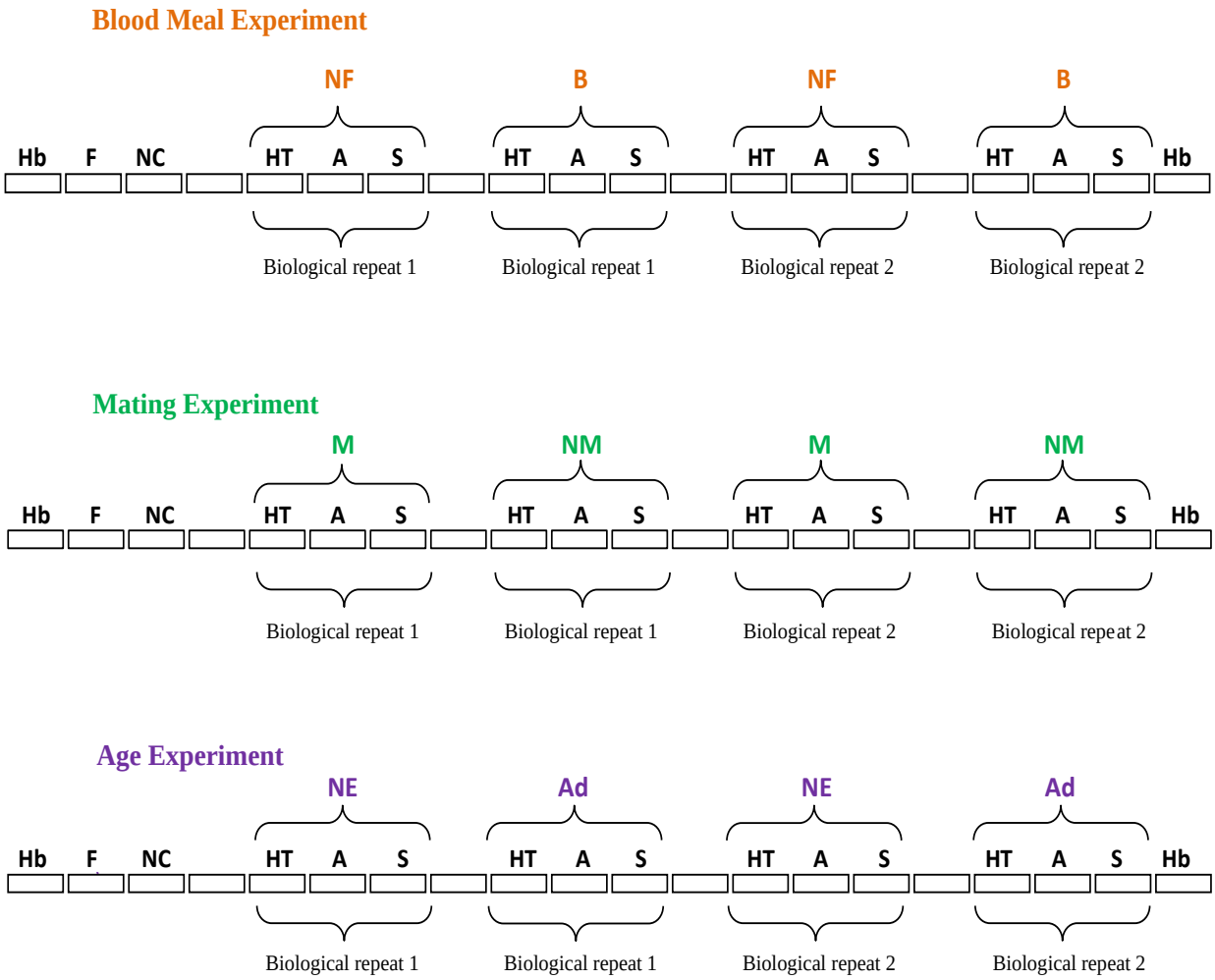

Figure 4.4. Isoenzyme gel set-up. NF: Control/non-blood fed**; B:** Blood-fed**; M:** Mated; **NM:** Non-mated; **NE:** Newly emerged; **Ad:** Adult (10 days); **Hb:** Haemoglobin marker; **F:** Whole female positive control; **NC:** Negative control; **HT:** Head and thorax; **A:** Abdomen; **S:** Spermatheca.

Subsequent to gel electrophoresis, the gels were incubated to induce the enzyme reaction by incubating the gel in a solution of 0.1 M sodium phosphate buffer (pH 6.4) with substrate (2 % of alpha naphthyl acetate in 50 % acetone), at 37 °C for 30-45 minutes in a shaking incubator. The gels were then stained in a second soaking solution of 0.1 % Fast Blue RR at 37 °C for 2 hours in a shaking incubator (Labcon, South Africa). When alpha-naphthyl acetate is hydrolyzed, it releases naphthol compound to which Fast Blue RR dye binds and precipitates it as an insoluble compound that appears black on the enzyme position on the gel. This results in the formation of a visible band.

Destaining of IEE gels

After staining, individual IEE gels were destained in a mixture of methanol, acetic acid, and water (5:1:5, v/v/v) as described (Kadry and Mohamad, 2014).

4.3.4. Data analysis

Esterases on each gel were enumerated according to their migrated position on the gel, numbered in descending order from the top of the gel, as per Dahan-Moss and Koekemoer (2016). Densitometry analysis of IEE gels to estimate esterase activity, was performed using the ImageJ application (ImageJ 1.53e, Java 1.8.0_172, 64-bit, NIH, USA) (Abràmoff *et al.*, 2004). Initially, an image of the gel was captured using the Bio-Rad Molecular Imager® ChemiDoc™XRS$^+$ whilst visualized under Oriole-UV transilluminator and analysed using Image Lab™ Software. The image, saved as TIF file, was then loaded onto the ImageJ application and converted to a grayscale. Every single band on the gel was selected by drawing a box around it and the bands are then analysed and the software plots the lanes by producing a graphical depiction of band intensity and peak. The proteins were further quantified/measured by marking with a straight line, the areas under the peaks of each lane, followed by band peak selection which produces a new window of values that represent esterase activity, which can be graphed (Rasband, ImageJ). The relative enzyme activity of all esterases in each body section was standardized according to the EST-9 activity observed in the whole FUMOZ female (F) control for each gel. EST-9 was selected for standardization because it produced similar activity across all three experiments. For individual gels, the EST-9 activity was set at one fold by dividing its activity value by itself.

The relative activity of all nine esterases in the test samples were then calculated by dividing their activity by the activity of the EST-9 of the control, as in the formula below:

$$\text{Relative activity of each esterases in each test sample} = \frac{\text{Esterase activity in test sample}}{\text{EST}-9\ \text{activity}}$$

Since four biological repeats for each body sections were processed, the average relative activity was calculated. The enzyme activity was analysed in three categories: 1. in blood-fed versus non-blood fed females; 2. in mated and non-mated females; 3. in newly-emerged versus adult

females. For each experiment, the average relative enzyme activity amongst the four biological repeats was calculated, and then plotted onto bar graphs. A fold difference of > 1.5 was considered important.

$$\text{Average relative activity} = \frac{\sum \text{esterase relative activity of biological repeats}}{\#\ \text{of biological repeats (n=4)}}$$

4.4. Results

4.4.1. Qualitative analysis of esterases in *An. funestus*

Effect of blood feeding on esterase activity:

In the blood meal experiment, the activities of all esterases, except EST-6 and EST-2, were detected in the upper body of both fed and non-fed females (**table 4.1**). In the abdomen, only activities of EST-5 and EST-3 were undetected in blood fed, whilst only activities EST-7, -4, -3 and -1 were detected in the abdomen of non-blood fed specimens. EST-2 activity in this experiment was only detected in the abdomen of blood fed females. Esterase activity was not detected in the spermatheca of both blood fed and non-blood fed specimens (**table 4.1**)**.** One other esterase, denoted EST-B, positioned just above EST-9 on the zymogram (**fig 4.5**), was detected only in blood-fed females and not in non-blood fed females. This position corresponds to the Hb control band and is very likely to be heamoglobin. The enzyme was detected strongly in the abdomen, followed by the upper body, and also in the spermatheca of blood fed females. Another additional esterase located between EST-2 and EST-1, was detected and denoted EST-2A. This esterase was only active in the abdomen of both blood-fed and non-fed females (**table 4.1)**.

Table 4.1. **Distribution of esterases in the different body parts of laboratory colonised *An. funestus* females.**

	Blood Meal Experiment							Mating Experiment							Age Experiment						
	Control	Upper body		Abdomen		Spermatheca		Control	Upper body		Abdomen		Spermatheca		Control	Upper body		Abdomen		Spermatheca	
		BF	NF	BF	NF	BF	NF		M	NM	M	NM	M	NM		NE	Ad	NE	Ad	NE	Ad
Esterase-B	-	+	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Esterase 9	+	+	+	+	-	-	-	+	+	+	+	+	-	-	+	+	+	+	+	-	-
Esterase 8	+	+	+	+	-	-	-	+	+	+	+	+	-	-	+	+	+	+	+	-	-
Esterase 7	+	+	+	+	+	-	-	+	+	+	+	+	-	-	+	+	+	+	+	-	-
Esterase 6	+	-	-	+	-	-	-	+	-	-	-	-	-	-	+	+	-	+	-	-	-
Esterase 5	+	+	-	-	-	-	-	+	+	+	-	+	-	-	+	-	-	-	-	-	-
Esterase 4	+	+	+	+	+	-	-	+	+	+	+	+	-	-	+	+	+	+	+	-	-
Esterase 3	+	+	+	-	+	-	-	+	+	+	+	-	-	-	+	-	-	-	-	-	-
Esterase 2	+	-	-	+	-	-	-	+	-	-	-	+	-	-	+	+	-	+	+	-	-
Esterase 2A	-	-	-	+	+	-	-	-	-	-	+	+	-	-	-	-	-	+	-	-	-
Esterase 1	+	+	+	+	+	-	-	+	-	-	-	-	-	-	+	-	-	+	+	-	-

+: esterase activity detected, -: esterase activity not detected, BF: blood fed, NF: non-blood fed, M: mated, NM: non-mated, NE: newly-emerged, Ad: adult.

Effect of mating on esterase activity:

In the first set-up of the mating experiment, 32 of 50 females were successfully inseminated. The experiment was repeated a second time, and 25/50 females were inseminated. A total of 57 inseminated females were successfully inseminated, and used for the isoenzyme assay. Activities of EST-9, -8, -7, -5, -4, and -3 were detected in the upper body of mated and non-mated females. Activities of EST-6, and -1 were not detected in all body parts of both mated and non-mated females. EST-5, and -2 activities were not detected in the abdomen of mated females whilst they were detected in the abdomen of non-mated females (**table 4.1)**. Activity of EST-3 was detected in the abdomen of mated females but not in non-mated females. EST-2A activity was only detected in the abdomen of both mated and non-mated females.

Effect of age on esterase activity:

Esterase activity was not detected in the spermatheca of both newly emerged and adult *An. funestus* females (**table 4.1**). The activities of EST-9, -8, -7 and -4 were detected in the upper body and abdomen of both newly-emerged and adult females. In the abdomen section, EST-6 activity was detected for newly-emerged females but not in adult females. EST-2 activity was detected in the upper body of newly-emerged females, and in the abdomen of newly-emerged and adult females. EST-2A was only detected in the abdomen of newly emerged and adult females.

4.4.2. Semi-quantitative analysis of esterases' activities in *An. funestus*

Effect of blood feeding on esterase activity:

The activity of EST-9 was 1.6 fold higher in the abdomen of blood fed females compared to non-blood fed (**fig 4.5** & **fig 4.6)**. EST-7 activity was 1.9 fold higher in the upper body of non-blood fed compared to blood-fed, whilst it was 7.5 fold higher in the abdomen of blood fed females compared to non-blood fed. EST-4 activity was higher in the abdomen of blood fed females by a 1.7 fold difference compared to non-blood fed (**fig 4.6)**. The activity of EST-1 was 2.2 and 9.1 fold higher in the upper body and abdomen respectively of blood fed versus non-blood fed *An. funestus* females (**fig 4.6**). The activity of EST-2 was only detected in the abdomen of blood fed females, but this relative activity was very low at 0.02.

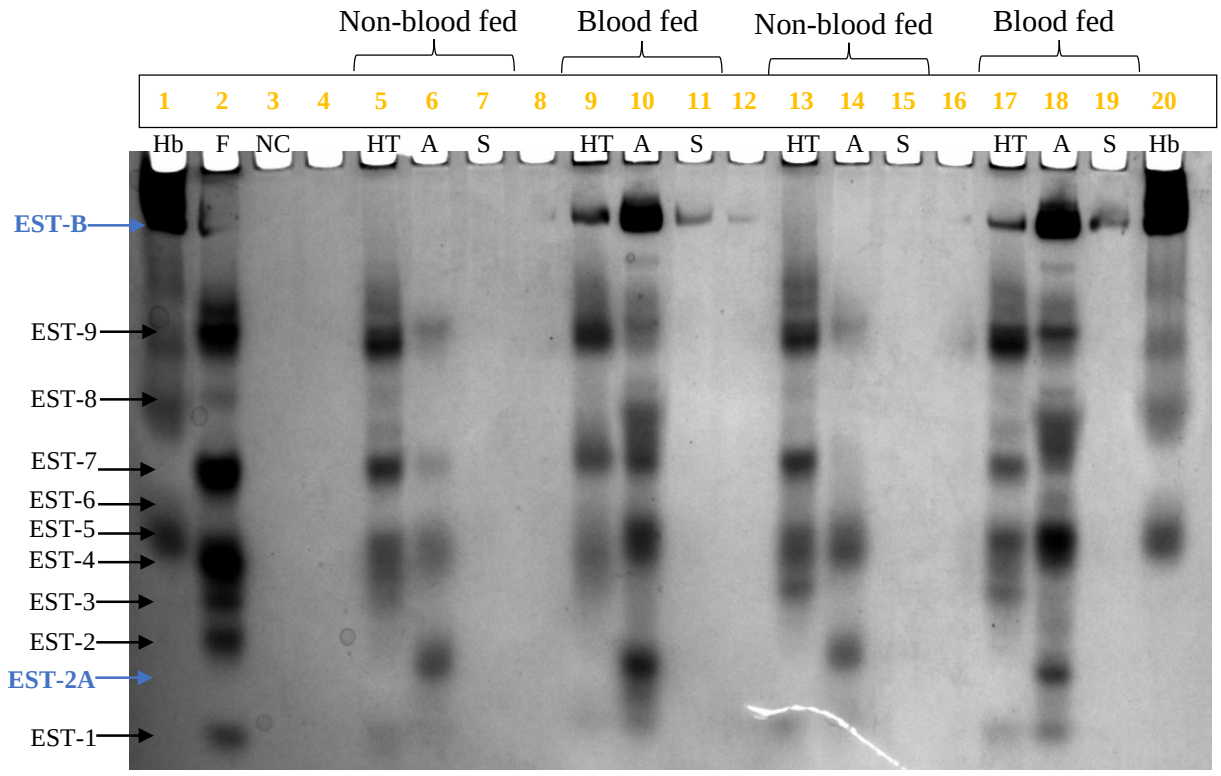

Figure 4.5. Zymogram for the blood meal experiment. Biological repeats 1 & 2. Lanes 1 & 20: Haemoglobin (Hb) marker. Lane 2: F-One whole female (positive control). Lane 3: NC: o template control. Lanes 9-11 and lanes 17-19: Blood-fed females. Lanes 5-7 and lanes 13-15: Non-blood fed females. HT: Head and thorax; A: Abdomen; S: Spermatheca.

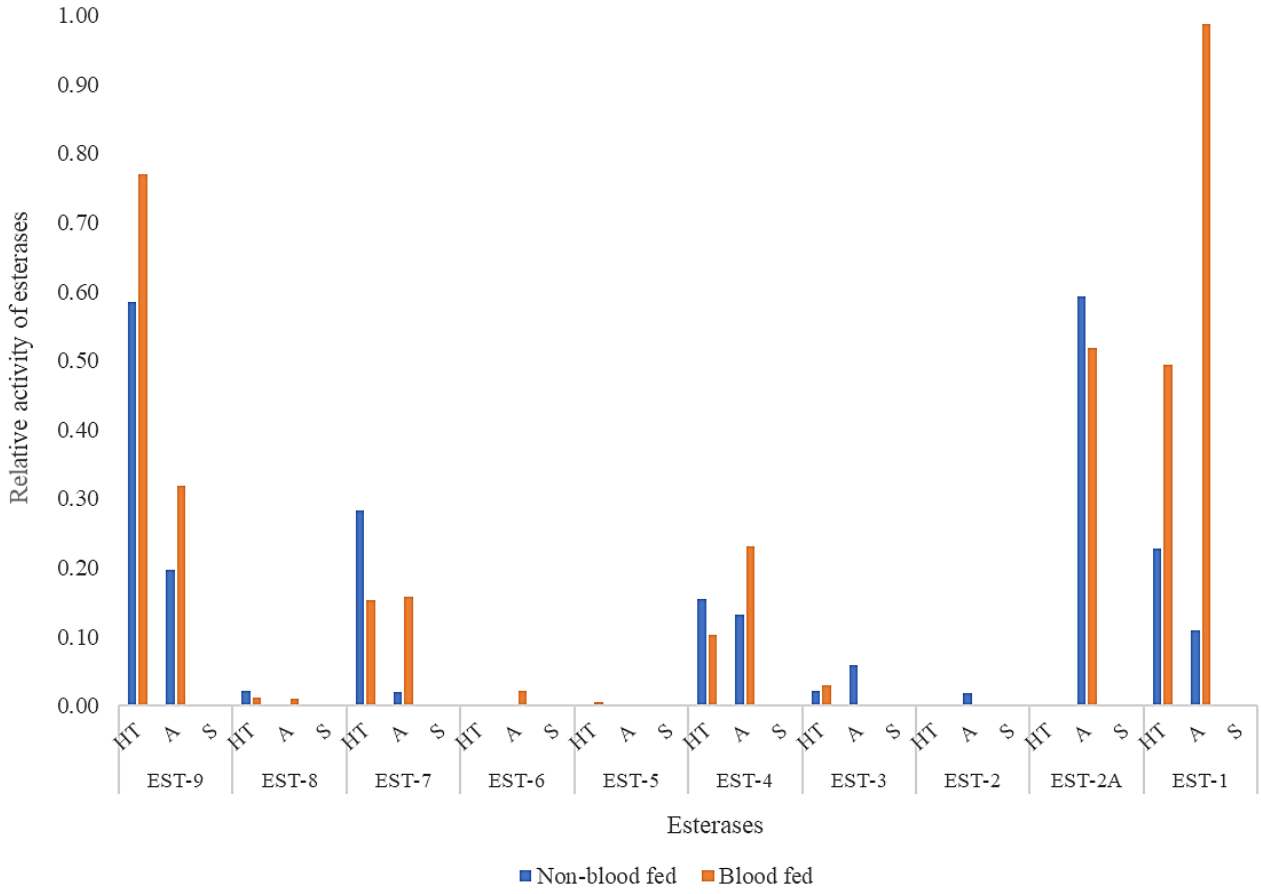

Figure 4.6. Average relative activity of esterases for the blood meal experiment. Activity of EST-9, -8, -7, -6, -5, -4, -3, -2, -2A and -1 in *An. funestus* females. HT: Head and thorax, A: Abdomen, S: Spermatheca.

Effect of mating on esterase activity:

For the mating experiment, EST-9 activity was 1.8 fold higher in the abdomen of non-mated females compared to mated females (**fig 4.7 & fig 4.8)**. In the upper body of non-mated females, EST-7 activity was 1.7 fold higher than in mated ones, whilst it was 1.8 fold higher in the abdomen of mated versus non-mated females (**fig 4.8)**. EST-4 activity was 2.8 fold higher in the upper body non-mated versus mated females (**fig 4.8)**. EST-2 was 124.5 fold higher in the abdomen of non-mated females (**fig 4.7B,** lane 10) compared to mated females. This esterase band was only observed in one out of four replicates of non-mated females and may have been an error of unknown source. The activity of EST-2A was 1.8 fold higher in the abdomen of non-mated females compared to the mated ones (**fig 4.8**). EST-2 band was visible in the spermatheca of mated females (**fig 4.7A**). However, this was only observed in one of four replicates and when averaged, its activity level was insignificant (<0.00).

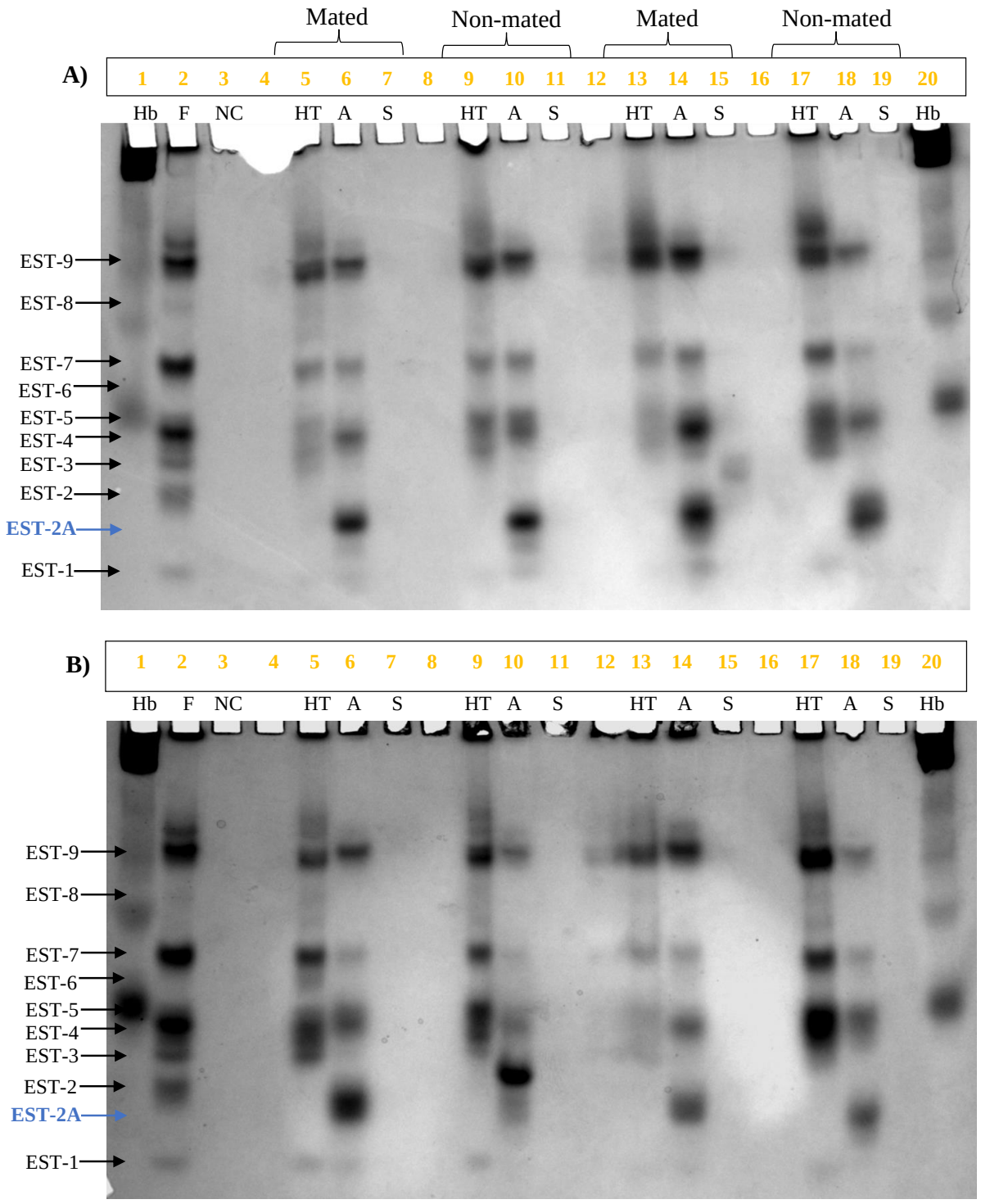

Figure 4.7. Zymogram for the mating experiment. **A)** Gel 1- Biological repeats 1 & 2. **B)** Gel 2- Biological repeats 3 & 4. Lanes 1 & 20: Haemoglobin (Hb) marker. Lane 2: One whole female (positive control). Lane 3: NC: No template control. Lanes 9-11 and lanes 17-19: Non-mated females. Lanes 5-7 and lanes 13-15: Mated females. HT: Head and thorax; A: Abdomen; S: Spermatheca.

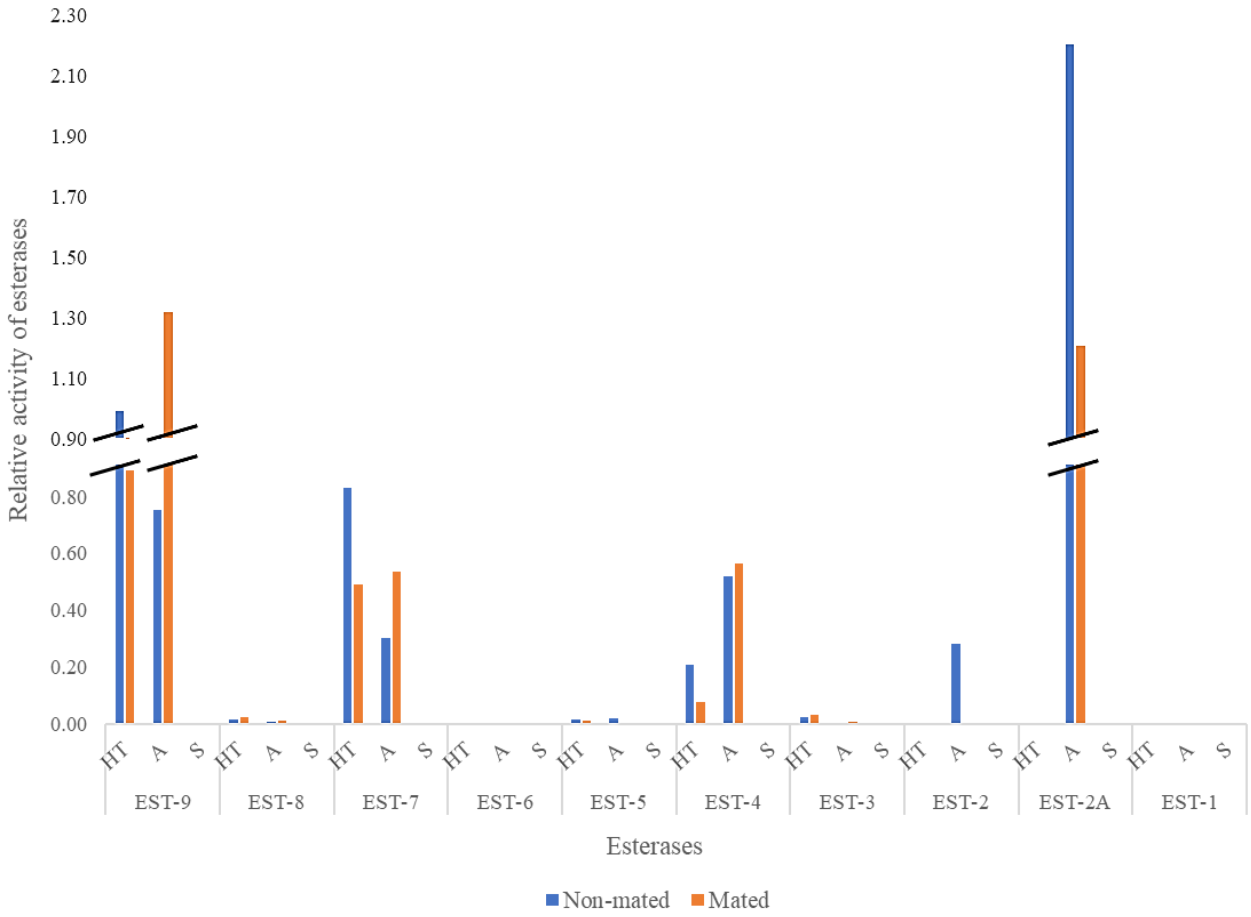

Figure 4.8. Average relative activity of esterases for the mating experiment. Activity of EST-9, -8, -7, -6, -5, -4, -3, -2, -2A and -1 in *An. funestus* females. HT: Head and thorax, A: Abdomen, S: Spermatheca.

Effect of age on esterase activity:

EST-9 activity was 2.7 and 5.5 fold higher in the upper body and abdomen respectively of newly emerged *An. funestus* females (8-12 hours post-eclosion) versus adult females (**fig 4.9**, **fig 4.10**). The activity of EST-7 was 6.6. and 7.8 fold higher in the upper body and abdomen respectively of newly emerged compared to adult females (**fig 4.10**). EST-4 activity was 2.8 fold higher in the upper body of newly emerged females compared to adult females. The activity of EST-2 was 6.1 fold higher in the abdomen of adult females versus newly emerged females (**fig 4.10**). EST-1 activity was 7.7 fold higher in the abdomen of newly emerged females compared to the adults (**fig 4.10**). EST-2A activity was only detected in the abdomen of newly-emerged *An. funestus* females, whilst completely absent in adult females.

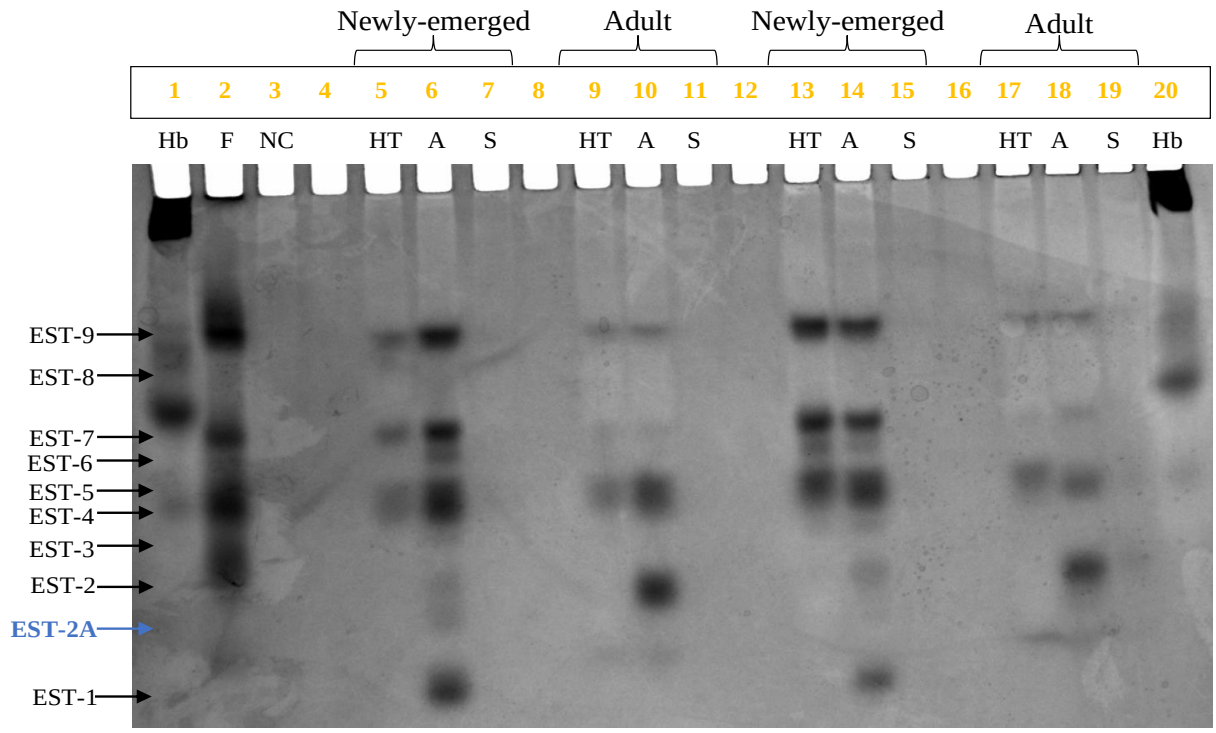

Figure 4.9. Zymogram for the age experiment. Biological repeats 3 & 4. Lanes 1 & 20: Haemoglobin (Hb) marker. Lane 2: One whole female (positive control). Lane 3: NC: no template control. Lanes 9-11 and lanes 17-19: Adult females. Lanes 5-7 and lanes 13-15: Newly emerged females. HT: Head and thorax; A: Abdomen; S: Spermatheca.

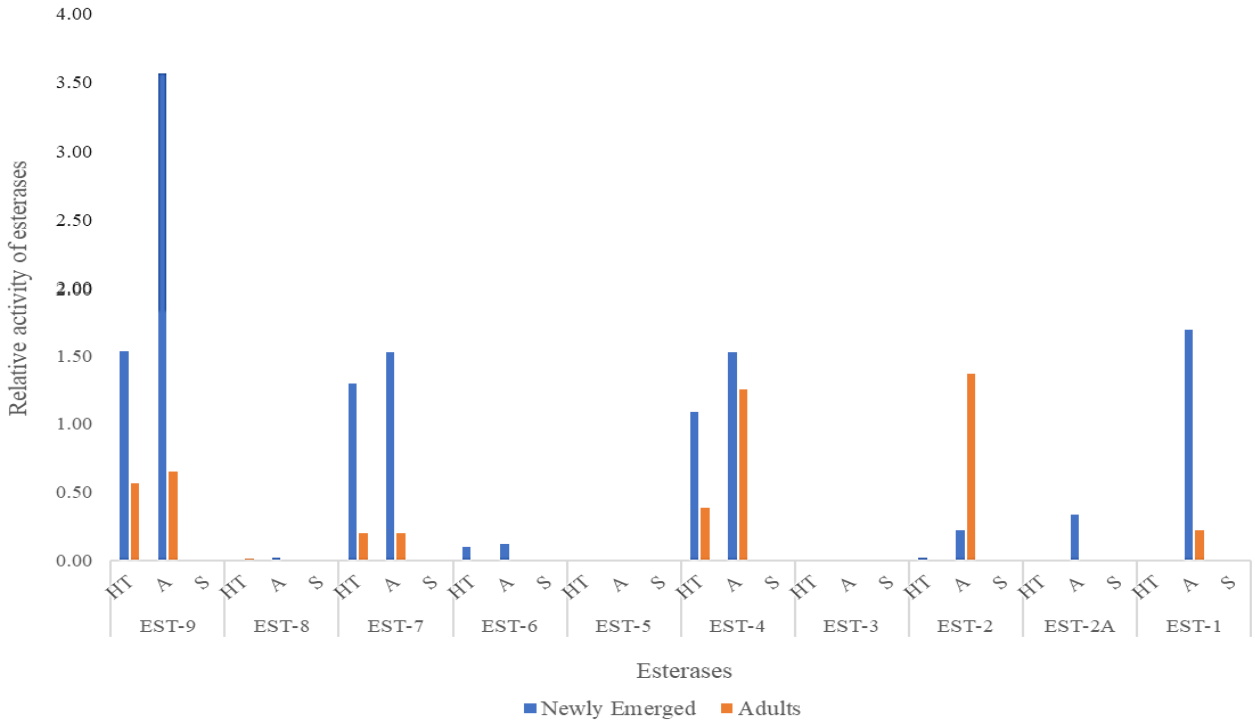

Figure 4.10. Average relative activity of esterases for the age experiment. Activity of EST-9, -8, -7, -6, -5, -4, -3, -2, -2A and -1 in *An. funestus* females. HT: Head and thorax, A: Abdomen, S: Spermatheca.

4.5. Discussion

The effect of blood feeding on the expression and activity of esterases in *An. funestus* females was investigated. Other variables such as age, and mating in the laboratory colonised *An. funestus* were also investigated. As with Dahan-Moss and Koekemoer (2016), this study also identified nine esterases common to the laboratory colonised *An. funestus* (FUMOZ). The special interest was on how a blood meal affects esterases' activities in the different body sections of *An. funestus* females. In so doing, one could also predict the function of those esterases based on their localization in the mosquito. However, age and mating were also investigated to determine whether or not the differential activities of esterases were only a result of blood feeding.

Selected esterases showed increased activities after blood feeding and include: EST-9, -7, -4, and -1. These were localized in the abdomen of blood fed *An. funestus* females. The activity of EST-9 was much higher in the upper body and abdomen of newly-emerged females when compared to adults. It appears that its activity decreases with age. This coincides with the result by Dahan and Koekemoer (2016), where EST-9 activity was at its maximum following emergence of *An. funestus* and decreased with age. The authors recognized that it may be essential throughout the life of *An. funestus* males and females. However, in this study EST-9 activity was increased in the abdomen of adult *An. funestus* females as a result of blood meal and mating. This means that EST-9 may play a role in the digestive and/or reproductive systems of female *An. funestus*. EST-9 is an acetylcholinesterase, and this group of esterases play several roles including promoting longevity, female reproduction, and development of insecticide resistance in insects (Lu *et al.*, 2012; Ibrahim *et al.*, 2016; Weedall *et al.*, 2020). Blood meal itself can increase the longevity of *Anopheles* (Oliver and Brooke, 2014). Hence, it could be that EST-9 increases the longevity of blood fed *An. funestus*. This would require further investigation.

EST-7 was also an important determinant in the abdomen of blood fed *An. funestus* females. This esterase belongs to the class of carboxylesterases, mainly recognized for their role in insecticide resistance (Weedall *et al.*, 2020). However, they also play a role in olfaction, reproduction and behaviour (Chertemps *et al.*, 2012). The broad range of function of this esterase explains why EST-7 activity was also increased in the abdomen of mated females.

Additionally, EST-7 activity was increased in the upper body and abdomen of newly-emerged compared to adult females, corresponding to the findings of Dahan-Moss and Koekemoer (2016). Although the authors found that this esterase fluctuated with age in FUMOZ adults between 2h and 30 days old, but the activity of EST-7 generally decreased with age in females. This means that its increased activity in the abdomen of mated females in this study, may be involved in reproduction. Interestingly, EST-7 activity was also increased in the upper body (head and thorax) of non-blood fed and non-mated females. Since carboxylesterases play a role in olfaction, it is possible that EST-7 activity is increased during the non-fed and non-mated states to aid in seeking a host using odours, as haematogenous insects are known for this behaviour (Dekker *et al*., 2005; Batista *et al*., 2014; Emami *et al.*, 2017; Wooding *et al.*, 2020; Stromsky *et al.*, 2021). The activity of EST-7 then decreases after a female has received a blood meal. It is possible that EST-7 is secreted in close proximity to olfactory receptors. In adult *Anopheles*, these receptors have been localized to their antennae, maxillary palps and proboscis (Pitts *et al*., 2004; Pitts *et al.*, 2011; Saveer *et al.*, 2018).

In the abdomen of *An. funestus*, EST-4 activity was detected irrespective of feeding, mating, or age. However, it had increased activity in the abdomen of blood fed specimens. EST-4 is an arylesterase, and other midgut arylesterases have been involved in reducing the virulence of pathogens to protect the vector (Stoltz *et al.*, 2008). Stoltz *et al.* (2008) found that increased arylesterase activity in the midgut of *D. melanogaster* reduced the virulence of *Pseudomona* species. These gram-negative bacteria, amongst others, are also present in midgut of *Anopheles* mosquitoes (Stoltz *et al.*, 2008; Tainchum *et al.*, 2020). Additionally, midgut reactive oxygen species (ROS) levels decrease post-blood meal in mosquitoes, and this state allows the establishment of viral or protozoan infections (Oliveira *et al.*, 2011; Tainchum *et al.*, 2020). However, blood meal also promotes bacterial growth in the midgut, which is an antagonist for infections with *Plasmodium* for instance (Dong *et al*., 2009). To counteract that, it is possible that the increased activity of EST-4 in blood fed *Anopheles* females in this study may help prevent bacterial growth, decreasing the virulence of dangerous bacteria such as *Pseudomonas*, and possibly decrease ROS levels. This can, in consequence, provide a favourable environment for *Plasmodium* parasites.

Contrary to its undifferentiated activity in the upper bodies of blood fed and non-blood fed females, EST-4 activity was higher in the upper body of non-mated and in newly-emerged females compared to mated females and adult females respectively. Our results in newly-

emerged FUMOZ females is different from Dahan-Moss and Koekemoer (2016), where EST-4 showed differential esterolytic activity patterns in FUMOZ colonies from 2 h post-eclosion up to 30 days old. These patterns were not associated with a specific condition, as they were all unmated and sugar fed (Dahan-Moss and Koekemoer, 2016). The results of EST-4 in the upper body of newly-emerged and non-mated females in this study could not be correlated to other findings.

Another esterase that had increased activity after blood feeding was EST-1. This esterase was classified as an alpha-esterase and further characterized as an acetylesterase (Dahan-Moss and Koekemoer, 2016). Outside of the blood feeding experiment, EST-1 activity was only detected in the abdomen of newly emerged females, and decreases in adults. It appears that blood meal increase the activity of EST-1 in the upper body of blood fed females. Dahan-Moss and Koekemoer (2016) did not identify EST-1 as a female-specific or age-specific esterase, instead its activity varied vastly. Dahan-Moss and Koekemoer (2016) proposed that this variability may be influenced by external or environmental factors such as food source, and in this study the food source was blood meal from guinea pig. It seems that blood meal had induced increased activity of EST-1, and based on its location in the mosquito, it may play a role in parasite transmission and blood meal digestion in *An. funestus* females. However, future research needs to clarify its function.

The other arylesterase, EST-2, which was identified as female-specific (Dahan-Moss and Koekemoer, 2016) was further investigated here. Its activity was consistently absent from the upper bodies of FUMOZ females in all three experiments investigated. This means that it is unlikely to have activity in the salivary gland of *An. funestus* and hence may not play a role in blood-feeding or transmission of malaria parasites. The activity of EST-2 rather appears to be localized in the abdomen of *An. funestus*. It was detected in all abdomens in the three experiments, but in very negligible levels in the abdomens of fed and mated females. A large fold difference in activity was observed in adult females (10-days old) where it was higher compared to newly-emerged females. Based on its localization and property, it may have similar functions to EST-4. Since its activity was higher in the adult female, it may have a role in development and/or maturation of their reproductive system. EST-2 activity seemed rather decreased in mated females compared to non-mated females. Further investigation is required.

An additional uncharacterized esterase, EST-2A, was detected only in the abdomen of female *An. funestus* in all three experiments. It may be a novel esterase for the species, its function is unknown but due to the localization of its activity, it may play a role in the digestive or reproductive system. This esterase was not identified by Dahan-Moss and Koekemoer (2016) where two mosquitoes for each time point were studied. In this study, multiple mosquito body sections were pooled which increased the possibility of identifying esterases in specific body parts. Additionally, EST-2A cannot be categorized as a strictly female-specific esterase, as only *An. funestus* females were studied here.

The esterase denoted EST-B, because it was only detected in blood-fed *An. funestus* females, was not further characterized in this study. However, this enzyme appears to originate from the blood meal source and is possibly the haemoglobin as stated earlier. The laboratory colonised *An. funestus*, were fed on guinea pig blood. According to a study by Monahan *et al.* (1981), a "non-specific esterase", classified as an α-naphthyl acetate esterase (ANAE), is found in vertebrate blood cells, including guinea pigs and humans. Such enzyme has cytoplasmic localization but its activity was also demonstrated on the cell surface of peripheral blood cells. Although the function of ANAE is not well elucidated, it is believed to play a role in inactivating toxic products amongst others, such as insecticides (Monahan *et al*., 1981; Shin and Smartt, 2016). It could be that the non-specific esterase in the blood meal mosquitoes received, has the ability to confer insecticide resistance, and contribute to longevity as compared to non-blood fed *Anopheles*. However, the effect of EST-B was only studied in the susceptible strain *An. funestus*, the study should be expanded to compare enzyme profile between insecticide-resistant versus susceptible strains. Not only does blood meal trigger a variety of metabolic mechanisms, such as the initiation of the gonotrophic cycle of mosquitoes (Lee *et al*., 2013; Shapiro *et al*., 2017; Agyekum *et al*., 2021), the content of blood itself may play a role in conferring insecticide resistance and promoting parasite transmission. Spillings *et al*. (2008) and Oliver and Brooke (2014) have already established that blood meal enhances insecticide resistance in *Anopheles* females, I propose that activity of esterases in resistant versus susceptible *Anopheles* strains as a function of blood meal be studied.

Apart from EST-B, the activities of all other esterases were not detected in the spermatheca, regardless of the experimental treatment. It was noted that the tissue sample of five pooled spermathecae was extremely minute to detect any esterase, given that the spermatheca is extremely small. The spermatheca of an *An. gambiae* is approximately $1/25^{th}$ its body size

(White and Muniss, 1972). One would need 20 or more mosquitoes to study esterase activity in the spermatheca. To determine how many spermathecae to be pooled would be suitable for isoenzyme electrophoresis, the Bradford protein assay is rapid and suitable in determining concentration of total protein in a pooled sample (Bradford, 1976).

CHAPTER FIVE

General discussion and conclusions

South Africa as a whole is experiencing low level malaria transmission, and elimination strategies should include identifying factors that drive the ongoing low-level malaria transmission. The province of Kwa-Zulu Natal (KZN) in South Africa is endemic for malaria, but has consistently been reporting the lowest number of cases for several years, as compared to Limpopo and Mpumalanga (Maharaj *et al.*, 2019; Raman *et al.*, 2020; Moonasar *et al.*, 2021). In light of this, KZN is considered closest to achieving local malaria elimination (Maharaj *et al*., 2019; Raman *et al*., 2020). According to Raman *et al*. (2020), KZN is being targeted for provincial elimination by 2023 (SANDoH, 2019; Moonasar *et al.*, 2021).

Some of the elimination strategies brought forward include cross-border monitoring of asymptomatic malaria carriers, which can drive residual local transmission in the province (Raman *et al*., 2020), "the introduction of transmission-blocking drug" called primaquine (Raman *et al*., 2020). On the vector arm of malaria, continued use of effective vector control methods is highly recommended (Maharaj *et al.*, 2019; Brooke *et al.*, 2020; Moonasar *et al.*, 2021). Additionally, it was suggested that other factors be studied. Factors such as status of secondary malaria vectors, population dynamics of the *An. funestus* group and correlating climate and vector abundance and ecology in KZN (Dr Patrick Moonasar, at the 4th malaria conference in 2018). It is against this background, that this study was carried out in the Mamfene area, located in Jozini municipality, within the uMkhanyakude district of KZN.

Analysis in this study showed that four species belonging to *An. funestus* group occur outdoors in Mamfene, namely *An. parensis* being the most common, followed by *An. vaneedeni*, *An. rivulorum*, and *An. leesoni*. *Anopheles funestus* was not identified during the collection period in this study. The species is generally considered eradicated from within South Africa, except for one *An. funestus* collected in 2018 in Limpopo (Braack *et al.*, 2020), but this was attributed to the bordering of the province to Zimbabwe where the species commonly occurs. This study also confirmed that *An. rivulorum-like* was not collected outdoors in Mamfene. None of the species identified in this study were infected with *P. falciparum* sporozoite. Therefore, neither one of the four *Anopheles* species could be incriminated as a secondary malaria vector for this study and could not possibly be implicated in the ongoing residual malaria transmission in the province.

However, *An. vaneedeni* and *An. parensis* collected in KZN were found to be infective with *Plasmodium* (Burke et *al.*, 2017; Burke *et al*., 2019). Since humans are the sole reservoir of *P. falciparum*, it's enough to conclude that these two anopheline species acquired the infection by feeding on humans. However, at the time of collection, these specimens had no blood meal evidence in their abdomens. Hence, it would be beneficial to closely monitor the vector capacity and host preferences of *An. vaneedeni* and *An. parensis*, as well as other outdoor feeding and resting species of the *An. funestus* group. A limitation encountered in terms of *P. falciparum* ELISA detection is that because samples which are positive the first time are repeated to confirm results, the volume of the positive homogenate decreases when it is time to perform nested-PCR confirmation. Simply put, this decreases the available sample volume to perform DNA extraction of *P. falciparum*, if present, for nested-PCR confirmation, thereby lowering the chances of detection. Based on this, it can be proposed that DNA be extracted directly from the head and thorax of female members of the *An. funestus* species for immediate *P. falciparum* specific-PCR. Other studies have successfully done so (Temu *et al.*, 2007; Cassiano *et al.*, 2011; Echeverry *et al.*, 2017).

As previously stated, a successful human malaria vector should meet certain important criteria. One should be the successful detection of *Plasmodium* CSP from its salivary glands (Ghosh *et al.*, 2009; Gunathilaka *et al.*, 2016). Detection of *Plasmodium* elements in the salivary glands of mosquitoes, with irrefutable confirmation, is indicative of an infectious stage and can be transmitted to a human host (Ghosh *et al*., 2009). This usually provides sufficient evidence of the vectorial capacity of a mosquito. In addition, evidence of feeding on humans based on blood meal identification provides another important link for human malaria transmission (Kent and Norris, 2005; Ghosh *et al.*, 2009; Gunathilaka *et al.*, 2016). This feeding behaviour should be exhibited over a sustained period of time to incriminate a new malaria vector. Therefore, identifying blood meal origin in malaria vectors, in addition to detecting the causative agent of malaria, is of great relevance. For future studies into the blood feeding preferences of *Anopheles* mosquitoes and other mosquitoes of medical importance, it would be beneficial to consider more sensitive methods such as real-time PCR, which has been described, and testing a wider range of vertebrate hosts (Gunathilaka *et al.*, 2016; Keven *et al.*, 2020). Additionally, utilising collection method(s) that maximise the number of blood-fed females collected can be considered. Evidence suggests that it is difficult to capture blood-fed females using CO_2 traps because they are not drawn to this attractant once they have had a blood meal (Roiz *et al.*, 2012). Favourable techniques for the capture of blood-fed female anophelines is advisable, such as ceramic clay

pots and cattle Kraal (animal pens) collections which were used in this study, or indoor collections to establish anthropophilic behaviour. Preservation methods of mosquito are also known to affect the success of blood meal identification (Reeves *et al.*, 2016). Reeves *et al.* (2016) showed that silica gel/dry storage was the least favourable, whilst frozen mosquitoes were more optimal for blood meal identification. However, since this study looked at archived specimens, protocols that maximised amplification success of silica-preserved specimens were described.

The host preferences of outdoor biting and resting species of *An. funestus* group in Mamfene area of KZN was investigated in this study, as well as the impact of climatic parameters (temperature, rainfall and humidity). Evidence show that *An. leesoni*, *An. parensis*, *An. vaneedeni*, and *An. rivulorum* prefer to take blood meals from cattle. The absence of human blood in these outdoor feeding and resting species in Mamfene, indicates that their role in malaria transmission could not be confirmed. It can be hypothesized that due to the wide range of alternative vertebrate hosts available outdoors, humans are less likely to be bitten as a food source. Our results show that blood feeding by species of the *An. funestus* group is seasonal. The mean feeding proportions of *An. parensis, An. rivulorum, and An. vaneedeni* are driven by relative humidity, whilst the mean feeding proportions of *An. vaneedeni* are also driven by rainfall. Relative humidity of 60-70 % increase the longevity of mosquitoes, and sufficient rainfall to allow formation of breeding sites that increases vector population (Yamana and Eltahir, 2013; Kumar and Reddy, 2014; Drakou *et al.*, 2020). Increased longevity of female *Anopheles* increases the chances of seeking blood meal to develop more eggs, which can subsequently affect malaria transmission dynamics.

Expanding the study into understanding blood feeding of anophelines in the other two endemic provinces would be beneficial in adding to the knowledge of malaria epidemiology within South Africa, and understanding human-vector-parasite interactions. Most importantly, additional host preference studies will help inform or monitor the effectiveness of vector control strategies by observing changes in biting behaviour over time or a reduction in human-blood feeding. Mathematical models have been developed to investigate the role of climatic parameters in malaria incidence and to assist in predicting future changes in the risk distribution and transmission of malaria, and for South Africa, scientists are using malaria early warning systems (MEWS) to develop seasonal malaria forecast models (Kim *et al.*, 2019; Landman *et al.*, 2020).

Malaria transmission during its peak seasons is dependent on the abundance of *Anopheles* vectors during those seasons, which means that climatic variables have a strong influence on their abundance as well as their biting activity. Temperature, rainfall and malaria cases to predict future incidence of malaria in Limpopo have been fitted in a weather-based model by Kim *et al.* (2019), whilst the malaria forecast model used by Landman *et al*. (2020) only took into account seasonal rainfall to predict malaria incidence. In this study, feeding by *Anopheles* species were influenced variably by climatic factors, with relative humidity and rainfall being the main driving factors. Based on these results, the afore mentioned models should could be adjusted to incorporate relative humidity into malaria prediction models. Additionally, I propose that prediction models also take into account *Anopheles* host preferences in relation to seasonality in Mamfene and other malaria endemic regions of South Africa, be developed and implemented. This will contribute to steering South Africa towards local malaria elimination

As elaborated in the introductory chapter, there is a need to implement vector control strategies that target outdoor biting *Anopheles* which contribute or suspected to contribute to low residual malaria transmission. There are many existing and emerging tools for vector control outdoors. For various types of settings, including rural settings such as Mamfene, the suggestion by Sougoufara *et al.* (2020) is encouraged, to use novel tools such as attractive toxic sugar baits (ATSB) and endectocides. These methods appear to be cost effective whilst employing wide range of angles of attack to control outdoor biting malaria vectors. The use of endectocides in particular, seems like an attractive approach and it involves the use of ivermectin drug treatment on human hosts and/or their domestic animals (Sougoufara *et al*., 2020). Application of this method has been shown to result in decrease of fertility and longevity of *Anopheles* species (Sylla *et al.*, 2010; Alout *et al.*, 2014; Pooda *et al.*, 2015). However, the implementation of these methods outside a research project present a challenge based on availability of trained personnel, funding and other resources to cover large testing areas.

Further understanding of the biology of *An. funestus* females was investigated, by studying activity of enzymes, which are known to mediate several life process in all life forms including *Anopheles*. Enzymes, specifically esterases, in adult *An. funestus* were identified and characterized (Dahan-Moss and Koekemoer, 2016). However, Dahan-Moss and Koekemoer (2016) concurred that one of the esterases, EST-2, was an important biomarker in female *An. funestus*. Hence, this study investigated the activity of EST-2, and the other 8 esterases, in the

different body parts of blood-fed *An. funestus* females. It is a pilot into understanding the mechanism of blood meal in *Anopheles* mosquitoes. In this study, EST-2 was barely detectable in the head or abdomen of blood fed females compared to the control group. The spermatheca, however, showed zero activity of EST-2 or of any other esterases. Further analysis should be performed with a larger number of pooled spermathecae, to evaluate its activity in both blood fed and non-blood fed female *An. funestus*. The spermatheca of *An. funestus* is approximately $1/25^{th}$ the size of its body length, hence a larger pool would be required to study the activity and potential roles of EST-2 and other esterases. Due to limited time and the subsequent shutdown as a result of the COVID-19 pandemic, the recommended further analysis could not be performed. The following additional esterases were also highly active in blood fed *An. funestus*. EST-1 in the upper body and abdomen, EST-2A in the abdomen, EST-4, -7, and -9 in the abdomen, were identified as important biomarkers in blood fed *An. funestus* females. A blood esterase (EST-B) of host source, possibly haemoglobin, was also detected in blood fed *An. funestus*. It is recommended that these esterases be further studied to understand their role in blood fed versus non-blood fed, for the susceptible and resistant strains of *An. funestus* females.

In addition to the small pool size of spermathecae for esterase activities in *An. funestus* females, another limitation was noted in the blood meal experiment in this study. Esterase activities could not be accurately delimited to a specific organ of the female *An. funestus*. For instance, an esterase activity in the abdomen could not be determined to have function in the digestive system (midgut) alone, because the abdomen section included the midgut as well as the reproductive system. Similarly, with the upper body which included the head (head, antenna, and salivary glands) and thorax. Future studies in *An. funestus* should consider isolating the different organs in the different body parts to further localize esterases' activities and functions following blood meal. This has been done in other *Anopheles* species. Rawal *et al*. (2016) isolated and pooled 100 pairs of salivary glands in *An. culicifacies* to study the importance of functional proteins following a blood meal. Marinotti *et al.* (2006) and Cui *et al.* (2020) pooled and studied midguts of *An. gambiae*. These researchers showed that blood meal in *An. gambiae* most commonly triggered midgut cells to secrete trypsin-like peptidases, a subclass of esterases, that aid in blood meal digestion. Isoenzyme gel electrophoresis approach to study esterases appear to be more cost-effective compared to genomic or proteomic approaches. Genomic or proteomic approach, however, provide a more useful and in-depth approach to gene-expression or protein activity, as well as their specific biological functions (Marinotti *et al.*, 2006; Rawal *et al.*, 2016; Cui *et al.*, 2020), and these approaches should be explored. More in-depth studies on

the specific roles of modified esterase activity as a result of blood meal, would further elucidate how blood feeding impact underlying physiological processes such as reproduction, mating and insecticide breakdown. Such studies could provide information that can be useful in targeting malaria vectors.

In this study, we could not confirm that *An. leesoni*, *An. parensis*, *An. rivulorum*, or *An. vaneedeni* collected outdoors in the Mamfene region of KZN, contribute to the low level residual malaria transmission. They are entirely zoophilic when outdoors, and were uninfected with *P. falciparum* parasites. However, since *An. parensis* and *An. vaneedeni* have been recently incriminated as potential secondary malaria vectors in South Africa, continued vector surveillance is warranted. More intensified surveillance methods should be implemented on the parasite as well as the vector arm of malaria intervention to achieve the WHO's GTS2025 target to eliminating malaria vector within South Africa. In terms of the biology of *An. funestus* females, further studies should be explored to add to the knowledge of how blood feeding influences their biological and underlying physiological processes. *Anopheles* malaria vectors exhibit differences in their ecology and behaviour. This plays a role in how malaria is transmitted and subsequently affects the success of vector control strategies. It has been repeatedly emphasised that accurate identification of malaria vector species, or potentially secondary vectors infected with *Plasmodium* CSP, is crucial to inform and devise efficient vector control interventions. In addition, distribution of such species and their bionomics prove essential to those interventions. As such, it can be proposed that host preference studies and impact of climatic variables, become integrated into vector surveillance activities in South Africa.

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7. APPENDICES

Appendix 1A

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

July 4, 2018

To whom it may concern,
Reference : Mwamba_Koekemoer ***Waiver Anopheles_NICD 20180702O***
Re: Waiver from the Animal Research Ethics Committee of the University of the Witwatersrand

Student : Tshiama Miriam Mwamba
Student number : 726360
Degree : MSc (Med)
Study Title : "Blood-meal identification and impact of blood feeding on femalespecific esterase in *Anopheles funsestus* group."

Department: Vector Control Reference Lab (VCRL), NICD

Supervisor: Prof Lizette Koekemoer
Co-investigator: Dr G Minhenga

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

Reason: No vertebrate animals will be used in the study.

Comment/Notes : Objectives are to

a) identify the wild caught species;
b) correlate the distribution of the mosquito with climate data from KZN;
c) investigate the role of the esterase enzyme during blood feeding in this species.

Please contact me should you require further information.

Yours sincerely

GP Candy

Geoffrey Candy
Chair : Animal Ethics Research Committee, University of the Witwatersrand
Geoffrey P Candy PhD
Professor, Department of Surgery, Faculty of Health Sciences, University of the Witwatersrand, *7 York Road Parktown 2193, Johannesburg; Tel: 27-11-717-2574; Email: geoffrey.candy@wits.ac.za*

Appendix 1B

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

Research Office

INSTITUTIONAL BIOSAFETY COMMITTEE
(R 14/16)

CLEARANCE CERTIFICATE **PROTOCOL NUMBER: 20180402Lab**

BRIEF DESCRIPTION OF APPLICATION:

Laboratory situated in room 10M02 10th Floor, Wits Research Institute for Malaria, Medical School

APPLICANT: Professors M/LL Coetzee and Koekemoer

SCHOOL/DEPARTMENT : Molecular Medicine and Haematology

DATE CONSIDERED: By Circulation 29 November 2018

DECISION OF COMMITTEE: Approved unconditionally

These laboratories are considered to meet the standards of the Institutional Biosafety Committee (IBC), for BSL2 approval, as specified in the IBC's published Standard Operating procedures.
Any change to the type of biohazardous agents being handled should be reported to the IBC
DAFF - Reg no 39.2/University of Witwatersrand - 18/041 expiry date 03 December 20-21

1. This clearance certificate expires on 03 December 2023 and may be renewed on application.

DATE OF APPROVAL: 03 December 2019

CHAIRPERSON: _____ James F. S. Larkin 2019.04.15 12:07:53 +02'00'

(Professor J Larkin)

DECLARATION OF APPLICANT:

To be completed in duplicate and **one copy** returned to the University of the Witwatersrand, Faculty of Health Sciences, Research Office, Office 301, Phillip Tobias Building, 29 Princess of Wales Terrace, Parktown,2193.

1. I have read, understood and accepted the approval conditions above
2. I note that the University Safety Officer, or his/her representative, may at any reasonable time inspect my laboratory or trial site to ensure compliance with current Health and Safety legislation. I undertake to offer my full co-operation in any such inspection.
3. I have read, understood and will comply with the *recommended standard operating procedures for the handling of biohazardous materials* posted at http://intranet.wits.ac.za/academic/uro/Pages/Institutional-Biosafety-Committee-(IBC).aspx

Signed: _____ **Date:** _____

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

Appendix 2A: Calculations for DNA extraction from mosquito using the ZyGEM prepGEM® Insect kit (Cat No.: PIN00200; ZyGEM™, New Zealand)

Reagents	Volume for 1 reaction
PCR water	8.75 µl
10X buffer black	1.0 µl
PrepGEM enzyme	0.25 µl
TOTAL	**10.0 µl**

NB: the protocol was optimised in-house and the reaction is still successful when 1/4th of the volumes recommended by the manufacturer is applied.

Appendix 2B: Mastermix calculations for the *An. funestus*-specific PCR

All reagents except primers (Cat No: R001, Takara. Separations, South Africa)

Primers were ordered from Macrogen Europe

Reagents	Volume for 1 reaction
10x PCR buffer	1.25 µl
10X dNTPs (2.5 mM each)	1.00 µl
25 mM $MgCl_2$ solution	0.75 µl
Primers (10 µM): UN	1.0 µl
FUN	1.0 µl
LEES	1.0 µl
PAR	1.0 µl
RIV	1.0 µl
RIV-LIKE	1.0 µl
VAN	1.0 µl
Taq (5 U/µl)	0.1 µl
PCR water	1.4 µl
TOTAL	**11.5 µl**

Add 1.0 µl sample DNA to 11.5 µl PCR master mix for a 12.5 µl total mastermix

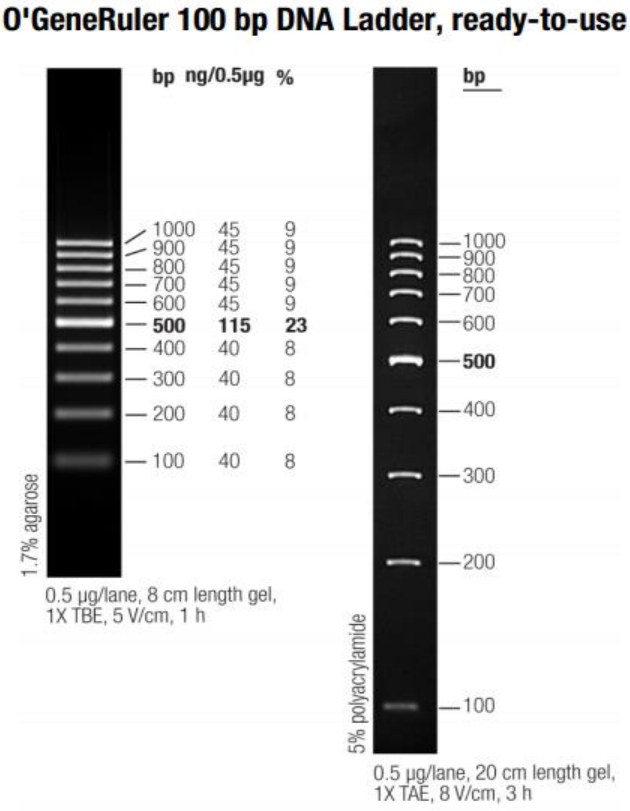
Appendix 2C: Resolving PCR products on 2.5% ethidium bromide-stained agarose gel

1. 2.5% Agarose gel:

10g SeaKem® LE Agarose Lonza (Cat No: 50004, WhiteSci, South Africa).

400 ml of 1x TAE buffer (1 L prepared from 20 ml of 50x TAE with 980 ml dH_2O)

12 µl ethidium bromide (Cat No: E1510, Sigma-Aldrich, South Africa)



100 bp DNA ladder (Cat No: SM0243, Thermo Scientific™, South Africa)

O'Range 6x Loading dye (Cat No: R0631, Thermo Scientific™, South Africa)

2. 50x TAE buffer preparation:

242.0 g Tris-base (Cat No: 77-86-1, Ace, South Africa)

57.1 ml Acetic acid glacial

100 ml 0.5M EDTA (Cat No: 60-004, Ace, South Africa)

Check pH = 8 and adjusted volume to 1000 ml with dH_2O

Autoclaved for 20 minutes

Appendix 2D: Reagents for *P. falciparum* ELISA

All reagents were kept in the fridge (2-8 °C) until ready to use.

1. 1x Phosphate Buffered Saline (PBS): (Cat No: 003002, Thermo Scientific™, South Africa)

One PBS tablet dissolved in 1L dH_2O or

PBS solution, pH 7.4 (Cat No: DMPA0457, DMP, South Africa)

2. PBS-Tween: 10 mM PBS, 0.05% Tween 20

500 µl Tween 20 (Cat No: 28352, Thermo Scientific™, South Africa)

added to 1L of 1x PBS

3. Blocking Buffer (BB): 0.5% w/v Casein, and 0.1N NaOH in 10 mM PBS, pH 7.4, plus 0.1% w/v phenol red

Reagents	**1L**
Casein (Cat No: C7078, Sigma-Aldrich, South Africa)	5 g
0.1N NaOH (Cat No: A4782602, Thermo Scientific™, South Africa)	100 ml
PBS (Cat No: DMPA0457, DMP, South Africa)	900 ml
Phenol Red* (Cat No: P3532, Sigma Aldrich, South Africa)	0.2 ml

Casein suspended in NaOH and boiled in microwave

Once casein dissolved, PBS was added

Solution adjusted to pH of 7.0-7.4 with concentrated HCl

*Phenol red added (1g/10ml water)

4. Grinding Buffer (GB): 0.5% w/v Casein, 0.05% Igpal/NP-40, 0.1% w/v phenol red, in 10 mM PBS, pH 7.4:

(1ml blocking buffer:5 µl Igepal/NP-40)

50 mL blocking buffer

250 µl Igepal/NP-40 (Cat No: CA-630, Sigma-Aldrich, South Africa)

50 µl used to homogenize each sample

5. Primary antibody mix: 0.200 µg/50µl/well or 20 µg/5ml

For a 96-well plate:

40 µl of the capture monoclonal antibody (Pf-2A10-28, Cat No: CDC-37-01-24-2: KPL), and

5 ml PBS

6. 2,2'-azino-biz-(3-ethylbenzothiazoline-6-sulphonic acid (ABTS 1) peroxidase substrate (Cat No: 37615, Thermo Scientific™, South Africa)

Appendix 2E: Reagents for confirmation of *P. falciparum* infectivity

1. Phenol-Chloroform DNA extraction

Phenol:Chloroform:Isoamyl (PCI) alcohol solution (25:24:1), pH 8.0 (Cat No: 77617, Sigma-Aldrich, South Africa)

Chloroform:isoamyl (24:1) (Cat No: C0549, Sigma-Aldrich, South Africa)

3M Sodium acetate (NaOAc, pH 5.2) (Cat No: R1181, Thermo Scientific™, South Africa)

2. Mastermix calculations for *Plasmodium falciparum* nested-PCR

Primary PCR:

Reagents	**Volume for 1 reaction**
10x PCR buffer	1.25 µl
10X dNTPs (2.5 mM each)	1.25 µl
25 mM $MgCl_2$ solution	0.75 µl
Primer rPLU5 (25 µM)	1.0 µl
Primer rPLU5 (25 µM)	1.0 µl
Taq (5 U/µl)	0.1 µl
PCR water	6.15 µl
TOTAL	**11.5** µl

Add 1.0 µl sample DNA to 11.5 µl PCR master mix for a 12.5 µl total mastermix

Secondary PCR:

Reagent	Volume for 1 reaction
10x PCR buffer	1.25 µl
10X dNTPs (2.5 mM each)	1.25 µl
25 mM $MgCl_2$ solution	0.75 µl
Primer rFAL1 (25 µM)	1.0 µl
Primer rFAL2 (25 µM)	1.0 µl
Taq (5 U/µl)	0.1 µl
PCR water	6.15 µl
TOTAL	**11.5** µl

Add 1.0 µl of primary PCR product to 11.5 µl PCR master mix for a 12.5 µl total mastermix

Appendix 2F:

Supplementary table 2.1. Number of *An. marshallii* misidentified as *An. leesoni* using *An. funestus* species-specific PCR

	Months	**Qty**
2018	July	16
	August	8
	September	11
2019	January	14
	Total	49

Appendix 3A: Collin's DNA extraction protocol and reagents (**Citation:** Collins *et al.* (1987))

1. Grinding buffer: (kept at 4 ℃ for up to 3 weeks)

Reagents	Qty for 20 ml solution
1M NaCl	1.6 ml
Sucrose	1.095 g
0.5M EDTA	2.4 ml
10% SDS	1 ml
1M Tris-HCl (pH 8.6)	2 ml
Distilled water	Make up to 20 ml

2. 8M KAc (potassium acetate):

Dissolve 7.851336 g in 10 ml dH_2O

3. TE buffer (pH 8.0):

Reagents	Qty for 100 ml solution
1M Tris-HCl	1 ml
0.5M EDTA	0.2 ml
Double distilled water	Make up to 100 ml

4. Collin's DNA extraction method:

a. A blood fed mosquito abdomen was placed into a 1.5 ml microcentrifuge tube, and grinded until very fine using a sterilised pestle.

b. Two hundred microliter of the grinding buffer was then added to the tube, and grinding continued to mix the blood in the buffer.

c. The pestle was then removed, ensuring that all grinding pieces were left in the homogenate solution.

d. The homogenate was then incubated at 70 ℃ in a heating block for 30 minutes.

e. The homogenate was then removed from the heating block and 28 µl of 8M KAc was added and mixed gently by tapping gently with a finger.

f. The mixture was incubated on ice for 30-60 minutes.

g. Following this, the sample was then centrifuged at 13 000 rpm for 20 minutes.

h. The liquid part after centrifugation contained DNA and was transferred into a new 1.5 ml microcentrifuge tube, without disturbing the pellet. The old microcentrifuge tube was discarded.

i. In the new microcentrifuge tube containing the liquid, 400 µl of ice cold 100% ethanol was added and mixed by inversion.

j. The mixture was then incubated at room temperature for 5 minutes, followed by centrifugation at 13 000 rpm for 30 minutes to collect a pellet.

k. The ethanol was then pipetted off and discarded, without disturbing the pellet.

l. To further clean the pellet, 200 µl of ice cold 70% ethanol was added to the tube without disturbing the pellet.

m. The tube was centrifuged at 13 000 rpm for 15 minutes, and the ethanol was pipetted off and discarded.

n. The pellet left in the microcentrifuge tube was allowed to air dry on the bench.

o. The pellet was then re-suspended in 200 µl 1x TE buffer (pH 8), and pipetting the buffer up and down to dissolve the pellet which contains the DNA.

p. Eluted DNA was used immediately or stored at -20 °C.

Appendix 3B: DNA extraction - Biological fluids & cells protocol with the Quick-DNA™ Microprep Plus kit

(Cat No. D4074, Inqaba Biotec™, South Africa), protocol extracted from page 13

Quick-DNA™ Microprep Plus Kit
Quick Protocol
Catalog Nos. D4074

ZYMO RESEARCH
The Beauty of Science is to Make Things Simple

Biological Fluids & Cells Protocol

Biological Fluids: ≤ 50 µl
Total DNA from whole blood, buffy coat, saliva, sputum, semen, etc. See the Instruction Manual page 2 for other samples and special considerations.

Cultured Cells: ≤ 1x10^8 cells
Total DNA from *E. coli*, insect, or mammalian cells (e.g. HeLa cells, buccal cells, HEK-293 cells, etc.). See the Instruction Manual page 2 for special considerations and sample preparation information.

Note: Pellet cells and discard supernatant. Resuspend ≤ 1 x 10^6 cell pellets using 50 µl DNA Elution Buffer or an isotonic buffer (e.g PBS).

**Add 260 µl of Storage Buffer to each 5 mg tube of Proteinase K. Store at -20°C.*

1. Add up to 50 µl sample to each microcentrifuge tube and add:
   50 µl **BioFluid & Cell Buffer** (Red)
   5 µl **Proteinase K**
   Note: For inputs < 50 µl biological fluid, increase the volume to 50 µl with DNA Elution Buffer or an isotonic buffer before continuing.
2. Mix thoroughly and then incubate the tube at 55°C for 10 minutes.
3. Add 1 volume **Genomic Binding Buffer** to the digested sample. Mix thoroughly.
   Example: Add 105 µl Genomic Binding Buffer to the 105 µl digested sample.
4. Transfer the mixture to a **Zymo-Spin™ IC-XM Column** in a **Collection Tube**. Centrifuge (≥ 12,000 *x g*) for 1 minute. Discard the collection tube with the flow through.
5. Add 200 µl **DNA Pre-Wash Buffer** to the column in a new Collection Tube and centrifuge for 1 minute. Empty the Collection Tube.
6. Add 700 µl **g-DNA Wash Buffer** and centrifuge for 1 minute. Empty the Collection Tube.
7. Add 200 µl **g-DNA Wash Buffer** and centrifuge for 1 minute. Discard the collection tube with the flow through.
8. To elute the DNA, transfer to a clean microcentrifuge tube. Add ≥ 10 µl **DNA Elution Buffer**, incubate for 5 minutes, and then centrifuge for 1 minute.

DNA Purification Made Simple

For the full Instruction Manual, visit
http://www.zymoresearch.com/m/D4074

Ver. 1.0.0

Appendix 3C: Kent and Norris (2005) blood meal PCR assay mastermix

Reagent		Volume for 1 reaction
10x PCR buffer		1.25 µl
10X dNTPs (2.5 mM each)		1.25 µl
25 mM $MgCl_2$ solution		0.75 µl
Primers (50 µM):	UNREV1025	0.5 µl
	HUM741F	0.5 µl
	DOG368F	0.5 µl
	COW121F	0.5 µl
	GOAT894F	0.5 µl
	PIG573F	0.5 µl
Taq (5 U/µl)		0.1 µl
PCR water		5.15 µl
Total		11.5 µl

Add 1.0 µl sample DNA to 11.5 µl PCR master mix for a 12.5 µl total mastermix

Appendix 3D: Cahyadi *et al.* (2018) blood meal PCR assay mastermix

Reagent		Volume for 1 reaction
10x PCR buffer		1.25 µl
10X dNTPs (2.5 mM each)		1.25 µl
25 mM $MgCl_2$ solution		0.75 µl
Primers (10 µM):	UN_F	0.5 µl
	Bov_R	0.5 µl
	Pig_R	0.5 µl
	Ch_R	0.5 µl
Taq (5 U/µl)		0.1 µl
PCR water		6.15 µl
Total		11.5 µl

Add 1.0 µl sample DNA to 11.5 µl PCR master mix for a 12.5 µl total mastermix

Appendix 3E:

Supplemental table 3.1. Tukey's HSD test for multiple comparisons of mean temperatures (2015-2019)

Cell No.	Tukey HSD test; variable DV_1 (Climatic parameters for 2014-2019) Approximate Probabilities for Post Hoc Tests Error: Within MS = ,77060, df = 44,000					
	YEARS	{1} **24,508**	{2} **24,250**	{3} **23,367**	{4} **22,825**	{5} **23,725**
1	2015		0,950622	0,021315	0,000367	0,204210
2	2016	0,950622		0,117619	0,002388	0,590144
3	2017	0,021315	0,117619		0,560823	0,854052
4	2018	0,000367	0,002388	0,560823		0,106585
5	2019	0,204210	0,590144	0,854052	0,106585	

*Values in blue are marked significant differences at $p < 0.05$

Values in bold represent annual mean temperatures

Supplemental table 3.2. Tukey's HSD test for multiple comparisons of mean relative humidity (2015-2019)

Cell No.	Tukey HSD test; variable DV_1 (Climatic parameters for 2014-2019) Approximate Probabilities for Post Hoc Tests Error: Within MS = 8,6838, df = 44,000					
	YEARS	{1} **68,400**	{2} **69,225**	{3} **69,583**	{4} **72,275**	{5} **70,808**
1	2015		0,958577	0,861275	0,019437	0,282149
2	2016	0,958577		0,998293	0,101248	0,682862
3	2017	0,861275	0,998293		0,185404	0,845664
4	2018	0,019437	0,101248	0,185404		0,740437
5	2019	0,282149	0,682862	0,845664	0,740437	

*Values in blue are marked significant differences at $p < 0.05$

Values in bold represent annual mean relative humidity

Appendix 3F:

Supplementary Table 3.1. Correlation coefficient (r-values) between annual feeding proportions on different vertebrate hosts per *Anopheles* female sampled and annual temperature, relative humidity, and total precipitation for 4 collection years (2015, 2016, May 2017-April 2018, and May 2018-April 2019).

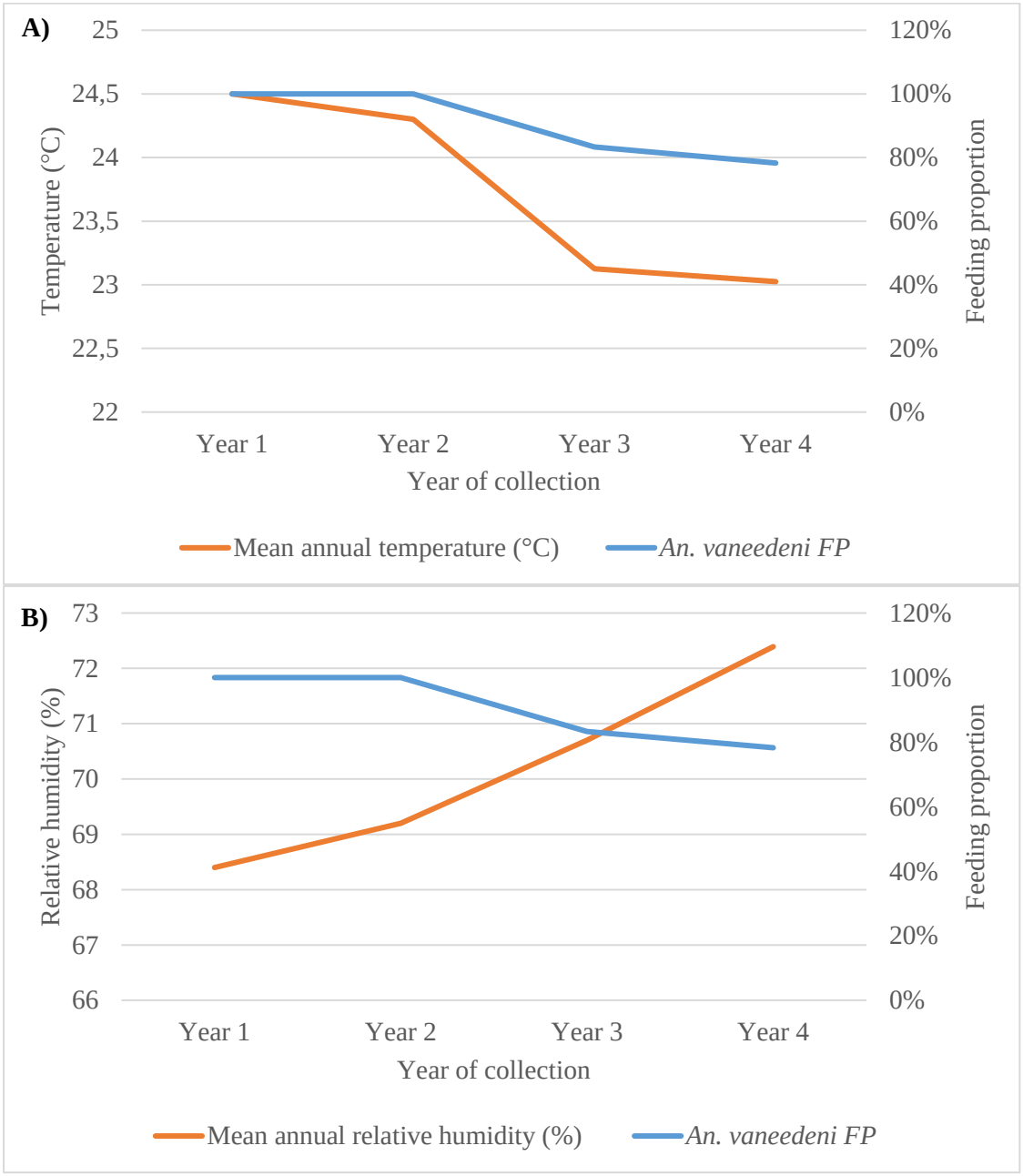
Year	Climatic variables	*An. leesoni* r-value	*An. parensis* r-value	*An. rivulorum* r-value	*An. vaneedeni* r-value
On cattle	Annual temperature (°C)	-0.738	-0.461	-0.266	0.986*
	Annual Average Relative Humidity (%)	0.738	0.118	0.215	-0.958*
	Annual Precipitation (mm)	0.738	0.854	0.404	-0.740
On goat	Annual temperature (°C)	0.775	0.258	0.469	-0.949**
	Annual Average Relative Humidity (%)	-0.775	-0.051	-0.398	0.949**
	Annual Precipitation (mm)	-0.775	-0.616	-0.571	0.738
On dog	Annual temperature (°C)	-	-0.993*	-	-
	Annual Average Relative Humidity (%)	-	0.901**	-	-
	Annual Precipitation (mm)	-	0.850	-	-
On pig	Annual temperature (°C)	-	-0.775	-0.738	-0.775
	Annual Average Relative Humidity (%)	-	0.775	0.738	0.775
	Annual Precipitation (mm)	-	0.258	0.949**	0.258

*Correlation is significant at the 0.05 level (2-tailed), $p < 0.05$, 95 %, as marked on STATISTICA

**Correlation is significant at the 0.1 level (2-tailed), $p < 0.1$, 90 %, as marked on STATISTICA

Values in green were calculated using Pearson's correlation, for normally distributed FP

Values in blue were calculated using Spearman's correlation. For non-parametric FP



Supplementary figure 3.1. Time series correlation between annual cattle feeding proportions of *An. vaneedeni* and climatic parameters in Mamfene. **A)** Correlation with annual temperatures (°C). **B)** Correlation with annual relative humidity (%). Year 1: 2015, Year 2: 2016, Year 3: May 2017-April 2018, Year 4: May 2018-April 2019. FP: feeding proportion.

Appendix 4A: 10% sucrose solution

10 g sucrose

100 ml deionized water

Cotton is then soaked with the sucrose solution, placed in a plastic cup and into mosquito cage for feeding.

Appendix 4B: Isoenzyme gel electrophoresis protocol and reagents

Collection, homogenisation, and storage of specimens. After specimens are collected, and homogenised as described in the text, in 80 µl grinding buffer using sterilised pestles.

1. Grinding buffer: (kept at 4 °C)

-2.5g sucrose (crystals)

-8 ml 1X TBE

-made up to 25 ml with dH_2O

Then ~4 µl of 1% bromophenol blue was added to each sample and controls. The dye allows visualization during loading and viewing.

2. 1% bromophenol blue – 10 ml (kept at 4 °C)

100 mg bromophenol blue

10 ml ddH20

Can be stored at room temperature

Homogenates were kept at -70 °C until ready for analysis.

3. Isoenzyme electrophoresis

3.1. 10x Tris-Borate EDTA buffer (pH 8.9)-TBE

Reagents	For 1 Litre	For 2 Litres	For 8 Litres
Tris; m.w.=	98.25 g	196.5 g	786 g
Boric Acid; m.w.=	17.5 g	35 g	140 g
EDTA, disodium salt; m.w. .= 372.7M	5.56 g	11.13 g	44.5 g
Distilled water	Make up 1L	Make up 2L	Make 8L

For 1L of 10x TBE:

Dissolve solutes in 800 ml water

Then adjust pH adjust to 8.9 with HCl before topping up with distilled water to 1L.

3.2. 1x TBE buffer that was used as an electrophoretic buffer:

5L 1x TBE:

-500 ml of 10X TBE buffer

-made up to 5L with distilled water (dH_2O)

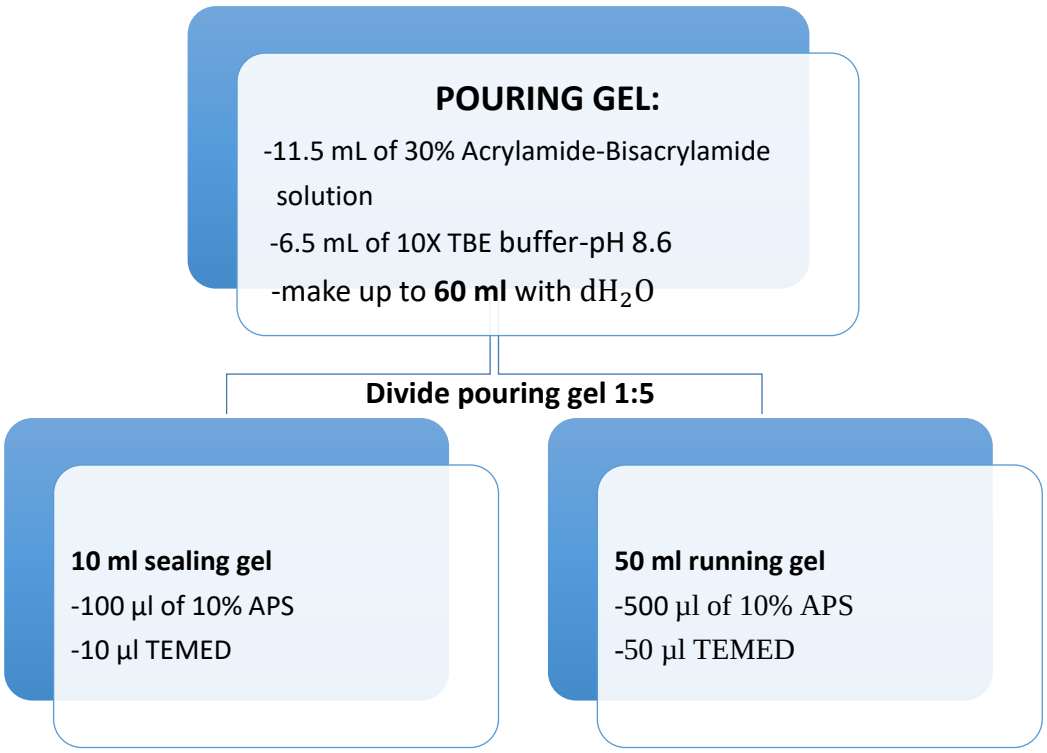
For Every electrophoretic reaction (4 large gels), 6L of 1X TBE is required.

(**Reagents are kept at room temperature**)

3.3. PAGE gel recipe-7.5% gel (for two gels)

Method:

1. Make **the pouring gel** as described in the diagram below
2. Separate the pouring gel 1:5
3. First make the sealing gel and add it into the plates from their corners.



Note:
-**N.B:** Always make 10% APS fresh for every use for best performance (hence always make a small amount: 0.1g in 1ml dH_2O)
-APS and TEMED are initiators of polymerization (**APS is the principal initiator**, and **TEMED is a catalyst**).
-**GB**: Grinding buffer
-*Running gel: adjusted calculations to allow timely gel polymerization.

4. Once the sealing gel has polymerized, make **the running gel** and pour it between the vertical plates. Immediately insert the 1mm combs between the plates.

5. Allow the gels to polymerize overnight.

6. Pre-run the polymerized gel for 1 hour at 150V with no regards to amperage

7. Cattle haemoglobin (Hb) was used as a marker for enzyme migration,

Hb marker preparation:

Lyze Cattle RBCs in blood to release Hb:

- Centrifuge ~150 µl blood at maximum speed (~13 000 rpm) for 5 minutes to lyze the RBCs and release Hb
- Remove the supernatant (plasma-~1/2) and leave the pellet containing RBCs
- In the 70µl pellet of RBCS, add 200 µl distilled water to create hypotonic solution.
- Leave the solution at room temperature for ~ 10 minutes to burst the RBCs and release Hbs
- Then 80 µl of the solution was added to 80 µl GB (grinding buffer) to increase density of the Hbs

8. Centrifuge the homogenates at 13 000 rpm for 5 minutes. This is to remove insoluble materials that may clog the pores of the acrylamide gel.

9. Slowly load 8 µl of each marker, control and homogenate into corresponding well using a syringe needle or pipette tip. Homogenates are added slowly to allow them to settle evenly at the bottom of the well. Then electrophorese

10. Initially start electrophoresis at 100 V with constant amperage of 400 mA for 15 minutes. Then 3 hours at a constant ampage of 40 mA, 50V with continuous cooling at 4 °C.

4. Staining isoenzyme gels:

4.1. 0.1M sodium phosphate buffer-pH 6.4

Containing 8% of alpha/1- naphthyl solution (2% alpha naphthyl acetate in 50% acetone)

Reagents required:

Reagents	**Measurements**
Sodium phosphate (monobasic) MW= 156.03	0.66g
Sodium phosphate (dibasic) MW = 268.07	0.27g
dH_2O	Make up to 50 ml
*2% α-Naphthyl acetate in 50 % acetone (substrate solution)	4 ml
Fast blue RR	0.05g or 0.1%

4.2. Sodium Phosphate buffer (Working buffer)
100 ml of 0.1 M sodium phosphate buffer mix-pH 6.4:
Mix 13.25 ml of 0.2M dibasic sodium phosphate (below) with 36.75 ml monobasic sodium phosphate (below). Dilute to 100 ml with ddH_2O.
Recipe source: https://www.ou.edu/research/electron/bmz5364/buffers.html

0.2 M dibasic sodium phosphate (1L) - stock
53.65 g made up to 1L with dH_2O

0.2 M monobasic sodium phosphate (1L) - stock
31.21 g made up to 1L with dH_2O

N.B:

The buffers are used to obtain the desired **pH 6.4 (do not use other chemicals to balance pH):**

-Dibasic sodium phosphate increases the pH.

-Monobasic sodium phosphate decreases the pH.

4.3. Substrate solution- 2% α-Naphthyl acetate:

Mix 1g of α-Naphthyl acetate plus 25 ml acetone

made up to 50 ml with dH_2O.

4.4. Soaking solution 1:

1. Mix 4ml of acetone solution with 46 ml of sodium phosphate buffer mix.

2. Take 25ml/50ml of the mixture and pour it onto the gel. Incubate at 37 °C for 30-45 minutes.

4.5. Soaking solution 2:

1. Measure 0.05g of Fast blue RR and combine it with the remaining sodium phosphate mix from step 3.4., by crushing the Fast Blue RR stain in the solution. Fast blue RR is a zinc stain (a negative stain).

2. Add the dye solution to the gel, and incubate the gel at 37 °C for 2 hours in a shaking incubator.

3. View and take a good image of the gel using the BioRad Gel doc system according to manufacturer's instructions (**Bio-Rad**: A guide to Polyacrylamide gel electrophoresis and detection, pp 41-43).

5. Destaining IEE gels

Solution mix of methanol, acetic acid, and water (5:1:5 ratio, v/v/v)

For a 1100 ml destaining solution:

500 ml acetic acid + 100 ml methanol + 500 ml water