Seasonal biology of malaria vectors in South Africa

Ashley Morgan Burke

A Thesis submitted to the Faculty of Health Science, University of the Witwatersrand, in fulfilment of the requirements for the degree of Doctor of Philosophy

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DECLARATION

DECLARATION –

I, Ashley M Burke, declare that this thesis is my own, unaided work. It is being submitted for the Degree of Doctor of Philosophy at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other University.

(Signature of candidate)

On this 1st day of October 2020

DECLARATION: Student's contribution to article(s) and agreement of co-author(s)

I, Ashley Morgan Burke, 1129903, declare that this Thesis is my own work and that I contributed adequately towards research findings published in the article(s) stated below which are included in my Dissertation.

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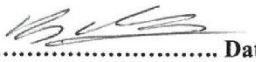

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Article 1: Title: A new malaria vector mosquito in South Africa

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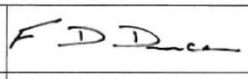
3rd author	Frances Duncan		25/3/2020
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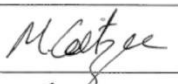
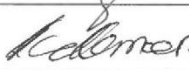
5th author	Maureen Coetzee		2-04-2020
6th author	Lizette Koekemoer		24-3-2020
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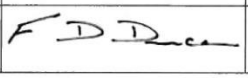
3rd author	Frances Duncan		25/3/2020

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None.

This thesis is dedicated to my parents, Brenda and Ivor.

You taught me the value of education and empowering myself through knowledge and I cannot thank you enough for that. Your unwavering support and encouragement from beginning to end afforded me the great honour of completing this degree.

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ABSTRACT

Residual malaria transmission refers to all forms of transmission beyond the reach of current control measures. Identifying the entomological drivers of residual transmission is a central component of South Africa's agenda to interrupt local transmission and to subsequently prevent re-establishment of transmission. This study aimed to 1) evaluate and compare the efficiency of different mosquito collection methods for routine surveillance, 2) determine the seasonal *Anopheles* assemblage within two malaria-affected regions, and 3) investigate winter dormancy in malaria vector mosquitoes. The merits of passive mosquito shelters (traditional clay pots and modified plastic buckets), used both indoors and outdoors, were assessed in parallel with active methods (human landing catches and CO_2 -baited nets) in two malaria affected villages. Passive vector surveillance was conducted weekly and active surveillance conducted monthly. The species of all *Anopheles* collected was determined using morphological keys and appropriate polymerase chain reaction (PCR) protocols. Every female mosquito was screened for *Plasmodium falciparum* circumsporozoite protein (CSP). The metabolic rate of wild anophelines, determined by CO_2 production during closed system respirometry, was measured. The effect of manipulated photoperiod on the metabolic rate of colonized *An. arabiensis* mosquitoes was determined by laboratory-based experiments. The outdoor clay pots accrued significantly more wild *Anopheles* than the plastic buckets and showed productivity alike to the active methods thus showing useful for routine vector surveillance. Changes in local population density of known vector and non-vector species was consistent with seasonal rainfall patterns. Malaria parasite screening of all females collected gave the first ever account of *P. falciparum* CSP-positive *An. vaneedeni* and *An. parensis* females in the wild. Although now considered secondary vectors, their role in malaria transmission in South Africa is likely minimal owing to their strong zoophilic tendency. There were no significant seasonal disparities in metabolic rate data obtained from wild-caught *Anopheles* nor amongst colonized *An. arabiensis* placed under simulated seasonal photoperiod conditions. The occurrence of vector species alongside malaria incidence throughout the dry season suggests that local vector populations do not enter a dormant phase in the adult stage. Due to the decrease in populations during winter leading to reduced malaria transmission, the study recommended control by winter larviciding of known breeding sites, which now forms part of South Africa's malaria vector control and elimination strategy for the period 2019 – 2023.

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PRESENTATIONS AND PUBLICATIONS

Presentations arising from this study

1. Oral presentation at the 2nd South African Malaria Research Conference, 31 July – 02 August 2016, University of Pretoria, South Africa.
2. Poster presentation at Health Sciences Research Day and Postgraduate Expo, 1 September 2016, University of the Witwatersrand, Johannesburg, South Africa.
3. Oral presentation at the combined congress of the Entomological Society of Southern Africa (ESSA) and the Zoological Society of Southern Africa (ZSSA), 3-7 July 2017, Pretoria.
4. Oral presentation at the 3rd Annual Southern Africa Malaria Research Conference, 7th – 9th November 2017, Johannesburg.
5. Oral presentation at the 4th Annual Southern African Malaria Research Conference, 30th July – 1st August 2018, Johannesburg.
6. Poster presentation at the Health Sciences Research Day and Postgraduate Expo, 06 September 2018, University of the Witwatersrand, Johannesburg.
7. Oral presentation accepted for the congress of the Entomological Society of Southern Africa, (talk delivered by Prof Frances Duncan), June 2019, Umhlanga, KwaZulu-Natal.

Publications arising from this study

1. Burke, A., Dandalo, L., Munhenga, G., Dahan-Moss, Y., Mbokazi, F., Ngxongo, S., Coetzee, M., Koekemoer, L. and Brooke, B. 2017. A new malaria vector mosquito in South Africa. *Scientific Reports*. 7:43779
2. Burke, A., Dahan-Moss, Y., Duncan, F., Qwabe, B., Coetzee, M., Koekemoer, L. and Brooke, B. 2019. *Anopheles parensis* contributes to residual malaria transmission in South Africa. *Malaria Journal*. 18:257
3. Burke, A.M., Brooke, B.D., Duncan, F.D. 2019. Metabolic rate does not vary with seasonal change in *Anopheles arabiensis* adults in South Africa. *Insect Physiology.* 118.

LIST OF ABBREVIATIONS AND SYMBOLS

%	percentage
µℓ	microlitre
°C	degrees Celsius
χ^2	chi squared
AMAL	*Anopheles arabiensis* strain originating from Malahlapanga, Kruger National Park, South Africa
ANOVA	analysis of variance
bp	base pairs
CI	confidence interval
cm	centimetres
CO_2	carbon dioxide
CSP	circumsporozoite protein
DDT	dichlorodiphenyltrichloroethane
dNTP	deoxyribonucleotide triphosphate
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
HCl	hydrochloric acid
HLC	human-landing collections
IRS	indoor residual spraying
ITS2	internal transcribed spacer 2
IVM	integrated vector management

KCl	potassium chloride
KWAG	*Anopheles arabiensis* strain originating from KwaZulu-Natal
KZN	KwaZulu-Natal
LLINs	long-lasting insecticide-treated nets
L	litre
M	molar
MAb	malaria antibody
MAFUS	*Anopheles merus* strain originating from Mafayene, Kruger National Park, South Africa
MBN-B	Susceptible *Anopheles arabiensis* strain originating from Mamfene, KwaZulu-Natal, South Africa
mg	milligram
$MgCl_2$	magnesium chloride
min	minute
ml	millimetre
mM	millimole
mm	millimetre
MR	metabolic rate
n	total number
nL	nanolitre
NaCl	sodium chloride
NHLS	National Health Laboratory Services
NICD	National Institute for Communicable Diseases

nM	nanomole
OD	optical density
p	probability level
PBS	phosphate buffered saline
PBS-TW	phosphate buffered saline- tween
PCR	polymerase chain reaction
Pf	*Plasmodium falciparum*
pH	potential Hydrogen
ppm	parts per million
rDNA	recombinant deoxyribonucleic acid
rFAL	*Plasmodium falciparum* specific primer
rPLU	*Plasmodium* (genus) primer
RMR	resting metabolic rate
Rpm	revolutions per minute
SA	South Africa
SANGWE	*Anopheles quadriannulatus* strain originating from Sangwe, Zimbabwe
SD	standard deviation
SE	standard error
SENN-B	Susceptible *Anopheles arabiensis* strain originating from Sennar, Sudan
SIT	sterile insect technique
s.l.	sensu lato (“in the broader sense” refers to a species complex as a whole)
s.s.	sensu stricto (“in the narrow sense” refers to a specific species)
ssRNA	single-stranded ribonucleic acid

TAE	Tris (hydroxymethyl) aminomethane-acetate
TE	Tris (hydroxymethyl) aminomethane ethylenediaminetetraacetic acid
UV	ultraviolet
V	voltage
VCRL	Vector Control Reference Laboratory
WHO	World Health Organisation
WRIM	Wits Research Institute for Malaria

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CHAPTER ONE – INTRODUCTION

1.1 Literature review

1.1.1 History and impact of malaria

Malaria has an ancient and ongoing association with humans. Despite having affected human civilisations for thousands of years, it was only from 1880 that a grasp of the cause of malaria was realised. The discovery of parasites belonging to the *Plasmodium* genus in the blood of malaria patients by Laveran and the incrimination of female *Anopheles* mosquitoes as the transmission vector by Ross and Grassi (Harrison 1978) forged the way for scientific studies on this disease. Malaria is still perhaps the most important and devastating threat to global public health despite the largest disease burden being concentrated in a select number of countries (Snow 2015).

Malaria is still largely endemic throughout Sub-Saharan Africa, parts of South America and South Asia. A staggering 228 million cases were reported in 2018 with an estimated 405 000 malaria-related deaths globally (WHO 2019). The WHO African region accounted for approximately 94% of total recorded deaths in 2018, 99.7% of which were attributed to infection with *Plasmodium falciparum* (WHO 2019). *Plasmodium falciparum* causes malignant tertian malaria and is considered the most important human malaria parasite species. Other *Plasmodium* species of importance to humans include *P. vivax* Grassi and Feletti, *P. malariae* Grassi and Feletti, *P. ovale* Stevens, and in some cases the simian malaria parasite *P. knowlesi* Sinton and Mulligan (Singh *et al.* 2004, Jongwutiwes *et al.* 2004, Cox-Singh *et al.* 2008). The global drive to eliminate malaria from affected countries has suffered slow progress in recent years. The African countries that make up the 10 highest impacted regions experienced an increase of approximately 3.5 million malaria cases in 2017 from 2016 (WHO 2018a). This increase threatens the two key 2020 milestones of the WHO Global Technical Strategy for Malaria 2016-2030, which aim to reduce case incidence and mortality by at least 40% from levels experienced in 2015 (WHO 2018a). There is still a preponderance of people at risk of malaria infection without adequate protection. Children under five years and pregnant women are particularly at risk of fatal infection with malaria. Young children have often not yet developed partial immunity to the disease and pregnant women experience weakened immunity, increasing their risk of malaria fatality (Reuben 1993). The World Malaria Report (WHO 2018a) revealed that not all people at risk of malaria infection have enough access to potentially life-saving tools and interventions. Rigorous vector control in

malarious areas coupled with early detection and treatment of infections is vital to the global malaria elimination agenda.

1.1.2 Malaria transmission between mosquito and host

The developmental program of the malaria parasite involves switching between extracellular motile invasion stages and intracellular replication phases (Matuschewski 2006). The cycle of human malaria transmission starts when a female *Anopheles* mosquito ingests male and female gametocytes with a blood meal from an infected human host. Fertilisation of the female gametocyte (macrogametocyte) follows to form a motile ookinete that then passes through the stomach wall of the mosquito and encapsulates on the coelomic surface forming an oocyst. There the oocyst develops into a sporocyst that later ruptures and releases sporozoites that migrate to the salivary glands, making the female *Anopheles* mosquito infective to her next meal. The bite of an infective mosquito inoculates the host's tissue with sporozoites that enter the bloodstream via the basal side of a blood capillary (Vanderberg and Frevert 2004, Amino *et al.* 2006) and migrate to the host's liver cells. The circulatory system allows the sporozoites to reach the liver within minutes (Matuschewski 2006). Within the liver sporozoites attach to the endothelial cell layer and move along it in search of hepatocytes to invade. Sporozoites then undergo asexual replication within the hepatocytes to form thousands of merozoites that then attach to and penetrate the red blood cells (Baldacci and Ménard 2004). The next stage, referred to as the blood stage or the erythrocytic phase of the cycle, is usually when clinical symptoms of the disease present. The merozoite develops and replicates asexually to form a mature schizont containing about 10 new merozoites (Baldacci and Ménard 2004). The mature schizont then ruptures the red blood cell to liberate the merozoites that go on to invade other uninfected red blood cells. Gametocytes will appear in the blood stream following several cycles of schizogony, making the human host infective to the next female *Anopheles* mosquito that takes a blood-meal.

Typical symptoms of uncomplicated malaria include fever, chills or rigors, headaches, nausea and/or vomiting, sweats, body aches, and general malaise. Infections may become severe and lead to death if left untreated and/or if serious complications arise. The rapid degenerative nature of malaria demands early diagnosis, rapid treatment and prevention of further infection for control and elimination purposes.

1.1.3 Biological characteristics and systematics of African malaria vectors

Accurate species identification of those mosquitoes involved in malaria transmission is essential to the success of vector control programmes. Primary identification is based on the external morphology of adult mosquitoes or larvae. Dichotomous keys are used to identify species within the *Anopheles* genus according to morphological characteristics and are useful for grouping potential vectors and non-vectors (Gillies and de Meillon 1968, Gillies and Coetzee 1987, Coetzee 2020). However, morphology alone cannot always provide an identification to species as many medically important taxa are members of species complexes or groups in which all members are either morphologically identical or differentiating characteristics between sibling species are obscure and easily confused (Spillings *et al.* 2009, Erlank *et al.* 2018). A species complex infers that all members are morphologically identical to one another, as evident in the *An. gambiae* complex, whereas a species group may contain some members that are actually morphologically distinguishable using two or more life stages (White 1977), as evident in the *An. funestus* group. In this case, further molecular methods of identification are required to distinguish between sibling species within a complex or group. Robust molecular identification tools have been established for the *An. gambiae* complex (Scott *et al.* 1993) and the *An. funestus* group (Koekemoer *et al.* 2002, Cohuet *et al.* 2003). Distinguishing the few malaria vector species from the hundreds of non-vector *Anopheles* species is important because certain bionomic characteristics, such as insecticide susceptibility/ resistant phenotypes, only have meaningful operational implications for control if they are linked to vector populations by species.

1.1.3.1 Basic description of members of the An. gambiae complex and An. funestus group

The *An. gambiae* complex comprises the major African malaria vector species *An. gambiae* Giles, *An. coluzzii* Coetzee and Wilkerson, and *An. arabiensis* Patton, as well as the saltwater-adapted species *An. melas* Theobald and *An. merus* Dönitz, which are less effective in malaria transmission but may become vectors when present in high numbers (Gillies and de Meillon 1968, Coetzee *et al.* 2013b, Sharp *et al.* 2007, Cuamba and Mendis 2009). Also included in this complex is the localised minor vector *An. bwambae, An. fontenillei* recently described from Central Africa (Barrón *et al.* 2019), and the primarily zoophilic species *An. quadriannulatus* Theobald and *An. amharicus* Hunt, Wilkerson and Coetzee that have not been implicated in malaria transmission (Gillies and Coetzee 1987, Hunt *et al.* 1998, Sinka *et al*, 2010, Coetzee *et al.* 2013a). The *An. funestus* group originally consisted of nine members

of which *An. funestus* Giles is the most important vector species (Gillies and Coetzee 1987, Coetzee and Koekemoer 2013). Subsequent systematic reclassification of the *An. funestus* species group allocated *An. funestus*, *An. aruni* Sobti, *An. parensis* Gillies, *An. confusus* Evans and Leeson, *An. vaneedeni* Gillies and Coetzee, and *An. funestus*-like from Malawi to the *An. funestus* subgroup, while *An. brucei* Service, *An. fuscivenosus* Leeson, *An. rivulorum* Leeson, and *An. rivulorum*-like were assigned to the *An. rivulorum* subgroup (Hackett *et al.* 2000, Cohuet *et al.* 2003, Harbach 2004, Garros *et al.* 2005, Spillings *et al.* 2009). *Anopheles leesoni* was reclassified to the Asian *An. minimus* subgroup (Harbach 2004).

Anopheles funestus is the most efficient transmission vector of the *An. funestus* group, but several other members (Coetzee and Koekemoer 2013) have emerged as secondary vectors in some regions. Secondary vectors include *An. rivulorum* which was implicated in malaria transmission in Tanzania (Wilkes *et al.* 1996) and in Kenya (Kawada *et al.* 2012), *An. vaneedeni* implicated in South Africa (Burke *et al.* 2017), and *An. parensis* also recently implicated in South Africa (Burke *et al.* 2019b). The role of secondary vectors in malaria transmission is not yet fully understood but is likely minor or localised.

Certain members of the *An. gambiae* species complex and *An. funestus* subgroup are of significant public health concern in southern Africa. Sibling species within these groups may look near identical, but their breeding site choice, adult behaviours, and roles in malaria transmission vary considerably (Gillies and De Meillon 1968). *Anopheles funestus* is the most anthropophilic of the two major vector species in southern Africa (Gillies and De Meillon 1968) and tends to breed in permanent water bodies located close to human settlements (Sinka *et al.* 2010). *Anopheles funestus* larvae prefer perennial or semi-perennial freshwater bodies that contain emergent vegetation. The adults are highly endophilic and usually bite indoors where humans are their primary blood source (Gillies and De Meillon 1968, Gillies and Coetzee 1987, Sinka *et al.* 2010). *Anopheles arabiensis*, like other members of the *An. gambiae* complex, opt to breed in smaller, non-perennial, shallow freshwater bodies, which may be slow-moving and partly shaded by vegetation (Edillo *et al.* 2002, Himeidan and Rayah, 2008). *Anopheles arabiensis* adults show highly variable behaviour, taking blood meals from both humans and animals, resting both indoors and outdoors, and biting both indoors and outdoors (White 1974, Gillies and Coetzee 1987, Sinka *et al.* 2010). The female adults of both *An. funestus* and *An. arabiensis* are most active at night and take blood meals between 19:00 and 03:00 (Tirados *et al.* 2006), with *An. funestus* showing peak biting after

22:00 (Oyewole *et al.* 2007, Oyewole and Awolola 2006). The variable behaviours of *An. arabiensis*, particularly their outdoor resting and biting habits, pose a significant challenge to current vector control methods that largely target indoor-resting mosquitoes. The indoor behavioural habits of *An. funestus* allow for very effective control through indoor residual spraying (IRS), which has reduced occurrence of this species in South Africa to an undetectable level (Coetzee *et al.* 2013b) using current surveillance methods.

1.1.4 General methods for malaria vector control

Vector control is the most important and effective tool for the suppression of vector-borne diseases. The planning of vector control operations requires careful consideration of several basic principles that include; i) reducing adult vector populations and thus disease transmission, ii) ensuring that interventions are ecologically, environmentally, socially, economically and politically viable, iii) establishing the environmental safety, risk of resistance development, and possible non-target effect of interventions, iv) understanding the parasite-vector-host transmission cycle, basic vector bionomics and survival strategies, v) developing descriptive and predictive vector population and transmission models, and vi) implementing evidence-based interventions that are responsive and able to adjust to feedback from surveillance systems as part of an integrated vector management (IVM) programme (Beier *et al.* 2008). The entomological component is fundamental to control planning and encompasses accurate vector species identification, insecticide susceptibility testing, and behavioural monitoring (Brooke 2019). The target should be unambiguously identified and enough of its ecology understood for measures towards its control to be implemented effectively. This information also aids the development of supplementary methodologies for the control of vector populations.

Current methods for malaria vector control rely heavily on the use of insecticides. Key control strategies include indoor residual spraying (IRS) of houses and the use of long-lasting insecticide-treated nets (LLINs) (Fig. 1.1, Benelli and Beier 2017). High coverage with either of these interventions, or a combination of both, has generally resulted in large-scale reduction of malaria-related cases and deaths (Pluess *et al.* 2010). Pyrethroids are still largely favoured for use in bednets, but the switch to alternative insecticides for IRS, especially non-pyrethroid products, is growing (Sherrard-Smith *et al.* 2018). The WHO currently recommends 16 insecticide formulations for use in IRS, including dichlorodiphenyltrichloroethane (DDT),

from five different chemical classes; organochlorines, organophosphates, pyrethroids, carbamates, and neonicotinoids (WHO 2018). The organochlorine, organophosphate, pyrethroid, and carbamate classes of insecticides used for IRS target two insect neurological sites, causing mortality on contact, whereas the recent addition of neonicotinoids to the approved list of chemicals has a novel mode of action that will likely reduce the risk of cross-resistance (IRAC, 2020). The chemical classes vary in residual activity, cost, and efficacy in field applications (Tangena *et al.* 2020). Insecticide spraying programmes throughout Africa initially favoured pyrethroids and DDT for IRS due to their low cost and longer residual decay rates, but the development of resistance to these insecticides necessitated the shift to alternative and more expensive insecticides such as carbamates and, later, organophosphates (Walker 2008, Oxborough 2016, Tangena *et al.* 2020).

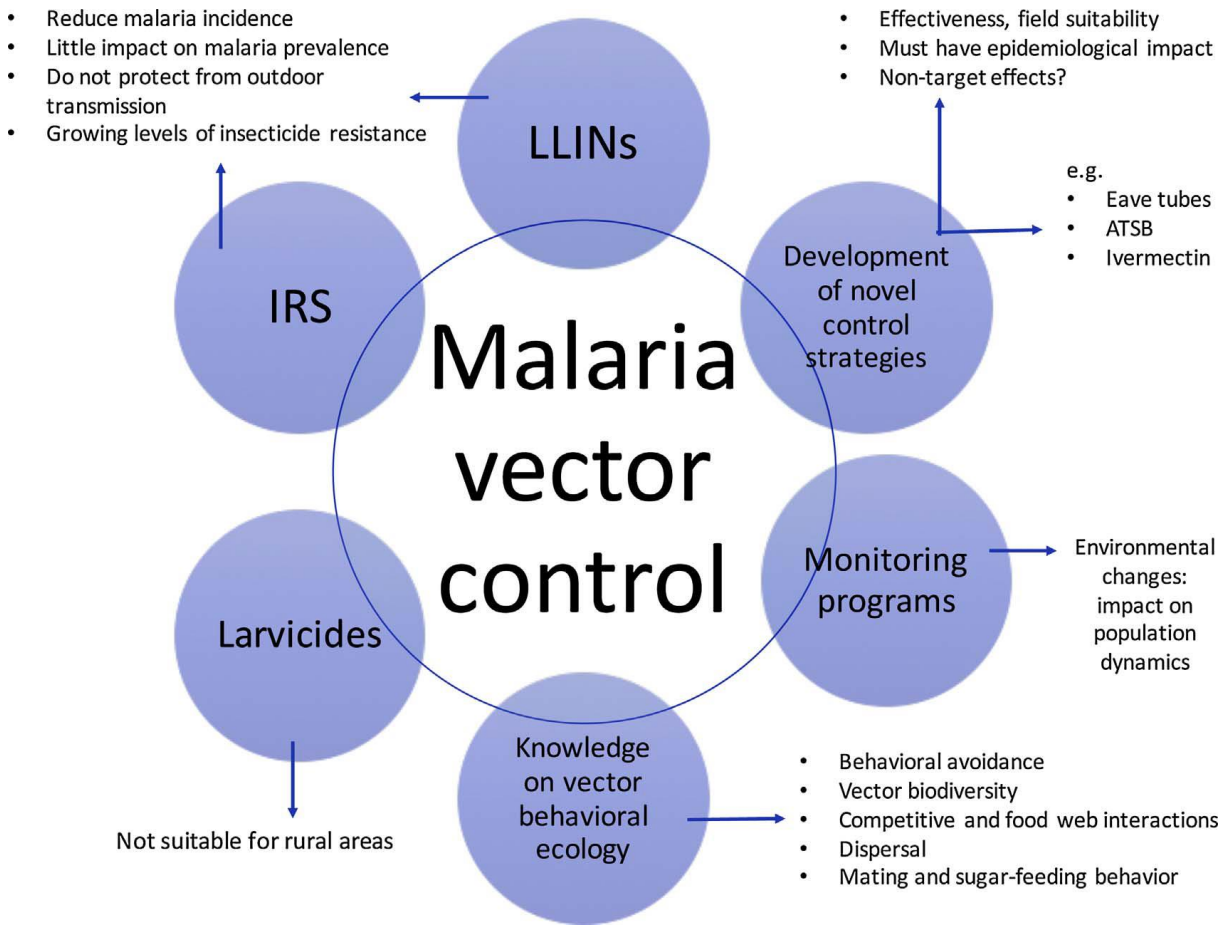

Figure 1.1. Main challenges and trends in current malaria vector control research. Source: Benelli and Beier, 2017.

1.1.4.1 Insecticide resistance, its impact and management

Extensive insecticide use in both vector-borne disease control and agricultural pest management has led to large-scale selection for resistance (Sparks and Nauen 2015). Insecticide resistance in a vector population refers to the ability of mosquitoes to withstand exposure to insecticide at the recommended dose through physiological adaptations or behavioural avoidance (WHO 2018b). Physiological traits of resistant mosquitoes allow them to either metabolise insecticide at a much higher rate or to absorb toxins at a much lower rate than do their susceptible counterparts. Simple behavioural changes from endophily to exophily are also effective evolutionary adaptations leading to resistance in vector

populations. Rapid generation turnover and large population sizes lead to high levels of genetic recombination and variation in mosquitoes. These factors in areas of strong insecticide selection pressure often lead to the development of insecticide resistance in wild vector populations. Resistance to the pyrethroid class of insecticides is almost ubiquitous in African malaria vector species (reviewed by Ranson *et al.* 2011). Evidence of resistance has been recorded in *An. funestus* and *An. arabiensis* in South Africa, particularly in KwaZulu-Natal (Hargreaves *et al.* 2000, Hargreaves *et al.* 2003), Mozambique (Brooke *et al.* 2001, Casimiro *et al* 2006a, Casimiro *et al.* 2006b), Zimbabwe (Munhenga *et al.* 2008, Sande *et al.* 2015) and in *An. funestus* alone in Zambia (Choi *et al.* 2014), Ghana (Okoye *et al.* 2008), and Burundi (Protopopoff *et al.* 2008). Pyrethroid resistance in *An. gambiae* is widespread throughout regions of central and western Africa (Santolamazza *et al.* 2008, Awolola *et al.* 2002, Corbel *et al.* 2007, Yawson *et al.* 2005) which threatens successful control of malaria transmission in high-risk areas.

1.1.4.2 Mechanisms of insecticide resistance

The two main mechanisms for resistance are modifications of the insecticide target-binding site (target site resistance) and increased rates of insecticide detoxification (metabolic resistance) (Ranson *et al.* 2010). Auxiliary modes of resistance include increased insecticide tolerance of resistant *An. funestus* females following a blood-meal prior to exposure (Spillings *et al.* 2008) and thickening of the cuticle in resistant phenotypes (Wood *et al.* 2010). The longevity of *An. funestus*, development of insecticide resistance in certain populations, and highly adaptable nature of both *An. arabiensis* and *An. funestus* contribute to their efficiency as malaria vectors, which poses a great risk to the public health of southern Africa (Gillies and De Meillon 1968, Gillies and Coetzee 1987, Gatton *et al.* 2013). The threat of insecticide resistance, particularly pyrethroid resistance, and artemisinin drug-resistance (Ashley *et al.* 2014) intensifies the risk of malaria outbreaks and epidemics, which necessitates effective vector surveillance and management in high-risk and endemic regions.

1.1.4.3 Insecticide resistance management

The WHO launched the Global Plan for Insecticide Resistance Management (GPIRM) to sustain existing vector susceptibility and curb the spread of insecticide resistance (WHO 2012). Recommendations by the GPIRM include mosaic spraying, insecticide rotation, integrated methods of control, and the use of combinations of insecticide compounds with different modes of action for vector control (WHO 2012). The overwhelming need for the development of new chemicals led the WHO to grant interim approval of chlorfenapyr, a pyrrole, for use in insecticide treated nets (ITNs) (WHO 2017a). Following evidence of improved malaria transmission control, increased use of the long-lasting insecticidal net incorporating a synergist piperonylbutoxide (PBO) has also been recommended by the WHO (Protopopoff *et al.* 2018, WHO 2017b). Recent studies on the efficacy and durability of a combination of pyrethroid, permethrin, and pyriproxyfen (an insect juvenile hormone mimic) treated nets showed promise for LLIN application in African countries with some improvements (Toé *et al.* 2019). Second-generation insecticides, such as pirimiphos methyl and clothianidin, have become available and have been prequalified by the WHO for use in IRS programmes (WHO 2018c, Sherrard-Smith *et al.* 2018). Clothianidin, a neonicotinoid, has shown promising efficacy following susceptibility testing in 16 African countries (Oxborough *et al.* 2019). Using a combination of two or more insecticides with different modes of action offers a robust attack on highly adaptable vector species. Insecticide combination field trials using clothianidin and deltamethrin showed effective control of pyrethroid-resistant vectors in Equatorial Guinea (Fuseini *et al.* 2019).

1.1.4.4. Alternative methods of control

Although considerable ground has been gained from the use of insecticides, the growing threat of resistance necessitates an integrated approach to vector control that includes alternative tools to supplement current methods. Integrated vector management is widely promoted by the WHO's Global Malaria Control Strategy and attention has turned to incorporating an array of alternative interventions into current control regimens. New control innovations that do not rely on insecticide-use are developing rapidly. There are now four classes of malaria intervention that have shown efficacy in the field: environmental modification, biological control, chemical treatment, and genetic modification (McGraw and O'Neill 2013). Alternative strategies range from the introduction of biological control agents

to the genetic manipulation of wild vector populations through the sterile insect technique (SIT) and gene drives. Larval source management (LSM) has re-emerged as a tool for vector population suppression following its previous disbandment in favour of IRS in the early twentieth century. Current interventions largely target the adult vector population, particularly indoor resting and biting mosquitoes, but targeting the aquatic stages by reducing larval habitats has a broader effect on both indoor and outdoor vectors (see Fillinger and Lindsay 2011). Mosquito breeding site management and microbial larviciding have shown great potential for success when implemented in conjunction with established IRS methods (Fillinger and Lindsay 2011). The logistical challenge of reaching all breeding pools with larvicide is a major drawback of this method in affected areas with extensive water bodies or floodplains (Majambere *et al.* 2010), but there remains potential for LSM as a part of IVM. The SIT relies on mass production and release of sterile male vector species in sufficient number to induce sterility of wild population females that eventually leads to a reduction in vector population density (Lees *et al.* 2015). The methods for mass-rearing, sterilization (by irradiation see Bakri *et al.* 2005, or using *Wolbachia* see Saridaki and Bourtzis 2010), and release are available and may be a useful supplement to current vector control programmes. Genetic manipulation of wild vector populations provides the beneficial features of area-wide control, self-dispersing control agents, and species-specific targets (Alphey 2014). There are still uncertainties associated with some of these methods that warrant further study of vector biology, consideration of ethical factors, and thorough training of personnel before these supplementary interventions can be included in routine control programmes.

1.1.5 Malaria transmission in South Africa

Malaria incidence is still largely driven by seasonal trends in South Africa. The main transmission season often parallels the high rainfall period from September to May when there is an abundance of potential breeding sites to sustain high vector population density (Dandalo *et al.* 2017) and peaks during the December and April holidays when there is high human cross-border traffic with our highly endemic neighbouring countries (Raman *et al.* 2016). Vector control strategies deployed in South Africa today are primarily insecticide-based and involve indoor residual spraying (IRS) of purpose-formulated insecticides and targeted larval source management (Brooke *et al.* 2013). These methods have reduced malaria incidence in South Africa by at least 95% (Brooke 2019) and, along with the mass distribution of

insecticide treated bed nets, have also achieved a global reduction in malaria incidence where used correctly (Pluess *et al.* 2010). Burgeoning insecticide resistance in several vector populations within South Africa continue to challenge vector control and elimination efforts (Brooke *et al.* 2013).

Malaria incidence was far more widespread in South Africa towards the beginning of the 20th century than it is today. Large-scale implementation of intensive malaria prevention and vector control programmes has confined malaria transmission to the low altitude border regions of Limpopo, Mpumalanga, and KwaZulu-Natal provinces in South Africa (Fig. 1.2). The introduction of IRS with DDT and robust surveillance strategies in the late 1940s significantly reduced *Anopheles* population density and thus malaria incidence levels to near zero by 1970 (Coetzee *et al.* 2013b). A brief resurgence in malaria brought on by high rainfall and flooding over the 1971/1972 malaria season was brought under control by intensified surveillance, rapid treatment with chloroquine, and application of DDT-based IRS (Coetzee *et al.* 2013b). Later in 1978, another severe epidemic brought on by heavy rains ravaged settlements east of Tzaneen, Limpopo province, which was again suppressed by an increase in resource allocation to the affected areas (Coetzee *et al.* 2013b).

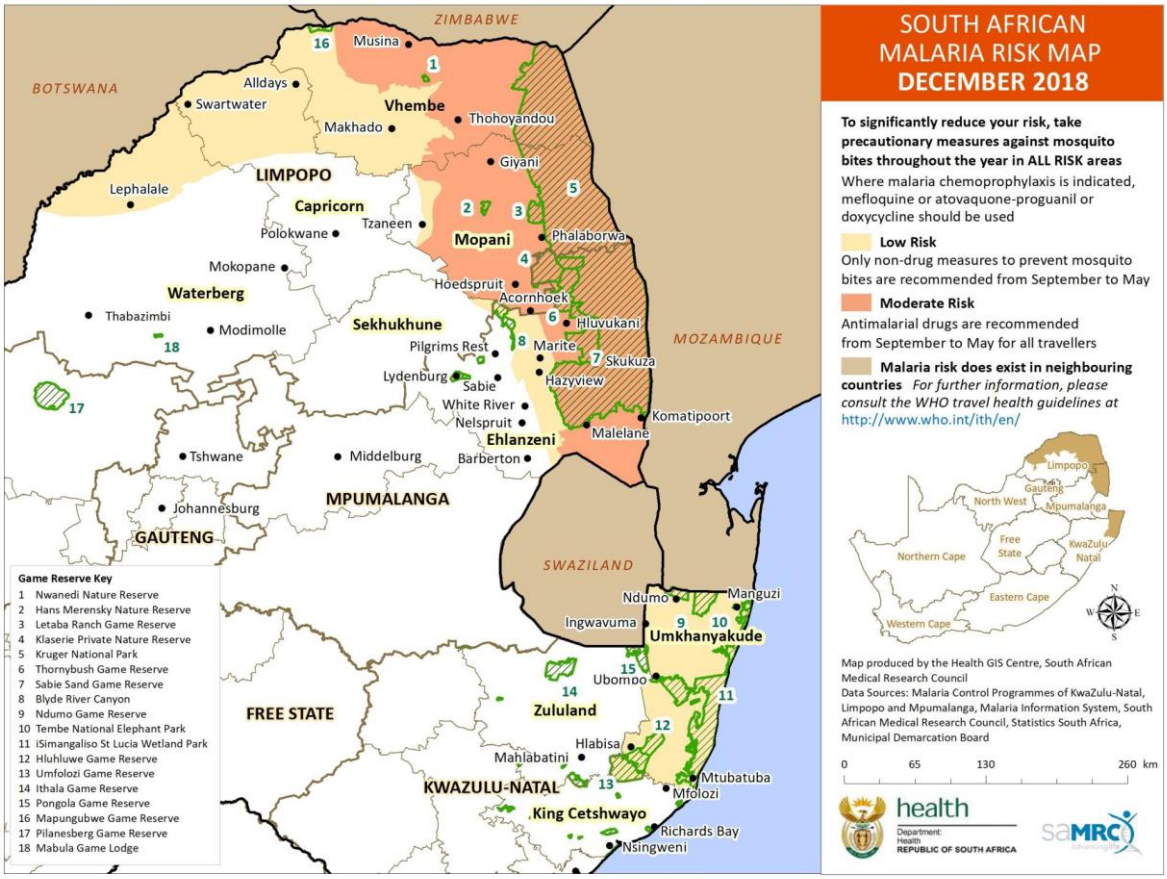

Figure 1.2. South African malaria risk map 2018. Source: National Department of Health, South Africa.

The worst malaria epidemic South Africa has experienced to date progressed from the late 1990s to its peak in 2000 which saw over 60 000 reported cases in the year 2000 (Maharaj *et al.* 2013). The cause of this vast epidemic was likely a perfect storm combination of favourable environmental conditions and a rise in cross-border human movement compounded by increases in drug-resistant parasites and insecticide-resistant vectors (Maharaj *et al.* 2012). The effects of the epidemic were mitigated by an increase in cross-border malaria control between the hyperendemic region of southern Mozambique and South Africa (Sharp *et al.* 2007), introduction of artemisinin-based combination therapy (ACT) to overcome drug-resistant parasites (Barnes *et al.* 2005), and re-introduction of the effective insecticide DDT for IRS activities (Maharaj *et al.* 2005). Transmission was subdued to a low level for just over a decade following the height of the 2000 outbreak and malaria elimination in South Africa seemed feasible, until the unexpected upsurge in locally acquired cases and

malaria-related deaths in 2017 (Figure 3; National Department of Health, 2019). Malaria cases increased more than three-fold from 2016 to 2017, which was facilitated by an unusually high rainfall period in 2017 together with delayed IRS activities in some regions (Christian *et al.* 2018). Transmission rates declined from 2017 to 2018 and remained at a low level going into 2019 (Fig. 1.3).

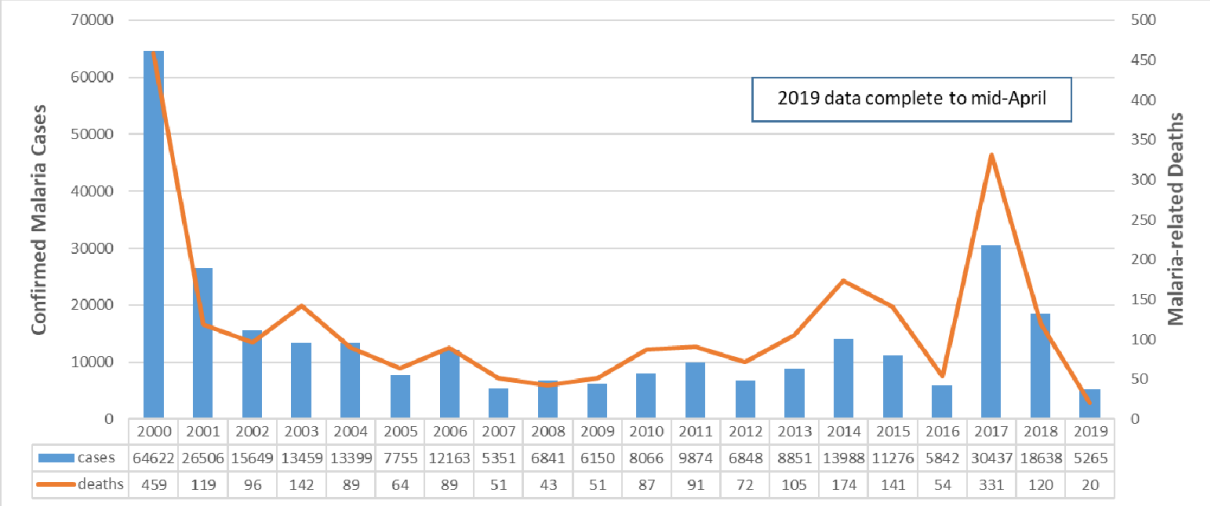

Figure 1.3. Malaria cases and deaths in South Africa from 2000 to 2019. Source: National Department of Health. Data subject to updating.

South Africa is steadily working towards the goal of malaria elimination within its borders. The Malaria Elimination Strategic Plan, developed by the National Department of Health (NDOH), aims to achieve zero malaria transmission within South Africa by 2023 (National Department of Health, 2019). The NDOH hopes to reach this target by completing a number of key strategic objectives that centre around ensuring optimal implementation of malaria interventions, strengthening entomological and malaria surveillance systems, providing broad-scale education and establishing communication pathways with 90% of people affected by malaria, achieving at least 95% coverage with key vector suppression strategies and interventions, together with ensuring universal access to accurate diagnosis and rapid treatment in both endemic and non-endemic regions of South Africa (National Department of Health, 2019). From an entomological perspective, there are several knowledge gaps about the vector that, when addressed, will significantly reinforce the effectiveness of the weaponry used in the fight against malaria.

1.1.6. Residual transmission in South Africa

1.1.6.1 Vector surveillance

Low-level residual transmission continues to impede malaria elimination in South Africa. Identifying the entomological drivers of residual transmission requires ongoing and intensified vector surveillance and research of mosquito behaviour and biology (see Section 3.1). Investigating the vector population density, species composition, breeding habits, resting and biting activities, and the extent of insecticide resistance of local vector populations is instrumental to developing appropriate novel control strategies to supplement existing methods in transmission regions. Live collected specimens can be used to assess vector species composition, relative and seasonal abundance, biting behaviour, blood-meal source by species and insecticide susceptibility by species, all of which contribute vital information required to develop and implement effective vector control. This information provides the base on which choices of insecticide for IRS, spraying priorities by region and complementary vector control strategies in high risk or epidemic-prone regions depend. Because of this, it is crucial to establish effective and efficient surveillance techniques for use in high-risk areas.

Strengthening surveillance techniques initially depends on assessing the performance of various passive and active vector-trapping techniques. Passive adult mosquito collection techniques of relevance to this project include ceramic pots and modified buckets placed both indoors and outdoors, and active trapping techniques include outdoor CO_2 traps and human landing catches. The human landing catch technique is generally efficient for high vector catch rates; however, this method carries the risk of exposing collectors to vector-borne diseases. Other active techniques such as CO_2 baited nets need to be cleared periodically throughout the night and require two field visits; one to setup the apparatus and one to collect the mosquitoes, which raises logistical problems. Sources of CO_2 would also need to be arranged for every sampling trip. Collection efforts have therefore shifted towards passive trapping techniques that can be installed and cleared early each morning as frequently as is logistically possible. Clay pots placed outdoors were found to be efficient for trapping blood-fed female *An. gambiae* in parts of Kenya (Wong *et al.* 2013) as well as Kwa-Zulu Natal (Dandalo *et al.* 2017), which is useful for behavioural studies and analysing blood-meal sources. The modified plastic buckets mimic the clay pots, to an extent, providing a cheaper and easily available collection method effective in trapping vector species. Comparisons between trapping methods enables the development of localised malaria vector species surveillance strategies based on the most suitable combinations of trapping techniques.

1.1.6.2 Seasonal activity of vectors

Because malaria transmission is seasonal in affected regions of South Africa, it is necessary to establish the underlying seasonal biology of the major vector species. Mosquito population density is largely dictated by changes in rainfall, temperature, and humidity. The *An. arabiensis* population shows a marked increase in population density during the wet summer season and a significant decrease during the dry winter season in South Africa (Dandalo *et al.* 2017). Interestingly, the vector population density declines during the dry season but does not disappear entirely and malaria transmission continues albeit at substantially lower levels (Munhenga *et al.* 2014, Dandalo *et al.* 2017). The physiological and/or behavioural changes that *Anopheles* undergo to accommodate unfavourable conditions remains largely unknown.

The known life history characteristics of African *Anopheles* do not advocate long-term survival in dry conditions. The lifespan of *Anopheles* is generally short (Maharaj 2003, Olayemi and Ande 2009, Oliver and Brooke 2014) and the aquatic stages of development are highly susceptible to desiccation (Koenraadt *et al.* 2003), which suggests survival in the egg stage during unfavourable conditions is unlikely. Hypotheses for vector survival during unfavourable conditions include: (i) the use of perennial breeding sites or refugia, (ii) long-distance migration between permanent breeding sites and seasonally arid areas, and (iii) entering a low metabolic state to extend longevity (winter diapause or summer aestivation) in sheltered dwellings (Omer and Cloudsley-Thompson 1968, Omer and Cloudsley-Thompson 1970, Dukeen and Omer 1986, Obala 1995, Charlwood *et al.* 2000, Donnelly *et al.* 2002, Lehmann *et al.* 2010, Munhenga *et al.* 2014, Huestis and Lehmann 2014). Because of the residual malaria case incidence and evidence of continued feeding and breeding activities during the dry season in South Africa, it is presumed that the vector populations do not enter a dormant state in unfavourable conditions. Insights into the seasonal biology of vector populations may elucidate their survival mechanisms and strategies that may present novel opportunities for targeted control.

2.1 Study Rationale

South Africa has made great strides in malarial control since adopting an elimination agenda in 2012, although many challenges have since been identified that have the potential to derail progress towards a country free of malaria. Some of the unaddressed challenges to control programmes today hinge on the lack of information about vector distribution and insecticide susceptibility (Raman *et al.* 2016). It is critical to address knowledge gaps in the complexities of *Anopheles* identification, vector incrimination, insecticide resistance, and behavioural diversity to sustain progress towards elimination. The entomological component of malaria programmes cannot be neglected. The collection of information about the vectors informs control procedures and contributes to the development of additional interventions and policies. Increasing our understanding of the biology and behavioural traits of *Anopheles* species directly enhances the efficiency and efficacy of control measures. The presence of outdoor resting and biting vectors, in combination with local pockets of parasite-infected hosts, is currently presumed to be a major driver of residual malaria in South Africa. Thorough entomological surveillance together with reliable vector incrimination will elucidate the contribution of outdoor-active vector populations. Very little is currently known about the dry-season physiology and behavioural adaptations of *Anopheles*, which may have important consequences for control strategies. Filling these gaps in our current understanding of vector biology may contribute significantly to finding alternative methods of control and to the grander implementation of integrated vector management strategies.

2.1.1 Study aim and objectives

The aim of this study was to clarify the entomological drivers of residual transmission in two malaria-affected regions of South Africa, Mpumalanga and KwaZulu-Natal, by assessing *Anopheles* surveillance in those regions, providing thorough vector incrimination tools, and investigating seasonal vector biology.

2.1.2 Specific objectives

1. Assess the merits of a series of passive and active mosquito collection techniques at sentinel sites in Mpumalanga, South Africa, to increase the effectiveness and reliability of malaria vector surveillance.
2. Assess the *Anopheles* species assemblage and their respective malaria vectorial capacities at selected sentinel sites in KwaZulu-Natal and Mpumalanga, South Africa, to identify the vectors of residual transmission
3. Assess the seasonal variations in *Anopheles* vector species composition and density of adult male and female mosquitoes at selected sentinel sites in KwaZulu-Natal, South Africa, to determine the extent of mosquito activity during the unfavourable dry period.
4. Investigate the overwintering strategy of adult mosquitoes by looking at the seasonal variation in metabolic rate (CO_2 output) of wild and colonized *Anopheles* species to determine whether there is any evidence of diapause in the adult stage during the dry season.

CHAPTER TWO – GENERAL METHODS AND MATERIALS

This chapter provides descriptions of the general methodologies used for entomological surveillance, mosquito species identification, and parasite detection during this study. These methods were common to results chapters 3, 4, and 5. Details of methodologies specific to objectives are included in the relevant results chapters.

2.1 Mosquito Surveillance Methods

2.1.1 Institutional infrastructure

Facilities at the Vector Control Reference Laboratory (VCRL) based at the National Institute for Communicable Diseases (NICD) in Johannesburg include fully equipped laboratories for all bionomic, biochemical, immunological and molecular procedures described below. The insectaries of the VCRL at the NICD house a collection of live mosquito colonies of the four major vector species in Africa: *Anopheles gambiae, An. coluzzii, An. arabiensis,* and *An. funestus,* as well as the minor vector species *An. merus,* and the non-vector species *An. quadriannulatus*. These colonies provide a reference resource for species identification and further research of important malaria vectors.

2.1.2 Study sites

Entomological surveillance took place at field sites located in the malaria-affected villages of Vlakbult (25° 38' 10" S, 31° 42' 33" E) and Block A (25° 41' 1.05" S, 31° 47' 27" E) that form part of the Ehlanzeni District of the Mpumalanga Province lowveld, and Mamfene (27° 24' 48" S, 32° 11' 60" E) which is part of the uMkhanyakude District in northern KwaZulu-Natal Province (Fig. 2.1). These field sites were selected based on historical entomological and malaria case data obtained from the provincial malaria control programmes and vector surveillance systems active in Mpumalanga and KwaZulu-Natal provinces, respectively. Malaria incidence in these regions is seasonal: low-level residual transmission occurs during the dry winter months (May to August) and comparatively high transmission occurs during the wet summer months (November to March). Warm tropical temperatures (average daily highs ranging from 25℃ to 32℃) and high summer rainfall (750mm to 800mm) at both localities allow for suitable mosquito breeding conditions. Warmer

temperatures increase the development rate of the aquatic stages of *Anopheles* mosquitoes (Lyons *et al.* 2013) which sustains their proliferation and increases malaria transmission potential (Patz *et al.* 2000). Winter conditions in Mpumalanga and KwaZulu-Natal are generally dry (0mm to 130mm rainfall) with lower temperatures (average high 15°C to 26°C), which usually leads to a reduction in mosquito population density and lower levels of malaria transmission. Cross-border movement between South Africa and the malaria endemic regions of Mozambique and Zimbabwe, especially during the festive seasons of March/April and December, is common and increases the risk of imported cases of malaria (Raman *et al.* 2016). Vector control interventions in these regions include focal-point IRS and targeted larval source management.

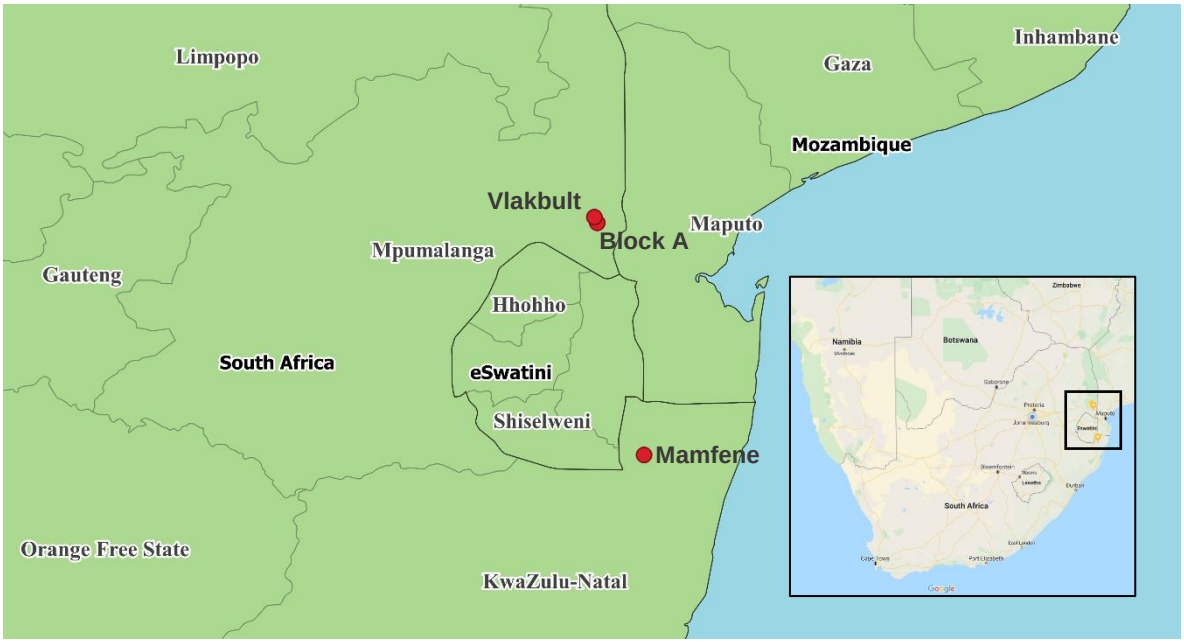

Figure 2.1 Map of the field surveillance sites located in the Ehlanzeni District of Mpumalanga Province, and the uMkhanyakude District in northern KwaZulu-Natal Province, South Africa. The red markers indicate the location of Vlakbult and Block A in Mpumalanga, and Mamfene in KwaZulu-Natal. Map of South Africa source: Map data ©2019 AfriGIS (Pty) Ltd.

2.1.3 Field adult mosquito collection

Ten households in each of the three field sites were identified for mosquito surveillance, following consent of the homeowners and relevant community leaders. Houses were selected based on their proximity to known mosquito breeding sites to ensure effective surveillance was carried out. Most houses were modernised brick and iron-roofed homes apart from a few traditional mud and thatched-roof structures. Both indoor and outdoor, passive and active mosquito collections, as described in sections 2.1.3.1 and 2.1.3.2, were conducted at the Mpumalanga sites, whereas only outdoor passive collection techniques were used at the KwaZulu-Natal sites

2.1.3.1 Passive mosquito collection techniques

Passive mosquito resting shelters consisted of locally sourced traditional clay pots (Fig 2.2) and modified plastic buckets (Fig 2.3). The clay material maintains a cool and dark environment inside the pot that is suitable for adult mosquitoes seeking rest after feeding. Plastic buckets were modified to somewhat mimic the clay pots as a cheaper and more easily accessible alternative to the pots. The interior of each bucket was roughened with course sandpaper to increase mosquito tarsal contact, and spray-painted black. The contrast between the dark entrance of the bucket and the white exterior serves as a visual cue to mosquitoes seeking cool and undisturbed shelter following feeding or mating activities. No further sensory bait was placed inside either of the passive resting shelters. At the field sites where both indoor and outdoor collection was conducted, one modified bucket and one clay pot was placed inside the house with the owner's consent (Appendix A), and a single bucket and pot were placed outdoors within the property boundary. Care was taken to place buckets and pots strategically within houses to avoid inconveniencing the residents and to lower the risk of disturbance of the shelters. Outdoor shelters were placed in shaded, vegetated areas (as far as possible) of the property and with the entrance directed either north or south to avoid direct sunlight infiltration. Pots and buckets were placed within 1 m of each other and were moved occasionally to avoid positional bias. Once installed, all shelters remained on-site until completion of the sampling period.

Figure 2.2 Traditional clay pots used as adult mosquito resting shelters for entomological surveillance in South Africa.

Figure 2.3 Modified plastic buckets used as adult mosquito resting shelters at surveillance sites in South Africa.

All available live adult *Anopheles* mosquitoes were collected from the resting shelters by mouth aspiration within two hours of sunrise. Collections were performed by the locally based and experienced provincial entomology teams and researchers from the Wits Research Institute for Malaria (WRIM) and NICD. All shelters at the Mpumalanga field sites were cleared weekly while the shelters at the KwaZulu-Natal field sites were cleared monthly. Live mosquitoes were transported from the field to the relevant provincial Malaria Control Programme laboratory in polystyrene cups with gauze covering the entrance of the cup, secured by a rubber band. All shelters were checked for live specimens at every site visit. Mosquito samples intended for experiments were kept alive and transported in polystyrene cups to the quarantine insectary at the Botha De Meillon insectary of the NICD in Johannesburg. Specimens collected for surveillance purposes only were knocked down using an insect kill jar containing ethyl acetate and placed into Eppendorf tubes with silica gel for identification and further analysis at the VCRL. Any collected specimens that died during transportation were preserved on silica gel.

2.1.3.2 Active mosquito collection techniques

Active trapping methods conducted at the Mpumalanga field sites included outdoor human-landing catches (HLC) (Fig. 2.4a) and the use of carbon dioxide (CO_2)-baited nets (Fig 2.4b). The sites for active trapping were determined from preliminary passive trap data and reported malaria cases within the surveillance sites. The two active trapping methods were conducted near one another for logistical ease and to maximize the total number of *Anopheles* mosquitoes caught. Field visits were conducted to performs the active trapping for at least one week every month during the malaria transmission season in Mpumalanga. Both staff and students from the VCRL based at the NICD and the local entomology teams conducted the active trapping. Adult *Anopheles* mosquitoes were collected by HLC and the use of CO_2-baited net traps from 18:00 (dusk) until 22:00, covering the first peak biting period for the major vector *An. arabiensis* (Oyewole *et al.* 2007, Oyewole and Awolola 2006). The CO_2-baited net was checked hourly for *Anopheles* mosquitoes. Live specimens were then transported back to the VCRL at the NICD in Johannesburg for identification and further analysis. No active trapping was performed at the KwaZulu-Natal field sites.

Figure 2.4a) A member of the Mpumalanga entomological surveillance team conducting night-time human landing catches in the foreground while a fellow team member checks the CO_2-baited net for mosquitoes in the background. b) a CO_2-baited net erected at early dusk in Vlakbult, Mpumalanga Province, South Africa. CO_2 is produced from blocks of dry ice placed into a polystyrene box with the lid slightly loose.

2.2 Identification of Wild Mosquitoes

2.2.1 Morphological identification

All *Anopheles* mosquito specimens collected during this study were initially identified by external morphology using dichotomous keys (Gillies and Coetzee 1987).

2.2.2. Molecular identification

Where indicated (i.e. for members of the *An. gambiae* complex and *An. funestus* group), morphologically identified specimens were further classified to species by polymerase chain reaction (PCR) (Scott *et al*.1993; Koekemoer *et al*. 2002; Cohuet *et al*. 2003). These PCR assays amplify species-specific regions within the internal transcribed spacer 2 (ITS2). The pertinent assay for each species complex/group is multiplexed so that each member species can be identified by the amplification of a uniquely sized PCR fragment.

2.2.2.1 Anopheles gambiae complex PCR

A single leg or wing from each test specimen, morphologically identified to the *An. gambiae* complex, was placed into a 0.2 ml PCR tube with the reagents listed in table 2.1. Primer working stock concentrations were at 10 μM. Every reaction included positive controls for *An. merus*, *An. gambiae*, *An. arabiensis*, and *An. quadriannulatus* and a negative control containing sterile distilled water instead of template DNA. The PCR cycling conditions were as follows: denaturation at 92°C for two minutes, 30 cycles at 94°C for 30 seconds, 50°C for 30 seconds, 72°C for 30 seconds, followed by extension at 72°C for five minutes, and 8°C hold until removal (Scott *et al*. 1993).

Table 2.1 *Anopheles gambiae* complex PCR master mix.

Reagent		**Volume per sample**
10× PCR buffer (100mM Tris-HCl (pH 8.3), 500mM KCl)		1.25 µl
10× dNTP mixture (2.5mM of each dNTP)		1.25 µl
$MgCl_2$ solution		0.5 µl
Primers:	QD (*An. quadriannulatus*) (R, 25 pmol/µl) [CAGACCAAGATGGTTAGTAT]	0.5 µl
	GA (*An. gambiae*) (R, 25 pmol/µl) [CTGGTTTGGTCGGCACGTTT]	1 µl
	AR (*An. arabiensis*) (R, 25 pmol/µl) [AAGTGTCCTTCTCCATCCTA]	1 µl
	ME (*An. merus*) (R, 25 pmol/µl) [TGACCAACCCACTCCCTTGA]	1 µl
	UN (Universal) (F, 25 pmol/µl) [GTGTGCCCCTTCCTCGATGT]	1 µl
Sterilized distilled H_2O		4.9 µl
r*Taq* (Takara)		0.1 µl
Total		**12.5 µl**

2.2.2.2. Anopheles funestus group PCR

Genomic DNA was extracted from a single *An. funestus* group test specimen leg or wing using the ZyGEM™ *prep*GEM® Insect kit according to the manufacturer's protocol. One mosquito leg or wing was added to a 0.2 ml PCR reaction tube containing a master mix of 8.75 µl PCR grade water, 1 µl of 10× Buffer (BLACK) and 0.25 µl *prep*GEM. The mixture was then incubated in a thermal cycler (G-Storm, Vacutec) at 75°C for 15 minutes followed by a cycle of 95°C for five minutes. A no-template control was included for every reaction. Extracted DNA was stored at -20°C until ready for use.

Extracted template DNA was placed into a 0.2 ml PCR tube along with the reagents listed in table 2.2. All reactions included a positive control for *An. funestus* s.s. and two negative controls: one no-template control for the extraction and PCR step, respectively. The cycling conditions were as follows: pre-denaturation 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 extension at 72°C for 40 seconds. A final extension cycle was performed at 72°C for 5 minutes, followed by a hold at 8°C until removal (Koekemoer *et al*. 2002, Cohuet *et al*. 2003).

Table 2.2 *Anopheles funestus* group PCR master mix.

Reagent		**Volume per sample**
10× PCR buffer (100mM Tris-HCl (pH 8.3), 500mM KCl)		1.25 μl
dNTP mixture (containing 2.5mM of each dNTP)		1 μl
$MgCl_2$ solution		0.75 μl
Primers:	UV (Universal) (F, 3.3pmol/μl) [TGTGAACTGCAGGACACAT]	1 μl
	FUN (*An. funestus*) (R, 3.3pmol/μl) [GCATCGATGGGTTAATCATG]	1 μl
	VAN (*An. vaneedeni*) (R, 3.3pmol/μl) [TGTCGACTTGGTAGCCGAAC]	1 μl
	RIV (*An. rivulorum*) (R, 3.3pmol/μl) [CAAGCCGTTCGACCCTGATT]	1 μl
	PAR (*An. parensis*) (R, 3.3pmol/μl) [TGCGGTCCCAAGCTAGGTTC]	1 μl
	LEES (*An. leesoni*) (R, 3.3pmol/μl) [TACACGGGCGCCATGTAGTT]	1 μl
	RIVLIKE (*An. rivulorum*-like) (R, 3.3pmol/μl) [CCGCCTCCCGTGGAGTGGGGG]	1 μl
Sterilized distilled H_2O		1.9 μl
r*Taq* (Takara)		0.1 μl
Template DNA		0.5 μl
Total		**12.5 μl**

2.2.3 Electrophoresis and visualisation of PCR product

Samples were loaded into individual wells of a 2.5% 1× Tris Acetate EDTA (TAE) agarose gel stained with ethidium bromide. A volume of 3 µl of loading buffer (Fermentas O'Range 6 X Loading Dye) was mixed with the 12.5 µl of PCR product before loading into the gel. At least one lane of the gel contained 5 µl of molecular weight marker (Fermentas O'Range Ruler 100 bp) as a size reference for PCR product fragments. Gels were electrophoresed at 120 volts (Bio-Rad PowerPac™) until enough separation of the DNA ladder was achieved to distinguish PCR product sizes. Gels were then visualised under an ultraviolet (UV) transilluminator in a gel documentation system (ChemiDoc™ XRS+ Imaging System, Bio-Rad).

2.3 Malaria Parasite Detection in Wild Specimens

2.3.1 Detection of *Plasmodium falciparum* sporozoites using an ELISA

The enzyme-linked immunosorbent assay (ELISA) is designed to determine the presence or absence of the *Plasmodium falciparum* circumsporozoite protein (CSP) in mosquito homogenate. Methods were adapted from Wirtz *et al.* (1987) and Burkot *et al.* (1984).

All wild female *Anopheles* were screened for the presence of *P. falciparum* by ELISA. The head and thorax of each mosquito was removed and placed into a 1.5ml Eppendorf tube with 50 µl grinding buffer (made up of Blocking Buffer and Igepal/NP-40). A washed and sterilised stainless-steel bead was added to the reaction tube and the sample was homogenised using a TissueLyser II (Qiagen) for four minutes. Once homogenised, the bead was removed and 150µl blocking buffer was added to make a total volume of 200 µl. The head and thoraces of seven insectary-reared females of the same species as the test specimens were ground in the same way and served as the negative controls. The homogenised samples were stored at -20°C until ready for use. A 96 well clear plate (NUNC®) was coated with 50 µl of antibody mix made up of 5 ml phosphate buffered saline (PBS) and 40 µl primary antibody (Pf2A10), covered with aluminium foil, and stored at 4°C overnight.

The plate wells were aspirated thoroughly the following morning and filled with blocking buffer before being covered with aluminium foil and incubated for one hour at room

temperature. The plate was aspirated again following the incubation step. A positive control was prepared by mixing 2 µl *P. falciparum* positive solution and 50 µl blocking buffer in a clean reaction tube. The positive control was added to well A1 of the plate, and 50 µl of each negative control was added to the last seven wells of the plate (H6 – H12). Fifty microlitres of each sample was added to the remaining wells. The plate was then covered with aluminium foil and incubated at room temperature for two hours. Following sample hybridisation, all unbound samples were shaken out of the wells and washed twice with PBS-Tween. Excess PBS was tapped out onto paper towel to ensure the plate was dry. The peroxidase antibody mix was then prepared by adding 10 µl stock peroxidase Pf 2A10 to a plastic trough with 5 ml blocking buffer. Once mixed, 50 µl malaria antibody mix (MAb) was added to each well and the plate was incubated for one hour under aluminium foil. A secondary wash with PBS-Tween was performed four times to remove unbound antibody. An aliquot of 100 µl ABTS 1 step peroxidase substrate was added to each well and the plate was left to stand uncovered for 30 to 60 minutes. The optical density (OD) was determined using a SpectraMax ABS Plus Microplate Reader (Molecular Devices) at 405 nm. The critical cut-off value was determined by the formula: X = ((sum of the seven negative OD values)/7) x 2). All samples with OD readings higher than the critical threshold were repeated in a new ELISA following a boiling step at 100°C for ten minutes. The boiling step reduces the risk of false positives presenting in the assay. All samples with OD values above the critical threshold in the repeat ELISA were considered positive for *P. falciparum* CSP.

2.3.2 Confirmation of *Plasmodium falciparum* sporozoite-positive specimens

In cases where wild *Anopheles* specimens indicated the presence of *P. falciparum* CSP in the repeat ELISA, but were members of species that have not been categorically incriminated as malaria vectors before, it was necessary to use additional sensitive molecular assays to confirm the positive status of the samples. The *P. falciparum*-specific nested PCR (Snounou *et al.* 1993) and DNA sequence analysis were used to further confirm positive samples as described below.

2.3.2.1 Phenol-chloroform DNA extraction from ELISA homogenate

DNA was extracted from the remaining ELISA homogenate of samples using the phenol-chloroform method. This protocol uses biohazardous chemicals; thus, care was taken to ensure that proper personal protective equipment (gloves, lab-coat etc.) was used and that waste products were appropriately disposed of.

A volume of 100 µl of 1× Tris-EDTA (TE) buffer was added to the remaining ELISA homogenate in its existing tube to make a total volume of 200 µl. Then 200 µl of 1:1 phenol-chloroform was added to the tube and mixed briefly before centrifugation for five minutes at 12 000g. Following separation of the solution within the tube, only the top aqueous phase was pipetted off without disturbing the middle layer and transferred to a clean 1.5 ml tube. All phenol waste was appropriately disposed of. A volume of 100 µl chloroform was added to the tube, mixed briefly, then centrifuged for one minute at 12 000g. The top layer was again pipetted off without disturbing the middle layer and placed into a clean 1.5 ml tube. Ten microlitres of 3M sodium acetate (pH 4.8) and 200 µl of cold 100% ethanol was added to the tube and mixed before storing at -20°C overnight.

Following overnight storage, the sample was centrifuged at 4°C for 35 minutes at 12 000g to form a DNA pellet. The 100% ethanol was then discarded from the tube without disturbing the DNA pellet. A volume of 200 µl cold 70% ethanol was added to the tube and centrifuged at 4°C for 20 minutes at 12 000g. The 70% ethanol was then discarded without disturbing the DNA pellet and the tube was left with the cap open to air-dry on the bench top. Once the DNA pellet was dry, it was re-suspended in 15 µl of 1× TE, ensuring the pellet dissolved. The sample was then stored at -20°C until ready for use.

2.3.2.2 Plasmodium sporozoite detection by nested PCR

Nested PCR amplification provides a higher sensitivity of *Plasmodium* species detection in mosquito samples (Snounou *et al.* 1993) which is particularly useful for incriminating new vector species. This technique was used as a confirmation test of results that indicated sporozoite-positive using the ELISA method. Two genus-specific primers, rPLU5 and rPLU6, were used for the first cycle of amplification (Table 2.3). The PCR cycling conditions for reaction one included a denaturation step at 95°C for five minutes, followed by 25 cycles of annealing at 58°C for two minutes, extension at 72°C for two minutes, and denaturation at 94°C for one minute, followed by an annealing step at 58°C for two minutes,

and a final extension step at 72°C for five minutes. An aliquot of the product thus obtained was used for a second amplification cycle, in which the *Plasmodium* species was detected using species-specific primers (rFAL1 and rFAL2) (Table 2.4). The PCR cycling parameters for reaction two comprised a denaturation step at 95°C for five minutes, followed by 30 cycles of annealing at 58°C for two minutes, extension at 72°C for two minutes, and denaturation at 94°C for one minute, followed by an annealing step at 58°C for two minutes, and a final extension step at 72°C for five minutes. Amplified PCR product was detected by ethidium bromide staining and agarose gel electrophoresis.

Table 2.3 PCR reaction one for *Plasmodium falciparum*

Reagent		**Volume per sample**
10× PCR buffer (100mM Tris-HCl (pH 8.3), 500mM KCl)		1.25 μl
dNTP mixture (containing 2.5mM of each dNTP)		1.25 μl
$MgCl_2$ solution		0.75 μl
Primers:	rPLU6 (*Plasmodium*) (F, 250 nM) [TTAAAATTGTTGCAGTTAAAACG]	1 μl
	rPLU5 (*Plasmodium*) (R, 250 nM) [CCTGTTGTTGCCTTAAACTTC]	1 μl
Sterilized distilled H_2O		6.25 μl
r*Taq* (Takara)		1 μl
Template DNA		1 μl
Total		**13.5 μl**

Table 2.4 PCR reaction two for *Plasmodium falciparum*

Reagent		**Volume per sample**
10× PCR buffer (100mM Tris-HCl (pH 8.3), 500mM KCl)		1.25 µl
dNTP mixture (containing 2.5mM of each dNTP)		1.25 µl
$MgCl_2$ solution		0.75 µl
Primers:	rFAL1 (*P. falciparum*) (F, 250 nM) [TTAAACTGGTTTGGGAAAACCAAATATATT]	1 µl
	rFAL2 (*P. falciparum*) (R, 250 nM) [ACACAATGAACTCAATCATGACTACCCGTC]	1 µl
Sterilized distilled H_2O		6.25 µl
r*Taq* (Takara)		1 µl
Template DNA		1 µl
Total		**13.5 µl**

2.3.2.3 ITS2 region isolation for DNA sequence analysis

For vector incrimination, it was necessary to unequivocally verify the species identification of the parasite-infected mosquito. The ITS2 region from the test specimen was amplified by ITS2 PCR with the aim of DNA sequence analysis to confirm the identification. The PCR master mix was prepared according to Table 2.5. An aliquot of 23 µl master mix was added to 0.2 ml PCR reaction tubes along with 2 µl of template DNA extracted from a mosquito leg or wing using the ZyGEM™ *prep*GEM® Insect kit according to the manufacturer's protocol. The PCR cycling parameters consisted of a denaturation step at 94°C for two minutes, followed by 40 cycles of 94°C for 30 seconds, 50°C for 30 seconds and 72°C for 40 seconds, with a subsequent extension step at 72°C for 10 minutes. A positive and negative control was included in every PCR reaction. A 10 µl aliquot of PCR product was mixed with 2 µl loading dye and loaded into a 2.5% TAE gel and electrophoresed at 120V until adequate separation of the molecular weight marker was achieved. The gel was visualised under a UV light to confirm that the relevant DNA fragments of the test specimen had amplified without contamination before the remainder of the PCR product was packaged and sent to Macrogen Europe for DNA sequence analysis. Multiple ITS2 PCR reactions of the same specimen were performed to ensure enough PCR product was obtained for DNA sequence analysis (Koekemoer *et al*. 2002).

Table 2.5 ITS2 PCR master mix

Reagent		**Volume per sample**
10× PCR buffer (100mM Tris-HCl (pH 8.3), 500mM KCl)		2.5 μl
dNTP mixture (containing 2.5mM of each dNTP)		2 μl
$MgCl_2$ solution		3 μl
Primers:	ITS2A (F, 25 mM) [TGTGAACTGCAGGACACAT]	0.5 μl
	ITS2B (R, 25 mM) [TATGCTTAAATTCAGGGGGT]	0.5 μl
Sterilized distilled H_2O		14.3 μl
r*Taq* (Takara)		0.2 μl
Template DNA		2 μl
Total		**25 μl**

2.3.2.4 DNA sequence analysis for vector incrimination

Sequence analysis of the respective ITS2 and nested *Plasmodium* PCR products was used to confirm the *Anopheles* species identity and infection with *P. falciparum.* Purification and sequencing of the PCR products was performed by Macrogen. The chromatograms of the sequences were manually edited in BioEdit version 7.2.5 (Hall 1999). The Emboss Needle pairwise sequence alignment tool was used to compare the analysed test mosquito ITS2 sequence with the established reference sequences on GenBank. The unknown *Plasmodium* sequence was compared with the reference *P. falciparum* Pf8 18S ribosomal RNA gene, partial sequence (GenBank accession number: KC428742.1) (Ngassa Mbenda and Das 2014).

CHAPTER THREE - ADULT ANOPHELES MOSQUITO SURVEILLANCE IN SOUTH AFRICA

3.1 Introduction

Reliable vector population monitoring is fundamental to planning and implementing effective and sustainable control. Knowing which vector species are present and actively transmitting malaria in a region is critical for planning the appropriate intervention methods and monitoring these vector species over time provides valuable information about the efficacy of the intervention. Sampling adult stages of *Anopheles* can be used to determine the local vector species composition, relative species abundance, mosquito biting behaviour and sporozoite infection rate, blood-meal source by species and insecticide susceptibility by species. This information is useful for malaria control programmes and helps decipher the entomological drivers of localised low-level residual malaria transmission. Studies of vector populations and the dynamics of malaria transmission also assist important decision-making for IRS activities and the prioritisation of high-risk or endemic transmission zones. Routine surveillance can be optimized by identifying the most beneficial, cost-effective, ethical, and efficient mosquito trapping techniques. Comparisons between trapping methods enables the development of optimal localised surveillance strategies based on the most suitable combinations of collection techniques.

All mosquito collection methods have advantages and disadvantages. Service (1993) reviewed various mosquito sampling methods that included both attractive or baited traps and non-attractive traps. Direct and indirect human-baited traps and other attractive traps that use CO_2 , light, or visual stimuli tend to attract mostly host-seeking, hungry female mosquitoes or specific species, which may not be representative of the local population as a whole (Service 1993), but may be useful for targeting medically important disease vector species. Non-attractive traps, however, accrue less bias than attractive traps and therefore provide a more representative sample of the local wild population (Service 1993). Attractive traps, or active collection methods, such as human landing catches (HLC), indoor aspiration of adults, pyrethrum spray catches (PSC), CDC light traps, suction-powered gravid traps (Dugassa *et al.* 2013, Dugassa *et al.* 2016), CO_2 -baited traps and others require a fair amount of logistical organising, disruption of home-owner's lives and households, and incur higher costs and safety risks to collectors. Although certainly effective for entomological sampling, active collection methods are not as suitable for long-term routine surveillance in developing

countries that require scalable, low-cost, standardized methods to form part of provincial malaria intervention programmes. The target species largely determines the type of trapping method used as many are designed to attract or collect mosquitoes at a specific life stage, physiological status, or with specific behaviours or resting preferences.

Various non-attractive, or passive, trapping techniques targeting the indoor and outdoor resting mosquito population have been used with varying efficacy. Passive traps include but are not limited to resting shelters and boxes (Meyer 1985), diurnal resting shelters (Morris 1981), pit shelters (Service 1993), fibre pots (Komar *et al.* 1995), woven baskets (Harbison *et al.* 2006), and window-exit traps (Service 1977, Odiere *et al.* 2007). These types of traps or shelters do not target only host-seeking female mosquitoes, but also collect unfed females, males, and gravid females (Service 1993). Resting traps are also useful for collecting mosquitoes that display both endo- and exophily (depending on where the trap is placed, of course) and anthro- and zoophily.

The increasing indoor application of insecticides and insecticide-treated nets has largely controlled indoor-resting malaria vector populations; thus surveillance of the outdoor biting and resting populations is necessary. Several active and passive trapping techniques were assessed during this study to ascertain a suitable long-term entomological surveillance method that can be standardized across all provincial malaria programmes in South Africa. The passive adult mosquito collection methods relevant to this project include ceramic pots and modified plastic buckets placed both indoors and outdoors. These resting shelters were selected because of their low-cost, easy installation, scalability, and low-risk safety appeal. Active trapping techniques comprised CO_2-baited net traps and HLC as a means of comparison to the passive methods. The HLC technique is generally efficient, but this method raises ethical concerns related to exposing collectors to potential vector-borne diseases and the general safety risks of night-time activity. The use of CO_2-baited nets has shown variable success (Mboera *et al.* 1997), but drawbacks include the labour-intensive hourly clearing of the tent throughout the night that again exposes collectors to potential vector-borne diseases and presents safety issues. To avoid the ethical and safety risks involved with active trapping, attention has shifted towards passive trapping techniques that can be installed permanently and cleared in the early morning as frequently as is logistically possible. Traditional clay pots placed outdoors were found to be efficient for trapping blood-fed female *An. gambiae* in parts of Kenya (Wong *et al.* 2013) as well as in KwaZulu-Natal province in South Africa (Dandalo

et al., 2017, Burke *et al.,* 2017, Burke *et al.* 2019b), which is useful for studies requiring live wild material. Modified plastic buckets aim to mimic the clay pots, to an extent, potentially providing a cheaper and easily available alternative to ceramic pots if found to be as effective in trapping vector species.

A comprehensive understanding of local disease vectors and the entomological drivers of residual transmission is pertinent to malaria management. Of interest are the outdoor-resting and biting vectors that are less susceptible to indoor-targeted control methods and who continue to complicate the control and elimination of malaria transmission in most southern African countries. The outdoor vectors are likely driving low-level residual malaria transmission in South Africa (Coetzee and Hunt 1998, Brooke *et al.* 2013, Christian *et al.* 2018, Brooke 2019).

The aims for this component of the study were to 1) evaluate and compare the efficiency of different mosquito trapping methods at local field sites in consideration for routine surveillance, and to 2) establish the *Anopheles* assemblage at sentinel sites within two malaria-affected regions of South Africa. The end-goal of such research is to establish effective and efficient surveillance techniques to carry out in low malaria transmission areas, such as South Africa. Once a reliable surveillance system is in place, investigations into the relevant biological and behavioural aspects of medically important mosquitoes is possible. This information helps towards improving vector surveillance in South Africa.

3.2 Materials and Methods

3.2.1 Comparison of mosquito collection techniques in Mpumalanga

3.2.1.1 Passive indoor and outdoor trapping

Resting shelters described in section 2.1. were installed at 10 households in Vlakbult and Block A in early March 2015 and remained on-site until April 2016. Sampling started in March 2015 and continued through to the non-transmission dry season from May to October 2015, and then the subsequent summer transmission season from November 2015 to April 2016. All available adult *Anopheles* mosquitoes were collected from shelters by mouth aspiration on a weekly basis between 07:00 and 09:00 by the experienced provincial entomology teams based in Tonga, Mpumalanga Province. All shelters were checked for live specimens at every site visit each week. Collected specimens were preserved on silica in

Eppendorf tubes and then transported to the VCRL based at the NICD in Johannesburg. Specimens were then identified to species using the methods described in section 2.2 and all wild-caught females were screened for *Plasmodium* sporozoites using a *P. falciparum* circumsporozoite ELISA, details of which are provided in section 2.3.

3.2.1.2 Active outdoor trapping

The sites for active trapping were determined from preliminary passive trap data and reported malaria cases within the surveillance sites. Staff and entomology students from the WRIM and NICD accompanied the provincial malaria control programme team for active mosquito trapping for at least one week every month during the malaria transmission season in Mpumalanga in 2015 and again in 2016. Adult *Anopheles* were collected by HLC and the use of CO_2-baited tent traps from 18:00 (dusk) until 22:00. Live specimens were then transported back to the VCRL at the NICD in Johannesburg for species identification and parasite detection according to methods described in section 2.2 and 2.3.

3.2.1.3 Statistical analysis of trapping method data

All statistical analyses were performed using Stata Statistical Software programme version 13.0 (College Station, TX: StataCorp LP, 2013). A proportion test of equality was used to ascertain whether the trapping efficiency, quantified as the number of *Anopheles* mosquitoes collected, differed significantly between the different trapping methods. Count data of positively identified *Anopheles* species from each of the different trap types were obtained, but in low numbers, thus the non-parametric test of equality of proportions was considered more appropriate than the chi-square test for analysis. Proportion tests of equality were performed to compare the collecting efficiency of passive shelters placed indoors to those placed outdoors, and of ceramic pots to plastic bucket shelters. Mosquito assemblage data collected from active traps; CO_2 –baited nets and HLC, were observed but not statistically analysed because the traps were not entirely independent from each other. Comparisons by species (where enough specimens were collected) were made between the different trap types using the proportions test of association. The number of male and female specimens collected from each of the different trap types were compared and analysed by Pearson's chi-square test of equality. All statistical analyses were performed at the 5% significance level.

3.2.2 Outdoor vector surveillance in KwaZulu-Natal

Adult *Anopheles* mosquito surveillance, using outdoor ceramic pots described in section 2.1, was conducted at sentinel sites in Mamfene KwaZulu-Natal Province, South Africa. Field site visits took place monthly from January 2017 to April 2018 during which all shelters were cleared of live adult mosquitoes. All collected specimens were appropriately packaged and transported live to the insectary based at the University of the Witwatersrand in Johannesburg. Live wild mosquitoes were used for CO_2 emission rate experiments that are described in chapter 5. All specimens were then identified to species level using methods described in section 2.2 and all females were screened for the presence of *P. falciparum* sporozoites according to the protocol in section 2.3. In addition to mosquito collection, monthly weather station data was also collected from a data logging HOBO® RX3000 (Onset Computer Corporation, Bourne, MA) stationed in the malaria camp within the Mamfene region. These data enabled analyses of seasonal fluctuations in species and abundance alongside seasonal weather fluctuations. The results from this study were made available to the Kwa-Zulu Natal Malaria control programme and were used for developing malaria control interventions.

3.3 Results

3.3.1 Entomological surveillance in Mpumalanga, South Africa

Collections conducted for one year at two separate sites in Mpumalanga using various trapping techniques yielded 141 adult *Anopheles* mosquitoes in total. The collection results from both sites, Vlakbult and Block A, are combined in figure 3.1. The total number of adult mosquitoes collected was very low, which was likely caused by the severe lack of rainfall in South Africa during the sampling period. The collections did however give an indication of the species composition of the local *Anopheles* populations, and which technique was most efficient for their collection. The efficacies of the different shelters were determined by the number of adult *Anopheles* mosquitoes collected from each trap over the sampling period. Specimens were only included in the analysis if they were identified as members of either the *An. gambiae* complex or the *An. funestus* species group. Very few specimens, and only members from the *An. gambiae* complex, were collected from indoor shelters. No specimens were collected from the indoor bucket shelters.

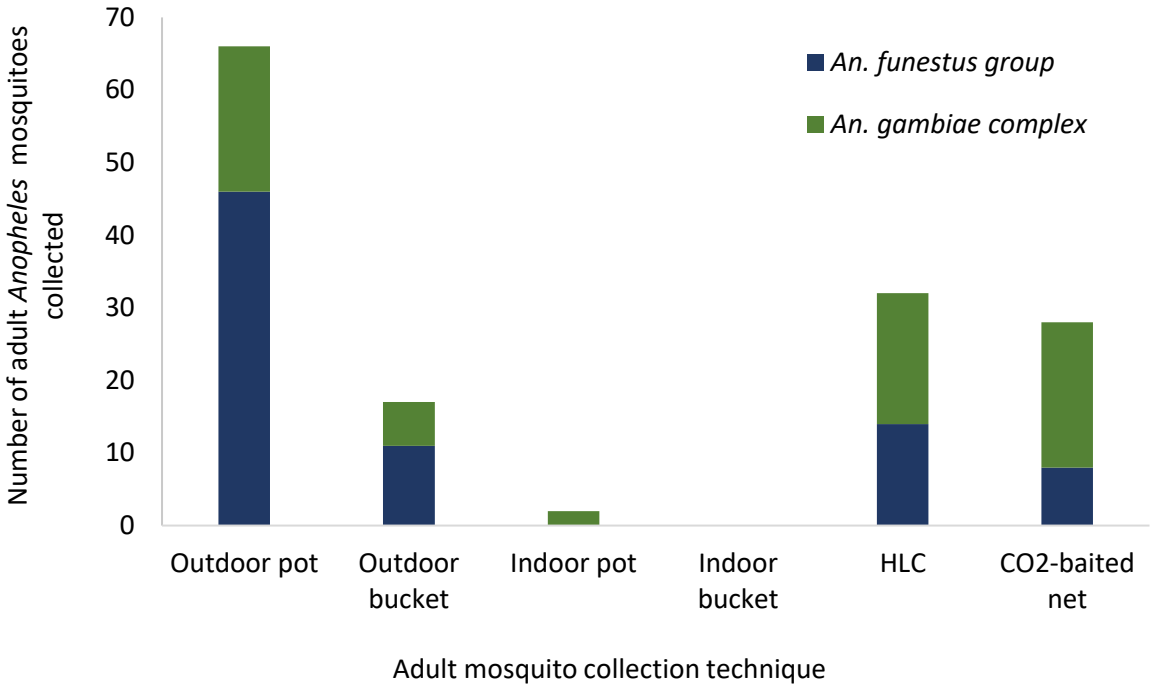

Figure 3.1. Total number of adult Anopheles mosquitoes by species complex/group collected per trapping technique from Vlakbult and Block A; two rural villages in Mpumalanga Province, South Africa from March 2015 to April 2016. HLC=human landing catch.

Anopheles gambiae complex specimens collected were *An. quadriannulatus* (*n* = 31), *An. merus* (*n* = 27), and *An. arabiensis* (*n*= 8). *Anopheles funestus* group specimens, which made up most of the collection yield, comprised *An. vaneedeni* (*n* = 53), *An. rivulorum* (*n* = 20), and *An. leesoni* (*n* = 2) (Fig. 3.2). Female adults from the *An. funestus* species group, particularly *An. vaneedeni*, made up most of the collection from Mpumalanga sites (Fig.3.1 and Fig. 3.2).

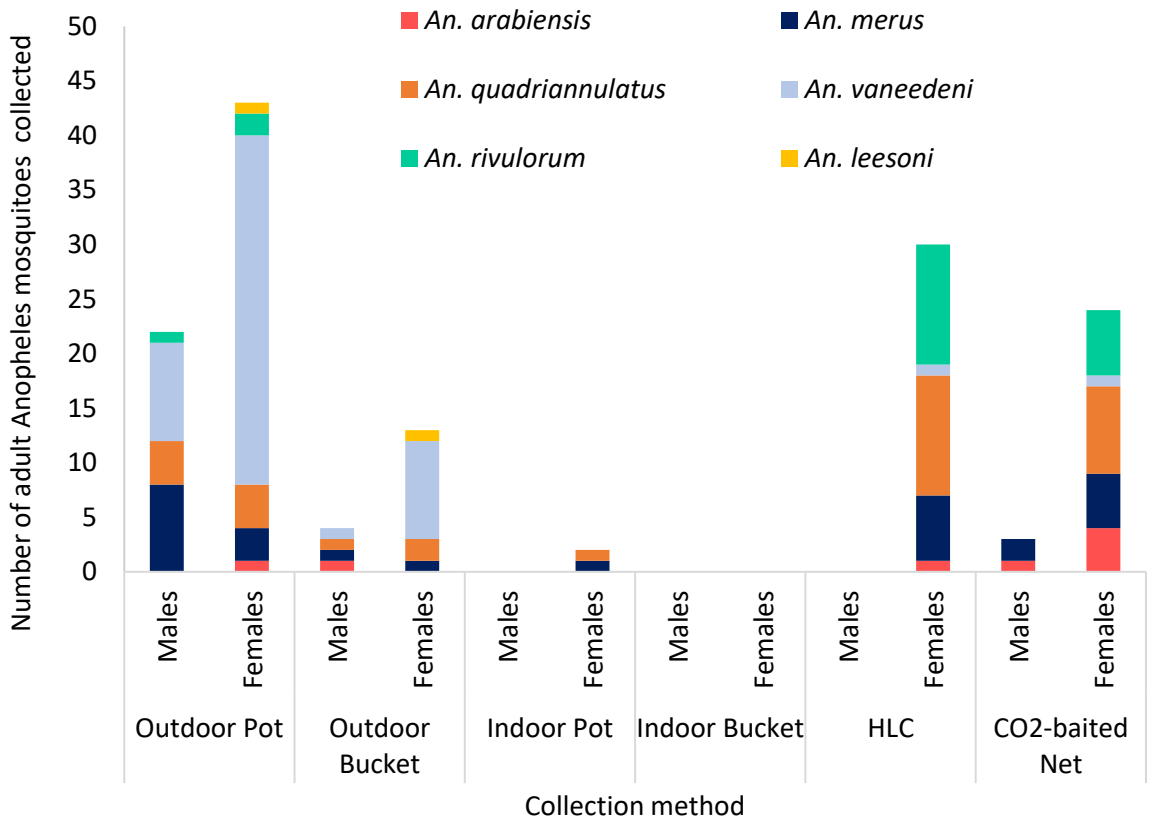

Figure 3.2. Species composition by sex and trap type from surveillance performed at households in Vlakbult and Block A, Mpumalanga Province, South Africa, from March 2015 to April 2016. HLC = human landing catch.

The unusually low rainfall in South Africa in 2015 and 2016 complicated the assessment of the seasonal variation in species composition as very few specimens were collected. What is typically the high season for malaria transmission and vector population density in the summer months of November to February, produced unusually low numbers of *Anopheles* (Fig. 3.3). Low *Anopheles* population densities are typical during the dry winter months from June to August, which was reflected by the vector surveillance data from the Mpumalanga sites over this period (Fig. 3.3). There was a sudden increase in *Anopheles* population density based on collections in September and October of 2015 (Fig. 3.3).

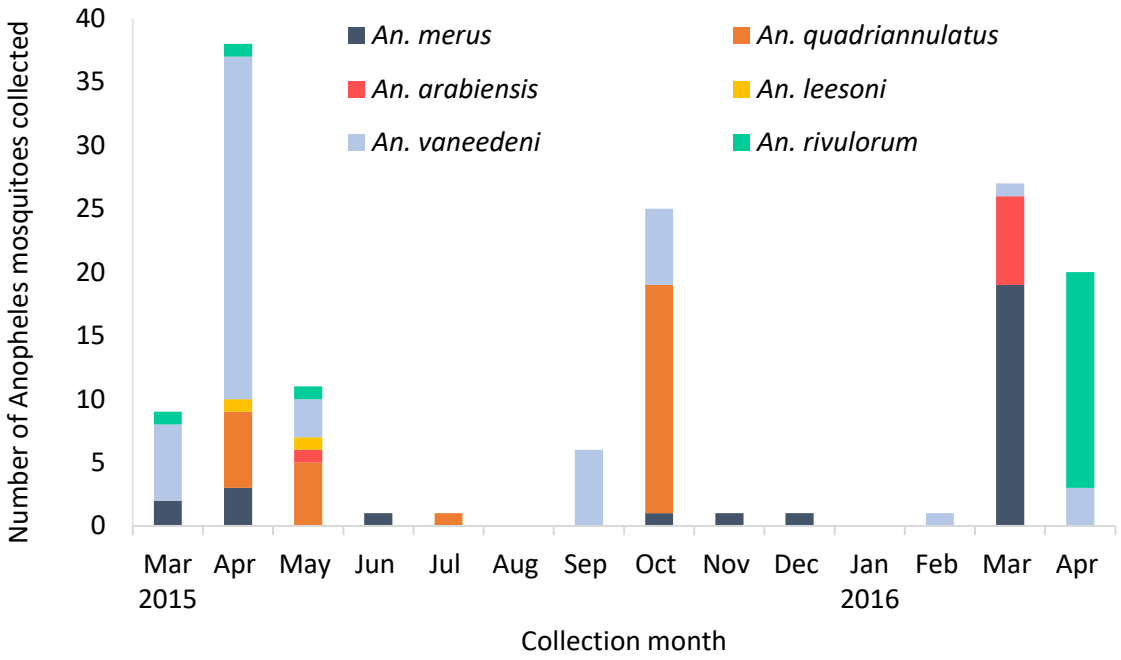

Fig 3.3. Seasonal variation of adult *Anopheles* mosquito species collected from various traps placed at households in Vlakbult and Block A, Mpumalanga Province, South Africa, from March 2015 to April 2016.

3.3.1.1 Comparison between indoor and outdoor collections

The activity of outdoor biting vectors and their contribution to malaria transmission was investigated by conducting indoor and outdoor entomological surveillance. The analysis indicated that the number of *An. gambiae* complex specimens collected from outdoor pots was significantly greater than those collected from indoor pots ($Z = 4.20$; $df = 65$; $p < 0.01$) (Fig. 3.1). No comparisons were made between the indoor and outdoor bucket collections as no specimens were collected from indoor buckets. Assessment of traps per species suggested that outdoor shelters were more effective for the collection of *An. merus* and *An. quadriannulatus* specimens than the indoor shelters (Fig. 3.2). *Anopheles arabiensis* were only collected from outdoor shelters, thus were not analysed statistically. *Anopheles funestus* group specimens were only collected from outdoor shelters (no specimens were collected indoors). No *An. rivulorum* specimens were collected from indoor- or outdoor-placed buckets, and only one *An. leesoni* was collected from an outdoor bucket.

3.3.1.2 Comparison between ceramic pot and plastic bucket collections

Significantly higher numbers of *An. gambiae* complex and *An. funestus* group specimens were collected from the outdoor-placed pots compared to the outdoor buckets (*An. gambiae* complex: Z = 3.06; df = 65; $p < 0.01$; *An. funestus* group: Z = 6.2929 ; df = 54; $p < 0.01$) (Fig 3.1). Too few specimens were collected from indoor shelters to make a meaningful comparison between trap types.

3.3.1.3 Passive and active collection techniques

It is not possible to directly compare the efficacies of the active *vs.* passive collections because the active techniques were only conducted over a few nights once per month during the summer months whilst the passive shelters were cleared every week for a whole year. The active trapping methods were conducted in close proximity to one another thus cannot be analysed as independent collection techniques. *Anopheles gambiae* complex member collected by CO_2 nets and HLC included *An. arabiensis*, *An. merus* and *An. quadriannulatus*. Active collection techniques appeared more effective for the collection of the major vector *An. arabiensis* over the passive methods (Fig. 3.2). *Anopheles funestus* group members collected included *An. vaneedeni* and *An. rivulorum* (Figure 3.2). No *An. leesoni* specimens were collected by active methods.

3.3.2 Entomological surveillance in Mamfene, KwaZulu-Natal

A total of 491 male and female *Anopheles* specimens were collected from outdoor clay pots deployed outside households in Mamfene, northern KwaZulu-Natal Province, during the period January 2017 to May 2018 (Fig. 3.4). *Anopheles arabiensis,* a member of the *An. gambiae* complex, was the most abundant species collected during the surveillance period in Mamfene ($n = 228$). Other members of the *An. gambiae* complex collected included *An. merus* ($n = 11$) and *An. quadriannulatus* ($n = 10$) (Fig. 3). Within the *An. funestus* group, *An. parensis* was most abundant (n = 194) followed by *An. leesoni* ($n = 29$), *An. rivulorum* ($n = 18$) and *An. vaneedeni (*$n = 1$) (Fig. 3.4). There was an overall preponderance of females across all species collected (F = 240.58, df = 1, $p = 0.004$); 163 females and 86 males of the *An. gambiae*

complex, and 154 females and 88 males of the *An. funestus* group (Fig. 3.4). The monthly and seasonal distribution of collected *Anopheles* showed a significant increase in mosquito numbers in March 2017 (Fig. 3.5).

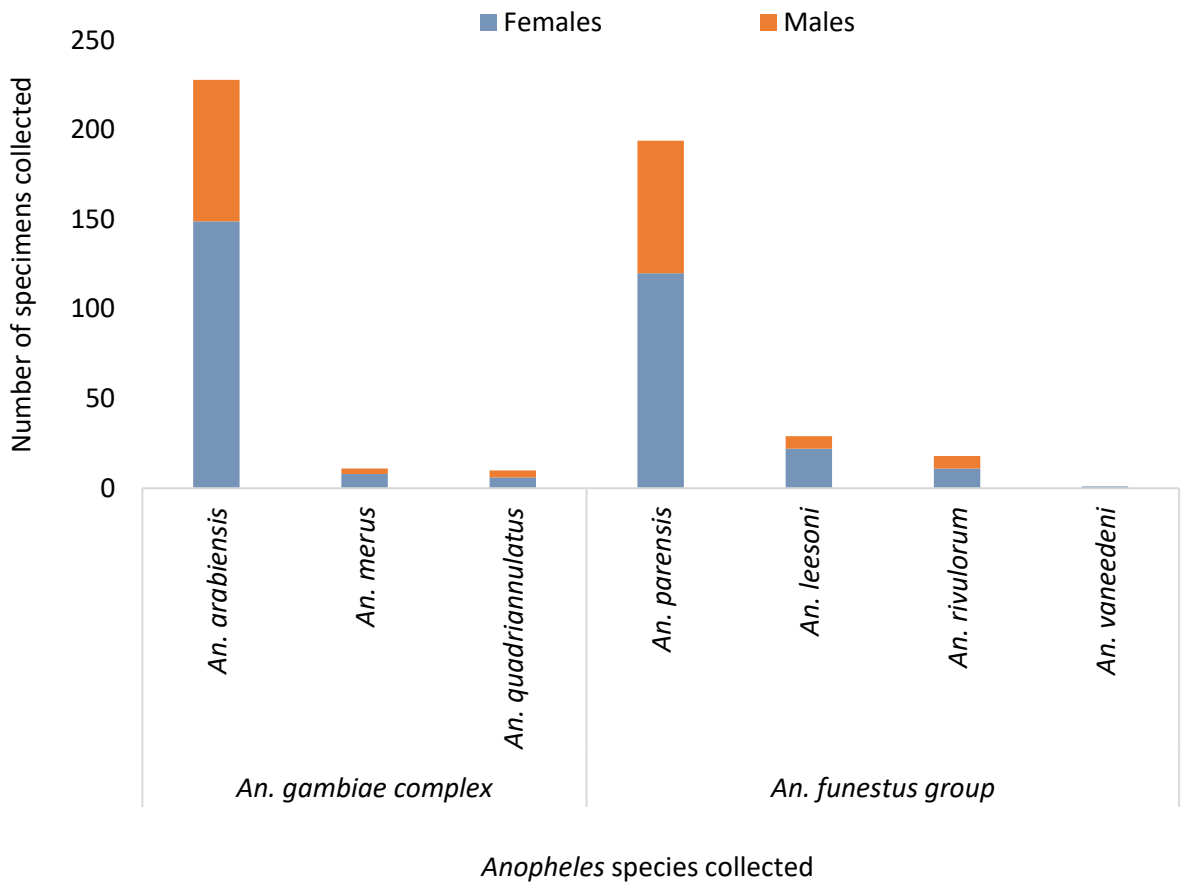

Figure 3.4. *Anopheles* mosquitoes by species group and sex collected using outdoor-placed clay pots deployed as resting shelters at a field site at Mamfene, KwaZulu-Natal Province, South Africa, from January 2017 to May 2018.

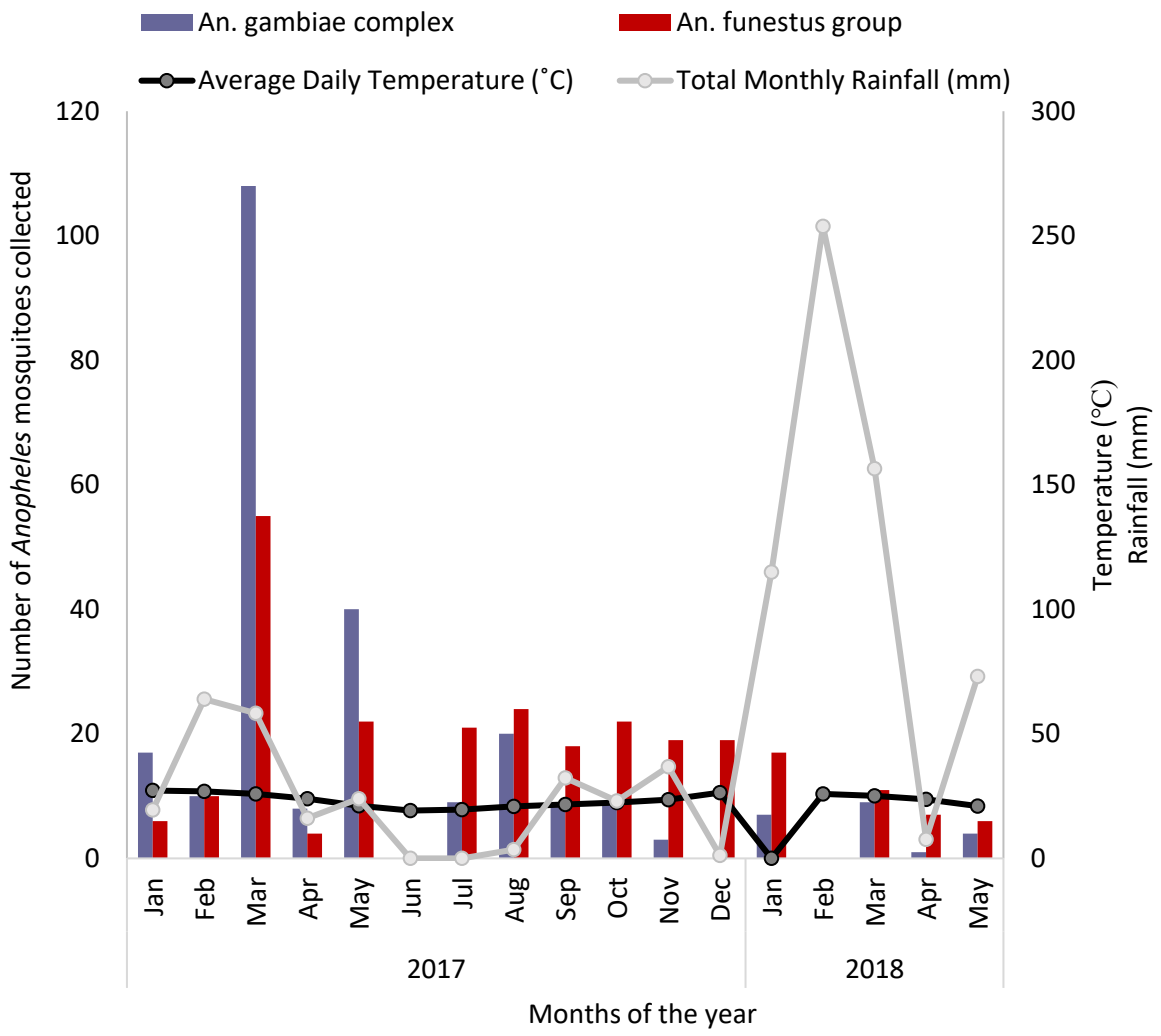

Figure 3.5 Monthly surveillance of male and female *Anopheles* by species group/complex in Mamfene, KwaZulu-Natal Province, South Africa, from January 2017 to May 2018. Mosquitoes were collected over a 3-day period each month. No fieldtrips were conducted in June 2017 and February 2018 due to logistical constraints.

3.3.3 *Plasmodium falciparum* infection rate of local mosquito populations in South Africa

One specimen from the Vlakbult site collections, collected in April 2015, was positive for the parasite after a repeat assay following repeat ELISA with a boiling step. This specimen was identified as *An. vaneedeni* of the *An. funestus* group, a species which had not previously been incriminated in malaria transmission. The infection was further confirmed by a nested

PCR and sequence analysis of the amplified fragment, which is discussed in chapter five. The infection rate of the local *An. vaneedeni* population at Vlakbult in Mpumalanga Province, South Africa, based on the surveillance period from March 2015 to April 2016, was 2.44% (n = 41).

Of the 163 female *An. gambiae* complex specimens screened for CSP, one *An. arabiensis* (collected in October 2017) showed positive by ELISA and *Plasmodium* nested PCR. As this *An. arabiensis* population has been implicated in malaria transmission before, no further DNA sequence analysis was considered necessary. Of the 154 female *An. funestus* group specimens screened, one female identified as *An. parensis* (collected in October 2017) showed positive for *P. falciparum* CSP by ELISA and nested *Plasmodium* PCR. These data indicate a *P. falciparum* infectivity rate of 0.67% (n = 149) for *An. arabiensis* and 0.83% (n = 120) for *An. parensis* at Mamfene, KwaZulu-Natal Province, South Africa.

All specimens were collected from outdoor ceramic pots as part of the local malaria vector surveillance programmes, which implicates *An. arabiensis*, *An. vaneedeni* and *An. parensis* as likely contributors to residual malaria transmission in South Africa.

3.4 Discussion

The sampling period for the first component of this study unfortunately coincided with a severe drought in South Africa. The total annual rainfall for the whole of South Africa was approximately 403mm in 2015, a historic low (South African Weather Service 2018), which was exacerbated by significantly warmer temperatures across of the interior of the country (National Department of Environmental Affairs 2017). The lack of rainfall was not favourable for mosquito breeding which likely caused the decreased population density and thus the poor catch-rate of specimens during sampling in Mpumalanga in 2015 and 2016. Moreover, the number of reported malaria cases in the Ehlanzeni district of Mpumalanga decreased with the decrease in rainfall over this period (Christian *et al*. 2018). The small mosquito sample size meant that it was difficult to compare certain collection techniques, particularly in the cases where no mosquitoes were collected from indoor shelters. There were no *An. funestus* group members and very few *An. gambiae* complex members collected indoors, which emphasizes the importance of considering the contribution of outdoor-resting and biting vector species in low-level transmission regions. The low indoor catch-rate was likely a consequence of the

IRS programme activities and individual homeowners use of commercial insecticide aerosols. Despite the small sample size and lack of comparison to other trapping methods, the outdoor ceramic pot proved as an effective collection method for *An. gambiae* complex and *An. funestus* group members, including known vector species, at the Mpumalanga sites. Ceramic pots have been used successfully as mosquito resting shelters in several field studies across South Africa (Brooke *et al.* 2012, Dandalo *et al.* 2017) and Kenya (Odiere *et al.* 2007) as well as in laboratory-based experiments (Farenhorst *et al.* 2008). The ceramic pots were more productive than the modified plastic buckets, although the plastic buckets did show promise as an alternative to the ceramic pot if required. It is likely that the microclimate of the ceramic pots is more appealing to mosquitoes seeking shelter because it is darker, cooler, and slightly more humid than that of the plastic buckets. This may explain superior collection performance of the ceramic pots over the buckets. The success of the clay pots in this study, albeit with a small sample size and not having tested it against other passive collection methods, suggests that they are certainly an efficient passive method for collection of male and female *Anopheles* mosquitoes in low-level transmission regions like South Africa. Besides their value as mosquito collecting devices, their appeal to vector species presents a novel method of insecticide and pathogen delivery to resting mosquitoes (Odiere *et al.* 2007).

The severe drought conditions during the sampling period may have influenced the local species composition at the Mpumalanga sites. Year-long surveillance determined that the most abundant species collected using the outdoor passive methods was *An. vaneedeni*, a member of the *An. funestus* group, followed by the *An. gambiae* complex member *An. merus*. Both species have been implicated as secondary vectors in southern Africa (Burke *et al.* 2017, Cuamba and Mendis 2009) and one *An. vaneedeni* specimen from the Mpumalanga sites was tentatively deemed positive for the presence of *P. falciparum* sporozoites, although it was not confirmed with molecular techniques. The presence of these minor vectors in relatively large numbers further emphasizes the necessity of monitoring the local population density and species composition through regular surveillance to pre-empt the risk of malaria incidence increases and epidemics. The outdoor active collections, which included HLC and CO_2-baited nets, produced mostly *An. quadriannulatus* specimens followed by *An. rivulorum*. The relatively large collection of the predominantly zoophilic species, *An. quadriannulatus*, using human-baited techniques suggests a shift to more opportunistic host-seeking behaviours in the absence of readily available animal blood sources, a possible effect of the drought. The relatively high number of *An. rivulorum* specimens, a species implicated in malaria

transmission in Western Kenya (Kawada *et al.* 2012), collected using outdoor active methods is important to note with the recent increase in activity of secondary transmission vectors in South Africa. The known major local vector, *An. arabiensis*, was collected in relatively low numbers from passive shelters but in slightly higher volumes using the CO_2 -baited tent. This species is recognised for its highly variable resting and biting behaviour (Hargreaves *et al.* 2003, Fettene *et al.* 2004, Tirados *et al.* 2006), characteristics which make this species difficult to control. It is generally accepted that *An. arabiensis* is the main vector species sustaining residual transmission in South Africa because of its outdoor resting and biting activities. The added contribution of secondary vectors, especially those found to be active outdoors, is important to consider when planning vector control strategies.

Following the usefulness of the clay pots for mosquito collections in Mpumalanga, the same style of trap was used for vector surveillance in Mamfene, KwaZulu-Natal. Year-round mosquito surveillance is useful for identifying seasonal trends in wild population density and species composition. The density of the local *An. gambiae* complex and *An. funestus* group population in Mamfene generally fluctuated with the seasonal rainfall patterns, although density did not always parallel the rainfall. Mosquito collections were only made for one week per month thus it is possible that small-scale fluctuations in population density were not captured. Generally, increases in rainfall lead to an increase in mosquito population density, and the opposite is true for the dry periods (Dandalo *et al.* 2017). Adult male and female members of both the *An. gambiae* complex and *An. funestus* group were collected throughout the year, including the dry season, implying that mosquito breeding activities are not halted during the dry season. The collection of blood-fed female *Anopheles* tear-round also suggests that host-seeking and biting continued throughout the unfavourable conditions. This has important implications for provincial vector control programmes as transmission is ongoing throughout the dry season, albeit at much lower rates.

The outdoor passive surveillance at households in Mamfene determined that *An. arabiensis* was the most abundant member of the *An. gambiae* complex present using this method of collection, and was followed closely by *An. parensis*, a member of the *An. funestus* species group. *Anopheles arabiensis* has been implicated as a major vector of malaria in South Africa (Dandalo *et al.* 2017), and *An. parensis* was recently incriminated in malaria transmission in this region because of this study (Burke *et al.* 2019b). The incrimination of *An. parensis* and *An. vaneedeni* is discussed in chapter five. These incriminated outdoor

vectors, together with *An. merus*, an incriminated vector in neighbouring Mozambique, draw much needed attention to the significance of outdoor transmission.

The major indoor vector *An. funestus* has not been detected in South Africa since the early 2000s owing to the use of DDT for IRS (Coetzee *et al.* 2013b). Although highly effective for the indoor-resting vector species, IRS has not been effective for outdoor biting and resting mosquito species, necessitating the inclusion of additional control methods for the outdoor vectors. Larval source management and winter larviciding have previously been shown to be effective for mosquito control and malaria prevention (Fillinger and Lindsay 2011). Targeting the immature aquatic mosquito life stages is particularly useful for reducing the adult vector density in areas with defined and accessible mosquito breeding sites and remains a valuable tool against malaria when managed correctly. Larval source management was recently reintroduced to the local provincial malaria programmes to supplement the current vector control interventions to combat the problem of outdoor biting vectors and residual transmission.

3.5 Conclusions

Clay pots were found to be more productive compared to the modified plastic buckets. However, without a choice, plastic buckets are still useful for entomological surveillance activities in areas of low population density (Dandalo, *et al.* 2017). Drawing conclusions about the productivity of indoor *versus* outdoor resting shelters is difficult as very few specimens were collected indoors. A combination of severe drought conditions and ongoing IRS activities in the sampling area resulted in a very low total catch, and substantially fewer mosquitoes were collected from indoor shelters than outdoor shelters. This also highlights the importance of outdoor resting vector surveillance in low-level residual malaria settings.

Active trapping techniques, specifically human landing catches (HLC) and CO_2 -baited nets, proved effective at the Mpumalanga sites, but are problematic for long-term routine vector surveillance due to the safety risks and clunky logistics involved in setting up the equipment. As useful as the active techniques are, the passive outdoor-based resting shelters are preferred for routine surveillance purposes in South Africa because they are inexpensive, can be installed for long periods, and pose little to no risk to collectors in terms of disease exposure and the accompanying safety concerns of night-time work. The passive mosquito

collection techniques assessed in this study performed equally to the active methods, making them a suitable alternative for use in routine surveillance.

Adult *Anopheles* mosquito surveillance at both Mpumalanga and KwaZulu-Natal sites indicated the presence of several *Anopheles* species, including known vectors and non-vectors, collected both indoors and outdoors in varying densities. The seasonal trend of malaria transmission in South Africa is certainly driven in part by seasonal fluctuations of local vector population densities. Rainfall and temperature largely affect the breeding site opportunities for *Anopheles* mosquitoes and have been shown previously to mirror malaria incidence patterns (Dandalo *et al.* 2017). Most specimens collected during the surveillance period in Mpumalanga and KwaZulu-Natal provinces were found during the warmer high-rainfall months (November to March) as opposed to the cool and dry winter months (May to August).

Vector surveillance activities, such as those conducted in this study, help unravel the complexities of malarial mosquito biology and behaviour with the aim to identify the entomological drivers of residual transmission with the goal of eliminating disease transmission entirely. Effective surveillance requires trained scientists with proficiency in wild mosquito sampling, species identification, vector incrimination, and insecticide susceptibility testing techniques. It is therefore critical for the national and provincial malaria control programmes to build capacity in entomological skills and numbers to make progress towards South Africa's malaria elimination agenda.

CHAPTER FOUR – SALINITY TOLERANCE OF COLONIZED ANOPHELES MOSQUITOES

4.1 Introduction

The larval and pupal life-stages of mosquitoes are completed in aquatic environments that range in salinity levels from salt marshes and hypersaline lakes to freshwater pools and streams (Smith *et al.* 2008). Most mosquito species live in freshwater bodies and have variable tolerances to low levels of salinity, however, a small number of species have adapted to live in brackish and saline aquatic environments (Clements 1992). Fluctuating environmental factors pose a challenge to mosquitoes occupying aquatic habitats as the salinity levels may be increased through evaporation during dry periods or decreased by rainfall during the wet season (Clements 1992). The salt-water adaptability of mosquito species, particularly those of public health importance, is a significant consideration for potential geographical range expansion in the context of malaria epidemiology.

Larval collections from shallow pools at sites in Mpumalanga indicated that a saltwater-adapted species of the *An. gambiae* complex, *An. merus,* was found in the same pools as the freshwater species *An. arabiensis, An. quadriannulatus,* and members of the *An. funestus* group (National Department of Health Malaria Control Programme). Larvae of *An. merus* are typically found in water bodies with a salinity range from 50% to 75% of seawater (Gillies and De Meillon 1968) and are able to complete development in 0-100% seawater under laboratory conditions (Mosha and Mutero 1982) while the sibling species *An. arabiensis* tends to inhabit and breed only in freshwater pools (Gillies and Coetzee 1987). Members of the *An. funestus* group vary in their salinity tolerance; *An. funestus* have a very limited salinity tolerance, but *An. rivulorum* can withstand increased salinity levels with no significant effect on certain life-history traits (Koekemoer *et al.* 2013). Knowing the salinity tolerance of major and secondary malaria vectors may provide important clues to the potential range expansion of local wild populations which could assist in larval source management prioritisation.

The collection of supposedly freshwater and salt-water adapted *Anopheles* species from the same water pools prompted this preliminary investigation into the salinity tolerance of malaria vector larvae. The aim was to investigate the salinity tolerance of the aquatic life-stages of medically important *Anopheles* species under laboratory conditions.

4.2 Materials and Methods

4.2.1 Salinity tolerance testing of aquatic stages of colonized *Anopheles* species

The larval salinity tolerance of colonized *Anopheles* species was tested following the protocol of Koekemoer *et al.* (2013). The laboratory strains tested were AMAL (*An. arabiensis*), MBN-BASE (*An. arabiensis*), SENN-BASE (*An. arabiensis*), SANGWE (*An. quadriannulatus*), and MAFUS (*An. merus*), which were all housed at the Botha De Meillon insectary based at the NICD in Johannesburg. The origin of each of the strains is shown in Table 4.1. All colonies were maintained in accordance with the standard insectary conditions specified by Hunt *et al.* (2005). Seawater concentration gradients were prepared by dissolving Cerebos^{TM} table salt (NaCl) in distilled water at different ratios in separate plastic bowls that were 15cm in diameter and 5cm in depth. The salinity concentrations tested were 0% seawater (distilled water), 15% seawater (0.435% NaCl), and 30% seawater (0.880% NaCl). Salt quantities in grams per each concentration were calculated using the standard that 31.7g/litre NaCl is equal to 100% seawater. Fifty to 60 eggs from each vector species were added to each salinity concentration replicate, and this was repeated in five replicates for each concentration. Because of the effect of evaporation on water salinity, larval rearing bowls were covered with plastic lids, removing them only when feeding larvae or removing dead specimens. The numbers of live larvae and/or pupae per bowl of each concentration and species, respectively, were counted daily until eclosion. Mortality rates of larvae and pupae were also recorded, and dead specimens were removed from bowls. The salinity tolerance of each mosquito strain was determined by the hatch rate of eggs, larval pupation rate, eclosion rate, and total development time. Five biological replicates were performed for each treatment.

Table 4.1 Colony strains

Colony Strain Name	Species	Colonised from	Year established as colony
SENN-Base	*An. arabiensis*	Sennar, Sudan	1980
MBN-Base	*An. arabiensis*	Mamfene, KwaZulu-Natal, South Africa	2002
AMAL	*An. arabiensis*	Malahlapanga, Kruger National Park, South Africa	2010
MAFUS	*An. merus*	Mafayene, Kruger National Park, South Africa	2010
SANGWE	*An. quadriannulatus*	Sangwe, Zimbabwe	1998

4.2.2 Statistical analysis of *Anopheles* salinity tolerance

All statistical analyses were completed using Stata Statistical Software programme version 13.0. The hatch rate, pupation rate, eclosion rate, and development rate at the different salinity concentrations was compared within species strains and between different species strains using the ANOVA test in Stata.

4.3 Results

4.3.1 Salinity tolerances of *Anopheles gambiae* complex mosquitoes

4.3.1.1 The effect of salinity on hatch rate

Hatch rate (HR) data was calculated as the number of first instar hatchlings divided by the number of eggs placed into the experimental larval bowls. The different laboratory colonies showed significantly different HR to one another at all the different salinity concentrations as determined by one-way ANOVA in Stata® 13.0; 0% seawater: $F_{(4,20)} = 3.51$, $p = 0.03$, 15% seawater: $F_{(4,20)} = 3.51$, $p = 0.03$, and 30% seawater: $F_{(4,20)} = 4.70$, $p = 0.008$ (Fig. 4.1). One-way ANOVAs were used to compare the HR within laboratory colonies at different salinity concentrations. There was a significant effect of salinity concentration on HR for AMAL ($F_{(2,12)} = 6.09$, $p = 0.02$) and SANGWE ($F_{(2,12)} = 4.09$, $p = 0.04$), but no significant effect on HR at different concentrations for SENN ($F_{(2,12)} = 0.73$, $p = 0.50$), MBN ($F_{(2,12)} = 0.75$, $p = 0.49$) or MAFUS ($F_{(2,12)} = 0.09$, $p = 0.91$). This suggests that water salinity influences the HR of AMAL (*An. arabiensis)* and SANGWE (*An. quadriannulatus*) colonies but does not affect the SENN (*An. arabiensis)*, MBN (*An. arabiensis)* or MAFUS (*An. merus*) colonies.

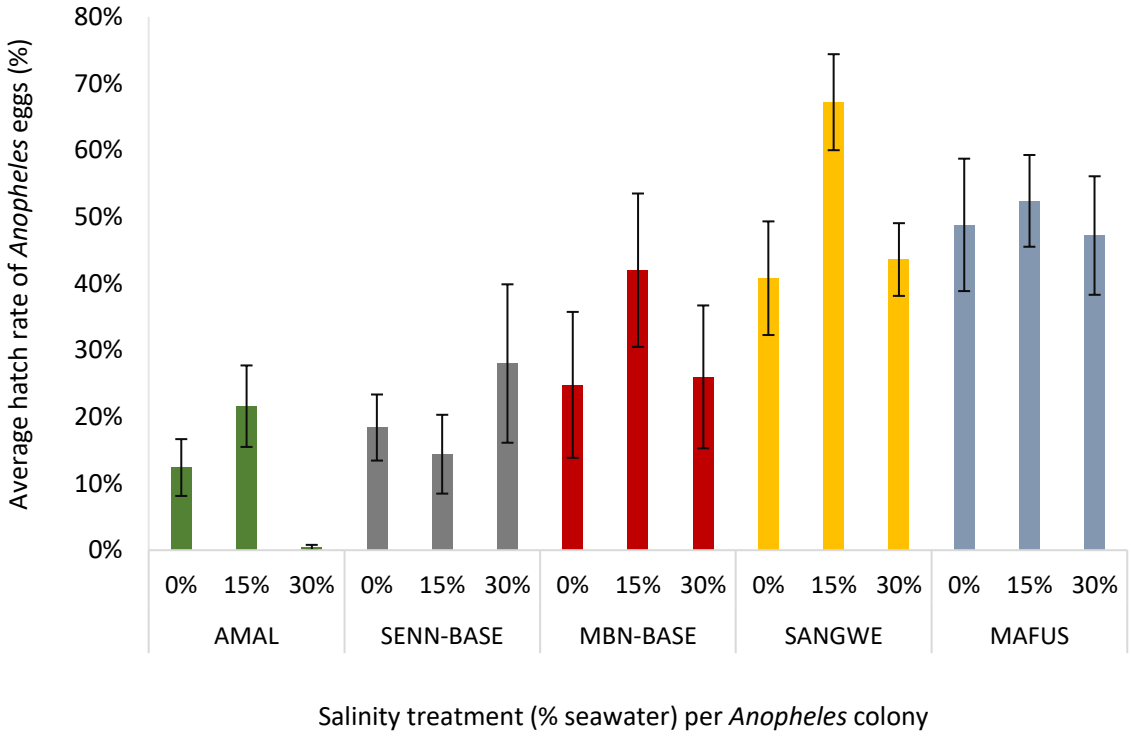

Figure 4.1 The average hatch rate (%) with SE error bars of eggs from colonized *Anopheles* strains placed into three different salinity concentrations: 0% seawater (distilled water), 15% seawater, and 30% seawater.

4.3.1.2 The effect of salinity on pupation rate

The pupation rate (PR) was calculated as the number of emergent adults divided by the number of pupae in each larval bowl once all surviving larvae had pupated. The different laboratory colonies were subjected to a one-way ANOVA to determine the effect of water salinity on PR between the colonies and within each colony, respectively. At 0% salinity there was no significant difference between the PR of different colonies ($F_{(4,20)} = 1.58$, $p = 0.22$), at 15% salinity the PR was close but not significantly different between colonies ($F_{(4,20)} = 2.78$, $p = 0.06$, and at 30% salinity there was a statistically significant difference in PR of the different colonies ($F_{(4,20)} = 7.24$, $p < 0.001$) (Fig. 4.2). No larvae pupated in the 30% salinity cohort of AMAL, SENN, and MBN, all of which are strains of *An. arabiensis*. Only one larva out of five replicates pupated from the 30% salinity cohort of SANGWE. This suggests a strong salinity effect on *An. arabiensis* and *An. quadriannulatus* strains. A one-way ANOVA

determined that PR was significantly higher at 15% compared to 0% salinity for *An. arabiensis* strains AMAL ($F_{(2,12)} = 18.57$, $p < 0.001$) and MBN ($F_{(2,12)} = 7.56$, $p = 0.008$). Besides no pupation taking place at 30% salinity, no significant difference was seen in the PR at 0% and 15% for SENN ($F_{(2,12)} = 2.84$, $p = 0.098$) and SANGWE ($F_{(2,12)} = 1.71$, $p = 0.22$). Salinity treatment had no significant effect on the PR of the salt-water adapted species *An. merus* strain MAFUS ($F_{(2,12)} = 0.07$, $p = 0.94$

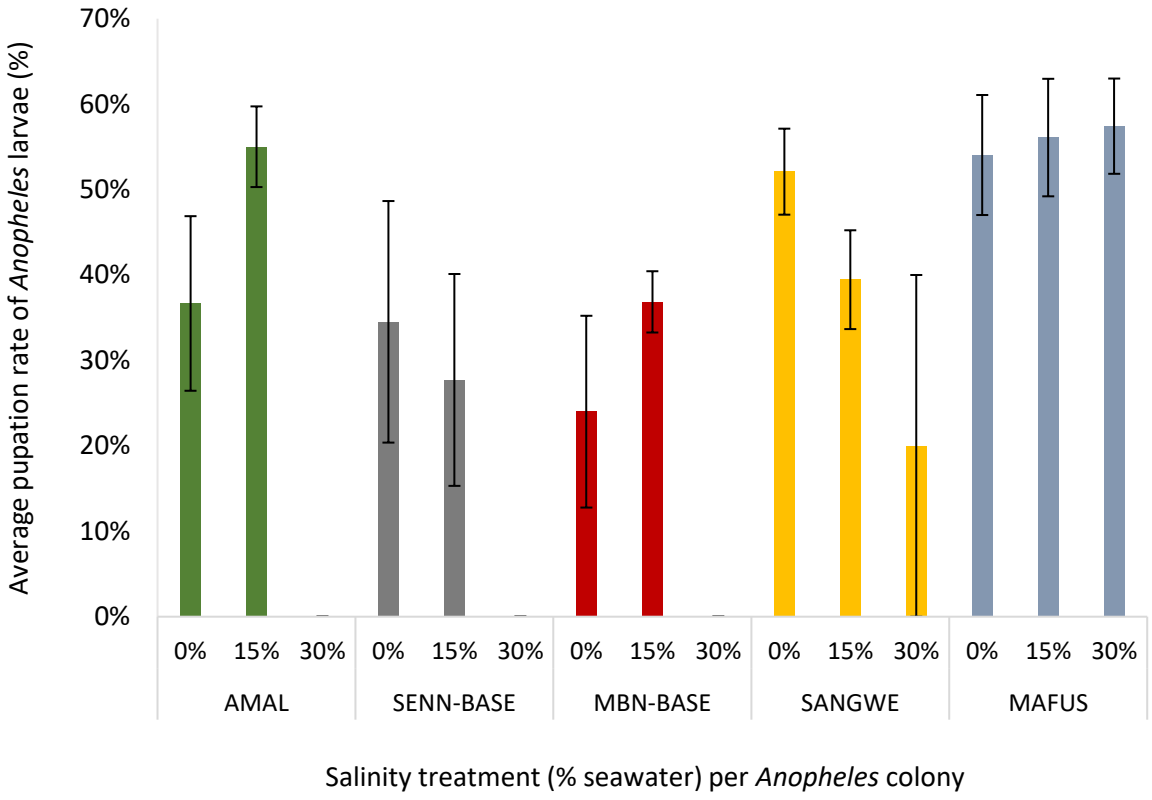

Figure 4.2 The average pupation rate (%) with SE error bars of colonized *Anopheles* larvae placed into three different salinity concentrations from the egg stage

4.3.1.3 The effect of salinity on emergence rate

The emergence rate (ER) was calculated as the total number of emergent adults divided by the total number of eggs placed in the larval bowls at the beginning of the experiment. A one-way ANOVA was used to determine significant differences in ER at different salinities within and between the experimental colonies.

One-way ANOVA determined no significant difference in the ER amongst the different colonies at 0% salinity ($F_{(4,20)} = 0.75$, $p = 0.57$) and at 15% salinity ($F_{(4,20)} = 2.05$, $p = 0.13$), but there was a statistically significant difference in ER at 30% salinity ($F_{(4,20)} = 279.87$, $p < 0.001$) (Fig. 4.3). No larvae pupated in the 30% salinity cohort of AMAL, SENN, and MBN (Fig. 4.3), thus no adults emerged from either of these salinity cohorts (Fig. 4.3). The ER was significantly higher at 15% compared to 0% salinity for AMAL ($F_{(2,12)} = 13.41$, $p < 0.001$) and MBN ($F_{(2,12)} = 7.90$, $p = 0.007$). Emergence rate at 0% and 15% salinity did not differ significantly for SENN ($F_{(2,12)} = 2.51$, $p = 0.12$) and SANGWE ($F_{(2,12)} = 1.07$, $p = 0.37$) colonies even if there was no emergence at 30%. Salinity concentration had no statistically significant effect on MAFUS ($F_{(2,12)} = 1.07$, $p = 0.37$). These data indicate that the ER is significantly affected by high salinity concentration (30%) for all species strains except for the salt-water adapted *An. merus* strain MAFUS.

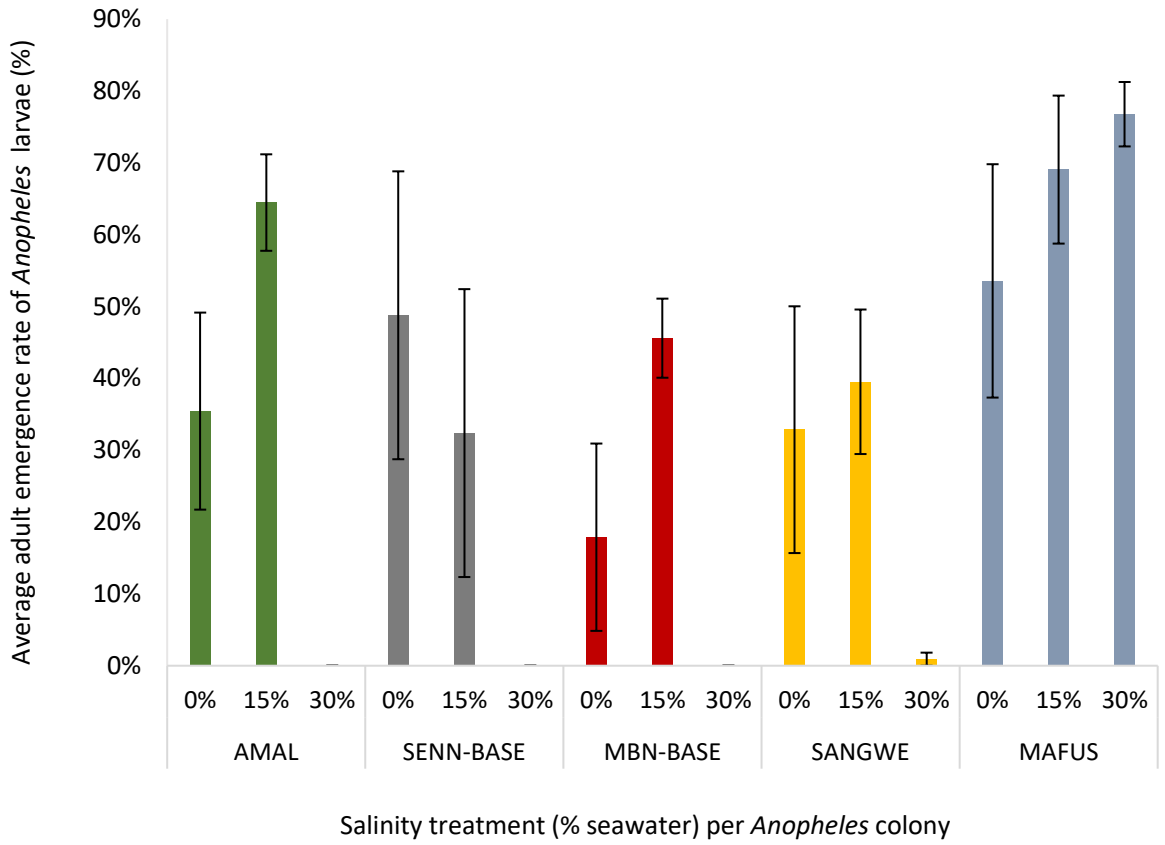

Figure 4.3 The average adult emergence rate (%) with SE error bars of colonized *Anopheles* larvae placed into three different salinity concentrations (0%, 15%, and 30% seawater) from the egg stage.

4.3.1.4 The effect of salinity on total larval development time

A Shapiro-Wilk test of normality, performed in Stata version 13.0, determined that the development time data for the different salinity treatments were not normally distributed ($\chi^2(2) = 23.66$, $p < 0.001$), thus data were further analysed using non-parametric statistical tests. A total of 514 colonized *Anopheles* larvae were successfully reared to adult eclosion during this salinity tolerance experiment (n = 150 at 0% seawater, n = 272 at 15% seawater, n = 92 at 30% seawater). A Kruskal-Wallis H test showed that development time in days of larvae reared in 15% seawater was significantly shorter than those reared in 0%, which was true for AMAL ($\chi^2(1) = 5.51$, $p = 0.02$), MBN base ($\chi^2(1) = 8.77$, $p = 0.003$), and SENN base ($\chi^2(1) = 9.171$, $p = 0.002$) (Fig. 4.4). The SANGWE larvae were the only strain besides

MAFUS that survived the 30% seawater treatment however development time was significantly longer at 0% seawater compared to the other concentrations ($\chi^2(1) = 29.99$, $p < 0.001$). The saltwater-adapted *An. merus* strain MAFUS showed no statistically significant difference in development time of larvae reared in the different salinity treatments ($\chi^2(1) = 4.563$, $p = 0.083$) (Fig. 4.4).

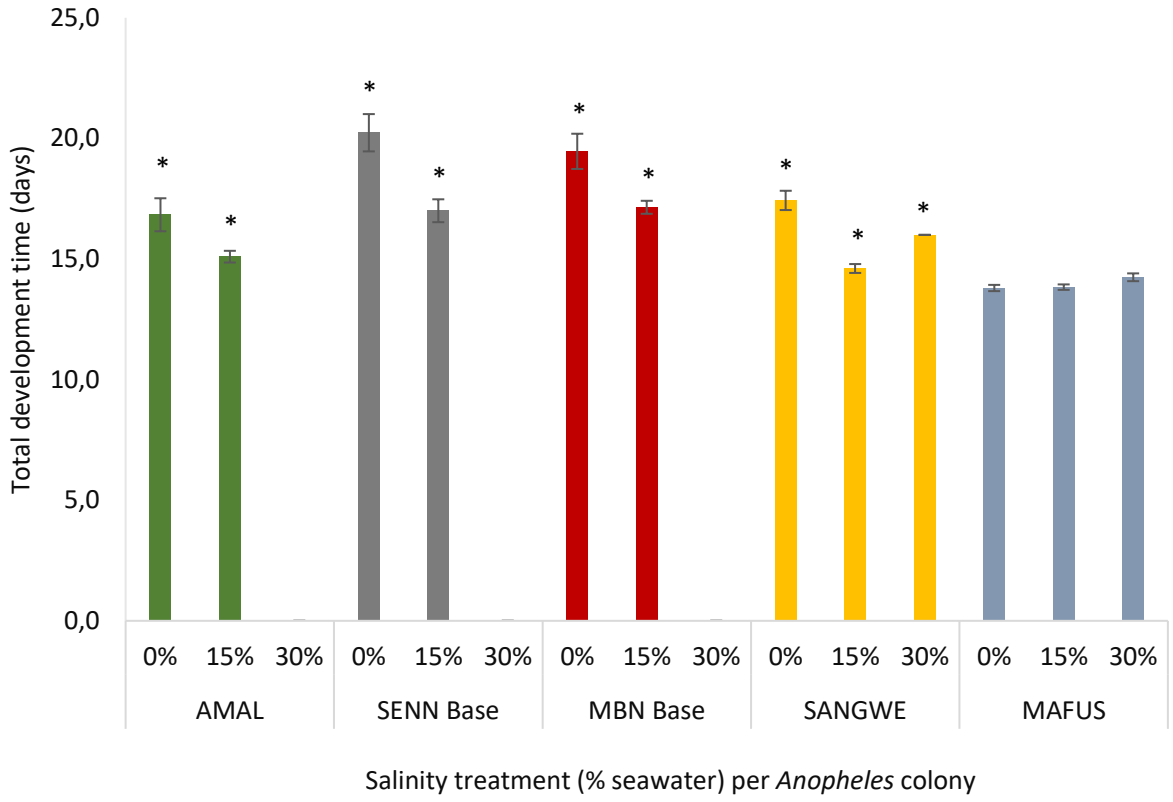

Figure 4.4 Averages of the total development time in days of *Anopheles* species exposed to different salinity treatments with SE error bars. The colonies AMAL, MBN base and SENN base are *An. arabiensis* strains, MAFUS is an *An. merus* strain, and SANGWE is an *An. quadriannulatus* strain. * Indicates a statistically significant difference at 95% CI of larval development time in different salinity treatments within the same colony.

4.4 Discussion and Conclusions

The salinity tolerance of *Anopheles* mosquitoes varies widely. The baseline salinity tolerance of important *Anopheles* species showed that only the *An. merus* MAFUS strain has an appreciable measure of salinity tolerance, particularly at 30% salinity. This is not unexpected as this species is adapted to breeding in coastal saltwater although it is equally tolerant of fresh water and is readily found breeding in inland freshwater localities (Mosha and Mutero 1982; Munhenga *et al.* 2014, Mbokazi *et al.* 2018). Not only was *An. merus* found at inland freshwater localities, it was found to be expanding its inland range in the Ehlanzeni district of Mpumalanga, South Africa (Mbokazi *et al.* 2018) which has important implications for local malaria transmission and control strategies. Changes in environmental factors, particularly rainfall, have shown to have significant seasonal effects on population density of *An. merus* along coastal regions of Kenya (Mosha and Mutero 1982) owing to changes in salinity of breeding pools with rainfall changes throughout the year. Generally, *An. merus* population density increased at the start of the rainfall season due to the formation of breeding pools, then declined as the rainfall began to dilute the salinity levels of the semi-permanent breeding sites, and finally increased again as the rainfall season came to a close and salinity levels of the breeding sites increased once more with the help of evaporation (Mosha and Mutero 1982). Seasonal vector population density fluctuations present a unique opportunity for targeted larval interventions in countries such as South Africa that are on the verge of malaria elimination.

The shifting environmental conditions along with vector species' rapid plasticity may lead to dire consequences regarding disease transmission of not monitored closely and adequately controlled. The rise in sea levels brought on by global climate change has been proposed to increase the available breeding sites of salinity-tolerant malaria vectors inhabiting coastal areas, thus expanding their natural range (Ramasamy and Surendran 2012). Considering the adaptable salinity tolerance of *An. merus* and *An. rivulorum* (Koekemoer *et al.* 2013), both considered minor malaria vectors in southern Africa, it is advisable to monitor the distribution and abundance of vector species found in both fresh and brackish to saline water bodies to maintain good vector control. It is also important to investigate the circumstances under which major and minor vector species breed to understand how best to control their proliferation, particularly now that larval source management and larvicide make up part of South Africa's malaria vector control programme. Larval source management

activities need to consider the different physiological tolerances of medically important mosquitoes so as to target vector species and apply interventions correctly.

CHAPTER FIVE – MALARIA VECTOR INCRIMINATION IN SOUTH AFRICA

This chapter draws largely from two co-authored published articles; "A new malaria vector in South Africa" (Burke *et al.* 2017), Scientific Reports https://doi.org/10.1038/srep43779 (Appendix B) and "*Anopheles parensis* contributes to residual malaria transmission in South Africa" (Burke *et al.* 2019b), Malaria Journal, https://doi.org/10.1186/s12936-019-2889-5 (Appendix C).

5.1 Introduction

Malaria is a parasitic disease caused by *Plasmodium* protozoa and transmitted by female *Anopheles* mosquitoes (Diptera: Culicidae) (Harrison 1978). The populations most at risk live in sub-Saharan Africa which accounts for 80% of cases and 90% of total deaths (WHO 2019). Malaria transmission in South Africa is limited to the low-altitude northern and north-eastern border regions of the country which span the Limpopo, Mpumalanga and KwaZulu-Natal (KZN) provinces (Morris *et al.* 2013). Historically, only the major malaria vector *Anopheles funestus* was directly implicated in malaria transmission in South Africa (Hargreaves *et al.* 2000). The malaria vectors *An. arabiensis* and *An. merus* were provisionally implicated based on their occurrence in South Africa's malaria affected regions and because they had been directly implicated in transmission in neighbouring southern Mozambique (Mendis *et al.* 2000, Cuamba and Mendis 2009, Maharaj *et al.* 2013, Brooke *et al.* 2013). Several *An. arabiensis* and one *An. merus* specimen collected outdoors during collections from 2014 to 2016 in KwaZulu-Natal, South Africa, were confirmed for infection with *P. falciparum* sporozoites (Dandalo *et al.* 2017), thus expanding the number of species directly implicated in malaria transmission within South Africa.

Members of the *An. funestus* group incriminated in malaria transmission in other African countries are *An. rivulorum*, *An. leesoni* and *An. parensis*, tentatively, in Tanzania (Wilkes *et al.* 1996, Temu *et al.* 2007, Kyalo *et al.* 2017), *An. rivulorum* in Kenya (Kawada *et al.* 2012) and *An. longipalpis* in Kenya (Kamau *et al.* 2003). *Anopheles parensis* and *An. vaneedeni* periodically appear as potential vectors. The perennial occurrence of several *An. funestus* group members at the Mpumalanga and KwaZulu-Natal field sites warrants investigation into their possible contribution to malaria transmission in these regions,

especially given the incrimination of some members in malaria transmission in other southern African countries.

Understanding the contribution of outdoor-resting *Anopheles* to residual malaria transmission is important in terms of scaling up vector control toward malaria elimination in South Africa. The aim of this project was therefore to assess the potential role of *Anopheles funestus* group species in residual malaria transmission using sentinel sites in the Ehlanzeni District of Mpumalanga and the uMkhanyakude District of northern KwaZulu-Natal Province, of which the latter currently experiences low malaria incidence and is the closest of all of South Africa's malaria endemic districts to achieving elimination status.

5.2 Materials and Methods

5.2.1 Entomological surveillance

Adult *Anopheles* mosquitoes were collected from sentinel field sites using the outdoor clay pot shelters described in Chapter 2, section 2.1. Entomological surveillance was conducted from March 2015 to April 2016 at sites in Mpumalanga Province, and from January 2017 to May 2018 at sites in KwaZulu-Natal Province, South Africa. The shelters were cleared weekly at sunrise by staff and students from the VCRL with assistance from the local malaria program entomology teams based near the collection sites in each province, respectively. Surveillance was conducted weekly at the Mpumalanga sites, and monthly at the KwaZulu-Natal sites apart from June 2017 and February 2018 when no collections were performed in KwaZulu-Natal due to logistical constraints.

5.2.2 Molecular identification

All specimens were identified by external morphology using dichotomous keys (Gillies and Coetzee 1987). Those identified as *An. gambiae* complex or *An. funestus* group were preserved on silica gel and transported to the Vector Control Reference Laboratory of the National Institute for Communicable Diseases in Johannesburg for molecular identification and parasite screening. Each specimen was identified to species using the appropriate PCR protocol for either *An. gambiae* complex (Scott *et al.* 1993) or *An. funestus* group (Koekemoer *et al.* 2002, Cohuet *et al.* 2003) using methods described in Chapter 2, section 2.2.

5.2.3 *Plasmodium* infectivity testing by ELISA

Every wild-caught female *Anopheles* mosquito was screened for the presence of *Plasmodium falciparum* CSP by means of an ELISA (Wirtz *et al.* 1987, Burkot *et al.* 1984) according to methods described in Chapter 2, section 2.3. The ELISA homogenates of all positive specimens were then boiled at 100°C for ten minutes followed by a repeat ELISA to confirm or refute the presence of CSP (Durnez *et al.* 2011).

5.2.4 *Plasmodium* infectivity confirmation by nested PCR and DNA sequence analysis

Samples that indicated ELISA-positive were verified using the highly sensitive nested *Plasmodium* PCR assay described by Snounou *et al.* (1993). Details of this methodology are provided in chapter 2, section 2.3.

To confirm *Anopheles* species identity, the internal transcribed spacer region 2 (ITS2) of the rDNA of each sporozoite positive sample was amplified using the appropriate primers. The amplified product was then purified, and the DNA sequenced. Sequences were then analysed and compared to established ITS2 reference sequences to confirm the mosquito species identity. To confirm *Plasmodium* species identity, *Plasmodium* ssRNA from the sporozoite positive samples was amplified using the appropriate primers. The amplified product was then purified, and the DNA sequenced. The edited sequences were then aligned with sequences stored in GenBank using nucleotide BLAST (BLASTn) (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The unknown *Plasmodium* sequence was compared with the reference *Plasmodium falciparum* Pf8 18S ribosomal RNA gene, partial sequence (GenBank accession number: KC428742.1) (Mbenda and Das, 2014).

5.3 Results

5.3.1 Vector incrimination

Certain positive samples from the first ELISA assay did not appear positive on the second assay that included a boiling step and were therefore considered negative for *P. falciparum* sporozoite infection. The specimens that tested positive for CSP in the first assay but negative in subsequent assays included *An. arabiensis* (n = 3), *An. parensis* (n = 9), *An.*

leesoni (n = 1), and *An. rivulorum* (n = 2). Of the 163 female *An. gambiae* complex specimens screened for CSP from the KwaZulu-Natal collections, one *An. arabiensis* (collected in October 2017) showed positive by two-step ELISA and *Plasmodium* nested PCR (Snounou *et al.* 1993) (Table 5.1). As this *An. arabiensis* population has been implicated in malaria transmission before (Dandalo *et al.* 2017), no further DNA sequence analysis was considered necessary. Of the 154 female *An. funestus* group specimens screened, one female identified as *An. parensis* (also collected in October 2017) showed positive for *P. falciparum* CSP by two-step ELISA and nested *Plasmodium* PCR (Table 5.1).

Entomological surveillance in two villages in Mpumalanga province yielded a total of 77 *An. funestus* group specimens, 45 of which were identified as *An. vaneedeni* (Table 5.1). One *An. vaneedeni* specimen tested positive for the presence of *P. falciparum* sporozoites by two-step ELISA.

Table 5.1 Total number of *Anopheles* collected from field sites in Mpumalanga (March 2015 – April 2016) and KwaZulu-Natal provinces (January 2017 to May 2018). Parasite infection with *Plasmodium falciparum* by ELISA, nested PCR and DNA sequence analysis is indicated.

Province	Site	Species	No. females	No. males	No. *P. falciparum* positive (ELISA)	No. *P. falciparum* positive (Nested PCR)	No. *P. falciparum* positive confirmed by DNA sequence analysis
Mpumalanga	Vlakbult	*An. arabiensis*	4	2	0		
		An. merus	3	2	0		
		An. vaneedeni	41	11	1	0	
		An. rivulorum	3	1	0		
		An. leesoni	3	0	0		
	Block A	*An. arabiensis*	2	0	0		
		An. quadriannulatus	27	5	0		
		An. merus	14	9	0		
		An. vaneedeni	3	0	0		
		An. rivulorum	16	0	0		
KwaZulu-Natal	Mamfene	*An. arabiensis*	149	79	1	1	
		An. quadriannulatus	6	4	0		
		An. merus	8	3	0		
		An. vaneedeni	1	0	0		
		An. parensis	120	74	1	1	1
		An. rivulorum	11	7	0		
		An. leesoni	23	7	0		

Not all procedures were performed on every specimen. A number or '0' value indicates the procedure was performed; however, a blank cell indicates the procedure was not done.

5.3.2 Validation of *Anopheles* species identity and the presence of a *Plasmodium* infection

Because no *An. funestus* group member besides *An. funestus* s.s. had ever been incriminated in malaria transmission in South Africa before, it was necessary to confirm the species identification unequivocally and to verify as far as possible the presence of *P. falciparum* CSP. The *Anopheles* species identification of these two specimens as *An. vaneedeni* and *An. parensis* was therefore confirmed by sequencing the internal transcribed spacer 2 (ITS2) region (Koekemoer *et al.* 2002). The *An. vaneedeni* specimen collected from

Mpumalanga province was not confirmed by nested PCR due to very low parasite DNA concentration levels, however a further *An. vaneedeni* specimen collected from Mamfene by colleague Dr Leonard Dandalo as part of the SIT project was positively confirmed for the presence of *P. falciparum* sporozoites by nested PCR and subsequent DNA analysis (Burke *et al.* 2017). There was a 99% sequence identity between the ITS2 region of the Mpumalanga and KZN *An. vaneedeni* specimens and the published *An. vaneedeni* sequence originating from South Africa (GenBank accession number JN994152.1) (Norris and Norris 2015). The presence of *P. falciparum* sporozoite DNA was confirmed by nested PCR for the KZN *An. vaneedeni* specimen (Snounou *et al.* 1993). Sequence analysis of the nested PCR product revealed that there was a 99% identity to the published sequence for *P. falciparum* (GenBank accession number KT991235.1) (Zhang *et al.* 2011).

The species identity of the infective *An. parensis* female was confirmed after standard PCR (Koekemoer *et al.* 2002) ITS2 sequences showed 99% homology between this specimen and that for a reference sequence of this species in GenBank (accession number: JN994144.1) (Norris and Norris 2015). The *P. falciparum* infection in this *An. parensis* specimen was confirmed by 95% sequence homology of the nested *Plasmodium* PCR product with the *P. falciparum* isolate Pf8 18S ribosomal RNA gene, partial sequence (GenBank accession number: KC428742.1) (Mbenda and Das 2014).

These data give *P. falciparum* a potential infectivity rate of 2.44% for *An. vaneedeni* at the Mpumalanga sites, and an infectivity rate of 0.67% for *An. arabiensis* and 0.83% for *An. parensis* at Mamfene, KZN. The *An. vaneedeni* specimen positively confirmed in Mamfene by Burke *et al.* (2017) indicates a *P. falciparum* infectivity rate of 1.96% for the Mamfene region. None of the *An. leesoni* or *An. rivulorum* female specimens from either field site (Table 4.1) showed positive for *P. falciparum* sporozoites based on ELISA analysis.

5.4 Discussion

Given that *An. parensis* and *An. vaneedeni* had not been unambiguously incriminated in malaria transmission before, it was considered necessary to ensure that all morphological, PCR, ELISA and sequence data were sufficiently aligned to confirm *Plasmodium* infectivity, and that all data were either double or triple-checked for quality assurance. The data summarised here represent the first record of wild-caught *P. falciparum* sporozoite positive

An. vaneedeni and *An. parensis* females, directly implicating these species in malaria transmission in South Africa. Although the *An. vaneedeni* specimen collected from Mpumalanga was not confirmed for *P. falciparum* CSP infection by nested PCR, the double positive ELISA result together with the molecular confirmation of an additional *An. vaneedeni* specimen from SIT collections in KwaZulu-Natal support the incrimination of this species in malaria transmission in South Africa.

It is reasonable to infer that the single *An. parensis* female that presented with *P. falciparum* CSP was infective for malaria. This at least shows that this species is a potential malaria vector. There are however other considerations that need to be made in terms of its contribution to malaria transmission. Available data show that *An. parensis* seldom takes blood from humans (Mouatcho *et al.* 2007, Muturi *et al.* 2009), being more inclined to feed on livestock animals and rest outdoors (Muturi *et al.* 2009, Gillies and De Meillon 1968), as has previously been demonstrated for the northern KwaZulu-Natal population (Mouatcho *et al.* 2007). This reduces its potential contribution to malaria transmission substantially, but not necessarily to zero. This is reinforced by earlier records that describe a high malaria incidence setting on the Kenyan coast in which *An. parensis* was abundant but no sporozoite positive specimens were found (based on salivary gland dissections) despite the sympatric *An. funestus* population showing a sporozoite positive index of 7% (Gillies and De Meillon, 1968). *Anopheles parensis* has nevertheless been recorded as an exophilic feeder on humans (Gillies and De Meillon 1968) with a comparatively high human blood index in some ecosystems (Muturi *et al.* 2009, Kweka *et al.* 2008), and can be found resting both indoors and outdoors (Kamau *et al.* 2003). Although *An. vaneedeni* is also considered primarily zoophilic (Gillies and Coetzee, 1987), it will readily feed on humans outdoors and has previously been experimentally infected with *P. falciparum* under laboratory conditions (De Meillon *et al.*, 1977). The outdoor-resting and feeding traits of this species are reinforced by the fact that most of the *An. vaneedeni* specimens collected in these surveys, including the two that tested sporozoite positive, were found in outdoor-placed ceramic pots deployed at randomly selected households at the two field sites (Burke *et al.* 2017).

Anopheles arabiensis is a major malaria vector throughout its distribution including northern KwaZulu-Natal (Dandalo *et al.* 2017, Sinka *et al.* 2012). It is worth noting that both the infective *An. arabiensis* and *An. parensis* specimens were collected in October 2017. Although October is generally a low-incidence month in South Africa, 2017 was an unusual year in that unexpectedly high numbers of cases were recorded from May onwards

(unpublished National Department of Health statistics). Incidence in KwaZulu-Natal was nevertheless low compared to other endemic provinces which precludes associating these sporozoite-positive specimens with any case or cluster of cases. It is important to note that both specimens were caught resting outdoors, highlighting the importance of addressing residual transmission in South Africa by targeting outdoor-resting vectors in addition to those targeted indoors by the provincial indoor residual spraying (IRS) programmes. Intensive provincial larval source management programmes, including winter larviciding, and community outreach programmes designed to educate on personal protection measures and treatment seeking, are currently under development to address this issue.

The collection of sporozoite-positive, outdoor-resting *An. vaneedeni*, *An. parensis* and *An. arabiensis* supports the hypothesis of ongoing outdoor residual malaria transmission in South Africa, as first proposed by De Meillon *et al*. (1977), and tentatively suggests that these species may also be contributing to malaria transmission in other malaria-endemic countries in which they occur. This information highlights the need to intensify malaria vector control in South Africa by including methods designed to target outdoor feeding vector populations without compromising the efficacy of the IRS programme. Intensive provincial larval source management programmes, including winter larviciding, and community outreach programmes designed to educate on personal protection measures and treatment seeking, are currently under development to address this issue.

5.5 Conclusions

It is concluded that *An. parensis* and *An. vaneedeni* are potential vectors of malaria but that their contribution to transmission in South Africa's endemic provinces is likely to be minimal at best owing to their strong zoophilic tendency. By contrast, *An. arabiensis* is a major vector throughout its distribution and is primarily responsible for the bulk of residual malaria transmission in South Africa. As all recently collected sporozoite-positive *Anopheles* mosquitoes were found in outdoor-placed resting shelters in Mpumalanga and northern KwaZulu-Natal provinces (Dandalo *et al.* 2017, Burke *et al.* 2017, Burke *et al.* 2019b), it is necessary to introduce additional interventions that can be used to control outdoor-resting vector populations while maintaining the efficacy of South Africa's IRS operations for the ongoing control of indoor-resting vectors.

CHAPTER SIX – MEASURING THE METABOLIC RATE OF ADULT MALARIA VECTORS BY SEASON IN SOUTH AFRICA

This chapter draws largely from the published article "Metabolic rate does not vary with seasonal change in *Anopheles arabiensis* adults in South Africa." (Burke *et al*. 2019a), Journal of Insect Physiology, https://doi.org/10.1016/j.jinsphys.2019.103942 (Appendix D).

6.1 Introduction

Seasonal mosquito density is directly correlated to malaria transmission in South Africa (Christian *et al.* 2018; Dandalo *et al.* 2017). Variations in rainfall, temperature and humidity by season play important roles in determining mosquito vector population densities. *Anopheles* mosquito density peaks during the high rainfall period from November to February when non-perennial surface water is available for breeding sites and declines to a low level during the dry period from June to August in South Africa. This is especially true for *An. arabiensis* (Dandalo *et al*. 2017). Although density declines to a low level, vector mosquitoes do not disappear entirely during the dry season, and malaria transmission still occurs albeit at substantially lower levels (Munhenga *et al*. 2014, Dandalo *et al*. 2017).

African a *Anopheles* have a relatively short lifespan (Maharaj 2003; Olayemi and Ande 2009; Oliver and Brooke 2014) and the aquatic stages (eggs, larvae, and pupae) are highly susceptible to desiccation (Koenraadt *et al*. 2003). These characteristics should theoretically hamper long-term survival in dry, stressful conditions. There is currently little information concerning the mechanisms that drive dry-season persistence of *Anopheles*. The major hypotheses for mosquito dry-season survival include: (i) retreating to local perennial breeding sites or refugia, (ii) migrating long distances from permanent breeding sites to seasonally arid areas at the onset of rainfall, and (iii) reducing metabolic expenditure to increase longevity by winter dormancy (diapause) or summer dormancy (aestivation) in sheltered dwellings (Omer and Cloudsley-Thompson 1968, Omer and Cloudsley-Thompson 1970, Dukeen and Omer 1986, Obala 1995, Charlwood *et al*. 2000, Donnelly *et al*. 2002, Lehmann *et al*. 2010, Munhenga *et al*. 2014, Huestis and Lehmann 2014). Understanding the seasonal biology of vulnerable dry-season vector populations and identifying their main survival tools presents valuable opportunities for targeted control.

Lowered resting metabolic rate reduces nutritional demands and the associated energy costs of resource foraging, and potential water loss associated with respiration, excretion, and

diffusion across the cuticle (Gray and Bradley 2005; Benoit and Denlinger 2007). This overwintering survival strategy is well documented in adult female *Culex pipiens* (Benoit and Denlinger 2007), but there is limited evidence for this in African *Anopheles*. The aim of this project was to investigate the seasonal change in metabolic rate to determine the presence or absence of winter dormancy in South African malaria vector mosquitoes. Seasonal metabolic rate variation, and consequent adaptations to overall energy expenditure, may be advantageous to mosquitoes during unfavourable conditions; however, the evidence of continued breeding in previous studies (Munhenga *et al*. 2014, Dandalo *et al*. 2017) during the winter season leads us to expect that these populations do not diapause. Disparities in resting metabolic rate between seasons were investigated (dry winter and wet summer) in *Anopheles* vector populations to assess whether this physiological trait contributes significantly to dry-season mosquito persistence in South Africa. It was hypothesized that no such seasonal disparity in metabolic rate would occur in South African malaria vector populations.

6.2 Materials and Methods

6.2.1 Collection of wild adult mosquitoes for metabolic rate analysis

Field collections of wild mosquitoes took place in the rural village of Mamfene, which forms part of the uMkhanyakude district of KwaZulu-Natal province South Africa. Collections were conducted monthly from January to December 2017 (excluding June 2017 due to logistical constraints). All available live *Anopheles* adult mosquitoes were aspirated from ten clay resting pots positioned outside consenting households in Mamfene. Two experienced entomologists checked and cleared every trap within two hours from sunrise, the period when mosquitoes are inactive, for two days every month. Collected specimens were identified to species complex level based on morphological features using dichotomous keys (Gillies and Coetzee, 1987) and only *An. gambiae complex* and *An. funestus* group specimens were retained for metabolic measurements. The live specimens were grouped by sex and species complex/group and then placed into polystyrene cups with a mesh gauze covering the opening of each cup. Mosquitoes had access to cotton pads saturated with 10% sucrose. Specimens were packaged and then transported by car to the Wits Insectary and Quarantine

facility based at the University of the Witwatersrand in Johannesburg within one to two days after initial collection from the clay pots. Sugar pads were refreshed where necessary upon arrival at the insectary and mosquitoes were left in their polystyrene cups in a room with natural light overnight. Metabolic rate measurements for all specimens collected were taken the following day. Rainfall and temperature readings for the period under review were captured by a HOBO® RX3000 (Onset Computer Corporation, Bourne, MA), data logging weather station kit positioned in Mamfene.

6.2.2 Respirometry of adult mosquitoes

The morphological identification, sex, and feeding status (females only) of each mosquito was determined before measurement took place. The metabolic rate of adult mosquitoes was determined by measuring CO_2 emission rates by closed system respirometry using techniques similar to those described by Woods and Singer (2001), Kambule *et al*. (2011), and Kaiser *et al*. (2014). It was possible to measure each mosquito individually because the CO_2 production of a single mosquito is sufficiently greater than the baseline noise to achieve an accurate reading. Measurements took place in a temperature-controlled laboratory at room temperature (25 ± 2°C) during daylight hours between 08:00 and 18:00.

Carbon dioxide production was measured using an infrared CO_2 analyser (LI-7000, LI-COR, Lincoln, NE, USA) based on the technique described by Woods and Singer (2001), Kambule *et al*. (2011) and Kaiser *et al*. (2014). Ambient air scrubbed of CO_2 and water was drawn through the CO_2 analyser at a flow rate of 100 $mL.min^{-1}$. Respirometry chambers were made of 30mL glass syringes (Becton Dickson, Franklin Lakes, NJ, USA) each fitted with a three-way stopcock. Three chambers were used for each measurement run; the first served as a control to monitor CO_2 leakage, and the second and third chambers housed a single mosquito sample in each. A live mosquito was aspirated into the barrel of the glass syringe with the plunger removed while cotton wool was held over the open rear end of the barrel to prevent the mosquito escaping the barrel during handling (Fig. 6.1). The plunger was then quickly inserted into the barrel while removing the cotton wool to ensure the mosquito did not escape. The chambers were then flushed with humid CO_2-free air for five minutes to remove excess CO_2 accumulated from the mosquito handling process. Following flushing, the plunger was depressed to the 20 mL mark and purged air was flushed through the needle. The stopcock between the syringe and the needle was closed to seal the chamber. After 25 minutes, a 5 mL

air bolus was injected into the CO_2-free air stream from each chamber in turn. Injections were performed in sequence, allowing enough time for the analyser to return to baseline CO_2 levels between each sample. Two CO_2 measurements were taken per syringe (six measurements in total) in a single run and recorded using ExpeData-P Data Analysis Software (Sable Systems, Las Vegas, NV, USA). The recorded CO_2 emissions were converted from parts per million to mL by dividing the values by 1 000 000 and multiplying by the flow rate of 100. The baseline of the CO_2 analyser was corrected in ExpeData to remove any residual analyser drift. The CO_2 peak recorded for each sample was integrated against time to give the volume of CO_2 in each injected sample. Carbon dioxide values were further scaled up to the volume of the chamber by multiplying the value by 4 and dividing by the time spent in the chambers by each sample to yield the CO_2 emission rate of each mosquito (Lighton, 2008). Mosquitoes were observed to be mostly only active (flying or walking) during the handling process prior to the flushing step. Once left to settle in the syringe most of the mosquitoes entered a quiescent state of less than 20% activity, any mosquitoes showing high activity were omitted from the analysis.

Measured mosquitoes were then aspirated from their respirometry chambers and placed into individual polystyrene cups. Each mosquito was knocked down in the freezer and weighed using a Libror AEG 45SM balance (Shimadzu Scientific Instruments, Japan) (±0.01 mg) to account for mass variation in the sample. The mass specific rate of CO_2 production (mL $CO_2.mg^{-1}.min^{-1}$) was calculated in Microsoft Excel (2016) by accounting for the mass (mg) of individual mosquitoes and the time interval between each CO_2 bolus injection. Specimens were dry-preserved on silica gel and identified further to species level using the appropriate PCR protocols for *An. gambiae* complex (Scott *et al*. 1993), and *An. funestus* group (Koekemoer *et al*. 2002, Cohuet *et al*. 2003) at the Vector Control Reference Laboratory of the National Institute for Communicable Diseases in Johannesburg.

Statistical analysis involved comparing the CO_2 production rates of wild adult mosquitoes between seasons (summer, autumn, winter, and spring) using the nonparametric Kruskal-Wallis rank test in Stata 13 (StataCorp LP, College Station, TX, USA). Analyses of the influence of mosquito sex and female blood-meal feeding status on metabolic rate were performed using the Wilcoxon matched-pairs signed-rank test in Stata 13. Nonparametric statistical tests were used because data were not normally distributed due to high variation in the dataset.

Figure 6.1. Female adult *Anopheles* resting inside the glass respirometry chamber

6.2.3 In-field metabolic rate measurements of wild mosquitoes

The effect of long-distance road travel on the metabolic rate of mosquitoes is unknown. To address this, CO_2 production measurements were performed at the KwaZulu-Natal Department of Health (DOH) laboratory in Jozini (Mamfene) using wild-collected mosquitoes that had not been subjected to long-distance transport and compared to those measurements taken from mosquitoes transported to the University of the Witwatersrand in Johannesburg. The same respirometry equipment used in the university laboratory was packaged, transported, and set up in the laboratory in Jozini. Wild specimens were collected in October 2018 from the same clay pots used before. Weather data collected from the local station in Mamfene for October 2017 and October 2018 is presented in table 6.1 and shows no significant variation in temperature, humidity, or rainfall between the two time points. Carbon dioxide production of the mosquitoes was measured using the respirometry techniques described in section 6.2.2. Once measured, mosquitoes were placed into individual polystyrene cups with access to 10% sucrose solution and were transported live to the

laboratory at the University of the Witwatersrand. Mosquitoes were knocked down in the freezer and weighed using the Libror AEG 45SM fine balance as before.

Mosquito metabolic rate measurements acquired in the field laboratory were statistically analysed against those measured at the university laboratory using the nonparametric Kruskal-Wallis rank test in Stata 13. Mosquitoes collected and measured in the field in October 2018 were compared to those collected and measured in the laboratory in October 2017 to eliminate the potential effect of seasonal differences. Statistical analyses were performed within mosquito species and sex to reduce the number of variables.

Table 6.1 Weather data collected from a HOBO® RX3000 (Onset Computer Corporation, Bourne, MA), data logging weather station kit positioned in Mamfene, KwaZulu Natal, South Africa.

Weather parameters	**October 2017**	**October 2018**
Monthly average humidity (%)	67,2	68,8
Monthly max humidity (%)	87	87
Monthly min humidity (%)	42	44
Monthly average temperature (°C)	23,1	21,4
Monthly max temperature (°C)	29,2	27,9
Monthly min temperature (°C)	17,1	14,8
Monthly total rainfall (mm)	36,2	38,4

6.2.4 Measuring photoperiodic effect on metabolic rate of colonised mosquitoes

The major variables that fluctuate between seasons in South Africa are temperature, humidity, and photoperiod. Because the focal malaria points in KwaZulu-Natal are in a low-rainfall hot semi-arid climate where the daily average temperature rarely falls below 18°C in winter, photoperiod changes were suggested as the most likely trigger for seasonal physiological changes in mosquitoes. Hence, the effect of seasonal photoperiod on mosquito metabolic rate was investigated.

The experiment was conducted in a temperature-controlled room at the University of the Witwatersrand, Johannesburg. The room was set to 25°C and maintained at 50-65% relative humidity. Three large steel-framed mesh enclosures ((H) 95 cm x (W) 55 cm x (L) 55 cm) were used to house smaller cages of mosquitoes (Fig. 6.2). One non-heat-producing 20 W LED floodlight (Eurolux (Pty) Ltd, Milnerton, RSA) was secured to the top of each mesh enclosure with the light source directed into the enclosure and plugged into an on/off weekly analogue programmable timer (Major Tech (Pty) Ltd, Johannesburg, RSA). Each enclosure was cloaked in dark cloth to prevent infiltration of natural light and/or ambient light from adjacent enclosures. The on/off timers were set to create daily light/dark cycles within each enclosure to represent the seasonal photoperiod cycles of winter (10 h light, 14 h dark) and summer (14 h light, 10 h dark) and a control (12 h light, 12 h dark).

Anopheles arabiensis (KWAG colony) mosquitoes – originally colonised using material collected from KwaZulu-Natal and reared at the Botha De Meillon insectary at the NICD, Johannesburg, were used for this experiment. Mosquitoes were collected from the insectary on the day of emergence and separated by sex to avoid mating. Male and female mosquitoes (100 of each) were placed into separate BugDorm© cages (model 4M3030, Mega View Science Co., Ltd., Taichung 40762, Taiwan) and provided with 10% sucrose solution that was refreshed every two days throughout the experiment. One cage of males and one of females was placed into each mesh enclosure and the entire enclosure was cloaked with black cloth. Each experiment was run for 15 days. The position of cages of males and females were swapped within the enclosure periodically to avoid positional bias. The metabolic rate of 10 males and 10 females from each treatment and control group was measured using the techniques described in section 5.2.2 every five days (amounting to three measurements at the end of 15 days). The measurement days were staggered to allow enough time to measure 20 samples in one day. Measured mosquitoes were not returned to the experimental cages,

thereby each mosquito was measured only once. The entire experiment was replicated three times and enclosures were repositioned after each replicate to avoid positional bias. The CO_2 production of colonised *An. arabiensis* mosquitoes placed under different photoperiod cycles were statistically analysed using the Kruskal-Wallis rank test in Stata 13.

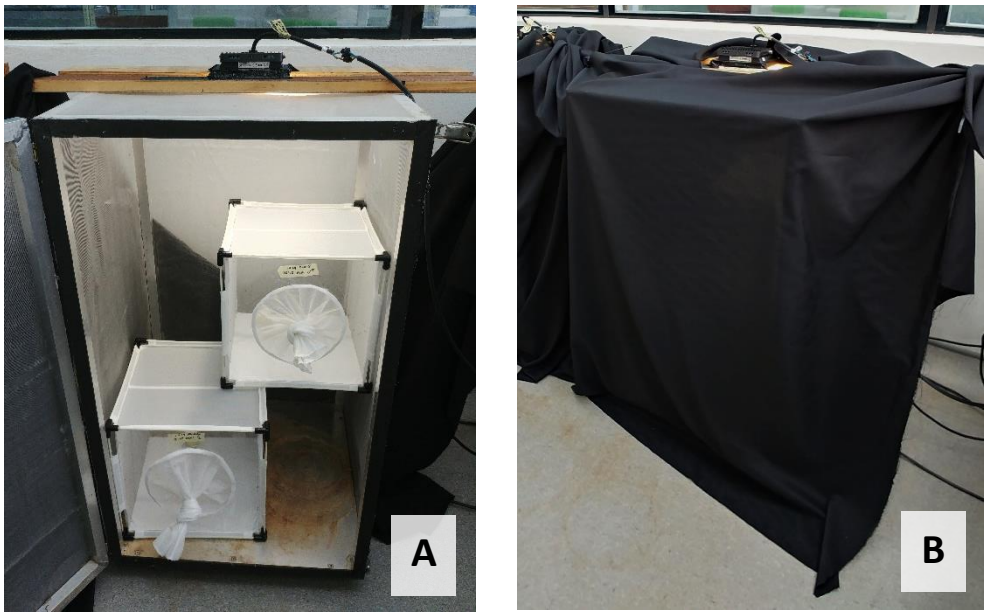

Figure 6.2 (A) Mesh enclosure with LED light-source set with a programmable on/off timer affixed to the top of the enclosure. BugDorm© cages containing male and female mosquitoes separately were placed inside the enclosures. (B) Enclosure cloaked in black cloth to prevent interference from ambient light.

6.3 Results

6.3.1 Seasonal abundance and metabolic rate of wild *Anopheles* mosquitoes

Weather station data indicated a total monthly rainfall range from 64 mm in February to 0 mm in June and July, and an average daily temperature range from a high of 27.2 °C to a low of 19.2 °C in January and June respectively (Fig. 6.3). A total of 234 *An. gambiae* complex and 203 *An. funestus* group specimens were collected during the period under review. *Anopheles* density was particularly high in March and mainly comprised members of the *An. gambiae* complex (n = 109) and, to a lesser extent, the *An. funestus* group (n = 55) (Fig. 6.3). The onset of high rainfall in February may have led to the mosquito density increase experienced in March. *Anopheles gambiae* complex numbers declined towards the end of 2017, while *An. funestus* group numbers remained relatively constant until the low-rainfall period in October (Fig. 6.3). *Anopheles* species collected included *An. arabiensis* (n = 216), *An. merus* (n = 10) and *An. quadriannulatus* (n = 8) of the *An. gambiae* species complex, and *An. parensis* (n = 162), *An. leesoni* (n = 29), *An. rivulorum* (n = 11) and *An. vaneedeni* (n = 1) of the *An. funestus* species group. Adult mosquitoes were still present during the dry winter period (June to August), but in lower densities compared to the high-rainfall period from February to May (Fig. 6.3).

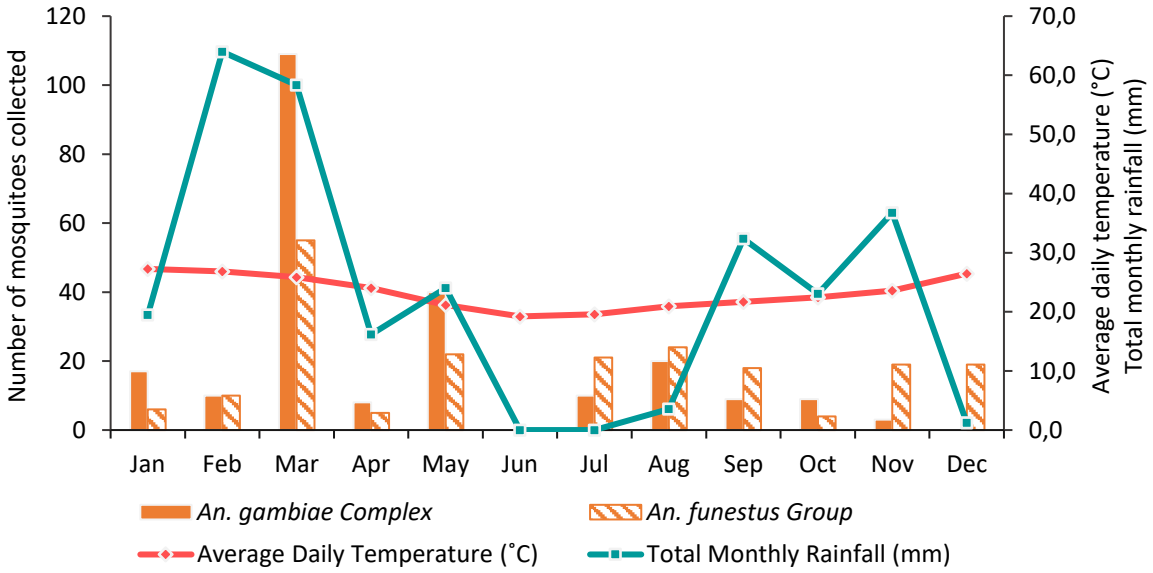

Figure 6.3 Total number of successfully identified *Anopheles* mosquitoes collected from clay pots from January to December 2017 in Mamfene, KwaZulu-Natal. Daily average temperature (°C) and total monthly rainfall (mm), respectively, over the sampling period are shown. *No mosquito collection data were obtained in June.

The metabolic rate (CO_2 production) of wild *Anopheles* collected from Mamfene varied considerably across the sampling period (Fig. 6.4). Monthly data were sorted into seasons: summer (January, February, and December), autumn (March, April, and May), winter (July and August) and spring (September, October and November) for data analysis. *Anopheles arabiensis* and *An. parensis* predominated the samples, and so these two species were used for seasonal comparisons of metabolic rates (Fig. 6.4). Statistical analyses revealed no significant differences in metabolic rate values of *An. arabiensis* females across all seasons ($\chi2 = 4.673$, df = 3, $P = 0.197$, n = 76), *An. arabiensis* males across all seasons ($\chi2 = 3.181$, df = 3, $P = 0.365$, n = 38), or between *An. arabiensis* females and males overall (z = -1.539, $P = 0.124$, n = 114). Similarly, *An. parensis* metabolic rate comparisons indicated no significant differences between females compared across all seasons ($\chi2 = 3.497$, df = 3, $P = 0.321$, n = 46), nor between *An. parensis* males and females overall (z = 0.089, $P = 0.929$, n = 82). A small number of male *An. parensis* collected in autumn (n = 7) exhibited significantly lower metabolic rate than males collected in winter (n = 17) (z = -3.588, $P = 0.0003$). The effect of feeding status on metabolic rate was not significant for wild *An. arabiensis* females

(z = -1.193, *P* = 0.233) nor for wild *An. parensis* females (z = 0.612, *P* = 0.541). The number of blood-meals taken and the gonotrophic stages of wild females was however unknown for wild material.

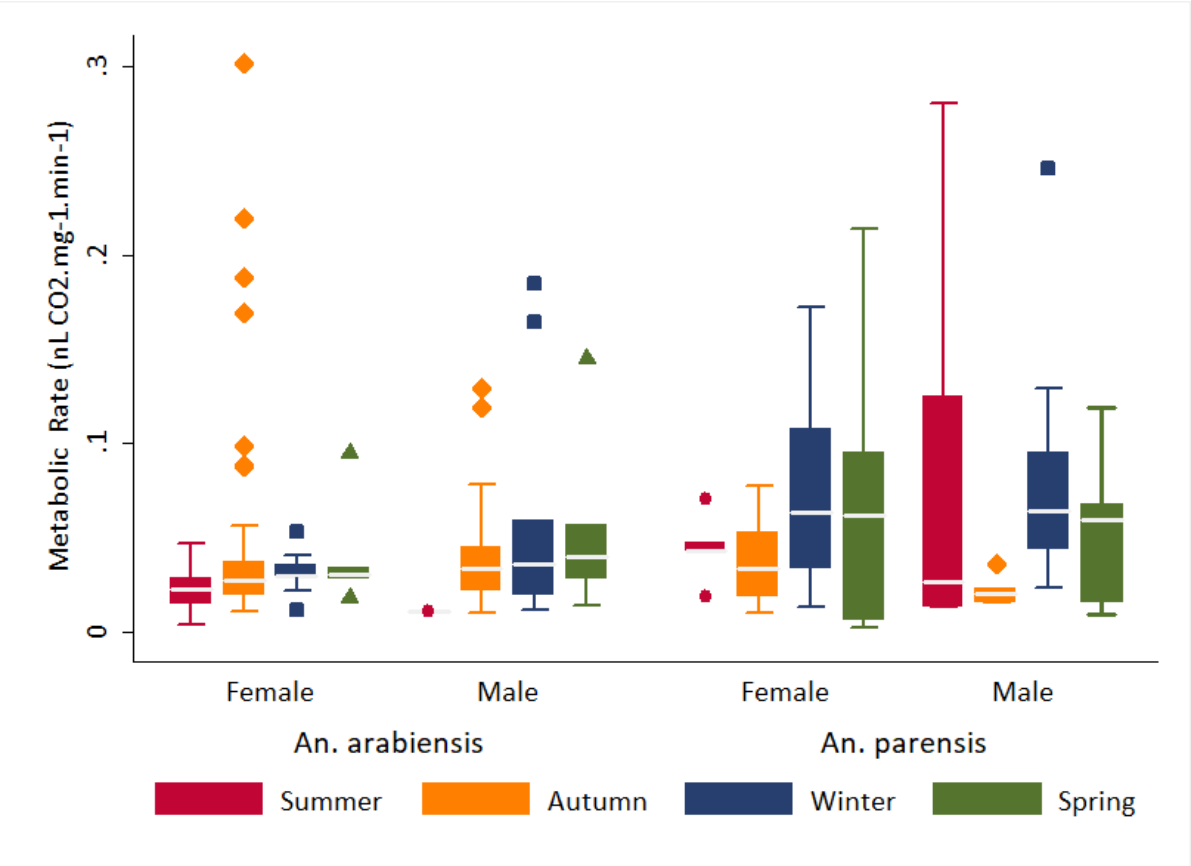

Figure 6.4 Seasonal metabolic rates (nL CO_2.mg^{-1}.min^{-1}) of wild adult male and female *An. arabiensis* and *An. parensis* mosquitoes collected from Mamfene, KwaZulu-Natal, from January to December 2017. Each box shows the interquartile range of metabolic rate with the median value indicated with a white line and outliers indicated with shaped markers.

6.3.2 Mosquito metabolic rate response to transportation

The distance between Mamfene and Johannesburg is approximately 500 km equating to approximately 6 hours travel time by road. Only *An. arabiensis* males and *An. leesoni* females were used for comparisons between the different measurement locations owing to insufficient sample sizes of other groups of species (Fig. 6.5). There was no apparent

significant effect of travel on metabolic rate for *An. arabiensis* males (z = -0.354, P = 0.724, n = 7), or on *An. leesoni* females (z = 1.567, P = 0.117, n = 15).

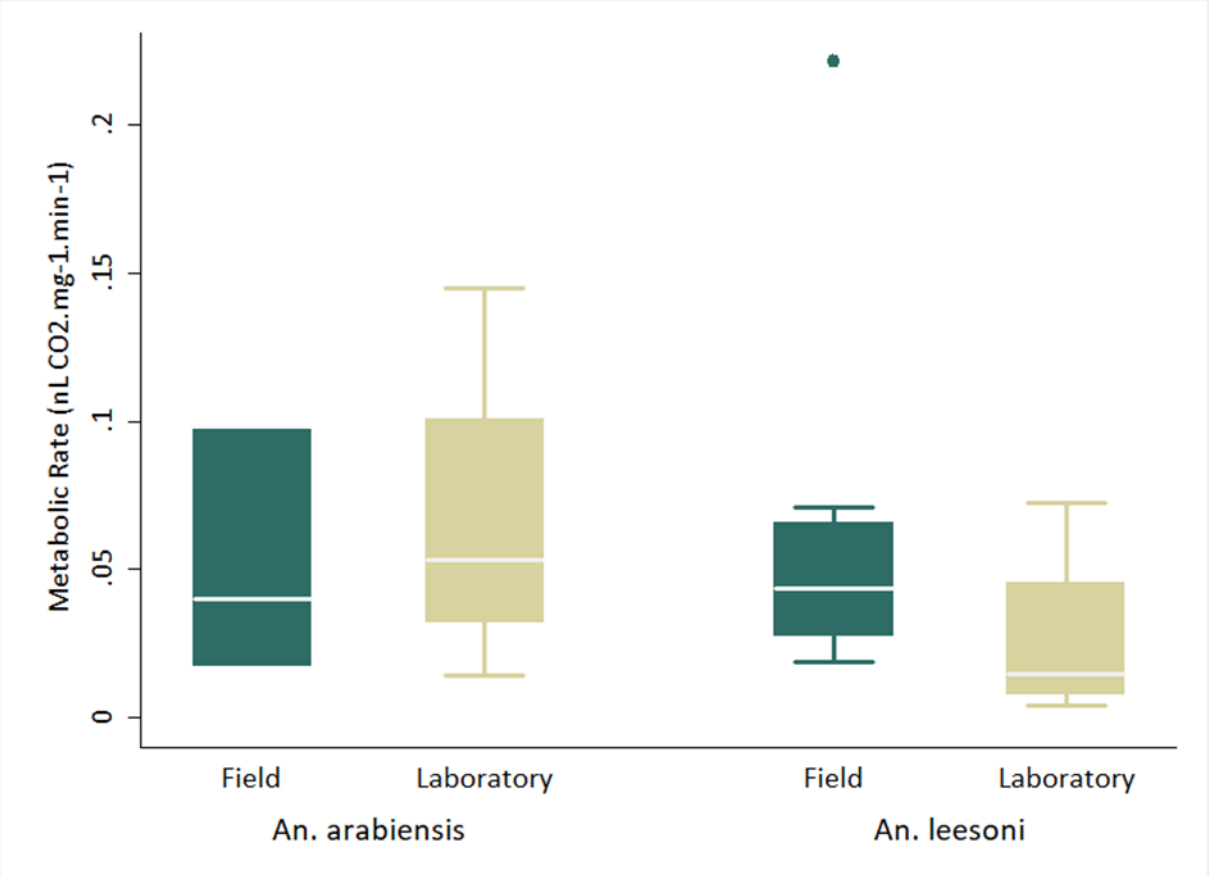

Figure 6.5. Comparison of metabolic rate (nL CO_2.mg^{-1}.min^{-1}) of wild *Anopheles arabiensis* males and *An. leesoni* females measured at different localities. Field = metabolic rate of wild *Anopheles* mosquitoes measured in a field-based laboratory immediately after collection from field sites in Mamfene, KwaZulu-Natal. Laboratory = metabolic rate of wild *Anopheles* specimens measured after transporting them to a laboratory at the University of the Witwatersrand in Johannesburg, Gauteng, after initial collection from field sites at Mamfene. Specimens measured in the field were collected in October 2018 and compared to specimens previously collected in October 2017. The box shows the interquartile range of the metabolic rates with the median values indicated with a white line and outliers indicated with shaped markers.

6.3.3 Mosquito metabolic rate response to photoperiod

The effect of photoperiod on mosquito metabolic rate was not significant following statistical comparisons of the three photoperiod conditions: midsummer (14 h: 10 h), midwinter (10 h: 14 h), and the control (12 h: 12 h) ($\chi 2 = 2.762$, df = 2, $P = 0.251$, n = 502). The sample showed high variation in metabolic rate throughout the experiment with many outlying data points (Fig. 6.6).

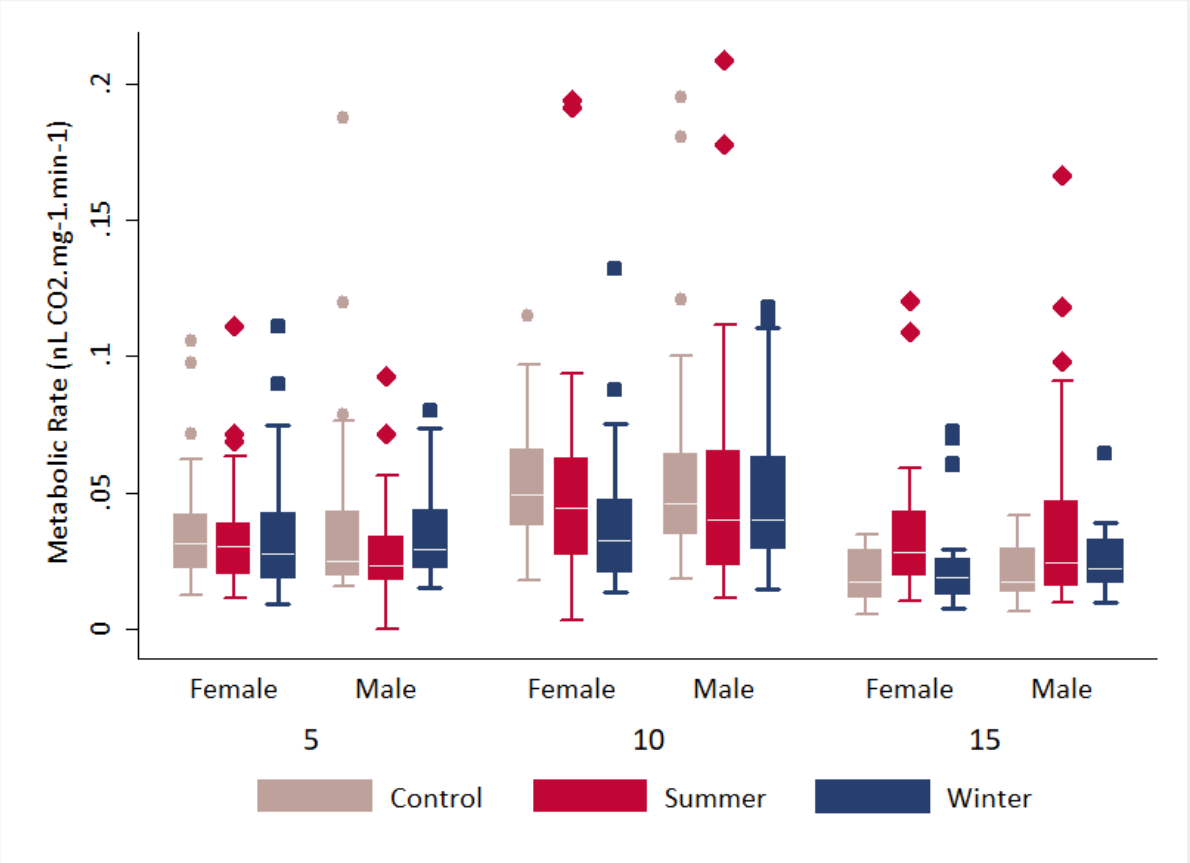

Figure 6.6 Metabolic rate (nL CO_2.mg^{-1}.min^{-1}) of colonised *An. arabiensis* male and female mosquitoes exposed to light-dark cycles mimicking midsummer (14 h light, 10 h dark)(n = 175), midwinter, (10 h light, 14 h dark) (n = 171), and a control (12 h light, 12 h dark) (n = 156) measured at 5, 10, and 15 days post adult emergence. Each box shows the interquartile range of the metabolic rate with the median value indicated with a white line and outliers indicated with shaped markers.

Mosquito age had a significant effect on metabolic rate within most of the photoperiod treatments and within genders as represented in Table 5.1. Metabolic rate of mosquitoes increased significantly from five to ten days post emergence (except for females under the winter photoperiod treatment), and then decreased significantly from ten to fifteen days post emergence (Table 5.1 and Fig. 5.6). However, for both female and male mosquitoes placed under the summer photoperiod treatment, the metabolic rate was not significantly different at the age of five and 15 days old.

Table 6.2 Pair-wise comparisons of the metabolic rate (nL $CO_2.mg^{-1}.min^{-1}$) of different adult age cohorts of colonised *An. arabiensis* mosquitoes. Significance is indicated by Two-sample Wilcoxon rank-sum (Mann-Whitney) test *P*-value < 0.05 at the 95% C.I.

Sex	Photoperiod treatment	Age cohort comparison	z statistic	*P*-value	sample size (*n*)
		5*vs*10days	3.205	0.001	58
	Control	10 *vs* 15 days	5.354	0.000	48
		15 *vs* 5 days	-3.367	0.001	50
		5 *vs* 10 days	1.074	0.283	58
Females	Winter	10 *vs* 15 days	3.501	0.001	58
		15 *vs* 5 days	-2.720	0.007	56
		5 *vs* 10 days	2.410	0.016	60
	Summer	10 *vs* 15 days	2.319	0.020	58
		15 *vs* 5 days	-0.140	0.889	58
		5 *vs* 10 days	3.003	0.003	58
	Control	10 *vs* 15 days	4.831	0.000	48
		15 *vs* 5 days	-2.515	0.012	50
		5 *vs* 10 days	2.011	0.044	55
Males	Winter	10 *vs* 15 days	4.125	0.000	60
		15 *vs* 5 days	-2.383	0.017	55
		5 *vs* 10 days	2.698	0.007	57
	Summer	10 *vs* 15 days	2.062	0.039	59
		15 *vs* 5 days	0.171	0.864	58

6.4 Discussion

Year-round malaria incidence in South Africa indicates continued vector biting and reproductive activity even in colder and drier winter conditions. This suggests that there is no necessity for winter diapause and/or long- distance migration to other breeding sites when year-round breeding is possible in local perennial refugia (see also Huestis *et al*. 2012). These observations are supported by the results from this study that show that the *Anopheles* populations tested do not appear to lower their metabolic rate in winter which leads to the tentative suggestion that diapause is not triggered in the adult mosquito stage as a result of photoperiod changes. In some cases, the metabolic rate of *An. arabiensis* and *An. parensis* increased over the winter period (although not significantly so), departing from predictions based on literature surrounding winter mosquito diapause (Benoit and Denlinger 2007). Should a significant decrease in metabolic rate have been found in the winter mosquito population sample, one could assume that the adult mosquitoes had entered a lowered energy-use state that may allude to diapause. The lack of a metabolic indication of a quiescent state in the samples collected in Mamfene, together with evidence of continued blood-meal foraging (obvious by observation of feeding state of females) and breeding supports the hypothesis that this population does not diapause.

The alternative explanation is that local mosquito populations decline in density and retreat to perennial breeding sites or refugia during the winter months when small temporary breeding sites are not readily available. The use of refugia in this way is typical of *An. arabiensis* in northern Sudan (Dukeen and Omer 1986), the Kilombero Valley in south-eastern Tanzania (Charlwood and Billingsley 2000), and in villages of the Baringo district in Kenya (Mala *et al*. 2011). This also means that local *Anopheles* species – especially within the *An. gambiae* complex and the *An. funestus* group - do not suspend their foraging and reproductive activities during the dry season. *Anopheles arabiensis* have shown significantly higher desiccation resistance facilitated by higher body water content compared to *An. gambiae* in other studies (Gray and Bradley 2005), which may facilitate their perseverance through the dry season with limited to no apparent behavioural changes.

Caveats of using data obtained from wild-caught mosquitoes is variation in age, male physiological status, and female gonotrophic status, all of which affect metabolic rate. The use of laboratory-reared colony material was designed to address these issues by controlling for these variables. Under these conditions, the metabolic rate responses of colonized *An.*

arabiensis to manipulated photoperiod showed no disparities between summer and winter daylight regimens, suggesting that variations in day length were not in this case a primer for changes in metabolic rate in adult *An. arabiensis*. Metabolic rate was significantly affected by mosquito age, which may account for the high levels of variation in the wild samples, along with other variables. Metabolic rate increased significantly at day 10 post emergence, which may suggest that mosquitoes were in an energy-demanding stage of development or maturation. Further experiments with shorter intervals between age cohorts may help tease out these small developmental effects on metabolic rate. The experimental data followed a parabolic trend with age of mosquitoes where metabolic rate was significantly higher at 10 days compared to 5 days, but then significantly lower at 15 days compared to 10 days.

Certainly, one cannot draw the conclusion that photoperiod does not trigger a metabolic response in any of the other mosquito life stages as this was only investigated post adult emergence in this study. Further experiments investigating the metabolic effect of photoperiod exposure from the mosquito egg stage and the possibility of epigenetic affects from the maternal line would be useful. There is marked evidence of a strong parental effect on progeny development time in the highly studied *Drosophila* species *D. simulans* (Giesel 1986) and *D. melanogaster* (Giesel 1988) which implies diapause. The roles of temperature and humidity, both alone and in combination with day length and each other, warrant further investigation as a combination of several factors may produce alternative results.

Because water plays a pivotal role in the aquatic stages of the mosquito lifecycle, rainfall trends often determine mosquito density fluctuations. The 2017 malaria season saw a dramatic increase in sporadic, locally acquired malaria - an estimated 31 000 cases were reported in South Africa, a significant increase from the 9478 cases reported in 2016 (Limpopo Department of Health, 2017; Christian *et al*. 2018). The unusually high incidence was likely attributed to a vector population upsurge associated with high rainfall, increased ambient temperature and humidity, combined with delayed control activities (Limpopo Department of Health, 2017). The ongoing occurrence of outdoor-resting components of the prominent vector species *An. arabiensis* together with the minor vectors *An. merus*, *An. parensis,* and *An. vaneedeni* in KwaZulu-Natal Province primarily explain ongoing residual transmission despite annual control. Outdoor-resting and biting vectors that are less amenable to control by indoor applications of insecticide pose a significant challenge to control. Cross-border human movement between South Africa and high malaria burden countries like

Mozambique and Zimbabwe contributes significantly to the malaria incidence rate (Maharaj *et al*. 2012, Maharaj *et al*. 2013). These factors stress the need to supplement South Africa's provincial indoor residual spraying-based vector control programs with methods that target outdoor-resting mosquitoes. Ongoing breeding during the winter months in reduced numbers of perennial breeding sites presents an important opportunity for enhanced control by winter larviciding. Based on these data, this recommendation has been made and now forms part of South Africa's malaria vector control and elimination strategy for the period 2019 – 2023.

6.5 Conclusions

Based on the year-round surveillance and metabolic rate measurements it is tentatively suggested that South African populations of the malaria vector species *An. arabiensis* and *An. parensis* do not curtail their breeding and foraging activities by reducing their metabolic rates during the colder and drier winter months. Their respective population densities do however decrease considerably during winter leading to reduced malaria transmission and the opportunity for control by winter larviciding of perennial breeding sites.

CHAPTER SEVEN – CONCLUSIONS

The more we understand about the seasonal behaviours and physiological traits of the mosquitoes transmitting malaria in South Africa, the better we can manage their local population density to reduce and eliminate transmission altogether. Residual malaria transmission in the South African context is one of ongoing, low-level, and sporadic incidence despite the efficient implementation of control interventions. Vector surveillance activities, such as those conducted during this project, help unravel the complexities of malarial mosquito biology and behaviour with the aim to identify the entomological drivers of residual transmission with the goal of eliminating disease transmission entirely.

Adult vector surveillance was carried out in malaria-affected regions of Mpumalanga and KwaZulu-Natal provinces, South Africa. The merits of different passive and active collection techniques were assessed at the Mpumalanga field sites only. Passive collection techniques included the use of traditional clay pots and modified plastic buckets as mosquito resting shelters. Clay pots were found to be more productive compared to the modified plastic buckets. However, without a choice, plastic buckets are still useful for entomological surveillance activities in areas of low population density (Dandalo, *et al.* 2017). Drawing conclusions about the productivity of indoor *versus* outdoor resting shelters is difficult as very few specimens were collected indoors. A combination of severe drought conditions and ongoing IRS activities in the sampling area resulted in a very low total catch, and substantially fewer mosquitoes were collected from indoor shelters than outdoor shelters. This also highlights the importance of outdoor resting vector surveillance in low-level residual malaria settings.

Active trapping techniques, specifically human landing catches (HLC) and CO_2 -baited nets, are productive mosquito collection methods in low incidence settings. Human landing catches are particularly useful for collecting human-biting mosquitoes and thereby members of vector populations. These collection methods proved effective at the Mpumalanga sites, but are problematic for long-term routine vector surveillance due to the safety risks involved. A relatively safer proxy for HLC was investigated by Tangena *et al.* (2015) in Laos for use outdoors. This method involves a human-baited double net that is representative of outdoor human biting rates but without exposing the collectors to mosquito-borne infections (Tangena *et al.* 2015). As useful as the active techniques are, the passive outdoor-based resting shelters

are preferred for routine surveillance purposes in South Africa because they are inexpensive, can be installed for long periods, and pose little to no risk to collectors in terms of disease exposure and the accompanying safety concerns of night-time work. The passive mosquito collection techniques assessed in this study performed equally to the active methods, making them a suitable alternative for use in routine surveillance.

Adult *Anopheles* mosquito surveillance at both Mpumalanga and KwaZulu-Natal sites indicated the presence of several *Anopheles* species, including known vectors and non-vectors, collected both indoors and outdoors in varying densities. Species collected over the sampling period included the major vector species *An. arabiensis*, along with the likely secondary vector species *An. merus* and the non-vector species *An. quadriannulatus.* Several *An. funestus* group members were collected which included large numbers of *An. parensis,* and, to a lesser extent, *An. vaneedeni, An. rivulorum,* and *An. leesoni.* It is important to note that ongoing use of DDT for IRS activities in South Africa's malarious areas has resulted in near complete suppression of the highly efficient vector species *An. funestus* (Coetzee *et al.* 2013b). This is supported by the collection of only one *An. funestus* specimen from the Limpopo Province during the 2018/2019 malaria season, being the first surveillance record of this species in South Africa in the past 18 years (Leo Braack, personal communication). In the absence of *An. funestus*, the major vector of concern in South Africa is *An. arabiensis.* The increasing prevalence of insecticide resistance in *An. arabiensis* in South Africa (Hargreaves *et al.* 2000, Hargreaves *et al.* 2003, Brooke *et al.* 2013, Brooke *et al.* 2015) and its highly varied resting and biting behaviour, which is predominantly outdoor-based, makes this vector difficult to control with indoor-based insecticidal control methods alone. Although IRS activities have been instrumental in supressing malaria incidence in South Africa by at least 95% (Brooke 2019), the threat of burgeoning insecticide resistance of vectors necessitates continued IR management strategies and the development of second-generation insecticides.

A significant component of entomological surveillance is determining the parasite infectivity rates of local vector populations. By screening all female *Anopheles* collected during this study, two *An. funestus* group members were identified and later incriminated as malaria transmission vectors. *Anopheles vaneedeni* was implicated in malaria transmission in Mpumalanga province, and *An. parensis* in KwaZulu-Natal province, South Africa (Burke *et al.* 2017, Burke *et al.* 2019b). This was the first record of *Plasmodium* infectivity of *An. parensis* and *An. vaneedeni* in the wild. Both specimens were collected from clay resting

shelters placed outdoors at their respective field sites. These species are considered secondary vectors as their contribution to malaria incidence in South Africa is likely low. It is suspected that *An. merus* is also a secondary vector in South Africa as it has been implicated in transmission in neighbouring southern Mozambique (Cuamba and Mendis 2009), however no wild sporozoite-positive *An. merus* specimens have been confirmed in South Africa to date. Preliminary salinity tolerance investigations of colonized *Anopheles* indicated that only *An. merus* had an appreciable tolerance for high levels of salinity, although they are also able to breed in fresh-water habitats. This adaptive behaviour allows for the range expansion of *An. merus* into areas that contain brine and/or fresh-water breeding habitats (Mbokazi *et al.* 2018), which has important implications for local malaria transmission.

Little is known about the insecticide susceptibility of *An. parensis*, *An. vaneedeni* and *An. merus*, although tentative resistance to DDT was noted in *An. merus* collected from breeding sites in Mpumalanga province (Davies *et al.* 2016). Certain *Anopheles* species found in South Africa have been incriminated as secondary vectors in other countries including *An. rivulorum* (Wilkes *et al.* 1996, Kawada *et al.* 2012), *An. leesoni* (Temu *et al.* 2007), *An. rufipes* (Da Silva *et al.* 2013, Tabue *et al.* 2017) and *An. coustani* (Mwangangi *et al.* 2013). It is, therefore, important to continue routine vector surveillance and parasite screening to detect all species transmitting the disease and to establish their contribution, whether major or minor, to residual malaria in South Africa.

The seasonal trend of malaria transmission in South Africa is certainly driven in part by seasonal fluctuations of local vector population densities. Rainfall and temperature largely affect the breeding site opportunities for *Anopheles* mosquitoes and are thus closely aligned with malaria incidence patterns. Most specimens collected during the surveillance period in Mpumalanga and KwaZulu-Natal provinces were found during the warmer high-rainfall months (November to March) as opposed to the cool and dry winter months (May to August). The assessment of metabolic rate (measured indirectly by CO_2 emission rate) of wild adult mosquitoes across the seasons, accompanied by experiments using colonized mosquitoes under laboratory simulated seasonal photoperiod, suggested no significant seasonal changes in the metabolic rate of adult *Anopheles* (Burke *et al.* 2019a). The effect of photoperiod, in conjunction with temperature and humidity, on the metabolic rate of different life stages of the mosquito would be useful to supplement the findings on the adult stage. Within local South African vector populations, no obvious physiological changes appear to take place to accommodate the seasonal changes, but there is certainly much still to be investigated. It is

important to note that although the local vector population density decreased during the dry winter months, vector species were still collected throughout the dry season owing to permanent breeding sites or refugia. Breeding and blood foraging activities, evident by the continuation of low-level local case incidence, are not interrupted during winter. The continual low-density breeding of local vector populations during winter presents a unique opportunity for targeted vector control interventions, such as winter larviciding. With fewer breeding sites and lower larval densities, winter larviciding may contribute significantly to the already established vector control operations. Based on these data, this recommendation has been made and now forms part of South Africa's malaria vector control and elimination strategy for the period 2019 – 2023. Larval source management has since become a key component of vector control programmes in South Africa's malaria areas as it targets both indoor and outdoor-biting vector populations. These data show that outdoor-resting vectors are significant contributors to ongoing residual malaria transmission in South Africa, thus effective control of their base populations brings us one step closer to total malaria elimination.

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APPENDICES

APPENDIX A

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"Baseline entomological surveillance in Vlakbult and Block A, Mpumalanga Province"

INFORMED CONSENT FORM

Name of Principle Investigator: Prof. Basil Brooke

Name of Organization: WITS Research Institute for Malaria and National Institute for Communicable Diseases, Johannesburg

Name of Sponsor: MRC-SA (Medical Research Council of South Africa)

Collaborators: Malaria Control Programme, Department of Health and Social Services, Mpumalanga Provincial Government, Nelspruit

Information sheet for the head of household in houses where adult mosquitoes are to be collected

Introduction

Malaria is a common illness in this community and in other parts of Mpumalanga and Mozambique. The germ causing malaria is spread from one person to another through mosquito bites. There are different types of mosquitoes in Mpumalanga but not all spread malaria.

An effective method of preventing malaria involves spraying of houses with insecticides. However, some mosquitoes that spread malaria may be resistant to the insecticides used for houses (i.e. the mosquitoes don't die even after been exposed to insecticides). This is a problem because it reduces the protective role of the insecticides. Furthermore, some mosquitoes rest outside and are not killed by the insecticides sprayed inside houses. We therefore need to identify good methods of collecting malaria transmitting mosquitoes inside and outside houses so that we can learn more about them which will enable us to improve malaria control. The main reasons for this research are: (i) to identify the mosquitoes that spread malaria in your community. (ii) to assess whether these mosquitoes die when exposed to the insecticides used for treating your home, (iii) to train people that will continue this research work in order to assist you in reducing malaria infection, iv) to see if we can develop new methods to kill these mosquitoes.

Procedures:

We wish to collect adult mosquitoes resting indoors and outdoors in selected houses. This entails having access to your house. We intend to visit your house during the day at least once a week for at least two years. During each visit, adult mosquitoes will be collected indoors and outdoors from pots and buckets that will be placed inside and outside the house. These mosquitoes will be transported to our research laboratory in Johannesburg where they will processed in order to identify those responsible for spreading malaria, and to test them to see if they still die when exposed to the insecticides used for spraying your homes. The expected duration of the study is 24 months.

If you accept, we would like you to collaborate with us for the full duration of the project. This will simply involve allowing us access to your house to collect adequate mosquito samples for the study.

Discomfort

As indoor resting mosquitoes prefer dark places such as under beds, mattresses, cupboards or toilet, our study may interfere with the privacy of your rooms. If this will make you uncomfortable, you may restrict our team to particular areas within your house.

Right to refuse

Giving us access to your home is entirely voluntary and you are not under any obligation if you do not wish to do so. This will not affect the privilege you derive from any the Government Institution such as Health Care in the community. You could still participate in other malaria related research work in the future.

Benefit

The study is of no direct benefit to you. However, your participation is likely to help us maintain and improve malaria control in your community. The information obtained from this project will be available in the public domain.

Incentive

You will not be provided with any incentive for giving us access to your home.

Confidentiality

The species identities of the mosquitoes collected from your house will be kept confidential. The mosquito samples to be stored will be coded and will not have your name or house number, but will be stored as part of sample collected from the entire community. Our teams collecting in your house will be requested to keep the house content confidential.

Who to contact

This proposal has been reviewed and approved by the Research Ethics Committee of the University of the Witwatersrand, and the Ethics Committee of Mpumalanga Province. The committees' task is to make sure that research collaborators are protected from harm. If you wish to find out more about the project, please contact: Prof. Basil Brooke (tel: 011 386 6483).

CERTIFICATE OF CONSENT

I have been invited to take part in the research project on malaria vectors by allowing Malaria Control Programme personnel and research officers direct access to my house in order to collect adult mosquitoes.

I have been informed that the purpose of this research study is to identify mosquitoes that spread malaria in our community and to test if these mosquitoes still die after exposure to the insecticide used for spraying our houses. This information will be used to maintain and improve malaria control in my community.

I am aware that the procedure will involve mosquito collection (at least once per week) in my rooms for a period of 24 months.

I have been informed that I will derive no personal benefit by participating in the study.

I have been informed that information relating to mosquitoes collected from my house will be kept confidential. The identification number or code will not include my name or house number and that the contents of my house will be treated as confidential.

I have been informed that allowing access to my house is purely voluntary and that I am under no obligation to participate in the study.

I have read the foregoing information/the foregoing information has been read to me.

I have had the opportunity to ask questions, and my questions have been answered to my satisfaction. I consent voluntarily to participate in the study by allowing the research team to have access to my house including my living room. I understand that I have the right to withdraw if I so desire.

Participant's Name _____ Signature of Participant _____

Moderator's Name _____ Signature of moderator _____

Date _____ Place _____

If illiterate,

Signed by the officer/researcher _____

In the presence of an independent literate witness _____ (selected by the participant)

Date _____ Place _____

COMMUNICATION OF STUDY OUTCOME TO PARTICIPANTS AND COMMUNITIES

At the completion of this project, a post survey visit will be made to each community by the research team. While expressing our appreciation for their cooperation during the period of the study, the community will be informed of the outcomes of the study.

APPENDIX B

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OPEN

A new malaria vector mosquito in South Africa

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South Africa aims to eliminate malaria within its borders by 2018. Despite well-coordinated provincial vector control programmes that are based on indoor residual insecticide spraying, low-level residual malaria transmission continues in the low-altitude border regions of the north-eastern sector of the country. In order to identify the underlying causes of residual transmission, an enhanced vector surveillance system has been implemented at selected sites in the Mpumalanga and KwaZulu-Natal (KZN) provinces. The collection periods for the data presented are March 2015 to April 2016 for Mpumalanga and January 2014 to December 2015 for KZN. The mosquito collection methods used included indoor and outdoor traps based on the use of traditional ceramic pots, modified plastic buckets and exit window traps (KZN only). All *Anopheles funestus* species group mosquitoes collected were identified to species and all females were screened for the presence of *Plasmodium falciparum* sporozoites. Two *An. vaneedeni* females, one from each surveillance site, tested positive for *P. falciparum* sporozoites. These are the first records of natural populations of *An. vaneedeni* being infective with *P. falciparum*. As both specimens were collected from outdoor-placed ceramic pots, these data show that *An. vaneedeni* likely contributes to residual malaria transmission in South Africa.

Malaria is a parasitic disease caused by *Plasmodium* protozoa and transmitted by female *Anopheles* mosquitoes (Diptera: Culicidae)[1]. The populations most at risk live in sub-Saharan Africa which accounts for 80% of cases and 90% of total deaths[2].

Malaria transmission in South Africa is limited to the low-altitude northern and north-eastern border regions of the country which span the Limpopo, Mpumalanga and KwaZulu-Natal (KZN) provinces[3]. Historically, only the major malaria vector *Anopheles funestus* was directly implicated in malaria transmission in South Africa[4]. In addition, the malaria vectors *An. arabiensis* and *An. merus* were provisionally implicated based on their occurrence in South Africa's malaria affected regions and because they have been directly implicated in transmission in neighbouring southern Mozambique[5–8]. Recently, several *An. arabiensis* and one *An. merus* specimen collected outdoors during 2014–2016 were also found to be infected with *P. falciparum* sporozoites in KwaZulu-Natal (Dandalo *et al.*, submitted), thus expanding the number of species directly implicated in malaria transmission within South Africa.

Malaria vector control in South Africa's malaria affected provinces is primarily based on indoor spraying of long-lasting residual insecticides[8]. The indoor residual spraying (IRS) method has been the mainstay of malaria vector control in South Africa since the 1940s and has remained effective owing to carefully co-ordinated provincial IRS programmes[9]. Despite this, low-level residual malaria transmission continues and is likely caused by outdoor feeding and resting *Anopheles* vector mosquitoes that are unaffected by indoor applications of insecticide[10]. Maintaining effective control whilst scaling up control methods to address ongoing residual malaria transmission within South Africa are high priority activities because the country has adopted a malaria elimination agenda and aims to eliminate malaria within its borders by 2018[11].

Continuing residual malaria transmission and the burgeoning incidence of insecticide resistance in malaria vector populations within South Africa's borders[4,12] have necessitated an intensification of vector surveillance activities in the affected provinces. The principle objectives of these enhanced surveillance activities are to compare and establish optimal methods of collecting adult *Anopheles* mosquitoes, to establish which *Anopheles* species

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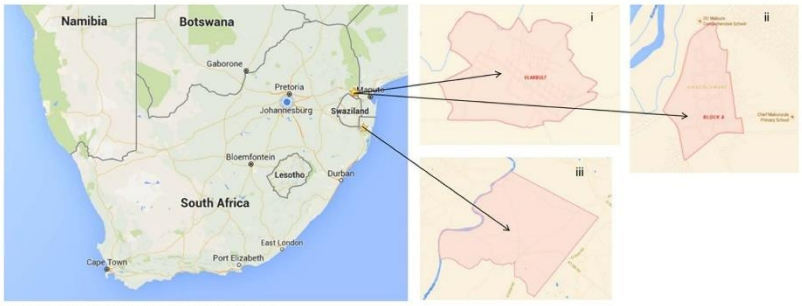

Figure 1. *Anopheles* mosquito surveillance sites at Vlakbult (i) and Block A (ii) (Ehlanzeni District of Mpumalanga) and Mamfene (iii) (KwaZulu-Natal) South Africa. Map source data were obtained from Map data (c) 2016 AfriGIS (Pty) Ltd, Google (https://www.google.co.za/maps/place/South+Africa/).

Province	Site	Species	Total	
			Males	Females
Mpumalanga	Vlakbult	*An. vaneedeni*	11	41
		An. rivulorum	1	3
		An. leesoni	0	2
	Block A	*An. vaneedeni*	0	3
		An. rivulorum	0	16
		An. leesoni	0	0
KwaZulu-Natal	Mamfene	*An. vaneedeni*	25	51
		An. rivulorum	5	13
		An. leesoni	13	53
		An. parensis	34	61

Table 1. Distribution of *Anopheles funestus* group collected by species and gender from the Mpumalanga (Vlakbult and Block A: March 2015–April 2016) and KwaZulu-Natal (Mamfene: January 2014–December 2015) *Anopheles* mosquito surveillance sites, South Africa.

are responsible for ongoing residual malaria transmission, to assess the extent of residual malaria transmission within South Africa and to assess the range and geographical extent of insecticide resistance in incriminated vector populations. Within these broad objectives, the aim of this project was to assess whether *An. vaneedeni*, a member of the *An. funestus* species group[13], contributes to residual malaria transmission in South Africa.

Results

Anopheles vector surveillance for indoor and outdoor-resting mosquitoes was conducted in two villages in Mpumalanga Province (Tonga - Block A and Vlakbult) (S25°42′03″; E31°48′31″ and S25°38′42″; E31°42′01″) for one year (March 2015–April 2016) and in Mamfene, KwaZulu-Natal (KZN) Province (S27°23′50.5″; E032°12′20.1″) for two years (January 2014–December 2015) (Fig. 1).

A total of 255 and 77 *An. funestus* group specimens was collected from the KZN and Mpumalanga sites respectively (Table 1). Of these, 45 of the adult females collected from Block A and Vlakbult (Mpumalanga) and 51 of the adult females collected from Mamfene (KZN) were positively identified as *An. vaneedeni*, first by morphology[10] to *An. funestus* group followed by PCR[14] to species (Table 1). Two of these *An. vaneedeni* specimens, one from each province, tested positive for the presence of *Plasmodium falciparum* sporozoites through two ELISA assays. These data give *P. falciparum* infectivity rates for *An. vaneedeni* of 2.44% and 1.96% for the Mpumalanga and KZN sites respectively.

The *Anopheles* species identification of these two specimens as *An. vaneedeni* was further confirmed by sequencing their internal transcribed spacer 2 (ITS2) region[14]. There was a 99% sequence identity between the ITS2 region of the KZN and Mpumalanga specimens and the published *An. vaneedeni* sequence originating from South Africa (GenBank accession number JN994152.1[15]). The presence of *P. falciparum* sporozoite DNA was confirmed by nested PCR for the KZN specimen[16]. Sequence analysis of the nested PCR product revealed that there was a 99% identity to the published sequence for *P. falciparum* (GenBank accession number KT991235.1[17]).

None of the *An. leesoni*, *An. rivulorum* and *An. parensis* female specimens from either field site (Table 1) showed positive for *P. falciparum* sporozoites based on ELISA analysis.

Figure 2. Ceramic pot (**A**) and modified plastic bucket (**B**) used for adult *Anopheles* mosquito surveillance, Mpumalanga and KwaZulu-Natal Provinces, South Africa.

Discussion

The perennial occurrence of several *An. funestus* species group members–*An. vaneedeni*, *An. rivulorum*, *An. leesoni* and *An. parensis*–at the Mpumalanga and KwaZulu-Natal field sites warrants investigation into their possible contribution to malaria transmission in these regions, especially given that *An. rivulorum* has been implicated in malaria transmission in Tanzania[18] and Kenya[19], and a previous survey has shown that *An. parensis* will readily rest indoors in the Mamfene region[20]. The evident absence of *An. funestus sensu stricto* at these sites can be attributed to ongoing annual IRS based control activities, particularly the use of DDT which has effectively eradicated this species from South Africa. This is because *An. funestus* in South Africa is highly susceptible to DDT[4,8] and has a strong tendency to rest indoors, making this species especially susceptible to IRS programmes that utilize DDT[7,9].

The data summarised here represent the first record of wild-caught *P. falciparum* sporozoite positive *An. vaneedeni* females, directly implicating this species in malaria transmission in South Africa. Although this species is considered to be to be primarily zoophilic[10], it will readily feed on humans outdoors and has previously been experimentally infected with *P. falciparum* under laboratory conditions[21]. The outdoor-resting and feeding traits of this species are reinforced by the fact that most of the *An. vaneedeni* specimens collected in these surveys, including the two that tested sporozoite positive, were found in outdoor-placed ceramic pots (Fig. 2) deployed at randomly selected households at the two sites.

The geographical range of *An. vaneedeni* primarily includes the north-eastern low-altitude regions of South Africa, and likely extends into southern and eastern Zimbabwe and southern Mozambique[22]. However, there are collection records of *An. vaneedeni* in the western highlands of Kenya[23], suggesting that its range may be substantially more extensive.

The collection of sporozoite-positive, outdoor-resting *An. vaneedeni* supports the hypothesis of ongoing outdoor residual malaria transmission in South Africa, as first proposed by De Meillon *et al.* in ref. 21, and tentatively suggests that this species may also be contributing to malaria transmission in other malaria-endemic countries in which it occurs. This information highlights the need to intensify malaria vector control in South Africa by including methods designed to target outdoor feeding vector populations without compromising the efficacy of the IRS programme.

Materials and Methods

Ethical statement. Informed consent was obtained from all household owners involved in this study. Ethical clearance for the collection of mosquito specimens was obtained from the University of the Witwatersrand (M141023 & W-CJ-150520-2) and the KwaZulu-Natal Department of Health (HRKM337/14).

Mosquito collections. Adult *Anopheles* mosquitoes were collected using traditional ceramic pots and modified plastic buckets (Fig. 2). These were placed both inside and outside selected households in Vlakbult and Block A in Mpumalanga (March 2015–April 2016), and only outside at households in Mamfene, KZN (January 2014–December 2015). Traps were not deployed indoors at Mamfene because homeowners consent did not include this provision and because exit window traps were instead used to collect indoor resting mosquitoes at this site (Fig. 3). The traps were cleared at sunrise weekly by the malaria vector surveillance teams based near the collection sites in each province.

***Anopheles* species identification and vector incrimination.** All *Anopheles* specimens collected were preserved on silica and initially identified by external morphology using dichotomous keys[10]. Those identified as belonging to the *An. funestus* group were subsequently identified to species level by PCR[14]. All females were screened for the presence of *Plasmodium falciparum* sporozoites by ELISA[24]. *Plasmodium falciparum* infectivity, where indicated by ELISA, was confirmed with a nested *Plasmodium* PCR[16].

Figure 3. Window exit trap used for adult *Anopheles* mosquito surveillance, KwaZulu-Natal Province, South Africa.

Sequence analysis of mosquito ITS2 and *Plasmodium falciparum* ssRNA. In order to confirm *Anopheles* species identity, the internal transcribed spacer region 2 (ITS2) region of the rDNA of each *An. vaneedeni* sporozoite positive sample was amplified using the following primers: ITS2A: 5′-TGTGAACTGCAGGACA-CAT-3′; and ITS2B: 5′-TATGCTTAAATTCAGGGGGT-3′. PCR conditions were the same as those used in the *An. funestus* species-specific PCR[14]. In order to confirm *Plasmodium* species identity, *Plasmodium* ssRNA from the KwaZulu-Natal *An. vaneedeni* sporozoite positive sample was amplified using the following primers: rPLU5: 5′-CCTGTTGTTGCCTTAAACTTC-3′; rPLU6: 5′-TTAAAATTGTTGCAGTTAAAACG-3 for the first amplification step, and rFAL1: 5′-TTAAACTGGTTTGGGAAAACCAAATATATT-3′; and rFAL2: 5′-ACACAATGAACTCAATCATGACTACCCGTC-3′ for the second amplification step and sequencing. PCR conditions were the same as those previously described[16]. All amplicons were electrophoresed on 2.5% agarose gels stained with ethidium bromide and product sizes were confirmed using a molecular weight marker (Thermo Scientific O'GeneRuler 100 bp DNA Ladder; 0.1 μg/μl concentration, supplied with 1Ml 6x Orange DNA Loading Dye). ITS2 PCR products were purified and sequenced by Macrogen. The sequences were manually edited by BioEdit version 7.2.5[25]. Subsequently, the sequences were aligned with sequences stored in GenBank using nucleotide BLAST (BLASTn) (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

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Author Contributions

M.C., L.K., B.B., F.M., S.N. and G.M. designed the mosquito surveillance system and associated experiments. A.B., L.D., G.M., L.K., B.B. and F.M. conducted the field work. A.B. and L.D. conducted the laboratory work and initial data analysis. Y.D. co-ordinated the sequence analyses. B.B., L.K. and G.M. finalised the data analysis. A.B., L.D., Y.D. and B.B. wrote the initial drafts of the manuscript. B.B. produced the final version of the manuscript. All authors read and approved the final manuscript.

Additional Information

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APPENDIX C

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Malaria Journal

RESEARCH Open Access

Anopheles parensis contributes to residual malaria transmission in South Africa

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Abstract

Background: Understanding the contribution of outdoor-resting *Anopheles* mosquitoes to residual malaria transmission is important in terms of scaling up vector control towards malaria elimination in South Africa. The aim of this project was to assess the potential role of *Anopheles parensis* and other *Anopheles* species in residual malaria transmission, using sentinel surveillance sites in the uMkhanyakude District of northern KwaZulu-Natal Province.

Methods: Monthly vector surveillance was conducted at the sentinel sites from January 2017 to May 2018. Outdoor-placed clay pot resting traps were used to collect male and female adult *Anopheles* mosquitoes. All *Anopheles gambiae* complex and *Anopheles funestus* group specimens collected were identified to species and all females were screened for *Plasmodium falciparum* circumsporozoite protein (CSP) by enzyme-linked immunosorbent assay (ELISA). Samples showing infectivity for *P. falciparum* were further verified by a nested PCR and subsequent DNA sequence analysis.

Results: From a sample of 491 anophelines, *Anopheles arabiensis* ($n = 228$) and *An. parensis* ($n = 194$) were the most abundant. Other species collected included *Anopheles merus* ($n = 11$), *Anopheles quadriannulatus* ($n = 10$), *Anopheles leesoni* ($n = 29$), *Anopheles rivulorum* ($n = 18$), and *Anopheles vaneedeni* ($n = 1$). Of the 317 female specimens screened for *P. falciparum* CSP, one *Anopheles arabiensis* and one *An. parensis* showed positive by ELISA and *Plasmodium* nested PCR. For the *An. parensis* specimen, confirmation of its species identity was based on sequence analysis of the ITS2 region, and the presence of *P. falciparum* DNA was further confirmed by sequence analysis.

Conclusions: *Anopheles parensis* is a potential vector of malaria in South Africa although its contribution to transmission is likely to be minimal at best owing to its strong zoophilic tendency. By contrast, *An. arabiensis* is a major vector that is primarily responsible for the bulk of residual malaria transmission in South Africa. As all recently collected sporozoite-positive *Anopheles* mosquitoes were found in outdoor-placed resting traps, it is necessary to introduce interventions that can be used to control outdoor-resting vector populations while maintaining the efficacy of South Africa's indoor house spraying operations.

Keywords: *Anopheles parensis*, Secondary vector, *Plasmodium falciparum*, Residual malaria, Incrimination

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Background

Malaria in South Africa is endemic in the low altitude northeastern border regions of KwaZulu-Natal, Mpumalanga and Limpopo provinces, and transmission follows a seasonal trend with peaks during the rainy season of November to April. Almost all infections are *Plasmodium falciparum* [1]. Malaria vector control operations in the affected provinces primarily utilize insecticide-based indoor residual spraying (IRS) with supplementary larviciding in select districts where incidence is highest. These interventions have reduced overall incidence to a point where elimination is a feasible prospect, leading to the development of an elimination strategy [2]. There was however an unexpected and substantial increase in cases in 2017 throughout the southern African region [3]. This increase coupled with ongoing residual transmission, despite control operations in South Africa, has necessitated intensified vector surveillance activities designed to better understand the entomological drivers of transmission [4].

The primary vectors of malaria in South Africa are *Anopheles funestus* and *Anopheles arabiensis* [5]. The former species is highly anthropophilic and endophilic, making it especially susceptible to control by IRS. As a result of South Africa's IRS operations, the incidence of *An. funestus* has been reduced to a point where it is almost entirely undetectable using a range of surveillance methods [6]. *Anopheles arabiensis* on the other hand shows variable feeding and resting habits, and will readily rest outdoors making it less amenable to control by IRS. This species has recently been directly incriminated in malaria transmission in South Africa [7] and is considered to be the primary contributor to residual incidence. Added to this list is *Anopheles merus*, which has been directly implicated in transmission in southern Mozambique [8] and which occurs in South Africa in all malarious provinces [9, 10]. *Anopheles vaneedeni*, an outdoor resting member of the *An. funestus* group [11], has recently been incriminated as a secondary vector of malaria in South Africa and a likely contributor to residual transmission [12].

Members of the *An. funestus* group that have been incriminated in malaria transmission in other African countries are *Anopheles rivulorum, Anopheles leesoni* and *Anopheles parensis* (tentative) in Tanzania [13–15], *Anopheles rivulorum* in Kenya [16], and *Anopheles longipalpis* in Kenya [17]. *Anopheles parensis* periodically appears as a potential vector. This species is predominantly zoophilic and rarely takes blood from humans even though some populations have shown a strong inclination to rest indoors [18, 19]. A single positive *An. parensis* (1 out of 4 specimens sampled) using nested PCR was detected in Bagamoya, Tanzania [14]. Samples of *An. parensis* from Uganda gave a *P. falciparum* infection rate of 4.2% (n = 94) using a Taqman assay but this was not confirmed by nested PCR, suggesting the detection of false positives [20]. Tentative evidence of malaria infectivity by this species was obtained from northern KwaZulu-Natal Province in South Africa based on a sample of 149 specimens of which 20 (13.4%) showed positive using the standard ELISA assay [19] for *P. falciparum* circumsporozoite protein. Follow-up PCR analysis however did not reveal any positives and it was subsequently shown that all ELISA positive specimens had taken blood from non-human sources [19]. The status of *An. parensis* as a malaria vector, therefore, remains to be confirmed.

Understanding the contribution of outdoor-resting anophelines to residual malaria transmission is important in terms of scaling up vector control toward malaria elimination in South Africa. The aim of this project was therefore to assess the potential role of *An. parensis* and other *Anopheles* species in residual malaria transmission using sentinel sites in the uMkhanyakude District of northern KwaZulu-Natal Province, which currently experiences very low malaria incidence and is the closest of all of South Africa's malaria-endemic districts to achieving elimination status.

Methods

Entomological surveillance

Monthly entomological surveillance was conducted for just over 1 year—January 2017 to May 2018—at sentinel sites in the rural village of Mamfene, in the uMkhanyakude District of KwaZulu-Natal Province, South Africa (Fig. 1). Traditional clay pots (Fig. 2) were deployed outside households (with the home-owners' consent) to serve as resting traps from which live *Anopheles* mosquitoes were collected in the cool early hours of each morning (between 06:00 and 08:00). Five households were recruited for this purpose and two pots were deployed in each (n = 10 pots). All pots were cleared of mosquitoes for 3 days each month during the surveillance period, except for June 2017 and February 2018. There were no collections during those two months owing to logistical constraints.

Molecular identification

All specimens were identified by external morphology using dichotomous keys [21]. Those identified as *Anopheles gambiae* complex or *An. funestus* group were dry-preserved on silica gel and transported to the Vector Control Reference Laboratory of the National Institute for Communicable Diseases in Johannesburg for further processing. Each specimen was identified to species using the appropriate polymerase chain reaction (PCR) protocol

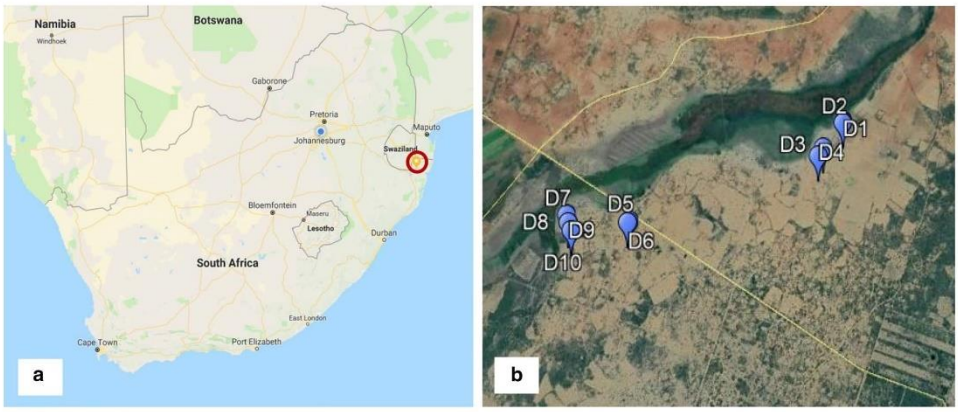

Fig. 1 **a** Location of the field site at Mamfene (yellow pin circled in red), KwaZulu-Natal Province, South Africa. Map data © 2018 AfriGIS (Pty) Ltd, Google. **b** Spatial distribution of 10 clay pots (blue pins) deployed as outdoor-placed resting traps at the field site in KwaZulu-Natal Province. Image © 2018 DigitalGlobe, Google Earth © Google 2018

Fig. 2 An example of the locally-sourced clay pots that were used as outdoor-placed resting traps for *Anopheles* mosquito collections in KwaZulu-Natal Province, South Africa

for either *An. gambiae* complex [22] or *An. funestus* group [23, 24].

Plasmodium infectivity testing

Every female anopheline mosquito was screened for the presence of *P. falciparum* circumsporozoite protein (CSP) by means of an enzyme-linked immunosorbent assay (ELISA) [25, 26]. The ELISA homogenates of all positive specimens were then boiled at 100 °C for 10 min followed by a repeat ELISA to confirm or refute the presence of CSP [27]. Those that remained ELISA-positive were verified by a nested *Plasmodium* PCR assay [28]. The internal transcribed spacer 2 (ITS2) region of an anopheline female showing positive for a *P. falciparum* infection was amplified by ITS2 PCR assay [23] to be used for DNA sequence analysis to verify the species identification.

DNA sequence analysis

Sequencing analysis of the ITS2 and the nested *Plasmodium* PCR products was used to confirm the anopheline species identity and infection with *P. falciparum*. Purification and sequencing of the PCR products was performed by Macrogen. Subsequently, the chromatograms of the sequences were manually edited by BioEdit version 7.2.5 [39]. The Emboss Needle pairwise sequence alignment tool (https://www.ebi.ac.uk/Tools/psa/emboss_needle/nucleotide.html) was used to compare the analysed *An. parensis* ITS2 sequence with the established *An. parensis* ITS2 reference sequences (GenBank accession number JN994144.1) [29]. The unknown *Plasmodium* sequence was compared with the reference *P. falciparum* Pf8 18S ribosomal RNA gene, partial sequence (GenBank accession number: KC428742.1) [30].

Results

Entomological surveillance

Adult *Anopheles* mosquitoes were collected from outdoor-resting traps deployed around households in Mamfene, northern KwaZulu-Natal Province (Figs. 1 and 2), during the period January 2017 to May 2018. A total of 491 male and female specimens were identified as either *An. gambiae* complex or *An. funestus* group using

morphological features [21]. These were subsequently identified to species using the standard PCR assays for each group [22–24]. *Anopheles arabiensis,* a member of the *An. gambiae* complex, was the most abundant ($n=228$). Other members of the *An. gambiae* complex included *An. merus* ($n=11$) and *An. quadriannulatus* ($n=10$) (Fig. 3). Within the *An. funestus* group, *An. parensis* was most abundant (n = 194) followed by *An. leesoni* ($n=29$), *An. rivulorum* ($n=18$) and *An. vaneedeni* ($n=1$) (Fig. 3). There was an overall preponderance of females across all species collected ($F=240.58$, $df=1$, $P<0.004$); 163 females and 86 males of the *An. gambiae* complex, and 154 females and 88 males of the *An. funestus* group (Fig. 3). The monthly and seasonal distribution of collected anophelines showed a significant increase in mosquito numbers in March 2017 (Fig. 4.). This increase in relative mosquito population density is likely attributable to high rainfall experienced in Mamfene from February to March 2017.

Vector incrimination

All female mosquitoes were screened for *P. falciparum* CSP by a two-step ELISA [25–27] and were subsequently validated by nested PCR and DNA sequence analysis where necessary. The majority of positive samples from the first ELISA assay did not appear positive on the second that included a boiling step. Of the 163 female *An. gambiae* complex specimens screened for CSP, one *An. arabiensis* (collected in October 2017) showed positive by ELISA and *Plasmodium* nested PCR [28]. As this *An. arabiensis* population has been implicated in malaria transmission before [7], no further DNA sequence analysis was considered necessary. Of the 154 female *An. funestus* group specimens screened, one female identified as *An. parensis* (also collected in October 2017) showed positive for *P. falciparum* CSP by ELISA and nested *Plasmodium* PCR. These data indicate a *P. falciparum* infectivity rate of 0.67% (n = 149) for *An. arabiensis* and 0.83% (n = 120) for *An. parensis* at Mamfene.

Validation of *Anopheles* species identity and the presence of a *Plasmodium* infection

The species identity of the infective *An. parensis* female was confirmed after standard PCR [23] ITS2 sequences showed 99% homology between this specimen and that for a reference sequence of this species in GenBank (accession number: JN994144.1) [25]. The *P. falciparum* infection in this *An. parensis* specimen was confirmed by 95% sequence homology of the nested *Plasmodium* PCR product with the *P. falciparum* isolate Pf8 18S ribosomal RNA gene, partial sequence (GenBank accession number: KC428742.1) [30].

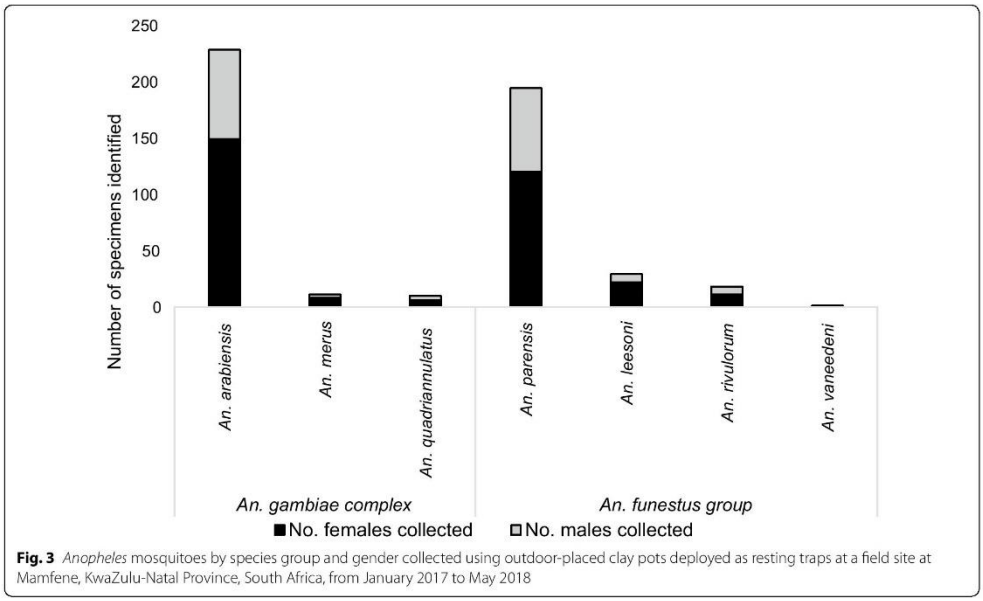

Fig. 3 *Anopheles* mosquitoes by species group and gender collected using outdoor-placed clay pots deployed as resting traps at a field site at Mamfene, KwaZulu-Natal Province, South Africa, from January 2017 to May 2018

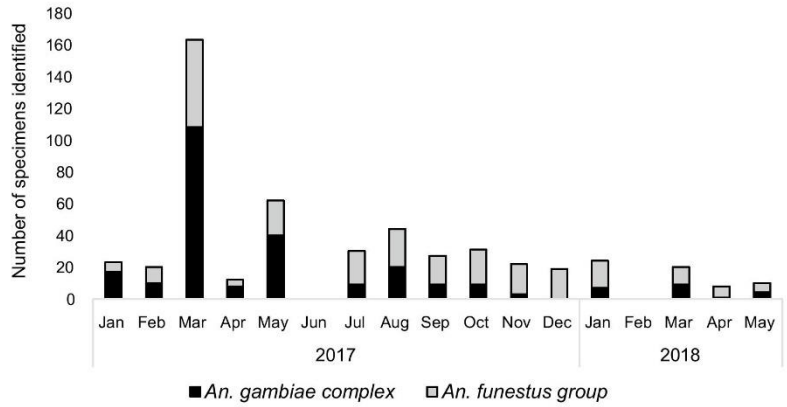

Fig. 4 Monthly sampling of male and female *Anopheles* by species group/complex in Mamfene, KwaZulu-Natal Province, South Africa, from January 2017 to May 2018. Mosquitoes were collected over a 3-day period each month. No mosquitoes were collected for June 2017 and February 2018 due to logistical constraints

Discussion

The morphological and molecular taxonomy of the *An. funestus* group is comparatively complex [11], making identification of member species potentially problematic. The need for morphological identification prior to molecular analysis has been highlighted [31], and is especially critical in terms of follow-on vector incrimination. This is because *Anopheles* species misidentification can easily occur when entirely reliant on nested PCR assays, and sequence information can be misleading in terms of the critical thresholds required for conspecific homology between unknown samples and banked sequences linked to voucher specimens. In addition to the complexities of *Anopheles* species identification, establishing *Plasmodium* infectivity (as distinct from infection) also requires careful use of methodologies and data interpretation. It is for this reason that the two-step ELISA assays for CSP detection were conducted prior to molecular verification for the *An. parensis* samples. Given that *An. parensis* has not been unambiguously incriminated in malaria transmission before, it was considered necessary to ensure that all morphological, PCR, ELISA and sequence data were sufficiently aligned to confirm *Plasmodium* infectivity, and that all data were either double or triple-checked for quality assurance. A similar approach was recently conducted for the incrimination of *An. vaneedeni* (also a member of the *An. funestus* group) as a secondary malaria vector in South Africa [12].

It should be noted that the single ELISA technique used to detect *Plasmodium* sporozoites can lack sensitivity and specificity. It is however more likely to give a false positive than a false negative result, especially in zoophilic species such as *An. parensis* [19, 27]. The false positive ELISA result that can occur in zoophilic *Anopheles* species is due to a cross-reacting heat-unstable antigen [27]. Heating the ELISA lysate, as was done in the experiments described here, reduces the possibility of obtaining false positives. Additionally, the use of nested PCR for the detection of *Plasmodium* sporozoites is a highly sensitive method [28] that further eliminates the chance of false positives. Although Taqman assays [32, 33] are also effective for detecting *Plasmodium* in anophelines, they can be prohibitively expensive. Owing to South Africa's comparatively low malaria incidence and the general rarity of *Plasmodium* infective mosquitoes, the two-step ELISA method followed by nested PCR is the most cost-effective and ideal method for detecting and confirming *Plasmodium* infectivity.

It is reasonable to infer that the single *An. parensis* female that presented with *P. falciparum* CSP was infective for malaria. This at least shows that this species is a potential malaria vector. There are however other considerations that need to be taken into account in terms of its contribution to malaria transmission. Available data show that *An. parensis* seldom takes blood from humans, being more inclined to feed on livestock animals and rest outdoors [34, 35], as has previously been demonstrated for the northern KwaZulu-Natal population [19]. This reduces its potential contribution to malaria transmission substantially, but not necessarily to zero. This is reinforced by earlier records that describe a high malaria incidence setting on the Kenyan coast in which *An.*

parensis was abundant but no sporozoite positive specimens were found (based on salivary gland dissections) despite the sympatric *An. funestus* population showing a sporozoite positive index of 7% [35]. *Anopheles parensis* has nevertheless been recorded as an exophilic feeder on humans [35] with a comparatively high human blood index in some ecosystems [34, 36], and can be found resting both indoors and outdoors [37].

Anopheles arabiensis is a major malaria vector throughout its distribution including northern KwaZulu-Natal [7, 38]. It is worth noting that both the infective *An. arabiensis* and *An. parensis* specimens were collected in October 2017. Although October is generally a low-incidence month in South Africa, 2017 was an unusual year in that unexpectedly high numbers of cases were recorded from May onwards (unpublished National Department of Health statistics). Incidence in KwaZulu-Natal was nevertheless low compared to other endemic provinces which precludes associating these sporozoite-positive specimens with any case or cluster of cases. Also important to note is that both of these specimens were caught resting outdoors, highlighting the importance of addressing residual transmission in South Africa by targeting outdoor-resting vectors in addition to those targeted indoors by the provincial IRS programmes. Intensive provincial larval source management programmes, including winter larviciding, and community outreach programmes designed to educate on personal protection measures and treatment-seeking, are currently under development to address this issue.

Conclusions

It is concluded that *An. parensis* is a potential vector of malaria but that its contribution to transmission in South Africa's endemic provinces is likely to be minimal at best owing to its strong zoophilic tendency [19]. By contrast, *An. arabiensis* is a major vector throughout its distribution and is primarily responsible for the bulk of residual malaria transmission in South Africa. As all recently collected sporozoite-positive *Anopheles* mosquitoes were found in outdoor-placed resting traps in northern KwaZulu-Natal Province [7, 12], it is necessary to introduce additional interventions, intensive larval source management in particular, that can be used to control outdoor-resting vector populations while maintaining the efficacy of South Africa's IRS operations for the ongoing control of indoor-resting vectors.

Abbreviations

CSP: circumsporozoite protein; DNA: deoxyribonucleic acid; ELISA: enzyme-linked immunosorbent assay; IRS: indoor residual spraying; ITS2: internal transcribed spacer 2; PCR: polymerase chain reaction; RNA: ribonucleic acid.

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Authors' contributions

AB collected and analysed the data, conducted the laboratory analysis of material, and assisted with writing the manuscript. YD-M interpreted the DNA sequence analysis results and assisted with writing the manuscript. BB, FD, MC, BQ and LK conceived the study, assisted with data interpretation and the drafting of the manuscript. BB interpreted the data and produced the final version of the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request. All data presented in this study is stored in the malaria vector database of the Vector Control Reference Laboratory, National Institute for Communicable Diseases, and is available on request.

Ethics approval and consent to participate

An ethics approval waiver was obtained from the animal ethics screening committee of the University of the Witwatersrand, Johannesburg, South Africa. Reference: A Burke Waiver (2) 24-02-2017-O. Informed consent was obtained from all home-owners affected by the surveillance portion of this study.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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APPENDIX D

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Metabolic rate does not vary with seasonal change in *Anopheles arabiensis* adults in South Africa

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ABSTRACT

An important component of South Africa's malaria elimination agenda is identifying the entomological drivers of residual transmission, especially those that present opportunities for enhanced vector control. Seasonal mosquito density correlates directly with malaria transmission in South Africa. Transmission is highest during the warm rainy season and lowest but not entirely absent during the cooler dry season. The factors that sustain dry-season mosquito survival remain unknown. The aim of this project was therefore to investigate seasonal change in metabolic rate to determine the presence or absence of winter dormancy in malaria vector mosquitoes. Metabolic rate, determined by CO_2 production during closed-system respirometry, was measured from wild anophelines collected from KwaZulu-Natal Province, South Africa. Monthly sampling spanned all four seasons (summer, autumn, winter, and spring) in 2017. *Anopheles arabiensis* and *An. parensis* specimens formed the majority of the total 437 identified specimens (n = 216 and n = 162, respectively). Metabolic rate data from wild-caught mosquitoes showed no significant seasonal disparities for *An. arabiensis* and *An. parensis* males and females. Further laboratory experiments assessed the effect of manipulated photoperiod, representing seasonal day-length changes, on the metabolic rate of colonized *An. arabiensis* mosquitoes. Simulations of midwinter (10 h:14 h light dark) and midsummer (14 h:10 h) day-length showed no significant effect on the metabolic rate of these mosquitoes. Age (in days) had a significant effect on the metabolic rate of both male and female colonized adult *An. arabiensis* mosquitoes which may be linked to developmental factors during maturation of adults. These data suggest that the South African populations of the malaria vector species *An. arabiensis* and *An. parensis* do not curtail their breeding and foraging activities during the colder and drier winter months. Overwintering by diapause does not appear to be triggered in the adult mosquito stage in *An. arabiensis*. However, their respective population densities do decrease considerably during winter leading to reduced malaria transmission and the opportunity for control by winter larviciding of known breeding sites.

1. Introduction

Malaria is a major public health risk in the Southern African region (World Malaria Report, 2018). South Africa's malaria-endemic areas include the north-eastern border regions of Limpopo, the Lowveld regions of Mpumalanga and the northernmost regions of KwaZulu-Natal all of which experience seasonal transmission. Most cases are caused by infection with *Plasmodium falciparum* (Maharaj et al., 2013) which is primarily vectored by *Anopheles arabiensis* and *An. funestus* (Maharaj et al., 2013; Dandalo et al., 2017). Use of the insecticide-based indoor residual spraying control method has reduced the indoor-resting and anthropophilic *An. funestus* population to undetectable levels in South Africa (Coetzee et al., 2013). The exophilic feeding and resting habits of *An. arabiensis*, however, present a challenge for indoor-focused control. This leads to persistent residual malaria transmission that can be attributed to *An. arabiensis* alongside other secondary vector species including *An. merus* (Cuamba and Mendis, 2009), *An. vaneedeni* (Burke et al., 2017) and *An. parensis* (Burke et al., 2019). Residual transmission presents a significant threat to South Africa's malaria elimination agenda.

Seasonal mosquito density is directly correlated to malaria transmission in South Africa (Christian et al., 2017; Dandalo et al., 2018). Variations in rainfall, temperature and humidity by season play important roles in determining mosquito vector population densities.

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Anopheles mosquito density peaks during the high rainfall period from November to February when non-perennial surface water is available for breeding sites, and declines to a low level during the dry period from June to August in South Africa. This is especially true for *An. arabiensis* (Dandalo et al., 2018). Although density declines to a low level, vector mosquitoes do not disappear entirely during the dry season, and malaria transmission still occurs albeit at substantially lower levels (Munhenga et al., 2014; Dandalo et al., 2017).

African anophelines generally live a relatively short lifespan (Maharaj, 2003; Olayemi and Ande, 2009; Oliver and Brooke, 2014) and the aquatic stages (eggs, larvae, and pupae) are highly susceptible to desiccation (Koenraadt et al., 2003). These characteristics should theoretically hamper long-term survival in dry and desolate conditions. There is currently little information concerning the mechanisms that drive dry-season persistence of anophelines. The major hypotheses for mosquito dry-season survival include: (i) retreating to local perennial breeding sites or refugia, (ii) migrating long distances from permanent breeding sites to seasonally arid areas at the onset of rainfall, (iii) reducing metabolic expenditure to increase longevity by winter dormancy (diapause) or summer dormancy (aestivation) in sheltered dwellings (Omer and Cloudsley-Thompson, 1968, 1970; Dukeen and Omer, 1986; Obala, 1995; Charlwood et al., 2000; Donnelly et al., 2002; Lehmann et al., 2010; Munhenga et al., 2014; Huestis and Lehmann, 2014). Understanding the seasonal biology of vulnerable dry-season vector populations and identifying their main survival tools presents valuable opportunities for targeted control.

Lowered resting metabolic rate reduces nutritional demands and the associated energy costs of resource foraging, and potential water loss associated with respiration, excretion, and diffusion across the cuticle (Gray and Bradley, 2005; Benoit and Denlinger, 2007). This overwintering survival strategy is well documented in adult female *Culex pipiens* (Benoit and Denlinger, 2007), but there is limited evidence for this in African anophelines. The aim of this project was to investigate the seasonal change in metabolic rate to determine the presence or absence of winter dormancy in South African malaria vector mosquitoes. Seasonal metabolic rate variation, and consequent adaptations to overall energy expenditure, may be advantageous to mosquitoes during unfavourable conditions; however, the evidence of continued breeding in previous studies (Munhenga et al., 2014; Dandalo et al., 2017) during the winter season leads us to expect that these populations do not diapause. Disparities in resting metabolic rate between seasons were investigated (dry winter and wet summer) in *Anopheles* vector populations in order to assess whether this physiological trait contributes significantly to dry-season mosquito persistence in South Africa. It was hypothesized that no such seasonal disparity in metabolic rate would occur in South African malaria vector populations.

2. Materials and methods

2.1. Collection of wild adult mosquitoes for metabolic rate analysis

Field collections of wild mosquitoes took place in the rural village of Mamfene, which forms part of the uMkhanyakude district of KwaZulu-Natal province, South Africa. Specifics of this field site are described in Dandalo et al. (2017). Collections were conducted monthly from January to December 2017 (excluding June 2017 due to logistical constraints). All available live anopheline adult mosquitoes were aspirated from ten clay resting pots positioned outside consenting households in Mamfene. Two experienced entomologists checked and cleared every trap within two hours from sunrise, the period when mosquitoes are inactive, for two days every month. Collected specimens were identified to species complex level based on morphological features using dichotomous keys (Gillies and Coetzee, 1987) and only *An. gambiae* complex and *An. funestus* group specimens were used for the metabolic measurements. The live specimens were grouped by sex and species complex/group and then placed into polystyrene cups with a mesh gauze covering the opening of each cup. Mosquitoes had access to cotton pads saturated with 10% sucrose. Specimens were packaged and then transported by car to the Wits Insectary and Quarantine facility based at the University of the Witwatersrand in Johannesburg within one to two days after initial collection from the clay pots. Sugar pads were refreshed where necessary upon arrival at the insectary and mosquitoes were left in their polystyrene cups in a room with natural light overnight. Metabolic rate measurements for all specimens collected were taken the following day. Rainfall and temperature readings for the period under review were captured by a HOBO® RX3000 (Onset Computer Corporation, Bourne, MA), data logging weather station kit positioned in Mamfene.

2.2. Respirometry of adult mosquitoes

The morphological identification, sex, and feeding status (females only) of each mosquito was determined before measurement took place. The metabolic rate of adult mosquitoes was determined by measuring CO_2 emission rates by closed-system respirometry using similar techniques described by Woods and Singer (2001), Kambule et al. (2011), and Kaiser et al. (2014). It was possible to measure each mosquito individually because the CO_2 production of a single mosquito is sufficiently greater than the baseline noise to achieve an accurate reading. Measurements took place in a temperature-controlled laboratory at room temperature (25 ± 2 °C) during daylight hours between 08:00 and 18:00.

Carbon dioxide production was measured using an infrared CO_2 analyser (LI-7000, LI-COR, Lincoln, NE, USA) based on the technique described by Woods and Singer (2001), Kambule et al. (2011) and Kaiser et al. (2014). Ambient air scrubbed of CO_2 and water was drawn through the CO_2 analyser at a flow rate of 100 mL.min^{-1}. Respirometry chambers were made of 30 mL glass syringes (Becton Dickson, Franklin Lakes, NJ, USA) each fitted with a three-way stopcock. Three chambers were used for each measurement run; the first served as a control to monitor CO_2 leakage, and the second and third chambers housed a single mosquito sample in each. A live mosquito was aspirated into the barrel of the glass syringe with the plunger removed while cotton wool was held over the open rear end of the barrel to prevent the mosquito escaping the barrel. The plunger was then quickly inserted into the barrel while removing the cotton wool to ensure the mosquito did not escape. The chambers were then flushed with humid CO_2-free air for five minutes to remove excess CO_2 accumulated from the mosquito handling process. Following flushing, the plunger was depressed to the 20 mL mark and purged air was flushed through the needle. The stopcock between the syringe and the needle was closed to seal the chamber. After 25 min, a 5 mL air bolus was injected into the CO_2-free air stream from each chamber in turn. Injections were performed in sequence, allowing enough time for the analyser to return to baseline CO_2 levels between each sample. Two CO_2 measurements were taken per syringe (six measurements in total) in a single run and recorded using ExpeData-P Data Analysis Software (Sable Systems, Las Vegas, NV, USA). The recorded CO_2 emissions were converted from parts per million to mL by dividing the values by 1 000 000 and multiplying by the flow rate of 100. The baseline of the CO_2 analyser was corrected in ExpeData to remove any residual analyser drift. The CO_2 peak recorded for each sample was integrated against the time to give the volume of CO_2 in each injected sample. Carbon dioxide values were further scaled up to the volume of the chamber by multiplying the value by 4 and dividing by the time spent in the chambers by each sample to yield the CO_2 emission rate of each mosquito (Lighton, 2008). Mosquitoes were observed to be mostly only active (flying or walking) during the handling process prior to the flushing step. Once left to settle in the syringe the majority of the mosquitoes entered a quiescent state of less than 20% activity, any mosquitoes showing high activity were omitted from the analysis.

Measured mosquitoes were then aspirated from their respirometry

chambers and placed into individual polystyrene cups. Each mosquito was knocked down in the freezer and weighed using a Libror AEG 45SM balance (Shimadzu Scientific Instruments, Japan) (± 0.01 mg) to account for mass variation in the sample. The mass specific rate of CO_2 production (mL $CO_2.mg^{-1}.min^{-1}$) was calculated in Microsoft Excel (2016) by accounting for the mass (mg) of individual mosquitoes and the time interval between each CO_2 bolus injection. Specimens were dry-preserved on silica gel and identified further to species level using the appropriate PCR protocols for *An. gambiae* complex (Scott et al., 1993), and *An. funestus* group (Koekemoer et al., 2002; Cohuet et al., 2003) at the Vector Control Reference Laboratory of the National Institute for Communicable Diseases in Johannesburg.

Statistical analysis involved comparing the CO_2 production rates of wild adult mosquitoes between seasons (summer, autumn, winter, and spring) using the nonparametric Kruskal-Wallis rank test in Stata 13 (StataCorp LP, College Station, TX, USA). Analyses of the influence of mosquito sex and female blood-meal feeding status on metabolic rate were performed using the Wilcoxon matched-pairs signed-rank test in Stata 13. Nonparametric statistical tests were used because data were not normally distributed due to high variation in the dataset.

2.3. In-field metabolic rate measurements of wild mosquitoes

The effect of long-distance road travel on the metabolic rate of mosquitoes is unknown. To address this, CO_2 production measurements were performed at the KwaZulu-Natal Department of Health (DOH) laboratory in Jozini (Mamfene) using wild-collected mosquitoes that had not been subjected to long-distance transport and compared to those measurements taken from mosquitoes transported to the University of the Witwatersrand in Johannesburg. The same respirometry equipment used in the university laboratory was packaged, transported, and set up in the laboratory in Jozini. Wild specimens were collected in October 2018 from the same clay pots used before. Carbon dioxide production of the mosquitoes was measured using the respirometry techniques described in Section 2.2. Once measured, mosquitoes were placed into individual polystyrene cups with access to 10% sucrose solution and were transported to the laboratory at the University of the Witwatersrand. Mosquitoes were knocked down in the freezer and weighed using the Libror AEG 45SM fine balance as before.

Mosquito metabolic rate measurements acquired in the field laboratory were statistically analysed against those measured at the university laboratory using the nonparametric Kruskal-Wallis rank test in Stata 13. Mosquitoes collected and measured in the field in October 2018 were compared to those collected and measured in the laboratory in October 2017 to eliminate the potential effect of seasonal differences. Statistical analyses were performed within mosquito species and sex to avoid compounding variables.

2.4. Measuring photoperiodic effect on metabolic rate of colonized mosquitoes

The major variables that fluctuate between seasons in South Africa are temperature, humidity and photoperiod. Because the focal malaria points in KwaZulu-Natal are located in a low-rainfall hot semi-arid climate where the daily average temperature rarely falls below 18 °C in winter, photoperiod changes were suggested as the most likely trigger for seasonal physiological changes in mosquitoes. Hence, the effect of seasonal photoperiod on mosquito metabolic rate was investigated.

The experiment was conducted in a temperature-controlled room at the University of the Witwatersrand, Johannesburg. The room was set to 25 °C and maintained at 50–65% relative humidity. Three large steel-framed mesh enclosures ((H) 95 cm × (W) 55 cm × (L) 55 cm) were used to house smaller cages of mosquitoes. One non-heat-producing 20 W LED floodlight (Eurolux (Pty) Ltd, Milnerton, RSA) was secured to the top of each mesh enclosure with the light source directed into the enclosure and plugged into an on/off weekly analogue programmable timer (Major Tech (Pty) Ltd, Johannesburg, RSA). Each enclosure was cloaked in dark cloth to prevent infiltration of natural light and/or ambient light from adjacent enclosures. The on/off timers were set to create daily light/dark cycles within each enclosure to represent the seasonal photoperiod cycles of winter (10 h light, 14 h dark) and summer (14 h light, 10 h dark) and a control (12 h light, 12 h dark).

Anopheles arabiensis (KWAG colony) mosquitoes – originally colonised using material collected from KwaZulu-Natal and reared at the Botha De Meillon insectary at the National Institute for Communicable Diseases, Johannesburg – were used for this experiment. Mosquitoes were collected from the insectary on the day of emergence and separated by sex to avoid mating. Male and female mosquitoes (100 each) were placed into separate BugDorm© cages (model 4M3030, Mega View Science Co., Ltd., Taichung 40762, Taiwan) and provided with 10% sucrose solution that was refreshed as required throughout the experiment. One cage of males and one of females was placed into each mesh enclosure and the entire enclosure was cloaked with black cloth. Each experiment was run for 15 days. Cages of males and females were swapped around within the enclosure periodically to avoid positional bias. The metabolic rate of 10 males and 10 females from each treatment and control group was measured using those techniques described in Section 2.2 every five days (amounting to three measurements within the 15 days). The measurement days were staggered to allow enough time to measure 20 samples in one day. Measured mosquitoes were not returned to the experimental cages so each mosquito sample was measured only once. The entire experiment was replicated three times and enclosures were repositioned after each replicate to avoid positional bias. The CO_2 production of colonized *An. arabiensis* mosquitoes placed under different photoperiod cycles were statistically analysed using the Kruskal-Wallis rank test in Stata 13.

3. Results

3.1. Seasonal abundance and metabolic rate of wild Anopheles mosquitoes

Weather station data indicated a total monthly rainfall range from 64 mm in February to 0 mm in June and July, and an average daily temperature range from a high of 27.2 °C to a low of 19.2 °C in January and June respectively (Fig. 1). A total of 234 *An. gambiae* complex and 203 *An. funestus* group specimens were collected during the period under review. *Anopheles* density was particularly high in March and mainly comprised members of the *An. gambiae* complex ($n = 109$) and, to a lesser extent, the *An. funestus* group ($n = 55$) (Fig. 1). The onset of high rainfall in February may have led to the mosquito density increase experienced in March. *Anopheles gambiae* complex numbers declined towards the end of 2017, while *An. funestus* group numbers remained relatively constant until the low-rainfall period in October (Fig. 1). Anopheline species collected included *An. arabiensis* ($n = 216$), *An. merus* ($n = 10$) and *An. quadriannulatus* ($n = 8$) of the *An. gambiae* species complex, and *An. parensis* ($n = 162$), *An. leesoni* ($n = 29$), *An. rivulorum* ($n = 11$), and *An. vaneedeni* ($n = 1$) of the *An. funestus* species group. Adult mosquitoes were still present during the dry winter period (June to August), but in lower densities compared to the high-rainfall period from February to May (Fig. 1).

The metabolic rate (CO_2 production) of wild anophelines collected from Mamfene varied considerably across the sampling period (Fig. 2). Monthly data were sorted into seasons: summer (January, February, and December), autumn (March, April and May), winter (July and August) and spring (September, October and November) for data analysis. *Anopheles arabiensis* and *An. parensis* predominated the samples, and so these two species were used for seasonal comparisons of metabolic rates (Fig. 2). Statistical analyses revealed no significant differences in metabolic rate values of *An. arabiensis* females across all seasons ($\chi^2 = 4.673$, df = 3, $P = 0.1974$, n = 76), *An. arabiensis* males across all seasons ($\chi^2 = 3.181$, df = 3, $P = 0.3646$, n = 38), or between *An. arabiensis* females and males overall ($z = -1.539$, $P = 0.1239$,

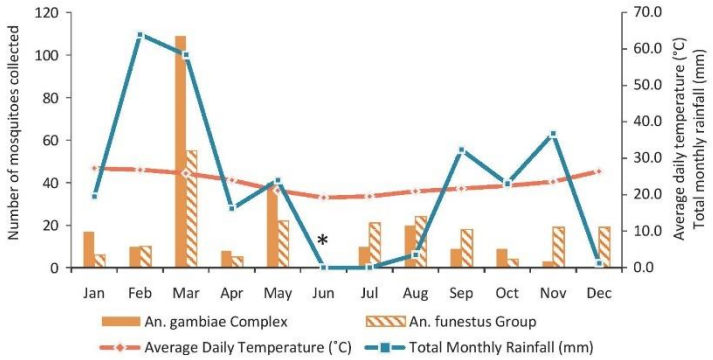

Fig. 1. The bars represent the total number of successfully identified *Anopheles* mosquitoes collected from clay pots from January to December 2017 in Mamfene, KwaZulu-Natal. The line graphs indicate the daily average temperature (°C) and total monthly rainfall (mm), respectively, over the sampling period. *No mosquito collection data were obtained in June.

n = 114). Similarly, *An. parensis* metabolic rate comparisons indicated no significant differences between females compared across all seasons (χ^2 = 3.497, df = 3, P = 0.3212, n = 46), nor between *An. parensis* males and females overall (z = 0.089, P = 0.9293, n = 82). A small number of male *An. parensis* collected in autumn (n = 7) exhibited significantly lower metabolic rate than males collected in winter (n = 17) (z = −3.588, P = 0.0003). The effect of feeding status on metabolic rate was not significant for wild *An. arabiensis* females (z = −1.193, P = 0.2330) nor for wild *An. parensis* females (z = 0.612, P = 0.5405). The number of blood-meals taken and the gonotrophic stages of wild females was however unknown for wild material.

3.2. Mosquito metabolic rate response to transportation

The distance between Mamfene and Johannesburg is approximately 500 km equating to approximately 6 h travel time by road. Only *An. arabiensis* males and *An. leesoni* females were used for comparisons between the different measure locations owing to insufficient sample sizes of other groups (Fig. 3). There was no apparent significant effect of travel on metabolic rate for *An. arabiensis* males (z = −0.354, P = 0.7237, n = 7), or on *An. leesoni* females (z = 1.567, P = 0.117, n = 15).

3.3. Mosquito metabolic rate response to photoperiod

The effect of photoperiod on mosquito metabolic rate was not significant following statistical comparisons of the three photoperiod conditions: midsummer (14 h:10 h), midwinter (10 h:14 h), and the control (12 h:12 h) (χ^2 = 2.762, df = 2, P = 0.2514, n = 502). The sample showed high variation in metabolic rate throughout the experiment with many outlying data points (Fig. 4).

Mosquito age had a significant effect on metabolic rate within most of the photoperiod treatments and within genders as represented in Table 1. Metabolic rate of mosquitoes increased significantly from five to ten days post emergence (except for females under the winter photoperiod treatment), and then decreased significantly from ten to fifteen days post emergence (Table 1 and Fig. 4). However, for both female and male mosquitoes placed under the summer photoperiod treatment, the metabolic rate was not significantly different at the age of five and 15 days old.

4. Discussion

Year-round malaria incidence in South Africa indicates continued vector biting and reproductive activity even in colder and drier winter

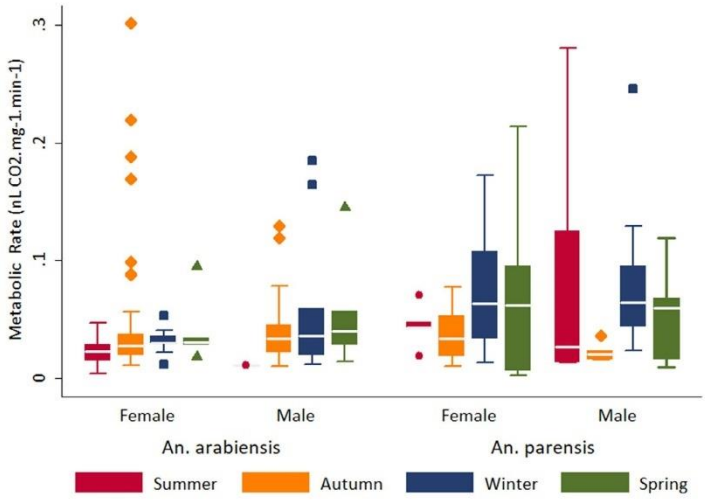

Fig. 2. The boxplot represents the seasonal metabolic rates (nL CO_2.mg^{-1}.min^{-1}) of wild adult male and female *An. arabiensis* and *An. parensis* mosquitoes collected from Mamfene, KwaZulu-Natal, from January to December 2017. The box shows the interquartile range of the metabolic rates with the median values indicated with a white line and outliers indicated with shaped markers.

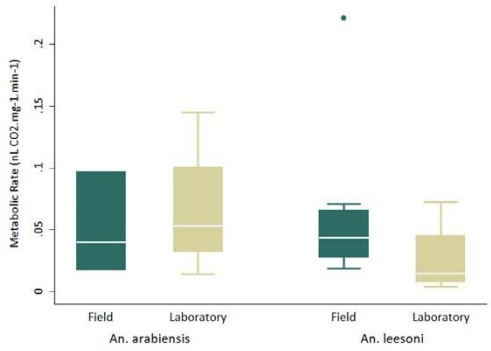

Fig. 3. Comparison of metabolic rate (nL $CO_2.mg^{-1}.min^{-1}$) of wild *Anopheles arabiensis* males and *An. leesoni* females measured at different localities. Field = metabolic rate of wild *Anopheles* mosquitoes measured in a field-based laboratory immediately after collection from field sites in Mamfene, KwaZulu-Natal. Laboratory = metabolic rate of wild *Anopheles* specimens measured after transporting them to a laboratory at the University of the Witwatersrand in Johannesburg, Gauteng, after initial collection from field sites in Mamfene. Specimens measured in the field were collected in October 2018 and compared to specimens previously collected in October 2017. The box shows the interquartile range of the metabolic rates with the median values indicated with a white line and outliers indicated with shaped markers.

conditions. This suggests that there is no necessity for winter diapause and/or long- distance migration to other breeding sites when year-round breeding is possible in local perennial refugia (see also Huestis et al., 2012). These observations are supported by the results from this study that show that the anopheline populations tested do not lower their metabolic rate in winter and do not appear to trigger diapause in the adult mosquito stage as a result of photoperiod changes. In some cases the metabolic rate of *An. arabiensis* and *An. parensis* actually increased over the winter period (although not significantly so), departing from predictions based on literature surrounding winter mosquito diapause (Benoit and Denlinger, 2007). Should a significant decrease in metabolic rate have been found in the winter mosquito population sample, one could assume that the adult mosquitoes had

Table 1
Pair-wise comparisons of the metabolic rate (nL $CO2.mg^{-1}.min^{-1}$) of different adult age cohorts of the colonized *mosquito An.* arabiensis. Significance is indicated by Two-sample Wilcoxon rank-sum (Mann-Whitney) test p-value > 0.05 at the 95% C.I.

Sex	Photoperiod treatment	Age cohort comparison	z statistic	*p-value*	sample size (*n*)
Females	Control	5 *vs* 10 days	3.205	0.001	58
		10 *vs* 15 days	5.354	0.000	48
		15 *vs* 5 days	−3.367	0.001	50
	Winter	5 *vs* 10 days	1.074	0.283	58
		10 *vs* 15 days	3.501	0.001	58
		15 *vs* 5 days	−2.720	0.007	56
	Summer	5 *vs* 10 days	2.410	0.016	60
		10 *vs* 15 days	2.319	0.020	58
		15 *vs* 5 days	−0.140	0.889	58
Males	Control	5 *vs* 10 days	3.003	0.003	58
		10 *vs* 15 days	4.831	0.000	48
		15 *vs* 5 days	−2.515	0.012	50
	Winter	5 *vs* 10 days	2.011	0.044	55
		10 *vs* 15 days	4.125	0.000	60
		15 *vs* 5 days	−2.383	0.017	55
	Summer	5 *vs* 10 days	2.698	0.007	57
		10 *vs* 15 days	2.062	0.039	59
		15 *vs* 5 days	0.171	0.864	58

entered a lowered energy-use state that may allude to diapause. The lack of a metabolic indication of a quiescent state in the samples collected in Mamfene, together with evidence of continued blood-meal foraging (obvious by observation of feeding state of females) and breeding supports the hypothesis that this population does not diapause.

The alternative explanation is that local mosquito populations reduce in density and retreat to perennial breeding sites or refugia during the winter months when small temporary breeding sites are not readily available. The use of refugia in this way is typical of *An. arabiensis* in northern Sudan (Dukeen and Omer, 1986), the Kilombero Valley in south-eastern Tanzania (Charlwood et al., 2000), and in villages of the Baringo district in Kenya (Mala et al., 2011). This also means that local *Anopheles* species – especially within the *An. gambiae* complex and the *An. funestus* group – do not suspend their foraging and reproductive activities during the dry season. *Anopheles arabiensis* have shown

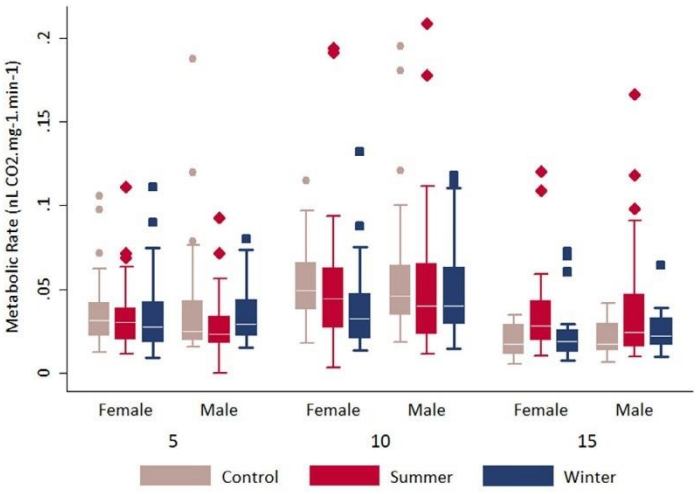

Fig. 4. Metabolic rate (nL $CO_2.mg^{-1}.min^{-1}$) of colonized *An. arabiensis* male and female mosquitoes exposed to light–dark cycles mimicking midsummer (14 h light, 10 h dark) (n = 175), midwinter, (10 h light, 14 h dark) (n = 171), and a control (12 h light, 12 h dark) (n = 156) measured at intervals of five days for 15 days post adult emergence. The box shows the interquartile range of the metabolic rates with the median values indicated with a white line and outliers indicated with shaped markers.

significantly higher desiccation resistance facilitated by higher body water content compared to *An. gambiae* in other studies (Gray and Bradley, 2005), which may facilitate their perseverance through the dry season with limited to no apparent behavioural changes.

A caveat of using data obtained from wild-caught mosquitoes is variation in age, male physiological status, and female gonotrophic status, all of which affect metabolic rate. The use of laboratory-reared colony material was designed to address these issues by controlling for these variables. Under these conditions, the metabolic rate responses of colonized *An. arabiensis* to manipulated photoperiod showed no disparities between summer and winter daylight regimens, showing that variations in day length are not necessarily a primer for changes in metabolic rate in adult *An. arabiensis*. Metabolic rate was significantly affected by mosquito age, which may account for the high levels of variation in the wild samples, along with other variables. The experimental data followed a parabolic trend with age of mosquitoes where metabolic rate was significantly higher at 10 days compared to 5 days, but then significantly lower at 15 days compared to 10 days.

Certainly, one cannot draw the conclusion that photoperiod does not trigger a metabolic response in any of the other mosquito life stages as this was only investigated post adult emergence in this study. Further experiments investigating the metabolic effect of photoperiod exposure from the mosquito egg stage and the possibility of epigenetic affects from the maternal line would be useful. There is marked evidence of a strong parental effect on progeny development time in the highly studied *Drosophila* species *D. simulans* (Giesel, 1986) and *D. melanogaster* (Giesel, 1988) which insinuates diapause. The roles of temperature and humidity, both alone and in combination with day length and each other, warrant further investigation as a combination of several factors may produce alternative results.

Because water plays a pivotal role in the aquatic stages of the mosquito lifecycle, rainfall trends often determine mosquito density fluctuations. The 2017 malaria season saw a dramatic increase in sporadic, locally acquired malaria – an estimated 31 000 cases were reported in South Africa, a significant increase from the 9478 cases reported in 2016 (Limpopo Department of Health, 2017; Christian et al., 2017). The unusually high incidence was likely attributed to a vector population upsurge associated with high rainfall, increased ambient temperature and humidity, combined with delayed control activities (Limpopo Department of Health, 2017). The ongoing occurrence of outdoor-resting components of the prominent vector species *An. arabiensis* together with the minor vectors *An. merus*, *An. parensis*, and *An. vaneedeni* in KwaZulu-Natal Province primarily explain ongoing residual transmission despite annual control. Outdoor-resting and biting vectors that are less amenable to control by indoor applications of insecticide pose a significant challenge to control. Cross-border human movement between South Africa and high malaria burden countries like Mozambique and Zimbabwe contributes significantly to the malaria incidence rate (Maharaj et al., 2012, 2013). These factors stress the need to supplement South Africa's provincial indoor residual spraying-based vector control programmes with methods that target outdoor-resting mosquitoes. Ongoing breeding during the winter months in reduced numbers of perennial breeding sites presents an important opportunity for enhanced control by winter larviciding. Based on these data, this recommendation has been made and now forms part of South Africa's malaria vector control and elimination strategy for the period 2019–2023.

It is concluded that South African populations of the malaria vector species *An. arabiensis* and *An. parensis* do not curtail their breeding and foraging activities by reducing their metabolic rates during the colder and drier winter months. Their respective population densities do however decrease considerably during winter leading to reduced malaria transmission and the opportunity for control by winter larviciding of perennial breeding sites.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The authors wish to acknowledge the support and assistance received from the KwaZulu-Natal Department of Health Malaria Control Programme entomological team, in particular Mr Jabulani Zikhali and Mr Dumisani Dlamini, as well as the director of the MCP Mr Bheki Qwabe. The staff and students from the Vector Control Reference Laboratory based at the National Institute for Communicable Diseases in Johannesburg are duly thanked for assistance with field collections, particularly Dr Maria Kaiser and Mrs Leanne Lobb. Mr Innocent Kambule is acknowledged for his assistance with training on the respirometry equipment and general assistance in the Ecophysiology laboratory at the University of the Witwatersrand. Mr Nic Venter is acknowledged for his assistance and general trouble-shooting with setting up the experimental enclosures at the Wits Insectary and Quarantine facility. Thanks also to biostatistician Dr Innocent Maposa from the University of the Witwatersrand for suggestions on the initial statistical analysis of data. Anonymous reviewers are thanked for their comments which improved this manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jinsphys.2019.103942.

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APPENDIX E

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG
7 York Road, Parktown, 2193 South Africa * Telephone (011)717-2363

24 February 2017

To: Whom it may concern,

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

This letter is to confirm that Ashely Burke (School of Pathology in the faculty of Health Sciences, University of the Witwatersrand) does not require full animal ethics clearance to undertake the study titled "Overwintering of Anopheles mosquitoes: surveillance techniques to detect numbers and mosquito survival strategy".

No animals or animal tissues will be used in the study which will be carried out on wild and colonised mosquitoes. The colonized mosquitoes are housed at the Botha De Meillon insectary at the NICD campus in Johannesburg. Routine vector surveillance will be conducted in Mamfene in KwaZulu Natal during the malaria transmission season.

The study is supervised by associate professor Basil Brooke and Professor Frances Duncan. If you would like any further or more specific information in this regard, do not hesitate to contact me.

Yours sincerely,

Kennedy Erlwanger
(Chairman: Animal Ethics Screening Committee, University of the Witwatersrand)

Reference: A Burke Waiver 24-02-2017-O

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APPENDIX F

PLAGIARISM FORM

PLAGIARISM DECLARATION TO BE SIGNED BY ALL HIGHER DEGREE STUDENTS

SENATE PLAGIARISM POLICY: APPENDIX ONE

I Ashley Morgan Burke (Student number: 1129903) am a student registered for the degree of Doctor of Philosophy in the academic year 2020.

I hereby declare the following:

- I am aware that plagiarism (the use of someone else's work without their permission and/or without acknowledging the original source) is wrong.
- I confirm that the work submitted for assessment for the above degree is my own unaided work except where I have explicitly indicated otherwise.
- I have followed the required conventions in referencing the thoughts and ideas of others.
- I understand that the University of the Witwatersrand may take disciplinary action against me if there is a belief that this is not my own unaided work or that I have failed to acknowledge the source of the ideas or words in my writing.
- I have included as an appendix a report from "Turnitin" (or other approved plagiarism detection) software indicating the level of plagiarism in my research document.

Signature: _____ Date: 15 April 2020

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