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Dandalo, Leonard Chikondi

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Characterization of a local genetic sexing strain as well as a wild population of *Anopheles arabiensis* from KwaZulu Natal, South Africa.

Leonard Chikondi Dandalo

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

Johannesburg, 2017

DECLARATION

I declare that this thesis hereby submitted by me for the Degree of Doctor of Philosophy at the University of Witwatersrand, Johannesburg is my own independent work and has not been previously submitted for any degree or examination in any other University. I further more cede copyright of the thesis in favour of the University of Witwatersrand.

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ABSTRACT

Malaria remains endemic in the north-eastern areas of KwaZulu-Natal (KZN), Mpumalanga and Limpopo provinces of South Africa (SA). *Anopheles arabiensis* is now implicated as the main malaria vector. This vector is not completely amenable to current vector control strategies which target indoor biting and resting mosquitoes. SA is moving towards malaria elimination and there is a need for additional vector control interventions to complement existing tools. The sterile insect technique (SIT) targeting *An. arabiensis* was selected as a potential intervention. In a mosquito SIT programme, only sterile males should be released because females are potential disease vectors. In order to achieve male releases only, a reliable sex separation strategy is needed. Additionally, it is imperative to gather entomological baseline information on the population density, species composition, and vectorial capacity of the targeted wild population. The aim of this study was to evaluate the use of a local genetic sexing strain for SIT and to determine the population dynamics of wild *An. arabiensis* in northern KZN. The following objectives were initiated in this study: development of a local genetic sexing strain (GSS), evaluation of the life history and reproductive effects of irradiation on *An. arabiensis*, and weekly mosquito surveillance was conducted over a period of 24 months.

A local GSS named GMK was established by introgressing a local wild-type population of *An. arabiensis* with an available GSS strain. The strain exhibited the following attributes: low egg hatch rates, fast developmental time, long adult survival and a high mating competitiveness. Dieldrin treatment of GMK eggs/larvae mainly produced males but this result remains controversial. The irradiation dose of 70 Gy induced male sterility without compromising their mating competitiveness and impacted negatively on female fitness, but not vectorial capacity. The perennial presence of *An. arabiensis*, the dominant anopheline species in Mamfene, was confirmed. Its population density fluctuated with season reaching a peak in summer. Clay pots were more productive than the other collection methods, collecting 16.3 mosquitoes per trap. This study recorded for the first time wild caught *An. arabiensis* and *An. vaneedeni* infected with *P. falciparum* in SA. *An arabiensis* sporozoite infection rates were 0.7% (2014) and 0.5% (2015). *Anopheles vaneedeni* has never been implicated as a malaria vector in nature. However, an infection rate of 1.96% was recorded (2014-2015), which implicate this species as a potential malaria vector.

These results highlight the importance of intensive mosquito surveillance to establish malaria vectors responsible for low level/residual malaria transmission. The data generated provides important baseline vector surveillance information and is valuable to stakeholders and researchers to make informed decisions regarding the use of SIT against vector mosquitoes in SA.

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PUBLICATIONS

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5) Ashley Burke, **Leonard Dandalo,** Givemore Munhenga, Yael Dahan, Frans Mbokazi, Maureen Coetzee, Lizette Koekemoer, Basil Brooke. **A new malaria vector mosquito in South Africa.** *Scientific Reports*, 7, 43779; doi: 10.1038/srep43779 (Chapter 5).

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PRESENTATIONS

1) **Leonard C. Dandalo**, Basil B. Brooke, Givemore Munhenga, Leanne Robertson, Oliver Wood, Malibongwe Zulu, Alan Kemp and Lizette L Koekemoer. **Population dynamics of *Anopheles arabiensis* from Mamfene, KwaZulu-Natal, and development of a local Genetic Sexing Strain (GSS)**. XIX Congress of the Entomological Society of Southern Africa, 12-17 July 2015, Grahamstown, South Africa. (Oral)

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4) Ashley Burke, **Leonard Dandalo**, Givemore Munhenga, Maureen Coetzee, Lizette Koekemoer & Basil Brooke. **Is *Anopheles vaneedeni* a new Malaria Vector in South Africa?** 2nd South African Malaria Research Conference, 31st July – 2 August, 2016, Pretoria, South Africa. (Oral)

5) **Leonard C Dandalo,** Givemore Munhenga, Maria Kaiser and Lizette L Koekemoer. **Development of a Genetic Sexing Strain (GSS) of *Anopheles arabiensis* (Diptera: Culicidae) for KwaZulu Natal, South Africa.** 1st September, 2016. Faculty of Health Sciences Research Day, University of Witwatersrand, Johannesburg, South Africa. (Poster)

6) **Leonard C Dandalo**, Annette Bennett, Dewaldt Engelbrecht, Alan Kemp, Givemore Munhenga, Theresa Coetzer and Lizette L Koekemoer. **Effect of ionising (gamma) radiation on female *Anopheles arabiensis*: An investigative study towards the development of the Sterile**

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NOMENCLATURE

1:1:1	Ratio of 1 to 1 to 1
1:1:3	Ratio of 1 to 1 to 3
1:1:5	Ratio of 1 to 1 to 5
%	Percentage
µg	Micrograms
µl	Microliters
µM	Micromole
1st use	First use
2nd use	Second use
3rd use	Third use
AB	Antibody
ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
ACTs	Artemisinin-based combination therapies
ANO IPCL	*Anopheles arabiensis* genetic sexing strain developed at the Food and Agriculture Organization/International Atomic Energy Agency Insect Pest Control Laboratory (IPCL, Seibersdorf, Austria)
ANOVA	Analysis of variance
ArwP$_{IT}$	*Aedes albopictus* strain originating from Italy
AW-IPM	Area-wide integrated pest management
AW-IVM	Area wide integrated vector management
BB	Blocking buffer
bp	Basepairs
C	Competitive Index
CI	Confidence Interval
cm	Centimeters
CO_2	Carbon dioxide
CS	Circumsporozoite
DDT	Diethyl diphenyl trichloroethane
DFID	Department for International Development
DNA	Deoxyribonucleic acid

dNTP	Deoxynucleotide triphosphate
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked Immunosorbent assay
et al	and others
Etc	et cetera
F	Filial
GAMA	*Anopheles arabiensis* strain derived from the genetic sexing strain, ANO IPCL strain (Austria) and *An. arabiensis* from Kruger National Park (named AMAL), South Africa
Gamma	γ (penetrating electromagnetic *radiation)*
GMK	*Anopheles arabiensis* strain derived from GAMA strain (South Africa) and wild type KWAG strain from Mamfene, KwaZulu-Natal, South Africa.
GSS	genetic sexing strain
Gy	Gray
IAEA	International Atomic Energy Agency
IPTp	Intermittent preventive treatment in pregnancy
IRS	Indoor residual spraying
IS	Induced Sterility
ITNs	Insecticide treated nets
ITS2	Internal transcribed spacer 2
IVM	Integrated vector management
KAc	Pottassiun Acetate
kb	Kilobase
KGB	*Anopheles arabiensis* strain originating from Kanyemba, Zimbabwe
KWAG	*Anopheles arabiensis* strain from KwaZulu-Natal
KZN	KwaZulu-Natal
L	Litre
L1	First instar larvae
M	Molar
Max	Maximum
mg	Milligrams

$MgCl_2$	Magnesium chloride
Min	Minimum
ml	Millilitre
mM	Millimole
mm	Millimetre
MMR	Mark-release-recapture
N	Total number
NaCl	Sodium chloride
NF54-GFP	A transgenic *P. falciparum* NF54 strain that expresses green fluorescent protein (gfp-luciferase)
NICD	National Institute for Communicable Diseases
nM	Nanomole
^oC	Degrees celcius
P	Probability level
PABA	Para- aminobenzoic acid
PBS	Phosphate buffered saline
PBS-TW	Phosphate buffered saline – tween
PCR	Polymerase chain reaction
Pf	*Plasmodium falciparum*
Pg	Pictogram
pH	Potential of hydrogen
Pmol	Per mole
Ppm	Parts per million
Rdl	Resistance to dieldrin
RdlF	Resistance to dieldrin forwad primer
Rdlr	Resistance to dieldrin reverse primer
rDNA	Ribosomal deoxyribonucleic acid
rFAL	*Plasmodium falciparum* specific primer
rPLU	*Plasmodium* (genus) primer
Rpm	Revolutions per minute
R^2	Square of the sample *correlation coefficient* in regression analysis

s.l.	Sensu lato
s.s.	Sensu stricto
SA	South Africa
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SEM	Standard error of mean
Ser	Serine
SIT	Sterile insect technique
SMFA	Standard membrane feeding assay
Spp	Species
SPSS	Statistical Package for the Social Sciences
SSA	Sub-Saharan Africa
ssRNA	Single-stranded ribonucleic acid
TAE	Tris(hydroxymethyl) aminomethane-acetate
Taq	*Thermus aquiticas* polymerase
TE	Tris(hydroxymethyl)aminomethane ethylenediaminetetraacetic acid
Tris-Cl	Tris(hydroxymethyl)aminomethane chloride
Tsl	Temperature sensitive lethal
V	Voltage
VCRL	Vector Control Reference laboratory
WHO	World Health Organization
WRIM	Wits Research Institute for Malaria
WT2	Wild Type 2
$\boldsymbol{X^2}$	Chi –square

CHAPTER 1

GENERAL INTRODUCTION

Malaria remains a major public health concern in South Africa (SA). Significant efforts to control malaria have successfully reduced the burden of malaria in SA and the transmission of the disease is now limited to the low-altitude northern and north-eastern regions of Limpopo, Mpumalanga and KwaZulu-Natal provinces (Morris *et al*., 2013). In 2015, 11 000 cases of malaria were officially recorded. Of these, 36% were locally acquired (Department of Health, unpublished records). Such transmission levels represent a malaria case incidence of <1/1000 population at risk which meets the World Health Organization (WHO) criteria for pre-elimination (Maharaj *et al*., 2013). The South African government is committed to eliminate malaria transmission by 2020 (Malaria Elimination Group, 2009; WHO, 2016a).

The success of reducing malaria transmission to pre-elimination levels in SA is largely attributed to sustained vector control efforts. These efforts primarily depend on indoor residual spraying (IRS) of households with either DDT (in traditional mud-walled houses) or synthetic pyrethroids (in modern cement-brick houses) (Brooke *et al*., 2013). However, as SA shifts towards malaria elimination it is unlikely that current malaria control efforts can successfully eliminate *Anopheles arabiensis* due to its variable feeding and resting behavior. This necessitated research into additional vector control tools such as the Sterile Insect Technique (SIT) to complement existing strategies.

The SIT entails artificial mass-rearing of the target vector species, sterilizing the males and releasing them in large numbers. The aim is to inundate the target population with sterilized males to a point where the vast majority of matings do not produce progeny, leading to target population suppression and a reduction in disease transmission (Vreysen, 2001). The successful implementation of the SIT as an insect intervention strategy is dependent on a number of factors. These include prior knowledge on the bionomics of the targeted species as well as a thorough understanding of the species diversity in the area targeted for releases. Another essential requirement for applying the SIT is the development of a sexing system to exclusively obtain males for sterilization and releases.

Female mosquitoes, even when sterilized with ionizing radiation, cannot be released, because of their capacity as disease vectors. It is against this background that this study was conceived.

This research consisted of four parts: establishing a local genetic sexing strain (GSS) that can be used to exclusively obtain males only for sterilization, characterizing fitness parameters of the strain, assessing the effect of irradiation on the physiological and reproductive fitness of GSS females, and understanding the population dynamics of *An. arabiensis*, its vector status and mosquito species diversity in an area targeted for pilot SIT releases.

1.1 Malaria parasites

Malaria is an infectious disease caused by parasitic protozoan in the genus *Plasmodium*. There are approximately 156 *Plasmodium* species that infect various vertebrate species (Berger and Marr, 2006), but only six of these cause human malaria. However, the five species that exclusively utilize human host as intermediates and are considered true parasites of humans are: *P. ovale curtisi, P. ovale wallikeri, P. malariae, P. vivax* and *P. falciparum*. The sixth species, *P. knowlesi* has been detected in humans and has an intermediate host; macaque monkeys (van Hellemond *et al*., 2009). No information is available to ascertain whether transmission from human to human via the mosquitoes occurs and it is therefore still regarded as zoonotic malaria (Singh and Daneshvar, 2013). Malaria symptoms include cyclical fever and shivering, joint pains, vomiting, headache and general body pain while severe cases include convulsions and/or kidney failure resulting in severe complications of acute anaemia, transient or permanent neurological effects, and death (Schumacher and Spinelli, 2012).

Plasmodium ovale curtisi and *P. ovale wallikeri* occurs in sub-Saharan Africa and some western Pacific islands. These two species differ in their biology with *P. ovale wallikeri* having a shorter latent period than *P. ovale curtisi*. These parasites cause milder illness with few fatalities in comparison with *P. vivax* and *P. falciparum* (Nevill, 1990).

Plasmodium malariae has a wide distribution, found in sub-Saharan Africa, South America, Asia, Indonesia and on islands of the western Pacific, but is less frequent than *P. falciparum* in terms of infection (Collins and Jeffery, 2007). Its asexual stages multiply every 3 days in blood and never cause severe infections (van Hellemond *et al*., 2009).

Plasmodium knowlesi occurs in long-tailed and pig-tailed macaque monkeys inhabiting forested areas in south-east Asia (van Hellemond *et al*., 2009). It was known to cause malaria among these monkeys but in recent years has also been discovered to cause malaria in humans (Nevill, 1990; WHO, 2003a). Asexual stages of *P. knowlesi* resemble those of *P. malariae* but differ in that they multiply daily and high parasitemia can cause death in humans (van Hellemond *et al*., 2009).

Plasmodium vivax is the most frequent and widely distributed species causing recurring malaria but is less virulent than *P. falciparum*. It is mainly found in South America, Asia and some parts of Africa with the exception of sub-Saharan Africa (Gething *et al*., 2012). This parasite is carried by at least 71 mosquito species most of which live in temperate climates. The hypnozoite form of the parasite can become dormant in the liver for days to years, causing no symptoms, and being undetectable in blood tests. The sexual stages can multiply in the bloodstream before the patient shows symptoms. The liver stages allow relapses up to 14 months after elimination from the bloodstream (Adak *et al*., 2001).

Plasmodium falciparum is the deadliest species, causing severe illness (Nevill, 2009). It is less widespread in comparison with *P. vivax*. It is found in many tropical and subtropical regions of the world and accounts for almost all malaria deaths that occur in sub-Saharan Africa (Snow and Omumbo, 2006). In 2015, estimated 429 000 malaria deaths cause by this species were reported worldwide and 92% of these occurred in Africa (WHO, 2017)

1.2 Malaria vectors

Mosquitoes in the genus *Anopheles* act as malaria vectors. In this genus, members of the *Anopheles gambiae* complex and *An. funestus group* are responsible for the bulk of malaria transmission in the Afro-tropical region (Gillies and De Meillon, 1968; White, 1974; Gillies and Coetzee, 1987). *Anopheles gambiae s.s., An. coluzzii* and *An. arabiensis* are the major malaria vectors from the *An. gambiae* complex (Coetzee *et al*., 2013), whereas *An. funestus* s.s. and *An. rivulorum* are the only

main malaria vectors from the *An. funestus* group (Gillies and De Meillon, 1968; Gillies and Coetzee, 1987; Coetzee and Koekemoer, 2013). The other members from the *An. gambiae* complex (*An. merus*, *An. melas* and *An. bwambae An. quadriannulatus* and *An. amharicus*) and (*An. parensis*, *An. vaneedeni*, *An. leesoni*) from the *An. funestus* group are either non-malaria vectors or play a very limited role (Gillies and De Meillon, 1968; Gillies and Coetzee, 1987; Wilkes *et al*., 1996; Kweka *et al*., 2013; Coetzee *et al*., 2013). This study focused on *An. arabiensis*, implicated as a likely major malaria vector in South Africa. The following literature review on malaria vectors is restricted to the *An. gambiae* complex.

1.2.1 *Anopheles gambiae* complex

The *An. gambiae* complex consists of eight sibling species namely: *An. gambiae* s.s., *An. coluzzii*, *An. arabiensis*, *An. merus*, *An. melas*, *An. bwambae*, *An. quadriannulatus* and *An. amharicus* (Gillies and Coetzee, 1987; Hunt *et al*., 1998, Coetzee *et al*., 2013). These sibling species are widely distributed in sub-Saharan Africa and surrounding islands and vary in their larval habitats, adult female resting behaviour and feeding preferences (Coetzee *et al*., 2000).

(i) *Anopheles gambiae sensu stricto* Giles

Populations of *An. gambiae* are thought to be undergoing speciation and have historically been defined in two ways: chromosomal forms and molecular forms (Lanzaro and Lee, 2013). There are five chromosomal forms that carry different strain specific combinations of inversions and differ in their vectorial capacity, namely, Bissan, Forest, Mopti, Savanna and Bamako. The forms occur sympatrically but are segregated environmentally (Coluzzi *et al*., 1979; Favia *et al*., 1994; Bayoh *et al*., 2001; Sogoba *et al*., 2008). Apart from chromosomal forms, this species has also been divided into two genetically distinct molecular forms, “M” and “S” (Lehmann and Diabate, 2008). The M form has been renamed *An. coluzzii* Coetzee and Wilkerson, sp.n [Described in 1.2.1(ii)] whereas the S form retains the nominotypical name *An. gambiae s.s* Giles described further in this section (Coetzee *et al*., 2013).

Anopheles gambiae s.s. has the broadest distribution occurring throughout sub-Saharan Africa and is regarded as one of the most efficient malaria vectors (Lanzaro and Lee, 2013). This species is often localized in areas with closed canopy and breeds in a great variety of shallow open sunlit

pools such as borrow-pits, drains, brick-pits, ruts, car-tracks, hoof prints, round ponds and water holes (Gillies and De Meillon, 1968). Although *An. gambiae* thrives under rather cool conditions, it is tolerant of relatively high temperatures (Gillies and De Meillon, 1968). In general terms, seasonal changes in *An. gambiae s.s* populations tend to follow the seasonal pattern of rainfall. Thus, in savanna zones with a single rainy season per year, numbers start to rise explosively soon after the first main rainfall, reaching a peak in the middle of the rainy season and declining steadily thereafter as the levels of water become stabilized and vegetation and predators become established (Gillies and De Meillon, 1968). Female *An. gambiae s.s.* are highly anthropophilic, feeding preferentially on humans (White, 1974; Coluzzi *et al*., 1979). The endophilic and anthropophagic behaviours of *An. gambiae s.s* create a very close association between the human reservoir and the insect vectors of malaria.

(ii) ***Anopheles* (Cellia) *coluzzii* Coetzee & Wilkerson, sp.n.**

This species was historically known as *An. gambiae* molecular m form (della Torre *et al*., 2001). Its distribution extends from northern Senegal in the west to east central Africa and south to coastal Angola. This species is found in the Zambezi valley in Zimbabwe and is also regarded as one of the most efficient vectors of *P. faciparum* (della Torre *et al*., 2001, Coetzee *et al*., 2013). Larval breeding sites originate from longer lasting human activities. In savannah areas these tend to be irrigated sites (e.g., man-made rice fields, reservoirs and drainage ditches) whereas in forest areas these tend to be urban pools (Favia *et al*., 1994; Sinka *et al*., 2010; Coetzee *et al*., 2013).

(iii) ***Anopheles arabiensis* Patton**

Anopheles arabiensis is widely distributed in Africa ranging from Madagascar to Senegal (Coetzee *et al*., 2000). Its range and relative abundance tend to be influenced by climatic factors, especially total annual precipitation. It prefers to breed in fresh temporary sunlit or rain water pools (Lindsay *et al*., 1998). This species is more tolerant to higher temperatures and is able to survive in drier conditions (Gillies and Coetzee, 1987; Petrarca *et al*., 2000). It has been observed that adult females persist in arid conditions by laying their eggs on damp surfaces, rather than water, with hatching being delayed in a proportion of eggs (Lindsay *et al*., 1998) and aestivate during periods of prolonged dryness (Omer and Cloudsley-Thomson, 1970). *Anopheles arabiensis* has a more opportunistic feeding behaviour, it can be entirely zoophilic, as recent studies from Madagascar

have shown (Duchemin *et al*., 2001). This species also tends to be more exophagic and exophilic (Gillies and Coetzee, 1987). The variable behaviour of *An. arabiensis* females, being anthropophilic and zoophilic as well as endophilic and exophilic makes them incompletely vulnerable to house-spraying (White, 1974).

The seasonal abundance of *An. arabiensis* with peaks following the onset of rains makes it largely responsible for malaria transmission in southern African countries including South Africa (Gillies and Coetzee, 1987; Hargreaves *et al*., 2003; Maharaj *et al*., 2013).

(iv) ***Anopheles quadriannulatus* Theobald**

Anopheles quadriannulatus species have a limited distribution associated perhaps with more subtropical climates than the other members of the complex. They are freshwater species and are adapted to lower developmental temperatures (Gillies and De Meillon, 1968). They are not known to transmit malaria parasites (Coetzee, 1989). The species that retained its nominotypical name *An. quadriannulatus s.s*. Theobad was previously called *An. quadriannulatus* s.s. species A and is widespread in Southern Africa (Gillies and De Meillon, 1968; Gillies and Coetzee, 1987). This species feeds on cattle and rests outside houses (Gillies and De Meillon, 1968).

(v) ***Anopheles amharicus* Hunt, Wilkerson & Coetzee, sp.n.**

This species was previously called *An. quadriannulatus* species B (Hunt *et al*., 1998) and has been named *An. amharicus* based on chromosomal, cross-mating and molecular evidence (Coetzee *et al*., 2013). Its distribution is limited to Ethiopia (Fettene *et al*., 2002; Coetzee *et al*., 2013) covering Bahir Dar, Bako, Bedele, Dejen and Jimma areas, and along Omo River. This species is associated with cattle and is found abundantly in animal shelters or mixed human/animal dwellings but no information is available for the larval habitats (Coetzee *et al*., 2013).

(vi) ***Anopheles merus* Dönitz**

Anopheles merus is a saltwater breeding member of the *An. gambiae* complex involved in low rate malaria transmission (Gillies and De Meillon, 1968; Gillies and Coetzee, 1987; Temu *et al*., 1998; Cuamba and Mendis, 2009). This species has a limited distribution occurring in the East coast of

Africa, as well as the adjacent inland areas and coastal islands (Gillies and De Meillon, 1968; Gillies and Coetzee, 1987; Sharp, 1983). This species also occurs inland in some malaria endemic areas in South Africa (Gillies and De Meillon, 1968; Gillies and Coetzee, 1987; Obala, 1995; Mouatcho *et al*., 2009; Munhenga *et al*., 2014). Larvae of *An. merus* are found in coastal *Avicennia* mangrove swamps, saltpans, brackish ponds (Gillies and De Meillon, 1968) or mineral springs (Coetzee and Le Sueur, 1988).

In the absence of domestic animals, *An. merus* bites man readily both indoors and outdoors. It has also been observed that in the presence of other hosts *An. merus* is more attracted to cattle (Gillies and De Meillon, 1968).

(vii) ***Anopheles melas*** **Theobald**

Anopheles melas is the West Coast salt-water member of the *An. gambiae* complex. It can utilise saline environments yet it does not require brackish water for its larval development (Sinka *et al*., 2010). It occurs in patches of salt grass in tidal swamps and in pools, ponds or lagoons flooded by spring tides, and also in *Avicennia* mangrove swamps (Gillies and De Meillon, 1968). *Anopheles melas* is generally restricted to coastal areas (Coetzee *et al*., 2000), with the exception of Gambia, where it occurs far inland along the Gambia River (Bøgh *et al*., 2003).

This species shows opportunistic biting behaviour being highly anthropophilic and zoophilic (Sinka *et al*., 2010). It feeds on humans and feeds as readily indoors as outdoors even in the presence of domestic animals (Gillies and De Meillon, 1968). This species generally rests outdoors after feeding. *Anopheles melas* is an important vector of malaria at many points along the West African coast but is considered to be a vector of lesser importance where it occurs in sympatry with *An. gambiae s.s* or *An. arabiensis* (Sinka *et al*., 2010). It is also capable of transmitting Brancoftian filariasis (Gillies and De Meillon, 1968).

(viii) ***Anopheles bwambae*** **White**

The distribution of *An. bwambae* is very limited being defined by its breeding sites in the Buranga brackish water hot mineral springs in the Semliki forest in Bwamba County, Toro district, Uganda (White, 1985; Sinka *et al*., 2010). The natural hosts of this species in the Semliki forest are

unknown but the adults readily bite humans from the nearby villages. *Anopheles bwambae* is regarded as a potential malaria vector due to its susceptibility to *P*. *falciparum*. *Plasmodium falciparum* infection rates of 0.7% were recorded in indoor collections from Bwamba, Uganda (White, 1985). This species is also regarded as a vector of filariasis (White, 1985).

1.3 Malaria transmission cycle

Malaria parasite life cycle involves two hosts: humans and female *Anopheles* mosquitoes. During a blood meal, a malaria infected female *Anopheles* mosquitoes inoculates sporozoites into the human host. The sporozoites infect liver cells and mature into schizonts, which rupture releasing merozoites into the blood stream. The merozoites infect red blood cells and are responsible for the clinical manifestation of the disease (usually fever, headache, chills and vomiting) (Aly *et al*., 2009). Female *Anopheles* mosquitoes ingest male and female's gametocytes during a blood meal. Inside the mosquito's stomach, the gametocytes generate zygotes, which develop into oocysts. The oocysts grow, rupture and release sporozoites which migrate to the mosquito's salivary glands (Huff, 1947). The inoculation of the sporozoites into a new human host perpetuates the malaria life cycle.

The susceptibility of anopheline mosquito species to *Plasmodium* infection varies depending on species. Some species are more permissive to infection than others (Hume *et al*., 2007). *Plasmodium* refractory lines of *Anopheles* have previously been selected in laboratories (Hurd *et al*., 2005; Voordouw *et al*., 2008; Eldering *et al*., 2016). Refractory mosquitoes are poor malaria vectors as they impede parasite development in their midguts (Hurd *et al*., 1995). Several parasite – inhibiting mechanisms have been reported and these include: 1) Failure of the parasite to exflagellate or to undergo syngamy in the midgut of mosquitoes (Nijhout, 1979), 2) Failure in activation of parasite chitinase for penetration of the peritrophic matrix (Shahabuddin *et al*., 1993), 3) Melanotic encapsulation of the developing oocyst that occurs between the basolateral epithelial cell membrane and the basement membrane (Collins *et al*., 1986), 4) Salivary gland barriers that prevent sporozoite penetration or reduce sporozoite survivourship (Roseberg, 1985), 5) The incompatibility between the mosquito and the parasite also referred to as a refractory condition. Loss of parasites without trace might be attributed to lysis in the midgut epithelial cells (Vernick *et al*., 1995; Yan *et al*., 1997; Hurd *et al*., 2005).

There is fitness cost associated with refractoriness in female mosquitoes. Previous laboratory and field studies have shown that malaria infected mosquitoes are less fit than uninfected ones. There is a reduction in the reproductive success (fecundity and fertility) for infected mosquitoes (Hogg and Hurd, 1995, 1997; Hurd *et al*., 2005) and also a reduction in survival rate (longevity) (Anderson *et al*., 2000; Ferguson and Read, 2002). However, Hurd *et al*., 2005 reported that parasite infection has no effect on survivourship of mosquitoes whereas Ve´zilier *et al*., 2012 reported that parasite infection increases longevity of mosquitoes. The increase in longevity arises through a trade-off between reproduction and survival. The elevated expression of oxidoreductive genes in refractory strains is responsible for causing fitness costs following infection. Hence refractoriness to parasites is as costly as tolerance (Hurd *et al*., 2005). The refractory population has a significantly shorter egg to adult developmental time and small body size. The reduction in body size allows the females to take smaller blood meal and subsequently lay fewer eggs than their susceptible counterparts (Yan *et al*., 1997).

The immune system of many insects decreases with an increase in age leading to the prediction that older females are more prone to infection than the young ones. In contrast to this theory, older female mosquitoes have a lower probability of becoming infected with the parasite. However, the age effect is entirely reversed when old mosquitoes have taken one previous non-infected blood meal (Pigeault *et al*., 2015).

1.4 Malaria burden in sub-Saharan Africa

Malaria is among the most dangerous human diseases in the world. An estimated 3.2 billion people are at risk of malaria, with populations living in sub-Saharan Africa (SSA) having the highest risk of acquiring malaria (WHO, 2016b).

Approximately 80% of cases and 78% of deaths occur in sub-Saharan Africa (SSA), with children under five years of age and pregnant women most severely affected (WHO, 2016b). Malaria causes death to young children in three ways. Firstly, an acute infection of cerebral malaria may kill the child directly and quickly. Secondly, repeated infections cause severe anaemia which increases the risk of death. Thirdly, malaria infection in pregnant women results in children born with low birth weight thereby increasing the risk of death (Steketee *et al*., 2001).

In addition, children who survive severe malaria may suffer long-term consequences of the infection such as reduced appetite, and restricted playing and social interaction, which may eventually affect their educational opportunities (Steketee *et al*., 2001). An estimated 2% of children who survive cerebral malaria suffer from learning impairments and disabilities due to brain damage (Murphy and Breman, 2001).

Despite causing mortality and morbidity, malaria is also a burden to African health systems expenditure. In some countries with heavy malaria burden, malaria may account for 40% of public expenditure, up to 25-35% of all outpatient clinic visits and 20-45% of all inpatient admissions (WHO, 2016b; DFID, 2010). Malaria also has direct costs to individuals in highly endemic areas. Individuals or families spend their income on insecticide treated nets (ITNs), antimalarial drugs, transport to health facilities, etc. The direct and indirect costs associated with malaria pose a substantial burden on poor households in SSA because people living in poorer households are more at risk of contracting malaria. This is most likely due to them living in dwellings that offer little protection against mosquitoes. They are also less likely to afford effective malaria treatment/ transport to health facilities or insecticide treated nets (Chima *et al*., 2003; Worrall *et al*., 2005).

1.5 Malaria control in sub-Saharan Africa

One approach used to reduce malaria transmission is through vector control. The most effective forms of vector control in SSA are the distribution of insecticide treated nets (ITNs) and indoor residual sprays (IRS). In this region, in 2014, 56% of the population received ITNs and 6% of the population at risk of malaria lived in households protected by IRS (WHO, 2015).

Administration of intermittent preventive treatment in pregnancy (IPTp) for malaria is another method of control used in SSA. However, the acceptance of ITNs and IPTp depends on cultural, behavioural and demographic factors (Govere *et al*., 2000).

There are several reasons why ITNs are not used in certain communities and these include: 1) Unpleasant smell produced by the nets (Sangare *et al*., 2012), 2) Chemicals used to treat ITNs are associated with family planning (Alaii *et al*, 2003), 3) Discomfort due to heat and humidity (Adam *et al*., 2008), 4) Educational status – low ITN usage is associated with low level education and the vice versa, 5) People from low density areas are more likely to own a bed net compared to those

from high density areas, 6) People from monogamous families are more likely to sleep under bed net than their counterparts from polygamous families (Aluko and Oluwatosin , 2012).

The number of women receiving IPTp in SSA remains below national targets (WHO, 2015). IPTp usage is limited mainly due to lack of knowledge regarding this method among pregnant women (Adam *et al*., 2008). The effective drugs being administered in SSA against *P. falciparum* are the artemisinin-based combination therapies (ACTs). In 2014, the African region accounted for 98% of ACT manufacturer deliveries (WHO, 2015). However, the progress in malaria control is being hampered by rapid development and spread of drug and insecticide resistance.

1.6 Malaria in South Africa

Despite great strides in malaria control, malaria remains a public health concern in SA. The disease is still endemic in the north-eastern areas of KwaZulu-Natal (KZN), Mpumalanga and Limpopo provinces. Members of the *An. gambiae* complex and *An. funestus* group are predominant malaria vectors in SA. *Anopheles gambiae s.s, An. arabiensis, An. merus* and *An. funestus s.s.* have been implicated in malaria transmission in this country (Maharaj *et al*., 2013). *Anopheles funestus s.s* was eliminated in the 1960s but resurfaced in 1999 and was re-eliminated after 2000. *Anopheles merus* only played a minor role in malaria transmission up to the present time (Coetzee *et al*., 2013). Currently, malaria transmission is presumed to be maintained predominantly by *An. arabiensis*, and approximately 90% of malaria cases in SA are attributed to *P. falciparum* infections (Maharaj *et al*., 2013).

1.7 Malaria vector control in South Africa

Malaria vector control constitutes the back-bone of successful malaria control in SA with insecticide residual spraying (IRS) of dwellings being at the center of this success. IRS has been in place for the past 70 years and various insecticides have been used (Coetzee *et al.*, 2013).

Currently, the South African malaria control programme uses synthetic pyrethroids to spray cement-brick structures and DDT is sprayed in traditional mud-walled structures, while in some instances carbamates are used (Brooke *et al*., 2013). Like any insecticide based vector control strategy the current approach has several shortfalls. The greatest challenge for vector control using

insecticides is the development of insecticide resistance in target vector populations because of the limited number of insecticides recommended for vector control. There are only four classes of insecticides (DDT, pyrethroids, organophosphates and carbamates) which share 2 modes of action approved by WHO for use in insecticide residual spray and insecticide treated bed nets (Constant *et al*., 2012). It has been known that insects, including mosquitoes, could become resistant behaviourally by avoiding exposure to lethal dose or physiologically by finding ways to survive a normally lethal dose to insecticides (Mullin and Scott, 1992). Behavioural mechanisms for resistance have been less studied than physiological (Sparks *et al*., 1989). Physiological resistance is divided into 3 mechanisms; reduced cuticular penetration, altered target size, increased metabolic detoxication or sequestration (Oppenoorth, 1985; Scott, 1991). Increased excretion is also a possible resistance mechanism though it has not convincingly demonstrated (Mullin and Scott. 1992).

However, multiple insecticide resistance may occur in the same mosquito population. Multiple insecticide resistance has been detected among major malaria vectors in Africa (WHO, 2012; Djouaka *et al*., 2016; Riveron *et al*., 2016). Multiple insecticide resistance mechanisms have been expressed in mosquitoes where a single mutation at a target site can result in mosquito resistance to DDT and pyrethroids or to organophosphates and carbamates (Perera *et al*., 2008). In *An. gambiae sl* mosquitoes, mutations in the DDT/pyrethroid target site [Knockdown resistance (*kdr*) alleles] have been found in conjunction with resistance acetylcholinesterase gene alleles (Ace-1^R), the target site of organophosphates and carbamates (Yewhalaw *et al*., 2011). This multiple resistance poses a big challenge in malaria control programmes. Hence, for effective vector control, there is need for application of intergrated vector control approach that can include SIT and other biological methods which are not insecticide based.

In addition, the adverse effects of these synthetic pesticides, such as high toxicity from residues in food, and contamination of water and the environment poses a health hazard to humans (Bhattacharyya and Mukhopadhyay, 2013). This approach is also proving to be challenging when applied in low malaria transmission situations where *An. arabiensis* is the main vector.

Anopheles arabiensis females feed on both cattle and humans, and rest both inside human habitations and outdoors (White, 1974) making it difficult to control them using IRS (Gillies and Coetzee, 1987). These concerns and the mandate by the South African government to eliminate malaria by 2020 necessitated the search for additional vector control strategies to complement the existing tools. One such vector control method is the Sterile Insect Technique (SIT) (Howell and Knols, 2009).

1.8 Other vector control strategies

Genetic control methods have been explored to achieve population suppression/ elimination e.g. Sterile insect technique (SIT) and to modify population achieved by replacement or alteration of the hereditary material with the aim of reducing the reproductive potential of insects e.g. Gene drives (Curtis and Graves, 1988). Advances in new genetic tools, such as gene drives and SIT have been explored for mosquito control. Gene drives are genetic systems that greatly increase the chance of inherited gene or set of genes to be passed on to offspring. The genes are subsequently spread to all members of the population (Macias *et al*.., 2017). Gene drives can be achieved by editing desirable site-specific genes with CRISPR/Cas9 systems (Macias *et al*.., 2017). Gene drive has been applied to prevent the spread of insects that carry pathogens such as mosquitoes that transmit malaria, dengue, and zika pathogens (Yen and Barr, 1973; Stouthamer *et al*., 1999). The first successful synthetic gene drive was demonstrated in the flour beetle (*Tribolium castaneum*) (Chen *et al*., 2007). In mosquitoes, the most successful gene drive was the development of "Flightless female" *Ae. aegypti*. This strain contains a genetic element that encodes a toxin to destroy the wing muscles of females.

Since the wings fail to function normally, these females are unable to mate, to reach food or to search places for ovipostion (Fu *et al*., 2010). The gene set that encodes these phenotypes is spread into the population by the males who are unaffected by the transgene.

These males are being released in Brazil and Florida as a control strategy to suppress *Ae. aegypti* population (Harris *et al*., 2012; Carvalho *et al*., 2015). Gene drive has also been successful in *Ae. albopictus* and *An. stephensi*, but have not yet been utilized to control wild population (Labbé *et al*., 2012; Marinotti *et al*., 2013).

Genetic control of mosquitoes using gene drives has an advantage over SIT in that gene drives can be applied to multiple mosquito species while as SIT can only target a specific species (Macias *et al*., 2017). However, gene drives might not be adopted under local settings due to technical complexity and challenges in public acceptability of releasing genetically modified mosquitoes coupled with tight regulatory approvals associated with them.

1.9 Monitoring and evaluation of vector control strategies

Knowledge of the local vector species and their susceptibility to insecticides as well as vector and human behaviours that may reduce contact is the key to effective malaria vector control (WHO, 2015). There is a need to periodically collect such data to evaluate the impact of vector control strategies on malaria transmission. It is also important to monitor the coverage, usage, quality and durability of vector control interventions following their deployment (WHO, 2015). Performance of vector control strategies varies greatly when employed in different settings and this has a bearing on the overall impact on malaria transmission. Therefore, evaluation of the impact of intervention methods on malaria outcomes is very important. There are a number of entomological parameters that can be assessed to evaluate the performance of vector control strategies in an area.

The entomological parameters (human biting rates (HBR), sporozoite rates (SR) and the entomological inoculation rates (EIR) determines intensity of malaria parasite transmission. The HBR is calculated as the total number of freshly fed mosquitoes of a particular species divided by the total number of human occupants in houses used for collection (Aju-Ameh *et al*., 2016). The SR is calculated as the number of sporozoites positive mosquitoes divided by the number of mosquitoes dissected (Omalu *et al*., 2015). The EIR is the average number of infectious bites, per person, per year in a given area; defined as the product of HBR and SR (Aju-Ameh *et al*., 2016). In experimental laboratory studies, the oocyst rate (OR) (calculated as total number of oocysts in female mosquito's mid gut divided by the total number of females examined) is used to determine mosquito infectivity (Ndiathi *et al*., 2011). The high rates of entomological parameters indicate high malaria parasite intensity and the vice versa.

In Africa, the EIR is variable and ranges from <1 to >1000 infection bites per person per year (Aju-Ameh *et al*., 2016). There are several factors that affect malaria transmission such as: (i) Human

factor- infectiousness of human carriers having gametocyte reservoir (Coosemans *et al*., 1992) (ii) Entomological factors – influenced by the natural environment and also its modification by man. Malaria transmission is enhanced by the increase in vector density, associated with high humidity, high temperatures (Omar *et al*., 2015) and availability of water pools due to irrigation or rainfall (Coosemans *et al*., 1992). (iii) Man-vector contact - the frequency of contact between man and vector is another important factor. High contact may lead to high malaria incidences (iv) Longevity of the vector also plays a significant role. Malaria transmission will only be possible if the vector lives long enough to complete sporogony (Coosemans *et al*., 1992). In Mamfene, malaria transmission is associated with irrigation schemes on the Makhathini flats. Stagnant water bodies in irrigation schemes act as mosquito breeding sites (Obala, 1995).

1.10 Sterile Insect technique

SIT relies on the mass production of target insects in mass-rearing facilities, sterilization, and mass release of the sterile insects into the natural habitat. The sterile insects are released in sustained numbers to outnumber the wild pest population (Vreysen, 2001). The released sterile males compete with wild males to mate with wild females. The sterile males inseminate wild females with sterile sperm, which results in the production of non-viable offspring leading to overall reduction of the target population. This technique was developed by Edwin F. Knipling as early as 1937 (Bartlett and Staten, 1996).

1.10.1 Advantages of the Sterile Insect Technique

As a vector control strategy, the SIT has several advantages as outlined below:

1) It is species-specific and the released sterile males can only mate with females of the same species. This enhances its efficacy in terms of reducing or eliminating the targeted insect/pest (Vreysen, 2001).

2) It is an environmentally friendly, non-polluting method. The chances of targeting other species beside the intended target species are minimal when employing this method and no toxic chemicals are released into the environment as is the case of using traditional insecticides (Alphey *et al*., 2010).

3) Released sterile males cannot establish in the released area since they provide no viable off-spring. This is unlike other releases in biological control programmes where introduced insects as control agents become established causing damage to crops (Benedict and Robson, 2003).
4) SIT can easily be integrated with other vector control methods already in place such as integrated vector management (IVM) programs using several approaches simultaneously (Dyck *et al*., 2005).
5) SIT can be cost effective since the sterile males released in targeted areas disperse themselves and can even invade protected areas or inaccessible areas, unlike chemicals where human intervention is required (Alphey *et al*., 2010; Bloem *et al*., 2005).

1.10.2 Disadvantages of the Sterile Insect Technique

Like any other vector control method, SIT also has limitations that need to be considered before project implementation. These are outlined below.

1) This technique is suitable in areas with a single dominant vector for a particular disease. Due to its species-specificity it might be very difficult to deploy in areas where several vector species need to be controlled simultaneously. Two vectors might be manageable but beyond that the technique might not be effective (Alphey *et al*., 2010).

2) Released sterile males can disperse anywhere in the area of release and dispersal cannot be controlled.
Insects with high dispersal range can migrate outside of the control area thereby reducing efficiency of the program while insects that do not disperse readily need to be released at fine spatial scale, which might increase program cost (Alphey *et al*., 2010).

3) SIT programmes do not show immediate effects on vector numbers. Reduction of vector population is observed in subsequent generations and hence impact is slow (Bloem *et al*., 2005).
4) Depending on the stage of the released insect, there might be density dependent effects. For example, when larval stages are released, they tend to compete for food and space thereby reducing their survival rate which may lead to reduction in efficiency of the SIT programme (Rogers and Randolph, 1984).

5) SIT requires cheap and alternative techniques that can effectively suppress populations to low densities on an area- wide basis. This also applies to areas where there is no human population or where there is very difficult terrain. The technique is less effective when the vector population is of very high density since this might require release of higher numbers of sterilized males (Rogers and Randolph, 1984; Vreysen, 2001).

1.10.3 Reasons for failures of SIT programs from mid-1950s to the mid-1970s

The reasons for the disappointing outcomes for SIT programmes that were conducted between mid-1950s to mid-1970s were commonly believed to be due to poor male competitiveness (as a result of the mass rearing and/or the effect of sterilization process), political interference and immigration of already mated males from nearby untreated areas (Becker *et al*., 2010). Some of the reasons are discussed below.

1) Low competitiveness of released sterile males

A field trial was conducted in 1969 on an island (5km^2) in Lake Kariba, Zimbabwe to control of *Glossina morsitans* Westwood. The adult flies that were sterilized after emergence and held in captivity suffered an 80% loss in field competitiveness (Klassen and Curtis, 2005). Another study was conducted in Pensacola, Florida where irradiated (110-180 Gy) *Aedes aegypti* were released for two seasons (1960-1961) by the Centers for Disease Control.

The released mosquitoes were not effective due to reduced competitiveness caused by irradiation in pupae stage (Dame *et al*., 2009).

2) Reduced longevity of released sterile males due to irradiation

In 1991, a large field trial to eradicate introduced Sea lampreys *Petromyzon marinus* from the Great Lakes (USA) was initiated and lasted for several years. Radiation sterilization was considered but yielded unsatisfactory results regarding male survival and competitiveness (Helinski *et al*., 2009).

3) Use of chemosterilants

Release of sterile male lampreys (*Petromyzon marinus*) in the Great Lakes (USA) using chemosterilant bisazir was also initiated. Chemosterilizing agents being mutagenic, present a potential hazard to humans during the treatment process, their use was abandoned (Helinski *et al*., 2006a). Concerns were raised about the effects of the chemicals on the environment and non-target organisms, particularly when large numbers of treated insect were released. Spiders that fed on a diet of only chemosterilized mosquitoes subsequently became sterile (Helinski *et al*., 2006a; 2009).

4) Change in mating behaviour of released sterile males

The release of sterile *Anopheles quadrimaculatus* males by the United States Department of Agriculture (USDA) in South Florida in 1959-1960 led to reduced incidence of mating with wild females due to a change in mating behavior influenced by colonization and this resulted in programme failure (Dame *et al*., 2009). Another study that showed assortative mating involved the release of *Culex tarsalis* in 1981. The released sterile males showed a significant inability to seek out and mate with wild females (Reisen *et al*., 1982).

5) Political interference/civil war

Large scale mosquito field trials were conducted in El Salvador and India in the mid-1970s. In El Salvador, the target was the malaria vector *Anopheles albimanus*. Unfortunately, the study was interrupted by eruption of civil war.

Similarly, in India, the study targeting *Aedes aegypti* never materialized due to the political problem encountered. There were false accusations that the project was intended to collect data on biological warfare (Klassen, 2009).

6) Influx of already mated females from outside the targeted release areas

The initial releases of sterile male *Culex quinquefasciatus* in India (mid-1970s) in targeted villages achieved only limited levels of sterility in eggs laid by wild females because of the influx of already- mated females from outside targeted release area (Robinson *et al*., 2009; Klassen, 2009).

1.10.4 Some of the major requirements for successful implementation of SIT programme

Successful implementation of a SIT programme depends on several factors and these need thorough investigation prior to project implementation. Some of these requirements are discussed below:

1) A comprehensive knowledge of the ecology and behaviour of the target population is required. Before SIT programme initiation, it is very important to know the ecology and behaviour of the targeted species. Mating barriers may exist between species complexes or cryptic species. In cases where strong pre-mating barriers exit between two or more sibling species of the target species, rearing and releasing only one type may be of limited benefit (Alphey *et al*., 2010). For example, in areas where *An. gambiae s.s.* and *An. arabiensis* are both major vectors and occur in sympatry, mating barriers between different chromosomal forms or types of these species may occur. However, SIT programs against agricultural pests have typically not found significant mating barriers between widely separated populations despite genetic differences (Cayol *et al*., 2002). Similarly, Girod *et al*., 2001 found no mating incompatibility between genetically distinguishable populations of *An. arabiensis*. It is also very important to know detailed information on population dynamics (spatial and temporal), mating behaviour, breeding sites and flight distance of the target population (Helinski *et al*., 2006b).

2) Colonization

The insect pest targeted for SIT should be amendable to colonization. The colony or strain characterization like fertility, fecundity etc. should be known. The cost of colonization and mass production of target insects should be reasonable in order to meet the required numbers for release (Helinski *et al*., 2006b). It is very important to have an estimate of the relative cost of the SIT programme before implementation. The cost will be determined by the area to be controlled and the number of sterile insects to be mass-reared and released. One way of reducing the cost is to release the sterile males when the targeted wild population is at its lowest because relatively low numbers of sterile males may be released to suppress the wild population (Alphey *et al*., 2010).

3) Sex separation system

It is preferable that insects released in an SIT programme shouldn't have unintended consequences (Klassen and Curtis, 2005). In a mosquito SIT programme, the release of sterile females is not

acceptable due to the risk of disease transmission (Robinson *et al*., 2009). It is therefore a prerequisite that all females be removed from the released population. A number of separating methods have been used in SIT programmes:

(i) *Morphological separation* – In most insects, the external morphology in pupal or adult stages has been used to separate males from females (Klassen and Curtis, 2005). Sexual dimorphism in size can be used to separate the sexes in some mosquitoes such as *Aedes* mosquitoes, *Culex quinquefasciatus* (Papathanos *et al*., 2009) and in Lepidoptera but the consideration overlap in the size renders the sorting inefficient (Parker, 2005).

(ii) *Classical genetics* – This approach confers a selectable trait to males with the aim of eliminating females. In mosquitoes, a selectable trait such as insecticide resistance is translocated to the male determining factor such as the Y-chromosome (Curtis *et al*., 1976). A Genetic Sexing Strain (GSS) has been successfully established in *An. gambaie* (Curtis *et al*., 1976), *An. albimanus* (Kaiser *et al*., 1978) and *An. arabiensis* (ANO IPCL) based on dieldrin resistance (Oliva *et al*., 2012; Yamada *et al*., 2012).

An effective sexing strain has been developed for Mediterranean fruit fly based on a temperature sensitive lethal mutation where a white pupae (wp) mutant is linked to the temperature sensitive lethal (tsl) mutation and male determining chromosome. Male pupae are brown and female pupae are white and thus provide a visual indicator for the GSS stability (Franz, 2005). Various GSS have been developed for 20 insect species (Robinson, 2002) but very few strains have been developed to the point of being mass reared and released in area wide integrated vector management (AW-IVM) (Papathanos *et al*., 2009). Elimination of females based on exposure of eggs to insecticide or high temperature can reduce rearing costs during mass production and gives flexibility for the insectlife stage to be released (e.g pupae or adult; Papathanos *et al*., 2009).

(iii) *Transgenic methods* – Several new methods have been proposed such as sex-linked expression of a fluorescence-protein, combined with fluorescence-based sorting. This has been achieved in a Mediterranean fruit fly GSS where separation was achieved by scoring newly hatched larvae for the fluorescent trait (Condon *et al*., 2007; Papathanos *et al*., 2009). Sex limited expression of transgenes has also been achievedin *An. stephensi* where a sperm specific promoter from the *An*.

gambiae β2-tubulin gene was used to express the fluorescent protein and conditional female-specific lethality where females die during larval development (Catterucia *et al*, 2000; Fu *et al*., 2007).

4) Low population density

There is an advantage to having very low population density of the target insect in the control area before program initiation. This can be achieved by employing economically feasible suppression techniques such as biological control methods or insecticide sprays before sterile insect release (Vreysen, 2001). The releases may also be timed to coincide with natural seasonal declines in the numbers of target pests, which is mostly observed in the winter months of the year (Becker *et al*., 2010).

5) Sterile male competitiveness

An SIT programme can only be successfully applied if the released sterile males can successfully compete for wild females against wild males.

Therefore, there is a need to thoroughly evaluate the competitiveness of the sterile male insects that are released. Competitiveness of sterile male insects is compromised by various factors such as colonization, sterilization (Helinski *et al*., 2006a), handling, distribution andgenetic manipulations. These factors may reduce quality of released males such as longevity, dispersal as well as the ability to find and successfully court females (Kaiser *et al*., 1979; Benedict *et al*., 2009; Alphey *et al*., 2010). For example, colonized males are homogeneous entities due to bottlenecking which select specific traits required to survive under artificial conditions and therefore not as genetically diverse as wild populations and this might alter mating ability (Norris *et al*., 2001). The modified insectary environment has been shown to lead in fixation of alleles or behaviour that can result in assortative mating between the released colonized sterile males and wild females (McDonald, 1979). This was observed in colonized *Cx. tarsalis* in which released sterile colony males swarmed in different arenas compared to wild vectors (Reinsen *et al*., 1982). This was also reported in *An. culicifacies* (Reinsen *et al*., 1981) and *An. albitarsis* (Lima *et al*., 2004) which had been under colonisation for several generationsGenetic manipulation during development of genetic sexing strains particularly mutations and chromosome rearrangements of the GSS strains have been shown to significantly reduce physiological fitness (Alphey *et al*., 2010).

However, despite these adverse effects of colonization, the mating competitiveness of colonized males can be enhanced in different ways which include:

(i) Creating an ideal rearing environmental conditions that can increase the mating competitiveness during laboratory rearing regimes (Ng'habi *et al*., 2005) i.e. increasing the mating rank. This can be done by reducing larval crowding to reduce competition of food. It has been shown that larval crowding has a strong effect on the mating competitiveness of male mosquitoes (Ng'habi *et al*., 2005).

(ii) Increasing adult male size – Low larval crowding results in emergence of large males (Bargielowski, 2010). It has been shown that body size has a strong effect on the mating competitiveness of male mosquitoes. Studies conducted by Ng'habi *et al*., (2005) and Bargielowski, (2010) on *An. gambiae* and *Ae. aegypti* mosquitoes respectively, showed that:

- Larger males were more likely to acquire mates than smaller individuals.
- Larger males were more likely to acquire the first mating. Large males of *An. gambiae* won females by approximately 11 times more often than the smaller males.
- The proportion of total matings between larger males and smaller ones did not differ. However, the females showed no propensity to re-mate over several gonotrophic cycles.

(iii) Increasing teneral reserves – Males with high teneral energy reserves have a competitive advantage over males with low teneral energy reserves (Kaspi and Yuval, 2000). In *An. gambiae s.s*., males with sugar reserves showed high insemination capacity and high survival rate at temperatures, 23˚C and 27˚C than males without sugar reserves (Gary Jr. *et al*., 2009). Females are more likely to oviposit when mated with young males (2-3 days) than when mated with older males (Chambers and Klowden, 2001). High teneral energy reserves in colonized males can be achieved by lowering larval density. However, some species like *An. gambiae s.s*. inherently build a very small teneral reserves (Briegel, 1990). Males that emerge from low teneral reserves are more vulnerable to starvation if they are unable to acquire sugar soon after emergence (Magnarelli, 1986; Foster and Takken, 2004).

(iv) Heterosis (Hybrid vigour) - Enhanced hybrid males can be produced in colonized insects to increase their mating ability. Heterosis has been achieved in *An. coluzzii* by crossing two colonized

strains, > 35 and 8 years old. Heterotic males were more fertile, increased female fecundity and showed increased longevity (Ekechukwu *et al*., 2015). Hybridization of two colonized *An. gambiae* strains evaluated for 20 generations led to increased performance in fecundity, body size and adult longevity (Menge *et al*., 2005). Heterosis can also be achieved by refreshing laboratory strain with newly wild caught material (Baeshen *et al*., 2014).

6) Capability of released males to disperse.

The males earmarked for release males should be able to disperse for effective SIT programme. The dispersal ability of males is crucial in determining appropriate release strategies before programme implementation. Male dispersal rates can be investigated using mark-release-recapture (MMR) techniques (Verhulst, 2013).

However, there is also a need to investigate environmental conditions of the area targeted for release because dispersal is affected by environmental factors such as wind/wind direction (Midega *et al*., 2012), rainfall (Hausermann, 1971), vegetation, humidity (Lacroix *et al*., 2012), topography (Thomas *et al*., 2013) etc. In mosquitoes, dispersal rates are greatly affected by human settlement (Trpis and Ha¨usermann, 1986). Both male and female mosquitoes travel longer distance in uninhabited areas than in inhabited areas (Harrington *et al*., 2005). Even though male mosquitoes do not blood feed, they are readily recaptured in households due to their mating behaviour (Hartberg, 1971; Harrington *et al*., 2005). Due to the effects of colonization, the mean dispersal rates of both male and female mosquitoes are lower than their wild counterparts (Lacroix *et al*., 2012; (Hausermann, 1971).

7) Isolated population

The SIT is very effective when applied to the total target population or part of the population that can be isolated by natural or artificial barriers such as mountain ranges, lakes/oceans etc. (Helinski *et al*., 2006b). The immigration of fertile insects from non-targeted breeding sites reduces programme efficiency hence the artificial or natural barriers play an important role in preventing immigration of fertile insects from neighbouring sites outside the area of intervention.

8) Effective collaboration with stakeholders

It is also very important to pay attention to the political climate, economic and environmental realities and community sensitivities in the intended control areas before programme implementation. Excellent communication skills with the public are needed as well as collaboration with local and international stakeholders to seek their assistance in programme implementation (Robinson *et al*., 2009).

1.11 Sterile Insect Technique for major agricultural pests

The SIT has been shown to be an effective and sustainable genetic approach to control populations of major pest insects when used with other conventional pest control methods as part of Area-Wide Integrated Pest Management (AW-IPM) programmes (Robinson *et al*., 2009).

Sterility in insects has been achieved by ionizing radiation (irradiation) as well as chemosterilization. Sterile insect technique releases have been performed in an effort to control or eradicate various agricultural insect pests which include: *Cochliomyia hominivorax, Zeugodacus cucurbitae, Glossina* species, *Pectinophora gossypiella* etc. Table 1.1 summarizes some of the SIT releases that have been performed globally against major agricultural insect pests. In South Africa, SIT was initiated in 1996 to create fruit-fly free areas in the Western Cape (Barnes *et al*., 2015). In 1997, an SIT pilot project was initiated in the Hex River Valley with a view to suppress Ceratitis capitata (Mediterranean fruit flies) in an economical and environmentally compatible manner. This valley was ideal for SIT because it is isolated from other areas that may have fruit flies and a cost-benefit study showed that there were reduced estimated SIT control costs of this pest compared to the conventional control methods (Barnes *et al*., 2015). This technique has also been employed to control false codling moth (Thaumatotibia leucotreta) on citrus fruits (Hofmeyr *et al*., 2015) and codling month (Cydia pomonella) on apples and pears (Barnes *et al*., 2015).

Table 1.1: Some of the SIT programmes employed against major agricultural pests around the world.

Target Pest	**Study area**	**Aim**	**Result**	**Reference**
New World screwworm fly (*Cochliomyia hominivorax* Coquerel)	North America, Central America and Panama	Population eradication	Successfully eradicated (1957 - 1966)	Bartlett and Staten, 1996; Dame *et al*., 2009
	Mexico	Population eradication	Successfully eradicated (1972 - 1991)	Bartlett and Staten, 1996; Dame *et al*., 2009
	Libya	Population eradication	Successfully eradicated (1988 - 1992)	Vargas-Terán, *et al*., 1994
Melon fly (*Dacus cucurbitae* Coquillett)	Okinawa, Japan	Population eradication	Successfully eradicated in 1990s	Hendrichs, 2000
Mediterranean fruit fly (*Ceratitis capitata* Wiedemann)	Chile, southern Peru and South Mexico	Population eradication	Successfully eradicated (1977 - 1982)	Hendrichs, 2000
	South Africa, Hex River Valley	Population suppression	Population reduction was observed (1997 – 2006)	Barnes *et al*., 2015
Tsetse fly (*Glossina austeni* Newstead)	Unguja Island in Zanzibar (Tanzania) and Upper Volta	Population eradication	Successfully eradicated (1994 – 1997)	Vreysen, 2001
Pink Bollworm (*Pectinophora gossypiella* Saunders)	California, USA	Population suppression	Population successfully suppressed (1970 – 1996)	Staten *et al*., 1993

Mexican Fruit Fly (*Anastrepha ludens* Loew*)*	Along the USA-Mexican border	Preventing migration (quarantine)	Migration has successfully been prevented since 1964	Hendrichs, 2000
Codling moth (*Cydia pomonella* Linnaeus)	British Columbia	Population eradication	Population reduction was observed (1990 – 1994)	Bloem *et al*., 1997; Hendrichs, 2000.
False codling moth (*Thaumatotibia leucotreta* Meyrick)	South Africa, Olifants River Valley	Population suppression	Population reduction was observed (2002 – 2010)	Hofmeyr *et al*., 2015

1.12 Sterile Insect Technique for mosquitoes

In the mid-1950s to the mid-1970s determined efforts were made to develop SIT for mosquito suppression. Field trials demonstrated the feasibility of SIT against mosquitoes (Alphey *et al*., 2010). These trials were conducted to explore and validate aspects of the technique and sometimes to attempt to control mosquito populations (Benedict and Robinson, 2003). The target species for mosquito SIT have included: *Ae. aegypti*, *Ae. albopictus*, *Cx. pipiens*, *Cx. tritaenio-rhynchus*, *Cx. quinquefasciatus*, *An. alimanus*, *An. culicifacies*, *An. gambiae* and *An. arabiensis* (Becker *et al*., 2010). Although results of these early initiatives were promising, the potential of SIT as a malaria vector control tool was never achieved mainly because the projects were never completed due to a variety of reasons, among them technical and operational problems (Klassen, 2009). Nevertheless, much biological and operational knowledge on mosquito SIT was gained. With recent advances in molecular biology which has helped in addressing several technical, biological and operational issues, the use of the SIT in mosquito control has regained new interest. Table 1.2 summarizes some of the mosquito releases related to SIT.

Table 1.2: Some of the SIT projects against different species of mosquitoes around the world.

Mosquito species	**Study area**	**Aim**	**Result**	**Reference**
An. quadrimaculatus	South Florida, USA (1959-1960)	Population reduction	There was a change in mating behavior of the colonized sterile males which resulted in lack of sterility in wild population and consequently programme failure.	Dame *et al*., 2009
An. gambiae	Burkina Faso (1968-1969)	Population reduction	Released sterile males lacked competitiveness and there was no significant effect on sterility of eggs.	Davidson *et al*., 1970
	Egypt (1942-1945)	Population eradication	Population was successfully eradicated	Shousha, 1948
An. albimanus	El Salvador (1977-1979)	Population reduction	No effect was observed due to immigration of females from untargeted areas.	Weidhaas, 1974
An. arabiensis	Austria (2006)	Evaluating the effect of irradiation (0 - 100 Gy) on adult emergence, male survival, induced	All doses tested had no effect on adult emergence. Survival of irradiated males was similar or slightly higher than non-irradiated males. There was negative	Helinski *et al*., 2006a

		sterility and insemination capability.	correlation between insemination rate and dose.	
	Khartoum, Sudan (2008)	Insemination capability.	Irradiated males were able to inseminate wild females equally the same as wild males.	Helinski *et al.*, 2008
	Merowe area, Sudan (2014)	Assessing the capability of released sterile males in swarming participation.	Irradiated males were able to participate in swarming and dispersed for a long distance.	Ageep *et al*., 2014
Cx. quinquefasciatus	New Delhi/ India in 1971	Population reduction	Sterile males were competitive and induced sterility in wild population but immigration of fertile females from other breeding sites reduced programme efficiency.	Dame *et al*., 2009
Cx. tarsalis	California, in 1981	Population reduction and assessment of mating behaviour	A change in mating behavior due to colonization (Assortative mating) was observed as a result there was no population reduction.	Reisen *et al*., 1982

Cx. pipiens	France (1970)	Population reduction and semi sterility	Population reduction was observed and population was eventually eliminated.	Laven *et al*., 1972
Ae. aegypti	New Delhi/ India in 1971	Population eradication	The project never materialized due to political interference. There were false accusations that the project was intended to collect biological warfare data.	Klassen, 2009
	Pensacola, Florida, USA (1960-1961)	Population reduction	Wild population was observed in contracted release areas but un-expected immigration of fertile females from other breeding sites reduced the effect.	Dame *et al*., 2009

To date no active SIT programs are in place in South Africa for public health pests such as mosquitoes. Currently there are laboratory and field studies being conducted to ascertain the feasibility of using SIT to control *An. arabiensis*. One of the sites being used to evaluate this technique is Mamfene in northern KwaZulu-Natal (KZN). Mamfene was chosen because it supports a seasonal population of *An. arabiensis* and is in a malaria endemic area.

Overall, literature on SIT depicts varying successes but it has proven to be a safe, effective and environmentally sound method to suppress, eliminate or contain particular insect populations (Dame *et al*., 1981; Helinski *et al*., 2008).

1.13 Study rationale

Amongst the three provinces where malaria is endemic, KZN meets the World Health Organization (WHO) criteria suggesting that malaria control can be scaled up towards the elimination phase in this area. In this province, all districts have a malaria incidence of <1/1000 population at risk (Maharaj *et al*., 2013). However, elimination is currently compromised by various factors amongst others, the presence of *An. arabiensis*. This species is pleiotropic in its behavior therefore additional vector control interventions are essential. In addition to this species behavioral challenge, insecticide resistance has also been reported (Brooke *et al*., 2015). For these reasons, SIT research has been initiated in the country (Munhenga *et al*., 2014; 2016). Investigations into a potential field site were initiated and basic entomological information on species presence, seasonal abundance, a genetic sexing strain and mating competitiveness were completed in the northeastern parts of the Kruger National Park (Munhenga *et al*., 2011; 2014: 2016). For evaluation of the impact of the SIT intervention, a control site with similar species dynamics are essential and this was not available in studies conducted by Munhenga and others (Munhenga *et al*., 2011; 2014; 2016) and the field site was abandoned. An additional field site, a reliable genetic sexing strain and fit sterilization of males are needed.

1.14 Study aim and objectives

The primary aim of this study was to evaluate the use of a local genetic sexing strain of *An. arabiensis* in SIT activities and to determine the population dynamics of *An. arabiensis* in Mamfene, KwaZulu-Natal, South Africa.

1.14.1 Specific objectives

1) Establish a local genetic sexing strain (GSS) of *An. arabiensis* through introgression with an existing GSS.

2) Assess the effect of irradiation on the *An. arabiensis* (males and females) fitness and susceptibility of females to *P. falciparum*.

3) Assess the mating competitiveness of the new local genetic sexing strain under laboratory and semi-field conditions.

4) Determine the population dynamics of *An. arabiensis* at Mamfene, KwaZulu-Natal, South Africa.

CHAPTER 2

DEVELOPMENT OF A GENETIC SEXING STRAIN OF *ANOPHELES ARABIENSIS* FOR KWAZULU-NATAL, SOUTH AFRICA

2.1 Introduction

The South African National Malaria Control Programme aims to eliminate malaria within South Africa by 2020 (Malaria Elimination Group, 2009). However, the current malaria vector control interventions are unlikely to achieve elimination. In search of additional vector control strategies to the existing tools, the use of sterile insect technique (SIT) was investigated.

In this chapter, the local genetic sexing strain of *An. arabiensis* (GMK) was established. The attributes of this strain (emergence rate, developmental time, survivorship, genetic stability) from immature to adult stage were assessed and compared to the parental strains in order to make an informed decision whether the strain could be ideal for use in SIT programme.

A manuscript emanated from this chapter, and has been accepted for publication in *Medical & Veterinary Entomology*. A copy of the manuscript is attached in section **2.3.1**

2.2 Materials and methods

A brief description of methods and materials are presented in the manuscript attached (section **2.3.1**). However, a detailed methodology on: (a) female elimination described by Yamada *et al*., (2012), (b) DNA extraction and detection of *rdl* mutation using hydrolysis probe assay with slight modifications described by Bass *et al*., (2008) and Du *et al*., (2005), and (C) DNA extraction as described by Collins *et al*., (1987), are presented Appendix 1.

2.3 Results

The results of this chapter are presented in the manuscript attached in section **2.3.1**.

2.3.1 Leonard C Dandalo, Givemore Munhenga, Maria L Kaiser and Lizette L Koekemoer· **Development of a genetic sexing strain of *Anopheles arabiensis* for KwaZulu-Natal, South Africa**. *Medical & Veterinary Entomology* (2017), doi: 10.1111/mve.12264).

1) **Contribution**

I conducted all the experiments outlined in the paper, did data analysis and interpretation of results, and wrote the first and subsequent revised drafts of the manuscript.

CHAPTER 3

MATING COMPETITIVENESS OF THE LOCAL STERILE GENETIC SEXING STRAIN MALES (GMK) OF *ANOPHELES ARABIENSIS* UNDER LABORATORY AND SEMI-FIELD CONDITIONS

3.1 Introduction

The successful implementation of any SIT is largely dependend on the ability of mass-reared and sterilized laboratory males to locate wild females and compete for mating with wild males (Dame *et al*., 2009; Alphey *et al*., 2010). However, the many developmental processes a strain under goes before it can be used as an SIT release strain alters its genotype and the corresponding phenotype leading to reduced physiological and reproductive fitness. The quality of sterile males to be used in SIT programme depends on several factors including capacity to survive (longevity), body size, actively disperse (flight ability) and mating ability. Most of these attributes except sexual competiveness were evaluated during the development of the GSS strain and are presented, and discussed in Chapter 2. Sexual competiveness and ability of mass-rered sterlised males to seek for mates under under feld conditions is an essential parameter which should be established prior any sterile releases. There are numerous factors which impact on the mating quality of mass-reared males which include loss of natural characteristics during adaptation to lab rearing and during induction of sterility using ionizing radiatio. A through review of these factors is given in Chapter 1.

There are several studies on the mating competitiveness of genetic sexing strains of anopheline mosquitoes such as *An. albimanus* (Kaiser *et al*., 1978), *An. arabiensis* (Yamada *et al*., 2014; Munhenga *et al*., 2016) and *An. coluzzii* (Maïga *et al*., 2014). In these studies, the competitiveness of males shows high variation between and within strains highlighting the importance of doing a specific competitive assay of the newly developed GSS strain.

The aim of this study was to evaluate the mating competitiveness of laboratory-reared and sterilized males drawn from locally established genetic sexing strain (GMK), as well as determining the optimal irradiation dose required to induce male sterility in the newly established strain.

3.2 Materials and methods

3.2.1 Study site

Competitive assays were divided into three components: dose optimizations, laboratory and semi-field assays. The laboratory competitive assays and dose optimization experiments were both carried out in the Botha De Meillon insectary. Semi-field competitive experiments were carried out in large field cages (made from Anti Thrip Netting (2.9m diameter x 2.0m high with floor) (Figure 3.1A) under natural environmental conditions at the NICD premises between November 2015 May 2016 targeting the onset and the end of the rainy season. Temperature and relative humidity were logged every 1 hour using HOBO data loggers (Cat No: 19088743, Onset Computer Corporation, United States of America). The NICD is situated in Gauteng Province, Johannesburg, South Africa. The site is surrounded by *Acacia robusta*, *Acacia xanthoploea* (Fever tree) which provided canopy and shade to the field cages during competitive experiments.



Figure 3.1: Experimental set-up for semi-field competitiveness assays A) semi-field cages under tree canopies which provided shading during experiments; B) a white tray filled with water for additional humidity and a plastic jar with sucrose solution soaked in cotton wool to provide energy to the mosquitoes; C and D) mosquito resting containers.

3.2.2 Biological material

For all experiments in this section two *An. arabiensis* laboratory strains were used. The first genetic sexing strain (GSS) used is denoted GMK. This strain was developed by mating **GAMA** males with KWAG females. The GAMA strain is an introgression of *An. arabiensis* GSS ANO IPCL1 males. [carrying a dieldrin resistance gene on the Y chromosome provided by the Insect Pest Control Laboratory (IPCL), Seibersdorf, Austria] with *An. arabiensis* females colonized from material collected in 2010 from Kruger National Park acronymed **AMAL** (Munhenga *et al*., 2011).

The KWAG strain is the wild *An. arabiensis*colonized from Mamfene, Kwa-Zulu Natal in 2005 (Mouatcho *et al*., 2009)].

Larvae were fed on a daily basis with approximately 30mg of larval food [(a mixture of brewer's yeast (Vital Health Foods, South Africa) and finely ground dog biscuits (West's traditional crunching biscuit treats, Martin and Martin, South Africa) prepared at a ratio of 1:3)]. All adults were fed on a 10% sucrose solution soaked on cotton pads. The females of all the strains in this insectary are mainly blood fed on anaesthetized guinea pigs twice a week for egg production (Ethical clearance M141023, Appendix 2A).

3.2.3 Mosquito separation

A standard sex separation method using terminalia morphology at pupae stage (Harbach and Knight, 1980) was used in all the experiments to ensure that the age of mosquitoes for both controls and treatments was relatively the same. In all cases 20–26 hours' pupae were physically separated using stereomicroscope. GMK_1 males were obtained by physically separating male pupae from the GMK colony. Similarly, wild type KWAG male and female pupae were separated from the wild type KWAG colony to obtain males and females respectively.

For the semi-field competitive assays pupae were separated daily for four consecutive days to obtain enough numbers/ sample size for the experiments and the mosquitoes were mixed to avoid the confounding effect of age. In all treatments mosquitoes (fertile males, sterile males and virgin females) were maintained in separate cages with sugar water soaked in cotton wool for 2 days (laboratory assays) and between 2-4 days (semi-field assays) after emergence to allow for sexual maturation. The females were kept in separate cages from the males to maintain their virginity.

3.2.4 Mosquito irradiations

Pupae aged 20-26 hours used during these experiments were exposed to ionizing (gamma) radiation using a Gammacell 220 (MDS Nordion, Ottawa, Canada) (Figure 3.2) that was thoroughly dose mapped (Appendix 3) to ensure that pupae evenly obtained the desired dose (Munhenga *et al*., 2016). In all experiments pupae were transported to an irradiation facility approximately 800m from the insectary in batches of 100-500 suspended in 150ml of distilled

water in a doughnut irradiation jig (Figure 3.3). For the laboratory and semi-field assays, the optimal irradiation dose obtained in section **3.2.5** was used to induce sterility to males.



Figure 3.2: Gammacell 220 (MDS Nordion, Ottawa, Canada) used during all irradiation procedure



Figure 3.3: Doughnut shaped irradiation jig used during pupal irradiations

3.2.5 Determining the optimal irradiation dose

To determine the optimal dose that induces sterility without affecting the mating competitiveness of adult males, the effect of ionising radiation on male pupae was assessed at five different doses based on dose-response curves for *An. arabiensis* obtained by Helinski *et al*., (2006a) where irradiation dose ranging from 25–100 Gy had little impact on pupal survival, adult longevity and fecundity of females mated with irradiated males. However, it is very important to determine the optimal irradiation dose because it has been shown that a lower irradiation dose results in insufficiently sterilized males whereas a high dose reduces competitiveness of sterilized males (Helinski *et al*., 2006a). Against this background, irradiations during this study were carried out at increasing doses of 0 (control), 65, 70, 75, 80 and 100 Gy. After irradiation, the irradiated males were mated with virgin wild type KWAG females and the adult emergence, adult longevity, female fecundity and egg hatch rates (fertility) were measured as fitness parameters to select the optimal dose. For each single irradiation dose, three replicates and two controls were set up. The first control (Baseline) consisted of wild type KWAG males mating with wild type KWAG females. This was a fertile control representing the wild type. The second (separation) consisted of unirradiated GMK_1 males mating with wild type KWAG females treated the same way as irradiated pupae except that they remained un-irradiated. This control was set up to estimate the effect of physical handling and stress exerted on pupae during sex separation.

i) **Emergence**

The irradiated pupae from each dose (described in section **3.2.5**) were allowed to emerge in 30cm x 30cm x 30cm BugDorm-1Insect Rearing Cages® (Mega View Science Co., Ltd, Taiwan) under standard insectary conditions (Hunt *et al*., 2005). For each treatment and controls, three replicates were set up. The adult mosquitoes emerging from irradiated pupae and were able to fly out of a bowl were recorded and emergence was calculated as percentage of adults emerged. Dead pupae or semi-emerged adults were scored as non-emerged. The emerging adults from each irradiation dose were separated into two groups to determine adult survivorship rates and fecundity.

ii) **Adult longevity**

For each irradiation dose tested, fifty newly emerged adult males from the GMK_1 irradiated pupae [obtained from **3.2.5** (i)] were placed in BugDorm-1Insect Rearing Cages®. Two controls were

also set up, the fertile and separation control as described in section **3.2.5** throughout all three replicates. Adults were maintained on 10% sugar solution soaked in cotton wool at standard insectary conditions. Survival in each cage was assessed daily by counting the number of dead individuals until 100% mortality was reached. The same experimental set-up was used for controls; wild type KWAG and unirradiated GMK_1 males.

iii) **Fecundity**

Fifty newly emerged virgin wild type KWAG females (obtained from section **3.2.3**) and 50 newly emerged irradiated GMK_1 males from each dose [obtained from **3.2.5** (i)] were allowed to mate for 4 days in BugDorm-1Insect Rearing Cages® (with three replicates) over a five-day period. After the second blood meal, females were left to digest blood for two days. On the second day 20 randomly selected females were individually tubed in oviposition glass vials with a damp filter paper disc to induce oviposition and left overnight (Choi *et al*., 2014). The following day, eggs laid by each female were counted using an Optical Glass Binocular Magnifier (Cat. No: DA-5, Donegam Optical Company, United States of America). Two controls were set for each replicate (i) 50 virgin males from wild type KWAG colony mating with 50 virgin females from wild type KWAG colony as a fertile control. (ii) 50 virgin GMK males from pupae handled as irradiated males except irradiation mated with 50 virgin wild type KWAG females (Separation control). In each treatment, the mean number of eggs laid was calculated (Total number of eggs laid divided by total number of females that laid eggs). Fecundity of females was compared between treatments and controls.

iv) **Fertility**

Eggs produced by each individual female from each treatment and controls were transferred into plastic bowls (27cm x 16cm x 6.5cm) containing 150ml of distilled water and allowed to hatch. Upon hatching, larvae were counted daily by removing any hatched larvae and recording for 10 consecutive days (Munhenga *et al*., 2016).

The larvae picked daily were then killed using hot water and discarded. The proportion of eggs hatching was determined and compared between treatments and controls.

3.2.6 Mating competitiveness of the irradiated GMK_1 males

An important aspect that needs thorough investigation before implementation of any mosquito SIT is the mating competitiveness of the strain earmarked for release. To assess the relative mating competitiveness of sterilized males and to measure the effect of increased release ratios on hatch rates, competitiveness experiments were conducted under both laboratory (controlled environmental conditions) and semi-field conditions (natural conditions). These experiments were conducted at increasing ratios of sterile to fertile males (normal females: normal males: sterile males): 1:1, 1:3 and 1:5. It has been shown that irradiation reduces the mating competitiveness of males (Helinski *et al*., 2006a) hence the need to assess competitiveness at different irradiated male ratios to determine the best ratio that can induce sterility in the wild population. The two controls were included to assess the fecundity and fertility between fertile and sterile cohorts. Controls for both fertile and sterile males were performed at a ratio of 1:1 males to females.

The mating competitiveness of sterile GMK_1 males was assessed by looking at parameters such as insemination rates and fecundity of the recovered wild type KWAG females from laboratory and semi-field cages. The competitiveness values and expected egg hatch rates were computed using the Fried Index (1971). Mean egg hatch rate data for treatments and controls were used to compare the observed fertility rate in the competition groups with the controls to give a value of competitive index 'C' for each treatment cage, as described by Fried 1971, using the formula:

$$C = \frac{N}{S} \times \frac{Hn - Ho}{Ho - Hs}$$

Where (Hn) = is the mean hatch rate for fertile control cages (Hs) = mean hatch rates for sterile control cages, (Ho) = mean hatch rate for treatment cages, N is the number of "normal" males (untreated) and S is the number of sterile males. Competitive values normally range from zero to one where one indicates full competitiveness.

i) **Competitiveness of irradiated GMK_1 males under laboratory conditions**

The sterile and fertile males were introduced into 5 liter cages (Figure 3.4) containing virgin females under the following ratios of sterile to fertile males: The mating competitiveness of irradiated mosquitoes under laboratory conditions was determined by releasing these mosquitoes in 5 litre cages using a mechanical aspirator in the following ratios:

Cage 1- Control fertile: 50 wild type (WT) KWAG females + 50 WT KWAG males

Cage 2- Sterile control: 50 WT KWAG females + 50 irradiated GMK_1 males

Cage 3- Treatment 1:1:1: 50 WT KWAG females + 50 WT KWAG males +

50 irradiated GMK_1 males

Cage 4- Treatment 1:1:3: 50 WT KWAG females + 50 WT KWAG males +

150 irradiated GMK_1 males

Cage 5- Treatment 1:1:5: 50 WT KWAG females + 50 WT KWAG males +

250 irradiated GMK_1 males

Two different experimental set ups were used to determine the fertility and fecundity. The first was as described in section **3.2.5** but insemination rate was also included. The insemination rates were determined by dissecting the spermatheca of a sub-sample of randomly selected females from each treatment and controls under a dissecting microscope (Wild, Heerbrugg M5-71661, Switzerland). Each female's spermatheca was dissected and the presence of spermatozoa was assessed at 200 X magnification. The proportion of inseminated females was then calculated. All experiments were replicated three times. All females that remained in treatment and control cages were killed using hot water.

The second experimental set up used 20 randomly selected gravid females from each treatment and controls and were allowed to lay eggs *en masse* (Mass egg plating) in each separate 5 litre cages to compare results to be obtained from the two experimental set ups. Eggs per female were calculated based on number of live females which were present on the night oviposition plates were put in the cages. Fecundity was scored as the number of eggs laid by females per single gonotrophic cycle.

Egg hatch rates (fertility) and insemination rates were determined as described in sections **3.2.5** and **3.2.6** respectively. This set of experiments was repeated three times.

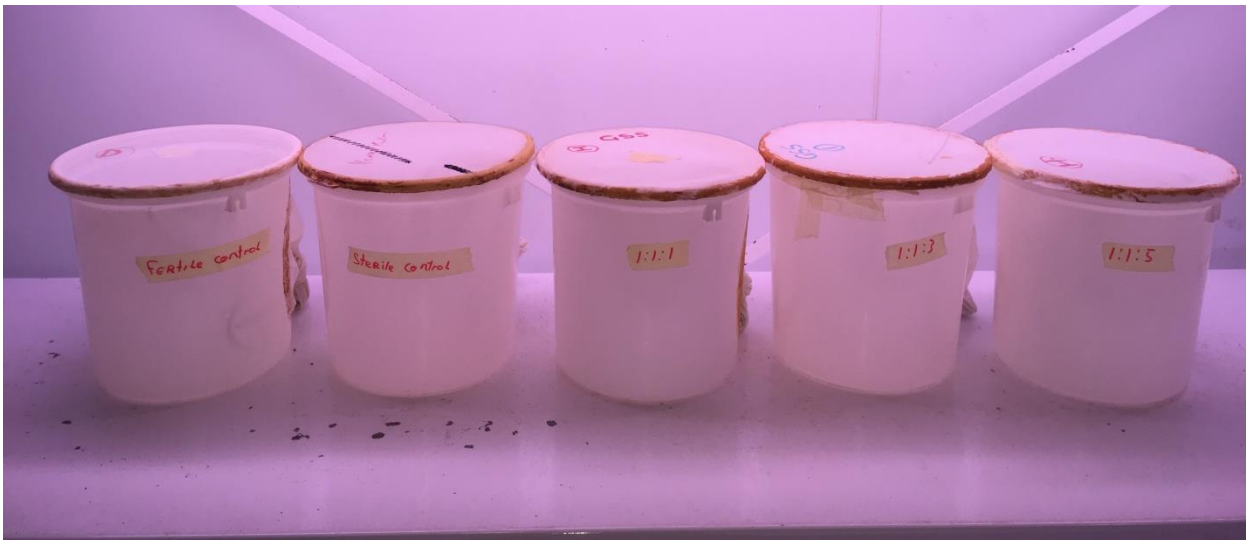


Figure 3.4: Experimental set up for the laboratory competitive assays.

ii) **Competitiveness of irradiated GMK_1 males under semi-field conditions**

A study of mating competitiveness of irradiated GMK_1 males under natural conditions in field cages was carried out to determine their mating vigour. Irradiated GMK_1 males, wild type KWAG males and wild type KWAG females were transported to appropriate site approximately 250 meters away from the Botha DeMeillon insectary as adults. These experiments were conducted from late spring (November) through summer (December) to late autumn (May) in large field cages. Temperature and humidity were monitored continuously using HOBO data loggers (Cat No: 19088743, Onset Computer Corporation, United States of America). These cages allowed simulation of prevailing ambient weather under natural conditions. There were two types of mosquito resting surfaces/containers in each cage. The first one was a wooden resting box (30cm x 30cm x 30cm) lined with black felt with one side having a hinged cover to allow mosquito entry. The box was covered with damp blankets to maintain a reasonable humidity (Figure 3.1C).

The second was a cylindrical tube (45cm long by 15cm diameter), made of black felt rolled around a black wire mesh (Figure 3.1D).

Each cage contained two white trays filled with 2.5 L water to add humidity and four plastic jars with cotton pads soaked in 10% sucrose solution to provide mosquitoes with an energy source (Figure 3.1B). The mosquitoes were released into these field cages in the following ratios:

Cage 1- Control fertile: 200 WT KWAG females + 200 WT KWAG males

Cage 2- Sterile control: 200 WT KWAG females + 200 irradiated GMK_1 males

Cage 3- Treatment 1:1:1: 200 WT KWAG females + 200 WT KWAG males +

200 irradiated GMK_1 males

Cage 4- Treatment 1:1:3: 200 WT KWAG females + 200 WT KWAG males +

600 irradiated GMK_1 males

Cage 5- Treatment 1:1:5: 200 WT KWAG females + 200 WT KWAG males +

1000 irradiated GMK_1 males

Similarly, fertility was calculated as described in section **3.2.5**, fecundity and insemination rates were calculated as described in section **3.2.6**.

3.2.7 Statistical analysis

Data on adult emergence, longevity, fecundity, fertility, number of mated females and number of females recovered after mating were summarized as mean number of adults emerging, mean number of mosquitoes surviving after mating, mean number of eggs produced, mean proportion of eggs hatching, mean number of mated females and mean number of females recovered after mating. One-way ANOVA was used to analyze differences between treatments and controls.

The data on adult survivorship, emergence and insemination rates of females was not normally distributed and normality test was conducted to ensure normal distribution before comparing their means using one-way ANOVA. Kaplan Meier survival analysis was used to plot survival curves and Cox's F test was used to compare mean difference in survivorship between treatments. In all cases data was analyzed in SPSS version 22 and a *P*-value of less than 0.05 was considered to indicate statistical significance. The competitiveness value and expected egg hatch rates were computed using methods described by Fried (1971).

3.3 Results

3.3.1 Dose optimization

The results obtained on adult emergence, longevity, fertility and fecundity were used to determine irradiation dose that can be applied to *An. arabiensis* pupae to cause sterility in emerging adult males without affecting their mating vigour.

i) **Emergence**

Statistically, there was no significant difference in emergence rates between the treatments and controls (One-ay ANOVA, df = 6; F = 0.8; *P* = 0.6). Mean emergence rate across all treatments was 84% (Figure 3.5).

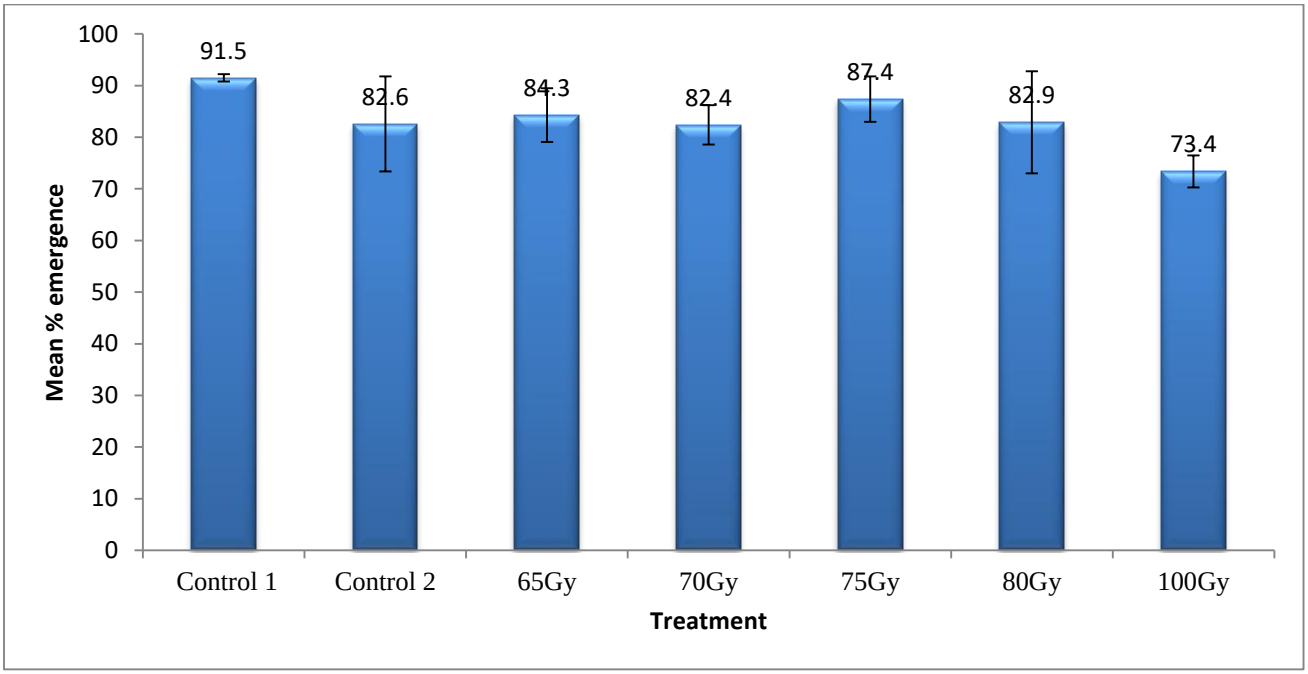


Figure 3.5: Mean % male adult emergence rate for GMK_1 pupae irradiated at different doses and reared under standard insectary conditions until emergence and corresponding controls. Control 1 (Baseline) consisted of wild type KWAG males mating with wild type KWAG females and Control 2 (Separation) consisted of unirradiated GMK_1 males mating with wild type KWAG females treated the same way as irradiated pupae except that they remained un-irradiated.

ii) **Adult longevity**

The highest mean survival rate (29 days) was observed in males which were not irradiated but handled in the same manner as the treatments (control 2, also known as separation) and the lowest mean survival rate (21 days) was recorded in males that emerged from pupae irradiated at 100 Gy (Figure 3.6). Log rank (Mantel-Cox) comparison of survival rates showed that there was a significant difference in survival between irradiated and uniradiated mosquitoes (X^2 = 41.1, DF= 5, P = <0.05). Pairwise comparisons showed that adults that emerged from pupae irradiated at the highest dose (100 Gy) showed significantly reduced survival rate in comparison with the other irradiation doses and the control cohorts.

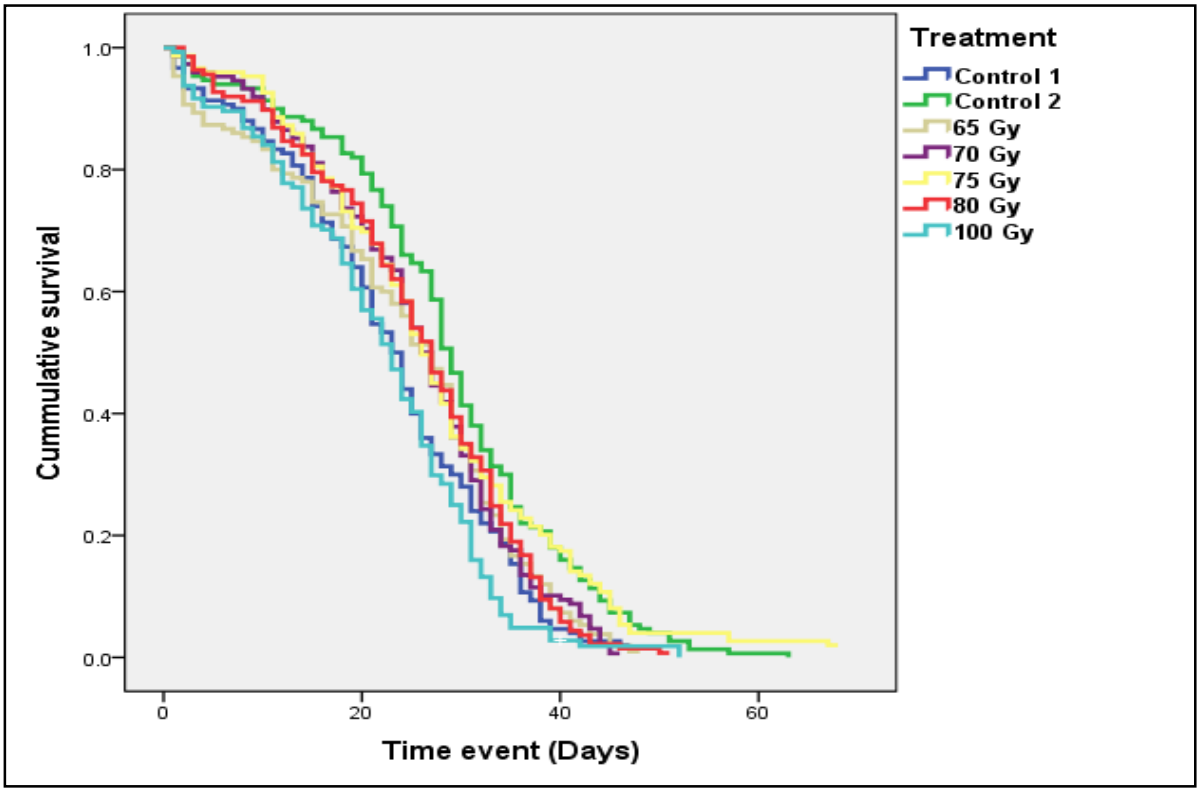


Figure 3.6: Kaplan – Meier survivorship curves for adult GMK_1 males reared under standard insectary conditions using pupae drawn from cohorts irradiated at varying doses and corresponding controls. Control 1 (Baseline) consisted of wild type KWAG males mating with wild type KWAG females and Control 2 (Separation) consisted of unirradiated GMK_1 males mating with wild type KWAG females treated the same way as irradiated pupae except that they remained un-irradiated.

iii) **Fecundity**

The fecundity data of wild type KWAG females mated with GMK_1 males irradiated at various increasing irradiation doses and corresponding controls is presented in Table 3.1. On average the percentage of females that successfully laid eggs ranged from 32-48% and 27-58% in control and treatment cohorts respectively. The highest proportion of females that laid eggs was recorded from females that mated with males irradiated at 75 Gy and the lowest proportion was recorded from females that mated with males irradiated at 80 Gy. Statistically there was a significant difference in the mean proportion of females laying eggs between control and treatment cohorts (One-way ANOVA, df = 6; F = 4.01; *P* = 0.02). Bonferroni pair wise comparison showed that a small proportion of females that mated with males irradiated at 80 Gy laid eggs compared to females that were mated with males exposed to 70 Gy and 75 Gy. The highest mean number of eggs per female was recorded from females that mated with males irradiated at 70 Gy and the lowest was recorded from females that mated with males unirradiated GMK_1 males (separation control). However, despite the observed variation in mean number of eggs laid per female between the control and treatment cohorts, statistically this difference was not significant (One-way ANOVA, df = 6; F = 1.58; *P* = 0.22).

Table 3.1: Fecundity of females mated with males drawn from pupae irradiated at different doses and corresponding controls

Treatment	**Total number of females tubed in oviposition vials**	**Mean % number of females laying eggs ± SD (95% CI)**	**Mean number of eggs laid per female ± SD (95% CI)**
Control 1*	60	48.3 ± 18.9 (1.3 – 95.4)^{ab}	61.2 ± 11.9 (31.7 – 90.8) ^{a}
Control 2**	60	31.7 ± 11.5 (3.0 – 60.3) ^{ab}	48.3 ± 15.9 (08.7 – 87.8) ^{a}
65 Gy	60	43.3 ± 12.6 (12.1 -74.6)^{ab}	57.7 ± 10.4 (31.7 – 83.7) ^{a}
70 Gy	60	56.7 ± 2.9 (49.5 – 63.8) ^{b}	74.0 ± 9.3 (51.0 – 97.0) ^{a}
75 Gy	60	58.3 ± 5.8 (44.0 – 72.7)^{b}	70.8 ± 9.5 (47.1 – 94.5) ^{a}
80 Gy	60	26.7 ± 15.8 (12.3 – 41.0)^{a}	64.2 ± 13.3 (31.1 – 97.4) ^{a}
100 Gy	60	45.0 ± 0.0^{ab}	64.1 ± 9.8 (57.1 – 88.4) ^{a}

Different superscript letters within columns show statistically significant differences (P <0.05, One-way ANOVA).

* Refers to the baseline control, newly emerged wild type KWAG males mated with wild type KWAG females.

**Refers to the separation control, newly emerged unirradiated GMK_1 males mated with wild type KWAG females treated the same way as irradiated pupae except that they remained un-irradiated

iv) **Fertility**

The egg hatch rates of wild type KWAG females mated with males irradiated at different irradiation doses and corresponding controls are summarized in Figure 3.7. The highest mean eggs that hatched were observed in control 1 cohort [14.8% (95% CI: 9.1% – 19.0%)], where wild type KWAG females (representative wild females) mated with wild type KWAG males (representative fertile wild males). The lowest egg hatch rate [0.3% (95% CI: -1.1% – 18.0%)] was observed from females that mated with GMK males irradiated at 80 Gy. Overall, there was a statistically significant difference in egg hatch rates between females irradiated at different dose and those for controls (One-way ANOVA, df = 6; F = 29.6; *P* = <0.05). Egg hatch rates decreased with an increase in irradiation dose.

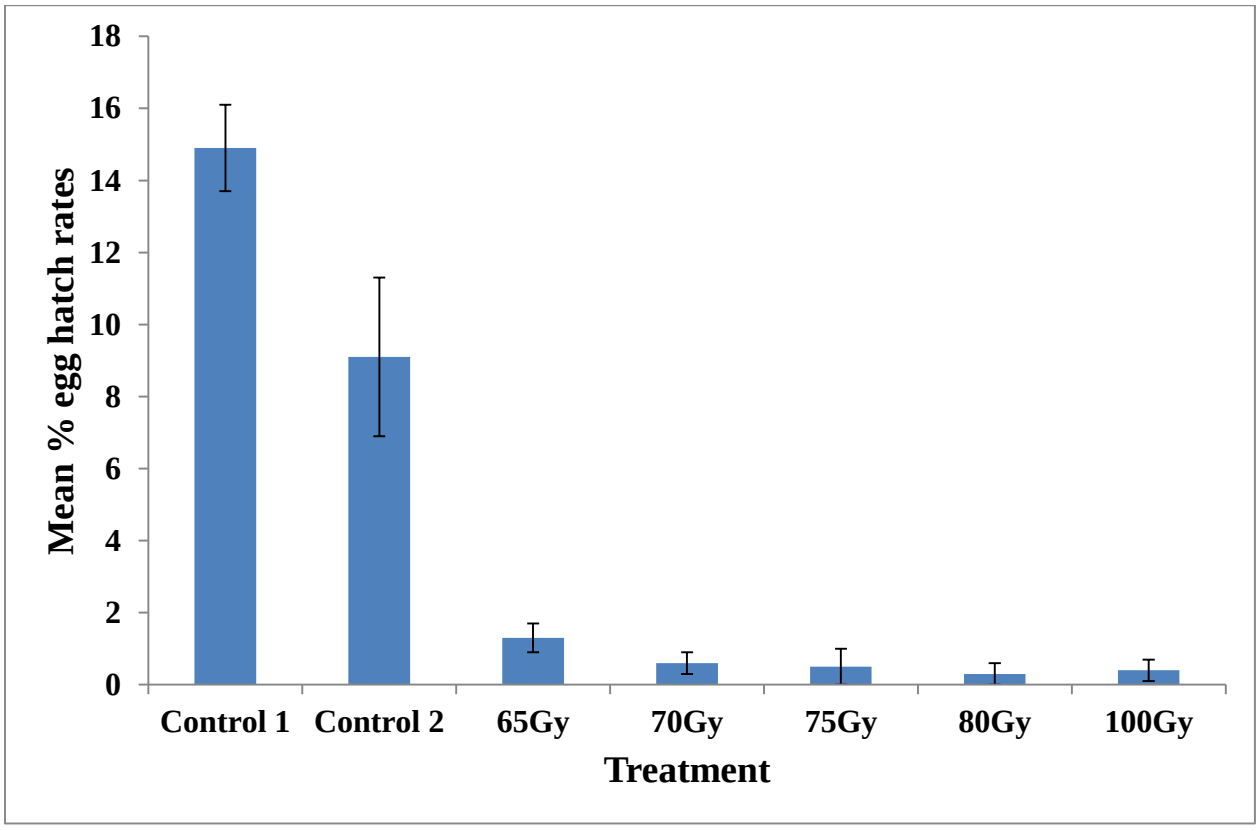


Figure 3.7: Mean percentage egg hatch rates of wild type KWAG females mated with GMK_1 males drawn from pupae irradiated at different dosages and their corresponding controls. Control 1 (Baseline) consisted of wild type KWAG males mating with wild type KWAG females and

Control 2 (Separation) consisted of unirradiated GMK_1 males mating with wild type KWAG females treated the same way as irradiated pupae except that they remained un-irradiated.

3.3.2 Mating competitiveness of the irradiated GMK_1 males

The effects of sterile male releases on the resulting fertility of caged populations were evaluated by observing the induced sterility (IS), described by Yamada *et al*., 2014, calculated as 100% minus the residual fertility value, which was obtained by dividing the observed hatch rate (Ho) by the control hatch rate (Hn). The formula: **IS = 100 – (Ho/Hn)** was used to calculate induced sterility in each cage.

Laboratory competitiveness assays were carried out in the insectary under controlled environmental conditions of 25ºC and 85% relative humidity, whereas the semi-field experiments were performed under different environmental conditions.

The mean temperature and relative humidity for the whole mating period were 18.1ºC and 75.9% respectively (Figure 3.8).

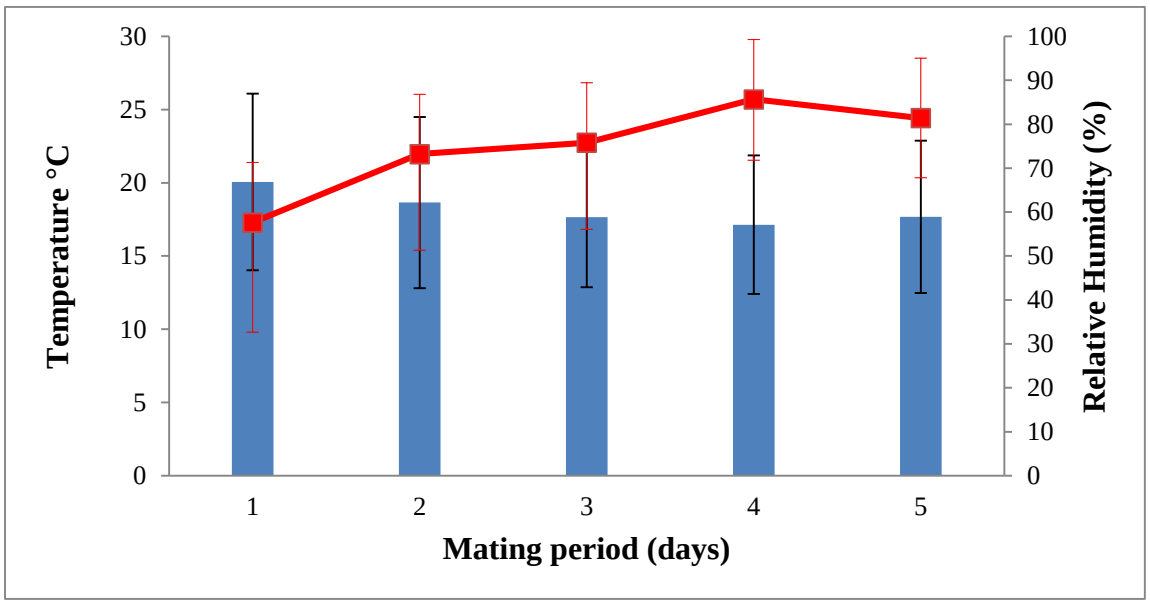


Figure 3.8: Mean temperature and average relative humidity recorded during the mating period of semi-field competitive assays (line graph represents humidity while bar graph shows temperature).

i) **Recovery of females**

The recovery rates of females after 5 nights of mating in cages are presented in Table 3.2. The average percentage of females recovered in laboratory competitive assays was high in both

experimental set ups in comparison with the recovery rates from the semi-field competitive assays. However, statistically there was no significant difference in percentage of females recovered from the treatment and control cages in all the experiments; laboratory assay (Experimental set up 1) (One-way ANOVA, df = 4; F = 0.72; *P* = 0.6), laboratory assay (Experimental set up 2) (One-way ANOVA, df = 4; F = 1.42; *P* = 0.3) and semi-field competitive assay (One-way ANOVA, df = 4; F = 1.23; *P* = 0.36).

Table 3.2: Percentage number of females recaptured after mating with males irradiated at different doses under laboratory and semi-field conditions

Treatment (fertile females: fertile males: sterile females)	**Laboratory assays**		**Semi-field assays**
	Experimental set up 1*	**Experimental set up 2***	
	Mean % of females recovered ± SD (95% CI)	**Mean % of females recovered ± SD (95% CI)**	**Mean % of females recovered ± SD (95% CI)**
1:1:0	81.3 ± 6.1 (66.2 – 96.5)[a]	84 ± 8.7 (62.3 – 105.7)[a]	41.3 ± 9.0 (19.1 – 63.5)[a]
1:0:1	76.7 ± 6.1 (61.5 – 91.8)[a]	80 ± 6.9 (62.8 – 97.2)[a]	32.2 ± 14.7 (-4.3 – 68.7)[a]
1:1:1	78.7 ± 12.0 (48.7 – 108.6)[a]	79.3 ± 8.3 (58.7 – 100)[a]	43.1 ± 3.3 (35 – 51.2)[a]
1:1:3	76.0 ± 6.0 (61.1 – 90.9)[a]	70.0 ± 6.4 (54.7 – 86.6)[a]	43.0 ± 8.2 (22.7 – 63.4)[a]
1:1:5	85.3 ± 7.0 (67.9 – 102.8)[a]	77.3 ± 4.2 (67 – 87.7)[a]	47.2 ± 9.0 (40.9 – 53.6)[a]

Different superscript letters within columns show statistically significant differences (P <0.05, One-way ANOVA).

*Females laid eggs in individual tubes

**Females laid eggs *en masse* (5-litre bucket)

ii) **Insemination rate and fecundity**

Insemination rate and fecundity data for females from the treatment and control cages for the laboratory and semi-field assays are summarized in Table 3.3 and 3.4 respectively. The insemination rates for the laboratory assays were high. Statistically, there was no significant difference in insemination rates between the control and treatment cohorts (One-way ANOVA, df = 4; F = 2.0; *P* = 0.17). The insemination rates for the semi-field competitive assays were lower

compared to the laboratory competitive assays. Statistically, there was no significant difference in number of females that were mated in control and treatment cages for the semi-field competitive assays (One-way ANOVA, df = 4; F = 3.0; $P = 0.07$).

The fecundity rate of females in experimental set up 1 (laboratory assays) ranged from 32 - 146. Statistically, no significant differences were detected between controls and treatments (One-way ANOVA, df = 4; F = 3.0; $P = 0.07$).

For the experimental set up 2, the fecundity rate ranged from 8 – 24 and similarly no statistically significant differences were observed in the fecundity rate between control and treatment cages (One-way ANOVA, df = 4; F = 1.3; $P = 0.3$).

The fecundity rate for the semi-field competitive assays ranged from 13 – 29% and there was no statistically significant difference in number of eggs laid per females in control and treatment cages (One-way ANOVA, df = 4; F = 2.6; $P = 0.1$).

Table 3.3: Mean percentage of inseminated females and fecundity of wild type KWAG females following mating competitiveness experiments under laboratory conditions.

Treatment (fertile females: fertile males: sterile females)	**Laboratory assays**			
	Experimental set up 1*		**Experimental set up 2****	
	% Insemination rate ± SD (95% CI)	**Fecundity (Mean no. eggs laid/female ± SD (95% CI))**	**% Insemination rate ± SD (95% CI)**	Fecundity (Mean no. eggs laid/female ± SD(95% CI))
1:1:0	100[a]	65 ± 5.6 (51.2 – 78.8)[a]	100[a]	24.7 ± 17.0 (-17.6 -66.9)[a]
1:0:1	93.3 ± 5.8 (79.0 – 107.7)[a]	31.7 ± 14.2 (-3.6 – 66.9)[a]	100[a]	13.7 ± 2.1 (8.5 – 18.8)[a]
1:1:1	100[a]	42.3 ± 7.6 (23.4 – 61.3)[a]	100[a]	13.7 ± 2.1 (-8.7 – 34.7)[a]
1:1:3	96.7 ± 5.8 (82.3 – 111)[a]	78.7 ± 22.5 (22.8 – 134.6)[a]	100[a]	9.7 ± 2.3 (3.9 – 15.4)[a]
1:1:5	100[a]	81.3 ± 40.1 (-18.2 – 180.9)[a]	100[a]	8.3 ± 4.6 (-3.1 – 19.8)[a]

Within columns, values followed by different lower case letters are statistically different ($P< 0.05$, One-way ANOVA).

*Females laid eggs in individual tubes

**Females laid eggs *en masse* (5 litre bucket)

Table 3.4: Mean percentage of inseminated femalesand fecundity of wild type KWAG females following mating competitiveness experiments under semi-field conditions.

Treatment (fertile females: fertile males: sterile females)	**Semi-field assays**	
	% Insemination rate ± SD (95% CI)	**Fecundity [Mean no. eggs laid/female ± SD (95% CI)]**
1:1:0	76.7 ± 20.8 (25.0 - 128.4)[a]	13.3 ± 8.0 (-6.6 – 33.1)[a]
1:0:1	46.7 ± 20.8 (-5.0 -98.4)[a]	11.7 ± 9.7(-10.9 – 34.2)[a]
1:1:1	67.3 ± 6.4 (51.6 – 83.1)[a]	13.7 ± 9.7 (-10.5 – 37.8)[a]
1:1:3	73.0 ± 5.8 (59.0 – 87.7)[a]	29.0 ± 7.2 (11.1 – 46.9)[a]
1:1:5	83.3 ± 5.8 (69.0 – 97.7)[a]	25.7 ± 8.6 (4.3 – 47.1)[a]

Within columns, values followed by different lower case letters are statistically different ($P< 0.05$, One-way ANOVA).

(iii) **Competitiveness index and induced sterility**

The results of mating competitiveness experiments conducted in the laboratory and semi-field conditions are presented in Tables 3.5 – 3.7. There was a marked statistically significant difference in egg hatch rates between the controls and treatments for the laboratory competitive assay (Experimental set up 1) (One-way ANOVA, df = 4; F = 13.0; P = 0.01) and no statistically significant difference was observed in egg hatch rates for controls and treatments in experimental set up 2 (One-way ANOVA, df = 4; F = 2.6; P = 0.1)

For the semi-field assays, a notable statistically significant difference in egg hatch rates between the controls and treatments was observed (One-way ANOVA, df = 4; F = 7.8; P = 0.01).

The average competitiveness indices for the treatment cages at ratios of 1:1:1, 1:1:3 and 1:1:5 (normal female: normal male: irradiated male) were greater than 1 in both experimental set ups for the laboratory competitive assays; 1.3 (Experimental set up 1) and 2.1 (Experimental set up 2). Competitiveness index of 1.3 was observed for the semi-field assays.

Table 3.5: Experimental set up 1: Mating competitiveness values for *An. arabiensis* GMK males irradiated at 70 Gy competing with wild type KWAG males for wild type KWAG females under laboratory.

Treatment (fertile females: fertile males: sterile females)	**S/N**	**Observed Hatch Rate (%) ± SD (95% CI)**	**Expected Hatch Rate (%)**	**Induced Sterility (%)**	**Competitiveness Index (C)**
1:1:0		37.6 ± 31.9 (-41.7 – 117.0)			
1:0:1		0.3 ± 0.6 (-1.1 – 1.8)			
1:1:1	1	12.5 ± 8.3 (-8.0 -33.1)	6.4	66	1.98
1:1:3	3	11.2 ± 8.4 (-9.6 – 31.9)	3	70.2	0.81
1:1:5	5	5.9 ± 3.1 (-1.8 – 13.6)	1.2	84.3	1.13
Average Competitiveness Index = 1.3 ± (-0.2 – 2.8)					

S/N refers to the ratio of sterile to fertile males in each treatment cage

Table 3.6: Experimental set up 2: Mating competitiveness values for *An. arabiensis* GMK_1 males irradiated at 70 Gy competing with wild type KWAG males for wild type KWAG females under laboratory conditions.

Treatment (fertile females: fertile males: sterile females)	**S/N**	**Observed Hatch Rate (%) ± SD (95% CI)**	**Expected Hatch Rate (%)**	**Induced Sterility (%)**	**Competitiveness Index (C)**
1:1:0		59.3 ± 23.3 (1.5 – 117.1)			
1:0:1		3.7 ± 1.1 (1.0 – 6.3)			
1:1:1	1	25.0 ± 1.2 (22.1 – 27.9)	14.4	57.8	1.55
1:1:3	3	12.3 ± 6.3 (-3.3 – 28.0)	5.6	79.3	1.81
1:1:5	5	7.3 ± 2.4 (1.1 – 13.6)	4.3	87.7	2.86
Average Competitiveness Index = 2.1 ± (0.3 – 3.8)					

Table 3.7: Mating competitiveness values for *An. arabiensis* GMK_1 males irradiated at 70 Gy competing with wild type KWAG males for wild type KWAG females under semi-field conditions.

Treatment (fertile females: fertile males: sterile females)	S/N	Observed Hatch Rate (%) ± SD (95% CI)	Expected Hatch Rate (%)	Induced Sterility (%)	Competitiveness Index I
1:1:0		87.3 ± 13.0 (55.0 – 119.6)			
1:0:1		4.6 ± 2.7 (-2.1 – 11.3)			
1:1:1	1	43.6 ± 15.8 (4.3 – 82.8)	41.4	50.1	1.08
1:1:3	3	23.6 ± 33.4 (-59.4 – 106.6)	18.4	73.0	1.12
1:1:5	5	23.3 ± 19.5 (-25.1 – 71.8)	10.7	73.0	0.68
Average Competitiveness Index = 1.3 ± (0.4 – 1.6)					

S/N refers to the ratio of sterile to fertile males in each treatment cage

3.4 Discussion

3.4.1 Dose optimization

Gamma radiation had varied effects on the physiological and reproductive fitness on GMK_1 males during this study. Irradiation ranging from 65-100 Gy did not affect adult emergence rate of 20-26 hours old pupae. The emergence rates observed during this study (ranging from 73- 92%) are similar to those obtained by Munhenga *et al*., (2016) during the dose optimization of *An. arabiensis* strain (GAMA) from South Africa which ranged from 79–97%. However, these emergence rates are slightly lower compared to those recorded for "KGB" strain of *An. arabiensis* originated from Zimbabwe which averaged 96 ± 0.6% (Helinski *et al*., 2006a) Overall, an average emergence rate of 84% was observed during this study which is a positive result for SIT when considering mass rearing and releases where more sterile males are needed (Munhenga *et al*., 2016). In this case, there is an assurance that over 80% of irradiated pupae will eventually emerge.

Irradiation had an effect on the survivorship of males irradiated at the highest dose of 100 Gy which exhibited a significantly reduced longevity. Results similar to ours were reported where a

decline in longevity was observed with an increase in irradiation dose on other mosquito species such as *An. stephensi* irradiated at 100 Gy (Yadav, *et al*., 2010), *Ae. aegypti* irradiated at 40 or 50 Gy (Shetty *et al*., 2016) and *An. arabiensis* irradiated at 80 or 100 Gy (Munhenga *et al*, 2016). Our findings differ from other studies where it was shown that an increase in irradiation dose increased male adult longevity of *An. arabiensis* (Helinski, *et al*., 2006a) and *An. pharoensis* (Abdel-Malek *et al*., 1966).

The results of this study also showed that the fecundity rates of females mated with males irradiated at different doses were not affected. There was no statistically significant difference in the mean number of eggs laid by females in the treatment cohorts. However, irradiation had a negative effect on egg hatch rates. Low egg hatch rates were observed at high doses and vice versa. A decline in fertility rate with an increase in irradiation dose beyond 70 Gy was also observed in other anopheline species such as *An. gambiae, An. stephensi* and *Ae. aegypti* (Sharma *et al*., 1978; Yadav *et al*., 2010; Shetty *et al*., 2016).

It is very important that sterile males be competitive in order to compete with wild males for wild females to successfully induce sterility in the wild population. However, it is known that irradiation affects the physiological and reproductive fitness of male mosquitoes (Henliski *et al*., 2006). Based on the dose optimization results obtained in this study, similar irradiation effects on physiological and reproductive fitness of *An. arabiensis* were observed in males irradiated at 70 and 75 Gy. The lower dose of 70 Gy was therefore chosen as an optimal irradiation dose and this tallies with other similar studies conducted on similar strain by Ndo *et al*., (2013); Yamada *et al*., (2014); Munhenga *et al*., (2016). This dose induced sterility in males without reducing their fitness and mating ability and was subsequently used in laboratory and semi-field experiments. Irradiation dose of 70 Gy or 75 Gy has previously been used in mating competitiveness experiments of different strains of *An. arabiensis* (Helinski *et al*., 2008; Yamada *et al*., 2014; Munhenga *et al*., 2016). However, mating competitiveness experiments on other mosquito species used different optimal doses compared to *An. arabiensis*.

A much lower optimal irradiation dose of 35 Gy was used on *Ae. albopictusas* (Atyame *et al*., 2016) and a higher dose of 90 Gy was used on *An. coluzzii* (Maïga *et al*., 2014). These studies clearly show that different irradiation doses can induce sterility in different mosquito species,

hence the need to determine the optimal irradiation dose of a particular species before performing mating competitiveness experiments.

3.4.2 Mating competitiveness

The main aim of performing semi-field competitiveness experiments is to determine whether laboratory reared mosquitoes will be able to survive and compete with wild males for females in the natural environment. It has been observed that colonization has a negative effect on mosquito fitness and mating ability (Kaiser *et al*., 1979). The females that were recovered from cages after a 5-day mating period varied between the laboratory and semi-field experiments. Few female mosquitoes were recovered during the semi-field competitiveness assays compared to the laboratory assays. This could be attributed to the adverse environmental conditions experienced during the study period. Temperature went as low as 9˚C in winter months reaching a maximum of 31˚C in summer. The cold dry spell resulted in high mosquito mortality. Lowest relative humidity recorded was 11% and maximum 100%. The high relative humidity recorded at times throughout the three replicates represented rainfall, which caused high mosquito mortality because some rainwater entered into the cages and affected their survivorship. However, the surviving adults during the semi-field competitive assays did not lose their mating vigour as exhibited by the insemination rates.

Despite having a notable low insemination rates in sterile control compared to the other treatments, statistically there was no significant difference in insemination rates between the controls and treatment cohorts due to high data variability. However, the insemination rates for the laboratory assays were much higher ranging from 93–100% compared to the semi-field assays which ranged from 47–83%. This could be attributed to differences in cage spaces. Possibly these mosquitoes were adapted to mating in confined spaces and found it difficult to locate swarms in larger cages under semi-natural conditions. Reduction in mating ability due to colonization has been reported in some mosquito species (McDonald, 1979; Asman *et al*., 1983; Knop *et al*., 1987; Benedict and Robison, 2003).

Another explanation could be that the low temperature prevented mosquitoes from forming swarms as they became inactive. It has been observed that low temperature tends to limit adult mosquito activity (Lyons *et al*., 2012).

Low insemination rates have previously been reported in other mosquito species during semi-field competitive assays: Yamada *et al*., (2014) reported insemination rates of ANO IPCL and Dongola strains of *An. arabiensis* ranging from 39–76%, and Atyame *et al*., (2016) reported insemination rate of *Ae. albopictus* ranging from 34–100%. High insemination rates have also been reported on *An. coluzzii* ranging from 67–95% (Maïga *et al*., 2014) and on *An. arabiensis* (GAMA strain), which ranged from 93–100% (Munhenga *et al*., 2016). Even though a high mean number of eggs were laid per female in laboratory assays (Experimental set up 1), statistically there was no significant difference in the mean number of eggs laid per female between the controls and treatments in both laboratory and semi-field competitive experiments.

In the current study the irradiated males were able to compete with wild males for wild females both in laboratory and semi-field assays and exhibited longer survivorship under natural conditions. Equal mating competitiveness between sterile and wild males was attained by the MACHO strain of *An. albimanus* (Kaiser *et al*., 1978) and *Wolbachia*-intogressed *Ae. polynesienis* strains (Chambers *et al*., 2011). The irradiated GMK_1 males attained a competitive index of >1 (where C of 1.0 indicate equal mating competitiveness) for both laboratory and semi-field competitive assays in all the mating ratios of normal female: normal male: irradiated male of fertile control, sterile control, 1:1:1, 1:1:3 and 1:1:5. The GMK_1 males irradiated at 70 Gy were highly competitive when confronted with the wild type KWAG males (wild type representatives) under the experimental conditions used especially high ratio of 1:1:3 to 1:1:5.. This could be attributed to prolonged colonization (Benedict *et al*., 2009) without infusion of field collected material which resulted in reduced mating vigour both under laboratory and semi-filed conditions. This strain has been under colonization for several generations for approximately 11 years from KwaZulu- Natal (Mouacho *et al*., 2009). It is therefore recommended that this colony be infused with new field material to evaluate if this will increase the insemination rate. The observed egg hatch rates for the fertile control (wild type KWAG males mating with wild type KWAG females) were very low in laboratory assays compared to those from semi-field competitive assays.

Possibly the wild type KWAG females do not like laying eggs in confined space of the glass vials. The high competitive index values obtained in this study show positive results for SIT because this strain will be able to compete with wild males for wild females and consequently induce sterility in the wild population. High C values of 0.785, 1.0 and 0.77 have been reported in *An. albimanus*

(MACHO) (Kaiser *et al*., 1979), *Ae*. *albopictus* (ArwP line) (Moretti and Calvitti, 2012) and *Ae*. *albopictus* ($ArwP_{IT}$ line) (Atyame *et al*., 2016). Other mosquito species have achieved even lower C values that ours; C values of 0.1 and 0.3 were achieved by *An. arabiensis* GAMA strain (Munhenga *et al*., 2016), a C value of 0.5 was achieved by *An. arabiensis* ANO IPCL1strain (Yamada *et al*., 2014) and a C value 0.215 was achieved by *Ae*. *albopictus* ($ArwP_{IT}$ line (Atyame *et al*., 2016). Due to differences in mosquito strains tested and experimental setups regarding cage sizes and release ratios used in this study, the C values obtained cannot be directly compared with those achieved in other mating competitiveness experiments since these variations are not transferrable to other situations. Results of this study show that the ratio of 1:1:3 can effectively be applied as the 1:1:5 ratios since the "IS" is similar and the CI is greater for 1:1:3. However, a high ratio of 1:1:5 is recommended for further semi-field mating competitive assays involving this local genetic sexing strain of *An. arabiensis* (GMK). Massive releases at the ratio of 1:1:5 are more desirable in order to maximize the chances of mating between the sterile males and wild type females to attain population suppression or elimination.

3.5 Conclusion

An irradiation dose of 70 Gy is sufficient to induce sterility in GMK_1 males without compromising their mating ability. GMK_1 males were more competitive than the wild strain type requiring an increase in the sterilized male ratio of 1:1:5 to induce sterility in the wild population. However, these results are not conclusive and will need to be repeated using a strain which is more representative to a wild strain.

CHAPTER 4

EFFECT OF IONISING (GAMMA) RADIATION ON PHYSIOLOGICAL AND REPRODUCTIVE FITNESS OF FEMALE *ANOPHELES ARABIENSIS*

4.1 Introduction

During mosquito mass production and release, only sterile males are required to be released into the field. However, it is inevitable that some females might escape the separation system and consequently be released together with sterile males. Some studies investigated the physiological effect of irradiation on male mosquitoes (Abdel-Malek *et al.,* 1966; Sharma *et al.,* 1978; Helinski *et al.,* 2006a) but its effect on females is not well documented. During this study an investigation was carried out to determine the effect of irradiation on physiological and reproductive ability of GMK_1 females. GMK_1 females were irradiated at the dose of 70 Gy, an optimal dose that induced sterility in GMK_1 males without compromising their competitiveness (Chapter 3). It has been shown that the optimal irradiation dose that induces sterility in male insects can equally induce sterility in females (Hallman, 1998). Females from over 20 species in the order Coleoptera (Brower and Tilton, 1985) and some spider mite (Tetranychidae) (Hallman, 1998) attained sterility at the same dose as their male counterparts but a higher dose is required in some species such as screwworm flies (Crystal, 1979) and the dermestid, *Anthrenus flavipes* (Brower and Tilton (1985). However, in tephritid fruit flies, females particularly the Mediterranean fruit fly (*Ceratitis capitata* Wiedemann) attain sterility at a lower dose than the male flies (Shelly *et al*, 2007). The physiological (adult emergence and longevity) and reproductive (fecundity, fertility, mating and blood feeding ability) fitness parameters were investigated during this study.

A further investigation was conducted to assess the effect of irradiation on susceptibility of female *An. arabiensis* mosquitoes. Susceptibility of anopheline mosquito species to *Plasmodium* parasites differs greatly (Frischknecht *et al*., 2006). The variability of anopheline mosquitos' susceptibility to *Plasmodium* infection depends on both genetic and environmental factors (Boissie`re *et al*., 2012; Mitri *et al*., 2015). Several studies have reported on the variability in susceptibility of *An. arabiensis* to *P. falciparum* originating from different localities such as Dakar, Senegal (Ndiath *et al*., 2011); Thies, Senegal (Awono-Ambene *et al*., 2001) and Goundry, Bukina Faso (Gnémé *et al*., 2013). In experimental infection studies, it is inevitable that some of the developed oocysts can fail to release sporozoites as a result of oocyst arrest.

Therefore, it is unlikely that all developed oocysts effectively contribute to mosquito infectiousness (Abduselan *et al*., 2016). Hence oocyst presence cannot reliably count as an effective measure of infectivity in mosquitoes (Vaughan, 2007). With this background, sporozoite infection rates were also evaluated to ascertain whether irradiated *An*. *arabiensis* female mosquitoes can support *P. falciparum* development to sporozoite stage, which is very important in determining mosquito's infectivity. A number of studies have assessed the effect of irradiation on sporozoites in malaria vaccine developmental studies (Orjih and Nussenzweig, 1980; Oakley *et al*., 2013; Syaifudin *et al*., 2014) but limited information is available on its effect on susceptibility of females to malaria parasites.

Two publications emanated from the results of this chapter. The first manuscript was published in *Transactions of the Royal Society Tropical medicine & Hygiene*. A copy of the manuscript is attached in section **4.3.1**. The second manuscript is under review (*Parasites & Vectors*) and is attached in section **4.3.2.**

4.2 Materials and methods

A brief description of materials and methods is presented in the first manuscript attached (**4.3.1**) and detailed methodology in Appendix 4. The detailed methodology for the second manuscript is presented in section **4.3.2**.

4.3 Results

The results of this chapter are presented in the manuscripts attached, sections **4.3.1** and **4.3.2**.

4.3.1. Leonard C Dandalo, Alan Kemp, Lizette L Koekemoer and Givemore Munhenga. **Effect of ionising (gamma) radiation on female *Anopheles arabiensis*:** *Transactions of the Royal Society of Tropical Medicine & Hygiene* 2017; 111: 38–40. doi:10.1093/trstmh/trx013.1).

Contribution

I conducted all the experiments for this paper, analyzed the data, interpreted the results, and wrote the first and subsequent revised drafts of the manuscripts.

4.3.2 <u>Leonard C Dandalo</u>, Annette Bennett, Noluthando S Nkosi, Belinda Bezuidenhout, Dewaldt Engelbrecht, Theresa L Coetzer, Alan Kemp, Givemore Munhenga and Lizette L Koekemoer. Susceptibility of irradiated *Anopheles arabiensis* to infection with *Plasmodium falciparum* (*Under review*, *Parasites & Vectors*).

1) **Contribution**

I participated by performing mosquito midgut and salivary gland dissections, analyzed the data, interpreted the results and wrote the first and subsequent drafts of the manuscript.

Materials and methods

(i) ***Plasmodium falciparum* culture preparations:**

Stage V gametocytes were made available by the Parasitology laboratory, Department of Molecular Medicine and Haematology, University of the Witwatersrand, Johannesburg, South Africa as per MR4 manual (2014).

(ii) **Mosquito preparation**

One hundred and fifty newly emerged irradiated GMK_1 females and 150 newly emerged unirradiated GMK_1 male separated at the pupal stage using terminalia morphology (Harbach and Knight, 1980; Harbach, 2016) were allowed to mate for 4 days in BugDorm-1® insect rearing cages at VCRL. The same experiment was set up for the unirradiated GMK_1 females and unirradiated males as a control. On the 5^{th} day the surviving irradiated and unirradiated females were separated from the males and put in two different cages and transported to Wits Research Institute for Malaria (WRIM) insectary a day before parasite infection feeding.

For oocyst examination, adult mosquitoes both at VCRL and WRIM were kept on 0.05% para-aminobenzoic acid (PABA) and 10% sugar solution soaked in cotton wool, and for sporozoite examination, adult mosquitoes were maintained on a mixed 0.05(%) Gentamicin - sulphate (Cat. No: 1289003, USP, United States of America) and 10% sugar solution soaked in cotton wool at standard insectary conditions (Hunt *et al*., 2005) in order to clear bacteria off the midguts and enhance visibility (Beier *et al*., 1994; Frischknecht *et al*., 2006).

(iii) **Mosquito infection to *P. falciparum* using the standard membrane feeding assay (SMFA)**

A Hemotek feeder with pig intestine as a membrane was used for the infections (Figure 4.1). A *P. falciparum* culture with gametocyte stage IV-V (strain NF54-luc) was used to infect freshly donated human sera. On the day of mosquito infection (day 0), all female mosquitoes placed in BugDorm-1Insect Rearing Cages® (30cm x 30cm x 30cm), were starved from 8:00 -12:30 hours before being provided with blood containing *P. falciparum*. All the unfed females were removed from the cages on the same day and all fed females were reared as normal to allow parasite development. Three biological repeats were performed.

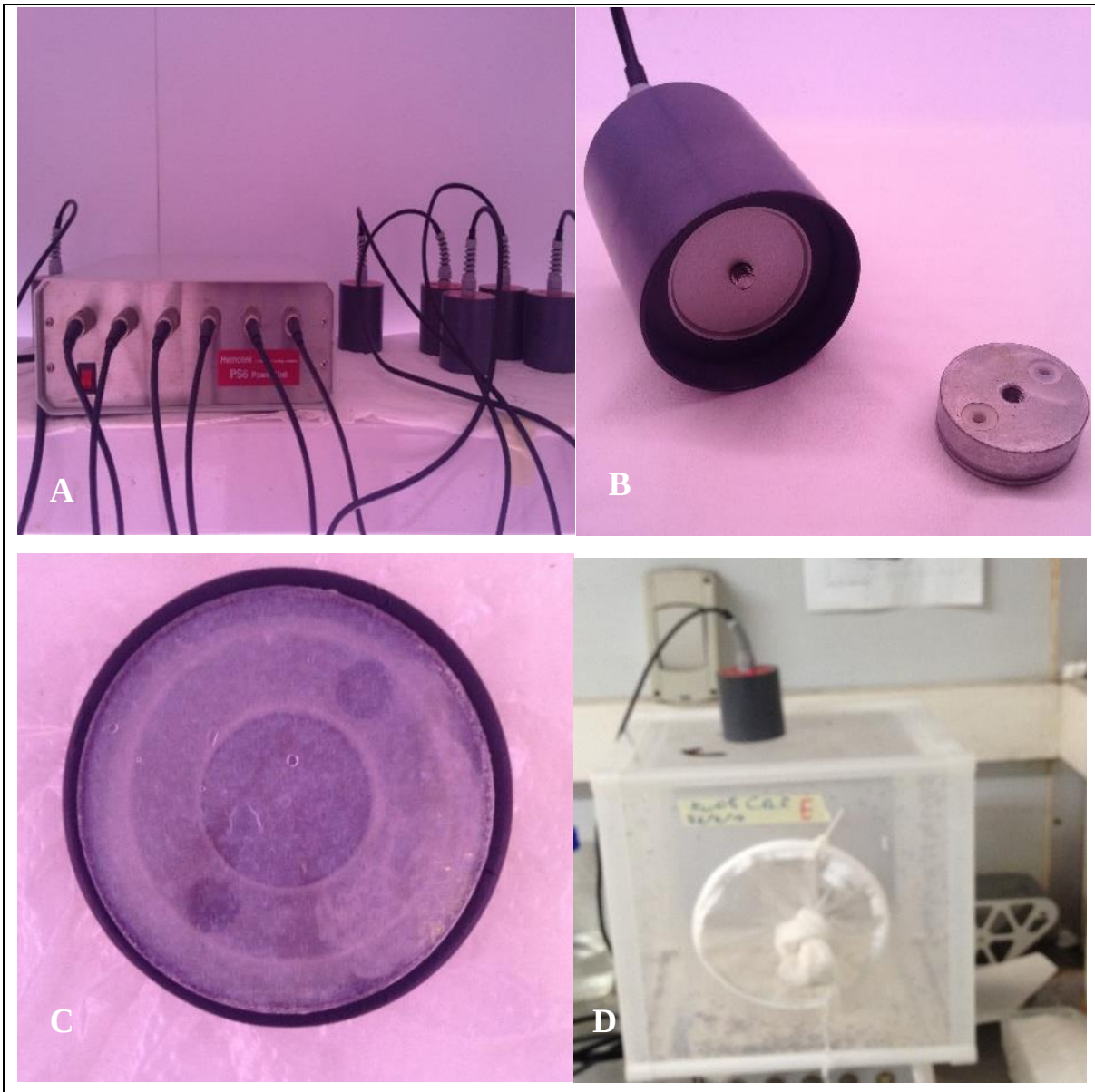


Figure 4.1: Hemotek feeder: A) Power unit; B) Parafilm membrane disk with holder; C) Bottom surface of the parafilm membrane disk; D) Blood feeding mosquitoes in a cage

(iv) **Mosquito midgut dissections**

Midgut dissections took place 10-12 days' post feeding, counting the first day after feeding as day 1. On the day of dissection, surviving females (irradiated and unirradiated) were killed by placing them in different falcon tubes (50ml) containing 70% alcohol and taken to the laboratory. Freshly killed specimens were put on slides and a few drops of phosphate buffered saline (PBS) were applied before being dissected under a dissection microscope to remove the midgut.

The midguts were put on a new slide (with a few drops of PBS) with a cover slip and observed under a fluorescent microscopy. The specimens were recorded as positive when the oocysts were observed in the midgut (Figure 4.2).

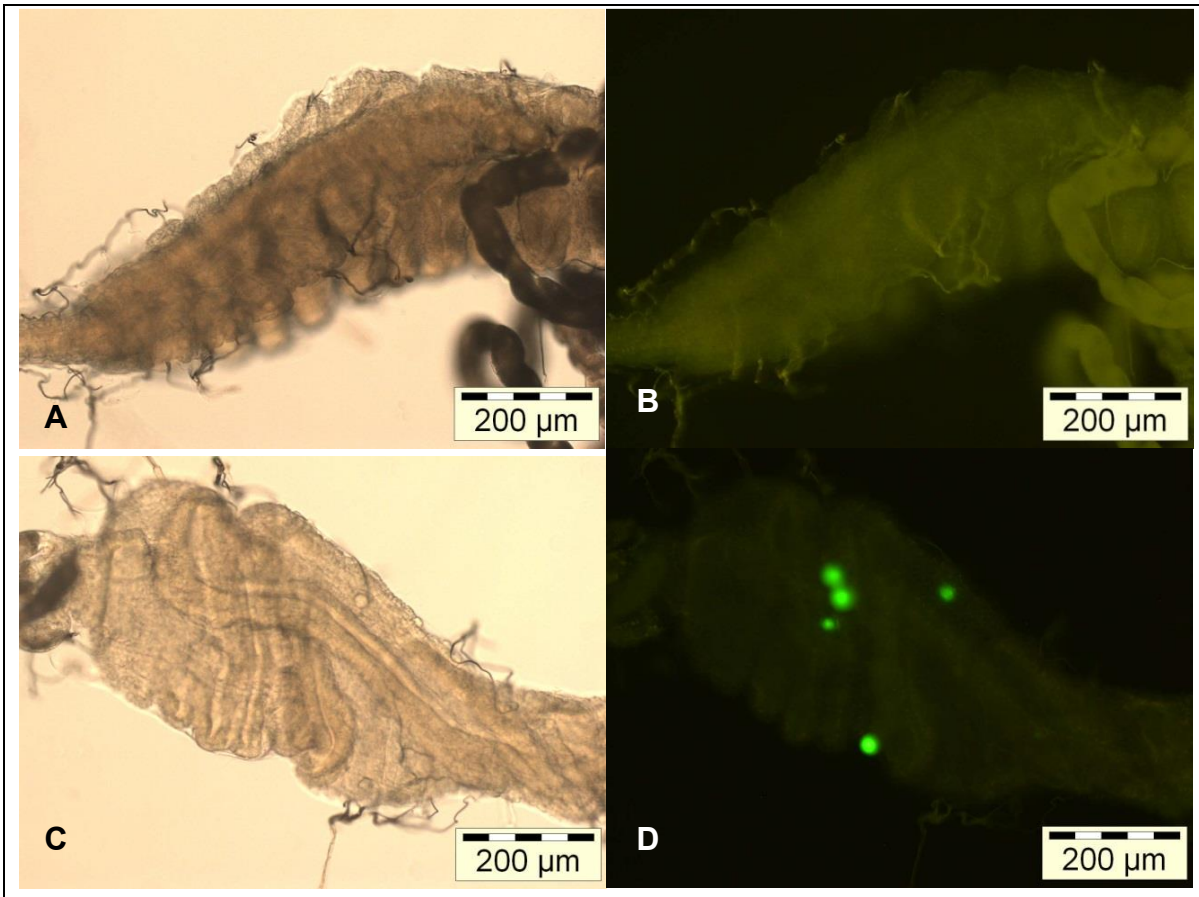


Figure 4.2: Midgut of uninfected mosquito (A and B) and infected mosquito with oocysts (C and D) under bright (A and C) and fluorescent fields (B and D).

(v) **Salivary gland dissections**

After 16 days' post infection, surviving females (irradiated and unirradiated) were killed by putting them in different falcon tubes (50ml) containing 70% alcohol and taken to the laboratory. All dead mosquitoes after oocyst infection (for replicates 4-6) were counted and their proportion calculated. Salivary glands were dissected using a stereo microscope and fluorescing sporozoites were viewed under a fluorescence microscope. In addition, to confirm the infection, salivary glands were ruptured on a microscope slide, stained with Giemsa, and left to dry (Beier *et al*.,

1990). The Giemsa stain used was diluted in the ratio of 1: 9; Giemsa Stock stain to filtered Sorensen's phosphate buffer at pH 7.2. Ruptured salivary glands (with a dissection needle) on a standard microscope slides were left overnight to dry.

The following day the slides were fixed in 100% Methanol for 4 seconds and left to dry for at least 10 minutes before staining them in the diluted Giemsa solution for 30 minutes.

After Giemsa staining, the slides were rinsed by running water down the back of the slide and left to dry before viewing. Slides were viewed under phase contrast with an Olympus microscope (Figure 4.3). The infection rate was based on the proportion of infected salivary glands in relation to uninfected glands, to indicate that the full infection cycle in mosquitoes has been established via the SMFA. Three biological repeats were conducted.

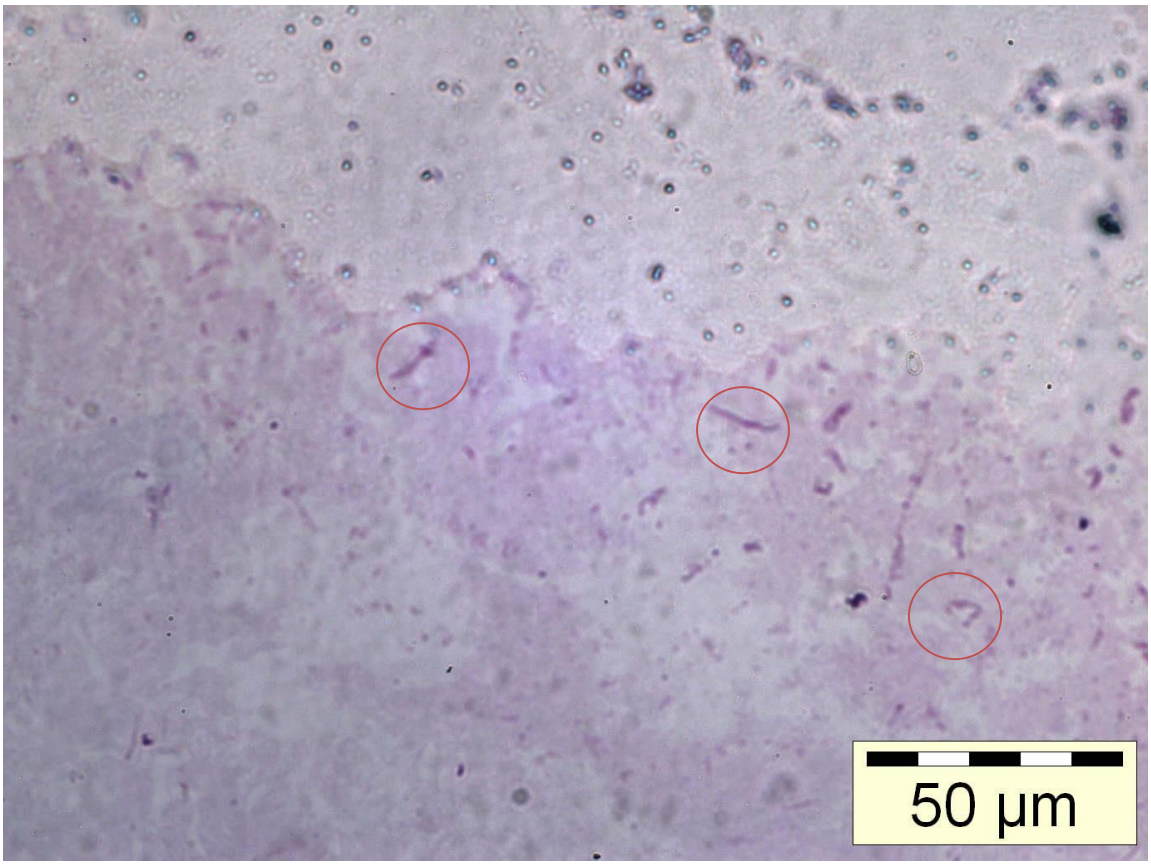


Figure 4.3: Salivary gland showing sporozoites highlighted in red circles

Statistical analysis

Data on female adult emergence, fecundity, egg hatch rates and female adult longevity was summarized as mean proportion of pupae surviving to adult stages, mean number of eggs produced, mean proportion of eggs hatching and mean female adult survivorship respectively.

The mating success of irradiated females was determined by calculating the mean proportion of mated females. Independent samples t - test were used compare adult emergence, fecundity, fertility, longevity, mating and blood feeding ability between irradiated and unirradiated cohorts. Survival curves were analyzed using Kaplan Meier survival analysis.

The total number of oocysts per midgut (oocyst prevalence rate) and total sporozoites in salivary glands per mosquito (sporozoite infection rate) were compared using Fisher's exact tests. The mean oocyst number between irradiated and unirradiated mosquitoes was compared using independent samples t - test. All statistical analyses were performed using SPSS® version 22. A P-value of 0.05 or less was considered as significant.

Results

i) **Blood feeding rate**

In total 473 *An. arabiensis* females successfully fed on blood infected with *P. falciparum* (Table 4.1). The mean feeding rate between unirradiated females and irradiated females was similar. There was no statistically significant difference in the blood feeding rate between unirradiated and irradiated females (Independent samples t - Test, df = 4; t = -2.4; P = 0.08).

Table 4.1: The feeding rate between irradiated and unirradiated females after standard membrane feeding.

Treatment	**Sample size (n)**	**% mean feeding rate ± SD (95% CI)**
Irradiated	348	77.0 ± 3.8 (67.6 – 86.4) [a]
Unirradiated	304	67.7 ± 5.6 (53.9 – 81.5) [a]

[a] no statistical difference (P >0.05)

ii) **Oocyst prevalence**

A total of 139 irradiated and 151 unirradiated mosquitoes were dissected to determine the oocyst prevalence and oocyst intensity (Table 4.2). *Plasmodium falciparum* gametocytes developed into zygotes, ookinetes and into rupturing oocysts in both irradiated mosquitoes and unirradiated ones in all the three replicates.

The oocyst infection rate per replicate did not vary greatly between the irradiated and unirradiated mosquitoes. High oocyst infection rate was observed in irradiated than in unirradiated ones (Figure 4.4). Statistically, there was a significant difference in the oocyst infection rate between irradiated and unirradiated females (Fisher's exact test, P = 0.04).

iii) Oocyst intensity

In total 162 and 42 oocysts were counted in all infected mid guts of irradiated and un-irradiated mosquitoes respectively. The mean oocyst intensity for the irradiated females was higher in comparison to the unirradiated ones (Figure 4.5). Statistically, there was no significant difference in the mean oocyst intensity between the irradiated and unirradiated females (Independent samples t - Test, df = 4; t = 1.4; P = 0.1).

iv) Sporozoite infection rates

A total of 29 irradiated females (n = 36) and 22 unirradiated females (n = 32) were infected by P. falciparum. The parasite infection rates at sporozoite stage are presented in Table 4.2. High percentage of infected salivary glands was observed in irradiated females than in unirradiated ones (Figure 4.6). No statistically significant difference was observed in the sporozoite infection rates between the irradiated and unirradiated mosquitoes (Fisher's exact test, P = 0.3).

Table 4.2: Infection rates of laboratory colonized *An. arabiensis* infected with *Plasmodium falciparum*

Biological replicate no.	**Treatment**	**Total number of fed mosquitoes**	**Oocyst infection**			**Sporozoite infection**	
			Total number of mid guts dissected	Infection rate (%) (Prevalence)	Mean number of oocysts (Intensity) (Min-Max)	Total number of mosquitoes dissected for salivary glands	Infection rate (%)
1	Irradiated	68	48	10.4	0.1 (1-1)	12	83
	Unirradiated	82	54	0	-	12	67
2	Irradiated	70	50	14	1.4 (1-25)	12	92
	Unirradiated	87	55	3.6	0.4 (1-11)	9	78
3	Irradiated	67	41	15.2	1.1 (1-32)	12	67
	Unirradiated	99	42	16.7	0.4 (1-5)	11	64

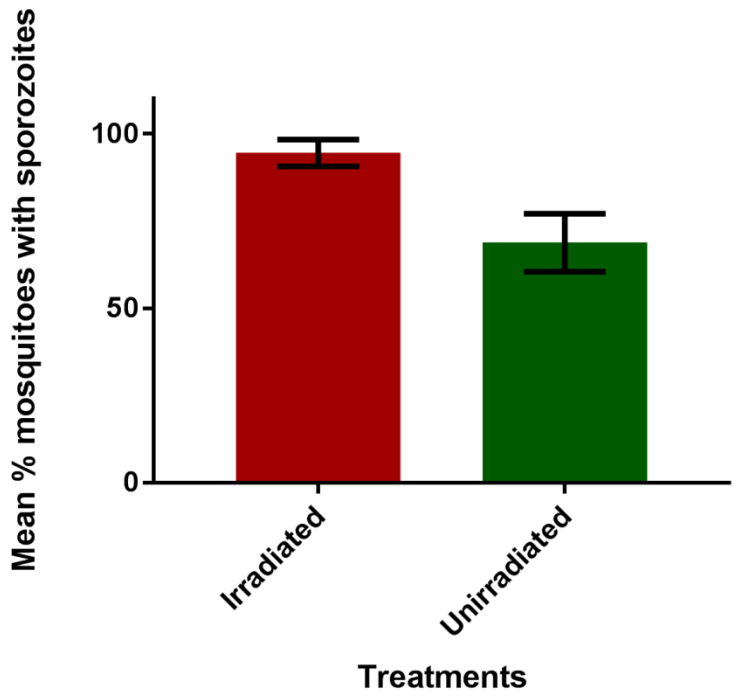


Figure 4.4: Mean percentage of irradiated and unirradiated *Anopheles arabiensis* females with oocysts post feeding *Plasmodium falciparum* gametocytes infected blood.

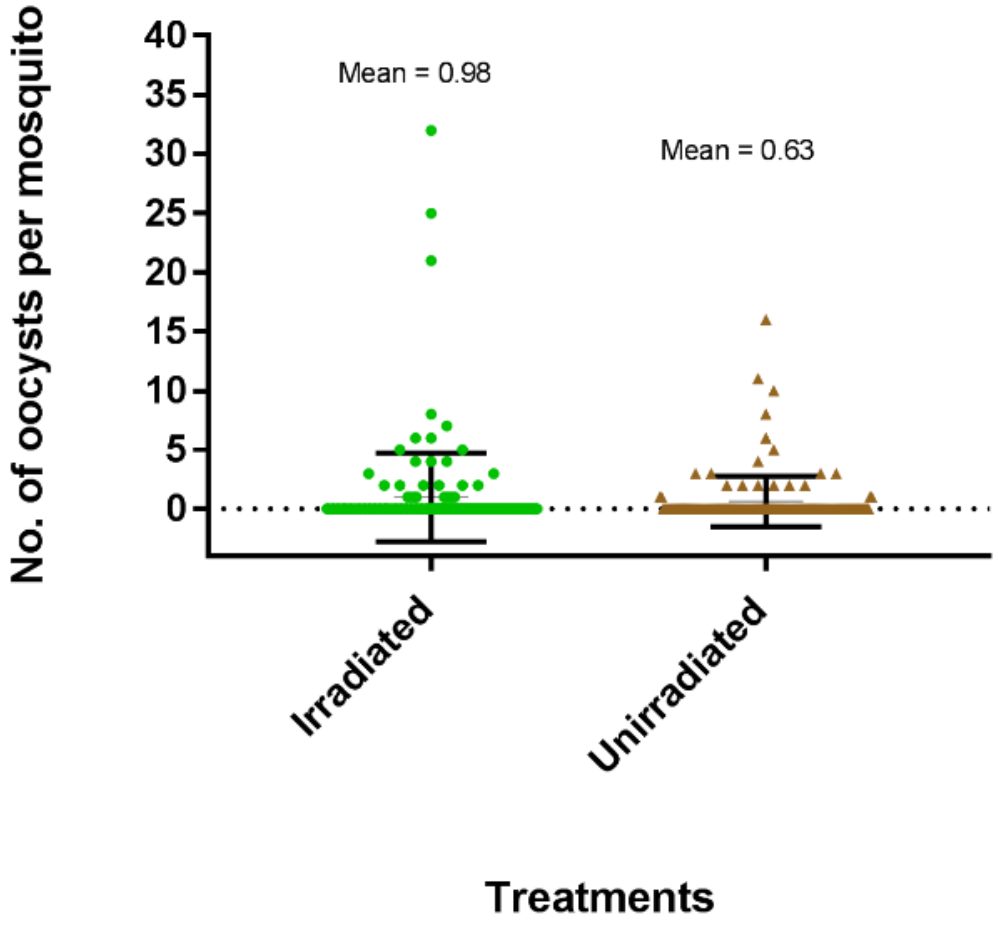


Figure 4.5: Distribution of oocysts per mosquito in infected mid guts of irradiated and un-irradiated mosquitoes over 3 biological replicates

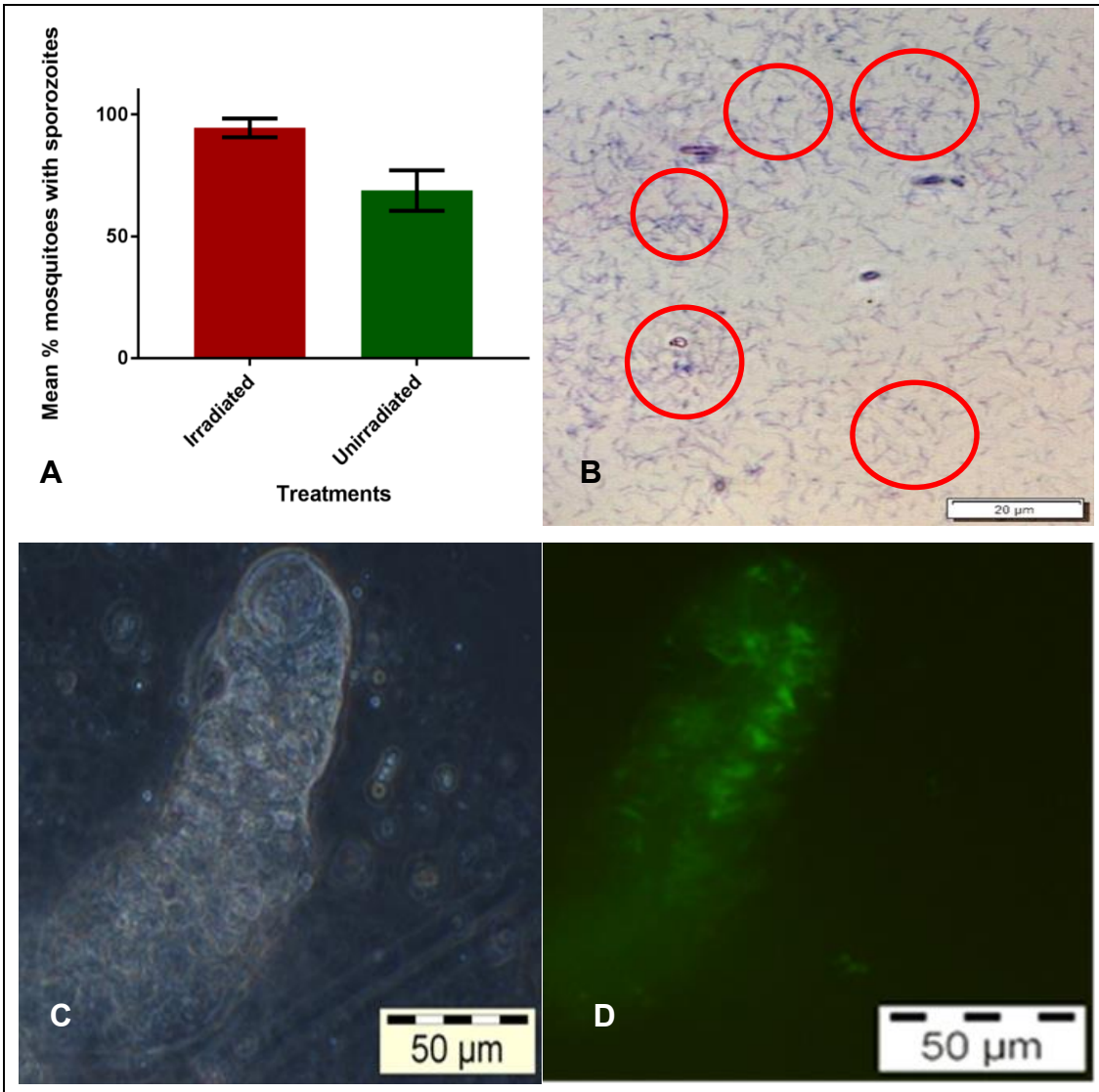


Figure 4.6: *An. arabiensis* sporozoites: A) The mean percentage of irradiated and unirradiated females with. B) Giemsa stained sporozoites. C) Salivary glands with sporozoites under phase contrast. D) Fluorescent sporozoites in salivary glands.

Discussion

This is the first detailed study to evaluate the susceptibility of irradiated *An. arabiensis* female mosquitoes to *P. falciparum* using a membrane feeding assay in South Africa. The results of our study showed that irradiation did not have any significant effect on the blood feeding ability of the irradiated females.

Even though the percentage of blood fed females was higher in the unirradiated cohort (77%) than the irradiated cohort (67.7%), statistically, there was no significant difference in the feeding ability between the irradiated and uniradiated females.

However, this result contradicts earlier finding where irradiation negatively affected voluntary blood feeding ability of *An. arabiensis* females on live rodents (Dandalo *et al*., 2017). The high feeding rate observed during this study could be attributed to the effect of starving the females for four and half hours since the females from the genetic sexing strain of *An. arabiensis* (GMK) used in irradiation studies were not weaned for membrane feeding.

The results of our study showed that both irradiated and unirradiated mosquitoes potentially supported parasite development. The parasite infection was much higher at sporozoite stage for both irradiated (80.7%) and unirradiated (69.7%) than at oocyst stage which was 13.8% and 6.8% for irradiated and unirradiated females respectively. Lower infection rates observed at oocyst stage could be due to lack of gentamicin in the sugar solution that was provided to females prior to infection. At oocyst stage, only para-aminobenzoic acid (PABA) was added to the sugar water prior to infection in relation to other infection studies which showed that PABA increases *Plasmodium* development in anopheline species (Peters and Ramkaran, 1980; Beier *et al*., 1994). However, during this study it was observed that very high infection rates were recorded after an addition of gentamicin to sugar solution prior to infection at sporozoite stage**.** Lower infection rates were also observed in *An. arabiensis* when only PABA was added to the sugar water of mosquitoes prior to infectious blood meals and a dramatic increase in oocyst infection rate was observed when gentamicin was added in some anopheline species such as *An. arabiensis* (Bennett *et al*., *in prep*), *An. stephensi* and *An. gambiae* s.l (Beir *et al*., 1994). It has previously been shown that the presence of bacteria in mosquito mid guts inhibits *P. falciparum* infectivity to other anopheline species such as *An. gambiae*, *An. stephensi* and *An. albimanus* (Pumpuni *et al*., 1993; 1996) but its mechanism still remains elusive (Boissie` re *et al*, 2012). However, addition of antibiotics such as gentamicin, penicillin or streptomycin to the sucrose solution provided to mosquitoes intended to be exposed to the parasites kills bacteria present in the mid gut thereby enhancing *P. falciparum* infectiousness (Beier *et al*., 1994; Frischknecht *et al*., 2016).

Despite having a significant difference in the *P. falciparum* infection prevalence at oocyst stage between the irradiated and unirradiated females, the parasite infection intensity at this stage was insignificant.

The high oocyst and sporozoite prevalence rate in irradiated females indicates that these mosquitoes are highly vulnerable to *P. falciparum* infection should they be released in the environment and might play an important role in transmitting malaria parasites. However, this does not take into account their migration patterns to reach a target host, their inherent immune system effect to combat the infection, as well as other genetic and environmental factors. The effect of irradiation for *Plasmodium* susceptibility should be evaluated in further studies where modelling of release programmes of irradiated mosquitoes are illustrated. The invasion of sporozoites in salivary glands is an important indicator of mosquito infectivity but is not an absolute prerequisite for infectivity and immunogenicity in the vertebrate host (Sato *et al*., 2014). It is recommended that further studies be conducted to investigate the infectiousness of mosquitoes with infected salivary glands in irradiated *An. arabiensis* female mosquitoes rather than irradiating sporozoites as the case in vaccine studies. Limited information is available on the infectiousness of irradiated mosquitoes and their ability to transmit parasites to vertebrate hosts. A study conducted by Pollock *et al*., (2011) only showed that mice subjected to bites of irradiated *An. stephensi* infected mosquitoes with *P. yoelli* 17XNL developed significantly higher levels of antibodies compared to mice subjected to the bites of unirradiated infected mosquitoes but did not look at their infectivity levels.

Conclusion

In this study irradiated *An. arabiensis* female mosquitoes displayed high capability of supporting *P. falciparum*. It is therefore, a prerequisite that all females be separated from sterile males earmarked for releases during mosquito SIT programmes to avoid accidental releases of irradiated female mosquitoes for human safety. Further studies need to be conducted to assess the transmission potential of mosquitoes with infected salivary glands in irradiated *An. arabiensis* female mosquitoes to ascertain the possible effect of releasing accidental irradiated mosquitoes in a SIT programme.

CHAPTER 5

Population dynamics and *Plasmodium falciparum* infectivity rates for the malaria vector *Anopheles arabiensis* at Mamfene, KwaZulu-Natal, South Africa

5.1 Introduction

Mamfene, KwaZulu-Natal has been earmarked for pilot releases of *An. arabiensis* to assess the applicability of SIT as an additional vector control tool in South Africa. Previous studies have reported the presence of *An. arabiensis* in this area (Obala, 1995; Mouatcho *et al*., 2009), currently being implicated as the main malaria vector in South Africa (Maharaj *et al*., 2013). However, limited information is available on the current *An. arabiensis* population density, seasonal variation, as well as the current species composition in this area. Since SIT is very effective when applied in an area where there is a single dominant vector (Alphey *et al*., 2010) with very low population density (Vreysen, 2001), it is important to know the baseline information of the target insect in the intended area of intervention. It is against this background that mosquito surveillance was initiated in Mamfene to assess the species composition, population density and seasonal variation of *An. arabiensis* in order to come up with current baseline information which is critical for decision making regarding the implementation of the SIT programme in this area.

In 2015, about five malaria cases (locally acquired) were reported in this area (Department of Health, unpublished records) despite there being active, intensive IRS programmes in malaria endemic areas (Coetzee *et al*., 2013). Since *An. arabiensis* is known to feed and rest both indoors and outdoors (White, 1974), during this study the productivity of two novel mosquito collection methods of Clay pots and Modified plastic buckets targeting outdoor feeding and resting mosquitoes were assessed. These novel mosquito collection tools target both male and female mosquitoes resting outside houses. A search for an efficient collection method targeting male mosquitoes is very important when considering mosquito SIT programme implementation where only males are released. Such a tool is vital for programme monitoring.

Two publications emanated from the results of this chapter. The first manuscript has been published in *Journal of Medical Entomology*. A copy of the manuscript is attached in section **5.3.1**.

The second manuscript has been published in *Scientific Reports* and the results are presented in section **5.3.2.**

5.2 Materials and methods

Details of materials and methods are presented in first and second manuscripts (Sections **5.3.1** and **5.3.2**). Additional information on materials and methods for the first manuscript is presented in Appendix 5.

5.3 Results

The results of this chapter are presented in the two manuscripts attached (Sections 5.3.1 and 5.3.2**)**

5.3.1 Leonard C. Dandalo, Basil D. Brooke, Givemore Munhenga, Leanne N. Lobb, Jabulani Zikhali, Sifiso P. Ngxongo, Phineas M. Zikhali, Sipho Msimang, Oliver R. Wood, Mohlominyana Mofokeng, Eunice Misiani, Tobias Chirwa and Lizette L. Koekemoer. **Population Dynamics and *Plasmodium falciparum* (Haemosporida: Plasmodiidae) Infectivity Rates for the Malaria Vector *Anopheles arabiensis* (Diptera: Culicidae) at Mamfene, KwaZulu-Natal, South Africa** *Journal of Medical Entomology*, XX(X), 2017, 1-9 doi: 10. 1093/jme/tjx169.

1) **Contribution**

I participated in mosquito collections, conducted species specific identification using PCR, conducted ELISA and wrote the first draft of the manuscript.

5.3.2 Ashley Burke, **Leonard Dandalo**, Givemore Munhenga, Yael Dahan, Frans Mbokazi, Maureen Coetzee, Lizette Koekemoer, Basil Brooke. A new malaria vector mosquito in South Africa. *Scientific Reports*, 7, 43779; doi: 10.1038/srep43779.

1) **Contribution**

I participated in mosquito collections, conducted species-specific identification using PCR and ELISA for all the specimens collected from Mamfene, KwaZulu-Natal and wrote the first draft of the manuscript.

CHAPTER 6

GENERAL DISCUSSION AND CONCLUSIONS

In order for South Africa to achieve malaria elimination apart from the current vector control strategies being implemented, additional resources and interventions will be needed. Furthermore, cross-border initiatives will be crucial as well as in-depth surveillance and monitoring systems (Malaria Elimination Group, 2009). Malaria elimination will have to be a cross-disciplinary approach and research into supplementary vector control interventions are one of these sections that can contribute towards this goal. Research into SIT was initiated and this research supplements those done by Munhenga *et al*., (2011; 2014; 2016). This research evaluated a local genetic sexing strain, the impact of irradiation on adults as well as the evaluation of a field site in respect of target species abundance and distribution over time.

The local genetic sexing strain established during this study (GMK) exhibited attributes characteristic for this strain (MacInnis *et al*., 2004). The fast growth observed in both immature and adult stages is a positive result although the inherited sterility due to chromosomal rearrangement (Willhoeft and Franz, 1996) and low egg hatch rates associated with this GSS strain remains a challenge for mass production (Curtis *et al*., 1976; Yamada *et al*., 2012). Fast growth can eventually lead to low cost of production since the numbers required for release can be reached within a short period of time. Long adult male survivorship exhibited by this strain is an advantage because during mass release there is an assurance that the males will live long in the field thereby increasing their chances of encountering and mating with wild females, which is critical for achieving wild population suppression or elimination. Multiple purification of GMK_1 resulted in low female emergence after exposing eggs or larvae to dieldrin (Lobb, unpublished data).

Despite successful establishment of this strain based on dieldrin resistance, dieldrin still remains environmentally unfriendly and hazardous to other animals in the ecosystem (WHO, 2003b).

Even though the results of this study have shown that dieldrin waste can be minimized by reusing it twice or treating four times more eggs than the number bythe current protocol described by

Yamada *et al*., (2012), it is very important that other alternative chemicals or plant derivatives with the same mode of action as dieldrin be explored.
This initiative is currently underway and will greatly improve the perceptions of the value of the strain (Koekemoer, personal communication). It is further recommended that alternative ways of developing efficient sex separating strains which are environmentally friendly be explored. A sexing strain based on temperature sensitive lethal (tsl) mutation is an option. This was successfully developed for Mediterranean fruit fly (Franz, 2005) and a similar technique is currently being explored for application in mosquitoes (Munhenga, personal communication).

During this study, an irradiation dose of 70 Gy was optimal to induce sterility in both males and females of the GMK strain. This dose did not compromise the mating ability of the males but prevented the females from laying eggs. It is therefore recommended that this dose be used in future SIT activities involving this strain. The mating competitiveness index for both laboratory and semi-field assays was >1, which means that the GMK_1 males were more competitive than the wild KWAG males. The average competitiveness index of 1.3 obtained during this study for both laboratory and semi-field assays indicates that even the ratio of 1:1:1 and 1:1:3 (wild female: wild male: sterile male) would be enough to induce sterility in the wild population and consequently reduce its population. However, during this study a ratio of 1:1:5 was recommended for future SIT activities involving this strain because this ratio induced high sterility in both laboratory and semi-field assays compared with the other ratios. The lower ratios could be of great advantage in that fewer sterile males would be required for release and this would reduce operational costs but compromise on the induced sterility of the target population. In this study, the ratio of 1: 5 (wild males to sterile males) can have significant impact by inducing sterility in the wild population thereby reducing its population density faster. Following the trend, the ratio of 1:1:10 (as recommended for the GAMA strain by in Munhenga *et al*., 2016) would drastically increase sterility in the wild population but may increase operational costs.

The results of this study further showed that irradiation had varying effects on the physiological and reproductive ability of female mosquitoes.

However, a very important finding was that irradiated females were capable of supporting the development of *P. falciparum* and hence have a potential for transmitting this parasite to humans. Therefore, it is recommended that all females be eliminated during mass production and release to avoid accidental female releases for human safety. The effect of irradiation on females physiological and reproductive fitness should be modelled in future studies to measure the epidemiological impact this will have on malaria transmission. The presence of sporozoites in salivary glands of mosquitoes does not imply that the mosquito will automatically transfer the sporozoites to vertebrate host when it bites (Sato *et al*., 2014). There are a number of factors that need further investigation on human -vector interaction. It is recommended that further studies be conducted to assess the infectiousness of irradiated females. Several factors have to be taken into account before drawing up conclusions regarding potential risks of accidentally releasing irradiated females because: 1) *An. arabiensis* will feed on both animals and humans and have a low infection rate in nature (0.5 – 0.7%), 2) not all infected mosquitoes will feed on humans, 3) not every person will carry the gametocytes, 4) as shown in this study irradiated females have a low blood feeding rate, and 5) a low impact of accidental releases is likely to be experienced in low malaria transmission settings e.g. South Africa.

Mosquito surveillance showed that Mamfene, KwaZulu-Natal is a suitable area to conduct a pilot SIT programme. The area is relatively isolated by the Lebombo mountain range to the West which runs from North to South, and the Indian Ocean to the East. The mountain range and the ocean form a barrier and can prevent immigration of mosquitoes from untreated areas. Mamfene has one dominant malaria vector *An. arabiensis* and the population of other minor vectors is very low. Overall, the population density of *An. arabiensis* in this area is very low and is suitable for SIT intervention. The area exhibits seasonal variation in abundance of *An. arabiensis* with very low numbers experienced in winter months. In addition to this, the vector population in this area is further reduced by the current malaria vector control (IRS) and this can make it easier to plan the time for mosquito releases in order to have a significant impact of SIT intervention.

This study provides evidence for the possible role of *An. arabiensis* and *An. vaneedeni* in malaria transmission in South Africa. Furthermore, this most likely implies that local malaria transmission

is likely due to outdoor biting from mainly *An. arabiensis* and minor vectors such as *An. vaneedeni* and *An. merus*. *Anopheles merus* has been confirmed as malaria vector in South Africa in 2016, (SIT report, unpublished).

Based on the current Mamfene data, it is recommended that section 9 be considered as a treatment area (release site) due to relatively low population density of *An. arabiensis*. This section will provide the best opportunity to obtain evidence for a significant impact of the SIT intervention. Furthermore, low numbers of sterile males will be required for releases and this has a significant impact on total cost of the entire pilot programme as it implies low operational costs.

Sections 8 is recommended to be the control site because the population density of *An. arabiensis* in this section is relatively high than in section 9. Section 2 has the least population density of *An. arabiensis* and cannot show significant impact of the SIT intervention.

An assessment of the novel mosquito collection methods of clay pots and modified plastic buckets revealed that clay pots were the most productive. Clay pots collected relatively high numbers of both male and female mosquitoes. The productivity of this method in collecting outdoor resting mosquitoes more especially males makes it an ideal method to monitor the SIT intervention in this area.

It is further recommended that mosquito surveillance be continued in this area as it brings new and viable vector information which can help researchers to come up with new and effective additional vector control tools. The results of this study will help policy makers/stakeholders to make an informed decision whether to consider SIT as an additional vector control method in South Africa and mobilize resources towards implementation of the pilot programme in Mamfene, KwaZulu-Natal.

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APPENDICES

APPENDIX 1

(1A) Genetic sex separation of *An. arabiensis* at egg/ larval stages, the method described by Yamada *et al*., 2012

1). **Identification of potentially hazardous procedures/operations/ substance where these may occur in the method:** Possible risk of exposure to dieldrin. Ensure safety precautions are taken (e.g. wear gloves. etc).

2) **The nature and volume of specimen required:**

- GSS eggs: Can range from 500 to 2000.
- GSS larvae: 25 fourth instar larvae or 50 first and second instar larvae

3) **Dieldrin storage**

1ml 1000ppm (Lot. No: LC12926V, Sigma Aldrich, Germany) diluted in acetone solution was stored in freezer at -20 ^{0}C.

4) **Dieldrin preparation (Dilution)**

The following formula was used to determine the desired concentration and volume:

$C_1V_1 = C_2V_2$; Where C_1 is the stock concentration (1000ppm), V_1 is the stock volume, C_2 is the desired concentration and V_2 is the desired volume

499ml distilled water was added to the stock solution (1ml 1000ppm) to reach the mark of 500ml to get the desired 2ppm concentration and store in opaque glass bottle or transparent bottle covered with aluminum foil at room temperature or use immediately.

5). **Dieldrin treatment and the required reagents and equipment**

a) 50ml 0.1ppm dieldrin solution when treating GAMA larvae or 50ml 2ppm dieldrin solution when treating GAMA eggs; b) IAEA/IPCL treatment tubes (mostly used when treating larvae); c)

Pipettes, d) Beaker; e) Measuring cylinder; f) Larval bowls; g) Petri dish; h) Filter paper; i) Polystyrene cups; J) Floating plastic rings.

Detailed step-by-step instructions on how to perform the sex separation at egg/larval stages

(i) Use dieldrin solution prepared for egg or larvae treatment (see point 4 above)

(ii) Pour ~50ml of solution into a petri dish that has filter paper around the edges in order to ensure that the eggs don't move up the edges of the dish and escape the solution

(iii) Rinse eggs (500-2000) with distilled water into filter paper making sure that all the water has drained/ larvae into IAEA/IPCL treatment tube held over separate larval bowl.

(iv) Use pipette and dieldrin solution to rinse eggs/ larvae off into larvae bowl. Eggs should be poured at the middle to ensure that eggs are not stuck around the larval bowl.

(v) Leave for at least two hours

(vi) Check after 1 hour to ensure no eggs on sides of dish

(vii) Remove eggs from solution by pouring through filter paper into larval bowl using distilled water. Larvae can be poured in the IAEA/IPCL tube.

(vii) Rear as normal

(viii) Poor used dieldrin in a dieldrin waste container

NB: Monitor sex ratio and no females are required those that emerge should be killed. The acceptable egg hatch rate should be ~ 27% or less to be monitored over 10 days.

(1B) DNA extraction using prepGEM® Insect kit (Bass *et al*, 2008)

DNA was extracted using DNA Extraction prep GEM insect Kit (Cat: PIN141106, BIOCM biotech®, New Zealand) following manufacturer's instructions with slight modifications. A mosquito leg was crushed and added to the DNA extraction master mix which contained 8.75µl of PCR grade water, 1µl of 10x Buffer (BLACK) and 0.25µl *prep*GEM. This mixture was then incubated in a Thermal Cycler machine (iCycler™, Biorad version 3.021) at 75ºC for 15 minutes followed by a cycle of 95ºC for 5 minutes.

The mixture was then stored on ice or in refrigerator at 4ºC. The DNA quantity and quality in the PCR tubes was determined using a spectrophotometer by measuring the optical density at A260 nm wavelength. High quality DNA preparations have A260/A280 nm wavelength ratio of 1.8 - 2.0.

(1C) DNA extraction using Collins method (Collins *et al*., 1987)

A mosquito leg was dissected and placed in an autoclaved 1.5ml microcentrifuge tube labelled using permanent marker. Each specimen was then homogenised in 200µl buffer (containing 1M NaCl, Sucrose, 0.5M EDTA, 10% SDS and 1M Tris-Cl (pH 8.6). The pestle was removed making sure that no mosquito parts remained on it. The microcentrifuge tube was incubated at 70ºC in a heating block for 30 minutes. Twenty-eight µl 8M KAc *(Pottassium Acetate)* was added and incubated on ice for 30 minutes. After the incubation period, the microcentrifuge tube was centrifuged for 20 minutes at 13 000 rpm. Subsequent to centrifuging, all the liquid was pipetted into a new microcentrifuge tube without disturbing the pellet. Four hundred µl of ice cold 100% ethanol was added to the aqueous phase, mixed by inverting the tube and incubated in a freezer at -20ºC overnight.

The microcentrifuge tube was centrifuged at 13000 rpm for 30 minutes and100% ethanol was discarded leaving the pellet undisturbed. Two hundred µl ice-cold 70% Ethanol was added before the sample was again centrifuged at 13000 rpm for 15 minutes. Thereafter, the 70% ethanol waste was removed. The DNA pellet was allowed to air dry. The pellet was re-suspended in 200µl 1x TE (pH 8) and DNA quantity and quality was determined as described in **2.1.5** (i).

(1D) Testing the presence or absence of Rdl mutation using the hydrolysis probe assay (Bass *et al*, 2008; Du *et al*., 2005)

One microliter of genomic DNA was added into a master mix containing: 4.5µl of PCR water; 10µl of IQ™ Supermix (Cat No: 170-8862, BIO-RAD, United States of America); 1.6µl each of RdlF and Rdlr primers (700nM); 0.4µl of WT2 probe and 0.4µl of Ser Rdl probe (200nM).

The presence of the *rdl* alanine to serine mutation (Du *et al*., 2005) was then detected by the Taqman hydrolysis probe assay in a C100 Touch Thermal Cycler CFX96 real time PCR machine (Biorad, Hercules, CA, USA). The cycling conditions for the PCR machine for the *rdl* assay were: 95ºC for 10 minutes followed by 39 cycles of: 95ºC for 15 seconds; 60ºC for 45 seconds.

APPENDIX 2

2A: University of the Witwatersrand Human Research Ethics Committee (Medical) ethical clearance certificate.

R14/49 Mr Leonard Dandalo

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M141023

NAME: (Principal Investigator)	Mr Leonard Dandalo
DEPARTMENT:	Wits Research Institute for Malaria Mamfene District, KwaZulu-Natal Province NICD
PROJECT TITLE:	Characterisation of Local Genetic Sexing Strain as well as a Wild Population of Anopheles Arabiensis from Kwazulu-Natal, South Africa
DATE CONSIDERED:	31/10/2014
DECISION:	Approved unconditionally
CONDITIONS:	
SUPERVISOR:	Prof Lizette Koekemoer

APPROVED BY: Professor P Cleaton-Jones Chairperson, HREC (Medical)

DATE OF APPROVAL: 07/01/2015

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

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Secretary in Room 10004, 10th floor, Senate House, University.

I/we fully understand the conditions under which I am/we are authorized to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated, from the research protocol as approved, I/we undertake to resubmit the application to the Committee. **I agree to submit a yearly progress report**.

Principal Investigator Signature Date

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

APPENDIX 2B: KwaZulu-Natal Department of Heatlth clearance certificate

health

Department:
Health
PROVINCE OF KWAZULU-NATAL

Health Research & Knowledge Management sub-component
10 – 103 Natalia Building, 330 Langalibalele Street
Private Bag x9051
Pietermaritzburg
3200
Tel.: 033 – 3953189
Fax.: 033 – 394 3782
Email.: hrkm@kznhealth.gov.za
www.kznhealth.gov.za

Reference : HRKM337/14
Enquiries : Ms G Khumalo
Telephone : 033 – 395 3189

Dear Mr L Dandalo

Subject: Approval of a Research Proposal

1. The research proposal titled **'Characterization of a local genetic sexing strain as well as a wild population of Anopheles arabiensis from KwaZulu- Natal, South Africa'** was reviewed by the KwaZulu-Natal Department of Health (KZN-DoH).

The proposal is hereby **approved** for research to be undertaken at Mamfene area provided that the relevant community approval has been obtained from the relevant authority.

2. You are requested to take note of the following:
   a. Make the necessary arrangement with the identified community before commencing with your research project.
   b. Provide an interim progress report and final report (electronic and hard copies) when your research is complete.

3. Your final report must be posted to **HEALTH RESEARCH AND KNOWLEDGE MANAGEMENT, 10-102, PRIVATE BAG X9051, PIETERMARITZBURG, 3200** and e-mail an electronic copy to hrkm@kznhealth.gov.za

For any additional information please contact Ms G Khumalo on 033-395 3189.

Yours Sincerely

Dr E Lutge
Chairperson, Health Research Committee
Date: 24/12/14

APPENDIX 3

Dosimetry

To ensure that test insects received the desired dose during irradiation, a thorough dose mapping of the irradiation chamber was carried out as described by Munhenga *et al*., 2016.

Petri dishes were set up inside the irradiation chamber as shown in figure 1. Dosimeters were placed in petri dishes 4, 5 and 6 (counting from the bottom upwards) filled with 40mls of distilled water. Petri dishes 3 and 7 were also filled with distilled water and were only used as back scatter and were not necessary for irradiations. The dosimeters were placed parallel to each other (Figure 1), one in the middle of the dish and the other at the periphery of the dish secure with colorless tape. All the dosimeters were placed in the same orientation (Figure 2) throughout the stack (3 positions). The surfaces of the dosimeters were marked with a fine marker pen to identify its position in the gamma cell. A polystyrene sleeve covering all the seven petri dishes was used for dosage build up.

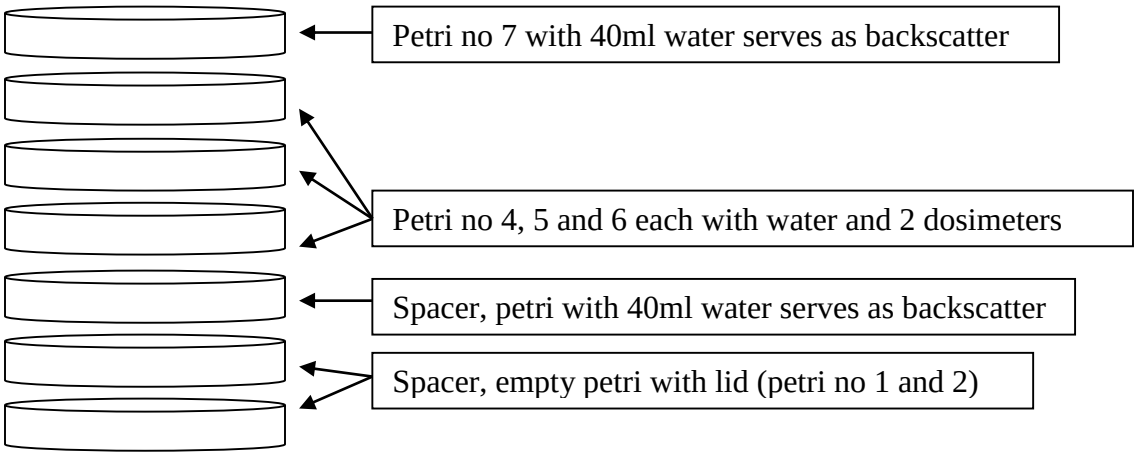


Figure 1: Dosimetry studies set up

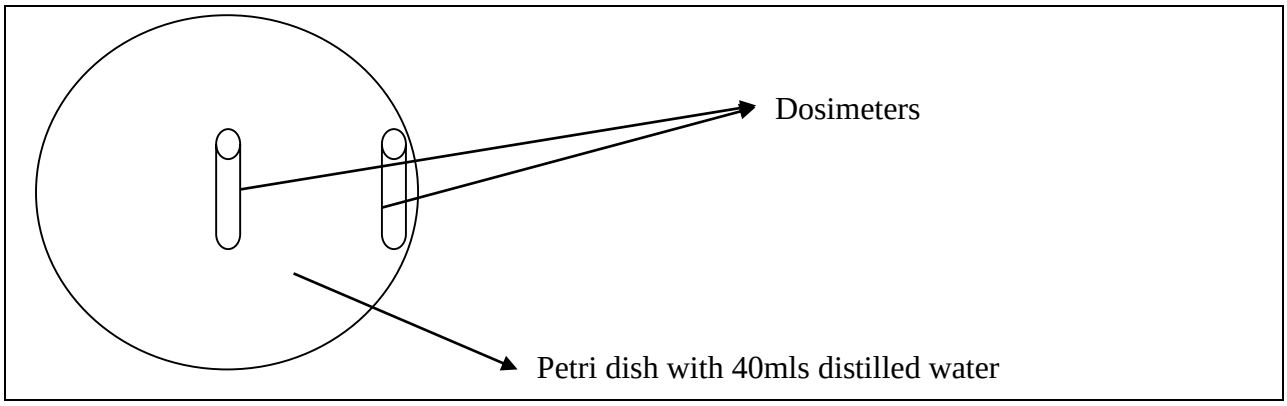


Figure 2: Orientation of dosimeters in petri dishes

Irradiation was carried out in five sets at irradiation times of 36 s, 57s, 78s, 99s and 120s. After exposure dosimeter from each set were carefully covered with a tissue paper and wrapped in aluminium foil to restrict ambient light exposure and send through overnight post to iThemba labs (Cape Town) for optical density readings.

RESULTS

Figure 3 summarizes the dosage at each of the six positions monitored. Results showed that the dose distribution in the irradiation volume of the chamber was not perfectly homogenous. The lowest dosage was recorded at the centre of petri dish number 4 while the highest dosage was recorded at the periphery of petri dish number 6. The variation in the dose rate across the diametrical positions (within petri dishes) was found to be 6%, 7% and 5% for petri dishes 6, 5 and 4 respectively. This falls within the acceptable dose error range for mosquito irradiation. The mean dosage rate variation between petri dish No.4 and 6 was 15%. This variation meant that all the 3 petri dishes could not be used concurrently for irradiation as the error range accepted for mosquito irradiation is 5% (Helinski *et al.,* 2006). The mean dose difference between petri dish number 5 and 6 was 9.8% which is also higher than 5% leaving an option of using one petri dish at any given irradiation. A nylon phantom (doughnut irradiation jig) that allows dose build-up and backscatter and a water filled target volume that ensures a dose variation of less than 6% was developed.

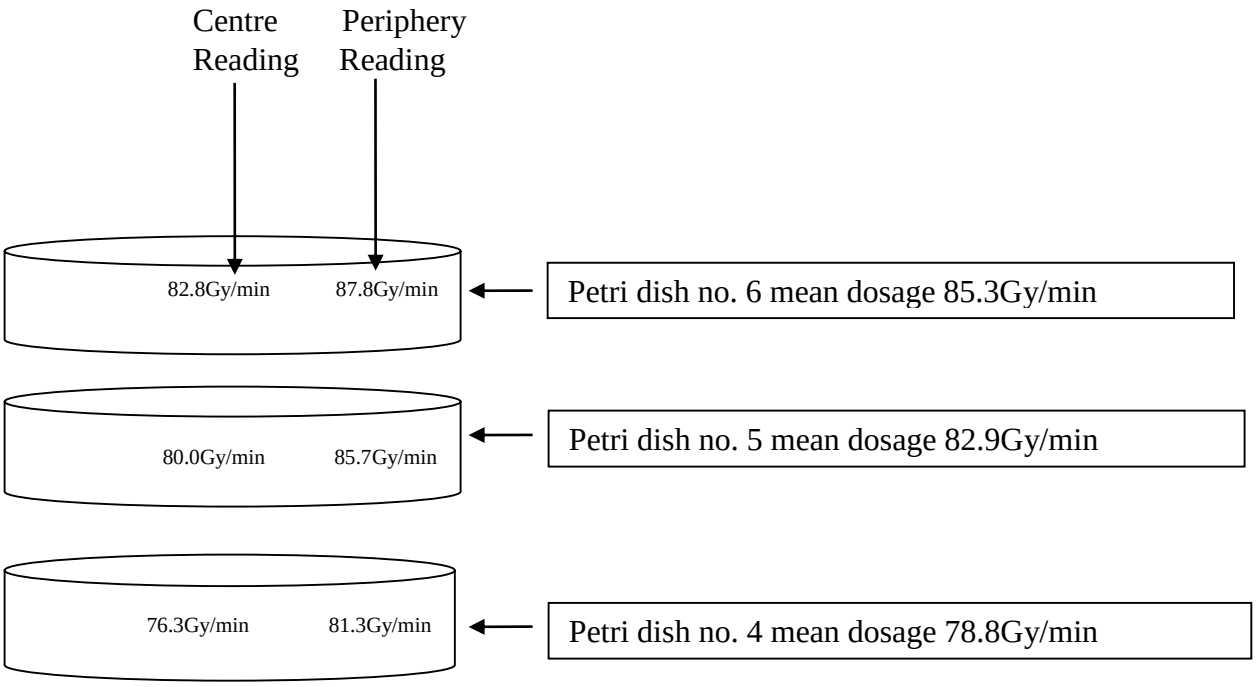


Figure 3: Dose distribution in the SPU gamma cell chamber

APPENDIX 4

Detailed methodology on the effect of ionizing (gamma) radiation on physiological and reproductive fitness of female A*nopheles arabiensis*

Mosquito strain

The local genetic sexing strain of *Anopheles arabiensis* (GMK) described in chapter 2 was used during this experiment. The only difference was that during this experiment female GMK mosquitoes were used instead of males.

Irradiation

GMK_1 females were irradiated at the pupal stage 20-26 hours by exposing them to ionizing (gamma) radiation using a Gammacell 220 (MDS Nordion, Ottawa, Canada) at a single optimal dose of 70 Gy. The irradiator was thoroughly dose mapped (Appendix 3) to ensure that pupae evenly obtained the desired dose (Munhenga *et al*., 2016). In all experiments pupae were transported to an irradiation facility approximately 800m from the insectary in batches of 100-500 suspended in 150ml of distilled water in a doughnut irradiation jig.

Effect of gamma radiation on female emergence

A total of 120 irradiated female pupae were allowed to emerge. The adult female mosquitoes emerging from irradiated pupae (able to fly out of a bowl) were recorded and emergence was calculated as percentage of adults emerged. Dead pupae or semi-emerged adults were scored as non-emerged for the treatment (irradiated) and control (unirradiated) females. The experiment was repeated three times. Within one day of emergence all emerged females were pooled and used for the longevity, insemination rates, fecundity and fertility experiments.

Effect of gamma radiation on the female longevity

Fifty newly emerged adult females from the treated cohorts (irradiated) and controls (unirradiated) were placed in separate BugDorm-1Insect Rearing Cages®. The mosquitoes were maintained on 10% sugar solution soaked in cotton wool at standard insectary conditions. Survival in each cage

was assessed daily by counting the number of dead individuals until 100% mortality was reached. The experiment was repeated three times.

Mating success of irradiated females

Fifty newly emerged irradiated GMK_1 females and 50 newly emerged unirradiated GMK_1 males were allowed to mate for 4 days.

On the fifth day, the insemination rates were determined by dissecting spermathecae of 20 randomly selected females from each treatment and controls under a dissecting microscope (Wild, Heerbrugg M5-71661, Switzerland). Each female's spermatheca was dissected and the presence of spermatozoa was assessed at 200 X magnification. The proportion of inseminated females was then calculated. The experiment was repeated three times.

Effect of gamma radiation on the female fecundity and fertility

Fifty newly emerged GMK_1 females originating from irradiated pupae and 50 newly emerged unirradiated GMK_1 males were allowed to mate for 4 days in BugDorm-1Insect Rearing Cages® (with three replicates) over a five-day period. After the second blood meal, females were left to digest blood for two days. On the second day 20 randomly selected females were individually tubed in oviposition glass vials with a damp filter paper disc to induce oviposition and left overnight (Choi *et al*., 2014). The following day, eggs laid by each female were counted using an Optical Glass Binocular Magnifier (Cat. No: DA-5, Donegam Optical Company, United States of America).

Fecundity was determined by dividing the total number of eggs laid by the total number of females that laid eggs. The mean number of eggs laid per females was then was then compared between treatment and control.

Fertility: Eggs produced by each individual female from each treatment and control cohorts were transferred into plastic bowls (27cm x 16cm x 6.5cm) containing 150ml of distilled water and allowed to hatch. Upon hatching, larvae were counted daily by removing any hatched larvae and recording for 10 consecutive days (Munhenga *et al*., 2016). The larvae picked daily were then killed using hot water and discarded. The proportion of eggs hatching was determined and compared between treatment and control

Effect of gamma radiation on blood feeding success of the females

Fifty newly emerged irradiated GMK_1 females and 50 newly emerged unirradiated GMK_1 males (all sexed at the pupal stage) were allowed to mate for 4 days before the females were offered a blood meal. The proportion of females successfully fed was calculated for both irradiated cohorts and controls. The experiments were repeated three times.

APPENDIX 5

Additional information on population dynamics and *Plasmodium falciparum* infectivity rates for the malaria vector *anopheles arabiensis* at Mamfene, KwaZulu-Natal, South Africa methodology.

Study area

The study was carried out from Mamfene, (S 27° 20'18; E 32° 12'.53), which forms part of Jozini municipality in northern KwaZulu-Natal. The study area lies at a relatively low altitude of 66m above sea level and it experiences an average rainfall of 600 mm – 800 mm per annum along the Lebombo Mountains with temperatures ranging from 23 - 40°C and 16 - 25°C in summer and winter respectively (Jozini Local Municipality, www.jozini.org.za).

Mamfene has a population of about 582,067 people (Census, 2011). Jozini municipality has approximately 38,530 households and most of these are headed by women (Jozini Local Municipality, www.jozini.org.za). The main occupation of most families in this area is subsistence farming along the Makhathini flats where they grow cash crops e.g. sugarcane and keep livestock e.g. cattle, sheep and goats (Obala, 1995). The Makhathini flats have a lot of swampy areas which provide suitable breeding sites for mosquitoes, and the area supports a number of mosquito species despite ongoing vector control activities such as IRS (Obala, 1995; Mouacho *et al*., 2005) (Figure 1). The households in this area are either made of bricks or mud, grass thatched or iron roofed (Figure 2).



Figure 1: Mosquito breeding sites: Swampy area along Makhathini flats in Mamfene



Figure 2: Mamfene households: A) Grass thatched house made of mud and B) iron roofed house made of cement bricks

Mosquito sampling

Figure 3 below shows the mosquito sampling methods that were used during the two year period. Mosquito catches from carbon dioxide baited tents; human landing and tyre were not included in the analysis of trap productivity. The traps were put under shade or near animal Kraal (Figure 4) in each of the randomly selected households.



Figure 3: Mosquito collection methods; A) Clay pot, B) Modified plastic bucket and C) Window exit trap, D) Tyre, E) Carbon dioxide trap and F) Human landing catches



Figure 4: Animal kraal and shrubs that provided shade to the traps

Mosquito identification

(a) *Morphological identification*

All collected mosquitoes were morphologically identified using dichotomous identification keys (Gillies and Coetzee 1987). Only anopheline species positively identified as members of the *Anopheles gambiae* complex and the *An. funestus* group were preserved on silica gel in microcentrifuge tubes and transported to the VCRL of the NICD in Johannesburg, South Africa, for further processing using PCR assays (Scott *et al*., 1993; Van Rensburg *et al*., 1996; Koekemoer *et al*., 2002).

(b) *Species-specific molecular identification using Polymerase Chain Reaction*

i) ***Anopheles gambiae*** **complex species-specific PCR identification**

All primers and PCR reagents used were procured from Inqaba Biotech South Africa. Table 1 shows the primer sequences of all primers used for *An. gambiae* complex species-specific PCR identification.

One leg per specimen was removed and placed in a microcentrifuge tube: 12.5µl of a master mix containing 1.25mM 10X PCR reaction buffer; 1.25mM 10X dNTPs; 25mM $MgCl_2$; 1.65pmol *An.*

quadriannulatus species A and 3.3pmol each of *An. gambiae s.s, An. merus, An. arabiensis* and universal primers; 4.9µl deionised water and 0.5 units of *Thermus aquaticus* (*Taq)* DNA polymerase enzyme were added.

The microcentrifuge tubes were placed into a centrifuge (Biofage, Haraeus), and centrifuged at 13,000 rpm for 10sec to release template DNA. The reaction mixture was then placed into a thermal cycler (Primus 96, MWG Biotech) with the following thermal profile: 30 cycles consisting of 94ºC denaturation for 30 seconds, 50ºC annealing for 30 seconds, and 72ºC extension for 30 seconds and a final auto extension step at 72ºC for 5 minutes (Koekemoer *et al*., 2002). Ten microlitres of the amplified products were loaded onto a 2.5% agarose gel, stained with 16µl ethidium bromide submerged in 1X Tris (hydroxymethyl) aminomethane-acetate (TAE) buffer and subjected to electrophoresis at 100 V for 1 hour 30 minutes or until bromophenol blue was 3cm from origin to allow for proper separation of amplicons. Each gel contained four positive control specimens drawn from known strains of *An. arabiensis, An. merus, An. quadriannulatus* and *An. gambiae s.s*. The negative control consisted of the reaction mixture only. The gel was photographed in a gel documentation box (Syngene G-box, sydr4/1152). The amplified fragment sizes are presented in Table 1.

Table 1: Primer sequences used for *An. gambiae s.l* PCR reactions (Scott *et al.*, 1993)

Species	Primer name	Primer sequence (5’ to 3’)	Transcript length
An. merus	ME	5’ TGA CCA ACC CAC TCC CTT GA 3’	464bp
s An. gambiae s.s	GA	5’ CTG GTT TGG TCG GCA CGT TT 3’	390bp
An. arabiensis	AR	5’ AAG TGT CCT TCT CCA TCC TA 3’	315bp
An. quadriannulatus	QD	5’ CAG ACC AAG ATG GTT AGT AT 3’	153bp
An. gambiae complex	UN*	5’ GTG TGC CCC TTC CTC GAT GT 3’	

* The UN primer anneals to the same position of rDNA for all five species

ii) ***Anopheles funestus*** **group species-specific PCR identification**

Table 2 shows the primer sequences of all primers used for *An. funestus* group species-specific PCR identification. One leg per specimen was removed and placed in a microcentrifuge tube.

Amplification was done in a 12.5µl reaction volume containing 8.25 pmol each of the following primers *An. vaneedeni*, *An. leesoni*, *An. parensis*, *An. funestus*, *An. rivulorum* and a universal primer; 1.5mM $MgCl_2$, 200µM of each dNTP and 0.75 units of *Taq* DNA polymerase. The volume was made up to 12.5µl with double distilled water.

Amplification was carried out in a thermal cycler (Primus 96, MWG Biotech) with the following thermal profile: denaturation at 94ºC for 30 sec, annealing at 50ºC for 30 sec and extension at 72ºC for 30 sec. Each cycle was repeated 40 times followed by a final extension at 72ºC for 5 minutes (Koekemoer *et al*., 2002). Ten microlitres of the amplified products were electrophoresed as described in b (i). Each gel contained 5 positive control specimens drawn from known strains of *An. vaneedeni, An. leesoni, An. parensis, An. funestus s.s* and *An. rivulorum*. The negative control consisted of the reaction mixture only. The gel was photographed in a gel documentation box (Syngene G-box, sydr4/1152). The amplified fragment sizes were 500 base pairs (bp) for *An. funestus s.s*, 400bp for *An. rivulorum*, 550bp for *An. vaneedeni*, 250bp for *An. parensis* and 153bp for *An. leesoni*. These were compared to molecular marker as described in (ii above.

Table 2: Polymerase chain reaction (PCR) primers and sizes of the amplified products for species-specific diagnostic tests for the *An. funestus* group (bp = base pair) (Koekemoer *et al*., 2002).

Species	Primer name	Primer sequence (5' to 3')	Fragment length
An. vaneedeni	VAN	TGT CGA CTT GGT AGC CGA AC	587bp
An. funestus s.s	FUN	GCA TCG ATG GGT TAA TCA TG	505bp
An. rivulorum	RIV	CAA GCC GTT CGA CCC TGA TT	411bp
An. parensis	PAR	TGC GGT CCC AAG CTA GGT TC	252bp
An. leesoni	LEES	TAC ACG GGC GCC ATG TAG TT	146bp
An. funestus group	UN*	TGT GAA CTG CAG GAC ACA T	

* The UN primer anneals to the same position of rDNA for all five species

(iii) *Plasmodium falciparum* screening

1) ***Plasmodium falciparum*** **detection using enzyme linked immunosorbent assay (ELISA)**

All female mosquitoes from the *An. gambiae* complex and *An. funestus* group were tested for the presence of *Plasmodium falciparum* circumsporozoite (CS) proteins using the ELISA method with slight modifications described by Wirtz *et al.*, (1987) and Durnez *et al*., (2011).

To determine *P. falciparum* infectivity, the head and thorax of each female was ground in separate microcentrifuge tubes containing 50µl grinding buffer (BB-NP-40) which was made up to 200µl aliquots of mosquito triturate by adding 150µl of Blocking Buffer (BB).
Wells of a disposable polyvinyl chloride, flat bottomed microtiter plate (Cat. No: 611F96, Thermo Fisher Scientific, Denmark**)** were coated with a 0.200µg/ 50µl volume of monoclonal antibody *P. falciparum* 2A10.28 (Cat. No. CDC-37-01-24-2, Kirkegaard and Perry Laboratories, United States of America). The plate wascovered with aluminium foil paper and incubated overnight at 4ºC. The following day the plate contents were aspirated and washed three times with PBS-Tween [PBS pH 7.4 with 0.05% Tween 20 (Ba. Number: 30023, LabChem, South Africa)]. The plates were then blocked with blocking buffer and incubated for 1 hour at room temperature (23-26ºC). After aspiration of the blocking buffer, 50µl aliquots of mosquito triturate were added to the respective wells. The first well of the microtitre plate (Figure 5) was designated for the positive control and last seven wells had negative controls in all cases. Negative controls consisted of seven 50µl aliquots of triturate of laboratory-reared uninfected *An. arabiensis*. A positive control: [Pf 2+ (Cat. No. Pf-PC, CDC, United States of America)] prepared at 100pg/50µl of BB consisted of a synthetic peptide standardised against *P. falciparum*. The plate was covered with aluminium foil for two hours to incubate, after which, well contents were aspirated and the plates washed twice with PBS-Tween 20, (PBS-Tw) before the addition of 9mg /µl of peroxidase-labelled mouse AB (Pf2A10-CDC; Cat No: 37-00-24-3, United States of America). After 1 hour of incubation in the dark at room temperature, the plates were washed three times with PBS-Tw, followed by addition of 100µl of freshly prepared 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid**)** (ABTS) peroxidase substrate (1-component) (Cat. No.50-66-18, KPL, Inc. United States of America) was added to each well. After 30 minutes of incubation in the dark, plates were read at 405 nm using an ELISA plate reader (Multiskan ascent, Thermo Electron Cooperation Ascent software version 2.6). After first ELISA, false positives also appear on ElLISA plate reader. In order to eliminate these false positives, a second ELISA was performed.

All the samples that tested positive during the first ELISA were confirmed by conducting the second ELISA as follows: The remaining 150µl aliquots of mosquito triturate of the positive specimens were boiled at 100ºC for 5-10 minutes. Fifty microliter aliquots of mosquito triturate from each boiled microcentrifuge tube were added to individual wells of a newly prepared microtitre plate as described above. The same procedure was followed until the samples were read on ELISA plate reader (Multiskan ascent, Thermo Electron Cooperation Ascent software version 2.6).



Figure 5: Microtitre plate showing some positive samples (green wells).

2) ***Plasmodium falciparum*** **detection using (nested)** ***PCR***

All female mosquito samples that tested positive from the ELISA test belonging to the *An. gambiae* complex and *An. funestus* group were tested to confirm the presence of *P. falciparum* circumsporozoite (CS) proteins using nested PCR following the procedure described by Snounou *et al*., (1993) with slight modifications. In order to confirm *Plasmodium* species identity, *Plasmodium* ssRNA from the positive sample was amplified using the following primers: rPLU5: 5'-CCTGTTGTTGCCTTAAACTTC-3'; rPLU 6: 5'-TTAAAATTGTTGCAGTTAAAACG-3 for the first amplification step, and rFAL1: 5'-TTAAACTGGTTTGGGAAAACCAAATATATT-3';

and rFAL2: 5'-ACACAATGAACTCAATCATGACTACCCGTC-3' for the second amplification step.

DNA from the ELISA extraction sample was extracted using phenol chloroform (Sambrook and Russell, 2001) as described below:

One hundred µl of ELISA homogenate was added to 100µl of 1xTris (hydroxymethyl) aminomethane ethylenediaminetetraacetic acid (TE) making a total volume of 200µl. An equal volume of phenol chloroform (ratio of 1:1) was added to mosquito homogenate and mixed. The mixture was centrifuged for 5 minutes. After centrifugation three layers were evident: top layer (DNA in aqueous phase), middle layer (white and contains proteins) and bottom layer (phenol chloroform). The top layer was pipetted off without disturbing the middle layer and transferred into new microcentrifuge tube. One hundred microliter chloroform was then added to 100µl of the DNA aqueous solution, mixed and centrifuged for 1 minute. The top layer was pipetted off without disturbing the middle layer and placed into a new microcentrifuge tube. Ten µl/ ($\frac{\mathbf{1}}{\mathbf{20}}^{\text{th}}$ of volume in supernatant) of 3M sodium acetate (pH 4.8) was added and mixed. Two hundred µl of cold 100% Ethanol was then added to the mixture and stored at -20˚C freezer overnight. The following day, the microcentrifuge tube was centrifuged for 35 minutes at 4˚C (hinge facing out- allows to keep track of the pellet). One hundred percent ethanol was then pipetted (from the opposite side of the tube without disturbing the DNA pellet) and discarded. To the microcentrifuge tube with the pellet, 200µl of cold 70% ethanol was added and centrifuged for 20 minutes at 4˚C. The 70% ethanol was then pipetted from the microcentrifuge tube leaving it to air dry on the lab bench (leaving the top open) until the pellet was completely dry. The pellet was then re-suspended in 30µl of 1xTE, ensuring the pellet dissolved. The DNA was then stored in refrigerator (1 month) or freezer (indefinite).

Two (nested) PCR reactions were performed to confirm *P. falciparum* infectivity. For the first nested PCR, 1µl of DNA (extracted using phenol chloroform) was added into 1.5ml microcentrifuge tube containing 1.25µl 10X PCR reaction buffer; 1.25µl dNTPs (125µM each); 0.75$MgCl_2$ (2mM); primer rPLU5 (250nM); Primer rPLU6 (250nM); 6.25 deionized water and 1 µl Taq DNA polymerase (0.4U).

A total volume of 13.5µl mixture in 1.5ml was then amplified in a thermal cycler (Primus 96, MWG Biotech) with the following cycling conditions: 95ºC for 5 minutes followed by 25 cycles consisting of 58ºC for 2 minutes; 72ºC for 2 minutes and 94ºC for 1 minute and the final steps of 58ºC for 2 minutes and 72ºC for 5 minutes. The second (nested) PCR was conducted using the product from the first PCR as DNA template. One µl DNA from the first PCR product was added into a 1.5ml microcentrifuge tube containing 1.25 µl 10X PCR reaction buffer; 1.25µl dNTPs (125µM each); 0.75$MgCl_2$ (2mM); primer rFAL1(250nM); primer rFAL2 (250nM); 6.25 deionized water and 1µl Taq DNA polymerase (0.4U). A total volume of 13.5µl mixture in 1.5ml was then amplified in a thermal cycler (Primus 96, MWG Biotech) with the following cycling conditions: 95ºC for 5 minutes followed by 30 cycles consisting of 58ºC for 2 minutes; 72º for 2 minutes and 94ºC for 1 minute and 58ºC for 2 minutes; and the final step of 72ºC for 5 minutes. Ten microliters of the amplified products, the positive control and negative controls were electrophoresed as described in b (i). The positive *P. falciparum* sample amplifies at 205bp and was compared with a molecular marker as described in b (i). A fragment of ~1.2 kb which is observed in all reactions corresponds to the product of the first amplification reaction. The first nested PCR products were then purified and sequenced by Macrogen. The sequences were manually edited by BioEdit version 7.2.5 (Hall, 1999). Subsequently, the sequences were aligned with sequences stored in GenBank (GenBank accession number KT991235.1) using nucleotide BLAST (BLASTn) (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

(3) **Sequence analysis of mosquito ITS2 region to confirm *Anopheles* species identity**

To confirm the identity of mosquito samples that tested positive to *P. falciparum* using nested PCR, the internal transcribed spacer rDNA region 2 (ITS2) was amplified using the following primers: ITS2A: 5′-TGTGAACTGCAGGACA-CAT-3′; and ITS2B: 5′-TATGCTTAAATTCAGGGGGT-3′ following the procedure described by Koekemoer *et al*., (2002) with slight modifications.

Two µl of DNA template sample (that tested positive to *P. falciparum* using nested PCR) was added to the mixture of: 2.5µl 10 x PCR buffer; 2µl 10 x dNTPs; 3µl $MgCl_2$; 0.5µl ITS2A primer; 0.5µl ITS2B primer; 14.3µl deionised water and 0.2µl *Taq* DNA polymerase.

A total volume of 23µl mixture in 1.5ml was mixed and then amplified in a thermal cycler (Primus 96, MWG Biotech) with the following cycling conditions: 94ºC for 2 minutes followed by 40 cycles consisting of 94ºC for 30 seconds; 50ºC for 30 seconds; 72ºC for 40 seconds and the final step of 72ºC for 10 minutes. Ten microliters of the amplified products, the positive control and the PCR negative control were electrophoresed as described in b (i). These were compared to molecular marker as described in 2.4.3 (ii) (a). The ITS2 PCR products were then purified and sequenced by Macrogen. The sequences were manually edited by BioEdit version 7.2.5 (Hall, 1999). Subsequently, the sequences were aligned with sequences stored in GenBank (GenBank accession number JN994152.1) using nucleotide BLAST (BLASTn) (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

APPENDIX 6

Givemore Munhenga, Basil D Brooke, **Leonard Dandalo**, Leyya Essop, Leanne Robertson, Power Tshikae, Oliver Wood, Alan Kemp and Lizette L Koekemoer (2016). **Mating competitiveness of laboratory-reared, sterilized males under laboratory and semi-field conditions: Steps towards use of the SIT to control *Anopheles arabiensis* under South African setting and context.** *Parasites & Vectors*, 9: 122.

Contribution

I participated in laboratory work (separating males from females using Terminalia morphology), helped in laboratory competitiveness experiments at VCRL and semi –field competitiveness assays in Kruger National Park.