FITNESS ASSESSMENTS OF *ANOPHELES ARABIENSIS* LABORATORY COLONIES FROM SOUTHERN AFRICA AND THEIR SUITABILITY FOR THE STERILE INSECT TECHNIQUE

Leyya Essop

A dissertation submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in fulfillment of the requirements for the degree of

Master of Science in Medicine

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DECLARATION

I, Leyya Essop declare that this dissertation is my own work. It is being submitted for the degree of Master of Science in Medicine in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other University.

Leyya Essop

17 day of JULY, 2014

DEDICATION

For my parents

and

Mohammed Ra’eel and Mohammed Umayr

“To infinity…and beyond!” (Buzz Lightyear, 1995)

PUBLICATIONS AND PRESENTATIONS ARISING FROM DISSERTATION

1. **Conference presentation**

   18th Congress of the Entomological Society of Southern Africa 2013

2. **Publication (Appendix I)**

   Givemore Munhenga, Basil D Brooke, Belinda Spillings, Leyya Essop, Richard H Hunt, Stephen Midzi, Danny Govender and Lizette L Koekemoer. 2014. Field study site selection, species abundance and monthly distribution of anopheline mosquitoes in the northern Kruger National Park, South Africa. *Malaria Journal*, 13:27.

   Contribution to publication:

   Leyya Essop carried out fieldwork and species-specific identification of specimens collected.

ABSTRACT

In order to employ the Sterile Insect Technique (SIT), biologically fit mosquitoes able to compete with their wild counterparts, suitable field sites for mass release of sterilized male mosquitoes, and appropriate methods of rearing genetic sex-separation (GSS) mosquito strains are required. The life history traits and biological fitness of four laboratory-reared, southern African *Anopheles arabiensis* strains were investigated. Despite being colonized at different times, the four strains demonstrated comparable levels of biological fitness. Three sites in the Kruger National Park were assessed as possible SIT field sites. Mosquito collections were conducted at each site during three summer months. *Anopheles arabiensis* was predominant at Malahlapanga during each collection period, establishing Malahlapanga as the most appropriate site for SIT field trials. A standard larval diet was shown to be appropriate for rearing *An. arabiensis* GSS. This work formed the laboratory basis for the evaluation of a SIT strategy for South Africa.

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To my family, thank you for your love, support, and patience. To Mr. Muhammed Aadil Ally thank you for your encouragement and love.

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%	Percent
µg	Microgram
µl	Microlitre
µM	Micro molar
bp	Base pair
cm	Centimeter
CO_2	Carbon dioxide baited net traps
DDT	1,1,1,-trichloro-2,2,-bis (p-chlorophenyl) ethane / Diethyl diphenyl trichloro ethane
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleotide triphosphates
EDTA	Ethylene diamine tetra acid (disodium salt)
et al.	And others
g	Relative centrifugal force
g	Gram
GSS	Genetic Sexing Strain
h	Hour (s)
HCl	Hydrochloric acid
HLC	Human landing catches
KCl	Potassium chloride
L1	First instar larva
mg	Milligram
mg/ml	Milligrams per milliliter

$MgCl_2$	Magnesium chloride
ml	Milliliter
mm	millimeter
mM	Millimolar
NaCl	Sodium chloride
°C	Degree Celsius
P	Probability level
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pH	Potential of hydrogen
rpm	Revolutions per minute
TAE	Tris Acetic EDTA

CHAPTER 1

LITERATURE REVIEW

1.1) Introduction

Mosquitoes are responsible for transmitting a range of helminthes, viruses, and protozoans to humans around the world. Malaria is a mosquito-borne protozoan infection (Sperança and Capurro, 2007). Annually there are approximately 216 million clinical cases of malaria reported corresponding to an estimated 655 000 deaths (Boete and Koella, 2002, 2003; WHO, 2011a). The tropical regions of the African continent are the most affected by the burden of malaria (fig.1.1) (Morlais *et al*., 2005). This is attributed to various factors such as high levels of poverty, low economic growth, extremely dangerous parasite strain and very effective malaria vectors. The movement of populations into malaria endemic areas, inefficient public health systems, deforestation, and the construction of dams has contributed to the growing incidence of this disease. The distribution of malaria is further aided by climatic changes related to global warming and El Nińo cycles (Gallup and Sachs, 2001; Sachs and Malaney, 2002; Hay *et al*., 2002; Takken and Lindsay, 2003; Caminade *et al*., 2014).

Malaria is particularly prevalent in sub-Saharan Africa where the majority (90%) of cases occur and 46 sub-Saharan countries are classified as malaria-endemic (Moreira *et al*., 2002; Christophides, 2005; Klassen, 2009; WHO, 2011a). There are five species of the *Plasmodium* parasite which cause malaria, namely: *P. falciparum, P. vivax, P. ovale, P.*

knowlesi, and *P. malariae*. *Plasmodium falciparum* is responsible for 90% of malaria cases, particularly in Africa (Snow *et al*., 2005; WHO, 2011a).

Anopheles is the only mosquito genus that is able to transmit malaria to humans (Clements, 2000; Service, 2000; Spitzen and Takken, 2005; Klassen, 2009). Female mosquitoes transmit the *Plasmodium* parasites to humans. In sub-Saharan Africa *Anopheles arabiensis*, *An. gambiae* sensu stricto, *An. coluzzii* and *An. funestus* are the main vectors of malaria (Takken and Lindsay, 2003; Morlais *et al.*, 2005; Klassen, 2009; CDC, 2010; Coetzee *et al.*, 2013a). Both an intermediate host (human) and a definitive host (mosquito) are required in order for malaria transmission to occur (Clements, 2000; Service, 2000).

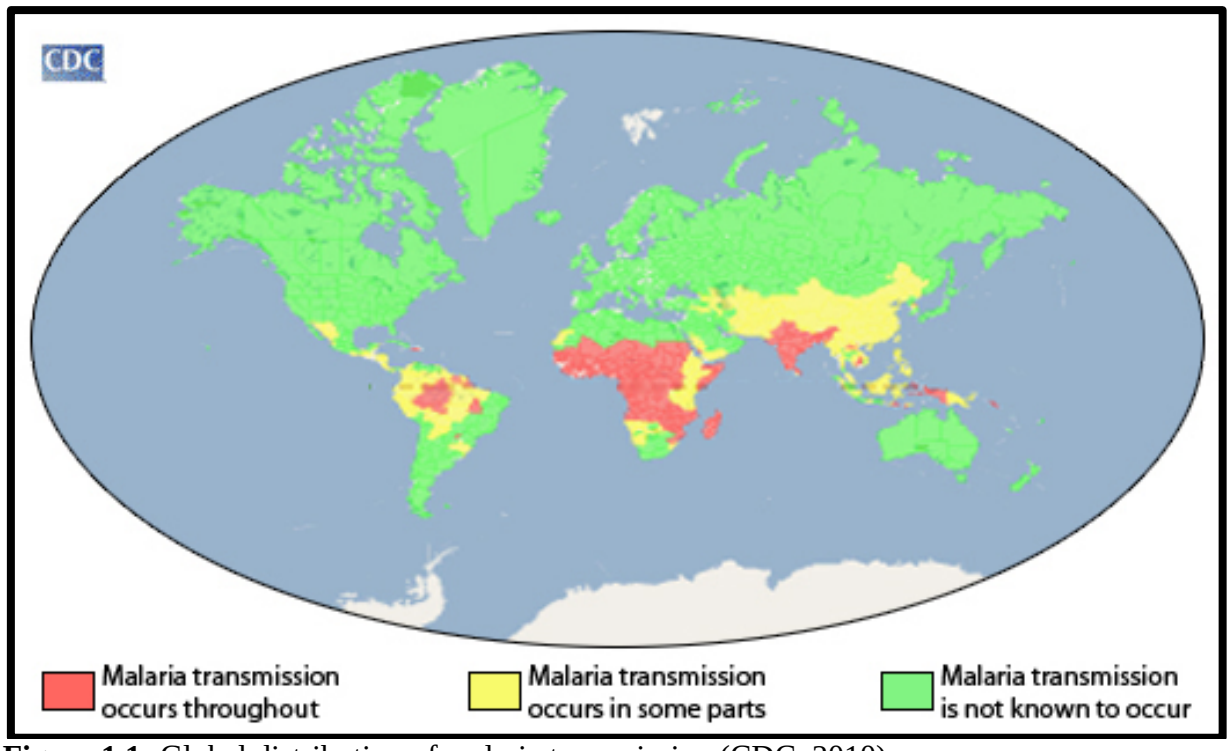

Figure 1.1: Global distribution of malaria transmission (CDC, 2010)

1.2) Malaria vectors

Mosquitoes belong to the order Diptera and are part of the Culicidae family. Anophelinae, Culicinae and Toxorhynichitinae are subfamilies of the Culicidae. *Anopheles*, *Culex*, and *Aedes* are genera which contain vectors of malaria, *Wuchereria bancrofti*, and dengue virus respectively (Clements, 1963; Service, 1980; Service, 2000). In addition to being highly efficient vectors, mosquitoes are also considered to be a nuisance. With the exception of Antarctica and a few islands, mosquitoes have a global distribution and are found throughout tropical and temperate regions, including the Arctic Circle (fig.1.2). Mosquitoes thrive at elevations of 5 500 meters as well as in mines which are 1 250 meters below sea level (Service, 1980; Service, 2000).

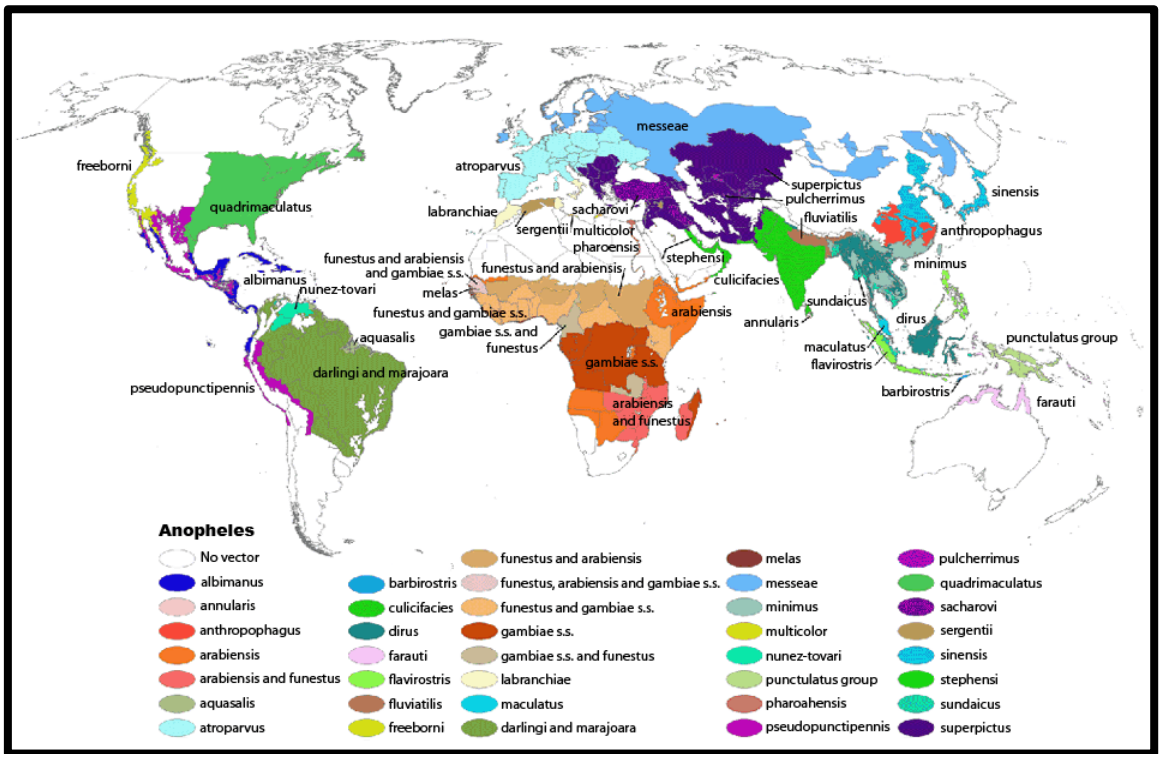

Figure 1.2: Global distribution of *Anopheles* mosquitoes (CDC, 2010)

There are 459 species of *Anopheles* mosquitoes belonging to the subfamily Anophelinae. However, only a few species are vectors of disease.

The *Anopheles gambiae* complex consists of eight species, namely: *An. gambiae* Giles, *An.coluzzii* Coetzee & Wilkerson sp.n., *An. arabiensis* Patton, *An. merus* Dönitz, *An. quadriannulatus* Theobald, *An. amharicus* Hunt, Wilkerson & Coetzee sp.n., *An. bwambae* White and *An. melas* Theobald (Gillies and Coetzee, 1987; Hunt *et al.*, 1998; Coetzee *et al.*, 2013a).

Of these, *An. gambiae*, *An. coluzzii* and *An. arabiensis* are major malaria vectors in Africa (Clements, 2000; Gillies and De Meillon, 1968; Gillies and Coetzee, 1987; Service, 2000; Takken and Lindsay, 2003; Coetzee *et al*., 2013a). During the rainy season *An. gambiae* and *An. coluzzii* are the major malaria vectors, while its tolerance for dry environmental conditions enables *An. arabiensis* to be the predominant vector in areas such as the Sahelian savannas (Gillies and De Meillon, 1968; Gillies and Coetzee, 1987; Morlais *et al*., 2005). Ideally, malaria control involves targeting the vectors (*Anopheles* mosquitoes) as well as *Plasmodium* parasites (Curtis, 2002; Spitzen and Takken, 2005; Sperança and Capurro, 2007). *Anopheles arabiensis* is the primary focus of this research, and will therefore be discussed in further detail.

1.2.1) Biology of Anopheles arabiensis

Anopheles arabiensis is a major vector of malaria in southern Africa, favouring arid regions. This vector species is present in South Africa's high-risk malaria provinces: Limpopo, Mpumalanga, and Kwa-Zulu Natal (Gillies and Coetzee, 1987; Coetzee *et al*., 2000; Sinka *et al*., 2010; Moonasar *et al*., 2012; Brooke *et al*., 2013).

1.2.1.1) Early developmental stages

Mosquitoes are holometabolous insects as a complete metamorphosis occurs during the pupal stage. The mosquito life cycle is comprised of four stages: egg, larva, pupa, and adult. The egg, larval, and pupal stages are completed in aquatic habitats (Clements, 1963; Gillies and Coetzee, 1987; Service, 2000; Takken and Lindsay, 2003).

The female mosquito oviposits 150 to 200 boat shaped eggs on water surfaces (Clements, 1963). The location of these water surfaces ranges from natural habitats such as streams, vegetated ponds and rock-pools, to man-made habitats including wells and clay pots. Temporary water surfaces such as rain pools and hoof prints also serve as oviposition sites (Gillies and Coetzee, 1987; Service, 2000; Takken and Lindsay, 2003; Sinka *et al*., 2010). Anopheline eggs are laid singly and one to three batches of eggs can be laid during the average lifetime of a female mosquito. The eggs are able to float on the water surface with the aid of a pair of lateral, air-filled chambers (fig.1.3). Hatching occurs two to three days post-oviposition, however environmental conditions can cause early and late hatching in certain egg batches (Gillies and De Meillon, 1968; Service, 2000; Yaro *et al*., 2006; CDC, 2010; Kaiser *et al*., 2010).

Figure 1.3: *Anopheles* eggs post-oviposition. The air-filled chambers (floats) are clearly visible on the sides of the eggs. (Modified from MR4, 2010)

Anopheline larvae undergo four instar stages of development (fig.1.4). Moulting occurs at the end of each instar allowing further growth of the larvae. The larvae are filter -, surface-feeders and spend most of their time lying parallel to the water's surface in order to feed and breathe (Clements, 1963; Service, 2000; Takken and Lindsay, 2003; Sinka *et al*., 2010). Disturbances of the larvae or the water's surface will cause the larvae to swim beneath the surface of the water and resurface soon afterwards. Factors such as population density, temperature, and nutrition can affect the growth rate of each larval instar (Lyimo *et al*., 1992; Service, 2000; Bayoh and Lindsay, 2003; Takken and Lindsay, 2003; Okoye *et al*., 2007; Sinka *et al*., 2010). Larval nutrition varies according to the nature of the aquatic habitat selected by the female. The diet of *Anopheles* larvae can include yeast, bacteria, detritus, biofilm and other micro-organisms (Clements, 2000; Service, 2000).

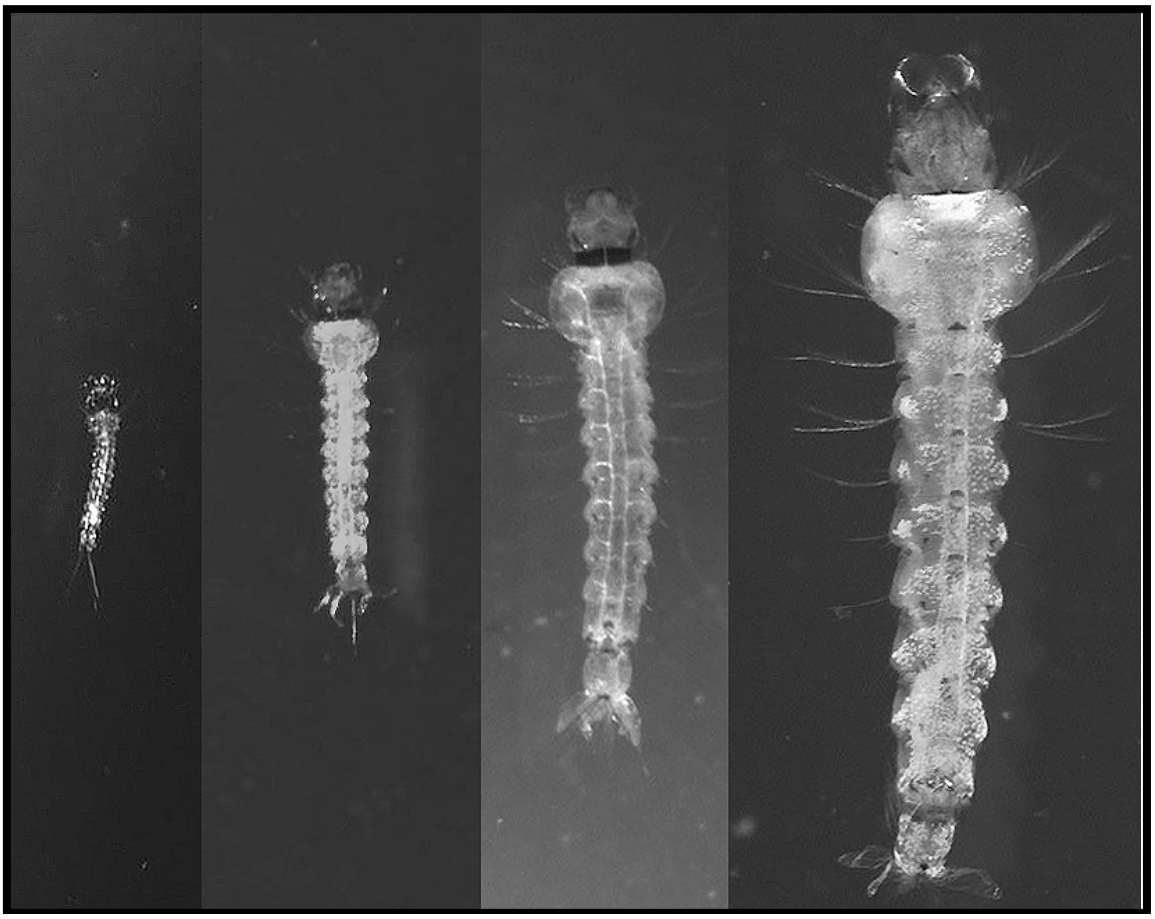

Figure 1.4: From left to right, *Anopheles* larvae first instar, second instar, third instar and fourth instar. (Modified from MR4, 2010)

The four larval instars develop over a period of approximately seven to ten days (Lyons *et al*., 2013). At the end of the fourth instar a comma-shaped pupa is formed (fig.1.5). Pupae do not feed but remain at the water's surface to breathe. A pair of palmate hairs located on the cephalothorax of the pupa allows the pupa to float at the surface of the water. Any disturbances will cause the pupa to swim beneath the water's surface (Clements, 1963; Service, 2000). The duration of the pupal stage is influenced by temperature and can last from one to three days. Metamorphosis takes place during the pupal stage and the transition from an aquatic habitat to a flying adult mosquito occurs (Clements, 1963; Service, 2000; Bayoh and Lindsay, 2003).

Figure 1.5: *Anopheles* pupae (Modified from MR4, 2010)

1.2.1.2) Adult stage

Post-emergence, the adult mosquito rests on the surface of the water until it is able to fly. Depending on environmental conditions, the lifespan of adult mosquitoes ranges from one week to longer than four weeks (Service, 2000). During periods of inactivity, adult mosquitoes rest indoors (endophilic) or outdoors (exophilic). Resting occurs with the body of the mosquito at an angle to the surface (Service, 2000; Takken and Lindsay, 2003; Sinka *et al*., 2010). Adult *Anopheles* mosquitoes have a functional pair of fore-wings with a distinct pattern formed by pale and dark bands (fig.1.6) (Gillies and Coetzee, 1987; Service, 2000). Male mosquitoes meet their nutritional requirements from nectar and other plant juices (Clements, 1963, 2000; Service, 2000). Mating occurs at dusk when males form swarms in order to attract females. Female mosquitoes mate only once during their lifetime. The spermatozoa from the male are stored in the female's spermathecae and are

usually sufficient to fertilize all the eggs laid for the duration of the female's lifetime (Service, 2000; Takken and Lindsay, 2003; Spitzen and Takken, 2005). *Anopheles* females are anautogenous as they require a blood meal in order for egg maturation to occur. Depending on the amount of blood ingested, a female mosquito can lay eggs every two to three days (Clements, 1963; Service, 2000). Blood meals can be obtained by biting animals (zoophagic) or humans (anthropophagic), either indoors (endophagic) or outdoors (exophagic) (Clements, 1963; Gillies and De Meillon, 1968; Service, 2000; Takken and Lindsay, 2003; Sinka *et al*., 2010). *Anopheles arabiensis* females are both anthropophagic and zoophagic, and they are able to blood feed and rest indoors as well as outdoors (Clements, 1963, 1999; Mnzava *et al*., 1995; Githeko *et al*., 1996; Service, 2000; Bøgh *et al*., 2001; Takken and Lindsay, 2003; Tirados *et al*., 2006; Sinka *et al*., 2010). When compared to *An. gambiae*, *An. arabiensis* is more exophagic and exophilic (Gillies and De Meillon, 1968; Takken and Lindsay, 2003; Sinka *et al*., 2010). The preference of *An. arabiensis* to rest and feed outdoors makes it difficult to control this vector species through conventional control methods such as indoor residual spraying (IRS) (Klassen, 2009; Sinka *et al*., 2010).

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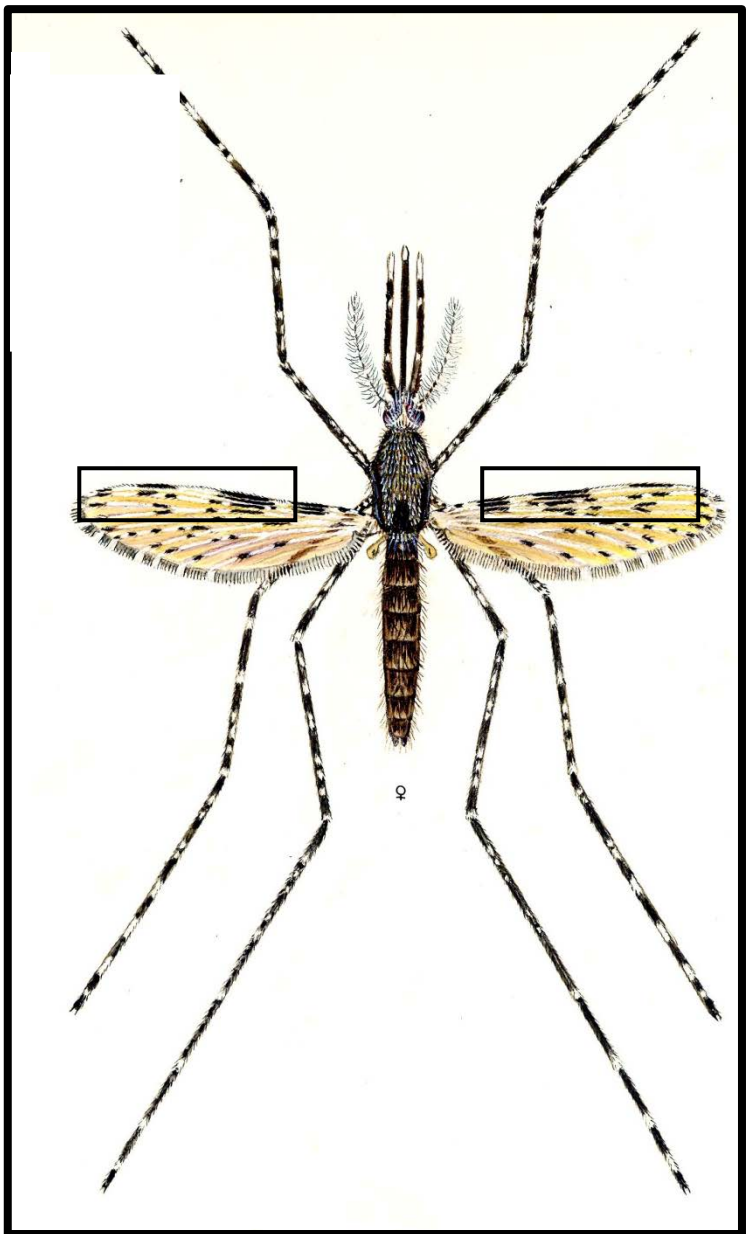


Figure 1.6: *Anopheles* adult mosquito. The patterned wings are indicated within the black rectangles. (N. Lighton, modified from Coetzee *et al*., 2009)

1.3) Vector control

The tendency of mosquitoes to rest on walls and ceilings within human dwellings after blood feeding led to the notion that the treatment of these surfaces with residual insecticides would be lethal to the mosquitoes (De Meillon, 1934, 1936; Klassen, 2009; CDC, 2010; Coetzee *et al*., 2013b). Trials in India and South Africa during the 1930s

proved that the spraying of pyrethrins onto the indoor resting surfaces of mosquitoes was an effective malaria control measure (De Meillon, 1934, 1936; Curtis and Townson, 1998; Curtis, 2002; Klassen, 2009). It was discovered that by applying DDT, the effectiveness of the treatments were prolonged (WHO, 1960; Klassen, 2009). Cyprus, Greece, Sardinia, USA, Taiwan, Italy, and Venezuela also implemented the use of residual insecticide treatment as a form of malaria control. This control measure led to a halt in the transmission of malaria in these countries and buoyed confidence in the idea of employing vector control as the means of global eradication of malaria (WHO, 1960; Klassen, 2009). Thus, the World Health Assembly initiated the Global Malaria Eradication Programme in 1955, with DDT spraying being the main control tool (WHO, 1960; Klassen, 2009). The programme achieved malaria eradication in the southern USA, the temperate regions of Asia and Europe, islands of the Caribbean, certain subtropical regions, and the Mediterranean Basin.

Malaria transmission was significantly reduced in India, Sri Lanka and Brazil (Gahan *et al*., 1945; Gratz, 1999; Klassen, 2009). The success of the programme led to the belief that malaria had been defeated hence residual insecticide treatments were no longer necessary and spraying with DDT was subsequently discontinued in 1964. However, a resurgence of malaria in Sri Lanka and India in 1967 called for the use of the residual treatments once again. Unfortunately, the targeted vector population had by this time become resistant to DDT (Gahan *et al*., 1945; Gratz, 1999; Klassen, 2009). Malathion was proposed as a possible replacement of DDT, however the cost associated with the more frequent applications that would be required, as well as community objections to the use of malathion, rendered this possibility void (Collins and Paskewitz, 1995; Klassen, 2009).

The incidence of malaria continued to rise as the changes associated with economic growth and development, such as mining; deforestation and the construction of dams, provided the ideal environment for the *Anopheles* species to thrive (Gratz, 1999; Klassen, 2009). From 1969 to 1976 the World Health Organization (WHO) conducted a large scale pilot project in the Garki District of northern Nigeria. This project demonstrated that while the residual spraying of insecticides achieved a 90% reduction in vectorial capacity, there was only a 25% reduction in malaria incidence (WHO, 1980; Klassen, 2009). This was attributed to the outdoor resting behaviour exhibited by mosquitoes once they have taken a blood-meal. It was also found that the combination of mass drug administration and residual insecticide treatment was not sufficient to interrupt malaria transmission in regions with a high vector population (WHO, 1980; Klassen, 2009).

To complement the residual treatments, the use of insecticide-impregnated bed nets was introduced on an area-wide basis. While these nets cannot provide protection against outdoor bites, they are able to significantly decrease the number of female mosquitoes and reduce infective bites (Curtis and Townson, 1998; Curtis, 2002; Walker and Lynch, 2007; Klassen, 2009). Africa is the continent which is most affected by malaria, thus in 1998 the attendees of the G8 Summit decided to increase funding aimed at the eradication of malaria, and in 2000 the Abuja Declaration (WHO, 2011b) served as evidence of the commitment of African governments to the support of malaria eradication (Klassen, 2009).

The mainstay of malaria control programmes in Africa has been the use of insecticide treated bed nets (ITNS), indoor residual spraying (IRS) with insecticides and anti-malarial drugs. To date, 45 countries worldwide have reported resistance to at least one of the four classes of insecticides which are used in malaria control programmes (Sperança and

Capurro, 2007; WHO, 2011a; WHO, 2012; Knox *et al*. 2014). While control measures implemented in developed countries achieved malaria eradication, Afrotropical regions are affected by socio-economic, political, and ecological factors which hamper the implementation and success of these same measures in these regions (Takken and Lindsay, 2003; Morlais *et al*., 2005; Sperança and Capurro, 2007). It has been predicted that, unless more effective or additional control measures are implemented, the incidence of malaria will double during the next 20 years (Gallup and Sachs, 2001; Sachs and Malaney, 2002).

Mosquitoes are capable of producing many generations during the year and, as such, there exists the potential for high levels of resistance to insecticides to develop rapidly (Karunamoorthi and Sabesan, 2013) This is highlighted by resistance being documented against insecticides which have been used for just a few years. There are approximately 125 species of mosquitoes which are resistant to at least one insecticide (Karunamoorthi and Sabesan, 2013). The development of resistance to insecticides by *Anopheles* mosquitoes as well as the ongoing global economic and medical burden of malaria has highlighted the need for new and more effective control measures to be developed (Miller and Greenwood, 2002; Hougard *et al*., 2002; Christophides, 2005; Phuc *et al*., 2007; Townson, 2009; Klassen, 2009).

1.4) Sterile Insect Technique (SIT)

The Sterile Insect Technique (SIT) is one such measure that has recently been re-investigated. The International Plant Protection Convention (IPPC) defines the SIT as: “A method of pest control using area-wide inundated releases of sterile insects to reduce fertility of a field population of the same species” (FAO, 2005; Klassen and Curtis, 2005).

Essentially, SIT involves the mass rearing and release of sterile male insects into the field. The sterile males then mate with wild females and reproduction is thus inhibited (Klassen and Curtis, 2005; Helinski *et al*., 2009; Robinson *et al*., 2009).

1.4.1) History of SIT

This idea of releasing sterile males belonging to a specific target species as a method of controlling and possibly eradicating the wild population was developed during the 1930's and 1940's when A.S. Serebrovskii, F.L. Vanderplank and E.F. Knipling developed the concept (Klassen and Curtis, 2005; Dame *et al*., 2009; Alphey *et al*., 2010).

This led to the integration of SIT in an Area-Wide Integrated Pest Management programme that was started during the 1950's and was aimed at eradicating the New World Screwworm, *Cochliomyia hominivorax* (Coquerel) (Craig, 1967; Knipling *et al*., 1968; Klassen and Curtis, 2005; Dame *et al*., 2009; Alphey *et al*., 2010).

The New World Screwworm programme continued for 43 years and achieved its goal of eradicating the New World Screwworm from the USA, Mexico, Central America and Panama. There is ongoing surveillance in these regions in order to prevent reintroduction of this pest species (Christy, 2013). Following the success of the New World Screwworm eradication programme, the SIT was implemented in programmes that targeted fruit flies (*Ceratitis capitata* Wiedemann), tsetse flies (*Glossina austeni* Newstead); the codling moth (*Cydia pomonella*) and the pink bollworm (*Pectinophora gossypiella*) (Craig, 1967; Knipling *et al*., 1968; Klassen and Curtis, 2005; Dame *et al*., 2009; Alphey *et al*., 2010). Currently SIT is employed in fruit fly eradication programmes in South Africa, Thailand, and Israel (Klassen and Curtis, 2005; Dame *et al*., 2009; Alphey *et al*., 2010).

Interest in mosquito SIT was first shown in the 1960's and 1970's and trials were conducted in El Salvador and India. The trial in India focused on the Culicine mosquito species *Culex quinquefasciatus*) (Rai *et al*., 1973; Breeland *et al*., 1974; Klassen and Curtis, 2005; Dame *et al*., 2009; Alphey *et al*., 2010). Studies revealed that this Culicine mosquito species was able to be mass-reared and that the sexes could be separated using pupal size. Separation of sexes and negligible female mosquito releases in mosquito SIT is of vital importance since it is the female mosquito which is responsible for transmitting disease (Rai *et al*., 1973; Breeland *et al*., 1974; Klassen and Curits, 2005; Dame *et al*., 2009; Alphey *et al*., 2010). This is still used as a mass sexing technique for the release of transgenic males in Brazil in 2011 (Harris *et al*., 2012). Sterility was induced with chemosterilants and field tests indicated that mating competitiveness of the males was successful (Rai *et al*., 1973; Klassen and Curtis, 2005; Dame *et al*., 2009).

In El Salvador, *An. albimanus* was targeted due to its multi-resistant status to insecticides, making it a problematic vector to control (Breeland *et al*., 1974; Weidhaas *et al*., 1974; Klassen and Curtis, 2005; Dame *et al*., 2009). Initial releases of sterile males induced 100% sterility in eggs that were laid by wild females. This trial separated the sexes by pupal size and by providing blood meals filled with Malathion to the females. However, in order for the females to receive the lethal blood meals, the males had to endure prolonged time in the cages. Time in the cages coupled with handling prior to release induced stress in the males which ultimately reduced their competitiveness (Breeland *et al*., 1974; Klassen and Curits, 2005; Dame *et al*., 2009).

Studies in India as well as El Salvador produced promising results. However, the outbreak of civil war in El Salvador and the false association of the project with biological warfare in India caused the trials in these countries to be halted. These events highlighted the importance of community involvement as well as the moral and ethical aspects of the SIT. The need for new control measures against malaria has renewed interest in mosquito SIT (Rai *et al*., 1973; Weidhaas *et al*., 1974; Breeland *et al*., 1974; Klassen and Curtis, 2005; Dame *et al*., 2009; Townson, 2009; Ferguson *et al*., 2010).

1.5) Vector Control in South Africa

During the 1920s, malaria control in South Africa comprised of land drainage to reduce mosquito breeding site availability and the treatment of breeding sites with oils. These control methods went on to include the weekly spraying of pyrethrum within houses. In 1946, DDT was selected to replace the use of pyrethrum for vector control (Gericke *et al*., 2002; Govere *et al*., 2002; Hargreaves *et al*., 2003; Brooke *et al*., 2013). This resulted in a significant decline in cases and eventually led to the eradication of malaria from most of the country, except the low-altitude areas of north-eastern KwaZulu-Natal, the Northern Province (currently called Limpopo), and Mpumalanga (Gericke *et al*., 2002; Govere *et al*., 2002; Brooke *et al*., 2013).

South Africa replaced DDT with pyrethroids for malaria vector control in 1995 due to various reasons. There was a growing international concern regarding the safety of DDT since 1976. In addition to this, the local community was objecting to its use due to resistance in the bedbug populations (Newberry *et al.*, 1984) as well as white deposits left on the walls (Coetzee *et al.*, 1999; Govere *et al.*, 2001).

Malaria vector control became reliant on insecticides such as carbamates, pyrethroids, and organophosphates. Between 1996 and 1999 there was a resurgence of malaria in South Africa. Upon investigation, *An. funestus* mosquitoes were found in pyrethroid-sprayed houses. These mosquitoes were discovered to be resistant to the pyrethroid insecticides, but remained susceptible to DDT (Hargreaves *et al*., 2000). The use of these insecticides for indoor spraying as well as for agricultural purposes led to the development of insecticide resistance in malaria vector mosquitoes. In 2002, the National Department of Health recommended that the use of DDT in combination with pyrethroids be used for IRS (Gericke *et al*., 2002; Govere *et al*., 2002; Maharaj *et al*., 2005).

Malaria remains endemic in the northern and eastern regions of South Africa bordering Zimbabwe and Mozambique. Currently, *An. arabiensis* is the main malaria vector in South Africa (Brooke *et al*., 2013; Maharaj *et al*., 2013). The majority of malaria transmission occurs in three provinces, KwaZulu-Natal, Limpopo, and Mpumalanga during the summer months (fig.1.7) (Maharaj *et al*., 2005; Gerritsen *et al*., 2008; Maharaj *et al*., 2012; Moonasar *et al*., 2012).

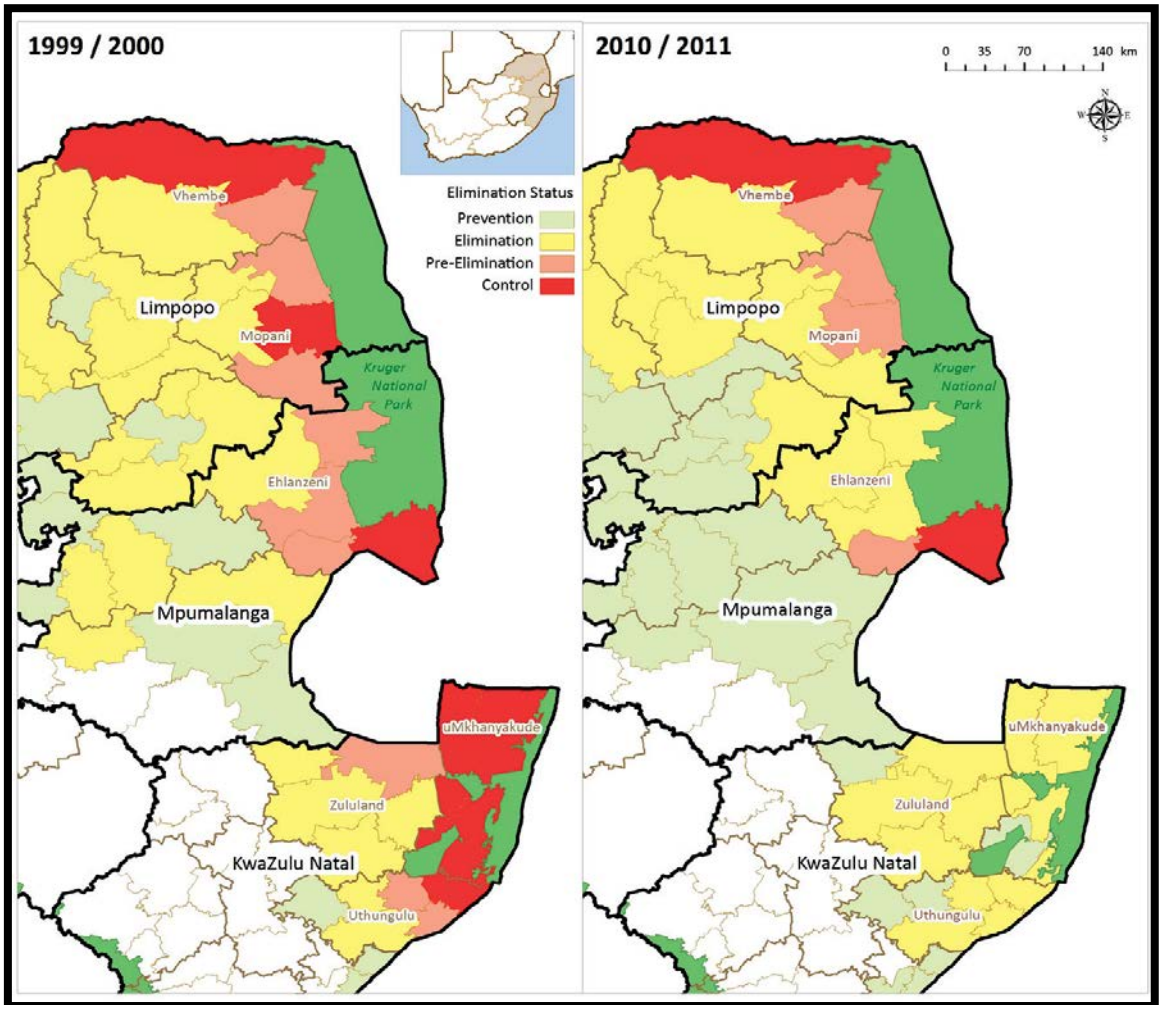


Figure 1.7: Phase of malaria elimination from 1999 to 2011 per district in Kwa-Zulu Natal, Mpumalanga and Limpopo provinces, South Africa. (Modified from Maharaj *et al*., 2013)

The mainstay of malaria vector control in South Africa has been the use of IRS with both DDT and pyrethroids. The use of ITNs is promoted for personal protection however, they do not form part of the National Department of Health's control programme and is mainly used by private individuals. While these control measures have been successful, the emergence of resistance to pyrethroids, particularly in *An. funestus* and *An. arabiensis*, and the limited ability of ITNs to provide protection emphasizes the need for further effective control measures (Hargreaves *et al*., 2000; Gericke *et al*., 2002; Govere *et al*., 2002;

Hargreaves *et al*., 2003; Maharaj *et al*., 2005; Maharaj *et al*., 2012; Moonasar *et al*., 2012; Brooke *et al*., 2013).

1.6) Rationale

Anopheles arabiensis is difficult to control using current vector control interventions in South Africa. This is due to the reliance of IRS on the female mosquito to feed indoors and, thereafter, to rest on the sprayed walls within the structure (Gericke *et al*., 2002; Govere *et al*., 2002; Brooke *et al*., 2013). However, it has been shown that *An. arabiensis* will feed and rest outdoors (Mnzava *et al*., 1995; Githeko *et al*., 1996; Service, 2000; Bøgh *et al*., 2001; Takken and Lindsay, 2003; Tirados *et al*., 2006; Mahande *et al*., 2007; Sinka *et al*., 2010). In addition to this, this species will readily feed on cattle as well (Service, 2000; Takken and Lindsay, 2003; Sinka *et al*., 2010). Therefore, it is clear that, with the current vector control interventions as well as insecticide resistance in this vector species, malaria elimination is not feasible. Additional control interventions are essential. SIT is an attractive tool as it will also target the outdoor feeding and resting proportion of the *An. arabiensis* vector population, which is currently neglected (Service, 2000; Hougard *et al*., 2002; Miller and Greenwood, 2002; Takken and Lindsay, 2003; Christophides, 2005; Phuc *et al*., 2007; Townson, 2009; Klassen, 2009; Ferguson *et al*., 2010; Sinka *et al*., 2010).

Prior to the implementation of SIT as a control intervention it is imperative to obtain baseline information on targeted species. Some of these include the understanding of the local vector populations, identification of appropriate testing field site and the identification of appropriate laboratory reared population that can be targeted for mass rearing and release in the field.

The efficacy of the SIT depends on the ability of the laboratory reared sterile males to successfully compete for mates against wild males of the target population. The processes involved in the SIT, such as mass rearing and irradiation, can adversely affect the fitness and therefore competitiveness of sterile males. It is therefore important to select the fittest and most competitive laboratory reared mosquito colony to ensure that good quality sterile male mosquitoes will be released (Benedict and Robinson, 2003; Klassen and Curtis, 2005; Helinski *et al*., 2009; Marrelli *et al*., 2006; Benedict *et al*., 2009; Dame *et al*., 2009; Robinson *et al*., 2009).

1.6.1) Aims and objectives

The overall aim of the project was to investigate if laboratory reared *An. arabiensis* colonies differ in their overall ability to reproduce. Secondly, in order to determine the feasibility of SIT for South Africa, a large natural population of *An. arabiensis* had to be identified. In 2010/2011 Munhenga *et al.* (2011) confirmed the observations of Braack *et al.* (1994) and Durrheim *et al.* (2001) of the presence of a wild *An. arabiensis* population at Malahlapanga, in the Kruger National Park. However, no data was available on the species distribution over consecutive months or if alternative sites in close proximity to Malahlapanga also host this species.

Specific objectives were:

1. To compare the relative fitness of four laboratory reared *An. arabiensis* colonies from southern Africa.
2. To assess the impact of larval food types on the development of the laboratory reared *An. arabiensis.*
3. To determine the mosquito species distribution at three sites in the northern Kruger National Park during the summer months.

CHAPTER 2

FITNESS COMPARISON BETWEEN FOUR LABORATORY-REARED *ANOPHELES ARABIENSIS* COLONIES.

2.1) Introduction

Laboratory-reared mosquitoes are prone to adapting to the artificial conditions in which they are being reared. This tends to reduce genetic variation and causes a reduction in heterozygosity leading to selection for those genotypes which best ensure survival in a laboratory setting. These effects may, however, adversely affect the fitness and competitive performance of these mosquitoes in the field (Parker, 2005; Huho *et al*., 2007; Robinson *et al*., 2009). Furthermore, as mosquitoes intended for the SIT will be subjected to processes such as mass rearing and irradiation, additional fitness costs might be incurred. It is important to obtain baseline data on the fitness of laboratory-reared colonies in order to determine which colony has maintained the best fitness with regards to those parameters important for mass rearing (Benedict and Robinson, 2003; Lance and McInnis, 2005; Benedict *et al.*, 2009; Howell and Knols, 2009).

2.1.1) Aims and objectives

The aim of these experiments was to determine the relative fitness of four laboratory reared *An. arabiensis* colonies from southern Africa. Fitness in these colonies was measured using specific life table characteristics including: Larval hatch rate (L1), pupation, adult emergence, fertility and fecundity, mating success and longevity of adult mosquitoes.

2.2) Materials and Methods

2.2.1) Laboratory reared material:

Anopheles arabiensis eggs were obtained from four laboratory reared colonies: MBN-BASE, KWAG-BASE, KGB, and MA. The MBN-BASE and KWAG-BASE colonies originate from material collected in Mamfene region of Kwazulu-Natal, South Africa, and were colonized in 2002 and 2005 respectively. Although both colonies originate from Mamfene, they were established during those instances when DDT resistance (2002, MBN-Base) and subsequently pyrethroid resistance (2005, KWAG-Base) was detected in the field (Hargreaves *et al*., 2003; Mouatcho *et al*., 2009). Note that neither colony was put under insecticide selection pressure and both are susceptible to insecticides (Nardini *et al*., 2012; 2013).

The MA colony originates from material collected in Maputo, Mozambique, and was colonized in 1990, while the KGB colony was colonized from field specimens collected in Kanyemba, Zimbabwe, in 1975. Both these colonies are also susceptible to insecticides.

These colonies are currently being reared under standard rearing conditions of 25ºC and ± 80% relative humidity in the Botha de Meillon Insectary (BDMI) at the Vector Control Reference Laboratory (VCRL) of the National Institute for Communicable Diseases (NICD) (Hunt *et al*., 2005). Larvae are fed a powdered diet consisting of crushed dog biscuits (West's Beeno Traditional Crunchy Biscuit Treats, Martin and Martin, South Africa) and Brewer's yeast (Vital Health Foods, South Africa) prepared at a ratio of 3:1. Adult female mosquitoes receive blood-meals from anaesthetized guinea pigs (Ethics Clearance REF:W-CJ-100510-1; Appendix II) twice a week. The insectary operates on a 12 h day/night cycle with a 30 min dusk/dawn transition period. All experiments were conducted in the BDMI of the VCRL using standard rearing equipment. Each experiment consisted of three replicates per colony.

2.2.2) Life table analysis:

Life table analyses were conducted according to a protocol developed by Dr. B.D. Brooke (2010, unpublished report) (Appendix III).

The following parameters were compared among the four laboratory-reared *An. arabiensis* colonies: fertility (hatch rate), larval and pupal survival, adult emergence, fecundity of females (egg production), mating success and longevity of adult mosquitoes.

2.2.3) Comparison of fertility, fecundity, and mating success between MBN-BASE, SENN-BASE, KGB, and MA colonies.

a) Fertility, fecundity, hatch rate, larval survival, pupation and adult emergence:

Eggs were collected from each of the above mentioned colonies and were reared through to the adult stage under standardized rearing conditions. Larvae received weighed amounts of food (Appendix III). Once adult mosquitoes emerged, 50 female and 70 male adult mosquitoes (per replicate) were transferred into 2 L adult cages (fig.2.1) and mating was

allowed to occur over a period of a week. These adults had continuous access to a 10% sugar solution (Appendix III) and the females received three blood-meals according to the insectary blood-feeding schedule. The first blood-meal was offered to females at four days old, and the second and third blood-meals at seven and nine days old respectively. The morning following the third blood-meal, 30 females per replicate were carefully transferred individually into oviposition vials (fig.2.2) while 20 females per replicate were used to calculate mating success (described below (b)).

Individual single-family egg batches were obtained during which time the females had continuous access to 10% sugar solution. Once oviposition had occurred, the number of eggs per female was counted. Each egg batch was transferred into a larval rearing bowl (27 cm x 16 cm x 6.5 cm) containing 150 ml of distilled water (fig.2.3). Fecundity was calculated as the mean number of eggs oviposited per female during the first gonotrophic cycle. Fertility was determined by the hatch rate of the first instar larvae.

Figure 2.1: 2 L adult rearing cages.

Figure 2.2: Oviposition vials with one female placed in each.

Figure 2.3: Larval rearing bowls.

Manual, daily counts of first instar larvae were conducted for each egg batch. The mean hatch rate for each colony was calculated from the proportion of first instar larvae accrued from 50 randomly selected eggs per female. First instar larvae were counted and

transferred to separate larval bowls on a daily basis. This was done to avoid double counting of newly hatched larvae. Larvae were fed thrice daily as they developed through each instar. Larval food was weighed for each instar to ensure that there was no variability in the amount of food received by each replicate or colony (Appendix III). Dead larvae were counted and removed on a daily basis. Pupae were removed daily using a pipette, counted, placed into pupal jars and transferred into 2 L adult cages. Pupae from each replicate and colony were caged separately. A daily count of adult mosquitoes emerging from the pupae was conducted.

b) Mating success

Twenty females were anesthetized using ethyl ether (MR4, 2010). Their spermathecae, located in segment VIII, were dissected out in Phosphate Buffered Saline (PBS) (Cat. No. 524650, CALBIOCHEM, Merck) (Appendix III), and viewed under a compound microscope (Olympus) at 100x magnification (MR4, 2010) in order to determine if sperm were present. The presence or absence of sperm within the spermathecae was recorded as an indicator of mating success (fig.2.4).

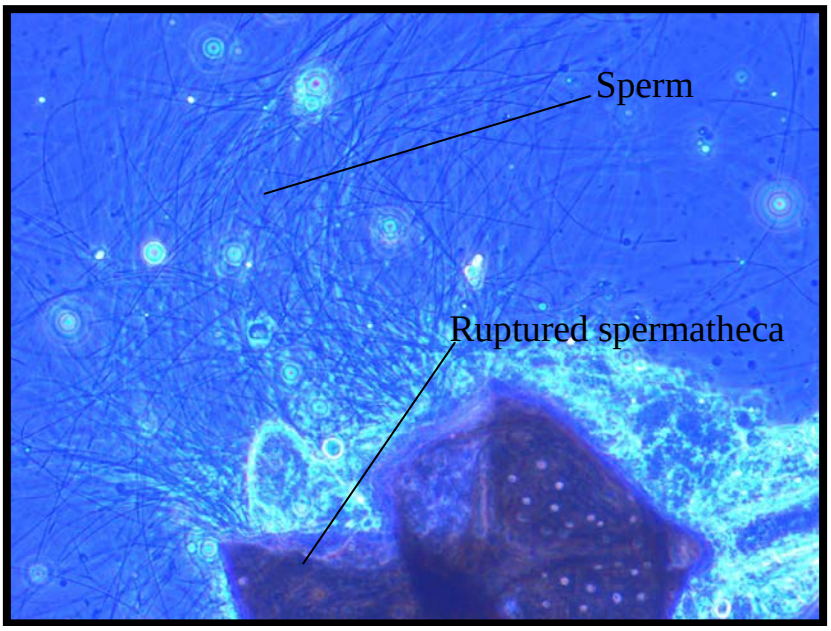


Figure 2.4: A spermatheca containing sperm.

2.2.4): Longevity and wing length measurements of adult male and female mosquitoes

In order to measure and compare adult longevities between colonies, larvae and pupae were reared as described in 2.2.3. Fifty adult male and female mosquitoes (constituting one replicate) from each colony were placed into separate 2 L adult cages. These adults had continuous access to a 10% sugar solution, refreshed every three days. A daily count and removal of dead adult mosquitoes was conducted until all the adults had died.

Wing length gives a sound approximation of mosquito body size which has previously been correlated to longevity (Lyimo *et al*., 1992; Lyimo and Takken, 1993; Yan *et al*., 1997; Blackmore and Lord, 2000; Ponlawat and Harrington, 2009). One wing from each dead adult from the longevity experiments was dissected and mounted onto a clean glass slide. The wing length (wing tip to thorax joint) was measured at 200 x magnification using an eyepiece micrometer mounted on a dissecting microscope (Olympus). Mean wing lengths were compared by gender and colony.

2.2.5) Data Analysis

Data were analyzed using the one way analysis of variance (ANOVA) and Kaplan-Meier survival analysis functions of the statistics programmes GraphPad Prism version 6.00 for Windows, GraphPad Software, La Jolla California USA (www.graphpad.com), and Statistix 7® program (Talahasee, Florida, USA), respectively. Results were interpreted at 95% confidence intervals. A P-value of less than 0.05 ($P<0.05$) indicated statistical significance. All fitness parameters assessed, excluding longevity, were represented in bar graphs. The longevity was represented in a Kaplan-Meier survivorship curve.

2.3) Results

The life history characteristics for each colony are summarized in Table 2.1. The fertility, determined by the hatch rate of first instar larvae, for each replicate of all four colonies was 100% (Table 2.1). The overall fecundity of the four colonies is shown in figure 2.5 and Table 2.1. The fecundity of MA, KGB, KWAG-BASE, and MBN-BASE was 80.33% (n=90); 81.53% (n=90); 85.67% (n=90); and 86.3% (n=90) respectively. There was no statistically significant difference between the fecundity ($P = 0.61$; $F=0.63$; $R^2=0.19$; $DF=3$) (fig.2.5, Table 2.1) of the four colonies.

Table 2.1: Life table characteristics by *An. arabiensis* laboratory colony.

Characteristic	MA	KGB	MBN-B	KWAG-B
Fertility (hatch rate) (%)	100	100	100	100
Mean fecundity	80.33	81.53	86.3	85.6
Survivorship, L1 to Pupa (%)	96	96.3	98	98
Survivorship, Pupa to Adult (%)	100	99.93	99.86	99.83
Mean wing length (mm)				
All adults	3.34	3.35	3.36	3.35
Female	3.37	3.37	3.39	3.36
Male	3.31	3.34	3.33	3.34
LT50 (days)	17.74	16.17	16.48	16.39

Fecundity = the number of eggs oviposited per female during the first gonotrophic cycle;

L1 = first instar larva; LT50 = Lethal Time (the time taken for 50% mortality to occur).

MBN-B=MBN-BASE; KWAG-B=KWAG-BASE

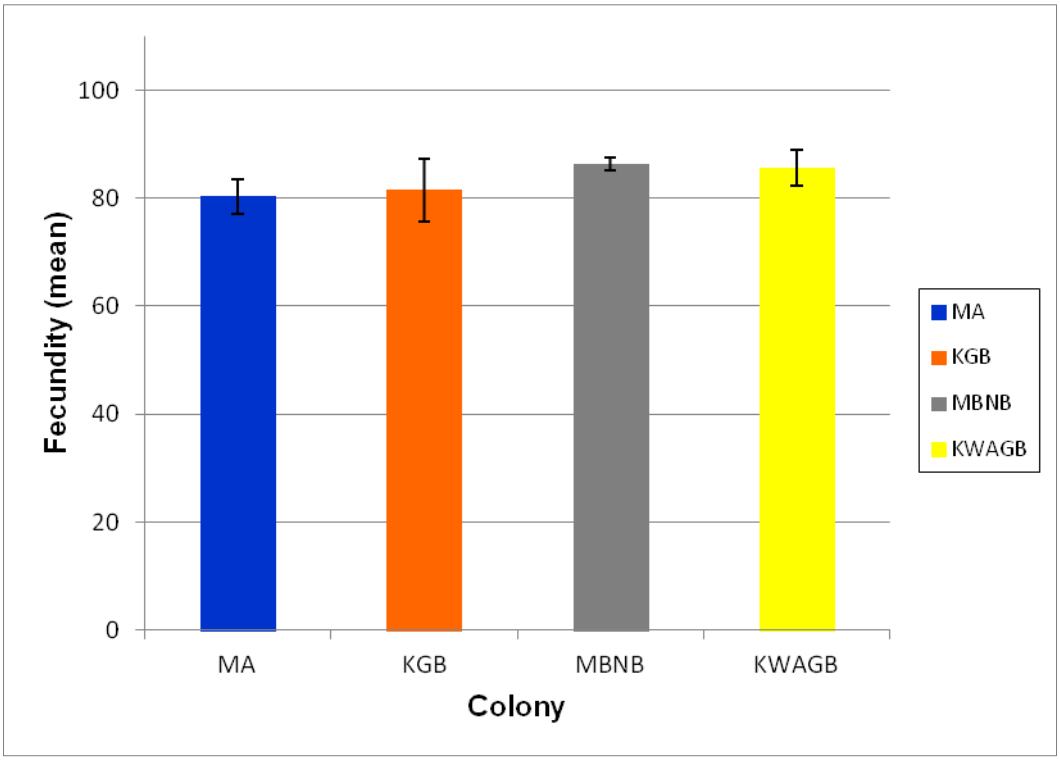


Figure 2.5: Fecundity of four *An. arabiensis* laboratory colonies. Fecundity was calculated as the mean number of eggs oviposited per female during the first gonotrophic cycle. MBNB=MBN-BASE; KWAGB=KWAG-BASE

Larval survival was calculated as the number of larvae which successfully pupated. For MA, KGB, KWAG-BASE, and MBN-BASE, the larval survival was 96% (n=1 120); 96.33% (n=2 555); 98% (n=3 087); and 98% (n=3 088) respectively (fig.2.6, Table 2.1). The proportion of larvae that pupated did not differ significantly between the four colonies (P= 0.37; F=1.21; R^2=0.31; DF=3) (fig.2.6, Table 2.1). Pupal survival was determined as the number of pupae which successfully emerged as adult mosquitoes, and did not differ significantly between the four colonies (P = 0.47; F= 0.94; R^2=0.30; DF=3) (fig.2.7, Table 2.1).

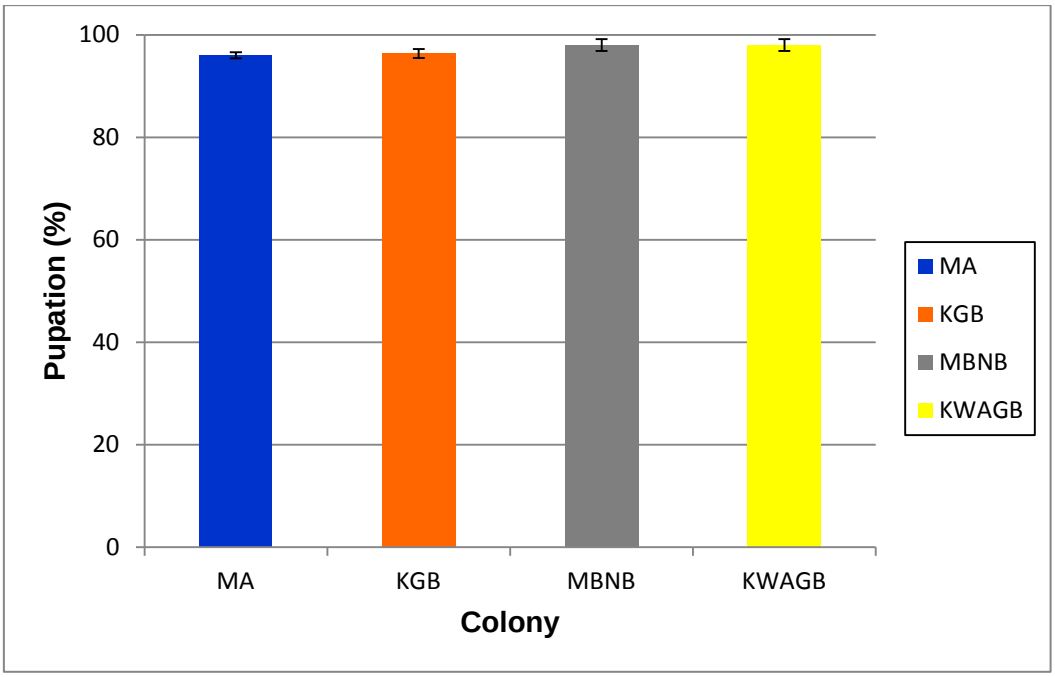


Figure 2.6: Mean larval survival (calculated as the proportion of larvae that pupated) of four *An. arabiensis* laboratory colonies. MBNB=MBN-BASE; KWAGB=KWAG-BASE

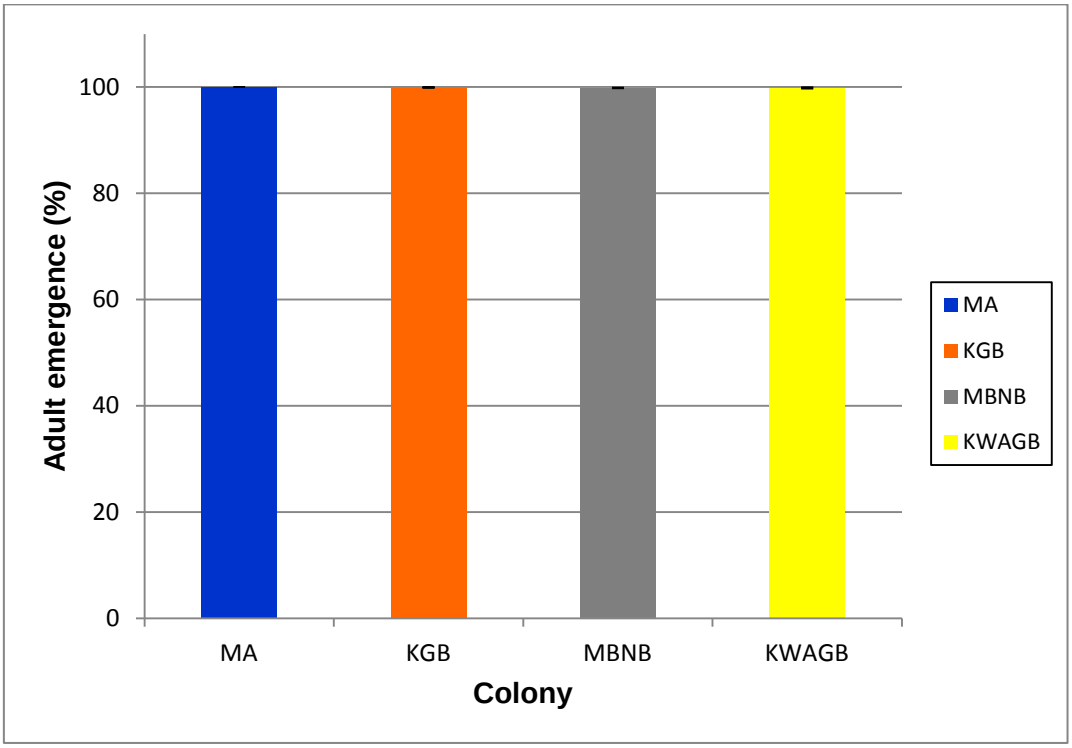


Figure 2.7: Mean pupal survival (calculated as those larvae that emerged as adults) of four *An. arabiensis* laboratory colonies. MBNB=MBN-BASE; KWAGB=KWAG-BASE

The presence of sperm was observed in 97% (n=60) MA females, 98% (n=60) KGB females, 97% (n=60) KWAG-BASE females, and 98% (n=60) MBN-BASE females. The mating success between the colonies was not statistically different (P = 0.94; F=0.13; R^2=0.50; DF=3) (fig.2.8, Table 2.1).

The combined mean wing lengths for adults (combined male and female; n=300) was 3.34 mm, 3.35 mm, 3.36 mm, and 3.35 mm for MA, KGB, MBN-BASE, and KWAG-BASE respectively (Fig.2.9), which did not differ significantly (P= 0.74; F=0.42; R^2=0.14; DF=3). The mean wing lengths for female mosquitoes (n = 150) were 3.37 mm, 3.37 mm, 3.39 mm, and 3.36 mm for MA, KGB, MBN-BASE, and KWAG-BASE respectively (Fig.2.9, Table 2.1), which did not differ significantly, (P = 0.17; F=2.14; R^2=0.45; DF=3). Adult males (n =150) had a mean wing length of 3.31 mm, 3.34 mm, 3.33 mm, and 3.34 mm for MA, KGB, MBN-BASE, and KWAG-BASE respectively (Fig.2.9, Table 2.1), which did not differ significantly (P= 0.69; F=0.50; R^2=0.20; DF=3).

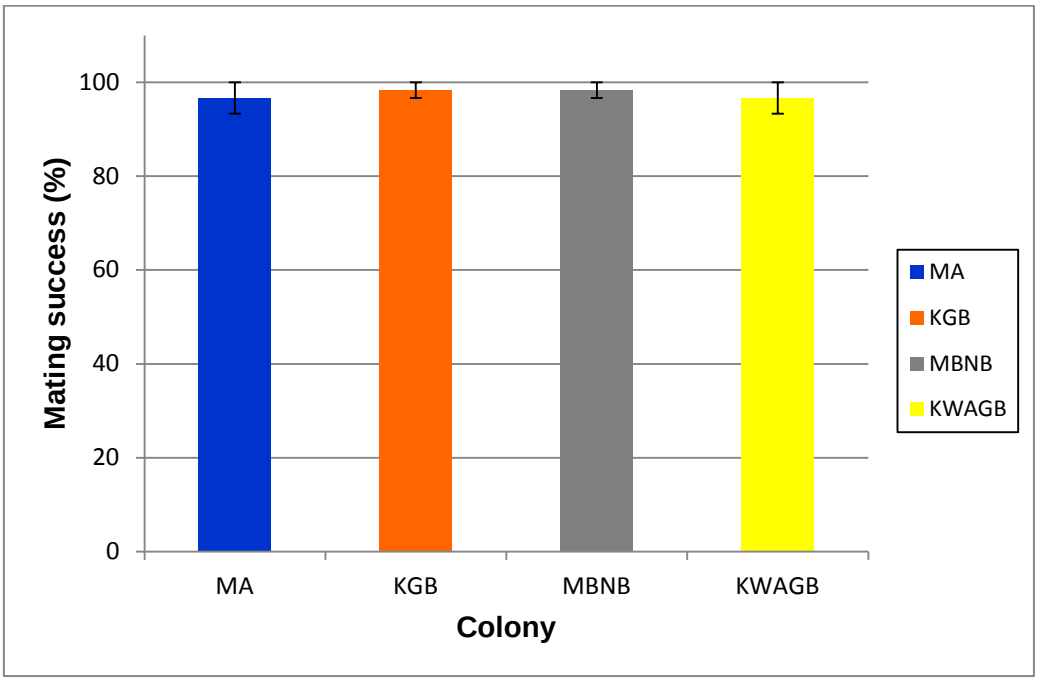


Figure 2.8: Mating success of four *An. arabiensis* laboratory colonies. MBNB=MBN-BASE; KWAGB=KWAG-BASE

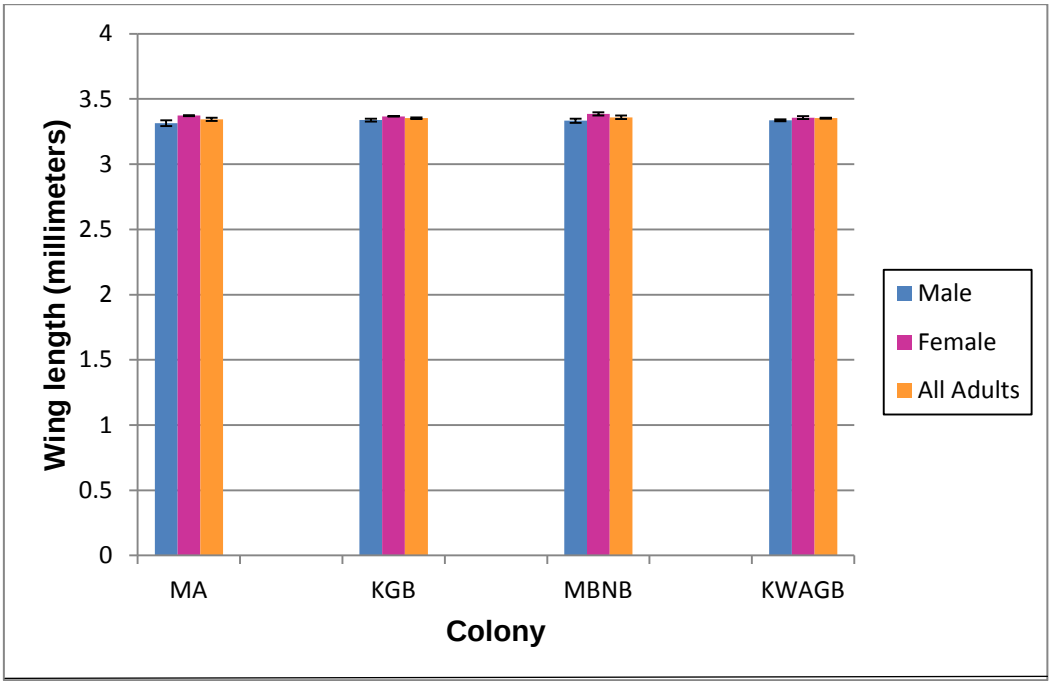


Figure 2.9: Wing length of adult male and female *An. arabiensis* from four laboratory colonies. MBNB=MBN-BASE; KWAGB=KWAG-BASE

Overall adult survivorship as a measure of longevity is shown in figure 2.10. Adult longevity did not significantly differ between the four colonies (P = 1) (fig.2.10). The LT50 for MA, KGB, MBN-BASE, and KWAG-BASE was 17.74, 16.17, 16.48, and 16.39 days respectively (fig.2.11, Table 2.1). Despite the LT50 of MA being one day longer than that of the other colonies, the LT50 between the four colonies did not differ significantly (P = 0.18; F=2.09; R^2=0.44; DF=3) (fig.2.11, Table 2.1). Mortality occurred from day two for KGB, MBN-BASE and KWAG-BASE, and from day eight for MA (fig.2.10, Table 2.1). At day 32 there was 100% mortality in all four colonies (fig.2.10). Survivourship according to gender was not reported as it was not part of this specific study.

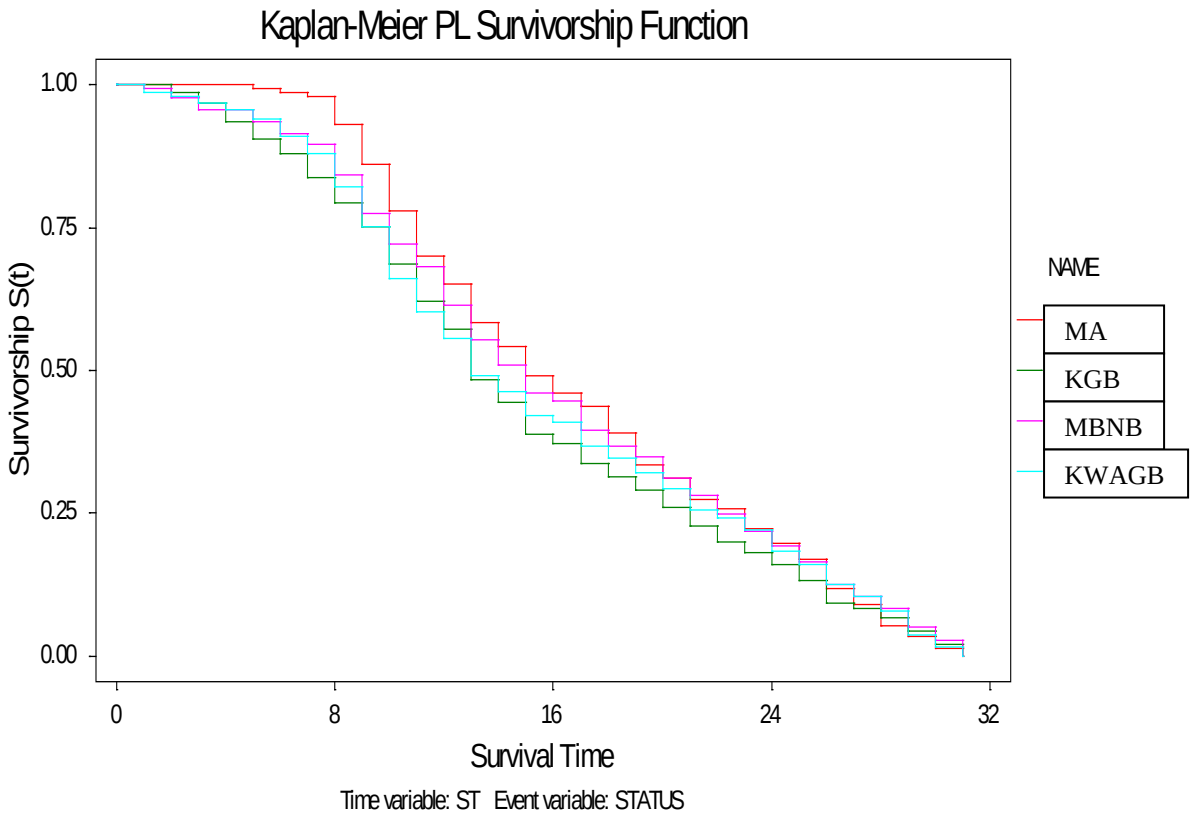


Figure 2.10: Adult survivorship curve for four *An. arabiensis* colonies. MBNB=MBN-BASE; KWAGB=KWAG-BASE

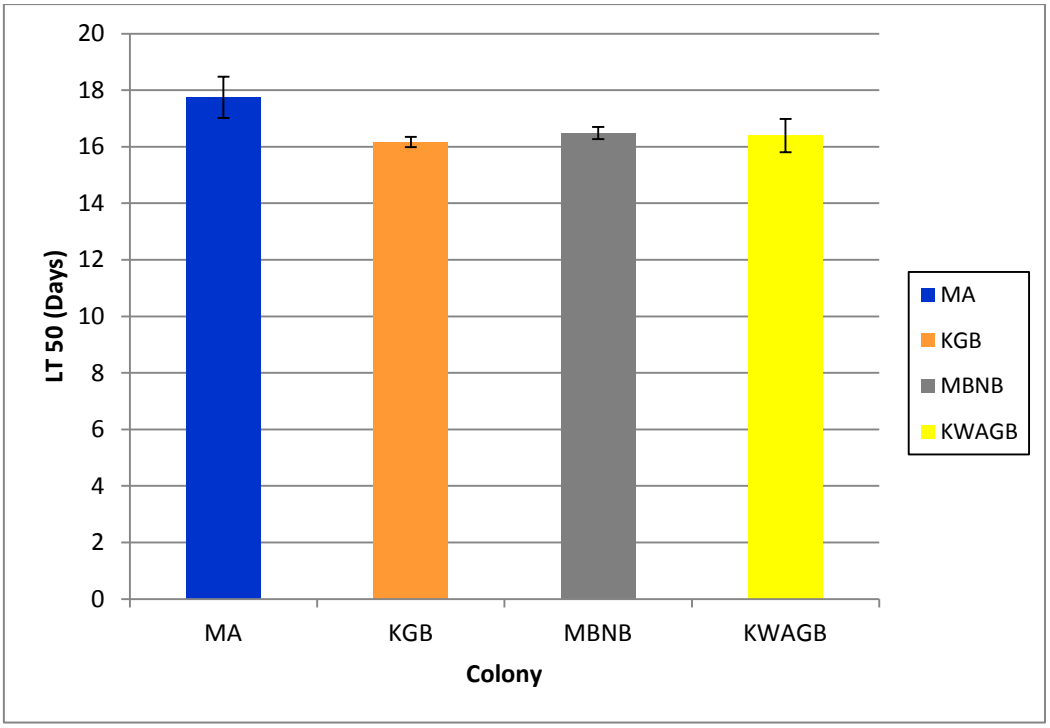


Figure 2.11: Lethal times to 50% mortality (LT50) in adults of four *An. arabiensis* laboratory colonies. MBNB=MBN-BASE; KWAGB=KWAG-BASE

2.4) Discussion

There were no differences in biological fitness between the four laboratory-reared *An. arabiensis* colonies: MA, KGB, MBN-BASE, and KWAG-BASE. All four colonies showed 100% fertility. Fertility was determined by the hatch rate of the first instar larvae. The proportion of eggs hatching under natural circumstances is variable and affected by many factors. Yaro *et al* (2006) discuss the egg hatching in natural populations, where hatches were a maximum of 70%. When a similar study was performed in a laboratory population by Kaiser *et al* (2010), 100% hatch rate was observed in several wild families. Therefore the observation of a 100% fertility (determined by hatch rate) is not unprecedented. A female mosquito lays and average of approximately 100 eggs per egg batch (Lane and Crosskey, 1993), therefore, 50 eggs are representative of a single clutch of eggs. With such a small batch of eggs and under laboratory conditions, the factors that would prevent all the eggs from hatching (such as desiccation) can be negated by stringent laboratory conditions. Although 100% hatch rate is not common in wild populations or routine colony maintenance, with a small egg batch, eggs can be carefully maintained and a 100% hatch rate can be obtained. While the SIT involves the release of sterilized males only, it is important for the female fertility of the colony providing the male mosquitoes to be high, as this will ensure that the colony can be sufficiently produce the numbers of males required for mass release. Larval survival did not differ significantly between the four colonies and there was no statistically significant difference in the pupal survival (adult emergence) between the four colonies. Mating success was determined by the number of females inseminated. Results showed no significant difference in the mating success of the four colonies.

Analyses of wing length measurements showed that adults from each colony did not differ significantly. This implies that adults from the MA, KGB, MBN-BASE, and, KWAG-BASE colonies are of a similar body size.

Survival analyses of adults from the MA, KGB, MBN-BASE, and KWAG-BASE colonies showed no significant difference in longevity. Mortality commenced on the first day (day 1) in KGB, MBN-BASE, and KWAG-BASE, and day 8 in MA. However, the duration to 100% mortality was 32 days in all four colonies.

Environmental factors such as temperature and diet can affect the development and survival of mosquitoes (Clements 1963, 1999; Service, 1980; 2000; Okoye *et al*., 2007). During this study however, temperature was maintained at 25°C ± 1°C, 80% ±10% RH, and larvae received weighed amounts of food (Appendix III).

The genetic profile, colonization, and exposure to laboratory rearing conditions can affect mosquito fitness and life history characteristics (Lyimo *et al*., 1992; Munstermann, 1994; Nylin and Cotthard, 1998; Armbruster *et al*., 2000; Blackmore and Lord, 2000; Huho *et al.*, 2007; Okoye *et al*., 2007; Howell and Knols, 2009). This study assessed four *An. arabiensis* colonies originating from three southern African countries. These colonies have dissimilar genetic backgrounds and were in colony for varying lengths of time at time of experimentation. However, they share similar life history characteristics and comparable levels of biological fitness. This suggests that each of these colonies have adapted to laboratory conditions to the same extent.

2.5) Conclusion

This study provides essential baseline data concerning the effects of colonization on the life history traits and biological fitness of *An. arabiensis* mosquitoes from southern Africa. Parameters such as fertility, fecundity, mating success, and longevity did not significantly differ between the laboratory –reared *An. arabiensis* colonies: MA, KGB, MBN-BASE, and KWAG-BASE. Even though the four colonies were introduced to laboratory settings at different times, they share similar life history characteristics and have equally adapted to laboratory conditions. MA, KGB, MBN-BASE, and KWAG-BASE show the same levels of biological fitness. This implies that, since there is no difference in the level of biological fitness, each of these four colonies has the potential to be utilized in an SIT programme. Furthermore, the ability of *An. arabiensis* to adapt to the laboratory environment, while maintaining appropriate levels of biological fitness, can be advantageous in the phases of SIT programmes which require mass rearing of mosquitoes under laboratory conditions.

CHAPTER 3

STERILE INSECT TECHNIQUE FIELD STUDY SITE SELECTION IN THE NORTHERN KRUGER NATIONAL PARK, INCLUDING *ANOPHELES* SPECIES ABUNDANCE AND DISTRIBUTION OF *ANOPHELES GAMBIAE* COMPLEX MOSQUITOES DURING SUMMER.

3.1) Introduction

The selection of a suitable field site in the feasibility phase of SIT is particularly important. An analysis of the proposed location for sterile male releases can provide vital information regarding the population structure and distribution of the vector species present in the area (Sperança and Capurro, 2007). The following criteria have been recommended as a guide for choosing an appropriate site:

1) *Isolated vector population*; an isolated field site prevents the re-invasion of the wild vector species because the influx of already inseminated females can offset any population suppression already achieved by the release of sterile males (Knipling *et al*., 1968; Malcolm *et al*., 2009; Robinson *et al*., 2009).

2) *One vector species*; The presence of a single vector species has the advantage of being cost effective as control methods would only be aimed at that specific species (Knipling *et al*., 1968; Dame *et al*., 2009; Malcolm *et al*., 2009).

3) *A low density of the vector population*; a low vector population density requires the release of fewer sterile males, thereby increasing the efficiency of SIT (Knipling *et al*., 1968; Malcolm *et al*., 2009)

4) *Actual or potential malaria transmission occurrence at the site*; monitoring the incidence of malaria cases in the presence of a vector control programme can provide information regarding the success of the control programme, as well as indication of imported cases of malaria and the influx of wild mosquitoes (Malcolm *et al*., 2009).

5) *Accessibility to the field site*; the use of conventional vector control techniques, such as insecticide spraying, is not a viable option for areas which are inaccessible or have limited access, making SIT a more attractive vector control option (Malcolm *et al*., 2009).

The efficient malaria control programmes in South Africa have reduced the number of sites which have an abundant *An. arabiensis* population. Areas such as Mamfene and Vlakbult, in Kwa-Zulu Natal and Mpumalanga respectively, do have *An. arabiensis* populations, however the population density and exact mosquito species composition of these areas has not been recorded and it is not known if these sites have a perennial population of *An. arabiensis* (Mouatcho *et al*., 2009; Mbokazi, 2012) .

The Kruger National Park (KNP) has a site which has a proven perennial population of *An. arabiensis* as demonstrated by previous studies conducted in the area by Braack *et al.* (1994) and Durrheim *et al.* (2001). These studies showed that *An. arabiensis* could be colonized from a site called Malahlapanga in the KNP (Braack *et al*., 1994; Durrheim *et al*., 2001). This makes the KNP an ideal region to conduct field site selection studies as it appears to be a region in South Africa which is most likely to meet the criteria for an appropriate SIT field site.

The Kruger National Park covers more than 1.949 million hectares and is one of the largest wildlife reserves in Africa. The park is located in the north-eastern region of South Africa and has a seasonal malaria-risk status (Braack *et al*., 1994; Durrheim *et al*., 2001). This study was conducted in the northern region of the KNP. The vegetation in this region is predominantly mopane-veld (*Colophospermum mopane*) and supports wildlife such as buffalo, antelope, and elephant. Summers are hot and wet, with the hottest days occurring during November through to February. During this period day-time temperatures exceed 40°C, with a drop to 18°C at night. The dry winter months from May to August have day temperatures of 26°C and night-time temperatures of 15°C. There is a distinct rainy season from October through to April. However, there are usually only four to five rainy days per month during this time (Braack, 2006).

This study forms part of an ongoing project to assess the species distribution, particularly members of the *An. gambiae* complex, in the northern Kruger National Park on an annual and seasonal basis.

3.1.1) Aim and objectives:

The aim of this study was to determine the species distribution of *An. gambiae* complex mosquitoes in the northern Kruger National Park during three summer months and to evaluate its potential as an SIT site.

Specific objectives were:

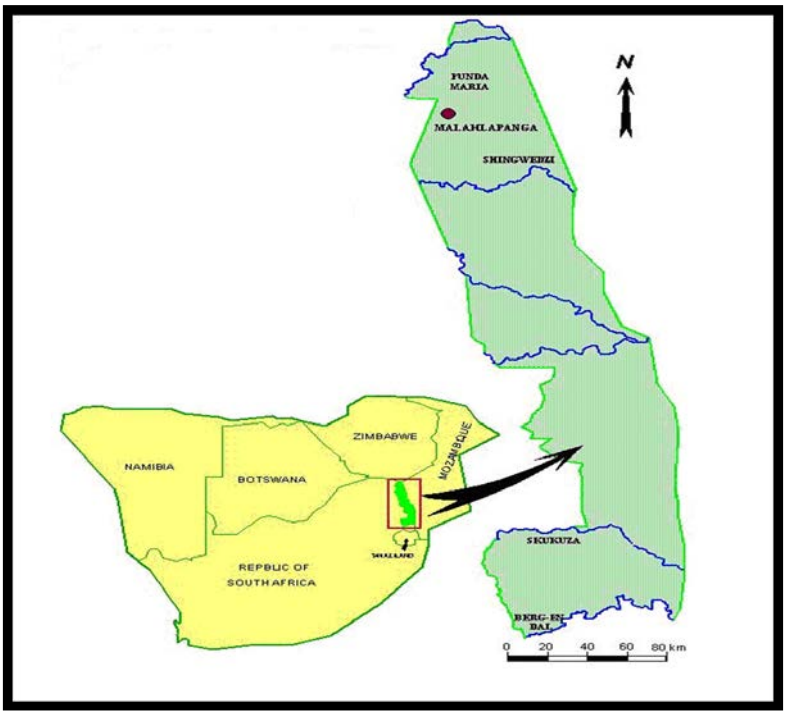
1. To collect *An. gambiae* complex mosquitoes from three sites: Malahlapanga; Sirheni bush camp; and Louis se gat, in the northern KNP during three summer months (November, January, and February).
2. Identify the mosquitoes collected to species level.
3. Compare the three sites as potential SIT sites

3.2) Materials and methods

3.2.1) Study sites

Louis se gat (23°06’39.88”S; 31°27’24.90”E), Sirheni bush camp (22°56’57.768”S; 31°13’51.348”E), and Malahlapanga (22°53’S; 31°02’E) are located in the northern region of the KNP (fig.3.1). *Colophospermum mopane* trees are found at both Sirheni bush camp and Louis se gat. Malahlapanga is a fresh water geothermal spring bordered by *Acacia nigrescens* and *C. mopane* trees. This isolated spring is known to support a perennial population of *An. arabiensis.* The population of *An. arabiensis* is sustained by various game animals such as buffalo and antelope which frequent the area as a water source (Braack *et al*., 1994; Durrheim *et al*., 2001; Munhenga *et al*., 2011).

(a)



(b)

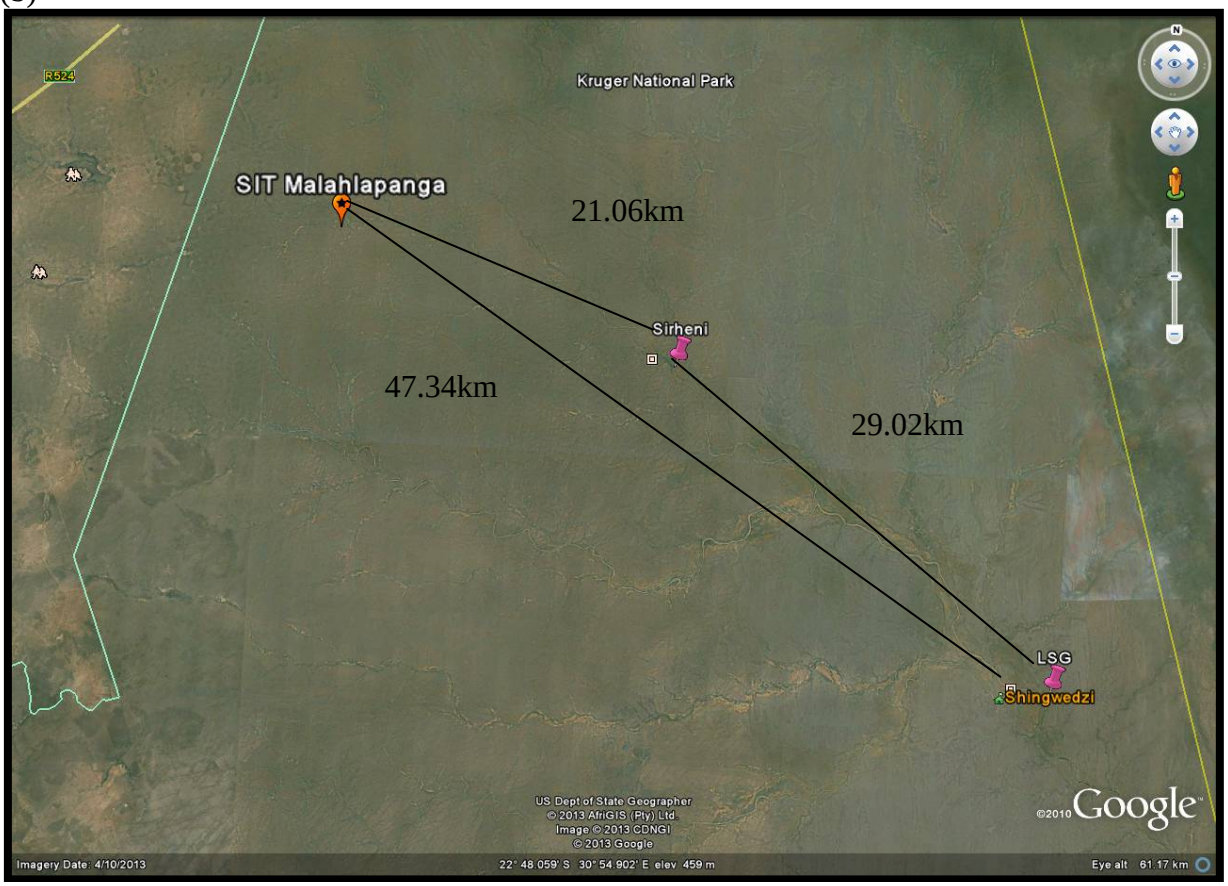


Figure 3.1: Map of the northern Kruger National Park (a), indicating the location and distance (km) of the three study sites (LSG = Louis se gat) (b) (Modified from http://www.tropmed.org/rreh/vol1_12 Google Earth http://www.google.com/enterprise/earthmaps/)

3.2.2) Mosquito collections

Mosquitoes were collected during the summer months of November 2011, January 2012, and February 2012 from Louis se gat, Sirheni bush camp, and Malahlapanga. Human landing catches (HLC) (fig.3.2) and carbon dioxide (CO_2) (fig.3.3) baited net traps were the two methods employed to attract and collect mosquitoes at each site. Human landing catches were conducted by collectors seated with exposed arms and legs. Carbon dioxide baited net traps were constructed by placing polystyrene boxes containing dry ice inside net traps. The traps were monitored for the presence of mosquitoes on an hourly basis. For each collection method, adult mosquitoes were collected using mouth aspirators. Collections were conducted between 18h00 and 23h00 over three successive nights in each of the collection months. Adult mosquitoes which were morphologically identified as members of the *An. gambiae* complex were collected separately, according to the collection method, in 250 ml paper cups placed in humid boxes, with continuous access to 10% sugar solution, and were then transported to the VCRL in Johannesburg.

Figure 3.2: Human landing catches to attract host-seeking mosquitoes.

Figure 3.3: Carbon dioxide baited net traps

3.2.3) Species identification

The Polymerase Chain Reaction (PCR) method described by Scott *et al*. (1993) was used to identify the collected specimens to species level (fig.3.4). Four positive controls were used in each PCR assay: *An. gambiae, An. arabiensis, An. merus, and An. quadriannulatus* from laboratory colonies currently maintained in the BDMI at the NICD. A negative control containing PCR mixture without DNA was used in each PCR assay. The diagnostic primers used are shown in Table 3.1.

Figure 3.4: PCR identification of members of *Anopheles gambiae* complex from the northern Kruger National Park. The amplicon size of *An. arabiensis* is 315 bp (bp = base pairs). Lane 1: DNA molecular weight marker. Lane 2: Negative control. Lane 3: *An. gambiae* positive control. Lane 4: *An. arabiensis* positive control. Lane 5: *An. merus* positive control. Lane 6: *An. quadriannulatus* positive control. Lanes 7 -8, 10 – 14: individual samples identified as *An. arabiensis.* Lane 9: No amplification.

Table 3.1: Diagnostic primer sequences, melting temperature (Tm) and PCR product size [base pairs (bp)] for the identification of members of the *Anopheles gambiae* complex (Scott *et al*., 1993). Species-specific primers: GA anneals to *An. gambiae*, AR anneals to *An. arabiensis*, ME anneals to *An. merus*, and QD anneals to *An. quadriannulatus*. UN is the universal primer which anneals at the same position on the rDNA in all four species

Primer name	**Primer sequence (5' to 3')**	**Tm (°C)**	**PCR Product size (bp)**
UN	GTG TGC CCC TTC CTC GAT GT	58.3	-
GA	CTG GTT TGG TCG GCA CGT TT	59.3	390
AR	AAG TGT CCT TCT CCA TCC TA	47.4	315
ME	TGA CCA ACC CAC TCC CTT GA	57.2	464
QD	CAG ACC AAG ATG GTT AGT AT	42.7	153

The PCR mixture had a total volume of 12.5 µl and contained: 1.25µl 10x reaction buffer [100 mM Tris-HCl (pH 8.3) (supplied by Acechem, Cat. No. 77-86-1; and Saarchem, Cat. No. SAAR306304OLP), 500mM KCl,), 1.2 mM of each dNTP (Takara Bio Inc., Cat. No. 4030), 1 mM $MgCl_2$, 0.015 µM QD primer, 0.03µM GA, AR, ME, UN primers (QD, GA, AR and ME primers were synthesized by Inqaba Biotechnical Industries, Cat. No. S3CF; UN primer synthesized by Macrogen, Cat. No. OG111220-09), 0.5 units of *Taq* DNA polymerase (Celtic Molecular Biosciences, Cat No. RR001), and 4.9 µl deionizer water. A single mosquito leg was added to the PCR mixture in a PCR microcentrifuge tube. Each microcentrifuge tube and its contents were centrifuged for 20 seconds at 13 226 x g prior to amplification in order to ensure that the mosquito legs were submerged in the PCR mixture.

The following cycling conditions were used for DNA amplification: initial denaturation at 94°C for 10 minutes, 30 cycles of denaturation at 94°C for 30 seconds, annealing at 50°C for 30 seconds, extension at 72°C for 30 seconds, and final extension at 72°C for 7 minutes. The amplicons were electrophoresed on an Ethidium Bromide (Sigma Aldrich, Cat. No. E1510) stained, 2.5% agarose gel (Seakem), with a final concentration of 0.125mg/ml Ethidium Bromide, submerged in 1 x TAE buffer (preparations of the agarose gel and TAE buffer are provided in Appendix III). DNA electrophoresis loading buffer was added to each PCR amplicon (Appendix III). The O'RangeRuler™ 100 bp DNA Ladder (Fermentas, Appendix III) molecular weight marker was loaded in the first lane of the gel. The negative control, consisting of PCR mix without any DNA, was loaded after the marker, followed by the positive controls and then the samples. Electrophoresis was conducted at 14 volts per cm until proper separation of the 7.5µl of molecular weight marker had occurred. Amplicons were visualized under ultra violet light. Each sample was identified to species level by comparing the size of the sample's amplicon to the molecular weight marker.

3.3) Results:

A total of 1 192 mosquitoes was collected during the three sampling months at three collection sites (Malahlapanga, Louis se gat and Sireni bush camp). The predominant species was *An. arabiensis*, comprising 71% (n=840) of the total collection (fig.3.5). The remaining 29% (n=352) of the collection was composed of *An. quadriannulatus* (17%, n=205) and other anophelines not belonging to the *An. gambiae* complex, such as *An. maculipalpis*, *An. rivulorum*, and members of the *An. coustani* group (12%, n=147) (fig.3.5).

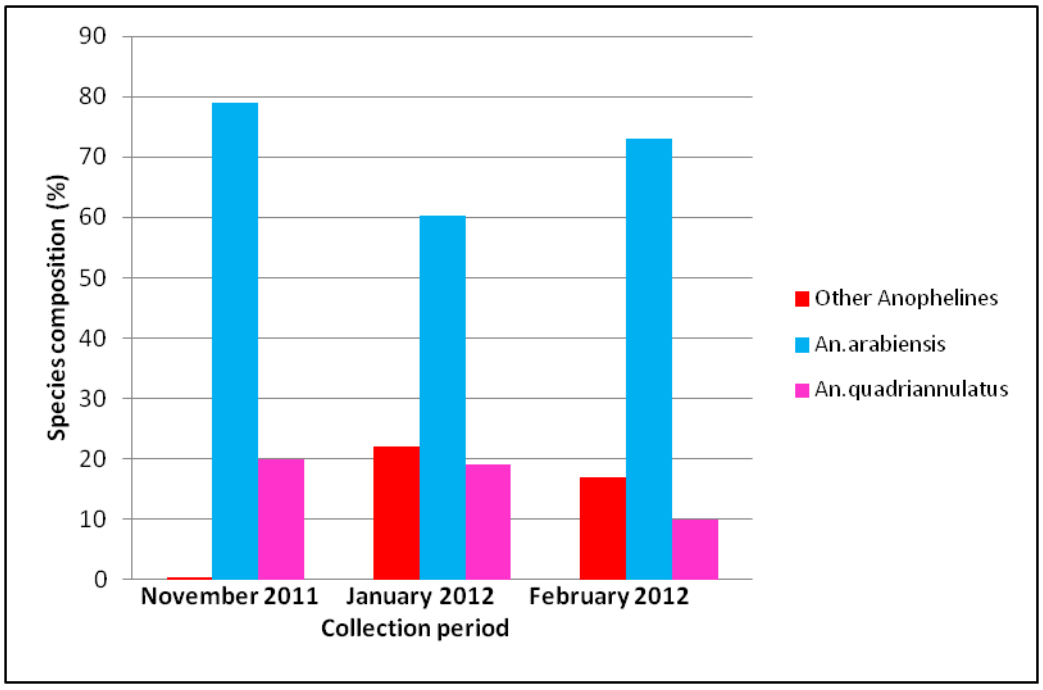


Figure 3.5: *An. gambiae* species composition from three sampling periods in the northern Kruger National Park (Other Anophelines = not members of *An. gambiae* complex).

During November 2011 *An. arabiensis* was the dominant species collected at Malahlapanga, contributing 94% (n=318) and 96% (n=64) of the CO_2 and HLC collections respectively, while *An. quadriannulatus* accounted for 100% (n=44) and 92% (n=23) of

the CO_2 and HLC collections at Louis se gat respectively. Only one species, *An. quadriannulatus* was collected at Sirheni bush camp during this month (Table 3.2).

Table 3.2: Anopheline mosquitoes caught at Malahlapanga, Sirheni bush camp, and Louis se gat during November 2011 (HLC = Human Landing Catches; CO2 = Carbon dioxide baited net traps; Other Anophelines = not members of *An. gambiae* complex)

Species	Malahlapanga		Sirheni		Louis se gat		Totals (%)
	HLC (%)	CO2 (%)	HLC (%)	CO2 (%)	HLC (%)	CO2 (%)	
An. arabiensis	64 (96)	318 (94)	0	0	0	0	382 (79.4)
An. quadriannulatus	3 (4)	20 (6)	0	7 (100)	23 (92)	44 (100)	97 (20.2)
Other Anophelines	0	0	0	0	2 (8)	0	2 (0.4)
Totals	**67**	**338**	**0**	**7**	**25**	**44**	**481**

It was not possible to collect samples in December 2011 due to SANParks regulations as it is their main tourist month and the second collection was only conducted in January 2012. During this month *An. arabiensis* continued to be the most abundant species and contributed 78% (n=229) and 84% (n=54) respectively of the CO_2 and HLC collections (Table 3.3). Other anophelines were predominant in the CO_2 collections at Sirheni bush camp and Louis se gat (93%, n=27) and 58%, n=39, respectively), as well as contributing 100% (n=8, n=17, respectively) of HLC at both of these sites, for the January sampling period (Table 3.3).

Table 3.3: Anopheline mosquitoes caught at Malahlapanga, Sirheni bush camp, and Louis se gat during January 2012 (HLC = Human Landing Catches; CO_2 = Carbon dioxide baited net traps; Other Anophelines = not members of *An. gambiae* complex)

Species	Malahlapanga		Sirheni		Louis se gat		Totals (%)
	HLC (%)	CO2 (%)	HLC (%)	CO2 (%)	HLC (%)	CO2 (%)	
An. arabiensis	54 (84)	229 (78)	0	1 (3)	0	3 (5)	287 (60)
An. quadriannulatus	7 (11)	52 (18)	0	1 (3)	0	25 (37)	85 (18)
Other Anophelines	3 (5)	11 (4)	8 (100)	27 (94)	17 (100)	39 (58)	105 (22)
Totals	**64**	**292**	**8**	**29**	**17**	**67**	**477**

During February 2012, the dominant species obtained from the CO_2 and HLC collections (81% and 95% respectively) at Malahlapanga was *An. arabiensis*, while both collection methods yielded only 29 specimens from Louis se gat (Table 3.4). Mosquito collections at Sirheni bush camp were unproductive for the November 2011 and February 2012 sampling periods, as only seven CO_2 specimens were collected in November 2011 (Table 3.1) and zero specimens during February 2012 (Table 3.4).

Table 3.4: Anopheline mosquitoes caught at Malahlapanga, Sirheni bush camp, and Louis se gat during February 2012 (HLC = Human Landing Catches; CO2 = Carbon dioxide baited net traps; Other Anophelines = not members of *An. gambiae* complex)

Species	Malahlapanga		Sirheni		Louis se gat		Totals (%)
	HLC (%)	CO2 (%)	HLC (%)	CO2 (%)	HLC (%)	CO2 (%)	
An. arabiensis	37 (95)	134 (81)	0	0	0	0	171 (73)
An. quadriannulatus	0	23 (14)	0	0	0	0	23 (10)
Other Anophelines	2 (5)	9 (5)	0	0	8 (100)	21 (100)	40 (17)
Totals	**39**	**166**	**0**	**0**	**8**	**21**	**234**

During the sampling periods of November 2011, January 2012, and February 2012, the recorded rainfall at Malahlapanga was 75.4 mm, 69.4 mm, and 12.1 mm respectively (Scientific Services, Kruger National Park, 2012, unpublished data) (fig.3.6). The number of mosquitoes (n=405) collected at Malahlapanga during November was the highest for the duration of this study (fig.3.6).

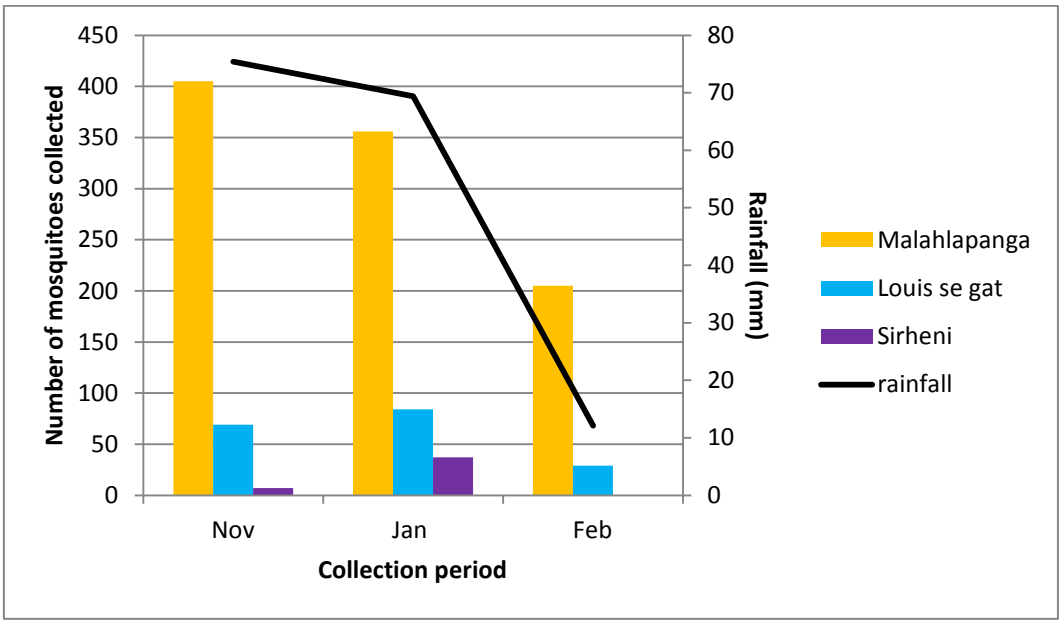


Figure 3.6: Rainfall in relation to the number of mosquitoes collected during the three collection periods at Malahlapanga.

Analyses of the relationship between the two collection methods (HLC and CO_2) and the number of mosquitoes as well as the species composition was conducted for Malahlapanga only due to the low number of mosquitoes collected at the other study sites. Results showed a statistically significant difference in the number of *An. arabiensis* collected, with carbon dioxide baited net traps yielding the highest number of *An. arabiensis* for the duration of the study (n=681, Students t-test t(4)=3.27, P=0.03) (fig.3.7). The number of

An. quadriannulatus collected at Malahlapanga did not significantly differ between the two collection methods (Students t-test t(4)=2.72, P=0.05) (fig.3.7).

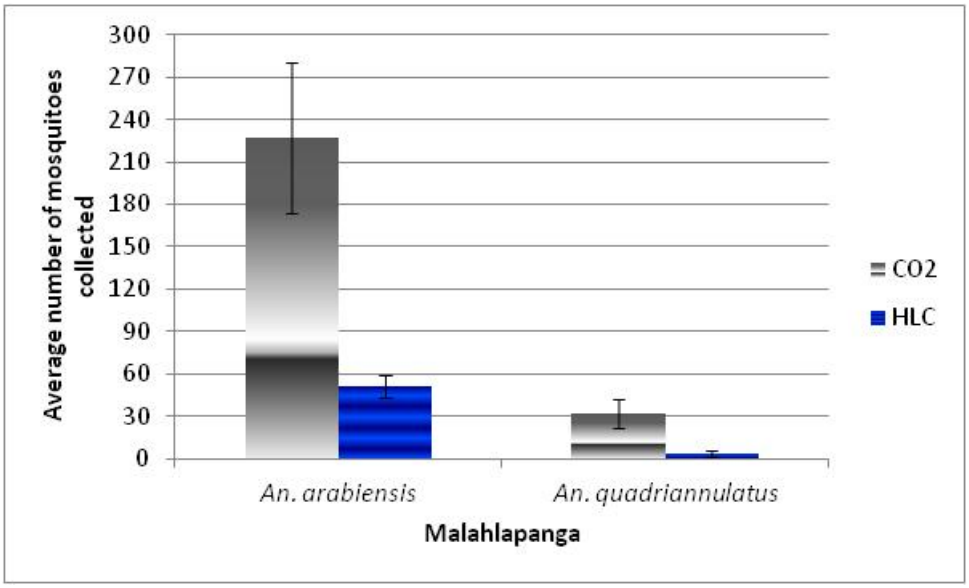


Figure 3.7: Average number of mosquitoes per collection method of *An. arabiensis* and *An. quadriannulatus* collected from Malahlapanga during November 2011, January and February 2012

The three study sites were evaluated based on the criteria recommended by Knipling *et al* (1968), Dame *et al* (2009), Malcolm *et al (*2009), and Robinson *et al* (2009) (Table 3.5). All three sites showed the presence of only one vector species, *An. arabiensis*. Malahlapanga demonstrated higher vector population numbers and seems to be isolated from the other two sites evaluated. Weather conditions such as drier summer months reduce vector population density. Malahlapanga does not have the potential for active malaria transmission due to the lack of a human population living in the area. Both Sirheni bush camp and Louis se gat have the potential for malaria transmission due to the presence of tourist camps and activities in close proximity to these sites.

Table 3.5: Evaluation of the three study sites based on criteria recommended by Knipling *et al* (1968), Dame *et al* (2009), Malcolm *et al* (2009), and Robinson *et al* (2009). A tick indicates satisfied criteria; a cross indicates unsatisfied criteria; a hash indicates the presence of an extremely low density isolated vector population; an upper case‘t’ indicates the presence of tourists.

Criteria	**Malahlapanga**	**Sirheni bush camp**	**Louis se gat**
Isolated vector population	✓	✓ #	✓ #
One vector species	✓	✓	✓
Low density	During winter	✓	✓
Potential malaria transmission	**X**	✓ T	✓ T
Accessibility	Difficult	✓	✓

Malahlapanga is the most inaccessible site, while Sirheni bush camp and Louis se gat are easily accessible, even during the rainy season. However, these two sites showed very low vector population numbers, with only one sample collected at Sirheni bush camp and three at Louis se gat for the duration of the overall study period.

3.4) Discussion:

This study is one aspect of a broader SIT field selection study with the main objective of employing SIT to attain a decline in a wild malaria vector population. To achieve this, factors such as the presence of one vector species, low density of vector population, isolated vector population, accessibility and actual or potential malaria transmission in the area were considered for field site selection (Knipling *et al*., 1968; Sperança and Capurro, 2007; Dame *et al*., 2009; Malcolm *et al*., 2009; Robinson *et al*., 2009).

Three sites (Malahlapanga, Sirheni bush camp and Louis se gat) in the northern Kruger National Park were assessed as prospective field sites as well as the presence of vectors during the summer months of November 2011, January 2012 and February 2012. Malahlapanga, Sirheni bush camp and Louis se gat differed in their potential as an SIT field site. Sirheni bush camp and Louis se gat are both easily accessible, however collections conducted at these sites yielded low sample numbers and almost no vector mosquitoes.

The highest number of mosquitoes was collected at Malahlapanga and *An. arabiensis* was the predominant anopheline mosquito species found during all three collection periods. This result is in agreement with previous research which found that Malahlapanga supports a perennial population of *An. arabiensis* and demonstrating that the site has a substantial population of this malaria vector species (Braack *et al*., 1994; Durrheim *et al*., 2001; Munhenga *et al*., 2011, Munhenga *et al*., 2014).

The peak of the *An. arabiensis* population appears to be during November. This is also the period with the most rainfall. This result supports that of previous studies conducted at Malahlapanga which have shown that the population of *An. arabiensis* is most abundant during the wet summer months and is at its lowest during the dry winter months (Coetzee *et al*., 2000; Takken and Lindsay, 2003; Malcolm *et al*., 2009; Munhenga *et al*., 2011; Munhenga *et al*., 2014).

Anopheles quadriannulatus are generally found in regions where there is >1000 mm annual rainfall, while *An. arabiensis* mosquitoes are usually distributed in regions which are lower rainfall zones (<1000 mm annually) such as the Nile valley in northern Sudan

(Coetzee *et al*., 2000; Takken and Lindsay, 2003). The average annual rainfall in the Kruger National Park was 537.2 mm during 2011 and 2012 (Scientific Services, Kruger National Park, 2012, unpublished data).

Comparison of collection methods showed that carbon dioxide baited net traps proved to be the most efficient method of attracting large numbers of host-seeking *An. arabiensis*. This could be due to a higher amount of carbon dioxide being released from the traps than from the humans involved in the human landing catches. The carbon dioxide baited net traps collected 4.5 times more samples than the HLC. This finding also applied at Sirheni bush camp and Louis se gat where sample numbers were extremely low (n=4) but were all collected by CO_2 baited net traps. Human landing catches at these sites produced zero specimens. When vector population numbers are extremely low it is useful to know which collection method is more effective in collecting samples to maximize resources.

Based on the results from this study, Malahlapanga is the most suitable field site to evaluate the effect of SIT on wild vector population. Malahlapanga appears to have an isolated or "island" population of *An. arabiensis*, contributing to the suitability of this area as a SIT field site. This will prevent the re-invasion of the wild vector species from neighbouring areas which can result in an influx of already inseminated fertile females. The population suppression already achieved by the release of sterile males can then be reversed (Knipling *et al*., 1968; Malcolm *et al*., 2009; Robinson *et al*., 2009).

In addition to the above, Malahlapanga has another advantage that the nearest human population are approximately 9 km away from the site (Braack *et al*., 1994; Coetzee *et al*., 2000; Durrheim *et al*., 2001; Takken and Lindsay, 2003; Malcolm *et al*., 2009; Munhenga

et al., 2011; Munhenga *et al*., 2014). This will ensure that the feasibility study can rapidly be conducted without involving communities during the feasibility phase. The results obtained will provide information needed to present to community leaders, National and Provincial malaria control personnel prior to implementation of the SIT intervention in communities.

The natural seasonal suppression of the wild *An. arabiensis* population during winter months (in South Africa and at Malahlapanga) will be advantageous for SIT as it will limit the number of sterilized males that needs to be released (Munhenga *et al*., 2014). Results to date further indicate that this is the only vector species present in this area (Munhenga *et al*., 2014).

Malahlapanga unfortunately has some shortcomings. The first is that there is no local malaria transmission taking place due to the lack of communities living in this area. Malahlapanga is a wilderness area in the park and human interference is restricted and might complicate the release of sterilized laboratory reared mosquitoes at the site. In addition to this, Malahlapanga is difficult to access, especially during the rainy season, as there are no tarred roads to the site and 4x4 vehicles are required to reach the site. It is located 47.34 km from Shingwedzi and is a two hour drive from the research camp (fig.3.1).

3.5) Conclusion

Anopheles arabiensis is the dominant species in Malahlapanga, Kruger National Park. The *An. arabiensis* population at Malahlapanga is highest during the rainy, summer months. While Louis se gat and Sirheni bush camp are sites which are more readily accessible than Malahlapanga, collections at both these sites were unproductive, indicating a lack of a substantial *An. arabiensis* population. The abundance of this vector species as well as the geographically isolated nature of the site makes Malahlapanga an appropriate field site for SIT feasibility studies.

This work contributed towards a larger study which has been submitted for publication in Malaria Journal (Appendix I).

CHAPTER 4

THE ASSESSMENT OF TWO LARVAL DIETS ON THE DEVELOPMENT OF A LABORATORY-REARED *ANOPHELES ARABIENSIS* GENETIC SEXING STRAIN (GSS)

4.1) Introduction

Female mosquitoes serve as vectors for human pathogens such as the malaria-causing *Plasmodium* parasite. Vector control programmes which involve the release of sterile male mosquitoes must therefore ensure that, ideally, zero female mosquitoes are released to prevent additional disease transmission. Separation between male and female Anophelines can be done at the pupal and adult phase. Pupae can be sexed by viewing their terminalia under a microscope (MR4, 2010; Barbosa, 1968; Papathanos *et al.*, 2009). Male and female pupae have distinct terminalia which enable the sexes to be distinguished from each other (fig. 4.1) (MR4, 2010; Barbosa, 1968). Adult male and female mosquitoes can be separated by visual inspection of the antennae (fig. 4.2). The antennae of female mosquitoes have short hairs with a non-plumose appearance and those of male mosquitoes have long hairs with a plumose appearance (fig.4.2) (Service, 1980; Gillies and Coetzee, 1987). However, both the above mentioned sex separation techniques are labour intensive and far from ideal.

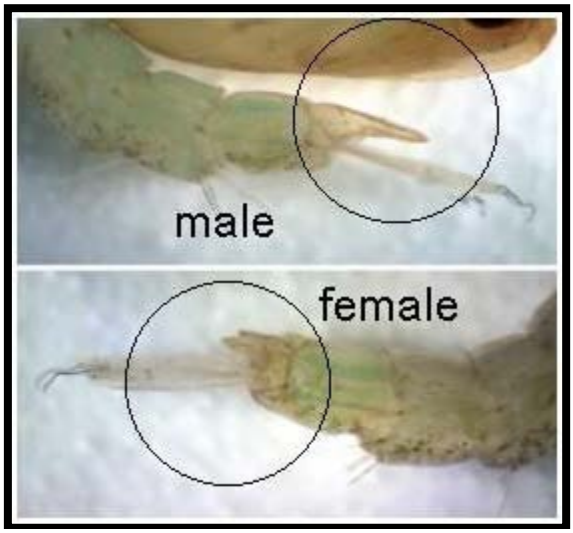


Figure 4.1: Sex separation in the pupal phase. The terminalia of male and female pupae are distinctive. (Modified from MR4, 2010)

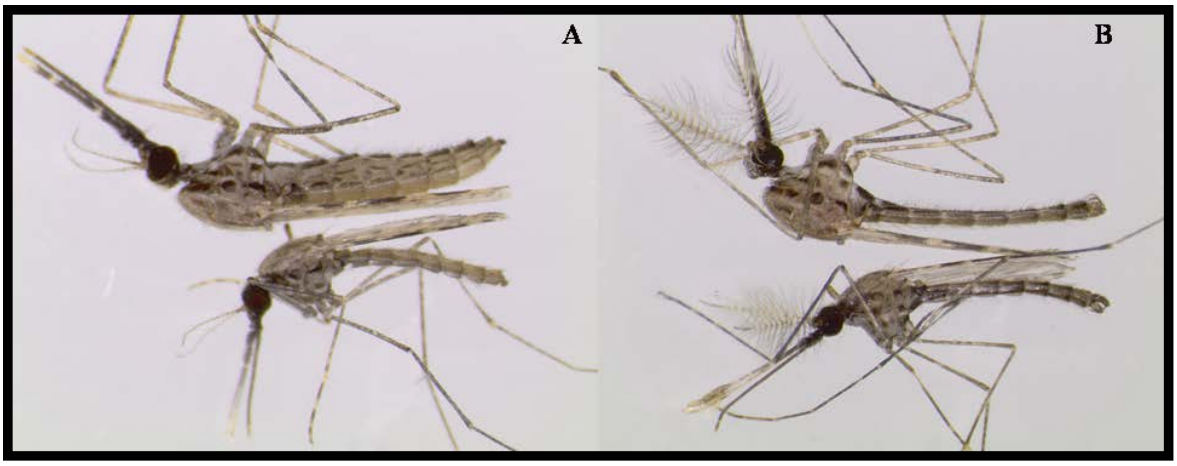


Figure 4.2: Sex separation in the adult phase. The antennae of adult mosquitoes differ between the genders. **A:** Adult female *An. arabiensis* showing non-plumose antennae; **B:** Adult male *An. arabiensis* showing plumose antennae. (Modified from Oliver and Brooke, 2013).

Effective, fast and easy sexing methods for a large scale SIT program involving mass production of mosquitoes would need to be employed to separate male and female mosquitoes before release. SIT programmes targeting *Aedes* mosquitoes can rely on the size disparity between male and female pupae as a method of sex separation (fig.4.3 B)

(Alphey, *et al*., 2010; MR4, 2010). Female *Aedes aegypti* pupae are visibly larger than their male counterparts, allowing for effective sex separation (fig.4.3 B) (Papathanos *et al*., 2009; Alphey, *et al*., 2010; MR4, 2010). This sexing method cannot be employed for Anopheline mosquitoes as the size difference between male and female pupae is not easily noticeable (fig.4.3 A); therefore anopheline mosquitoes require an alternate sexing method (Papathanos *et al*., 2009; Alphey, *et al*., 2010; MR4, 2010).

The *An. arabiensis* Genetic Sexing Strain (GSS) is one such method (Curtis *et al*., 1976; Curtis, 1978; Lines and Curtis, 1985; Papathanos *et al*., 2009; Yamada *et al*., 2012). Curtis (1978) created an *An. arabiensis* GSS by mating a dieldrin resistant *An. arabiensis* strain from Senegal with a dieldrin susceptible *An. arabiensis* strain from Nigeria in order to produce F1 progeny with a Y-linked translocation (Curtis, 1978). This translocation conferred dieldrin resistance to males only, allowing susceptible females to be eliminated by exposure to dieldrin (Curtis, 1978). The strain created by Curtis (1978) provided the basis for the development of the *An. arabiensis* GSS, ANO IPCL1, used in this study. ANO IPCL1 carries a Y-chromosome linked resistance to dieldrin. When the strain is exposed to dieldrin at either the egg or larval stage of development, the dieldrin-susceptible females are killed, and only the dieldrin-resistant males develop through to the adult stage (Yamada *et al*., 2012). These adult male mosquitoes can then be used in SIT studies; therefore it is important to rear this strain in a manner which produces high-quality mosquitoes, capable of successfully competing with their wild counterparts, as required for the SIT (Curtis, 1978; Franz, 2006; Papathanos *et al*., 2009; Yamada *et al*., 2012).

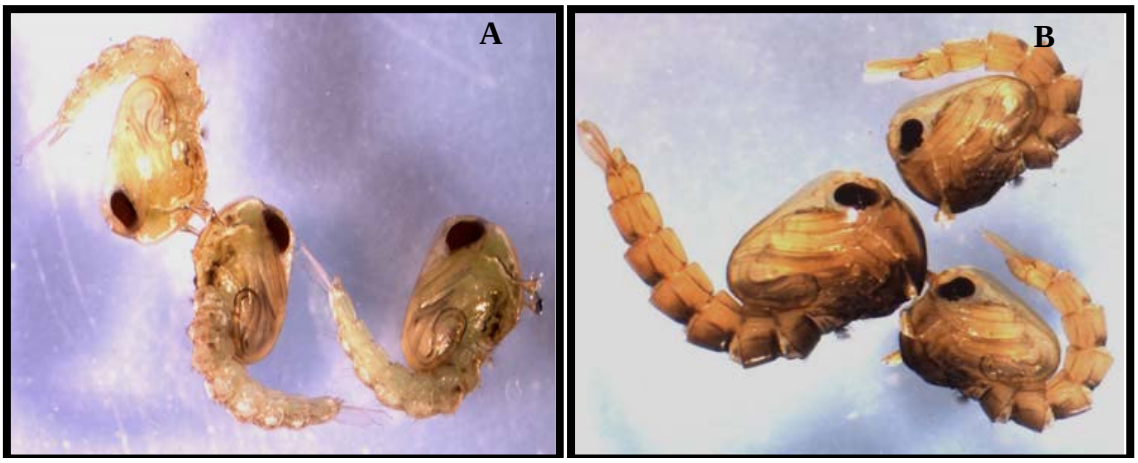


Figure 4.3: Sex separation by pupal size. **A**: *An. gambiae* pupae; two females (bottom right and centre) and one male (top left); size difference is not obvious. **B**: *Ae. aegypti* pupae; two males (top and bottom right) and one female (left); size difference is obvious. (Modified from MR4, 2010)

Adult mosquito traits such as fertility and fecundity are directly affected by the rearing conditions experienced during larval development (Foko Dadji *et al*., 2007; Benedict *et al*., 2009; Khan, 2010; Gilles *et al*., 2011; Damiens *et al*., 2012; Hood-Nowotny *et al*., 2012; Puggioli *et al*., 2013). In the wild, mosquito larvae develop in various habitats in which their dietary requirements are met by feeding on microorganisms, degraded plant material, bio film, and detritus (Service, 1980; Clements, 1999; Service, 2000). Anopheline larvae lie parallel to the water surface and are filter-, surface-feeders. Feeding usually occurs at the air/water interface during which the larval head rotates 180º in order to allow the ventral mouth brushes to sweep beneath the water surface (Service, 1980; Service, 2000).

When maintaining mosquitoes in a laboratory, optimal rearing conditions relating to climate and diet are sought which ensure sustained productivity of a population with fairly synchronized pupation and consistent adult size (Foko Dadji *et al*., 2007; Benedict *et al*., 2009; Khan, 2010; Hood-Nowotny *et al*., 2012). Research has shown that mosquito development requires a larval diet which contains proteins, sugar, polyunsaturated fatty

acids, sterols, vitamins, and nucleotides (Khan, 2010; Gilles *et al*., 2011; Damiens *et al*., 2012; Hood-Nowotny *et al*., 2012; Puggioli *et al*., 2013). The quality of mosquitoes is determined by factors such as size and fecundity. It is imperative that an adequate larval diet which provides the nutritional components necessary for the production of good quality mosquitoes be employed during larval rearing in a laboratory setting. Cost, availability, and quality should be taken into consideration when selecting a larval diet (Calkins and Parker, 2005; Parker, 2005; Foko Dadji *et al*., 2007; Benedict *et al*., 2009; Khan, 2010; Gilles *et al*., 2011; Damiens *et al*., 2012; Hood-Nowotny *et al*., 2012; Puggioli *et al*., 2013).

The *An. arabiensis* Genetic Sexing Strain ANO IPCL1 (GSS) was obtained from the colony which has been maintained since 2008 in the Insect Pest Control Laboratory (IPCL) of the FAO/IAEA Agriculture & Biotechnology Laboratories, Seibersdorf, Austria. The rearing conditions experienced at the IPCL differ from those at the BDMI of the VCRL. One such point of difference is the larval diet employed by each laboratory. The IPCL maintains the ANO IPCL1 strain on a liquid larval diet consisting of tuna meal, bovine liver powder, vitamins, and Brewer's yeast. Mosquito larvae reared in the BDMI receive a powdered diet of crushed dog biscuits and Brewer's yeast. This diet is currently used to maintain 27 mosquito colonies in the BDMI. The larval diets used by both the IPCL and the BDMI was assessed in the rearing conditions experienced in the BDMI in order to determine if the ANO IPCL1 (GSS) strain can be successfully reared in the BDMI.

4.1.2) Aim and objectives:

To assess the impact of two larval diets on the development of a laboratory – reared *An. arabiensis* GSS.

Specific objectives were:

1. To monitor larval development until adult emergence.
2. To determine adult mosquito body size as measured by wing length.
3. To determine fecundity of adult female mosquitoes.

4.2) Materials and methods

4.2.1) Laboratory reared material:

The *An. arabiensis* GSS, originally obtained from the Insect Pest Control Laboratory (IPCL) of the FAO/IAEA Agriculture & Biotechnology Laboratories, Seibersdorf, Austria, was reared under standard rearing conditions, as described by Hunt *et al*. (2005), of 25ºC and ± 80% relative humidity in the BDMI of the VCRL at the NICD. The insectary operates on a 12 h day/night cycle with a 30 min dusk/dawn period.

This experiment was conducted in the BDMI of the VCRL using standard rearing equipment. Three replicates were conducted per larval diet.

4.2.2) Larval diet preparation

Two larval diets were assessed: the diet routinely used in the BDMI (VCRL Diet) and the diet supplied by the IPCL (IAEA Diet). The VCRL Diet is a powdered mixture of crushed dog biscuits (West's Beeno Traditional Crunchy Biscuit Treats, Martin and Martin, South

Africa) and Brewer's yeast (Vital Health Foods, South Africa) prepared at a ratio of 3:1 (Appendix III). The IAEA Diet consists of powdered tuna meal, vitamins, Brewer's yeast, and bovine liver powder. This diet was prepared in liquid form (according to IPCL specifications) by dissolving the dry ingredients (6.25 g of tuna meal, 2.50 g of bovine liver powder, 1.25 g of Brewer's yeast, 2 g of vitamin mix) in 1 L of distilled water (Appendix III). Aliquots were prepared in 14 ml Falcon tubes and stored in a freezer at - 20°C.

4.2.3) Effect of larval diet type on the development of GSS

4.2.3.1) Larval duration and survivorship

600 hundred first instar (L1) GSS larvae (100 per replicate; three replicates per diet) were transferred into 2 L plastic containers with 500 ml distilled water. Two groups of larvae (each of three replicates) were fed with either VCRL Diet or IAEA Diet. Anopheline mosquitoes are sensitive to over-feeding; consequently larvae were fed on demand, and based on the colour and state of the water in the rearing container (approximate weighed amounts are given in Appendix III) (Gilles *et al*., 2011; Damiens *et al*., 2012; Hood-Nowotny *et al*., 2012; Puggioli *et al*., 2013). Larval duration from L1 to pupa and L1 to adult was monitored. Dead larvae were removed on a daily basis. Pupae were removed daily using a pipette, counted, transferred into pupal emergence vials (35 mm x 57 mm) filled with 50 ml distilled water and placed in 2 L adult cages. Larval duration was calculated as the number of days taken for L1 larvae to pupate. Larval survivorship was calculated as the proportion of larvae which pupated. The mean larval duration and survivorship was compared between the VCRL Diet and IAEA Diet cohorts.

4.2.3.2) Adult emergence

Pupae from each replicate and colony were counted and transferred into pupal emergence vials (35 mm x 57 mm) filled with 50 ml distilled water and placed in 2 L cages for adult emergence. A daily count of the number and gender of adult mosquitoes emerging from the pupae was conducted for each replicate. The gender of emerging adults was determined by using the external morphology of the antennae. The mean proportion of pupae surviving to adult emergence was calculated for each replicate and compared between the VCRL Diet and IAEA Diet cohorts.

4.2.3.3) Wing length measurements

Wing length has been shown to give a sound approximation of adult mosquito body size and is routinely used when referring to body size (Lyimo *et al*., 1992; Lyimo and Takken, 1993; Yan *et al*., 1997; Blackmore and Lord, 2000; Ponlawat and Harrington, 2009). Sixty adult mosquitoes (20 adults per replicate; 10 males and 10 females per replicate) were randomly selected post emergence, stored in Eppendorf tubes, and their wing length measurements were taken. One wing from each adult mosquito was dissected and placed on a clean glass slide. The wing length (wing tip to thorax joint) was measured at 200 x magnification using an eyepiece micrometer mounted on a dissecting microscope. The mean wing length was compared according to gender as well as the entire adult mosquito group that were fed on either the VCRL Diet or IAEA Diet.

4.2.3.4) Fecundity

Adult mosquitoes which emerged from each replicate were transferred into 2 L adult cages and mating was allowed to occur over a period of a week. These adults had continuous access to a 10% sugar solution (Appendix III) and the females received three blood-meals from anaesthetized guinea pigs (Ethics Clearance REF:W-CJ-100510-1; Appendix II) according to the BDMI blood-feeding schedule. The first blood-meal was received when the female mosquitoes were four days old, and the second and third blood-meals at seven and nine days old respectively. Once the third blood-meal had been received, egg plates were placed in each cage to obtain eggs. Once oviposition had occurred, fecundity was determined (total number of eggs laid/total number of females). The mean fecundity was compared between the VCRL Diet and IAEA Diet cohorts.

4.2.4) Data Analysis

Data on larval duration and survivorship, adult emergence, sex ratio, adult size (wing length), and fecundity were summarized as mean larval duration and survivorship (L1 to pupa; L1 to adult), mean proportion of pupae surviving to the adult stage, mean sex ratio, and mean wing length respectively. The differences in mean larval duration and survival, adult emergence, sex ratio, adult size (wing length), and fecundity, between VCRL Diet and IAEA Diet cohorts, was analyzed using a Student t-test performed in GraphPad Prism version 6.00 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com. Results were interpreted at 95% confidence. Results were represented in bar graphs.

4.3) Results:

The mean larval duration from L1 to pupation was 10.2 days for larvae (n = 300; three replicates of 100 larvae each per diet cohort) which were reared using either the VCRL Diet or the IAEA Diet (Table 4.1; fig.4.4). The larval duration from L1 to pupation was not significantly different for larvae which received either the VCRL Diet or the IAEA Diet (Student's t-test t (4) = 0.8, P=0.46). Of the 300 first instar larvae reared per diet, 99.67% of larvae survived till pupation for each experimental group (Table 4.1). Pupal survival was 100% (n = 299 per diet cohort) for each of the diets assessed. Larvae which were fed with either the VCRL Diet or the IAEA Diet had a mean L1 to adult duration of 11.2 days (Table 4.1; fig.4.4). There was no statistical difference between the larval duration from L1 to adult stage for larvae which received either the VCRL Diet or the IAEA Diet (Student's t-test t (4) = 0.9, P= 0.43).

Table 4.1: Assessment of VCRL Diet and IAEA Diet on the development of *An. arabiensis* GSS

Parameters	**VCRL Diet**	**IAEA Diet**
Larval duration to pupa (days)	10.2	10.2
Larval duration to adult (days)	11.2	11.2
Larval survival (%)	99.67	99.67
Pupal survival (%)	100	100
Number of adults (male and female)	299	299
Sex ratio	1:1	1:1
Fecundity (No. eggs laid per female)	148.6	149
Wing length (mm):		
All adults	3.43	3.39
Female	3.5	3.43
Male	3.36	3.35

Larvae which received either the VCRL Diet or the IAEA Diet produced an equal number of total adults (both male and female) and a sex ratio of 1:1 (Table 4.1). The mean fecundity (number of eggs laid per female) for the VCRL Diet cohort was 148.6 eggs per female (n = 120), and the mean fecundity for the IAEA Diet cohort was 149 eggs per female (n = 120) (Table 4.1). These values were not statistically different (Student's t-test t (4) =0.07, P= 0.9).

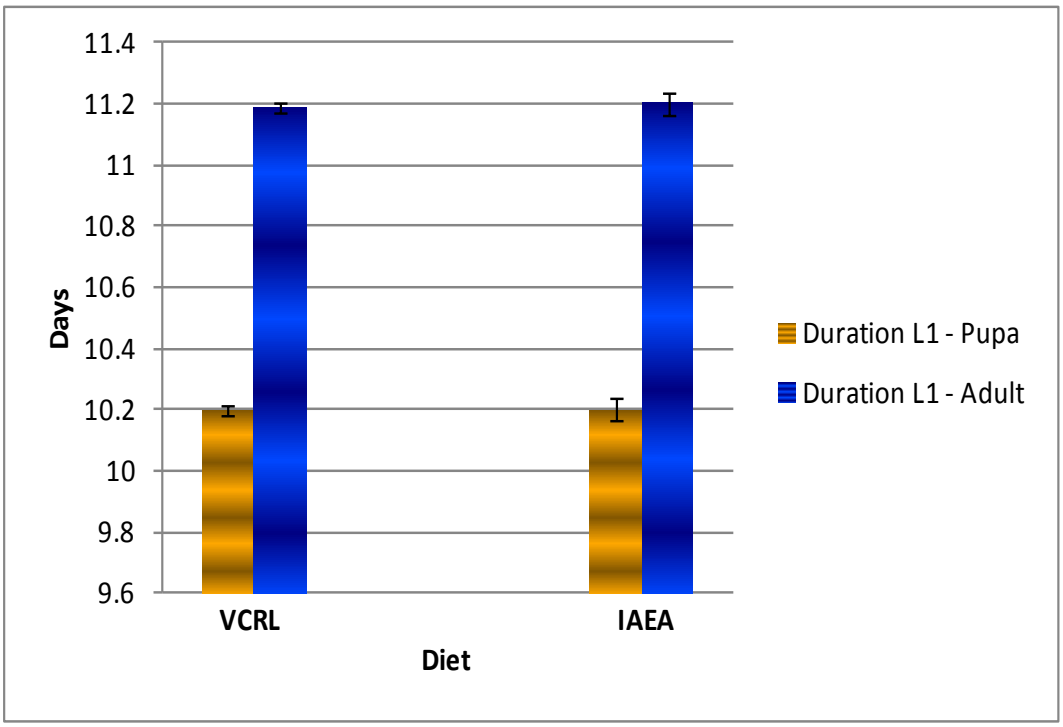


Figure 4.4: Comparison of larval duration of *An. arabiensis* GSS from first instar to pupa and to adult stage using either the VCRL Diet or IAEA Diet

The mean wing length for adults (both male and female; n=60; three replicates of 20 adults each per diet cohort) was 3.43 mm and 3.39 mm for the VCRL Diet and IAEA Diet cohorts respectively (Table 4.1, fig.4.5). The means for adult (both male and female) body size (wing length) were not significantly different (Student's t-test t (4) = 0.5, P= 0.7). The mean wing length for female mosquitoes (n = 30; three replicates of 10 females each per diet cohort) was 3.5 mm and 3.43 mm for the VCRL Diet and IAEA Diet cohorts respectively (Table 4.1, fig.4.5). The means for female body size (wing length) were not significantly different (Student's t-test t (4) = 0.7, P= 0.5). Adult male mosquitoes (n = 30; three replicates of 10 males each per diet cohort) had a mean wing length of 3.36 mm and 3.35 mm for the VCRL Diet and IAEA Diet cohorts respectively (Table 4.1, fig.4.5). The means for male body size (wing length) were not significantly different (Student's t-test t (4) =0.08, P= 0.9).

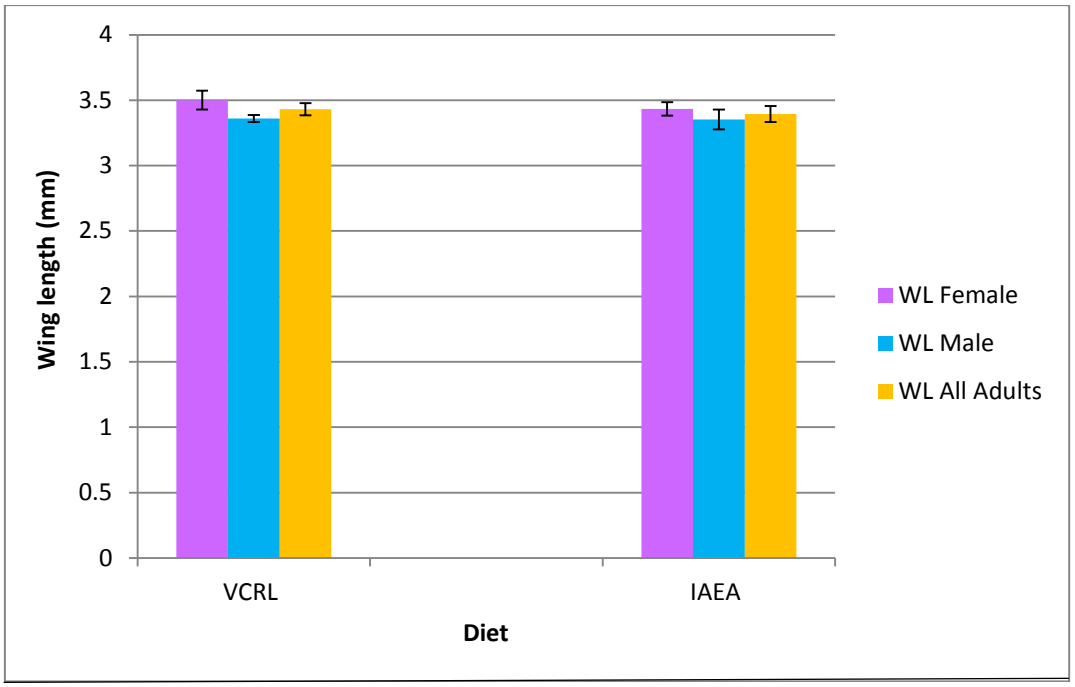


Figure 4.5: Wing length (mm) of adult male and female *An. arabiensis* GSS mosquitoes fed with different diets at the larval stage i.e. VCRL or IAEA Diet.

4.4) Discussion:

The nutritive components of an insect larval diet can manipulate developmental factors such as larval duration, fecundity, and adult body size (Lance and McInnis, 2005). The Anopheline larval and pupal period is normally 7-14 days and 2-3 days respectively (Clements 1963; Service, 1980; Service, 2000). Under the experimental conditions experienced in this study, the duration of GSS L1 larva to pupa, and L1 larva to adult was within the normal range. The two larval diets which were assessed did not have an impact on adult emergence or the sex ratio, with each diet cohort producing a sex ratio of 1:1.

Soon after mating and insemination, female adult Anopheline mosquitoes require a blood meal in order for eggs to develop. Females are able to lay 50-200 eggs per oviposition (Clements 1963; Service, 1980; Service, 2000). The fecundity of the GSS female

mosquitoes in this study was unaffected by the larval diets being assessed, and remained within the expected range for Anophelines.

Anopheles wing length measurement is often used to provide a reliable indication of body size. Adult mosquitoes belonging to this genus are usually 3-6 mm in length (Service, 1980; Lyimo and Takken, 1993; Service, 2000). The wing length, and thereby body size, of the GSS adult mosquitoes in this experiment was within the normal body size parameters, regardless of the larval diet they received (VCRL Diet or IAEA Diet). Previous studies conducted on larval diet composition and the effect of larval diet quality on insect development have demonstrated that the composition of the diet received in the immature life stages influences adult competitiveness as well as having lasting effects which are transferred to succeeding generations (Dadd *et al*., 1988; Lance and McInnis, 2005; Hood-Nowotny *et al*., 2012).

The impact of larval diet on the development of mosquitoes can have far reaching consequences for SIT programmes. If the mosquitoes being mass reared for release receive an inadequate larval diet, the competitiveness of these mosquitoes in the field may be adversely affected, and since the effects of poor nutrition are passed to the next generation, the ability of rearing facilities to produce sufficient numbers of mosquitoes could possibly be reduced. It is, therefore, imperative that the diet used to rear larvae meets the nutritional requirements for the adequate development of mosquitoes.

While mosquito development is of paramount importance when selecting a larval diet, factors such as economy, availability, and convenience should also be taken into

consideration. The cost of the IAEA diet (approximately R1 347 per kg) exceeds that of the VCRL Diet (R99 per kg), and has to be imported from Europe, whereas the VCRL Diet is locally available.

The IAEA Diet could not be stored in the BDMI due to the combination of water, yeast, and climatic conditions in the BDMI, which caused the diet to ferment. For this reason, the diet was stored in a freezer and daily aliquots which needed to thaw overnight were prepared. This made the preparation and storage of the IAEA Diet inconvenient in a small scale set up, especially for the weekend feeding of larvae. Comparatively, the VCRL Diet is a dry diet which can be easily prepared and stored in the BDMI. However, during large scale rearing, using specifically designed equipment, the IAEA Diet will be more practical to use. This liquid diet can be efficiently delivered to larvae using a mechanical system (Benedict *et al*., 2009; Gilles *et al.*, 2011).

4.5) Conclusion:

The two larval diets assessed in this study did not have a significant effect on the larval development, adult emergence, sex ratio, fecundity, and body size of the GSS colony. This suggests that both larval diets meet the nutritional requirements necessary for the optimal laboratory rearing of the GSS colony, and that the GSS colony does not require a specialised diet. The VCRL Diet is more cost effective and readily available for use in the BDMI where 27 colonies are being successfully reared, making this the diet of choice for rearing the GSS at the facility. It is essential to maintain a high quality GSS strain at the VCRL as this strain will be employed in further SIT feasibility studies.

CHAPTER 5

SUMMARY AND CONCLUSION

The Sterile Insect Technique (SIT) is a novel vector control method that is environmentally friendly and species specific. This study forms part of a larger project aimed at investigating the feasibility of the SIT as a control method for the malaria vector *Anopheles arabiensis* in southern Africa.

Mosquitoes used in an SIT programme are laboratory reared. Adaptations to the artificial rearing conditions experienced in the laboratory can adversely affect the fitness and competitiveness of these mosquitoes in a field setting (Parker, 2005; Huho *et al*., 2007; Robinson *et al*., 2009). In addition to these fitness costs, further fitness costs might be incurred by the exposure of the mosquitoes to SIT processes such as mass rearing and irradiation. Baseline data on the fitness of laboratory-reared *An. arabiensis* colonies is essential to SIT projects targeting this vector. This study determined the fitness of four laboratory-reared *An. arabiensis* colonies: KGB; MA; MBN-BASE; and KWAG-BASE. Each colony was colonized at different times and from three countries in southern Africa. Parameters such as fertility; fecundity; pupation, adult body size; mating success; and longevity were assessed for each of the four colonies. The results obtained showed that there was no significant difference between the colonies for the parameters assessed.

SIT reduces the wild population of a target species by the mass release of sterilized males of the same population. During the initial or planning phase of an SIT project, it is essential to select a suitable field site and to determine the species and distribution of the vector at

the proposed site (Sperança and Capurro, 2007). This study determined the species distribution of *Anopheles gambiae* complex mosquitoes in the northern Kruger National Park (KNP) during three summer months. Mosquito collections were conducted at three sites in KNP: Sirheni bush camp; Malahlapanga; and Louis se gat. The mosquitoes were identified to species level using the polymerase chain reaction (PCR). For each of the collection periods, Malahlapanga yielded the highest number of mosquitoes, with *An. arabiensis* being the predominant species collected. This confirms previous studies which reported that Malahlapanga supports a perennial *An. arabiensis* population (Braack *et al*., 1994; Durrheim *et al*., 2001; Munhenga *et al*., 2011, Munhenga *et al*., 2014). Collections at Louis se gat and Sirheni bush camp were unproductive. A comparison of two collection methods, carbon dioxide baited net traps and human landing catches, was conducted at the three sites during the each sampling period. The carbon dioxide baited net traps attracted the largest number of host-seeking *An. arabiensis*.

Since it is only female mosquitoes which serve as vectors for human pathogens such as the *Plasmodium* parasite, it is essential for mosquito SIT projects to have an efficient sex separation technique. This will ensure that female mosquitoes are not released during the release of sterilized males. An *An. arabiensis* Genetic Sexing Strain (GSS) was created to provide a quick and convenient method of sexing *An. arabiensis* mosquitoes (Curtis *et al*., 1976; Curtis, 1978; Lines and Curtis, 1985; Papathanos *et al*., 2009; Yamada *et al*., 2012). The strain used in this study (GGS, ANO IPCL1) carries a Y-chromosome linked resistance to dieldrin gene. Exposing the strain at either the egg or larval stage kills the dieldrin-susceptible females. The dieldrin-resistant males will develop to the adult stage and can be used for SIT studies. The males produced by this strain must be able to

successfully compete with their wild counterparts; therefore the rearing of this strain in order to produce high-quality mosquitoes is of paramount importance.

Factors such as size and fecundity determine the quality of mosquitoes. An inadequate larval diet can adversely affect these factors and negatively impacts on the quality of the mosquitoes produced (Dadd *et al*., 1988; Lance and McInnis, 2005; Hood-Nowotny *et al*., 2012). As discussed in chapter four, the impact of two larval diets (IAEA Diet and VCRL Diet) on the development of the ANO IPCL1 strain was assessed. The IAEA Diet is a liquid diet consisting of tuna meal; bovine liver powder; Brewer's yeast; vitamins; and distilled water. The VCRL Diet is a powdered diet consisting of crushed dog biscuits and Brewer's yeast. The two larval diets did not produce a significant difference in the development of ANO IPCL1 from first instar larvae to adult mosquitoes. These adult mosquitoes did not differ in body size. There was no significant difference in the fecundity of female adult mosquitoes which received either of the two diets as larvae.

Conclusion

The baseline data produced by this study provides insight into the effects of colonization on the biological fitness of mosquitoes form southern Africa. Although the laboratory colonies MA, KGB, KWAG-BASE and MBN-BASE were introduced to laboratory settings at different times, they show the same levels of biological fitness. This indicates that these colonies have adapted equally well to the conditions experienced in the laboratory.

Louis se gat and Sirheni bush camp do not have substantial *An. arabiensis* populations. However, a perennial population of this vector species is present at Malahlapanga. While

Malahlapanga is the least accessible of the three sites, the abundance of *An. arabiensis* and the geographically isolated nature of the site make Malahlapanga an attractive field site for the SIT feasibility study.

This study has shown that the VCRL diet meets the nutritional requirements of the ANO IPCL1 strain. The VCRL diet is cost effective and did not adversely affect the development of the strain when compared to the IAEA diet. The ANO IPCL1 can be successfully maintained in the BDMI, and will be used for further SIT feasibility studies. However, for mass rearing the IAEA diet will be operationally superior especially with current mass rearing equipment (Benedict *et al*., 2009).

The need for novel vector control techniques increases the appeal of SIT. While SIT can be successful, it is important to conduct thorough investigations. SIT feasibility studies are time and labour intensive, and comprise various phases. However, it is essential to obtain data on the target species, both in the lab and the field. This work contributes to one phase of a larger SIT feasibility project currently being conducted.

APPENDIX I

Publication arising from dissertation

Givemore Munhenga, Basil D Brooke, Belinda Spillings, Leyya Essop, Richard H Hunt, Stephen Midzi, Danny Govender and Lizette L Koekemoer. 2014. Field study site selection, species abundance and monthly distribution of anopheline mosquitoes in the northern Kruger National Park, South Africa. *Malaria Journal*, 13:27.

Contribution to publication:

Leyya Essop carried out fieldwork and species-specific identification of specimens collected.

APPENDIX II

Ethics clearance certificate:

University
of the Witwatersrand,
Johannesburg

Human Research Ethics Committee (Medical)
(formerly Committee for Research on Human Subjects (Medical)

Secretariat: Research Office, Room SH10003, 10th floor, Senate House • Telephone +27 11 717-1234 • Fax +27 11 339 5708
Private Bag 3, Wits 2050, South Africa

Ref: W-CJ-100510-1

10/05/2010

TO WHOM IT MAY CONCERN:

Waiver: This certifies that the following research does not require clearance from the Human Research Ethics Committee (Medical).

Investigators: **Professor Maureen Coetzee**

Project title: **Research on mosquitos.**

Reason: This waiver covers all research using mosquitos and mosquito parasites by Professor Coetzee, her staff and students as long as no humans or human tissues are involved. The waiver lasts 5 years and may be renewed.

UNIVERSITY OF THE WITWATERSRAND
2010 -05- 10
JOHANNESBURG

Professor Peter Cleaton-Jones
Chair: Human Research Ethics Committee (Medical)

copy: Anisa Keshav, Research Office, Senate House, Wits

APPENDIX III

MATERIALS AND METHODS

A. Fitness protocol (Brooke, 2010)

Assessing the relative fitness of laboratory reared Anopheline mosquito colonies.

Procedures

Test mosquitoes: Ensure samples of *Anopheles arabiensis* and *An. funestus* can be drawn as needed from the following: laboratory colonies; laboratory reared sterilized males (irradiated from a cobalt source); wild-caught males and females; F1 progeny of wild-caught females.

Male fitness:

Wing-length: Wing-length has been shown to be a good indicator of body size and mass which, in turn, serves as an indicator of physiological and therefore reproductive fitness (Huho et al., 2007; Koella and Lyimo, 1992).

- Draw samples of 50 male mosquitoes from each of the laboratory colonies to be assessed as well as samples of wild-caught *An. funestus* and *An. arabiensis* males (as many as possible up to 50).
- Dissect one wing from each individual and place on a clean glass slide.
- Measure their wing-lengths (wing tip to thorax joint) using a calibrated dissecting microscope.
- Comparisons of mean wing-length between samples can be verified for statistical significance using two-sample t tests and ANOVA.

Longevity:

- Draw samples of 100 newly emerged males from each of the following: laboratory *Anopheles* colonies to be assessed; sterilized laboratory reared males; males emerging from wild-caught larvae or from the progeny of wild caught females (in the case of the latter, the sample of 100 males should be representative of as many mothers/families as possible).
- Subdivide each of these samples into two sub-samples of 50 males each.
- Place one sub-sample in a cage containing virgin females allowing mating to take place. The other sub-sample should be kept in a cage without females. A 10% sucrose solution should be available to all males for the duration of the experiment.
- Record and remove dead males daily until all males have expired.
- Cox' regression analysis using SPSS 15.0 software can be used to compare survival between samples as well as between sub-samples characterized as either mated or unmated.

Reproductive success in females:

***Mating Experiments*:**

- Draw samples of newly emerged mosquitoes from each of the following: laboratory colonies to be assessed; laboratory reared sterilized males; F1 progeny of wild-caught females.
- Set up mating experiments using at least 100 males and 70 females per cage according to the following scheme:

1. Laboratory colony males X laboratory colony females
2. Laboratory colony males X F1 females
3. Sterilized laboratory reared males X laboratory colony females
4. Sterilized laboratory reared males X F1 females

1) *Egg production*:

- Offer three blood-meals over a period of one week to the females from each mating experiment.
- Two days after the third blood-meal, randomly select 30 blood-fed females from each mating experiment.
- Isolate each female in a glass vial lined with moistened filter paper to enable her to lay eggs.
- Count the number of eggs (if any) produced by each female.
- Calculate the mean number of eggs produced per female from each experiment.
- Compare mean egg production per female between experiments using two-sample t tests and ANOVA.

2) *Mating success*:

- Draw a random sample of 20 females from the remainder of each mating experiment.
- Dissect their spermathecae under a dissecting microscope in order to determine whether insemination has occurred. The presence of spermatozoa will serve as confirmation of mating success.
- Spermatheca dissection: Using fine forceps, remove the last segment of the abdomen and place the tissue on a clean glass slide. Add a drop of saline and then lightly press the segment with a blunted needle to eject the spermatheca. Use dissecting needles to remove any fatty tissue obscuring the spermatheca. Any spermatozoa in the spermatheca should be easily visible allowing also for an assessment of sperm motility.
- Compare the proportions of successfully mated females between experiments using two-by-two tables and Chi-square contingency tests.

3) *Egg hatch proportions:*

- Place the eggs produced by each isolated female from each mating experiment (see *Egg production*) in a separate bowl of distilled water.
- Monitor each egg batch for hatching and record the total hatch over a period of one week.

- Calculate mean hatch proportion per female from the proportion of first instar larvae accruing from each egg batch.
- Compare mean hatch proportion per female between experiments using two-sample t tests and ANOVA.

4) *Pupa and adult accrual*:

- Rear larvae accruing from each family from each mating experiment through to the pupa phase and collect emerging adults.
- Record the proportions of larvae that pupate as well as the proportions of pupae that emerge as either male or female adults.
- Compare mean proportions of pupae and mean proportions of emerging adults by gender between experiments using two-sample t tests and ANOVA.

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B. Larval food amount per instar per day (Gilles *et al*., 2011; Damiens *et al*., 2012; Hood-Nowotny *et al*., 2012; Puggioli *et al*., 2013):

Instar	**Food (mg/larva/day)**
$1^{st} – 2^{nd}$	0.2
$3^{rd} – 4^{th}$	0.4 - 0.8

C. VCRL larval diet:

- West's Beeno Traditional Crunchy Biscuit Treats (Martin and Martin, South Africa)

- Brewer's yeast (Vital Health Foods, South Africa)

Crush Crunchy Biscuit Treats and combine with Brewer's yeast at a ratio of 3:1.

D. IAEA larval diet:

- 6.25g Tuna meal

- 2.50g Bovine liver powder

- 1.25g Brewer's yeast

- 2g Vitamin mix

Dissolve tuna meal, bovine liver powder, Brewer's yeast, and vitamin mix in 1L of distilled water.

E. Sugar solution:

For a 10% sugar solution, dissolve 100g of sugar in 1L of distilled water.

F. 50x TAE (Tris Acetic EDTA) Buffer (pH 8.0) (Sambrook *et al*., 1989):

- 2M Tris

- 5.7% Glacial acetic acid

- 0.5M EDTA

Make up to 1L

G. Agarose gel:

For a 2.5% agarose gel, dissolve 10g agarose in 400ml 1 x TAE.

H. Preparation of Phosphate Buffered Saline (PBS):

Dissolve one PBS tablet in 1L distilled water to yield 10mM phosphate buffer, pH 7.4, 140mM NaCl, 2.7mM KCl.

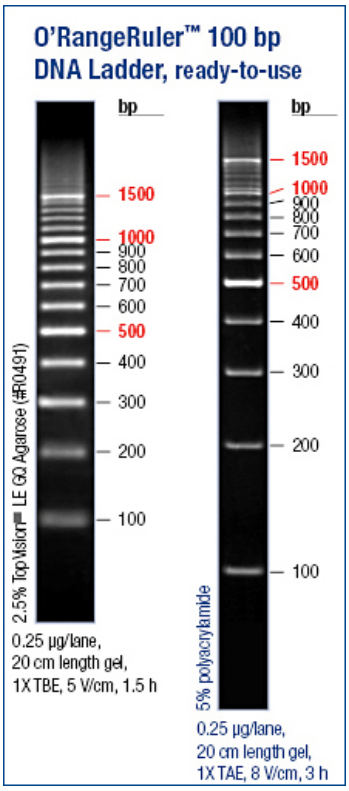
I. List of specialised equipment:

- Primus 96 Plus MWG AG Biotech

- G: Box Chemi XL gel documentation system (Syngene)

- Olympus SZ51 microscope

I. O'RangeRuler™ 100 bp DNA Ladder (Fermentas, supplied by ThermoScientific, Cat. No. SMO623):



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