An Investigation into the Robustness of Insectary-reared *Anopheles arabiensis* for use in the Sterile Insect Technique for Controlling Malaria

by

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PREFACE

The experimental work described in this thesis was carried out in the insectary and molecular research laboratories of the Malaria Unit, South Africa Medical Research Council, Durban. This work was carried out under the supervision of Professor R. Maharaj and Dr T. Olckers.

The studies represent original work by the author and have not otherwise been submitted in any other form to another University. Where use has been made of the work of others it is duly acknowledged in the text.

Signed:

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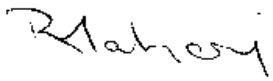

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Prof. R. Maharaj (Supervisor)

ABSTRACT

Human malaria is one of the deadliest vector-borne diseases in the world and is caused by parasites of the genus *Plasmodium* that are transmitted via mosquitoes of the genus *Anopheles.* The highest impact of malaria can be seen in Africa, where 90% of worldwide deaths occur. Although current vector control strategies include biological control, chemical application and environmental management, there is renewed interest in the Sterile Insect Technique (SIT). SIT involves the mass production of the target population, in this study *Anopheles arabiensis* Patton, sterilizing the males with ionizing radiation and, thereafter, the mass release of these sterile males into the natural environment. The subsequent mating of the sterile males with the wild females should result in a decrease, and ultimately the elimination, of the natural *An. arabiensis* population. However, for SIT to be successful, the insectary-reared males need to compete effectively with their wild counterparts for female insemination. This study was conducted to determine if the laboratory-reared males would be able to compete successfully with the wild male population in northern KwaZulu-Natal. Standard testing protocols were taken from the Malaria Research Unit, World Health Organization, as well as methods proposed by the National Health Laboratory Services. The collection of mosquitoes from the target area indicated that *An. arabienis* is a seasonal species with populations increasing during warmer conditions. The mating compatibility between the three tested strains of *An. arabiensis*, namely the Old Mamfene strain (laboratory strain), New Mamfene Strain (wild strain) and the Genetic Sexing Strain, proved favorable due to statistically non-significant insemination rates. However, the results indicated that the laboratory-reared colony displayed greater fecundity and mean numbers of larvae hatched than the wild colony. Within strains, overcrowding of larvae affected the size of the male adults, although reduced size did not affect mating within each strain, as insemination rates were not statistically affected ($p>0.05$). Dyes were tested to track mating between sterile males and wild females. However, dye transfer from male to female during copulation resulted in mating compatibility being negatively affected. Further investigations are thus needed to determine a better approach to tracking females that have copulated with released males. Although the results indicate that laboratory-reared males can compete successfully with their wild counterparts, field studies are required to verify these laboratory results.

Keywords: *Anopheles arabiensis*, Mosquito control, Malaria management, SIT

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CONTENTS

CHAPTER 1: Introduction and Literature Review

1.1 General Introduction

Malaria, an important mosquito-borne disease, has a devastating impact on human populations throughout the world, especially sub-Saharan Africa (Lindsay et al., 1998; Oliva et al., 2011). Figure 1.1 indicates countries with ongoing malaria transmission in 2015 and emphasizes the importance of the disease in Africa (WHO, 2016). This disease is caused by parasitic protozoan species in the genus *Plasmodium* and is transmitted to humans by mosquito species in the genus *Anopheles* (Klassen, 2009).

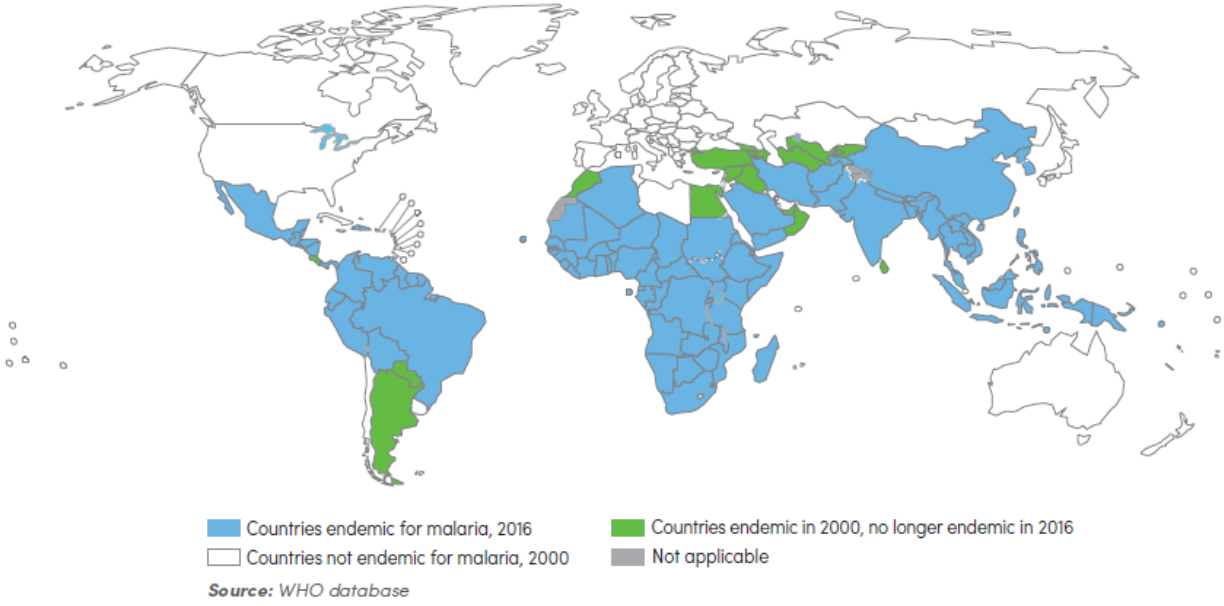

Figure 1.1: Countries with ongoing malaria transmission in 2015 (from WHO, 2016).

The *Plasmodium* species that cause malaria in humans are *P. falciparum* Welch, *P. vivax* (Grassi & Feletti), *P. ovale* Stephens, *P. malariae* (Feletti & Grassi) and *P. knowlesi* Sinton and Mulligan (Lee et al., 2011). *Plasmodium falciparum* is reportedly the most lethal and prevalent of this group of parasites (Laishram et al., 2012). Severe or complicated malaria is defined by at least one of the following clinical manifestations: unrousable coma, convulsions, malarial anemia, haemoglobinuria, hypoglycemia, metabolic acidosis, acute pulmonary oedema, acute renal failure, jaundice, circulatory collapse, hyperparasitaemia, high fever electrolyte disturbance and/or spontaneous bleeding (Laishram et al., 2012). Individuals with mild or uncomplicated malaria

present with a fever and/or chills, sweats, headaches, vomiting, watery diarrhea, anemia, jaundice and swelling of the spleen (Laishram et al., 2012).

1.2 Malaria: A Global Disease

The World Health Organization (WHO) Report of 2016 stated that globally an estimated 212 million cases of malaria occurred with some 429 000 deaths in that year. An estimate of 90% of malaria cases and 92% of deaths occurred in the WHO African region. Approximately 303 000 malaria deaths occurred in children under 5 years of age. The vast majority of deaths (99%) resulted from *P. falciparum* infection (WHO, 2016).

1.3 Regional Impact of Malaria

Malaria is recognized as both a disease of poverty and a cause of poverty in Africa (Enato and Okhamafe, 2005). This disease has measurable direct and indirect costs and is a major constraint to economic development. Economic growth has increased in countries such as the United States of America and Italy where malaria has been eliminated (Enato and Okhamafe, 2005). The effects of malaria on households are substantial and burdensome for the poor. The costs of prevention and treatment, and the loss of productivity due to malaria-related morbidity and mortality, can represent a considerable portion of the annual income of poor agricultural households (Malaney et al., 2004). Malaria-endemic regions, as opposed to malaria-free zones, are also undesirable to foreign investors due to costly health interventions. Trade within an economy is affected by malaria because visitors to endemic areas often lack immunity, which may inhibit traders from travelling within and between such areas. The risk of contracting the disease also negatively affects the tourism industry and its potential to achieve high profits (Malaney et al., 2004). Education in affected areas is also disrupted because malaria causes high rates of absenteeism which in turn causes increased failure and dropout rates (Malaney et al., 2004).

A major obstacle to improved disease management in sub-Saharan Africa is access to healthcare. Historically, the diagnosis of malaria in sub-Saharan Africa was mainly done clinically without laboratory confirmation (Castillo-Riqueime et al., 2008). The rapid diagnostic test (RDT) is a device that detects malaria antigens in a small amount of blood, typically between 5-15 µl, by immuno-chromatographic assay with monoclonal antibodies directed against the targeted parasite antigen and impregnated on a test strip (Wongsrichanalai et al., 2007). RDTs are being used as an

alternative to microscopic diagnosis in malaria-endemic regions (Gillet et al., 2011). These tests detect the antigens that are specific to a *Plasmodium* species (e.g. *P. falciparum*) in blood, by the antibody-antigen interactions on a nitrocellulose strip. RDTs have demonstrated up to 100% success for *P. falciparum* detection at densities above 100 asexual parasites/ul or 0.002% of parasitized erythrocytes (Gillet et al., 2011).

In at least 38 African countries, the primary treatment policy is Artemisinin-based combination therapies (ACT). The first health authority to implement this policy was in KwaZulu-Natal province, South Africa (Castillo-Riqueime et al., 2008). The implementation of these combination therapies, whose overall effectiveness depends on achieving high coverage levels, has relied largely on the available healthcare infrastructure for delivery. Effective treatment and equity in access to malaria services is a concern, especially with the introduction of a more expensive drug (Castillo-Riqueime et al., 2008).

1.4 Malaria in South Africa

According to the WHO Report of 2014, South Africa falls under the sub-region of Low Transmission Southern African Countries, together with Botswana, Namibia, Swaziland and Zimbabwe. It also states that, with the exception of Zimbabwe, there was a reported decrease of >75% in malaria case incidence in these countries, between 2000 and 2013. In this regard, South Africa is more fortunate than other African countries, for several reasons (Hlongwana et al., 2011). Firstly, South Africa is located at the southern extreme of the malaria distribution on the continent. Secondly, the relatively small areas that are affected experience seasonal transmission (i.e. malaria in South Africa is unstable and epidemic prone). Thirdly, the country has a well-organized national malaria control program, as well as a relatively well-developed scientific, economic and health infrastructure (Hlongwana et al., 2011).

South Africa has three malaria-endemic provinces, namely Limpopo, Mpumalanga and KwaZulu-Natal with an estimated 10% (4.9 million people) of the country's population living in these regions and at risk of contracting malaria (Hlongwana et al., 2011; Moonasaret al., 2012). *Anopheles arabiensis* Patton is the major malaria vector in South Africa, following the elimination of *Anopheles funestus* sensu stricto Giles by means of indoor residual spraying (IRS) involving dichlorodiphenyltrichloroethane (DDT) through the years (Brooke et al., 2015; Burke et al., 2017.

Plasmodium falciparum is the most prevalent parasite causing approximately 95% of malaria cases in South Africa (Hlongwana et al., 2011).

1.5 Bionomics of the Vector

The human malarial protozoa are transmitted by mosquitoes in the genus *Anopheles*, which include 465 formally recognized species with an estimated 70 species having the capacity to transmit these parasites (Sinka et al., 2012).

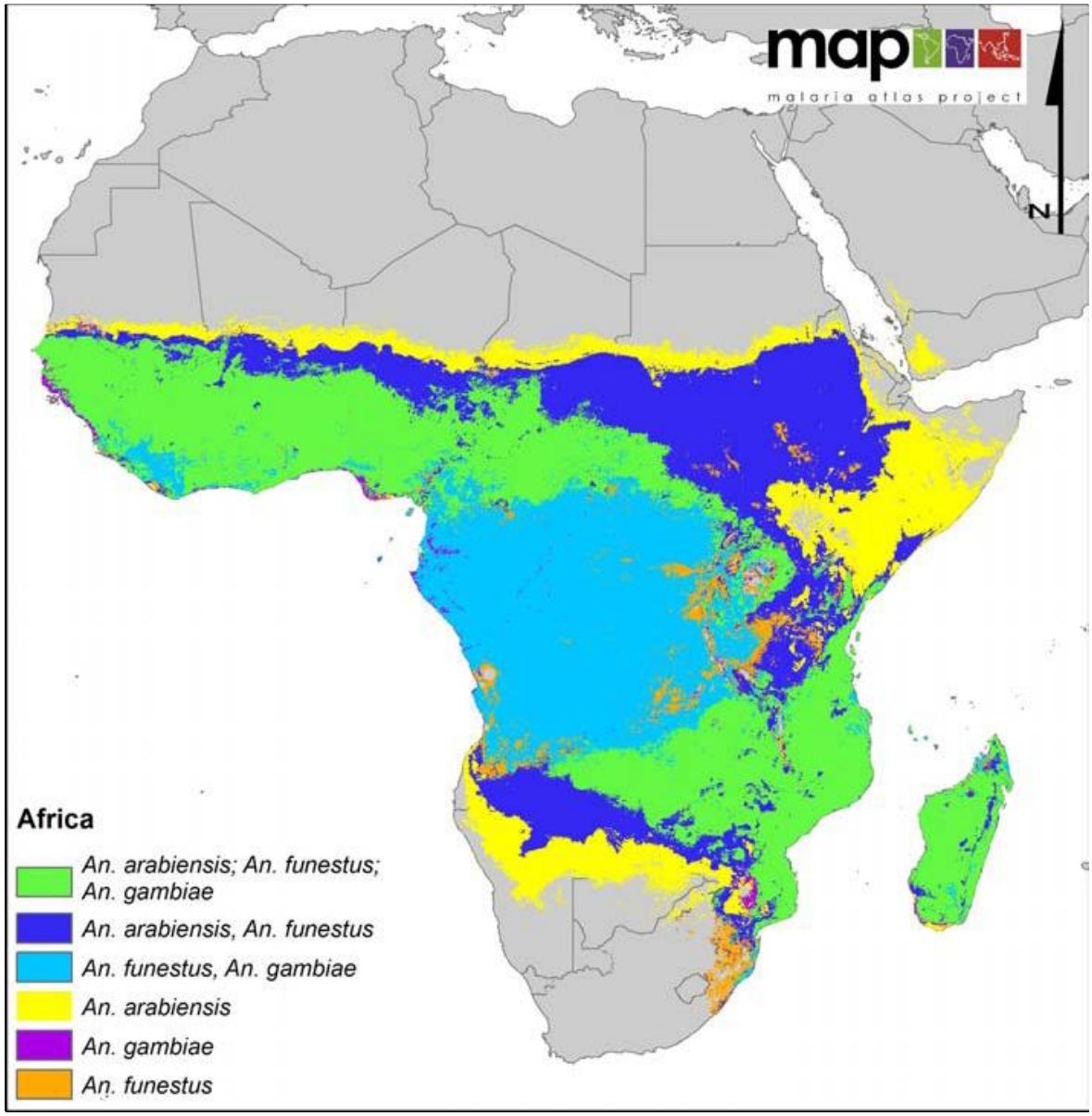

Figure 1.2: Historic distribution of dominant malaria vector species in Africa (from Sinka et al., 2012).

In sub-Saharan Africa, most transmission is attributed to four major vectors, namely *Anopheles gambiae* s.s. Giles, *An. arabiensis*, *An. coluzzii* and *An. funestus* (Sougoufara et al., 2017; Coetzee et al., 2013). *Anopheles arabiensis* is the one of the dominant malaria vectors in Africa and was, until recently, the only vector in South Africa (Moonasar et al., 2012; Oliver and Brooke, 2013). However, Burke et al. (2017), reported the emergence of *Anopheles vaneedeni* Gillies & Coetzee, a member of the *An. funestus* species group, as additional vector in South Africa. *Anopheles arabiensis* falls within the *Anopheles gambiae* s.l. species complex, together with seven other sibling species (Coetzee et al., 2013; Ebenezer et al., 2014). These sibling species are morphologically indistinguishable, with up to four species that may be sympatric. Therefore the most common method used for species identification is based on polymerase chain reaction (PCR) amplification of ribosomal DNA sequences (Bass et al., 2007). The principal malaria vectors within the complex are *An. gambiae* s.s. and *An. arabiensis*. The zoophilic, non-malaria species that form part of the complex are *An. quadriannulatus* Theobald, which is widespread in southern Africa, and *An. amharicus* Hunt, Wilkerson & Coetzee, which is found in Ethiopia (Bass et al., 2007; Coetzee et al., 2013). Also included are *An. merus* Dönitz and *Anopheles melas* Theobald, both originally known as salt water breeders (Coetzee et al., 2013). *An. merus* has been found to breed along the eastern coastal salt-water areas of Africa, but has also been isolated further inland in both saline and freshwater habitats in Mozambique, Zambia, Zimbabwe, Swaziland and South Africa (Mbokazi et al., 2018). The remaining species in the complex is *An. bwambae* White, which is restricted to a region close to the Buranga hot springs in Uganda (White, 1985; Bass et al., 2007).

The vector species in this study, *An. arabiensis*, can be described as zoophagic (feeds on animals), exophilic (rests outdoors) and exophagic (feeds outdoors) when compared to *An. gambiae* (Sinkaet al., 2010). However, it has been reported that *An. arabiensis* can be extremely anthropophagic (feeds on humans) in some localities (Fornadel et al., 2012). *Anopheles arabiensis* is not as anthropophagic as *An. gambiae* s.s. and not as an efficient a vector as *An. funestus* with which is it regularly found in sympatry (Oliver and Brooke, 2013). *Anopheles arabiensis* is variable in its foraging behaviour and although it can be found feeding and resting indoors in some areas, in other regions it mainly feeds and rests outdoors (Fornadel et al., 2012). This variability in behaviour

causes difficulties in controlling the vector by the indoor residual spraying of insecticides (Oliver and Brooke, 2013).

Anopheles arabiensis is considered to be a species of dry savannah environments and sparse woodland but is also known to occur in forested areas affected by land disturbances or clearances (Sinka et al., 2010). Larval habitats comprise mainly small, temporary, sunlit, clear and shallow fresh water pools, but can include slow flowing, partially shaded streams and a variety of large and small natural and man-made aquatic habitats. In addition, larvae of *An. arabiensis* have been found in turbid waters and occasionally in brackish aquatic habitats (Sinka et al., 2010).

The distribution and abundance of mosquito larvae results from the availability of aquatic oviposition sites, the oviposition preferences of the female and the ability of the larvae and pupae to survive and develop after the eggs are laid (Mwangangi et al., 2006). *Anopheles arabiensis* is associated with the "paddies paradox" (Oliver and Brooke, 2013). Malaria transmission is reduced in irrigated areas compared to non-irrigated areas, because *An. arabiensis* replaces *An. funestus* which is a more efficient vector (Oliver and Brooke, 2013). *Anopheles arabiensis* readily makes use of irrigated rice fields, where larval densities are related to the height of the rice plants; larval populations increase when the plants are relatively short, and decrease when the plants mature (Sinka et al., 2010). Oliver and Brooke (2013) state that the most dramatic example of agricultural impact on the life history of *An. arabiensis* is the effect of maize farming. *Anopheles arabiensis* larval populations are increased due to their consumption of maize pollen to the degree that the effect of larval crowding is reduced (Oliver and Brooke, 2013). The blood-feeding behaviour of the adults is variable, with biting generally occurring at night. The peak biting times can begin in the early evening (19:00) or early morning (03:00) (Sinka et al., 2010).

A major factor that influences the fitness of an adult mosquito is the density at which the larvae in the population developed (Ng'habi et al., 2008). In the absence of predators and pathogens, and within the genetic hereditary limits, the number of larvae in a particular aquatic habitat, together with the availability of food, determines the number of emerged adults, their survival and body size (Yuval et al., 1993; Ng'habi et al., 2008). Crowded larvae are faced with greater competition for food and are exposed to higher levels of toxic waste products, crowding chemicals and interference from other larvae (Ng'habi et al., 2008). The study of Ng'habi et al (2005) indicated that larval crowding affected the mating competitiveness of male *An. gambiae* mosquitoes. Their

results indicated that males reared under low crowding conditions were eleven times more likely to appear first in a mating swarm, compared to males from high crowding situations. However, there was no evidence that male copulation frequency was affected by larval crowding (Ng'habi et al., 2008).

Swarms are mating aggregations which are formed by most mosquito species (Yuval et al., 1993). These swarms are formed during dusk and are composed mainly of males. Female mosquitoes that approach these swarms are rapidly mated with (Yuval et al., 1993). The female mosquito is monogamous, while an individual male *An. gambiae* can fertilize up to four females (Charlwood et al., 2002). Charlwood et al. (2002) stated that larger insects are reproductively more successful than smaller ones, while Yuval et al. (1993) observed that larger males mated more successfully than smaller males. The latter authors hypothesized that larger males return to the swarm after mating while smaller males do not, possibly due to exhaustion of energy reserves. Smaller males may also refrain from swarming or begin swarming earlier than the larger males.

In most anopheline mosquitoes, it is believed that mating occurs only in swarms; however, male mosquitoes are not competent to mate at emergence (Howell and Knols, 2009). The male mosquito is not sexually mature immediately after emergence (Oliva et al., 2011), since the terminalia, sexual organs and antennal fibrillae must first mature (Howell and Knols, 2009). A 180° inversion of the male terminalia occurs within the first 12-24 hours post emergence. A further delay of one day in sexual activity after the inversion indicates that males are still not sexually mature (Howell and Knols, 2009). The complete functioning of the male antennal fibrillae, which are essential for mating, does not occur until 12 hours post emergence. The antennal fibrillae are required to locate females by responding to their flight tones. *Anopheles gambiae* males with one or both antennae removed were unable to locate females, indicating that reception of female wing beat tones is vital in their sexual maturation (Howell and Knols, 2009). Claspers tipped with claws are located on the tenth abdominal segment and enable the male to grasp the female for copulation. The claws of newly-emerged males are rotated dorsally, therefore preventing copulation from occurring prior to this (Oliva et al., 2011).

When mating occurs, the male and female interlock when the male grasps the female with his tarsal claw located on his first pair of legs. The male then swings his abdomen up to clasp the female's genitalia (Howell and Knols, 2009). Thereafter, the tarsal claw grasp is released, the

venter-to-venter position is assumed and flying is resumed. The pair then depart the swarm to complete mating, after which the male rejoins the swarm (Howell and Knols, 2009). Females of most mosquito species are unreceptive during the first 30-60 hours post emergence. Although some females may allow premature copulation, they will not become inseminated (Oliva et al., 2011).

Differences between laboratory-reared mosquitoes and wild mosquitoes, such as low fitness, low mating success, reduced heterozygosity and reduced competitiveness, accumulate during laboratory culturing (Benedict et al., 2009). These differences are formed due to selection, genetic drift and inbreeding (Oliva et al., 2011).

1.6 The Malaria Parasite

Human malaria was presumed to be caused by four species of the plasmodium parasite (*P. falciparum, P. ovale, P. malariae* and *P. vivax*). However, a fifth species, *P. knowlesi*, was later found to cause malaria in humans (see Lee et al., 2011). According to White (2008), Knowles and Das Gupta successfully transmitted the monkey malaria they had discovered (i.e. *P. knowlesi*) to humans in 1932, while in 1967 Chin indicated that this could be transmitted from monkeys to humans by *Anopheles balabacencis. Plasmodium falciparum* is the most virulent and common cause of malaria across sub-Saharan Africa and it accounts for 90% of malaria cases in the region (Enato and Okhamafe, 2005; Snow and Omumbo, 2006).

The life cycle of the plasmodium parasite (see Cox, 2010) begins when sporozoites are introduced into the human body via the female anopheline mosquito. The sporozoites are passed through the body until they invade the liver hepatocytes. A phase of asexual multiplication (exoerythrocytic schizogony) occurs in the hepatocytes which results in uninucleated merozoites. These merozoites exit the hepatocytes and flood the bloodstream where they invade the red blood cells. A second phase of exoerythrocytic schizongy occurs in the liver, which produces more merozoites which in turn invade new red blood cells. This process occurs almost indefinitely and is responsible for malaria. As the infection progresses, the young merozoites develop into male and female gametocytes that circulate in the peripheral blood. These gametocytes are then taken up by the female anopheline mosquito when it ingests blood. The gametocytes within the mosquito mature into male and female gametes; thereafter, fertilization occurs and a motile zygote (ookinete) is

formed within the lumen of the mosquito gut, which is the beginning of the process called sporogony. The ookinete then penetrates the gut wall and becomes a conspicuous oocyst, within which another multiplication occurs resulting in the formation of sporozoites that migrate to the salivary glands of the female mosquito and are injected into a new host during feeding (Cox, 2010).

1.7 Malaria Control and Prevention of Transmission

Malaria control in South Africa is largely two-pronged, broadly comprising vector control and case management, during which the vectors and parasites are targeted in separate operations. These operations are generally adopted across the African continent and elsewhere in the world.

1.7.1 Vector Control

The goal of vector control is to reduce the vectorial capacity of the local vector population to below the critical threshold to obtain a malaria reduction rate (The malERA Consultative Group on Vector Control, 2011). When it was discovered that mosquitoes are responsible for the transmission of malaria, early attempts at vector control focused on the larval stages of the *Anopheles* mosquito, using environmental management and larviciding (Russel et al., 2013). Larvicides act on a single, non-transmitting stage in the mosquito life-cycle (i.e. the larvae) and can only affect the disease by reducing vector abundance (Devine and Killeen, 2010). The use of larvicides and the drainage of aquatic breeding habitats have historically proven to be successful in reducing mosquito density (Maheu-Giroux and Castro, 2013). Examples of this success are the elimination of *An. arabiensis* in Egypt and Brazil, malaria control in the Zambian copper belt from 1930 to 1950 and the vector control program of the Tennessee Valley Authority in Tanzania (Maheu-Giroux and Castro, 2013).

The Larval Source Management (LSM) approach is often regarded as a secondary control strategy. This is due to it being labour-intensive, requiring managerial support and oversight for monitoring and evaluation, and it often being beyond the financial and operational capabilities of most endemic malaria areas in sub-Saharan Africa (Worrall and Fillinger, 2011; Maheu-Giroux and Castro, 2013). However, the WHO released an interim position statement in April 2012, stating that larviciding should be considered for malaria control, but only in areas where breeding sites are few, fixed and findable (Maheu-Giroux and Castro, 2013).

The challenges of larviciding are the countless and cryptic nature of the aquatic oviposition habitats and the identification and targeting of the most productive sites. However, Devine and Killeen (2010) discussed a new method that could be used for larviciding of malaria vectors. This technique utilizes adult female mosquitoes as larvicide-disseminating vehicles to transfer a potent larvicide between resting and oviposition sites. The technique requires a highly effective and persistent insecticide, limited aquatic habitats, the predictability of sites where adult mosquitoes could be exposed and a sufficient mosquito density. Only those aquatic habitats visited by adults were contaminated and the more popular the site, the greater the number of larvicide-transfer events (Devine and Killeen, 2010).

Environmental management or source reduction involves the concept of modifying vector habitats to discourage larval development and/or human vector contact (Walker and Lynch, 2007). Environmental modifications include drainage of aquatic habitats, land leveling, filling in of depressions and contouring of reservoirs. Environmental manipulation also includes vegetation management, safe storage of domestic water, managing peri-domestic waste and, reducing the contact between humans and the disease vector by means of behavioural changes (Pruss-Ustun and Corvalan, 2007).

When studies discovered that female vector mosquitoes rested indoors after feeding, the adult stage of the vector was targeted using insecticides. In the 1930s, indoor spraying using the insecticide pyrethrum was effective in South Africa and India (Russel et al., 2013). However, this insecticide lacked residual activity and weekly spraying was implemented, resulting in logistical and financial challenges (Russel et al., 2013). In 1939, the residual activity of DDT was discovered which resulted in financially viable operations that focused on large scale indoor residual spraying (IRS). Historically the use of IRS has been an important tool in the prevention of malaria. Several countries within sub-Saharan Africa have added IRS to their malaria control plan. The WHO Global Malaria Eradication program regarded IRS as a critical component and from 1955 to 1969, IRS was the main intervention contributing to the elimination, or dramatic reduction, of malaria in parts of Europe, Asia and Latin America (Kigozi et al., 2012). In 2006, the WHO began recommending increased implementation of IRS (Beer et al., 2013). In southern African countries, a significant decrease in malaria burden was observed due to the large scale and sustained

application of IRS. Mainly due to IRS, 98% of children under the age of five were protected (Beer et al., 2013).

Presently, the most effective vector control strategies in use today rely on IRS and long lasting insecticide-treated nets (LLINs) (Okumu and Moore, 2011; The malERA Consultative Group on Vector Control, 2011). Insecticide-treated nets (ITNs) were shown to reduce malaria cases by 39% to 62% and child mortality by 14% to 29% (Okumu and Moore, 2011). ITNs have been used in rural Africa since the 1980s; however, there was a low implementation of this method (Zhou et al., 2013). In 2000, only an estimated 3% of households in sub-Saharan Africa owned at least one ITN (Zhou et al., 2013). In 2006, there was a massive community-based distribution of ITNs, in Africa, which were provided free of charge or heavily subsidized through health facilities. This distribution was supported by the Global Fund to fight HIV/AIDS, Tuberculosis and Malaria along with many other donors (Zhou et al., 2013). In 2011, due to the downward trends in malaria cases there was another distribution campaign that issued free ITNs to everybody at risk of malaria within sub-Saharan Africa. The percentage of households in sub-Saharan Africa owning at least one ITN was estimated to be 50% before the 2011 distribution campaign and included countries such as Tanzania, Ethiopia, Kenya, Nigeria and the Democratic Republic of Congo (Zhou et al., 2013).

The greatest challenge regarding the use of insecticides is the development of resistance (Osse et al., 2012). According to Corbel and N'Guessan (2013), the WHO defines resistance as the ability of an insect to withstand the effects of the insecticidal toxins by means of natural selection and mutations. Gatton et al. (2013) stated that intensive chemical interventions have frequently caused the emergence of physiological/biochemical resistance due to the elevated selective pressure exerted on the target populations.

Insecticides used in malaria control include organochlorines, organophosphates, carbamates and pyrethroids (Corbel and N'Guessan, 2013). These four classes of insecticides are licensed for the control of adult mosquitoes for public health purposes (Gatton et al., 2013). Insecticide resistance is increasing worldwide as a result of increased selection pressure on the mosquito populations. However, resistance is not uniformly distributed among populations of the vector species and differs from one area to another. Africa has been reported to have the highest level of insecticide resistance worldwide (Corbel and N'Guessan, 2013). Gatton et al. (2013) reported that results from

experimental hut studies in West Africa exhibited a noticeable reduction in vector mortality in areas with high levels of physiological resistance.

Pyrethroids are the most popular insecticides for mosquito control because they are the most frequently used insecticides in IRS and are the only class of insecticide approved for the treatment of bed nets due to their low mammalian toxicity (Nardini et al., 2013). Pyrethroid resistance in *Anopheles* species is thus widespread but not uniformly distributed among the different countries (Corbel and N'Guessan, 2013).

Intact bed nets can provide a protective barrier in the absence of effective chemical control (Gatton et al., 2013). However, in areas where there are resistant vectors, damaged bed nets (e.g. torn or with holes) provide inadequate protection. In endemic areas that have susceptible vectors, damaged bed nets decreased the chances of being bitten by 66% and the majority of the mosquitoes were killed by the insecticide treatment (Gatton et al., 2013). Globally, there are 40 malaria endemic areas that have reported resistance to insecticides, mostly to pyrethroids (Gatton et al., 2013). However, multiple insecticide resistance is common with some regions reporting resistance to all four classes of insecticides used in public health protection. It was estimated that more than half of the benefits gained from the coverage of LLINs and IRS in Africa would be lost if pyrethroids lost their effectiveness, resulting in approximately 120 000 additional deaths per year (Gatton et al., 2013).

Behavioural resistance refers to any modification in the behaviour of the mosquito vector that facilitates the avoidance of insecticides (Gatton et al., 2013). This includes direct contact excitation or irritancy and non-contact spatial repellence, when the vector moves away from the insecticide-treated area before making direct contact. These changes in behaviour are a result of prolonged exposure to insecticides (Corbel and N'Guessan, 2013). The most common behavioural change in malaria vectors is the development of early outdoor-feeding phenotypes among anopheline populations in areas of extensive IRS use. These mosquitoes avoid LLINs and IRS control through preferential feeding, resting outside human homes and being active earlier in the evening before people have gone to sleep. Other behavioural changes include increased zoophagy (i.e. animal feeding) that may evolve in response to intensive chemical interventions (Gatton et al., 2013).

1.7.2 Parasite Control

Malaria is caused by parasites of the genus *Plasmodium* with *P. falciparum* causing 250-500 million clinical cases and up to 1.2 million deaths annually (Pasini et al., 2013). Most cases of *P. falciparum* malaria are uncomplicated and are treated with short courses of oral antimalarial drugs (Achan et al., 2009). Parasite control is reliant on the use of antimalarial drugs for both malaria prophylaxis and treatments of infection (Pasini et al., 2013). However, most affected human populations have limited access to modern health care facilities and therefore do not have access to early diagnosis and prompt treatments (Guerin et al., 2002; Tipke et al., 2008). Consequently, malaria is recognized as a disease of poverty (Worrall et al., 2005).

The WHO (2012), recommended three strategies which target specific groups that are at high risk of *P. falciparum* malaria, predominantly in sub-Saharan Africa. Firstly, in regions of moderate to high malaria transmission, intermittent preventative treatment with sulfadoxine-pyrimethamine (SP) is recommended for all pregnant women at each scheduled antenatal care visit. Secondly, in countries within sub-Saharan Africa, where malaria transmission is moderate to high and parasitic SP resistance is low, infants are treated through the Expanded Program on Immunization with the co-administration of SP and the second and third vaccinations of diphtheria-pertussis-tetanus. Thirdly, seasonal preventive treatments with amodiaquine and SP is recommended for children aged 3-59 months in areas of highly seasonal malaria transmission across the sub-Sahel sub-region in Africa (WHO, 2012). WHO (2016) reported that, in sub-Saharan Africa, intermittent preventative treatment of malaria in pregnancy with SP has reduced maternal anemia, low birth weight and perinatal mortality. Intermittent prevention treatment in infants (IPTi) with SP also provides protection against clinical malaria and anemia; however, as of 2015, no countries have reported the implementation of an IPTi policy (WHO, 2017).

There are two main classes of antimalarial drugs, the antifolates and the quinolone-containing drugs (Phillips, 2001). The antifolates include diaminopyrimidines, bigunides and sulfa drugs and the quinine-containing drugs include the cinchona alkaloids, quinine and quinidine, the aminoalcoholquinine analogues, mefloquine and halofantrine, and the 8-aminoquinolineprimaquines (gametocidal effect), 4-aminoquinolines, chloroquine and its relative amodiaquine (Phillips, 2001). Chloroquine was the mainstay of therapy for uncomplicated *P.*

falciparum malaria until the late 1990s (Anchan et al., 2009). Artemisinin has been used in traditional Chinese medicine for centuries, but is poorly absorbed and therefore many derivatives have been prepared and evaluated. There are three semi-synthetic derivatives in use, namely a water-soluble artesunate and two oil-soluble compounds, artemether and artheether. The artemisinins are the fastest acting antimalarial drugs available (Phillips, 2001).

The malaria parasite has an extremely complex life cycle, with sexual development in the mosquito and asexual replication in the human host erythrocytes (Birkholtz et al., 2012). Most of the antimalarial drugs target the asexual erythrocytic stages which essentially cause pathogenesis of the disease (Phillips, 2001; Birkholtz et al., 2012). The sexual stages of the parasite will not be affected by some antimalarial drugs and therefore transmission from these patients might still occur after treatments. However, the artemisinin drug group controls the asexual stages of the parasite but also significantly reduces opportunities for transmission from the patients (Phillips, 2001). In uncomplicated malaria, inhibition of parasite multiplication is important and this will prevent the progression of the disease. The inhibition of parasite multiplication is a first order process which would lead to a log-linear reduction in the parasite numbers (White, 2004).

Resistance to almost all antimalarial drugs has been recorded (Phillips, 2001). Quinine was the first established antimalarial drug and intravenous applications of quinine is the standard therapy for severe *P. falciparum* malaria in African countries (Achan et al., 2009). According to Phillips (2001), quinine has had the longest effective use, but resistance to it has been reported. As a result of decreased efficacy of older agents and the limited availability of Artemisinin Combination Therapy (ACT), quinine is increasingly used as a first-line treatment for uncomplicated malaria in Africa (Achan et al., 2009). The low cost and easy availability of chloroquine has contributed to the development of resistance and *P. falciparum* is highly resistant to chloroquine in most malaria affected areas (White, 2004). Antifolate resistance developed quickly after its introduction, but the combination of pyrimethamine with the sulfa drugs delayed resistance to pyrimethamine (Phillips, 2001). White (2004) stated that resistance to SP has also become widespread.

Predictions of the emergence and spread of resistance to current antimalarial drugs and newly introduced compounds are necessary for planning malaria control operations and instituting strategies that could delay the emergence of resistance (White, 2004). Numerous organizations and associations emphasize the need for novel antimalarial drugs that are: (i) effective against

erythrocytic and exo-erythrocytic stages of the parasite; (ii) effective against resistant forms of the parasite; (iii) chemically distinct, with new mechanisms of action against the malaria parasite; (iv) safe without associated toxicities; (v) pharmaco-kinetically amendable to once-daily oral dosing and; (vi) economically viable (Birkholtz et al., 2012).

1.8 Malaria Control in South Africa

South Africa, before the implementation of malaria control strategies, was typified by malaria transmission which extended as far south as Durban and Port St Johns and inland as far as Pretoria (Blumberg and Frean, 2007). In the 1930s, indoor spraying began using non-residual pyrethrum and in the 1940s, DDT became available which led to IRS as the favoured control strategy (Cliff et al., 2010). South Africa is one of 34 malaria-endemic countries targeting elimination of the disease, but malaria transmission still occurs in nine South African districts, including Capricorn, Mopani, Sekhukhune, Vhembe and Waterberg in Limpopo province; Ehlanzeni in Mpumalanga; and uMkhanyakude, uThungulu and Zululand in KwaZulu-Natal (Moonasar et al., 2013). According to Khosa et al (2013), South Africa currently represents a low transmission region that is characterized by a lower incidence of confirmed cases. In these low transmission areas, malaria is mostly seasonal, unstable and prone to epidemics. However, the country has a well-established malaria control program which includes vector control, health promotion, case management and cross-border strategies (Moonasar et al., 2012; Khosa et al., 2013).

The current vector control strategy implements IRS (Moonasar et al., 2012; Brooke et al., 2013). IRS intervention has proven to be a successful tool for vector management in KwaZulu-Natal since 1932. The use of DDT was phased out due to negative perceptions in the community but in 2000, following reports of pyrethroid resistance, DDT was subsequently reinstated (Moonasar et al., 2012). South Africa currently relies on DDT, deltamethrin and carbamates for IRS use (Moonasar et al., 2012). Insecticide resistance is widespread and relatively recent in South Africa (Brooke et al., 2013). In 2002, *An. arabiensis* collected from northern KwaZulu-Natal displayed resistance to DDT and susceptibility to deltamethrin; however, subsequent collections in 2005 uncovered resistance to permethrin and suspected resistance to deltamethrin (Brooke et al., 2013). The current insecticide susceptibility assays for adult mosquitoes are direct response-to-exposure tests and provide little information on the underlying genetic mechanisms of resistance; however, this information can be obtained using various molecular and biochemical assays (Brooke et al., 2013).

The health promotion strategy is used to educate and influence communities to take preventative precautions against malaria (Moonasar et al., 2012). This strategy also ensures that communities comply with instructions from spray operators during IRS campaigns. The strategy enables communities to recognize the signs and symptoms of malaria and seek early treatments (Moonasar et al., 2012).

The case-management strategy consists of diagnosis, malaria case confirmation and treatment (Moonasar et al., 2012). Public health facilities in South Africa provide free diagnosis and treatment. Historically, uncomplicated malaria was treated with chloroquine and complicated malaria was treated with quinine. In 1987, resistance to chloroquine was reported in KwaZulu-Natal and sulphadoxine-pyrimethamine (SP) was thus used in KwaZulu-Natal in 1988 and later in Mpumalanga and Limpopo in 1997. However, resistance to SP resistance reached approximately 80% in 2000 and treatments with Coartem were subsequently implemented (Moonasar et al., 2012).

The cross-border malaria strategy is used to monitor malaria transmission along the northern and eastern borders of South Africa (Moonasar et al., 2012). The Trans-Limpopo Malaria Initiative (TLMI) is an initiative between Zimbabwe and the Limpopo Province of South Africa aimed at reducing malaria transmission between the two countries. The main strategy has been to ensure policy harmonization and the synchronization of malaria interventions (Moonasar et al. 2012). The Lubombo Spatial Development Initiative (LSDI) was similarly a joint program between Mozambique, Swaziland and South Africa (Lubombo Spatial Development Initiative, 2010).This initiative was aimed at the development of the Lubombo region of eastern Swaziland, southern Mozambique and the north-eastern region of the KwaZulu-Natal province of South Africa into a globally competitive economic zone. The reduction of malaria in these areas should result in an increase in tourism, thus resulting in economic development. A secondary effect of this initiative is a decrease in malaria transmission in the border areas of South Africa (Moonasar et al., 2012).

According to Moonasar et al. (2013), there were several gaps identified in the South African Malaria Control Programme and these include the following: active surveillance in response to confirmed cases and treatment of identified cases, to interrupt local transmission; sensitive diagnostic tests to detect low level parasitaemias; maintaining a high level of malaria awareness by communities and health workers as malaria prevalence decreases; monitoring parasite drug and

vector resistance, as these risks increases when malaria case numbers decrease; supporting malaria control measures in neighboring countries more effectively and; sustained funding in the face of reduced case numbers.

1.9 Influence of Climate on Transmission

The climatic conditions of an area play a vital role in the transmission of malaria due to the influence of climate on mosquito development. Rainfall is largely responsible for the creation of breeding sites for mosquitoes. Temperature regulates the rate of mosquito larval development and influences the survival of adult mosquitoes (Grover-Kopec et al., 2006). Lyons et al. (2013) reported that low temperatures reduce larval development and adult activity of some *Anopheles* species, while extremely high temperatures cause excessive mortality. However, within the intermediate temperature range, there is a positive correlation between an increase in temperature and larval development rate, adult feeding rate and adult survival (Lyons et al., 2013). Humidity also affects the survival of malaria mosquitoes and when it is consistently less than 60%, mosquitoes will generally not live long enough to complete the transmission cycle (Grover-Kopec et al., 2006).

In endemic regions, these climatic variables create favourable conditions which support mosquito development and thereby increase malaria transmission. It is therefore essential that vector management strategies are in place to reduce transmission rates.

1.10 Integrated Vector Management

Malaria control strategies are of critical importance and include prevention as well as treatment in order to reduce mortality and morbidity. Mharakurwa et al. (2011) reported a decline in malaria cases in southern Africa, which was attributed to increased efforts by national malaria control programs as well as interventions funded under the Roll Back Malaria Partnership, the President's Malaria Initiative and other public-private organizations. The Roll Back Malaria (RBM) initiative was launched in Zambia in 1998 and focused on the promotion of insecticide-treated nets as the main, and in most cases the only, preventative measure (Chanda et al., 2008). However, there were many health system challenges that constrained the implementation of effective malaria vector control and a new approach was needed; consequently, since 2001, the WHO has been promoting integrated vector control (IVM). IVM is defined as the targeted use of different vector control

methods, alone or in combination, to prevent or reduce human-vector contact in a cost-effective manner, while addressing sustainability issues (Chanda et al., 2008).

According to Beier et al. (2008), IVM is not a new concept and the operations involved in IVM are designed to protect people from nuisance-biting and vector species of mosquitoes and are guided by the several principles. Adult vector populations and pathogen transmission should be reduced by interventions that are ecologically, environmentally, socially, economically and politically acceptable. Management strategies should not create negative side effects such as environmental contamination, the development of resistance or adverse impacts on non-target organisms. These strategies require an understanding of the transmission cycle, the life history of the vector species and the natural factors regulating vector survivorship, in order to develop descriptive and predictive models for vector population dynamics and transmission potential. Strategies should be dynamic and flexible by being able to respond to data from active and sensitive mosquito/pathogen surveillance programmes.

An IVM approach offers a variety of vector control methods, which can be applied in many combinations to suit different ecological and socio-economic settings (Mutero et al., 2012). It is also possible to target vectors at different stages in their life cycle, for example as larvae or pupae at the mosquito breeding habitats or at certain times during the host seeking and resting behaviour of adult mosquitoes (Mutero et al., 2012). The main vector control interventions in place in Africa are indoor residual spraying (IRS) and the prevention of bites using long-lasting insecticide-treated nets (LLINs). Other basic control measures utilized at a community level include environmental management, larval control using chemicals, and biological control. These form part of a comprehensive integrated vector control strategy (Walker, 2002, WHO, 2012).

Beier et al. (2008) firstly recommended strengthening the capacity building for IVM at national levels, improving the scope and quality of regional IVM training initiatives and supporting post-graduate education. A second recommendation was to promote interdisciplinary integration and inter-sectoral cooperation, by engaging appropriate stake-holders at the national level, including community groups and NGOs, and engaging experts from outside traditional entomological and public health frameworks for vector-borne disease control.

1.11 Innovative New Technologies

Due to the limitations of current vector control methods, new and alternative methods have been sought. Limitations include the cost of insecticides which is prohibitive in some transmission settings, legitimate environmental and human health concerns about the use of older insecticides (e.g. DDT), the limited number of insecticides available for use in public health situations and insecticide resistance (Hemingway et al., 2006).

1.11.1 Biological control

Interest in biological control as an alternative approach has heightened with increases in insecticide resistance and drug resistance. The WHO report of 2012 stated that insecticide resistance had been detected in 64 countries, affecting all major vector species and all classes of insecticide. Biological control of malaria vectors has considered the use of entomopathogenic fungi, bacterial agents, larvivorous fish and other agents like parasites, viruses and nematodes (Kamareddine, 2012).

The use of entomopathogenic fungi formulated as bio-pesticides have recently received a significant amount of attention (Blanford et al., 2012). Fungal species belonging to the genera *Coelomomyces*, *Culicinomyces*, *Beauveria*, *Metarhizium*, *Lagenidium* and *Entomophthora* have been considered for malaria vector control (Kamareddine, 2012). Fungi do not require host ingestion, since external contact with the insect's cuticle is sufficient to cause an infection. Fungal spores can be applied to various surfaces and substrates such as outdoor-attracting odour traps, indoor house surfaces, cotton pieces hanging from ceilings, bed nets and curtains and the fungal spores can persist for a several months on these surfaces (Blanford et al., 2012; Kamareddine, 2012). Fungal infections can act alone or in synergy with various insecticides and are effective on both insecticide-resistant and insecticide-susceptible mosquito vectors (Kamareddine, 2012). Fungal bio-pesticides are slower acting than conventional chemical insecticides, but fungal infections can cause substantial reductions in the transmission potential of a range of vector species (Blanford et al., 2012). Although their precise mechanism of action has not been clarified, many studies have indicated that fungi play a role in disrupting the nutritional balance of mosquitoes, elevating their immune responses, and/or resulting in the production of secondary metabolites in their haemolymph (Kamareddine, 2012). Even though entomopathogenic fungi appear promising there are concerns about the development of certain fungal resistant *Anopheles* strains

(Kamareddine, 2012). Also, the efficacy of microbial products depends on several factors, such as product stability, the storage potential of spores after production and the persistence of spores after application. Studies indicate that the particular strain of fungi, the production conditions, temperature, humidity and spore moisture content can all influence fungal viability during long-term storage, and thus their field efficacy (Blanford et al., 2012).

Over the past decade, bacterial-based larvicides, known as biocides or bio-larvicides have become more popular (Poopathi and Tyagi, 2006). *Bacillus thuringiensis* var. *israelensis* (*Bti*) and *Bacillus sphaericus* (*Bs*) have proved to be highly effective for the control of mosquito larvae, as well as some other dipterans (Mittal, 2003). Both *Bti* and *Bs* form spores which produce a parasporal crystal which is toxic to some invertebrates, mostly insects and nematodes (Poopathi and Tyagi, 2006). The major advantages of bio-larvicides are the reduction in application costs and their safety to the environment, human beings, animals and other non-target organisms (Poopathi and Tyagi, 2006). When the spore crystal of *Bti* containing toxic proteins is ingested by larvae of a susceptible species, the pro-toxins are solubilized in the alkaline pH of the larval gut and are activated (Mittal, 2003). The main target of these activated toxins is the plasma membrane of the mid-gut epithelium. The interaction between the activated toxin and the receptors of the plasma membrane lead to the disruption of membrane integrity and cytolysis (Mittal, 2003). The insecticidal protein of *Bs* is located in the spore wall. The mode of action of *Bs* is similar to that of *Bti*, which is through the larval gut. The activated protein toxin binds to the cells of the gastric caecum and posterior mid-gut (Mittal, 2003). The efficacy of *Bti* and *Bs* larvicides depends on the formulation that is suited to the biology and habitat of the target mosquito vector species (Mittal, 2003). The importance of using low-dosage formulations is recognized since it keeps operational costs low, especially if applications are on a weekly basis (Kamareddine, 2012).Although only a few studies have tested the effect of *Bti* and *Bs* on African malaria vectors, the results have been promising, but have highlighted the need for additional work (Kamareddine, 2012).

Certain protozoan (microsporidian) parasites such as *Vavraia culicis* and *Edhazardia aedis* can be used to terminate the development of other parasite species like *Plasmodium* or to target the mosquito vector itself (Kamareddine, 2012). The effectiveness of these parasites lies in their ability to exert combined effects on several important epidemiological traits of the mosquito. These microsporidian parasites moderately decrease larval survival rates which in turn decreases the

population densities of adult mosquitoes. The parasites also moderately affect adult mosquito longevity, the development of the malaria parasite in the mosquito and the biting rates of the mosquito (Kamareddine, 2012).

Riehle et al. (2007) suggested the use of a para-transgenic approach to control malaria, which utilizes a microbial organism capable of colonizing the mosquito's mid-gut to produce effector molecules that kill or inhibit the development of the *Plasmodium* parasite. A suitable microbial candidate should be readily propagated and stably engineered to express certain genes of interest, without imposing fitness costs on the mosquito. It should also exhibit a parasitic, commensal, or mutualistic relationship with its host and should be easily transported into the wild mosquito populations (Kamareddine, 2012).The engineered microbe should also have the ability to be sustained in the host's microenvironment with minimal or negative impacts on non-target species (Kamareddine, 2012). The densonucleosis viruses or "denso viruses" (DNVs), which belong to the Parvoviridae family and infect arthropods including mosquitoes, fulfill these requirements. The *A. gambiae* denso virus is highly infectious to *Anopheles* species at the larval stages, but is able to circulate in adult mosquito tissues and undergo vertical transmission from mother to offspring (Kamareddine, 2012). The importance of these viruses lies in their ability to transduce certain anti-*Plasmodium* genes or *Anopheles*-specific toxins in mosquito cells (Kamareddine, 2012).

Parasitic nematodes in the family Mermithidae have been used as biological agents for malaria control (Kamareddine, 2012). Approximately 25 species infect the larval stages of different mosquito species, but very little is known about their effects on adult mosquitoes. These nematodes interfere with the reproductive system of the host species and can reduce mosquito populations, which will in turn decrease disease transmission rates (Kamareddine, 2012).

The use of larvivorous fish is an older suggested method for malaria control (Kamareddine, 2012).The advantages of this method are that it can be used in low doses, is harmless to humans, cheap to implement and has minimal risks of mosquitoes developing resistance (Kamareddine, 2012). However, over time, the introduction of new fish species into aquatic environments has exerted negative impacts on the native invertebrate faunas. Therefore, pre-application studies are required which include the determination of the amount of larvae eaten by fish in different water bodies and the appropriate conditions of the aquatic environment where new fish species are introduced (Kamareddine, 2012). Fish are highly effective when the vegetation does not interfere

with their feeding habits and when mosquito breeding sites are restricted in number and are well defined (Kamareddine, 2012).

1.11.2 The Sterile Insect Technique

The Sterile Insect Technique (SIT) is a form of genetic control; the fundamental principle of which is to utilize factors that hinder transmission of the parasite or lead to reproductive failure of the vector (Knipling et al., 1968; Windbichler et al., 2012). The principle of SIT is to induce sterility in the pest populations, by rearing large numbers of males of the target pest (*An .arabiensis* in this study), reproductively sterilizing them using radiation and releasing them into the wild. When the sterile males mate with the wild females, the females are unable to produce viable offspring (Knipling, 1959; Parker and Mehta, 2007; Helinski et al., 2008, 2009; Oliva et al., 2011). SIT is species-specific, environmentally friendly and does not have negative impacts on human health. However, since it is species-specific, only one vector species can be controlled at a time (Wilke et al., 2009; Alphey et al., 2010). SIT has been used to control the New World screwworm fly (*Cochliomyia hominivorax* (Coquerel)) in the USA, Mexico and Central America, tsetse flies (*Glossina austeni* (Newstead)) in Zanzibar and Mediterranean fruit flies in the Hex River Valley region of South Africa (Papathanos et al., 2009; White et al., 2010; Munhenga et al., 2011).

Benedict and Robinson (2003) reported that most SIT initiatives against mosquitoes have been directed at answering a specific research question. The first major success was achieved against *Culex quinquefasciatus* Say in Myanmar and this species was also successfully eliminated on an island off Florida in the United States of America (Benedict and Robinson, 2003; Wilke et al., 2009). A joint approach between the WHO and the Indian Council of Medical Research was directed against *Ae. aegypti* (Linnaeus), *Cx. quinquefasciatus* and *An. stephensi* Liston in India. Methods in sex sorting, packaging, marking, transporting and distributing of adult males were developed. However, this project failed due to the unexpected migration of mated females into the control area, and the negative perceptions in the community that arose when media and politicians accused the scientists of carrying out research on biological warfare (Benedict and Robinson, 2003; Wilke et al., 2009). The elimination of *An. albimanus* Wiedemann in El Salvador is the first successful program against an anopheline mosquito species (Benedict and Robinson, 2003). However, when larger scale releases against the same vector were performed on the Pacific coast of El Salvador, population suppression was only possible once the release area was smaller than

originally planned and a sex-separation gene was introduced (Benedict and Robinson, 2003). According to Benedict and Robinson (2003), various technical factors contributed to the failure of mosquito releases and included: mosquito production below the desired levels, due to the absence of sexing strains or delays in production; loss of male fitness in the released males and; immigration of mated females into the release areas.

The challenges for using SIT in mosquito control include: (i) the production of high numbers of male mosquitoes for mass release; (ii) efficient gender sorting; (iii) an efficient method for the sterilization of large numbers of males with minimal effects on fitness; (iv) an effective distribution method for sterile males; (v) an efficient method to identify released individuals and; (vi) maintaining the competitiveness of the sterilized male mosquitoes (Wilke et al., 2009). In particular, laboratory culturing and mass rearing of insects for SIT can lead to a significant loss in physiological and reproductive fitness, as a consequence of severe reductions in genetic variation. Reduced fitness and sexual isolation as a consequence of culturing is an important consideration for the implementation of SIT (Munhenga et al., 2011). The success or failure of SIT programmes will depend largely on whether the sterile males can compete successfully with wild males for mates (Ng'habi et al., 2005). According to Dame et al. (2009), when the released males are not fully competitive, the numbers released must be increased substantially to compensate for this deficiency.

Curtis (1978) developed a genetic sexing strain (GSS) for *An. arabiensis* by translocating the semi-dominant autosomal gene for dieldrin resistance onto the Y chromosome, resulting in male resistance to the insecticide while females remained susceptible. Yamada et al. (2012) developed a new strain of male dieldrin-resistant *An. arabiensis* at the International Atomic Energy Agency (IAEA) in Austria, because the older strains no longer existed. Since malaria is transmitted via female mosquitoes, it must be possible to eliminate the females to achieve a successful SIT programme (Yamada et al., 2015). The genetic sexing strain ANO IPCL1 was developed by using two pure bred strains of *An. arabiensis*: the Sennar strain carrying the semi-dominant gene conferring resistance to dieldrin and the Dongola strain containing the dieldrin-susceptible allele (Yamada et al., 2015). The ANO IPCL1 strain showed no difference in life history characteristics apart from a 73% natural semi-sterility when compared to the Dongola strain of *An. arabiensis* (Yamada et al., 2015). Since the GSS strains are of Sudanese genetic backgrounds, if directly

released in a different geographical region besides Sudan, there might be challenges of mating compatibility and competitiveness between the GSS and the wild strains present in the release area (Dandalo et al., 2018).

The ecology and population biology of the target species throughout the proposed control area must also be well understood, since SIT will be more effective when population levels are low (Dame et al., 2009). However, excessive levels of sterility in released males could reduce their effectiveness, as a result of somatic cell damage due to exposure to radiation (Dame et al., 2009). Ionizing radiation could reduce male competitiveness when pupae are exposed to sterilizing doses. Relatively simple packaging, transport methodology, release containers and shelters have been devised for pupal and adult releases of sterilized males (Dame et al., 2009). Quality control has to be ensured for certain factors during laboratory rearing for SIT programs to be successful and these include the mean number of pupae produced in standard rearing containers, sex ratios, adult longevity and sexual aggressiveness of pre-and post-released males, eggs per colony per female, and blood and food quality (Dame et al., 2009).

The successful suppression of the target insect species using SIT also depends on the premise that the laboratory-reared males used for mass-rearing are genetically compatible with the wild target population (Munhenga et al., 2011). However, a drawback of SIT is the challenge of developing a laboratory strain which is both reproductively compatible and competitive with the target population. Colonization in the laboratory may also result in sexual isolation leading to sexual incompatibility (Munhenga et al., 2011). Therefore, fundamental knowledge about the fitness and sexual compatibility between the laboratory-reared colony that will be used for mass releases and the targeted wild population must be investigated as a means of assessing the feasibility of SIT (Munhenga et al., 2011). Munhenga et al (2011) found that the laboratory-reared *An. arabiensis* males had a greater longevity than the F1 progeny of the wild strain. Their study also indicated that there was mating compatibility between the laboratory-reared males and the F1 progeny of the wild strain.

1.12 Statement of the Problem

The theoretical basis for successful SIT is that the area containing the vector population is flooded with sterile males in order to decrease the mating success of the wild male population and

subsequently decrease the vector population (Benedict et al., 2003). The ability of released males to locate, copulate with and transfer sterile sperm to wild females is thus of great importance (Helinski et al., 2009). However, Howell and Knols (2009) reported a fundamental lack of knowledge with regard to the mating biology of mosquitoes, particularly the compatibility between laboratory-reared and wild individuals. These data are important in determining whether or not the insectary-reared male mosquitoes are likely to survive in the natural environment, locate females and successfully compete with wild males for female insemination.

1.13 Aims and Objectives of the Study

In theory, SIT is a potentially powerful control measure that could eradicate one of Africa's major malaria vectors, *An. arabiensis*. However, inappropriate extrapolation of laboratory results to the field could be the difference between success and failure. The mating ability and survival of the laboratory-bred mosquitoes, when released into the wild, is thus critical to this endeavour. Therefore, comparisons of fitness and compatibility between laboratory-bred male mosquitoes and wild-type mosquitoes are critical (Huho et al., 2007). The aim of the study was thus to investigate the robustness of laboratory-reared *An. arabiensis* mosquitoes for use in SIT applications for malaria control.

The specific objectives of this study included; (i) a comparison of the survival and fecundity of laboratory and wild strains of *An. arabiensis* (Chapter 2); (ii) an assessment of the effects of larval population density (in the context of mass-rearing) on adult size and the effects of adult size on mating success in *An. arabiensis* (Chapter 3) and; (iii) a determination of insecticide resistance in wild *An. arabiensis* populations in northern KwaZulu-Natal, South Africa and the transference of powdered dye (as a mating marker) between male and female mosquitoes during copulation.

CHAPTER 2: Comparison of the survival and fecundity of laboratory and wild strains of *Anopheles arabiensis*

2.1 Introduction

Malaria remains a global health threat (Palmer et al., 2003). According to the World Health Organization (WHO) Malaria Report of 2015, an estimated 212 million malaria cases occurred globally in 2015, with 90% having occurred in the WHO African Region. In 2015, 92% of malaria deaths occurred in the WHO African Region with an estimated 303000 malaria deaths in children under the age of five (WHO, 2016). Malaria was the fourth highest cause of death, accounting for 10% of child deaths in sub-Saharan Africa (WHO, 2015). Although South Africa lies at the southernmost tip of the continent, malaria still remains a significant disease of public health importance. There are three malaria endemic provinces in South Africa namely Limpopo, Mpumalanga and KwaZulu-Natal with an estimated 10% of the country's population living in these regions who are at risk of contracting the disease (Hlongwana et al., 2011; Moonasar et al., 2012).

Implementation of control measures is of critical importance in eradicating malaria. The main control interventions in place globally, are indoor spraying with residual insecticides (IRS) and the prevention of bites using long-lasting insecticide-treated nets (LLINs). Other basic control measures utilized at a community level include the use of larvicides, larval source management and environmental management (Walker and Lynch, 2007; Worrall and Fillinger, 2011; Maheu-Giroux and Castro 2013). The larval aquatic habitat has proven challenging for larviciding as it is numerous, widespread and temporary. This is compounded by the fact that the identification and targeting of the most productive sites is very difficult (Devine and Killeen, 2010). Larval-source management is considered as a secondary control strategy. This is because this strategy is labour intensive, requiring managerial support and is beyond the financial capabilities of most malaria endemic regions (Worrall and Fillinger, 2011; Maheu-Giroux and Castro 2013). Environmental management is the modification of vector habitats in order to hinder larval development and/or human vector contact (Walker and Lynch, 2007).

Southern Africa relies mainly on the use of insecticides and IRS for malaria vector control together with timely diagnosis and effective treatment with artemisinin-based combination therapy (ACT)

to control the parasite. Although these strategies have resulted in reductions of malaria cases and a 99% decline of malaria incidence in South Africa, the transmission of malaria has not ceased (Muhenga et al., 2011; Maharaj et al., 2012; Blumberg et al., 2014). This can be attributed to the emergence of insecticide and drug resistance which has also led to a heightened interest in alternative control methods (White et al., 2010; Munhenga et al., 2011). According to Blumberg et al. (2014), the occurrence of insecticide resistance is relatively recent occurrence. Insecticide resistance coupled with environmental and health concerns of insecticide use has led to the development of alternative vector control methods and the implementation of integrated vector control strategies (Blumberg et al., 2014). Genetic control of the target vector, based on the reproductive failure of wild populations (Knipling et al., 1968), is a promising alternative strategy.

The sterile insect technique (SIT), a means of genetic control of mosquito populations, is focused on causing sterility in wild populations by rearing large numbers of males, reproductively sterilizing them using radiation and releasing them into the target area. When the sterile males mate with the wild females, the females are unable to produce viable offspring (Knipling, 1959; Parker and Mehta, 2007; Helinski et al., 2008; 2009). Yamada et al. (2012) state that the use of SIT for mosquito control is still in its infancy and many of the fundamental components of the technique still need to be developed, validated and optimized. These include aspects of mass rearing of the target vectors, the quality of the sterilized males produced and the methods of handling, transporting and releasing the sterile insects within the target area. It is essential that female mosquitoes are not part of the released material because they compromise the disease vectors and even sterile females could theoretically transmit the parasite. Also, the sterilized males could copulate with the released females instead of the wild females thus reducing the efficacy of the program (Alphey et al., 2010).

For a SIT program to be successful, the mass-released males, which originate from a laboratory culture (strain), have to be able to compete successfully with the wild male population for female insemination. Laboratory culturing of insects can result in significant genetic divergence between the cultured and wild populations (Munhenga et al., 2011). Loss of physiological and reproductive fitness, caused by laboratory culturing, could be due to a lack of genetic variation, bottlenecking and uniformity of the laboratory rearing environment (Munhenga et al., 2011). The mating ability and survival of the laboratory-bred mosquitoes are critical for SIT to succeed; therefore,

verifications of fitness and compatibility between laboratory-bred male and wild type mosquitoes are critical (Huho et al., 2007). Howell and Knols (2009) reported a fundamental lack of knowledge with regard to the mating biology of mosquitoes. Therefore, the aim of this study was to compare the mating success between laboratory-bred and wild strains of *Anopheles arabiensis* Patton to determine if culturing does hinder mating success. As part of this study, adult and larval *Anopheles* populations were sampled monthly in northern KwaZulu-Natal during 2012 to determine their seasonal abundance.

2.2 Materials and Methods

2.2.1 Mosquito collections

Field collections of malaria vectors belonging to the *Anopheles gambiae* complex were conducted monthly over a 12-month period in the Mamfene area (Figure 2.1A) of northern KwaZulu-Natal (27°19'60S, 32°13'00E) during 2012. A total of 10 window traps (Figure 2.1B) were placed in sections 8 and 9 of the Mamfene area. Mosquitoes were collected from these window traps every morning between 6am and 7am. Night collections of mosquitoes were also carried out, using the human landing catches sampling method, between 7 pm and 10 pm, at the locations where most mosquitoes were collected that morning. Collected mosquitoes were placed in individual breeding tubes and transported to the insectary of the Malaria Research Unit at the Medical Research Council (MRC) in Durban.

Larval collections were also carried out monthly all around the Mamfene area over a 12-month period during 2012. Larvae were located in water-filled animal hoof prints (Figure 2.1C), in puddles along the Pongola River and in puddles formed in vehicle tyre tracks. Collected larvae were placed in distilled water (dH_2O) and transported to the MRC insectary.

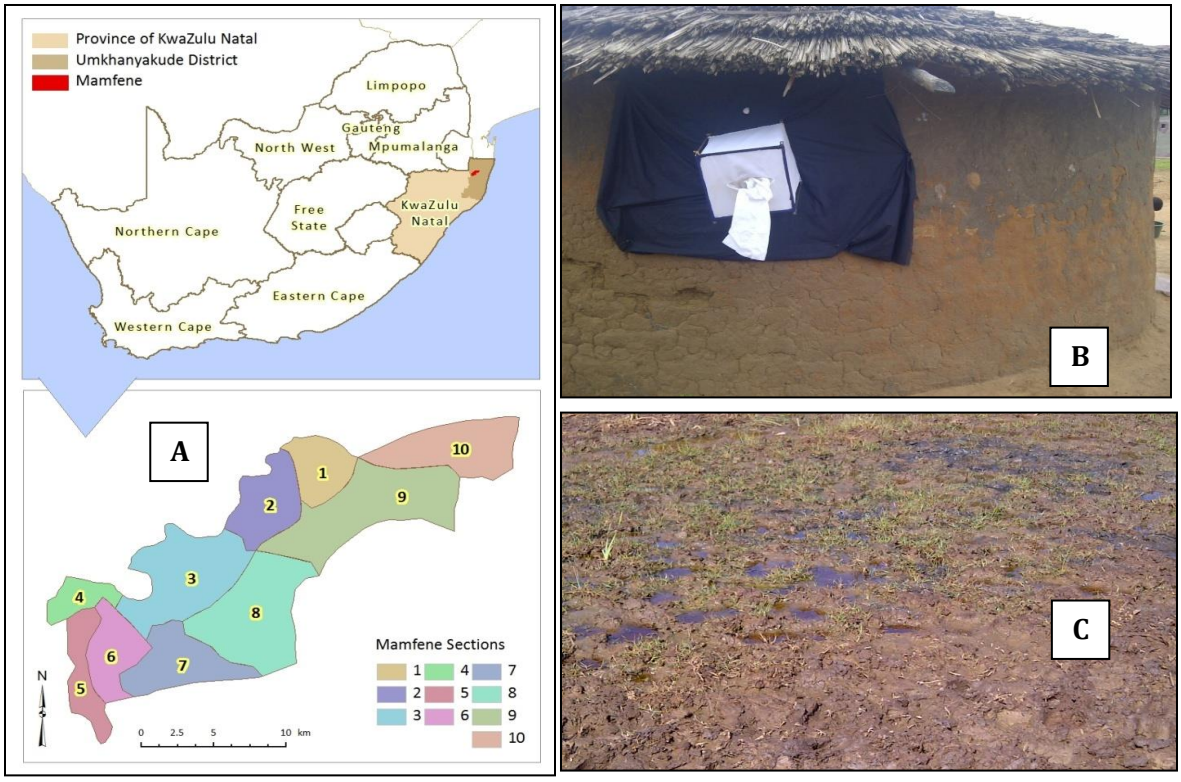

Figure 2.1: A) Map of the Mamfene area in northern KwaZulu-Natal; B) Window trap installed on a mud home; C) larvae breeding sites in animal hoof prints**.**

Field-collected female mosquitoes were placed into individual breeding tubes to allow oviposition. The eggs laid by field-collected females were transferred to dH_2O to allow hatching and development to adulthood. Eggs from each female mosquito were maintained as separate family groups until confirmatory identification was done using the DNA extraction method of Collins et al. (1987) and polymerase chain reaction (PCR) method of Paskewitz and Collins (1990). Once it was determined that the mosquitoes were *An. arabiensis*, the family groups were pooled together into one colony. The progeny of the wild type mosquitoes (designated as the New Mamfene strain) were reared in the insectary at a temperature of 27°C and a humidity of 70%. Adult mosquitoes were fed with a 10% sucrose solution, females were blood-fed using guinea pigs and larvae were fed with a diet consisting of powdered dog food (Purina Alpo® manufactured by Nestle Purina Petcare).

2.2.2 Mosquito colonies

The three colonies (strains) used during this study included the New Mamfene Strain, recently established from the wild colony (F8), and two laboratory strains, namely the Old Mamfene Strain, which is the MRC laboratory strain (F300), and the Genetic Sexing Strain GSS (F20). The GSS used in this study was created by the International Atomic Energy Agency (IAEA) in Austria (Yamada et al., 2012).

All three colonies were maintained in the MRC insectary under the same conditions (27°C and 70% RH) and adult mosquitoes were fed the same diet (10% sucrose solution and the blood of guinea pigs). The larvae were maintained in dH_2O and fed a diet of powdered dog food. Genetic sex separation for *An. arabiensis* was accomplished using the method of Curtis (1978), whereby the males in the selected strain became resistant to dieldrin exposure while the females remained sensitive. This allowed easy separation of males and females at the larval stage.

2.2.3 Larval survival and mating compatibility of the three *Anopheles arabiensis* strains

A population of 300 first instar larvae was placed into 2ℓ containers with 500ml dH_2O and fed 0.2mg of food per larva (i.e. 60mg per container) and this was adjusted accordingly as the larvae developed from first instars to fourth instars. Three replicates of this procedure were carried out for each strain. Larval survival to pupation was monitored and dead larvae were counted and removed on a daily basis. The resultant pupae were counted and separated into another container. Newly emerged adults were recorded and separated according to sex.

Once enough adults had emerged from pupation, a collective of 70 males and 50 females of each strain were placed together in a cage and allowed to mate for a 7-day period. A ratio of 7:5 males to females was used to ensure mating success. A 10% sucrose solution was made available to the mosquitoes during mating. At the end of the 7-day mating period, the females were given three blood meals, three days apart, using guinea pigs and thereafter 30 randomly selected females from each strain were placed individually into oviposition tubes to allow them to lay eggs.

The eggs laid by each female were recorded and placed into dH_2O. The larvae that hatched from each batch of eggs laid were counted, and the spermathecae of females that did not lay eggs were dissected to determine if mating had occurred.

2.2.4 Statistical analysis

The Stata Intercooled version 11.0 software (Stata Corporation, College Station, Texas) was used to statistically analyze the data obtained from the study. A Pearson's Chi-squared test was used to determine whether there were differences in the survival of the larvae and pupae to adult emergence between the three strains. Differences between the strains regarding the mean numbers of eggs laid and larval hatch were analyzed using One-way ANOVA. Significance was assessed at the $P < 0.05$ level. Following the ANOVA, t-tests were used for paired comparisons between the strains in order to determine which were significantly different from each other. No adjustments were made for multiple comparisons as these were pre-specified in the analysis plan.

2.3 Results

2.3.1 Species identification and seasonal abundance

Figure 2.2 indicates the total number of all *Anopheles* mosquitoes collected monthly in the study area throughout 2012. Although this study focused on *An. arabiensis*, other species in the *An. gambiae* complex, namely *An. merus* and *An. quadriannulatus*, were also collected from the same area but in very low numbers. *Anopheles arabiensis* was the most abundant of the three species in this complex (Figure 2.2). The "unknown" mosquitoes, which were sometimes more abundant than *An. arabiensis* and comprised most of the total mosquito catch (Figure 2.2), were anophelines that are not members of the *An. gambiae* species complex. The highest numbers of *Anopheles* mosquitoes were recorded in mid-summer (February) with later peaks in June (early winter) and December (early summer). Populations of *An. arabiensis* similarly displayed peaks in February, May and December, with no catches recorded in the March-April and June-September months of 2012. Thereafter, the numbers of *An. arabiensis* displayed a gradual increase from October to December of 2012 which, given the earlier trend (Figure 2.2), would likely have been sustained into the January-February period of 2013.

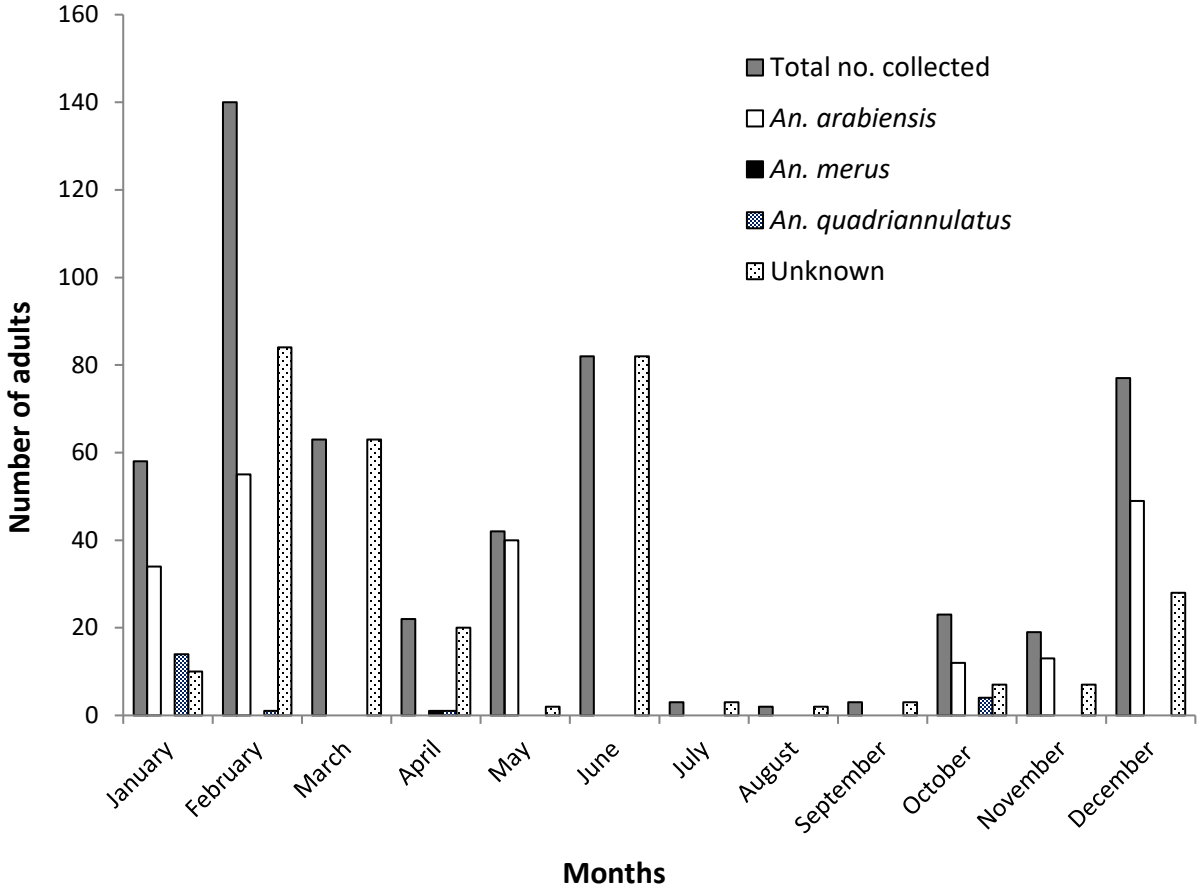

Figure 2.2: The total number of *Anopheles* mosquitoes collected in the study area throughout 2012.

Figure 2.3 indicates the total number of larvae of all *Anopheles* species that were collected between January and December 2012 in the Mamfene area. The highest numbers of larvae were collected in January, after which there was a gradual decrease in larval populations. No larvae were recovered during October because of high temperatures which caused the breeding sites to dry out. November and April yielded very small larval populations. Further investigations by entomologists at the Jozini Department of Health indicated that in April the sluice gates of the Jozini Dam were opened, resulting in the flooding of the main breeding sites and the larvae being washed away.

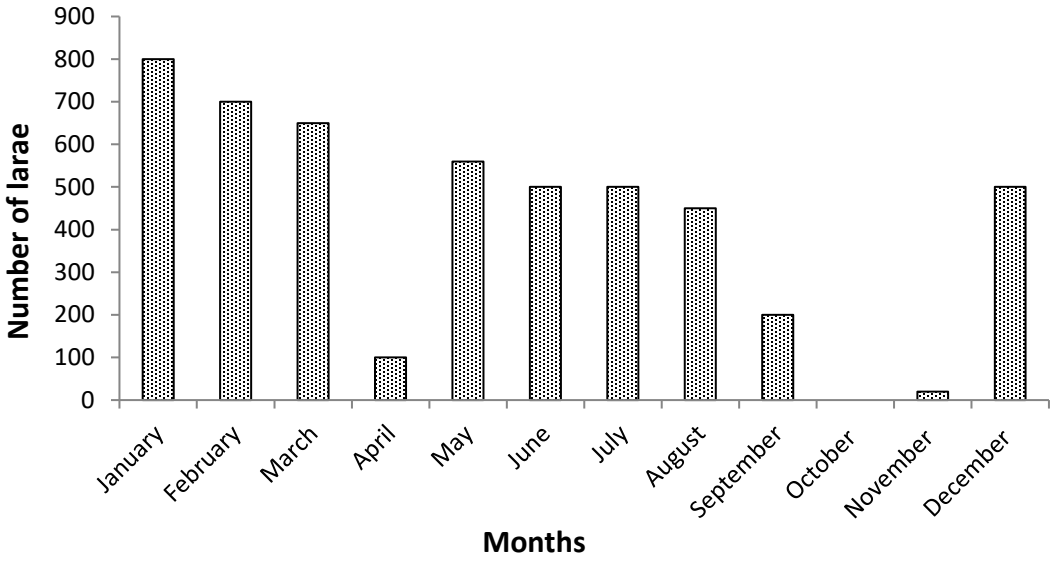

Figure 2.3: Total number of larvae of all *Anopheles* mosquitoes collected monthly in the study area during 2012.

2.3.2 Larval survival and mating success

The Old Mamfene strain displayed the highest percentage larval survivorship to pupation (72.7%) relative to the New Mamfene (39.7%) and GSS (43.4%) strains (Figure 2.4). There were significant differences in larval survivorship between the Old Mamfene and both the New Mamfene and GSS strains ($P < 0.001$) but no significant differences between the New Mamfene and GSS strains ($P > 0.05$).

The New Mamfene strain displayed the lowest percentage pupal survivorship to adulthood (67.2%) relative to the GSS (82.1%) and Old Mamfene (75.1%) strains (Figure 2.4). There were significant differences in pupal survivorship between all three strains of *An. arabiensis* (Old Mamfene vs. New Mamfene; $P < 0.01$, Old Mamfene vs. GSS; $P < 0.01$ and New Mamfene vs. GSS; $P < 0.001$).

The Old Mamfene strain supported the highest adult emergence with a female emergence of 52.1% and a male emergence of 47.9%, while the New Mamfene strain had a female emergence of 41.7% and a male emergence of 58.3% and the GSS had a female emergence of 56.4% and a male emergence of 43.6% (Figure 2.4). The survival of male and female larvae to emergence was similar within the Old Mamfene strain; however, the New Mamfene strain had a greater male survival of

larvae to emergence and the GSS had a greater female survival of larvae to emergence (Figure 2.4). A significant difference was recorded for adult emergence between the Old Mamfene and New Mamfene strains ($P < 0.01$) and between the New Mamfene and GSS strains ($P < 0.001$).

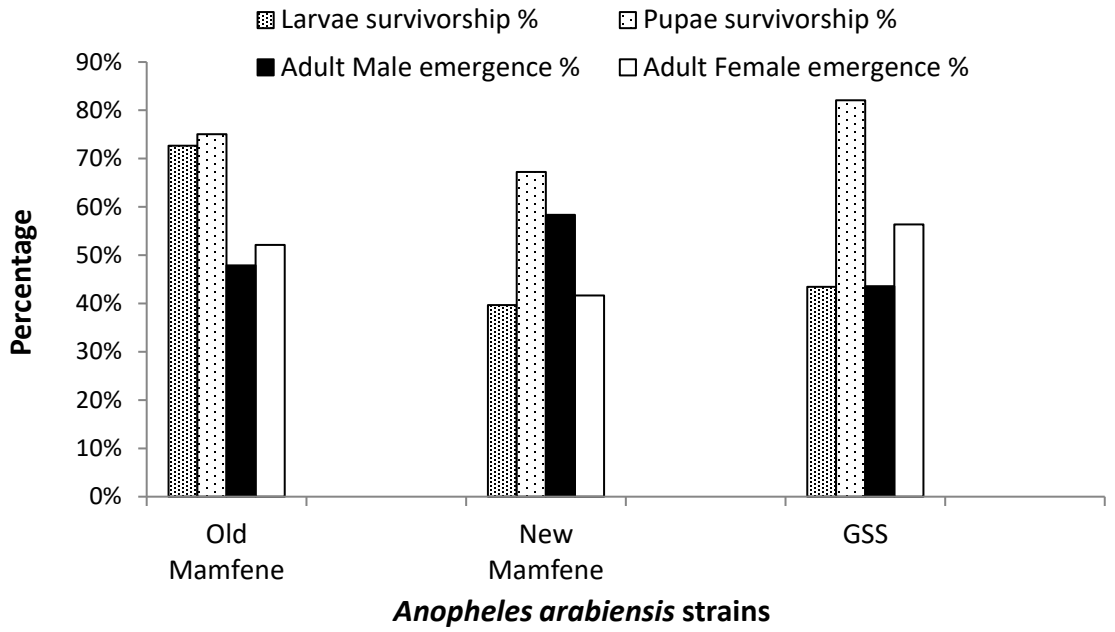

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Figure 2.4: Percentage survival of larvae and pupae and emergence of adult males and females of the three strains of *An. arabiensis.*

The fecundity data indicated that viable mating and insemination occurred in all three *An. arabiensis* strains (Figure 2.5). The viability of the eggs was determined by the numbers of first instar larvae that hatched from the eggs.

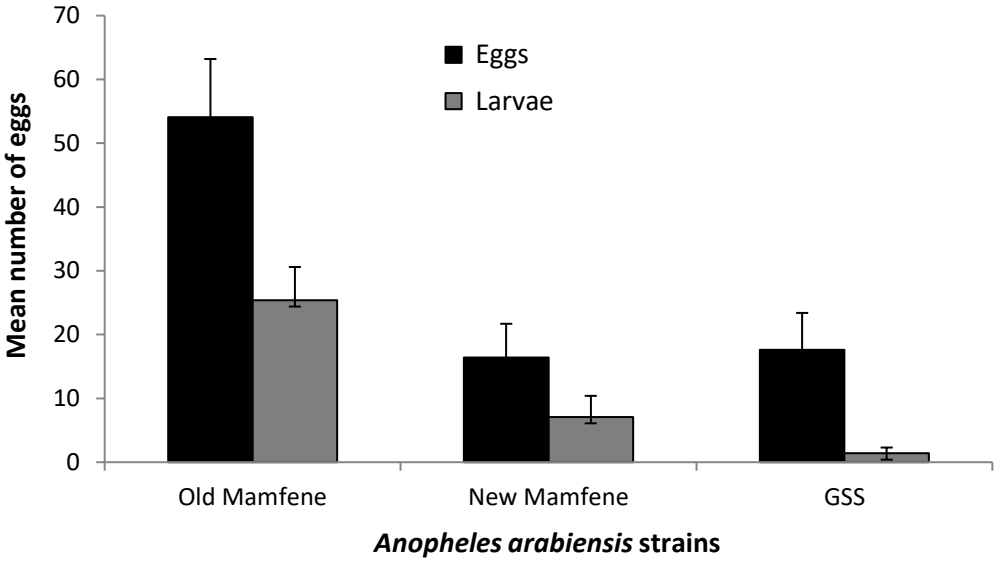

Figure 2.5: Mean numbers of eggs laid per female mosquito and larvae that hatched, for each of the three strains of *An. arabiensis*, with 95% confidence intervals.

The Old Mamfene strain displayed the highest fecundity of the three strains, with the highest mean number of eggs laid per female (54.1) compared to the New Mamfene (16.4) and GSS (17.6) strains, where fecundity was considerably lower (Figure 2.5). There were significant differences in the mean female fecundity between the Old Mamfene and New Mamfene strains ($P < 0.001$) and between the Old Mamfene and GSS strains ($P < 0.01$).

The Old Mamfene strain also displayed the highest egg viability of the three strains, with the highest mean number of larvae hatched per female (25.4) compared to the New Mamfene (7.1) and GSS (1.5) strains, where egg viability was considerably lower (Figure 2.5). There were significant differences in egg viability between the Old Mamfene and New Mamfene strains ($P < 0.01$), and between the Old Mamfene and GSS strains ($P < 0.001$).

The highest percentage of eggs that hatched was recorded in the Old Mamfene strain (46.9%) which was slightly higher than that of the New Mamfene strain (43.3%), while the GSS had the lowest hatch rate of only 8.5% (Figure 2.6).

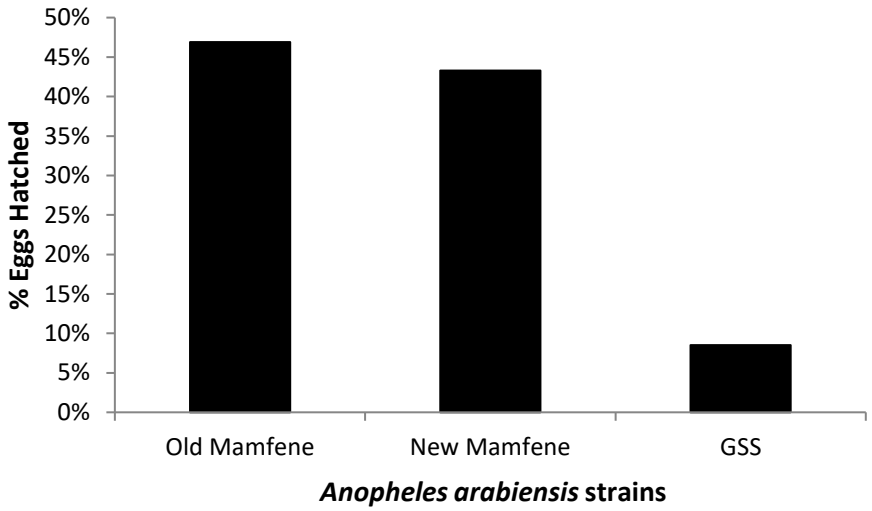

Figure 2.6: Percentage of eggs that hatched for each of the three *Anopheles arabiensis* strains.

The percentages of females of the three strains that were inseminated were determined by those that laid eggs and by dissection of the spermathecae of those that did not lay eggs.

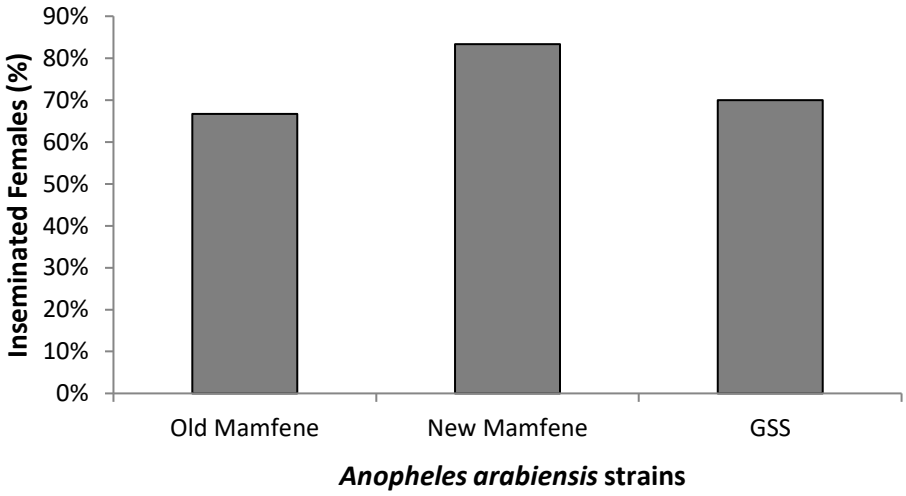

Figure 2.7: Percentage of females (n = 30) of the three strains of *Anopheles arabiensis* that were inseminated during the trials.

Although the New Mamfene strain displayed the highest insemination rate of 83%, which was higher than that of the GSS (70%) and the Old Mamfene (67%) strains (Figure 2.7), the differences between the three strains were not significant of ($P > 0.05$).

2.4 Discussion

The study was carried out in Mamfene, northern KwaZulu-Natal, which is a high risk malaria area. According to the Jozini Local Municipality 2012/13 to 2016/17 Integrated Development Plan, this area is characterized by seasonal dry winters and wet summers with periodic flooding. The document highlights climatic conditions that are conducive to malaria transmission, namely the hot summer temperatures that range from 23°C to 40°C and the relatively warm winter temperatures that range from 16°C to 25°C.

The survey of mosquito species in the area (Figure 2.2) indicated that *An. arabiensis* is the main vector for malaria transmission. During the colder and drier months, the adult mosquito populations drop while during the wet summer months they are much higher. The larval collections (Figure 2.3) indicated higher larval recoveries during summer than in winter, which is presumably due to the limited rainfall (and therefore lack of breeding sites) in winter. April 2012 reflected lower than expected larval numbers because of the flushing away of larval breeding sites in the study area. October and November 2012 also showed low larval numbers; this was presumably because of abnormally higher temperatures that caused the drying out of breeding sites. However, during October and November 2012 the adult populations of *An. arabiensis* began to increase (Figure 2.2), indicating a seasonal trend in the area. This seasonal change in the population density of *An. arabiensis* is an important consideration for a successful SIT programme, because the best time to mass-release sterile males is when the target population is at its lowest and the ratio between the sterile and wild males can be best exploited (Munhenga et al., 2011).

SIT depends on the released material being compatible with the target population and the mating competitiveness of the released males being, at the very least, comparable to that of the wild population (Benedict and Robinson, 2003; Oliva et al., 2011). The data indicated that larval survival to adulthood (Figure 2.4) and fecundity (Figure 2.5) was better in the Old Mamfene strain (laboratory-reared) than in the New Mamfene (wild colony) and GSS (genetically modified) strains during the laboratory trials. Another prerequisite for SIT in relation to malaria control is

that females are eliminated from the released material as the females transmit the disease (Yamada et al., 2015). The GSS strain of *An. arabiensis* has been genetically modified to ensure that males exhibit dieldrin resistance while females remain susceptible, thereby providing a mechanism to eliminate females (Yamada et al., 2015. Despite the high insemination rate, the GSS strain displayed a low egg production and the lowest hatch rate because this strain is semi-sterile due to the genetic modification (Yamada et al., 2015). Since SIT is directed at causing a decrease in the target species' population, this might be seen as advantageous. However, SIT requires the production and release of males in large numbers and therefore the Old Mamfene would be the most suitable strain to achieve this. A low fecundity would be a practical constraint.

There were no statistical differences between the three strains in relation to insemination rates which indicated that mating had occurred without major difficulties. However, given the significantly higher fecundity and fertility of the Old Mamfene strain, it is possible that the New Mamfene and GSS strains are less adapted to the laboratory conditions in the MRC insectary and the diet provided. Huho et al. (2007) reported that insect development and demography is strongly regulated by climate and other environmental variables and can vary significantly in response to considerable differences in diets. The differences in larval survival and adult reproductive fitness between the three strains could be a result of laboratory culturing. Strains that have been cultured for long periods (Old Mamfene) have become adapted to laboratory conditions, whereas their wild counterparts (New Mamfene) are adapted to harsher and more variable conditions. Culturing can alter the mating behaviour of laboratory-reared mosquitoes and generate selection for assortative mating traits, thereby increasing genetic relatedness within the laboratory population (Huho et al., 2007). Therefore, field studies should be carried out in order to fully grasp the mating compatibility between the wild mosquitoes and the laboratory-reared mosquitoes.

This study indicated that the Old Mamfene strain displayed the highest larval and adult fitness and suggested that it is the most suitable strain from which sterile males of *An. arabiensis* could be mass-reared for field releases. However, the study was conducted in a laboratory setting with controlled variables such as temperature, humidity and diet. The accuracy with which these laboratory results could be extrapolated to the field, particularly in relation to the mating competiveness of the laboratory-reared mosquitoes in the wild, is thus uncertain (Huho et al., 2007). Also, SIT requires males to be mass-released in the wild, where there are several climatic

and environmental variables which are largely unpredictable and cannot be controlled. Therefore, a similar study should be done in the field or at the study site which could provide a more accurate estimate of mating competitiveness between the laboratory-reared and wild mosquitoes.

According to the results of this study (Figures 2.4 to 2.7) laboratory culturing does not hinder mating success (i.e. insemination of females). The laboratory-bred (Old Mamfene) mosquitoes can perform successfully when compared to their wild counterparts (New Mamfene) and the genetically modified GSS under laboratory conditions. However, Benedict et al. (2009) warned that prolonged culturing may result in homogenous colonies that are very different from wild populations and display reduced competitiveness. SIT relies on the mass production as well as the mass release of sterilized males that are able to survive and be reproductively competitive in the field. Since the Old Mamfene strain displayed the highest larval and adult fitness, the GSS gene could potentially be introduced into the Old Mamfene population, thereby forming a hybrid strain where males are easily differentiated from females.

In order to ensure a successful SIT program, the mosquito colonies from which the sterile males are propagated have to be managed properly. Genetic variation should be added to the laboratory-cultured mosquitoes by allowing them to mate with their wild counterparts.

CHAPTER 3: Effects of larval population density on adult size and effects of adult size on mating success in *Anopheles arabiensis*

3.1 Introduction

The WHO (2016) reported an estimated 212 million malaria cases and 429 000 deaths that occurred in 2015, globally. Although, there was a 14% decrease in malaria cases and a 22% decrease in deaths from malaria worldwide, 90% of malaria cases and 92% of deaths occurred in the WHO African Region. It was also reported that an equivalent of 70% of the total deaths occurred in children aged under 5 years. South Africa, Botswana, Namibia, Swaziland and Zimbabwe are classified as "Low Transmission southern African Countries" (WHO, 2012). An estimated 10% of South Africa's population resides in provinces where malaria is endemic, namely, KwaZulu-Natal, Limpopo and Mpumalanga (Hlongwana et al., 2011; Moonasar et al., 2012).

Mosquito vector control strategies in South Africa are centered chiefly on indoor residual spraying (IRS) (Maharaj et al., 2013). During the past 80 years, IRS has been a critical factor in decreasing the malaria burden to levels that make elimination possible; however, insecticide resistance has provided a major challenge (Maharaj et al., 2013). The increasing use of insecticides for malaria vector control and the use of agricultural pesticides have led to the selection of resistant genes within populations of malaria vectors (Brooke et al., 2013).

The Sterile Insect Technique (SIT) is a genetic method that was developed by Edward F. Knipling (Klassen, 2009). He proposed the concept of releasing laboratory-reared sterile insects into field populations of important insect pests in order to compromise mating success and cause population crashes (Benedict and Robinson, 2003). For malaria vector control in northern KwaZulu-Natal, the principle objective of SIT is to induce sterility in the males of *Anopheles arabiensis*, by rearing large numbers of this target vector, reproductively sterilizing them using radiation and releasing them into wild populations of *An. arabiensis*in the study area. When the sterile males mate with the wild females, the females will be unable to produce viable offspring, thereby decreasing the *An. arabiensis* population in the target area. Continuous release of sterile males has the potential to achieve the elimination of the local vector population (Parker and Mehta, 2007; Helinski et al., 2008, 2009; Oliva et al., 2011). The advantages of SIT are that it is species specific,

environmentally friendly and does not have a negative impact on human health (Alphey et al., 2010). However, SIT does have challenges such as maintaining the competitiveness of the sterilized male mosquitos, since its success would be determined largely by whether or not the sterile males can compete successfully with the wild males for mates (Ng'habi et al., 2005).

Due to mass rearing for SIT, laboratory-reared males could be at a disadvantage by having reduced fitness or competitiveness when released into the wild population. In particular, the density at which larvae develop to adulthood is an important factor in the life history of anopheline mosquitoes. Larvae that develop in crowded habitats are confronted with greater competition for food and are exposed to higher levels of toxic waste products, crowding chemicals and physical interference from other larvae (Ng'habi et al., 2005). According to Jannat and Roitberg (2013), the larval environment is a major factor in determining the adult survivorship, fecundity and vector capacity of mosquitoes. Food availability and space determine the levels of competition between larvae. Increased larval densities have been shown to cause reduced larval metabolic rate, prolonged larval development and reduced larval survival (Tsurim et al., 2013). An increase in larval density has also been shown to cause decreased adult size and survival (Tsurim et al., 2013).

This study focuses on the effect of larval crowding on the body size and mating competitiveness of adult *An. arabiensis*. Food availability, which would otherwise have influenced the results, was controlled since each larva was given the same measured unit of food. Since SIT involves the mass production of the target vector, larval overcrowding could be a constraining factor. For an effective SIT program, the mass release of sterilized males that are equivalent to wild males in competitive ability is required. Larval overcrowding may compromise this process causing the production of smaller and less competitive adult males. Therefore, it was important to determine whether larval population densities affect *An. arabiensis* adult body size and whether this, in turn, affects mating success.

3.2. Materials and Methods

3.2.1 Mosquito colonies

Three strains of *An. arabiensis* were used in this study, namely Old Mamfene (F302), an existing laboratory colony, New Mamfene (F9), a wild colony, and the genetically modified Genetic Sexing Strain (F22). These colonies were maintained at the laboratories of the Medical Research Council

in Durban. The New Mamfene colony was specifically cultured for this study using wild mosquitoes that were collected from northern KwaZulu-Natal. The GSS colony was established at the Insect Pest Control Laboratory at the International Atomic Energy Agency in Austria. The colonies of larvae and adults were maintained under insectary conditions at a temperature of 27°C and relative humidity of 65%. All larvae were maintained in distilled water and fed a diet of dog food (Purina Alpo, manufactured at Nestle Purina Petcare), while adult mosquitoes were provided with a 10% sucrose solution. Females were fed with the blood of guinea pigs.

3.2.2 Population density and mating success

First instar larvae from each of the three *An. arabiensis* strains were maintained at low (200), medium (300) and high (500) densities in 2ℓ plastic containers with 500ml distilled water and fed a diet of 0.2 mg of dog food per larva. Three replicates were carried out for each population density per mosquito strain. The dead larvae were counted and removed on a daily basis, resultant pupae were separated and recorded and newly emerged adults were counted and separated according to sex. This was done for each replicate per density treatment for all strains (i.e. 27 containers were monitored).

Thereafter, 70 males and 50 females from each population density treatment for each strain were placed together in cages and allowed to mate for a 7-day period. These adults were provided with a 10% sucrose solution and the females were fed with the blood of guinea pigs. Once the mating period was over, 30 randomly selected females from each density treatment per strain were placed individually into oviposition tubes in order to record the number of eggs laid by each female. The eggs laid by each female from each population density treatment for each strain were placed in 500ml distilled water. The numbers of hatched larvae were recorded for each female cohort.

The spermathecae of females that did not lay eggs were dissected to determine whether mating had occurred, by evaluating if sperm was present within the spermathecae. The dissection of the spermathecae was carried out according to the MR4 Methods in *Anopheles* research (2014) (Chapter 6: Dissection Techniques). Each dead female mosquito was placed on a microscope slide under a compound microscope and treated with a phosphate buffer solution (PBS). The terminalia of the female was removed by pulling it away slowly with a fine tip forceps and the spermathecae were located in the 8th segment of the removed terminalia, where they appeared as a dark sphere.

In order to determine the size of the male mosquitos for each population density treatment within each strain, their wing lengths were measured using a calibrated micrometer at 40x magnification. The wing was measured from the wing tip to the wing joint on the thorax. The wing lengths were measured for 50 males for each larval density treatment per strain.

Male longevity for each of the three strains at each population density treatment was determined by placing 30 males into cages, providing them with a 10% sucrose solution and recording the number of dead males on a daily basis. This experiment was replicated three times.

3.2.3 Statistical analysis

Statistical analysis was done within each strain, and between the strains, to compare the different variables recorded for the low, medium and high densities. Pearson's Chi squared test was done on the life history characteristics (i.e. the larval and pupal survivorship and adult emergence). A two-way ANOVA was done on the mean numbers of eggs laid and larvae hatched and the mean male wing lengths, between and within strains, to determine if the results were statistically significant. A Pearson's Chi squared test was done to analyze the insemination rates. The level of significance was set at $P < 0.05$. A non-parametric Kaplan-Meier curve was done to estimate the survival curves for male longevity for each of the three *Anopheles* strains. A log-rank test was done to compare the Kaplan-Meier curves for the three *Anopheles* strains where the level of significance was set at $P < 0.05$.

3.3. Results

3.3.1 Life history characteristics

Data on the survival of first instar larvae to pupation and adult emergence as well as the sex ratios of the emerging adults, for each larval density treatment within each mosquito strain are presented in the figures below.

3.3.1.1 Larval survivorship

Larval survivorship, determined by the percentage of first instar larvae that survived to pupation, decreased significantly as larval density increased and this trend was consistent with all three *An. arabiensis* strains (Figure 3.1). Within the Old Mamfene, New Mamfene and GSS strains, the low,

medium and high larval population densities were all significantly different (P< 0.05) from each other, with the highest larval survival in the low density treatment and the lowest larval survival in the high density treatment.

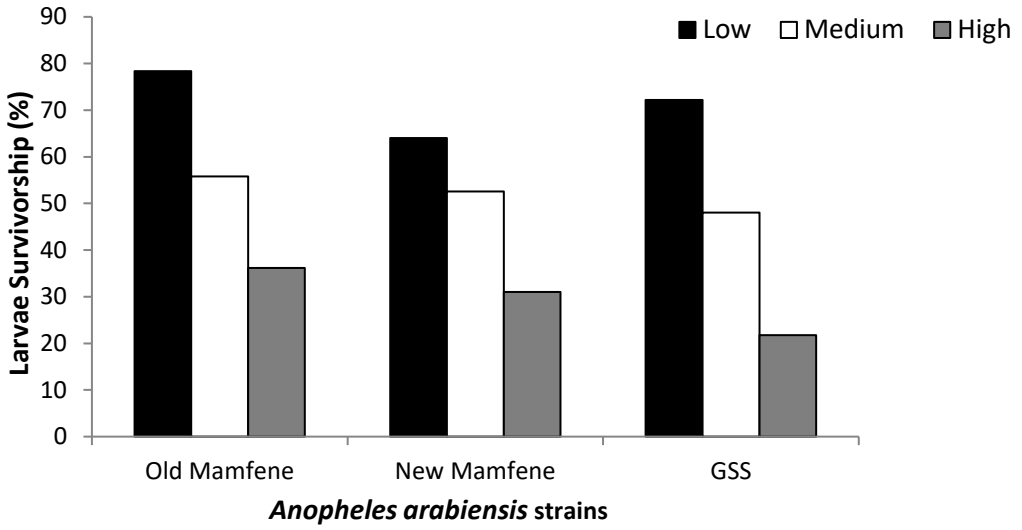

Figure 3.1: Larvae survivorship of the three *Anopheles arabiensis* strains at different population densities.

When comparing within population densities between strains (Figure 3.1), the only significant differences were in the low population density treatments of the Old Mamfene and New Mamfene strains (P =0.001), and in the high population density treatments of the New Mamfene and GSS strains (P =0.001) and Old Mamfene and GSS strains (P < 0.001). Overall, the New Mamfene strain displayed the lowest larval survival in the low density treatment (64%) while the GSS strain displayed the lowest larval survival in the high density treatment (21.7%).

3.3.1.2 Pupal survivorship

The numbers of pupae that survive to the adult stage depend largely on the number of larvae that develop into pupae as the pupae are presumably less affected by overcrowding. Consequently, there were significant differences in pupal survivorship only between the low density and medium density treatments (P =0.035), and between the low density and high density treatments (P <0.001) of the GSS strain (Figure 3.2). When comparing within population densities between strains there were significant differences in pupal survivorship in the low population density treatments of the

New Mamfene and GSS strains (P =0.007) and in the high density treatments of the Old Mamfene and GSS strains (P =0.003). It is of interest to note that the highest pupal survivorship was achieved in the high density treatment for the Old Mamfene (85.1%) and New Mamfene (80.9%) strains while in the GSS this was recorded in the low density treatment (88.9%). It was expected that the highest pupal survivorship would have occurred in the low density treatments due to less intra-specific larval competition for space and food.

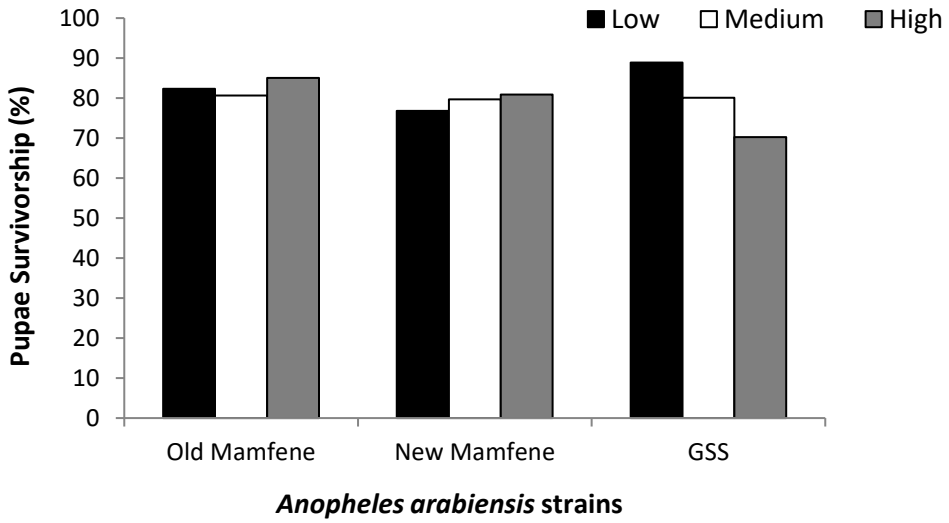

Figure 3.2: Pupal survivorship of the three *Anopheles arabiensis* strains at different larval population densities.

3.3.1.3 Sex ratios of emerging adults

The sex ratios of the emerging adults were observed within each strain in order to determine which strain produced more males versus females. Since SIT relies on the mass production and release of sterilized males, it is important to determine which strain and density affects the sex ratio. There were no statistically significant differences (P > 0.05) in the sex ratios (i.e. percentage of males to females) of emerging adults, between the strains and within the strains, where larval densities were concerned. Overall, the ratios of emerging males to females approached equality, indicating an absence of skewed sex ratios (Figure 3.3).

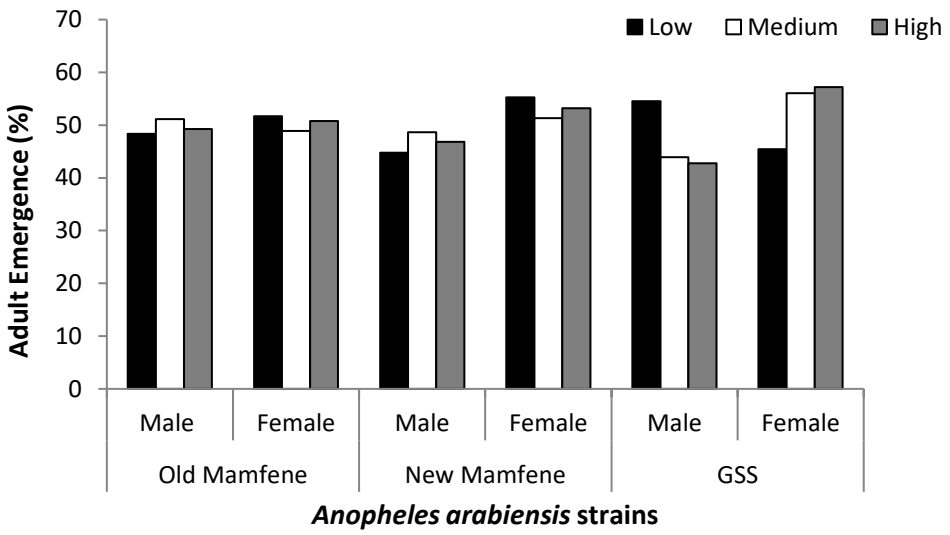

Figure 3.3: Sex ratios of emerging adults of the three *Anopheles arabiensis* strains reared at different larval population densities.

3.3.2 Impact of larval density on male body size

Wing length was used as a proxy indicator for the body size of male mosquitoes that were reared under the different larval density treatments. An overall significant difference was found ($P = 0.015$) between the three strains and population densities. Within the Old Mamfene strain, there were significant decreases in male wing length with increasing larval densities (Figure 3.4) ($P < 0.0001$ for low density and medium density; $P < 0.05$ for medium density and high density). The same trend was observed with the New Mamfene strain (Figure 3.4) with significant differences between all three larval population densities ($P < 0.001$ for low density and medium density; $P < 0.0001$ for low density and high density; $P < 0.05$ for medium density and high density). Within the GSS strain (Figure 3.4), there were significant decreases in male wing length between the low density and high density ($P < 0.005$), and between the medium density and high density treatments ($P < 0.005$). However, the differences between the low density and medium density treatments were not significant ($P > 0.05$).

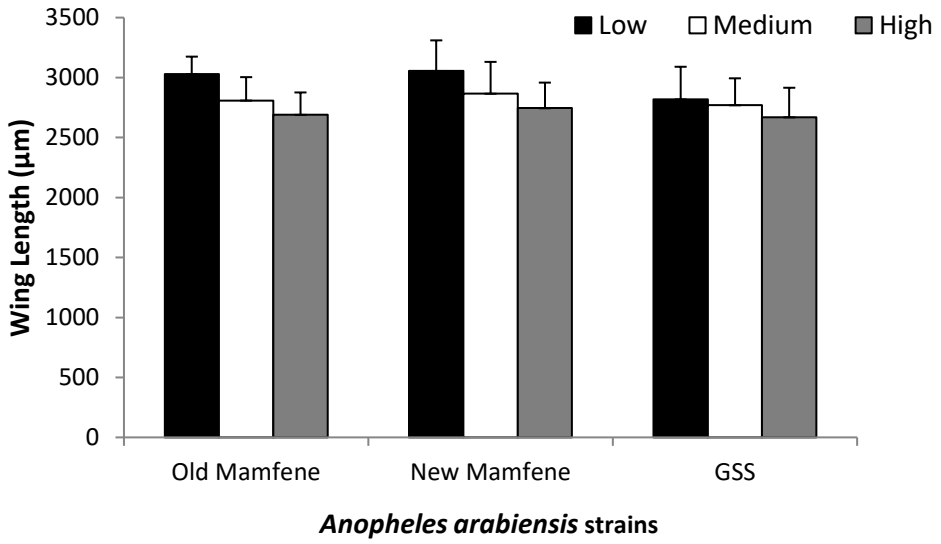

Figure 3.4: Mean (±SD) wing length (µm) of male *Anopheles arabiensis* of three different strains that were reared under increasing larval population densities.

Comparisons within population densities and between strains revealed significant differences only between the New Mamfene and GSS strains ($P < 0.0001$) and between the Old Mamfene and GSS strains ($P < 0.0001$) where the low density treatments were concerned (Figure 3.4). Significantly smaller males in the GSS strain could be due to it being a genetically altered strain. There were no significant differences in male size between the Old Mamfene and New Mamfene strains where any of the density treatments were concerned (Figure 3.4). This could be due to the F9 progeny of the New Mamfene strain that were used in the study and which could have become adapted to laboratory conditions. New Mamfene males appeared to consistently have a slightly larger wing length than Old Mamfene males (Figure 3.4) and with larger sample sizes these differences might become significant.

Overall, there was a clear and significant trend within each strain of *An. arabiensis* that as the larval population density increased, male wing length, and therefore body size, decreased.

3.3.3 Impact of larval density on female fecundity

The mean number of eggs produced by females that were reared at the different larval densities suggested a trend of decreased fecundity with increasing larval densities, within each strain of *An. arabiensis* (Figure 3.5). However, results were not statistically significant ($P > 0.05$).

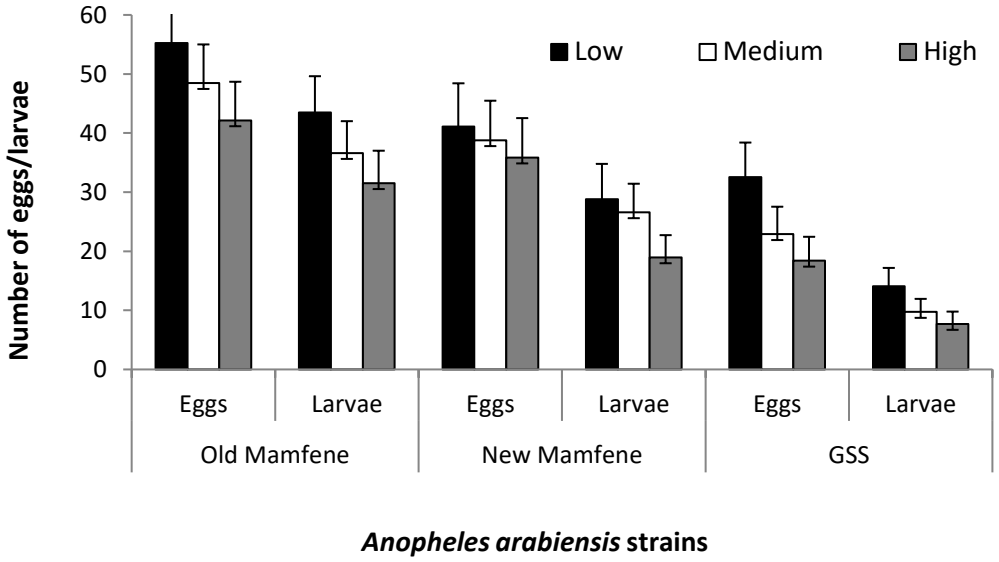

Figure 3.5: Mean (±SE) number of eggs and larvae produced by each strain of *An. Arabiensis* at different larval population densities.

However, significant differences were found in female fecundity between the three strains within the same larval density treatments (Figure 3.5). The mean female fecundity was significantly higher in the Old Mamfene strain than in the GSS strain at the low larval density (P =0.0125), medium density (P =0.0023) and high density treatments (P =0.0030). Fecundity was also significantly higher in the New Mamfene strain than in the GSS strain (P =0.0291) within the high density treatments.

The same trends were observed for the larvae that were produced in each strain, in that the mean numbers of larvae decreased with increasing larval densities (Figure 3.5). Within each mosquito strain, the differences in the mean numbers of larvae produced between the three larval population densities were significant (P = 0.043). At each population density, the differences in mean larval production between the three strains were significant (P <0.0005). There was no significant interaction between strain and larval density (P > 0.05). Significant differences were found in mean larval production between strains in the low density treatment (New Mamfene and GSS, P= 0.0159; Old Mamfene and GSS, P =0.0013), medium density treatment (New Mamfene and GSS, P< 0.0001; Old Mamfene and GSS, P <0.0001) and the high density treatment (New Mamfene and GSS, P= 0.0002; Old Mamfene and GSS, P =0.0001).

3.3.4 Insemination rates

Females that were produced at the different larval population densities were allowed to mate and their rates of insemination were compared (Figure 3.6).

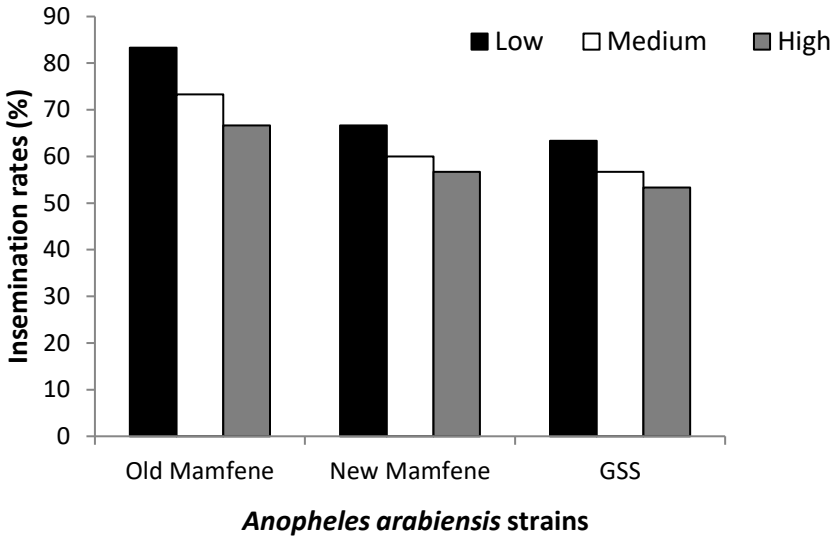

Figure 3.6: Insemination rates (%) of females (n=30) from each strain of *Anopheles arabiensis* that were reared at different larval population densities.

There appeared to be a trend of decreasing female insemination rates with increasing larval population densities, within each strain of *An. arabiensis* (Figure 3.6). There also appeared to be differences between the strains at the same density treatments (Figure 3.6). However, these differences were not significant ($P > 0.05$), either between the larval population densities or between the strains.

3.3.5 Male longevity

Survival curves generated for males from the three different mosquito strains (Figure 3.7) indicated that males from the Old Mamfene strain survived best ($P < 0.05$) at specific times (e.g. after 10 days). The survival rates of males from the New Mamfene and GSS strains were very similar (Figure 3.7).

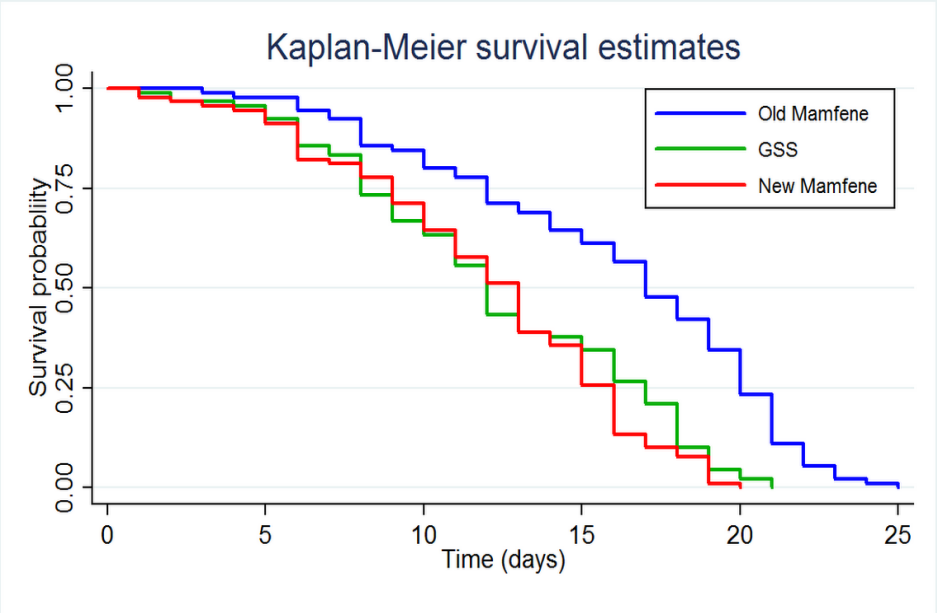

Figure 3.7: Probability of survival of males from each strain of *Anopheles arabiensis* over time, indicating their longevity.

3.4 Discussion

The results of this study indicate that within the confined laboratory conditions, larval survivorship within each of the three strains of *An. arabiensis* increased with a decrease in larval population size. The number of pupae that survived and the numbers of adults that emerged depended largely on the survival success of the larvae. Therefore, the highest survival success rate (egg to adult) within all strains was achieved in the low larval density treatments. In the study of Macia (2009) on *Aedes aegypti* (Linnaeus), mortality in larval cohorts developing at low densities was low but increased drastically at the higher larval densities. These observations are consistent with the findings of this study.

The environment in which the larvae develop is considered to be the determining factor for adult mosquito survivorship, fecundity and vector capacity, since density-dependent effects are most likely to occur during the immature stages (Gilles et al., 2011; Jannat and Roitberg, 2013). Overcrowding in mosquito habitats typically results in scarcity of larval resources, such as food and space, which in turn causes increased developmental times, increased mortality and smaller-sized adult individuals (Macia, 2009). These density-dependent effects are caused by larger numbers of individuals having less access to food, increased food portioning, or toxins induced by

stress which are released into the larval environment (Yoshioka et al., 2012). Also, larvae in higher density cohorts are more exposed to intraspecific chemicals (see below) and/or waste products (Ng'habi et al., 2005). Larvae of *Ae. aegypti* have been reported to release chemical retardants in high density populations when food availability is below a certain threshold. These chemical retardants are able to regulate the number of adults that emerge in overcrowded situations (Ng'habi et al., 2005). Even though these chemicals were not assayed for in the present study, their existence could explain why, in the absence of food limitations, mosquitoes cultured at higher larval densities performed poorer than those cultured at lower densities (Ng'habi et al., 2005).

The results in Figure 3.4 indicate that, in each strain of *An. arabiensis*, as the larval population density increases the wing length, and therefore the body size, of the males decreases. Although results were not significant, female fertility and fecundity follow a similar pattern, with decreasing insemination rates and decreased egg and larval production with an increase in larval population density (Figures 3.5 and 3.6). The wild strain, New Mamfene, produced the largest males (Figure 3.4) which could be a consequence of the culturing of the Old Mamfene and GSS strains and a subsequent reduction in their genetic variation (Muhenga et al., 2011). Relationships between large adult body size and high reproductive potential have been demonstrated in mosquitoes (Cator et al., 2010), since larger insects are generally more successful than smaller ones (Charlwood et al., 2002). However, a few published studies on anopheline mosquitoes have provided conflicting results. While Yuval et al. (1993) reported that large *Anopheles freeborni* Aitken males mated with more females than smaller ones, Charlwood et al. (2002) suggested that male size does not affect the chances of *An. gambiae* males mating at least once. Ng'habi et al. (2008) also suggested that intermediate-sized males were more likely to mate with females than larger or smaller males.

SIT for *An. arabiensis* control requires the mass production and release of very large numbers of male individuals. The results of this study indicate that while mass production (i.e. culturing under high larval densities) would yield males with a smaller body size, this will not necessarily cause a significant reduction in female insemination rates (Figure 3.6). Therefore, male mosquitoes cultured at higher larval densities would still be able to carry out female insemination successfully, irrespective of the *An. arabiensis* strain. This study indicates that the Old Mamfene strain had the highest egg and larval production compared to the other two strains suggesting that it would be best suited for SIT applications (Figure 3.5). The results also indicated that the different larval

densities and strains did not skew the sex ratio of the emerged adults (Figure 3.3). SIT requires mass releases of sterilized males into the target site and an equivalent sex ratio indicates that the mass-produced laboratory population would typically be 50% male.

The longevity of mosquitoes can be negatively affected by intraspecific competition (Reiskind and Lounibos, 2009) and other factors. The Old Mamfene strain displayed the greatest male longevity when compared to New Mamfene and GSS strains, with the New Mamfene strain having the lowest longevity (Figure 3.7). This could be due to the Old Mamfene strain being a laboratory adapted population. Differences between laboratory-reared and natural mosquito populations often develop and accumulate during the culturing process (Benedict et al., 2009). Effects such as population bottlenecks, genetic drift and the formation of homogenous colonies, could cause advantageous genetic changes in laboratory-reared mosquitoes, resulting in the greater life span of males from the Old Mamfene strain under laboratory conditions.

These studies were carried out in a laboratory environment to establish a fundamental knowledge of *An. arabiensis* characteristics, in order to move forward with studies on the deployment of SIT in malaria control. In order for SIT to be successful, it is essential that the laboratory-reared males are able to out-compete the wild males for female insemination. Although there were no significant reductions in female insemination rates that resulted from larval overcrowding, there were consistent trends in each *An. arabiensis* strain that indicated a decrease in female insemination with an increase in larval population density (Figure 3.6). There were also signs of decreasing insemination rates between the three *An. arabiensis* strains, although these differences were also not significant. It is possible that differences in female insemination rates could prove to be significant if higher larval population densities are used. The apparent decrease in insemination rates in the New Mamfene strain and GSS could be due to Old Mamfene being a laboratory-adapted strain with advantageous attributes.

The limitations experienced in this study are very similar to those discussed in the previous chapter and could have affected the outcomes of this study. It could be argued that larger sample sizes could have yielded significant differences in some of the trends that did not prove to be significant. According to Howell and Knols (2009), it was hypothesized that genetically bottle-necked populations are able to alter their mating selection standards by accepting mates that would not normally be accepted in the field, in order to avoid extinction. Natural mating habits may also be

compromised by space limitations in cages which can also affect the outcomes of laboratory trials. If modified mating habits become established during laboratory culturing, this may compromise the competitive abilities of mass-released individuals in the wild (Howell and Knols, 2009).

As discussed in the previous chapter, bottle-necking can be reduced by proper quality control protocols in laboratory cultures. Also, field studies should be conducted to provide a better understanding of the competitiveness of laboratory-reared versus wild mosquitoes, and thereby ensure a successful SIT program. These field studies should be carried out with larger population densities to determine the true competitive nature between the wild mosquitoes and the laboratory-reared mosquitoes. Since field conditions are very different to controlled laboratory conditions, the mass-released males would not only need to out-compete the wild males, but also be able to survive in the field for sufficient time to be effective. Field studies will ultimately indicate the true longevity and competitiveness of laboratory-reared male *An. arabiensis*.

Chapter 4: Investigations into insecticide resistance in *Anopheles arabiensis* in the study area and the transfer of powder dyes from males to females during copulation

4.1 Introduction

Malaria vector control relies on the use of insecticide treated nets (ITN) and the indoor residual spraying (IRS) of homes (Kleinschmidt et al., 2015). The majority of malaria mortality and morbidity occurs in sub-Saharan Africa and the World Health Organization (WHO) recommends the universal usage of long-lasting insecticide nets that are treated with pyrethroids to reduce malaria transmission (Bagi et al., 2015). According to the WHO Malaria report (2014), the proportion of the human population that utilizes ITNs has increased noticeably over the past 10 years, although not all households have enough nets to protect entire families. IRS for vector control has been widely adopted by 90 countries worldwide and 42 malaria-endemic countries in the WHO African region (WHO Malaria Report, 2014).

Pyrethroids are the only class of insecticides that are approved for ITNs because of their effectiveness, based on a strong excite-repellent effect on mosquitoes, and their lower mammalian toxicity (Ndiath et al., 2015). Other classes of insecticides used for vector control include organochlorines, organophosphates and carbamates (Abdalla et al., 2014). Pyrethroids and organochlorines (e.g. DDT) have a similar mode of action based on the opening of sodium channels in the nerve cells, which results in continuous nerve excitation, paralysis and death of the vector (WHO, 2011). Organophosphates and carbamates inhibit the enzyme cholinesterase, preventing the breakdown of the neurotransmitter acetylcholine and resulting in neuromuscular overstimulation (WHO, 2011). Due to these insecticides having similar modes of action, cross resistance can occur (WHO, 2011). Pyrethroid resistance is present in all major malaria vectors on the African continent (Kleinschmidt et al., 2015). Resistance to insecticides from the other three chemical classes that are used for IRS is also emerging in many regions where insecticides are used for vector control (Kleinschmidt et al., 2015). Due to increasing insecticide resistance, alternative methods for vector control need to be implemented.

The Sterile Insect Technique (SIT) is an environmentally friendly and species-specific pest management concept which focuses on the competition for mating partners between the wild and sterile males (Ant et al., 2012). When successful, this results in a reduction of the target pest's

population size due to the decrease in the number of fertile matings (Benedict and Robinson, 2003; Oliva et al., 2011; Ant et al., 2012). Alphey et al. (2010) discussed numerous aspects that SIT needed to improve on, in order to achieve success. These include the ability to mass-rear the target species, the dispersal range of the target species, its mating habits, density-dependent effects and infrastructure availability. However, a crucial aspect for SIT is to determine whether the released males can compete successfully with their wild counterparts in the target area, and assess the viability of the eggs laid by the mated females. To achieve this, a suitable marker is required that can be transferred from the sterile, released male to the wild female. In this way, the number of females that have mated with the released males can be determined as well as the viability of the eggs that were laid.

There are numerous methods for marking insects, including tags, mutilation (clipping, punching, notching or etching a mark), dyes, dusts, genetic markers, radioactive isotopes and protein markers (Verhulst et al., 2013; Dickens and Brant, 2014). An ideal marker should be inexpensive, non-toxic, easily applied, visible and should not affect the behaviour, development, longevity or reproduction of the target insect (Dickens and Brant, 2014). Marking methods such as tags and mutilation are only suitable for small numbers of insects. These marking methods are tedious and time consuming and will not be suitable for marking very large numbers of insects (Hagler and Jackson, 2001). The tag and mutilation techniques of marking would not be relevant to this study, as they would not help in tracking the females which have copulated with the tagged males.

For this study, fluorescent dust markers were tested since dust or powder marking is the most common marking technique used for mosquitoes (Hagler and Jackson, 2001). The advantages of dust marking are that it is inexpensive, readily available, environmentally safe, easily applied and easily detected. There are also a variety of colours which can be used for different cohort groups within a study (Hagler and Jackson, 2001). However, dust markers have affected insect longevity and behavioural responses in some studies, while in other studies there were no effects of the marker on the insects (Verhulst et al., 2013).

The dust can be applied by various methods which include the use of a syringe or a bulb duster, placing the mosquitoes in a bag containing the dust and shaking gently it, or by creating a dust storm within a cage (Jones et al., 2012; Dickens and Brant, 2014). However, the application of larger quantities of dust can cause adverse effects on the insect. The area of marking on the male

is important because the marker has to be transferred to the female during copulation. Copulation studies in *Anopheles* mosquitoes have shown that as the male approaches the female, he grasps her with the tarsal claw on his first pair of legs and then swings his abdomen up to clasp her genitalia (Howell and Knols, 2009). Once the two are interlocked, the male releases his tarsal grip and they take up the venter-to-venter position and resume flying. In order for the marker to be successfully transferred from the male to the female, the marker must thus be situated either on the tarsal claw or the abdomen of the male.

Preliminary studies are required to confirm the extent to which selected markers are successfully transferred during mating and thus whether they have practical value. This study was therefore conducted to determine whether a powdered dye would serve as an appropriate marker for male *Anopheles arabiensis*. Similarly, preliminary studies had to be conducted to determine the insecticide resistance status of the wild *An. arabiensis* population within the study area, in order to determine whether insecticide-resistant males would need to be released. Therefore, insecticide susceptibility and insecticide detection assays were carried out.

4.2 Materials and Methods

4.2.1 Field collections

Adult female mosquitoes were collected using window traps and aspirators from 10 households in Sections 8 and 9 in Mamfene, northern KwaZulu-Natal (see Figure 2.1). Only *An. arabiensis* mosquitoes were reared to the F10 generation and used for the purpose of this study.

4.2.2 Insecticide susceptibility assays

The susceptibility status of laboratory-reared adult mosquitoes was determined following the WHO protocol. Twenty mosquitoes were exposed to insecticide impregnated and control filter papers for 1 hour and then kept in holding tubes with access to a 10% sugar solution for 24 hours, after which the percentage mortality was determined. Four insecticides, each belonging to a different class, were tested. These included DDT (4%) (Organochlorine), Deltamethrin (0.05%) (Pyrethroid), Bendiocard (0.01%) (Carbamate) and Malathion (0.05%) (Organophosphate). Three replicates of each insecticide trial were carried out.

4.2.3 Detection assays

DNA was extracted from 178 individual mosquitoes according to the extraction method explained by Collins et al. (1987). Real-time Polymerase Chain Reaction (qPCR) was used to detect knockdown resistance (kdr) in 178 mosquitoes that were exposed to DDT and Deltamethrin. Tests were conducted to detect two types of kdr point mutations occurring in East and West African populations, namely kdr-east and kdr-west. Acetylcholinesterase (AChE) susceptibility to inhibition in mosquitoes exposed to Bendiocarb and Malathion was also determined in 172 mosquito DNA samples. Detection assays were carried out using a standard 96-well test plate.

4.2.4 Dye transference

One-day old males from the New Mamfene colony (wild mosquito colony) were used for this preliminary study, which was done in three replicates. Each replicate contained 30 males and 10 males were dyed at a time. A yellow powdered dye (Arc Yellow Day Glo), which was provided by the International Atomic Energy Agency (IAEA) in Seibersdorf, Austria, was used as a marker.

During the marking process, 10 males from the New Mamfene strain were placed in 500ml cups and dyed with the yellow powdered dye. Around 10g of the dye was blown into the cup using an aspirator until all the males were covered. A control group of 30 unmarked males was used in which the males were not exposed to the dye.

Thereafter, the 30 males that comprised a replicate were placed in a cage with 30 females, where they were provided with a 10% sugar solution and allowed to mate for a 7-day period. The females were given three blood meals (using guinea pigs) to stimulate egg maturation. An egg bowl containing water was placed in the cage after the first blood meal for oviposition. After the 7-day period, the egg bowls were removed and the eggs were counted for each experimental and control replicate. The females were then removed from the cage and inspected under a dissecting microscope to determine whether there had been any dye transfer from the males during copulation. The spermathecae of the females were then dissected to determine whether insemination had occurred.

4.3 Results

4.3.1 Insecticide susceptibility assays

Following treatment of the adult mosquitoes with the four insecticides, all displayed high susceptibility, with 100% knock down after one hour and 100% mortality after 24 hours (Figure 4.1). This suggests that the mosquitoes within the study area are currently not resistant to any of the insecticides used for malaria control.

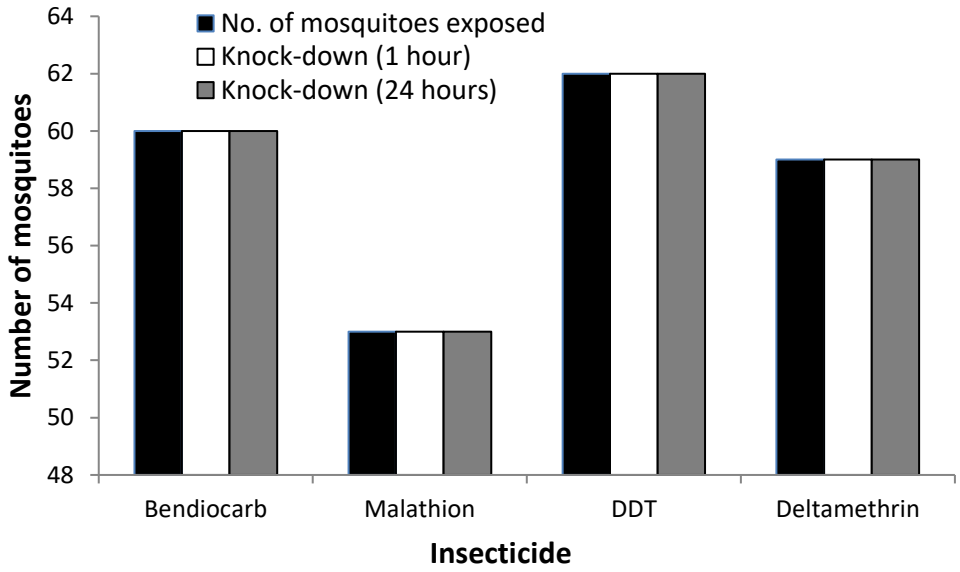

Figure 4.1: Total numbers of mosquitoes that were exposed to all four insecticides and were knocked down after 1 hour and 24 hour intervals.

4.3.2 Detection assays

The results from the real-time PCR indicated that all 178 mosquitoes that were exposed to DDT and Deltamethrin were determined to be heterozygous resistant, while none were homozygous resistant for the two kdr mutations (Table 4.1). However, when exposed to Bendiocarb and Malathion, all samples were determined to be completely susceptible to these two insecticides (Table 4.1: Wild type SS) as the mosquitoes did not possess any mutations to prevent the inhibition of the cholinesterase enzyme.

Table 4.1: Numbers of mosquitoes that were determined to be homozygous susceptible, homozygous resistant, and heterozygous resistant for three different alleles.

Alleles	Sample size (n)	Homozygous Susceptible (SS)	Homozygous resistant (RR)	Heterozygous resistant (SR)
kdr-East	178	0	0	178 (100%)
kdr-West	178	0	0	178 (100%)
AChE	173	173 (100%)	0	0

Real-time PCR makes use of two probes; VIC and FAM. An increase in VIC fluorescence, which is specific for the wild-type allele, indicates a homozygous sensitive (SS) individual. A peak in FAM fluorescence, which is specific for the mutant allele, indicates heterozygous resistance (SR). Each mosquito sample was exposed to the real-time PCR and Figure 4.2 indicates an example of a homozygous sensitive individual while Figure 4.3 indicates a heterozygous individual.

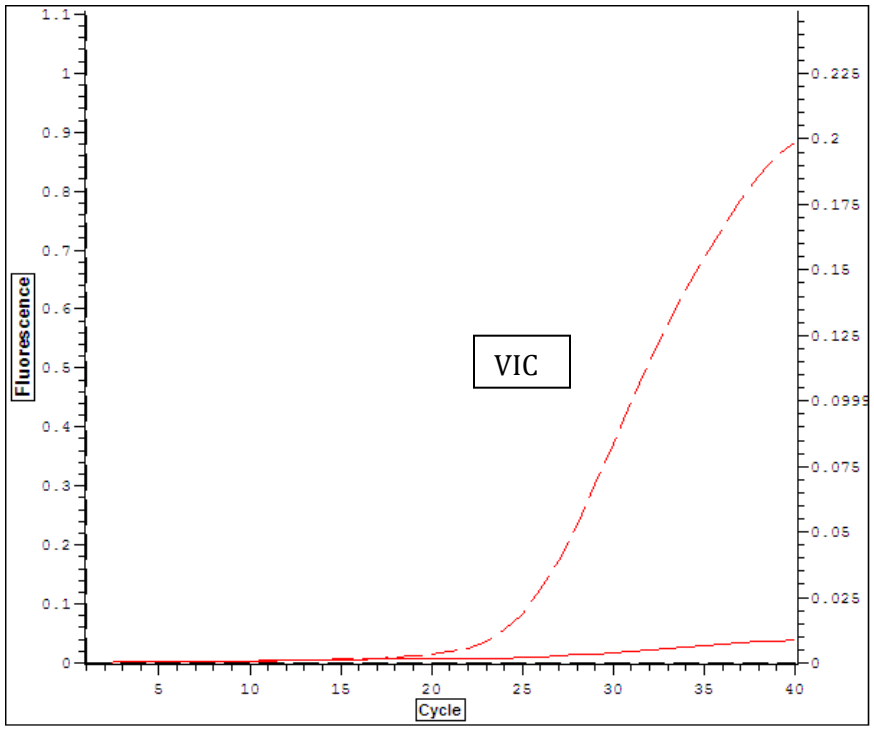

Figure 4.2: Cycling of the VIC labelled probe specific for the wild type allele (SS). An increase in VIC fluorescence only indicates a homozygous sensitive individual.

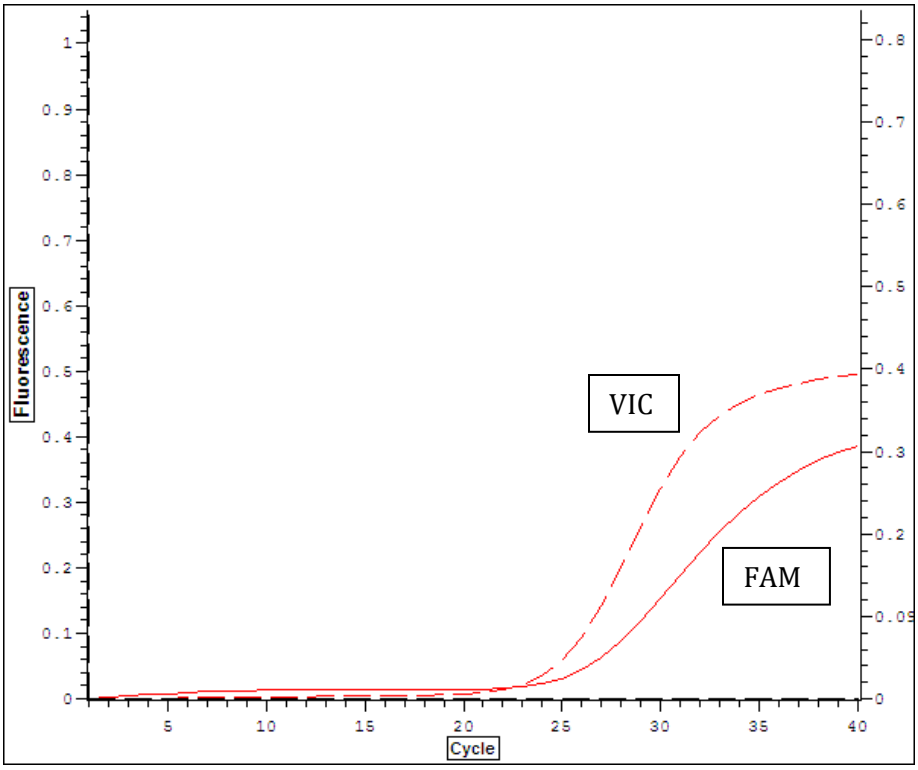

Figure 4.3: Cycling of the FAM and VIC labelled probes. An increase in fluorescence in both signals indicates a heterozygous resistant individual (SR).

4.3.3 Dye transference

The results in Table 4.2 indicate that the powder dye transference from male to female was largely unsuccessful, with 0-16.7% of the females (average of 8.9%) in the three replicates showing traces of the marker. Although all of the males were marked, there was either insufficient contact between the males and females for the dye to be transferred or the dye was mostly unable to attach to the females during copulation.

Table 4.2: The percentage of females that displayed dye transfer from the males during copulation.

Replicates	**Females with dye transfer**
1	10%
2	16.67%
3	0%
Control (no dye)	0%

Copulation did occur between the marked males and the females of the New Mamfene strain, with some 35-40% of the females inseminated during the three replicates (Figure 4.4). However, the percentages of females inseminated during each of these trials were much lower than that in the control group (around 75%). In addition, the number of eggs laid and number of larvae produced was substantially higher in the control group than in all of the trial replicates (Table 4.3), suggesting some form of debilitation in the marked males.

Table 4.3: The number of eggs laid and larvae produced for each replicate in which 30 marked males were exposed to 30 females of *Anopheles arabiensis* versus that in the control where the males were unmarked.

Replicates	**Eggs**	**Larvae**	**Hatch Rate (%)**
1	287	163	56.79%
2	130	107	82.31%
3	75	68	90.67%
Control	855	723	84.56%

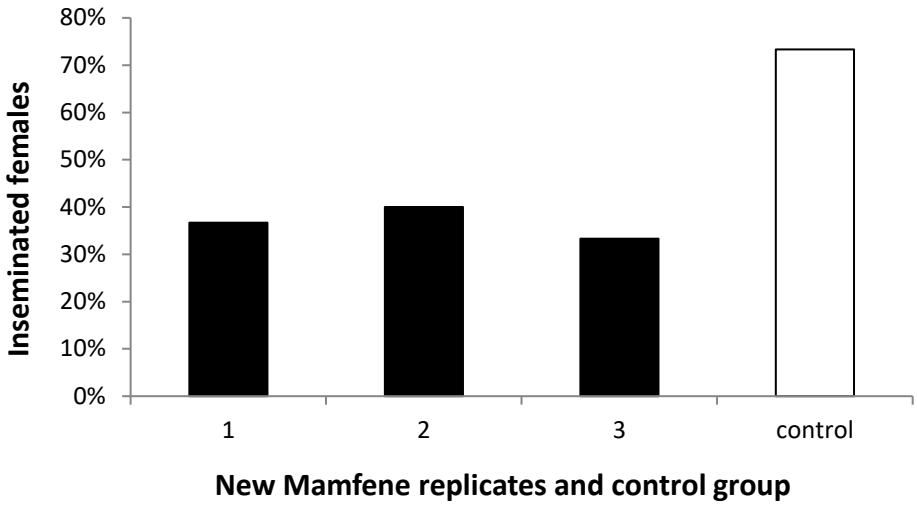

Figure 4.4: The percentage of inseminated females of *Anopheles arabiensis* when exposed to males for each trial replicate (marked males) versus the control group (unmarked males).

4.4 Discussion

The continuous monitoring of insecticide resistance in malaria endemic-areas is crucial as the development of resistance poses a serious threat to the success of vector control programs. The WHO released the Global Plan for Insecticide Resistance Management in Malaria Vectors (WHO, 2014). This comprises five strategies, namely: (i) undertake resistance monitoring; (ii) implement resistance management strategies; (iii) fill in knowledge gaps on the mechanism of resistance, and the impact of resistance management; (iv) develop new vector control tools and; (v) ensure that key enabling mechanisms are in place (WHO, 2014).

This study indicated that *An. arabiensis* populations around Mamfene, in northern KwaZulu-Natal, are in the process of developing resistance towards DDT and Deltamethrin (i.e. samples were 100% heterozygous resistant). In Mamfene, the insecticide currently being used for IRS is the pyrethroid K-Othrine which is similar to Deltamethrin. Regular exposure to the insecticide used in IRS programmes could be the cause of mosquitoes in the study area developing resistance. Switching to DDT may not be a viable option as cross-resistance to these two insecticides may occur, since they share the same mechanism of action. Although no potential for resistance to

Bendiocarb and Malathion was found (i.e. samples were 100% homozygous sensitive), these insecticides are more expensive and need to be applied more often. The problem is further compounded by the limited number of insecticides recommended by the WHO Pesticide Evaluation Scheme (WHOPES) for use in IRS operations (Casimiro et al., 2007).

As a result of the risk of insecticide resistance, research into the potential use of SIT for the management of mosquito vectors has increased. In order to successfully implement SIT into field populations, a major requirement is the introduction of sexually-competent genetically sterile males (Benedict and Robinson, 2003) that must be able to out-compete their wild counterparts for females. Therefore, the introduction of male-specific insecticide resistance (MIR), along with sterility, into field populations could prove advantageous because the wild population is still susceptible to the insecticides (Figure 4.1 and Table 4.1) which would further reduce the wild population. The study area has a malaria control program that implements IRS because it includes homes in rural settlements that are close to breeding sites where males were found to enter homes. Therefore, it would be best to release sterile, insecticide-resistant males into the study area in order to out-compete the wild male population. During mass-rearing, female mosquitoes should be eliminated early in their development as their presence in releases decreases the efficiency of the technique and poses a risk of disease transmission (Benedict and Robinson, 2003).

In order to monitor the sterility of the released males and determine whether mating with the wild females is successful, the males need to be marked with a dye that can easily be transferred to the females during copulation. If this is achieved, the females that have mated with the sterile males can be identified. However, the powdered dye used in these preliminary trials did not demonstrate adequate transference and alternate marking methods should be investigated. The results of these trials also suggested that powdered dyes did hinder the mating success of the mosquitoes. Fine sand and inert dusts can be used to protect stored grain from insect infestation, since they damage the outer wax layer of the insect cuticle, which prevents water loss, causing the insect to desiccate (Groot, 2004; Vani and Brindhaa, 2013). It is thus possible that the dye used in this study could have had a similar effect on the marked males and therefore hindered mating success.

Dickens and Brant (2014) recommended the use of the dust storm method (i.e. using a fan to create a dust storm within the exposure cage) to ensure dye adhesion to the males, since it caused the least mortality, while the recommended marker dyes included BC Pink and BC Red. The amount

of dye used (10g) could have had a negative impact as too much dust can decrease mobility, interfere with sensory organs and increase mortality (Dickens and Brant, 2014). Another method for monitoring or detecting mating is the use of a nitrogen-stable isotope semen label (Helinski et al., 2008). The study of Helinski et al. (2008) confirmed that a nitrogen-stable isotope can be used as a semen label to detect inseminated females. Isolation of the female after mating did not result in the loss of the label and this method may thus be better than the use of powdered dyes.

Chapter 5: General Discussion and Conclusions

5.1 Introduction

Human malaria is a global disease transmitted by *Anopheles* mosquito species and caused by several species of *Plasmodium* parasites (Klassen, 2009; Lee et al., 2011). According to the WHO (2016), an estimated 212 million malaria cases occurred worldwide in 2015, with 191 million of these in the WHO African Region. Globally, these led to an estimated 429000 deaths in 2015 with 394 000 deaths in the WHO African Region (WHO, 2016). Although there has been a decrease in malaria cases during the last decade (Snetselaar et al., 2017), the disease is estimated to take the life of a child every two minutes and thus remains a major killer (WHO, 2016).

South Africa represents a low transmission region and is characterized by a low incidence of confirmed cases (Khosa et al., 2013). The malaria endemic provinces in South Africa are Limpopo, Mpumalanga and KwaZulu-Natal with an estimated 10% of the country's population living in these areas who are at risk of contracting malaria (Hlongwana et al., 2011; Moonasar et al., 2012). Malaria in South Africa is seasonal, unstable and prone to epidemics. South Africa has a malaria control program which consists of vector control, health promotions, case management and cross-border strategies (Moonasar et al., 2012; Khosa et al., 2013). The confirmed malaria vector in South Africa is *Anopheles arabiensis* (Killeen et al., 2013). Global vector control strategies consist of IRS implementation, larvicidal applications and the distribution of long-lasting insecticide-treated bed nets (LLINs) (Moonasar et al., 2012; Brooke et al., 2013). The implementation of IRS has proven successful for the control of *An. arabiensis* in South Africa (Moonasar et al., 2012). However, the WHO (2016) warned that resistance of malaria vectors to the four insecticide classes currently used in IRS and ITNs threatens malaria prevention efforts. It was confirmed that 60 out of 73 malaria-endemic countries have reported insecticide resistance to at least one insecticide, with pyrethroids (the only class used in ITNs) being the most commonly reported (WHO, 2016).

The Sterile Insect Technique (SIT) is a form of genetic control that is deployed to cause reproductive failure of the vector (e.g. *An. arabiensis*), thereby decreasing the size of vector populations (Knipling et al., 1968; Windbichler et al., 2012). In a successful SIT program, the area containing the vector population is flooded with sterile males which decrease the mating success of the wild male population and subsequently decrease the vector population (Benedict et al.,

2003). To achieve this, the sterile males must have the ability to locate, copulate and transfer sterile sperm to wild females (Helinski et al., 2009). The mating ability and the survival of the laboratory-bred mosquitoes when released into the wild is of critical importance. Therefore, comparisons of fitness and compatibility between laboratory-bred male mosquitoes and wild-type mosquitoes are crucial to a successful SIT strategy (Huho et al., 2007).

5.2 Survival and fecundity of laboratory and wild strains of *An. arabiensis*

The *An. arabiensis* population within the study site fluctuated seasonally, with an increase during the warmer months when there is adequate rainfall to provide sufficient larval habitats. Grover-Kopec et al. (2006) stated that the climatic conditions of an area play an important role in the transmission of malaria, because rainfall is responsible for creating breeding sites for mosquitoes while temperature regulates the rate of larval/pupal development and influences adult survival. This information could be helpful with regards to SIT in determining the numbers of sterile, laboratory-reared males that need to be released in the target area (Oliva et al., 2011). These males could be released when the *An. arabiensis* population is relatively low (i.e. during the winter months), thereby requiring fewer males for releases and increasing the impact on the population even further. Alphey et al. (2010) stated that sterile males should be released periodically to match the phenology of the wild target population and thereby maintain a permanent standing population of the sterile males in the target area, so that females seeking mates always have a high probability of mating with a sterile male. During the winter months, the *An. arabiensis* populations in northern KwaZulu-Natal are typically low and this provides an opportune time for the release of sterile males.

The greatest percentage of larval survivorship to adult emergence was achieved with the Old Mamfene laboratory strain when compared to the New Mamfene (wild) strain and the GSS. This strain could thus be beneficial for SIT where large numbers of male *An. arabiensis* would need to be reared. The larger the population of released males, the higher the probability of a released male mating with a wild female, thereby improving the potential of reducing wild *An. arabiensis* populations (Benedict and Robinson, 2003; Oliva et al., 2011). The strain that performed the poorest overall was the GSS, making it unsuitable for mass-rearing for SIT. This could be due to the genetic modification within the strain which results in the elimination of females and survival of males when exposed to dieldrin (Yamada et al., 2015). The Old Mamfene strain also displayed

the best fecundity relative to the New Mamfene and GSS strains, which also supports its suitability for mass-rearing. However, accurate elimination of the females needs to be implemented before the initiation of releases. The relatively poorer performance of the New Mamfene strain in this study could be due to it being a wild colony which was poorly adapted to laboratory conditions.

5.3. Effects of larval population densities on adult size and thereby mating success in *An. arabiensis*

The results in Chapter 3 indicate that larval survivorship is affected by their density within the confined laboratory conditions. Larval survivorship decreased with an increase in larval density for each of the three *An. arabiensis* strains tested. This could be due to intra-specific competition between the larvae for food and space, although each larva was provided with an equivalent amount of food. The effects of larval crowding was investigated because SIT requires the mass production of sterile males and space would be a limiting factor (Ng'habi et al., 2005). Also, an increase in larval populations would lead to an increase in metabolic waste production which could affect larval survivorship (Macia, 2009; Ng'habi et al., 2005; Yoshioka et al., 2012).

The adult body size of male *An. arabiensis* individuals was also affected by larval density. As larval density increases, the average male body size decreases. This could be due to competition between the larval stages for space, and its associated effects (Macia, 2009). Of the three strains tested, the New Mamfene (wild) strain produced the largest males which could be a result of increased genetic variation in the wild population. Benedict et al. (2009) stated that differences between mass-produced and wild mosquitoes accumulate during laboratory culturing and that laboratory colonies become increasingly homozygous entities that differ genetically from their wild counterparts.

This study indicated that female fecundity was not affected by the different larval density treatments but rather by the strain of *An. arabiensis*. There were no significant differences in insemination success between the larval density treatments or between the strains. This suggests that even though higher larval densities result in smaller males, this would not necessarily hinder mating success or female fecundity. The Old Mamfene strain displayed the highest female fecundity and insemination success in this study and, given other indications (see above), should

be considered as the strain for SIT applications, since this is dependent on the production of high larval densities in the laboratory.

The Old Mamfene also strain displayed the greatest male longevity. This is also advantageous for SIT as the life span of laboratory-reared males is an important aspect for successful SIT. However, a field study should be conducted to verify the lifespan of these laboratory-reared males in uncontrolled (field) conditions.

5.4. Insecticide resistance in *An. arabiensis* in the study area and the transfer of powder dyes from males to females during copulation

The study indicated that although *An. arabiensis* collected from the Mamfene area were susceptible to the four main classes of insecticides used in mosquito control (organochlorines, pyrethroids, carbamates, and organophosphates), they are in the process of developing resistance to DDT (organochlorine) and Deltamethrin (pyrethroid). The pyrethroid K-Othrine, a formulation of Deltamethrin, is currently the insecticide used for IRS. Pyrethroids and organochlorines have the same mechanism of action by modulating the voltage-gated sodium channels in nerve cells, resulting in rapid knock-down properties. Therefore, switching to DDT may not be a viable option as cross-resistance may occur (Nauen, 2007). Effective malaria vector control is hindered by increasing insecticide resistance and, therefore, techniques like SIT that are environmentally friendly, effective and more sustainable, are increasingly needed (Lees et al., 2015; Wilke et al., 2009).

SIT requires the mass release of sexually competent, genetically sterile males that can out-compete their wild counterparts for females (Benedict and Robinson, 2003). Introducing male-specific insecticide resistance, along with sterility, into the released populations could prove advantageous for SIT given that the wild population in the study area is still susceptible to insecticides. SIT along with male-specific insecticide resistance could work synergistically with the implemented IRS in the study area, to significantly reduce the *An. arabiensis* population, provided that the females are eliminated early in the process (Benedict and Robinson, 2003).

Mating success in the released males will need to be monitored to achieve a successful SIT program. In this preliminary study, powered dye was used to mark the male mosquitos and it was investigated whether the dye would be transferred to the female mosquitos during copulation. The

results indicated inadequate transference of the powdered dye and a hindering of mating success. Alternate dye methods should thus be investigated since the powdered-dye procedure did not succeed. The dye method and the type of dye used should be easy to implement and also cheap due to financial constraints within the sub-Saharan African region. Helinski et al. (2008) suggested the use of a nitrogen-stable isotope semen label that can be used to detect inseminated females. The isolation of the female after mating did not result in the loss of the label and this marking method could thus be used as an alternative to dyes. However, the cost effectiveness of this approach will need to be evaluated before implementation.

5.5 Conclusions and recommendations

This study indicated that larval-rearing densities negatively affect larval survivorship and adult male body size within each of the three *An. arabiensis* strains. However, size of the male *An. arabiensis* did not affect their fertility as mating success was not hindered. Female fecundity was also not affected by larval population densities, but was affected by the strain of *An. arabiensis* tested. The Old Mamfene laboratory strain displayed the best survival and female fecundity when compared to the wild strain (New Mamfene) and the genetically modified strain (GSS). The Old Mamfene strain also displayed the highest insemination rates and female fecundity within the high larval density cohort. Therefore, the Old Mamfene strain should be considered for the mass-production of *An. arabiensis* males for SIT. However, since this study was conducted within a controlled laboratory environment, the results need to be verified in the field. A detailed field study should thus be conducted to confirm that the laboratory-reared males can compete successfully against their wild counterparts.

SIT is an important, alternative method for mosquito vector control in northern KwaZulu-Natal, especially since the *An. arabiensis* population tested in the Mamfene region is developing resistance to DDT and pyrethroids. These two insecticides display the same mechanism of action and resistance to these two chemical classes could result in cross-resistance to other classes with the same mode of action.

In conclusion, the following recommendations are offered:

i. Studies on the effects of larger larval population densities should be conducted. Since SIT requires high insect production and massive releases of sterile males, larval population densities are likely to exceed 500 larvae per 500ml of distilled water. Also, there appeared to be a trend of decreasing insemination rates with increasing larval population densities, although these results were not statistically significant at the levels tested.

ii. Due to laboratory culturing, genetic bottlenecking can occur and lead to homogenous mosquito colonies. Therefore, investigations should be conducted on the genetic variance between the laboratory strain (Old Mamfene) and the wild strain (New Mamfene) of *An. arabiensis* to assess the extent of this.

iii. The influence and impact of the environment (i.e. temperature and humidity in the field) on the survival and mating competitiveness of the laboratory strain versus the wild strain of *An. arabiensis* must be investigated, to confirm the strain that is best suited for SIT in the study site. This can be achieved with a detailed comparative study on the field ecology of the two strains.

iv. Given the failure of the powder-dye marking method, alternative methods to track the released males and inseminated females should be investigated, so that the efficacy of the released males can be monitored.

v. Insecticide resistance must be monitored continuously in the study area so that an integrated malaria control program, which utilizes SIT and IRS, can be devised and implemented effectively.

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APPENDIX

Appendix 1: Numbers of anopheline mosquitoes collected throughout 2012 in the Mamfene area.[1]WT: Window Trap; HLC: Human Landing Collection

Month (2012)	Collection method[1]	Total no. collected	*An. arabiensis*	*An. quadriannulatus*	*An. merus*	Other anophelines
January	WT + HLC	58	34	1	0	10
February	WT + HLC	140	55	0	0	84
March	WT + HLC	63	0	1	0	63
April	WT + HLC	22	0	0	0	20
May	WT + HLC	42	40	0	1	2
June	WT + HLC	82	0	0	0	82
July	WT + HLC	3	0	0	0	3
August	WT + HLC	2	0	0	0	2
September	WT + HLC	3	0	0	0	3
October	WT + HLC	23	12	4	0	7
November	WT + HLC	19	13	0	0	7
December	WT + HLC	77	49	0	0	28
Total		534	203	20	1	311