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

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

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

<table>
<thead>
<tr>
<th>Target Pest</th>
<th>Study area</th>
<th>Aim</th>
<th>Result</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>New World screwworm fly (Cochliomyia hominivorax Coquerel)</td>
<td>North America, Central America and Panama</td>
<td>Population eradication</td>
<td>Successfully eradicated (1957 - 1966)</td>
<td>Bartlett and Staten, 1996; Dame et al., 2009</td>
</tr>
<tr>
<td></td>
<td>Mexico</td>
<td>Population eradication</td>
<td>Successfully eradicated (1972 - 1991)</td>
<td>Bartlett and Staten, 1996; Dame et al., 2009</td>
</tr>
<tr>
<td>Melon fly (Dacus cucurbitae Coquillet)</td>
<td>Okinawa, Japan</td>
<td>Population eradication</td>
<td>Successfully eradicated in 1990s</td>
<td>Hendrichs, 2000</td>
</tr>
<tr>
<td></td>
<td>South Africa, Hex River Valley</td>
<td>Population suppression</td>
<td>Population reduction was observed (1997 – 2006)</td>
<td>Barnes et al., 2015</td>
</tr>
</tbody>
</table>
| **Mexican Fruit Fly**  
*Anastrepha ludens* Loew | Along the USA-Mexican border | Preventing migration (quarantine) | Migration has successfully been prevented since 1964 | Hendrichs, 2000 |
|--------------------------|-----------------------------|----------------------------------|--------------------------------------------------|----------------|
| **Codling moth**  
| **False codling moth**  
1.12 Sterile Insect Technique for mosquitoes

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

<table>
<thead>
<tr>
<th>Mosquito species</th>
<th>Study area</th>
<th>Aim</th>
<th>Result</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>An. quadrrimaculatus</em></td>
<td>South Florida, USA (1959-1960)</td>
<td>Population reduction</td>
<td>There was a change in mating behavior of the colonized sterile males which resulted in lack of sterility in wild population and consequently programme failure.</td>
<td>Dame <em>et al.</em>, 2009</td>
</tr>
<tr>
<td><em>An. gambiae</em></td>
<td>Burkina Faso (1968-1969)</td>
<td>Population reduction</td>
<td>Released sterile males lacked competitiveness and there was no significant effect on sterility of eggs.</td>
<td>Davidson <em>et al.</em>, 1970</td>
</tr>
<tr>
<td></td>
<td>Egypt (1942-1945)</td>
<td>Population eradication</td>
<td>Population was successfully eradicated</td>
<td>Shousha, 1948</td>
</tr>
<tr>
<td><em>An. albimanus</em></td>
<td>El Salvador (1977-1979)</td>
<td>Population reduction</td>
<td>No effect was observed due to immigration of females from untargeted areas.</td>
<td>Weidhaas, 1974</td>
</tr>
<tr>
<td><em>An. arabiensis</em></td>
<td>Austria (2006)</td>
<td>Evaluating the effect of irradiation (0 - 100 Gy) on adult emergence, male survival, induced</td>
<td>All doses tested had no effect on adult emergence. Survival of irradiated males was similar or slightly higher than non-irradiated males. There was negative immunity.</td>
<td>Helinski <em>et al.</em>, 2006a</td>
</tr>
<tr>
<td>Study Area</td>
<td>Main Activity</td>
<td>Outcome</td>
<td>Reference</td>
<td></td>
</tr>
<tr>
<td>----------------------------</td>
<td>----------------------------------------------------</td>
<td>---------------------------------------------------------------------------------------------------</td>
<td>-----------------------</td>
<td></td>
</tr>
<tr>
<td>Merowe area, Sudan (2014)</td>
<td>Insemination capability.</td>
<td>Irradiated males were able to inseminate wild females equally the same as wild males.</td>
<td>Ageep et al., 2014</td>
<td></td>
</tr>
<tr>
<td>New Delhi/ India in 1971</td>
<td>Assessing the capability of released sterile males in swarming participation.</td>
<td>Irradiated males were able to participate in swarming and dispersed for a long distance.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cx. quinquefasciatus</td>
<td>Population reduction</td>
<td>Sterile males were competitive and induced sterility in wild population but immigration of fertile females from other breeding sites reduced programme efficiency.</td>
<td>Dame et al., 2009</td>
<td></td>
</tr>
<tr>
<td>Cx. tarsalis</td>
<td>Population reduction and assessment of mating behaviour</td>
<td>A change in mating behavior due to colonization (Assortative mating) was observed as a result there was no population reduction.</td>
<td>Reisen et al., 1982</td>
<td></td>
</tr>
<tr>
<td>Species</td>
<td>Location</td>
<td>Method</td>
<td>Outcome</td>
<td>Reference</td>
</tr>
<tr>
<td>-------------</td>
<td>------------------------------</td>
<td>-----------------------</td>
<td>--------------------------------------------------------------------------------------------------------------------------------------------</td>
<td>-------------------</td>
</tr>
<tr>
<td><em>Cx. pipiens</em></td>
<td>France (1970)</td>
<td>Population reduction and semi sterility</td>
<td>Population reduction was observed and population was eventually eliminated.</td>
<td>Laven et al., 1972</td>
</tr>
<tr>
<td><em>Ae. aegypti</em></td>
<td>New Delhi, India in 1971</td>
<td>Population eradication</td>
<td>The project never materialized due to political interference. There were false accusations that the project was intended to collect biological warfare data.</td>
<td>Klassen, 2009</td>
</tr>
<tr>
<td></td>
<td>Pensacola, Florida, USA (1960-1961)</td>
<td>Population reduction</td>
<td>Wild population was observed in contracted release areas but unexpected immigration of fertile females from other breeding sites reduced the effect.</td>
<td>Dame et al., 2009</td>
</tr>
</tbody>
</table>
To date no active SIT programs are in place in South Africa for public health pests such as mosquitoes. Currently there are laboratory and field studies being conducted to ascertain the feasibility of using SIT to control *An. arabiensis*. One of the sites being used to evaluate this technique is Mamfene in northern KwaZulu-Natal (KZN). Mamfene was chosen because it supports a seasonal population of *An. arabiensis* and is in a malaria endemic area.

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

### 1.13 Study rationale

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

### 1.14 Study aim and objectives

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

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

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

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

4) Determine the population dynamics of *An. arabiensis* at Mamfene, KwaZulu-Natal, South Africa.
CHAPTER 2

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

2.1 Introduction

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

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

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

2.2 Materials and methods

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

2.3 Results

The results of this chapter are presented in the manuscript attached in section 2.3.1.
2.3.1 **Leonard C Dandalo**, Givemore Munhenga, Maria L Kaiser and Lizette L Koekemoer

1) **Contribution**

I conducted all the experiments outlined in the paper, did data analysis and interpretation of results, and wrote the first and subsequent revised drafts of the manuscript.
Development of a genetic sexing strain of Anopheles arabiensis for KwaZulu-Natal, South Africa

L. C. Dandalo1,2, G. Munhenga1,2, M. L. Kaiser1,2 and L. L. Koekemoer1,2

1Wits Research Institute for Malaria, MRC Collaborating Centre for Multi-Disciplinary Research on Malaria, School of Pathology, Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, South Africa and 2Vector Reference Laboratory, Centre for Emerging Zoonotic and Parasitic Diseases, National Institute for Communicable Diseases, National Health Laboratory Services, Johannesburg, South Africa

Abstract. An efficient sexing system is important for the release of sterile males for any control programme using the sterile insect technique. This study describes the development and characterization of a new genetic sexing strain from South Africa (GMK), needed for the planned implementation of such a programme in northern KwaZulu-Natal Province. The base colony used was a locally modified laboratory strain of Anopheles arabiensis containing a sex-linked gene conferring dieldrin resistance to male mosquitoes. Female A. arabiensis mosquitoes from northern KwaZulu-Natal were mated with these males and backcrossed to introduce the dieldrin resistance gene to the Y chromosome. The resulting strain therefore had an overall genotype representing the local population but with the Y chromosome containing the dieldrin resistance gene. Life-history characteristics, stability of the sex-linked resistance marker, and reduction in dieldrin waste were investigated. The strain showed semi-sterility exhibited by low egg hatch rates, faster development in the immature stages and longer adult survivorship compared with the parental strains. While the GMK strain carrying the dieldrin-resistant gene was successfully established, the stability of the gene is limited, requiring periodic purification. Dieldrin waste can be limited by treating many more eggs than currently recommended.

Key words. Anopheles arabiensis, Genetic sexing strain, sterile insect technique.

Introduction

The South African National Malaria Control Programme aims to eliminate malaria transmission within its borders by 2018 (Malaria Elimination Group, 2009). Some malaria-affected districts, such as Mamfene in northern KwaZulu-Natal (KZN), have a malaria case incidence of <1/1000 population at risk, meeting the World Health Organization (WHO) criteria for pre-elimination and suggesting that malaria control initiatives can now be shifted towards malaria elimination (Maharaj et al., 2013). However, malaria control activities, particularly current vector control interventions using indoor residual spraying (IRS), are insufficient and unlikely to achieve malaria elimination without supplementation (Maharaj et al., 2013). This is because IRS predominantly targets indoor biting and resting mosquitoes and is not very effective against vectors that feed and rest outdoors, such as Anopheles arabiensis (Gillies & Coetsee, 1987). Therefore, there is a need for additional vector control strategies to supplement existing tools; the sterile insect technique (SIT) was identified as one such tool and is under investigation.

Investigation of the feasibility of a SIT programme as a malaria vector control technique in South Africa was initiated in 2011 (Munhenga et al., 2011). Over the past 6 years, extensive baseline studies on the population dynamics of wild target A. arabiensis populations have been carried out in the Kruger National
Park and northern KZN, culminating in the identification of a pilot release site (Munhenga et al., 2011, 2014). In addition, mating competitiveness of representative laboratory strains has been established (Munhenga et al., 2016). Apart from this baseline information, there are other aspects of the SIT programme that require optimization before pilot releases can be done. One critical step is the development of a sexing system to exclusively obtain males for sterilization and releases (Papathanos et al., 2009). In a mosquito SIT programme, it is critical that female releases are avoided due to their capacity as disease vectors (Alphby et al., 2010). Moreover, male-only releases increase SIT programme efficiency because released males will only focus on mating with wild females. Therefore, it is essential to have an efficient sex separation method for any mosquito SIT programme.

There are various methods potentially available for separating males and females during mosquito SIT mass productions (Benedict & Robinson, 2003). Unfortunately, most of these sex separation methods, such as pupal sexual dimorphism and protandry, are species-specific and cannot be directly transferred to A. arabiensis where pupal size and sex developmental differences are not significant enough, making sexing using these approaches difficult (Papathanos et al., 2009). The use of sex-sorting systems based on the sex linkage of a readily selectable gene, also known as genetic sexing mechanisms (GSS), arguably remains the best approach currently available. This technique is based on the fact that anopheles males are heterogametic for sex-determining chromosones. Therefore, translocation between the male-determining Y chromosome and an autosomal region containing conditional lethal genes can be used as a selectable marker. This, in turn, will allow the production of mosquitoes under conditions that genetically favour production of males, consequently providing the basis for separating males from females (Curtis et al., 1976). This approach was used to develop a number of sexing strains in anopheles (Curtis et al., 1976; Kaiser et al., 1978); however, most of these strains are no longer available for use. Using the same principle, a GSS for A. arabiensis called ANO IPC1 was developed in 2008 at the Insect Pest Control Laboratory of the International Atomic Energy Agency (Yamada et al., 2012). This GSS strain has been evaluated for reliability in female elimination, radiation sensitivity and mating competitiveness (Oliva et al., 2012; Yamada et al., 2014) and shows potential as a candidate for use in a SIT programme. The greatest challenges facing this strain are environmental concerns, stability and productivity concerns associated with its use (Yamada et al., 2013a, 2015; G. Munhenga, unpublished data). Furthermore, this strain cannot be directly transferred for SIT releases under a different country setting because it contains an exclusively Sudanese genetic background. If directly released in a different geographical setting besides Sudan there might be challenges of mating compatibility and competitiveness between the strain and resident wild populations. There is also the danger of driving through foreign genomes such as insecticide-resistant alleles and superior vector competence characteristics in the release country. Before introducing a strain with a different genome to the wild population in areas where releases are planned, it is necessary to introduce the local genetic background to the strain being targeted for release. This study describes the introgression of a dieldrin resistance mutation (rdd) selectable marker into a local A. arabiensis strain with the objective of maintaining an indigenous genetic make-up in the sexing strain. Concurrently, experiments to minimize the impact of releasing a persistent bio-accumulative toxicant such as dieldrin into the environment were carried out by reusing the dieldrin solution and increasing larval and egg batch sizes to be treated. Additionally, as the major drawback with sexing systems using chromosomal translocation is instability due to unlinking of the selectable marker from the Y chromosome, which results in production of recombinants (Kerremans & Franz, 1995), this work assessed the stability of the dieldrin resistance marker on the Y chromosome of the newly established strain.

Materials and methods

Biological material

Two local A. arabiensis laboratory strains were used to develop the GSS strain. The first strain (GAMA) was developed by the introgression of the Sudanese A. arabiensis GSS ANO IPC1 males (carrying the dieldrin resistance rdd gene on the Y chromosome) with A. arabiensis females colonized from material collected in 2010 from the Kruger National Park (Munhenga et al., 2016). The GAMA strain had not been purified for the rdd gene for a number of generations prior to this study. A second strain (KWAG) is an A. arabiensis strain with rdd susceptible genotype that was colonized in 2005 from Mamtene, KZN (Mouatcho et al., 2009). Both colonies are maintained at the Vector Control Reference Laboratory (VCRL) in Johannesburg under standard insectary conditions as described by Hunt et al. (2005). Data for the introgressed line (GMK) and the first backcross (GMK1) are presented in this paper.

Establishment of the genetic sexing strain, GMK

Introgressed line (GMK). To remain with GAMA males only, all females were eliminated at the larval stage using procedures described by Yamada et al. (2012). A total of 35 replicates (23 replicates of 50 second/third-instar larval batches, 11 replicates of 25 fourth-instar larval batch and one replicate of nine fourth-instar larval batches) were exposed to 50 mL of a 0.1 p.p.m. dieldrin solution for 2h. Immediately after the 2h dieldrin exposure, larvae were rinsed in distilled water and transferred into clean larval-rearing bowls and reared through to adulthood. In total, 218 GAMA males were obtained after dieldrin exposure and these males were mated with an equivalent number of virgin KWAG females to establish the GMK strain. The KWAG females used were separated at the pupal stage using terminaliae morphology (Harbach, 2016) to ensure their virginity.

Purification of GMK strain. GMK was reared for four generations to obtain enough material to establish GMK1. After rearing for four generations, all GMK1F2 eggs were treated with 50 mL 2 p.p.m. dieldrin, using the procedure described by
Yamada et al. (2012). After treatment, the eggs were reared to adults. Males emerging after this treatment were backcrossed with virgin KWAG females. The resultant strain was named GMK.<sub>1</sub>.

Life-history characteristics of A. arabiensis laboratory strains

Egg batch rates. Egg hatch rates for the introgressed lines were monitored per generation and the pooled egg hatch rate data were compared against the baseline colonies. To determine egg hatch rates, 50 eggs from each colony of GMK<sub>0</sub> (generations F<sub>1</sub>–F<sub>3</sub>), GMK<sub>1</sub> (F<sub>3</sub>–F<sub>6</sub>), GAMA and KWAG were transferred into plastic bowls containing 150 mL distilled water and allowed to hatch. Upon hatching, first-instar larvae were counted daily by transferring to new rearing bowls for 10 consecutive days. These experiments were repeated three times and mean egg hatch rates were calculated.

Comparison of developmental time of GMK<sub>1</sub>F<sub>4</sub> against GAMA and KWAG immature/semiaquatic stages. A total of 100 first-instar larvae (L1) from each strain—GMK, F<sub>4</sub>, GAMA and KWAG—were transferred into different plastic bowls (27 cm x 16 cm x 6.5 cm) containing 150 mL distilled water and fed on a mixture of finely crushed dog biscuit and brewer’s yeast at a daily rate per tray until pupation (days 1 and 2, 30 mg; days 3 and 4, 60 mg; days 5 and 6, 120 mg; days 7–12, 180 mg). The time to pupation and number pupating were recorded daily. The pupae were immediately transferred into plastic urine jars (80 mm x 80 mm x 80 mm) containing distilled water and placed into 30 cm x 30 cm x 30 cm BugDorm<sup>®</sup> Insect Rearing Cages (Mega View Science Co., Ltd, Taiwan, China) for adult emergence. Time taken to emerge, number of pupae emerging into adults, and the sex of adults were recorded daily basis. The overall mean number of days taken to reach the next developmental phase (L1 to pupae and adult emergence) was calculated. The effect of variable insectary conditions was minimized by rotating positions of the trays on a daily basis. Three replicates for each strain were set up.

Adult male survivorship of GMK<sub>1</sub>F<sub>4</sub>, GAMA and KWAG strains. During this objective, only male longevity was investigated as they are the active agents of a mosquito SIT programme. Batches of 50 newly emerged adult males from the three strains (GMK, F<sub>4</sub>, GAMA and KWAG) were placed separately in BugDorm<sup>®</sup> cages in three replicates to monitor adult survivorship. These were maintained on 10% sugar solution at standard insectary conditions. Survival in each cage was assessed daily by counting the number of dead individuals until 100% mortality was reached. Female adult longevity was not investigated because females are not the active agents of a mosquito SIT programme.

Optimizing deltamethrin use to reduce its waste

Due to the toxic effects of deltamethrin (WHO, 2003), attempts were made to minimize the amount of deltamethrin waste generated during egg/larval treatments by carrying out experiments to improve the standard protocols for egg and larval deltamethrin treatments developed by Yamada et al. (2012). GAMA larvae were used during these experiments as the GMK strain was not large enough during this phase of the project.

To evaluate the effectiveness of reusing deltamethrin during larval treatments, procedures for larval treatments described by Yamada et al. (2012) were employed, except that the deltamethrin solution was reused up to four times, i.e. four different treatments. For each treatment, 50 second/third-instar or 25 fourth-instar larvae batches were used. The first treatment used freshly prepared deltamethrin (first use) and this was repeated 27 times (14 replicates of 50 second/third-instar larvae batches and 13 replicates of 25 fourth-instar larvae batches). The second treatment used deltamethrin which had been used for the first treatment (second use) and was repeated 10 times (five replicates of 50 second/third-instar larvae, one replicate of 34 second/third-instar larvae and four replicates of 25 fourth-instar larvae batches). The third treatment used deltamethrin residues from the second treatment (third use) and was carried out 11 times (eight replicates of 50 second/third-instar larvae batches and three replicates of 25 fourth-instar larvae). The final treatment reused deltamethrin for the third time (fourth use) and comprised of five replicates of 50 instar larvae per treatment batch. For each treatment, the numbers of male and female adults emerging were recorded.

The second part of this objective tested the maximum number of eggs that could be treated with the same volume and concentration of deltamethrin used by Yamada et al. (2012). This was carried out concurrently with the strain purification process described earlier. Eggs were treated with 50 mL of 2 p.p.m. deltamethrin in batches of 500, 1000, 2000 or 3000. For the first treatment, newly counted 500-egg batches were treated with: (a) freshly prepared deltamethrin (first use); (b) first reused deltamethrin (second use); and (c) second reused deltamethrin (third use). The same setup was used for the subsequent treatments but the only variation was the number of eggs being treated. The second treatment used 1000-egg batches, the third treatment used 2000-egg batches and lastly the fourth treatment used egg batches within the range of 2000–3000. Three replicates were conducted for each treatment. The numbers of males and females emerging after each treatment were recorded.

Genetic stability of each strain

To confirm successful introgression and monitor recombination, the presence of the rdl mutation was checked for each generation. A random subsample of 10–30 males and females from each GMK<sub>0</sub> generation (generations 1–8) were randomly selected and tested for the presence or absence of the rdl mutation using the hydropysis probe assay as described by Bass et al. (2008). Briefly, DNA was extracted from one leg per mosquito sample using the ZyGEM prepGEM insect DNA extraction kit (Cat: PIN14106, ZyGEM NZ Ltd., Ruakura, New Zealand) following the manufacturer’s protocol except that the reaction volume was quartered. In some instances, the extraction method described by Collins et al. (1987) was used. The presence of the
Statistical analysis

Data on egg hatch rates, adult emergence, number and developmental time of immature stages and male adult longevity were summarized as mean proportion of eggs hatching, mean % of adults emerging, mean proportion of immature stages developing to the next developmental stage, mean survivorship to the next developmental stage and mean male adult survivorship, respectively. Percentage values for larval and pupal survivorship and percentage of females emerging were checked for normality and transformed where applicable to achieve normal distribution. Where this was not possible non-parametric tests were used to compare difference in proportions. A Student's independent t-test in spss was used to analyse differences in mean egg production between the two baseline colonies (KWAG and GAMA), and for comparison between introgressed strains (GMK\textsubscript{G} and GMK\textsubscript{K}) and the two parental strains (KWAG and GAMA) a one-way ANOVA was used to compare differences. A univariate general linear model (two-way ANOVA) was used to compare the interaction between egg batch size and rank of dieldrin treatment. Survival curves were analysed using Kaplan–Meier survival analysis in spss and Cox’s F-test was used to compare mean difference in survivorship between KWAG, GAMA and GMK\textsubscript{K}, male cohorts. In all cases a P-value < 0.05 was considered to indicate statistical significance.

Results

The establishment of a local introgressed line (GMK\textsubscript{G}) used a total of 1409 GAMA larvae, which were treated with dieldrin. Of the treated larvae only 15.5% survived to the adult stage. After rearing dieldrin-treated larvae through to adults, the proportion of males emerging ranged from 80% to 100% with a mean male emergence of 96.7%.

Purification of GMK\textsubscript{K} was initiated on the fourth filial generation (F\textsubscript{4}) to produce GMK\textsubscript{K}. After the purification process there was a reduction in egg hatch rate from a mean of 29.2% in GMK\textsubscript{K} to 19.2% in GMK\textsubscript{K} (Table 1). This difference was statistically significant (independent samples test, d.f. = 4, T = 5.7, P < 0.05).

Life-history characteristics of A. arabiensis laboratory strains

The mean egg hatch rates for the newly introgressed strains GMK\textsubscript{G} and GMK\textsubscript{K} were lower than those of the two parental strains of KWAG (80.7%) and GAMA (42%), (Table 2). This difference was statistically significant (one-way ANOVA, d.f. = 3, F = 87.2, P < 0.05). Pairwise comparison using Bonferroni showed that mean egg hatch rates of the introgressed strains GMK\textsubscript{G} and GMK\textsubscript{K} were different from those of the parental strains KWAG and GAMA (P < 0.05). The mean egg hatch rates for KWAG and GAMA were statistically different from each other (P < 0.05).

The mean developmental time from L1 to pupation and adult emergence was investigated. GMK showed the shortest developmental time from L1 to pupation (~9 days), and GAMA had the longest developmental time (~11 days) (Table 2). Statistically the difference in larval development to pupation was significant between the three strains (χ² = 160.68, d.f. = 2, P < 0.05). Pairwise comparisons showed that GMK developmental time to pupation was significantly shorter than those of GAMA (χ² = 155.06, P < 0.05) and KWAG (χ² = 38.38, P < 0.05), whereas GAMA larval developmental time was significantly longer than that of KWAG (χ² = 34.7, P < 0.05). Overall, it took an average of 12 days for GMK to develop from L1 to the adult stage, whereas KWAG and GAMA took 13 and 14 days, respectively. Statistically, the difference in overall developmental time from L1 to the adult stage between the three strains was significant (χ² = 181.83, d.f. = 2, P < 0.05). Post hoc analysis showed that GMK developmental time to adulthood was significantly shorter than those of KWAG (χ² = 93.22, P < 0.05) and GAMA (χ² = 159.68, P < 0.05). However, this analysis showed no statistically significant difference in the developmental time to adulthood between GAMA and KWAG (χ² = 3.22, P = 0.07).

The mean percentage of mosquitoes surviving to the next developmental stage as well as the sex ratio of the resultant adults were investigated for the three strains. The mean percentage of larvae surviving to pupation was greater in the introgressed strains (GMK, and GAMA) than in the
Table 3. Survival rate of immature stages during development to the adult stage of introgressed *Anopheles arabiensis* strains (GMK and GAMA) and the corresponding baseline strain (KWAG), and sex ratio of emerging adults by strain.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Percentage survivorship from L1 to pupae (95% CI)</th>
<th>Percentage survivorship from pupae to adult (95% CI)</th>
<th>Sex ratio (95% CI) (number of males/total adults emerging)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GMK</td>
<td>80.0 ± 7.0 (63.0–96.0)</td>
<td>95.0 ± 0.4 (84.0–106)</td>
<td>0.45 ± 0.06 (0.31–0.59)</td>
</tr>
<tr>
<td>GAMA</td>
<td>80.0 ± 4.0 (69.0–90)</td>
<td>95.0 ± 0.6 (81.0–107)</td>
<td>0.53 ± 0.08 (0.35–0.72)</td>
</tr>
<tr>
<td>KWAG</td>
<td>59.0 ± 6.0 (43.0–75)</td>
<td>98.0 ± 0.3 (89.0–107)</td>
<td>0.53 ± 0.04 (0.44–0.61)</td>
</tr>
</tbody>
</table>

Different letters within columns show that the values were statistically different (*P* < 0.05). Data are shown as ± SD.

Fig. 1. Kaplan–Meier survivorship curves for GMK, GAMA and KWAG males. [Colour figure can be viewed at wileyonlinelibrary.com].

unintrogressed strain KWAG (Table 3). This difference was statistically significant (one-way ANOVA, d.f. = 2, *F* = 12.7, *P* < 0.05). Pairwise comparison showed that mean survival from larvae to pupae was not different between GMK and GAMA whereas there was a difference between KWAG and the two introgressed strains GMK and GAMA. There was a high mean percentage survival from pupa to adult in all three strains with no statistically significant differences in mean survival rates between the three strains (one-way ANOVA, d.f. = 2, *F* = 0.4, *P* = 0.68). The sex ratios (males: females) of adults emerging from GMK, GAMA and KWAG were 0.45, 0.53 and 0.53, respectively, for the three strains and there was no statistically significant difference in the sex ratio between the three strains (*χ²* = 4.4, d.f. = 2, *P* > 0.05).

Adult male longevity of the three strains was investigated and compared. The mean survival times for GMK, GAMA and KWAG males were 29, 12 and 23 days, respectively (Fig. 1). Male adult longevity experiments showed that GMK males survived significantly longer than both GAMA (*χ²* = 110.87, *P* < 0.05) and KWAG males (*χ²* = 16.146, *P* < 0.05).

Optimizing dieldrin treatments to reduce waste

Efficacy of dieldrin decreased, as evidenced by the decrease in males and increase in females emerging after each dieldrin reuse (Fig. 2). The mean percentage adult emergence by gender showed a significant difference between the different dieldrin treatments (one-way ANOVA, d.f. = 3, *F* = 4.02, *P* < 0.05). Bonferroni comparison showed two groups of mean proportion of male : female emergences which were not significantly different from one another (*α* = 0.05 and a critical *t*-value of 3.15). First dieldrin use belonged to the first group (high proportion of males emerging) while fourth dieldrin use belonged to the second group (high proportion of females emerging). However, second and third dieldrin uses overlapped between both groups. Pearson correlation analysis showed a positive correlation between dieldrin reuse and sex ratio of adult emerging after each respective dieldrin treatment. As the frequency of dieldrin reuse increased, the proportion of females emerging increased (Pearson correlation coefficient, *R* = 0.68).

Current protocols (Yamada et al., 2012) allow for treatment of eggs in batches of 500. In this study, the treatments of batches of 500, 1000, 2000 and 3000 eggs were investigated and the results are presented in Table 4. There was an interaction between egg batch size and rank of dieldrin used (Fig. 3). Generally, dieldrin efficacy decreased as the egg batch size increased above 1000 regardless of dieldrin rank used. More males emerged after treatment of egg batches up to 1000 eggs regardless of the dieldrin rank used. Thereafter, the proportion of males emerging decreased with increasing egg batch size. Statistically this interaction between egg batch size and rank of dieldrin used on the proportion of males emerging was significant [two-way ANOVA, *F* *(1,24) = 3.24, *P* < 0.05, partial *η²* = 0.14]. There was a strong relationship between dieldrin reuse and batch size on gender proportion (adjusted *R*² = 0.7). Post hoc analysis with
Table 4. Male *Anopheles arabiensis* GMK1 emergence after exposure of varying quantities of eggs to different dieldrin ranks.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Total number of eggs treated</th>
<th>Mean ± SD percentage of males emerging (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Egg batches</td>
<td>Dieldrin rank</td>
<td></td>
</tr>
<tr>
<td>500</td>
<td>First use</td>
<td>1500</td>
</tr>
<tr>
<td></td>
<td>Second use</td>
<td>1500</td>
</tr>
<tr>
<td></td>
<td>Third use</td>
<td>1500</td>
</tr>
<tr>
<td>1000</td>
<td>First use</td>
<td>3000</td>
</tr>
<tr>
<td></td>
<td>Second use</td>
<td>3000</td>
</tr>
<tr>
<td></td>
<td>Third use</td>
<td>3000</td>
</tr>
<tr>
<td>2000</td>
<td>First use</td>
<td>6000</td>
</tr>
<tr>
<td></td>
<td>Second use</td>
<td>6000</td>
</tr>
<tr>
<td></td>
<td>Third use</td>
<td>6000</td>
</tr>
<tr>
<td>2000–3000</td>
<td>First use</td>
<td>7500</td>
</tr>
<tr>
<td></td>
<td>Second use</td>
<td>7500</td>
</tr>
<tr>
<td></td>
<td>Third use</td>
<td>9000</td>
</tr>
</tbody>
</table>

Different letters within columns show that the values were statistically different (P < 0.05).

![Graph](image-url)

**Fig. 3.** Two-way ANOVA profile plot of male *Anopheles arabiensis* GMK1 emergence after exposure of varying quantities of eggs to different dieldrin ranks. [Colour figure can be viewed at Wileyonlinelibrary.com].

A Bonferroni correction showed that there were no significant differences in the number of females emerging between the 500 and 1000 batch sizes (P > 0.05). However, there was a significant difference in the number of females emerging at higher egg batch sizes. For dieldrin rank there was a significant difference in the number of females emerging between first use and all the other reuses (P < 0.05) and there was no significant difference between the second and third dieldrin ranks (P > 0.05).

**Genetic stability of the GMK1 strain**

The genetic stability of the newly established GMK1 strain was monitored in every filial generation by taking a subsample of males and females to check for the presence of the dieldrin resistance *rdl* marker using a standard PCR assay through generations F1–F8. The *rdl* allele was not detected in any of the females screened. The majority of the males screened were hemizygous resistant. However, a small proportion was susceptible (13.3%), most notably in the eighth generation (Table 5).

Table 5. *Rdl* genotype subsamples of *Anopheles arabiensis* males and females of the genetic sexing strain GMK1 drawn from generations F1–F8.

<table>
<thead>
<tr>
<th>Generation</th>
<th>Sex</th>
<th>Number tested</th>
<th>Susceptible genotype, SS (%)</th>
<th>Resistant (rdl) genotype, RS (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>Female</td>
<td>8</td>
<td>8 (100)</td>
<td>0</td>
</tr>
<tr>
<td>F1</td>
<td>Male</td>
<td>10</td>
<td>0</td>
<td>10 (100)</td>
</tr>
<tr>
<td>F2</td>
<td>Female</td>
<td>10</td>
<td>10 (100)</td>
<td>0</td>
</tr>
<tr>
<td>F2</td>
<td>Male</td>
<td>10</td>
<td>0</td>
<td>10 (100)</td>
</tr>
<tr>
<td>F3</td>
<td>Female</td>
<td>10</td>
<td>10 (100)</td>
<td>0</td>
</tr>
<tr>
<td>F3</td>
<td>Male</td>
<td>10</td>
<td>1 (10)</td>
<td>9 (90)</td>
</tr>
<tr>
<td>F4</td>
<td>Female</td>
<td>10</td>
<td>10 (100)</td>
<td>0</td>
</tr>
<tr>
<td>F4</td>
<td>Male</td>
<td>7</td>
<td>0</td>
<td>7 (100)</td>
</tr>
<tr>
<td>F5</td>
<td>Female</td>
<td>10</td>
<td>10 (100)</td>
<td>0</td>
</tr>
<tr>
<td>F5</td>
<td>Male</td>
<td>9</td>
<td>0</td>
<td>9 (100)</td>
</tr>
<tr>
<td>F6</td>
<td>Female</td>
<td>9</td>
<td>9 (100)</td>
<td>0</td>
</tr>
<tr>
<td>F6</td>
<td>Male</td>
<td>5</td>
<td>0</td>
<td>5 (100)</td>
</tr>
<tr>
<td>F7</td>
<td>Female</td>
<td>15</td>
<td>15 (100)</td>
<td>0</td>
</tr>
<tr>
<td>F7</td>
<td>Male</td>
<td>15</td>
<td>0</td>
<td>15 (100)</td>
</tr>
<tr>
<td>F8</td>
<td>Female</td>
<td>20</td>
<td>20 (100)</td>
<td>0</td>
</tr>
<tr>
<td>F8</td>
<td>Male</td>
<td>30</td>
<td>4 (13.33)</td>
<td>26 (86.66)</td>
</tr>
<tr>
<td>Total</td>
<td>Females</td>
<td>92</td>
<td>92 (100)</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>Males</td>
<td>96</td>
<td>5 (5.2)</td>
<td>91 (94.8)</td>
</tr>
</tbody>
</table>

**Discussion**

During this work, a genetic sexing strain for *A. arabiensis* containing a South African genetic background was successfully developed. In a typical genetic sexing strain, a mutation is used as a selectable marker for sex separation and the allele is linked to the male-determining factor through chromosomal translocations (Franz, 2005). The GSS strain developed is based on the dieldrin resistance *rdl* allele as a selectable marker and this is linked to the Y chromosome through chromosomal translocations (Yamada et al., 2012).

Genetic sexing based on insecticide resistance as a selectable marker and associated chromosomal translocations have previously been developed in a number of mosquito species (Curtis et al., 1976; Kim et al., 1987). The basic characteristic of a GSS are high levels of semi-sterility caused by segregation during meiosis which results in the production of both genetically balanced and unbalanced individuals; the unbalanced individuals are inviable and die early as embryos (Willhoft & Franz, 1996). Another important phenotypic characteristic of a GSS is that it is resistant to the insecticide used in the sexing programme. In this study, the newly developed sexing strain (GMK1) expressed most of the characteristics associated with such a GSS. The mean egg hatch rates for both strains of GMK (GMK1 and GMK1)
were lower than those of the parental strains, indicating intrinsic semi-sterility caused by complex chromosomal rearrangements which were inherited during the introgression process from one of the parental strains (GAMA). This semi-sterility, which is common in most GSS strains (Curtis, 1978; Yamada et al., 2012) poses serious productivity challenges during mass rearing as more eggs will be needed for dieldrin treatments in order to have enough males for sterilization and eventual releases. Additionally, low fertility levels of this strain are a cause of concern during mass production, as any contamination with a normal fertile strain can easily result in the loss of the sexing strain within a few generations because it can easily be outcompeted.

An ideal sexing system should effectively eliminate all females as early in the developmental cycle as possible (Franz, 2005). Results from this study showed that approximately 97% of the females in the newly developed GMK strain can be eliminated by dieldrin exposure at either the egg or larval stage, thus reducing the laborious process of eliminating females at the parental strain moves. The female elimination rate is lower than those obtained from similar GSS strains (Curtis et al., 1976; Kim et al., 1987; Yamada et al., 2012). The slightly lower female elimination rate obtained during this study might be attributed to reduced dieldrin efficacy due to reuse. During GMK establishment dieldrin solution was, in some instances, reused during dieldrin treatments, and we speculate that this might have resulted in treatment failure. Another possible explanation for a higher female emergence might be the treatment procedures used. It could be that some of the females that emerged where not adequately exposed to the dieldrin solution. These two hypotheses are supported by PCR genotyping of female survivors, which showed that they did not carry dieldrin resistance alleles.

In the evaluation of a GSS, it is important to consider the different developmental stage characteristics, as they have a bearing on the productivity and quality of a strain to be released. The life-history characteristics of the GMK strain showed a faster developmental time from the immature stages to adult emergence than did the parental strains. The relatively faster developmental rate for GMK observed in this study is an important characteristic from a mass-rearing perspective. Reduced developmental time in insects being mass-reared ultimately means reduced running costs and high turnaround time of adults during mass rearing. Another important characteristic observed was the proportion of mosquitoes surviving through to each developmental stage. The proportion of larvae surviving to pupation was higher in the GSS strains (GMK and GAMA) than in the wild-type strain KWAG. This faster development time seen in all immature stages shows that the proportion of egg hatching might be the only rate-limiting step during mass rearing using this strain.

In a SIT programme, released males need to survive longer in the field, as this increases their chance of mating with wild virgin females. The GMK, adult males survived longer than the parental strain males, which makes the GMK, strain a potential candidate for mass production and release, as these males are likely to survive long enough to mate with wild females. However, this still needs to be verified by conducting survival tests under field conditions.

The main concern of using insecticides as selectable markers for sex separation is to do with environmental issues associated with use of insecticides. The developed strain depends exclusively on the use of dieldrin to eliminate females. Dieldrin is a persistent organochloride that has the potential to indirectly affect other organisms through bio-accumulation in the environment (Pecotreccoli et al., 1975; WHO, 2005). Furthermore, it might pose serious health concerns and contamination of the mass-rearing facilities if not properly handled. Because of these negative effects, it is important that dieldrin residues and exposure to high concentrations of dieldrin are kept to a minimum.

In this study we explored ways of reducing dieldrin waste by increasing the number of eggs that can be treated, and explored the possibility of reducing the dieldrin concentration being used for treatments by reusing the dieldrin solution up to three times. The results showed that freshly prepared dieldrin is effective when treating egg batches of up to 2000 eggs, which are four times more than the standard protocol developed by Yamada et al. (2012). Reuse of dieldrin was effective up to egg batch sizes of 1000 for both second and third uses, with the number of females emerging not significantly different from the number emerging after using freshly prepared dieldrin solution. Although the results of this study are inconclusive and require further optimization, data show that dieldrin might be reused once when treating larvae and twice when treating eggs up to a maximum 1000 eggs. Therefore, with further optimization of the egg treatment procedures there is the potential to reduce dieldrin waste by increasing the size of egg batches. However, there are still a small percentage of females inevitably emerging. We suggest that these females can potentially be eliminated through the use of ivermectin. Females can be provided with bloodmeals spiked with ivermectin at a concentration of 7.5 p.p.m. to ensure total elimination within 4 days (Yamada et al., 2013b).

Strain stability is a major concern of any genetic sexing system. During mass rearing for SIT releases, insects are reared in their millions under stressful conditions and it is inevitable that the integrity of the strain will be compromised. This means that strains earmarked for a SIT programme should be periodically purified to maintain the selectable marker in the males. To our knowledge this is the first report concerning the use of molecular tools to monitor the stability of a selectable marker in mosquitoes. Previous studies relied on phenotypic expression of the selectable marker (Curtis, 1978), which is labour-intensive and in some instances unreliable at picking up heterozygous resistant males. In this study the stability of the rdl marker in males was monitored during each generation using PCR. Results showed that there were no recombinants observed in the first seven generations. In the eighth generation, 13.3% of males were genotyped as susceptible. Because no female recombinants were recorded, it is most likely that the sudden presence of susceptible males was due contamination by a susceptible strain housed in an adjacent room to the sexing strain. However, a definite conclusion could not be reached, as the number of samples analysed was relatively low and the number of generations available for monitoring is still limited. Nevertheless this result re-confirms the need for periodic purification of the strain, especially under mass-rearing conditions, to avoid accumulation of recombinants or strain contamination which might lead to strain deterioration. A positive observation in this study was that the rdl marker seemed to drift out of the population and was not transferred.
to females, suggesting that the dieldrin resistance is unlikely to spread to a wild target population.

Conclusion
The development of a GSS strain with a South African genetic background was successful. The attributes of this strain, such as fast development of the immature stages and high survival rates in both immature and adult stages, make it a good candidate for mass rearing for future SIT releases. However, there are a number of aspects, such as treatment procedures and genetic stability, that need further investigation and optimization.

Acknowledgements
Professor Basil Brooke is thanked for his comments on the manuscript. This project was funded by the International Atomic Energy Agency (research contract no. 17904; 19099 and SAF 501350141), the Industrial Development Corporation and the South African Nuclear Energy Corporation (Necsa) through its Nuclear Technologies in Medicine and the Biosciences Initiative (NTeMBI) – a national platform funded by the Department of Science and Technology – a Global Diseases Detection/CDC grant (U19GH006622-01 MAL01) and the National Research Foundation (NRF) through the Medical Faculty Research Endowment Fund (00140184612015121105RMVRL015) and a DST/NRF South African Research Chairs Initiative grant to Prof Maureen Coetzee and an NRF Rating Track Thuthuka Funding award 107428 awarded to Dr. Givemore Munhenga. Mr Malibongwe Zulu is thanked for helping with the GMK colony maintenance. We thank all staff and students at VCRL.

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© 2017 The Royal Entomological Society, Medical and Veterinary Entomology, doi: 10.1111/mve.12264


Accepted 30 June 2017

CHAPTER 3
MATING COMPETITIVENESS OF THE LOCAL STERILE GENETIC SEXING STRAIN MALES (GMK) OF ANOPHELES ARABIENSIS UNDER LABORATORY AND SEMI-FIELD CONDITIONS

3.1 Introduction

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

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

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

3.2.1 Study site

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

For all experiments in this section two *An. arabiensis* laboratory strains were used. The first genetic sexing strain (GSS) used is denoted GMK. This strain was developed by mating GAMA males with KWAG females. The GAMA strain is an introgression of *An. arabiensis* GSS ANO IPCL1 males, [carrying a dieldrin resistance gene on the Y chromosome provided by the Insect Pest Control Laboratory (IPCL), Seibersdorf, Austria] with *An. arabiensis* females colonized from material collected in 2010 from Kruger National Park acronymed AMAL (Munhenga *et al.*, 2011).
The KWAG strain is the wild *An. arabiensis* colonized from Mamfene, Kwa-Zulu Natal in 2005 (Mouatcho et al., 2009)]. Larvae were fed on a daily basis with approximately 30mg of larval food [(a mixture of brewer’s yeast (Vital Health Foods, South Africa) and finely ground dog biscuits (West’s traditional crunching biscuit treats, Martin and Martin, South Africa) prepared at a ratio of 1:3)]. All adults were fed on a 10% sucrose solution soaked on cotton pads. The females of all the strains in this insectary are mainly blood fed on anaesthetized guinea pigs twice a week for egg production (Ethical clearance M141023, Appendix 2A).

### 3.2.3 Mosquito separation

A standard sex separation method using terminalia morphology at pupae stage (Harbach and Knight, 1980) was used in all the experiments to ensure that the age of mosquitoes for both controls and treatments was relatively the same. In all cases 20–26 hours’ pupae were physically separated using stereomicroscope. GMK$_1$ males were obtained by physically separating male pupae from the GMK colony. Similarly, wild type KWAG male and female pupae were separated from the wild type KWAG colony to obtain males and females respectively.

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

### 3.2.4 Mosquito irradiations

Pupae aged 20-26 hours used during these experiments were exposed to ionizing (gamma) radiation using a Gammacell 220 (MDS Nordion, Ottawa, Canada) (Figure 3.2) that was thoroughly dose mapped (Appendix 3) to ensure that pupae evenly obtained the desired dose (Munhenga et al., 2016). In all experiments pupae were transported to an irradiation facility approximately 800m from the insectary in batches of 100-500 suspended in 150ml of distilled
water in a doughnut irradiation jig (Figure 3.3). For the laboratory and semi-field assays, the optimal irradiation dose obtained in section 3.2.5 was used to induce sterility to males.

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

Figure 3.3: Doughnut shaped irradiation jig used during pupal irradiations
3.2.5 Determining the optimal irradiation dose

To determine the optimal dose that induces sterility without affecting the mating competitiveness of adult males, the effect of ionising radiation on male pupae was assessed at five different doses based on dose-response curves for *An. arabiensis* obtained by Helinski et al., (2006a) where irradiation dose ranging from 25–100 Gy had little impact on pupal survival, adult longevity and fecundity of females mated with irradiated males. However, it is very important to determine the optimal irradiation dose because it has been shown that a lower irradiation dose results in insufficiently sterilized males whereas a high dose reduces competitiveness of sterilized males (Helinski et al., 2006a). Against this background, irradiations during this study were carried out at increasing doses of 0 (control), 65, 70, 75, 80 and 100 Gy. After irradiation, the irradiated males were mated with virgin wild type KWAG females and the adult emergence, adult longevity, female fecundity and egg hatch rates (fertility) were measured as fitness parameters to select the optimal dose. For each single irradiation dose, three replicates and two controls were set up. The first control (Baseline) consisted of wild type KWAG males mating with wild type KWAG females. This was a fertile control representing the wild type. The second (separation) consisted of unirradiated GMK₁ males mating with wild type KWAG females treated the same way as irradiated pupae except that they remained un-irradiated. This control was set up to estimate the effect of physical handling and stress exerted on pupae during sex separation.

i) Emergence

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

ii) Adult longevity

For each irradiation dose tested, fifty newly emerged adult males from the GMK₁ irradiated pupae [obtained from 3.2.5 (i)] were placed in BugDorm-1 Insect Rearing Cages®. Two controls were
also set up, the fertile and separation control as described in section 3.2.5 throughout all three replicates. Adults were maintained on 10% sugar solution soaked in cotton wool at standard insectary conditions. Survival in each cage was assessed daily by counting the number of dead individuals until 100% mortality was reached. The same experimental set-up was used for controls; wild type KWAG and unirradiated GMK\(_1\) males.

iii) Fecundity
Fifty newly emerged virgin wild type KWAG females (obtained from section 3.2.3) and 50 newly emerged irradiated GMK\(_1\) males from each dose [obtained from 3.2.5 (i)] were allowed to mate for 4 days in BugDorm-1 Insect Rearing Cages\(^\circledR\) (with three replicates) over a five-day period. After the second blood meal, females were left to digest blood for two days. On the second day 20 randomly selected females were individually tubed in oviposition glass vials with a damp filter paper disc to induce oviposition and left overnight (Choi et al., 2014). The following day, eggs laid by each female were counted using an Optical Glass Binocular Magnifier (Cat. No: DA-5, Donegam Optical Company, United States of America). Two controls were set for each replicate (i) 50 virgin males from wild type KWAG colony mating with 50 virgin females from wild type KWAG colony as a fertile control. (ii) 50 virgin GMK males from pupae handled as irradiated males except irradiation mated with 50 virgin wild type KWAG females (Separation control). In each treatment, the mean number of eggs laid was calculated (Total number of eggs laid divided by total number of females that laid eggs). Fecundity of females was compared between treatments and controls.

iv) Fertility
Eggs produced by each individual female from each treatment and controls were transferred into plastic bowls (27cm x 16cm x 6.5cm) containing 150ml of distilled water and allowed to hatch. Upon hatching, larvae were counted daily by removing any hatched larvae and recording for 10 consecutive days (Munhenga et al., 2016).
The larvae picked daily were then killed using hot water and discarded. The proportion of eggs hatching was determined and compared between treatments and controls.
3.2.6 Mating competitiveness of the irradiated GMK₁ males

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

The mating competitiveness of sterile GMK₁ males was assessed by looking at parameters such as insemination rates and fecundity of the recovered wild type KWAG females from laboratory and semi-field cages. The competitiveness values and expected egg hatch rates were computed using the Fried Index (1971). Mean egg hatch rate data for treatments and controls were used to compare the observed fertility rate in the competition groups with the controls to give a value of competitive index ‘C’ for each treatment cage, as described by Fried 1971, using the formula:

\[ C = \frac{N}{S} \times \frac{H_n - H_o}{H_o - H_s} \]

Where \( H_n \) = is the mean hatch rate for fertile control cages, \( H_s \) = mean hatch rates for sterile control cages, \( H_o \) = mean hatch rate for treatment cages, \( N \) is the number of “normal” males (untreated) and \( S \) is the number of sterile males. Competitive values normally range from zero to one where one indicates full competitiveness.

i) Competitiveness of irradiated GMK₁ males under laboratory conditions

The sterile and fertile males were introduced into 5 liter cages (Figure 3.4) containing virgin females under the following ratios of sterile to fertile males: The mating competitiveness of irradiated mosquitoes under laboratory conditions was determined by releasing these mosquitoes in 5 litre cages using a mechanical aspirator in the following ratios:
Cage 1- Control fertile: 50 wild type (WT) KWAG females + 50 WT KWAG males

Cage 2- Sterile control: 50 WT KWAG females + 50 irradiated GMK₁ males

Cage 3- Treatment 1:1:1: 50 WT KWAG females + 50 WT KWAG males +
  50 irradiated GMK₁ males

Cage 4- Treatment 1:1:3: 50 WT KWAG females + 50 WT KWAG males +
  150 irradiated GMK₁ males

Cage 5- Treatment 1:1:5: 50 WT KWAG females + 50 WT KWAG males +
  250 irradiated GMK₁ males

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

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

Egg hatch rates (fertility) and insemination rates were determined as described in sections 3.2.5 and 3.2.6 respectively. This set of experiments was repeated three times.
ii) Competitiveness of irradiated GMK₁ males under semi-field conditions

A study of mating competitiveness of irradiated GMK₁ males under natural conditions in field cages was carried out to determine their mating vigour. Irradiated GMK₁ males, wild type KWAG males and wild type KWAG females were transported to appropriate site approximately 250 meters away from the Botha DeMeillon insectary as adults. These experiments were conducted from late spring (November) through summer (December) to late autumn (May) in large field cages. Temperature and humidity were monitored continuously using HOBO data loggers (Cat No: 19088743, Onset Computer Corporation, United States of America). These cages allowed simulation of prevailing ambient weather under natural conditions. There were two types of mosquito resting surfaces/containers in each cage. The first one was a wooden resting box (30cm x 30cm x 30cm) lined with black felt with one side having a hinged cover to allow mosquito entry. The box was covered with damp blankets to maintain a reasonable humidity (Figure 3.1C). The second was a cylindrical tube (45cm long by 15cm diameter), made of black felt rolled around a black wire mesh (Figure 3.1D).

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

Cage 1- Control fertile: 200 WT KWAG females + 200 WT KWAG males
Cage 2 - Sterile control: 200 WT KWAG females + 200 irradiated GMK₁ males

Cage 3 - Treatment 1:1:1: 200 WT KWAG females + 200 WT KWAG males + 200 irradiated GMK₁ males

Cage 4 - Treatment 1:1:3: 200 WT KWAG females + 200 WT KWAG males + 600 irradiated GMK₁ males

Cage 5 - Treatment 1:1:5: 200 WT KWAG females + 200 WT KWAG males + 1000 irradiated GMK₁ males

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

3.2.7 Statistical analysis

Data on adult emergence, longevity, fecundity, fertility, number of mated females and number of females recovered after mating were summarized as mean number of adults emerging, mean number of mosquitoes surviving after mating, mean number of eggs produced, mean proportion of eggs hatching, mean number of mated females and mean number of females recovered after mating. One-way ANOVA was used to analyze differences between treatments and controls. The data on adult survivorship, emergence and insemination rates of females was not normally distributed and normality test was conducted to ensure normal distribution before comparing their means using one-way ANOVA. Kaplan Meier survival analysis was used to plot survival curves and Cox’s F test was used to compare mean difference in survivorship between treatments. In all cases data was analyzed in SPSS version 22 and a P-value of less than 0.05 was considered to indicate statistical significance. The competitiveness value and expected egg hatch rates were computed using methods described by Fried (1971).