Development of an *Anopheles arabiensis* sex separation strain and optimisation of mosquito handling, packaging and transport conditions for the South African Mosquito Sterile Insect Technique programme

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

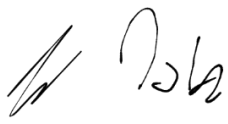
Doctor of Philosophy (PhD) in Medicine

School of Pathology, Faculty of Health Science, University of the Witwatersrand

Johannesburg, 2023

DECLARATION

I, Thabo Mashatola, declare that this dissertation is my work. It is being submitted for the degree of Doctor of Philosophy (PhD) in Medicine at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other University.



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…… 25th …. day of …… March ……., 2024

PUBLICATIONS AND PRESENTATIONS ARISING FROM THESIS

Publications

1) Ntoyi, N.L., **Mashatola, T**., Bouyer, J., Kraupa, C., Maiga, H., Mamai, W., Bimbile-Somda, N.S., Wallner, T., Carvalho, D.O., Munhenga, G. and Yamada, H., 2022. Life-history traits of a fluorescent *Anopheles arabiensis* genetic sexing strain introgressed into South African genomic background. *Malaria Journal*, 21(1), pp.1-12.

   Role: I contributed to the data analysis and the writing of all manuscript versions.

2) **Mashatola, T**., Ndo, C., Koekemoer, L.L., Dandalo, L.C., Wood, O.R., Malakoane, L., Poumachu, Y., Lobb, L.N., Kaiser, M., Bourtzis, K. and Munhenga, G., 2018. A review on the progress of sex-separation techniques for sterile insect technique applications against *Anopheles arabiensis*. *Parasites and Vectors*, 11(2), pp.127-137.

   Role: I performed the literature review, conducted the experiments on pupal sexual dimorphism, blood spiking and exploitation of *tsl* as a potential selectable marker, collected and analysed the data, and produced the first and final version of the manuscript.

Conferences and meetings

1) **Mashatola, T**., Koekemoer, L.L., Bourtzis, K., Munhenga, G. Expanding the South African sterile insect technique project's toolbox of sex separation systems for the malaria vector *Anopheles arabiensis.* 23rd Entomological Conference of Southern Africa, 11 – 14 July 2023, Stellenbosch, South Africa.

   Role: I gave a 10 minutes' talk followed by 5 minutes of questions and answers.

2) **Mashatola, T**., Koekemoer, L.L., Munhenga, G. Development of genetic sexing systems for the malaria vector, *Anopheles arabiensis*: progress made by the South African sterile insect technique project. ANTI-VeC Network Meeting: Genetic and Symbiont-based Control Approaches for Vector Control, 26 – 28 February 2023, Kilifi, Kenya.

   Role: I was awarded a travel bursary to give an 8 minutes' talk followed by 2 minutes of questions and answers.

3) **Mashatola, T**., Koekemoer, L.L., Munhenga, G. Development of methods to separate males and females during sterile insect technique applications against the malaria vector, *Anopheles arabiensis*. 8th Biennial Ecohealth Conference, 14 – 18 November 2022, Durban, South Africa.

Role: I gave a 10 minutes' talk followed by 5 minutes of questions and answers.

4) **Mashatola, T**., Koekemoer, L.L., Munhenga, G. Development of optimal handling, packaging and transport conditions of radiation-sterilised (γ-ray) *An. arabiensis* adult male mosquitoes earmarked for pilot sterile insect technique releases in South Africa. 8th Pan-African Mosquito Control Association (PAMCA) Annual Conference and Exhibition, 23 – 25 September 2022, Kigali, Rwanda.

Role: I designed the poster, summarised the contents and readied it for presentation. Dr Munhenga presented the poster to the conference delegates on my behalf.

5) **Mashatola, T**., Koekemoer, L.L., Munhenga, G. Development of temperature sensitive alleles to eliminate females during mosquito mass production: steps towards development of the sterile insect technique against Anopheles. Generic approach for the development of genetic sexing strains for SIT applications. 2nd research coordination meeting, 18 – 22 October 2021, Vienna, Austria.

Role: I gave a 15-minute presentation as the principal investigator of the coordinated research project titled 'Generic approach for the development of genetic sexing strains for SIT applications'. I specifically spoke about the development of temperature sensitive alleles to eliminate females during mosquito mass production.

6) **Mashatola, T**., Ndo, C., Koekemoer, L.L., Dandalo, L., Wood, O. Malakoane, L., Poumachu, Y., Lobb, L., Kaiser, M., Bourtzis, K., Munhenga, G. Progress on the development of sex-separation methods for Sterile Insect Technique applications against the Malaria Vector, *Anopheles arabiensis.* 6th Pan-African Mosquito Control Association (PAMCA) conference, 23 – 25 September 2019, Yaoundé, Cameroon.

Role: I gave a 10-minute talk followed by 5 minutes of questions and answers.

7) **Mashatola, T**., Ndo, C., Koekemoer, L.L., Dandalo, L., Wood, O. Malakoane, L., Poumachu, Y., Lobb, L., Kaiser, M., Bourtzis, K., Munhenga, G. 2019. Progress on the development of sex-separation methods for Sterile Insect Technique applications against the Malaria Vector, *Anopheles arabiensis.* BioAfrica Conference. 26 – 28 August 2019, Durban, South Africa.

Role: I gave a 10-minute talk followed by 5 minutes of questions and answers.

8) **Mashatola, T**., Ndo, C., Koekemoer, L.L., Dandalo, L., Wood, O. Malakoane, L., Poumachu, Y., Lobb, L., Kaiser, M., Bourtzis, K., Munhenga, G. Progress on the development of sex-separation methods for Sterile Insect Technique applications against the Malaria Vector, *Anopheles arabiensis.* 5th Southern Africa Malaria

Research Conference: Co-operation for Elimination, 30 July – 1 August 2019, Pretoria, South Africa.

Role: I gave a 10-minute talk followed by 5 minutes of questions and answers.

9) **Mashatola, T**., Ndo, C., Koekemoer, L.L., Dandalo, L., Wood, O. Malakoane, L., Poumachu, Y., Lobb, L., Kaiser, M., Bourtzis, K., Munhenga, G. Progress on the development of sex-separation methods for Sterile Insect Technique applications against the Malaria Vector, *Anopheles arabiensis.* 21st Entomological Conference of Southern Africa, 8 – 11 July 2019, Durban, South Africa.

Role: I gave a 10-minute talk followed by 5 minutes of questions and answers.

DEDICATION

I dedicate this work to my family, friends and everyone who has contributed to this journey.

Much love, and thank you very much for everything.

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ABSTRACT

South Africa is taking significant strides towards eliminating malaria transmission within its borders. However, existing vector control strategies that focus on indoor mosquito management face challenges with *Anopheles arabiensis*, the primary malaria vector. To bolster these efforts, the sterile insect technique (SIT) is being considered as an additional vector control strategy. SIT involves mass-rearing and sterilising specific pest insects, which are then released to mate with wild insects, effectively reducing the pest population.

The South African SIT project faces a crucial challenge of efficiently separating female mosquitoes from the production line. This is because female mosquitoes are capable of transmitting the malaria parasite, making their elimination vital. Current methods like manual separation and resistance-based sorting have operational limitations and require further optimisation for field trials.

To address this challenge, this thesis conducted optimisation and acclimatisation experiments on adult *Anopheles arabiensis* females, aiming to transition them to an artificial membrane feeding technique. Comparative assessments demonstrated that artificial blood-feeding methods utilising a Hemotek® membrane feeding system and hog casing could effectively replace conventional methods without significant detriment to reproductive fitness.

Subsequently, the study explored the use of ivermectin, a toxicant, to spike blood during artificial feeding to target and eliminate females. An optimal concentration of ivermectin (7.5 ppm) was identified, showing potential for segregating females from males during laboratory rearing. However, complete female elimination within the desired timeframe was not achieved, indicating that this method serves as a secondary phase for female elimination.

The study also investigated the use of genetic sexing strains (GSSs) to selectively eliminate females. Although efforts to induce temperature-sensitive lethal mutations temperature sensitive lethality mutations and random morphological variations were unsuccessful, further cross mating studies and insights from previous studies on thermosensitive strains from Cameroon informed future research aimed at developing GSSs tailored to the South African genetic background.

Another challenge addressed was the optimal temperature and compaction conditions for chilling and immobilising sterile males during handling and transport. This is crucial for maintaining the quality of sterile males. Optimal knockdown temperature ranges (4°C – 8°C for 20 minutes) and packaging conditions (1000 sterile, marked adult males per 27000 cm^3 Bugdorm-1® cage) were identified for laboratory handling and transport, facilitating recent small-scale pilot trials with successful packaging and transportation of sterile males to field sites.

These advancements signify significant strides in South Africa's malaria elimination endeavours, while also playing a pivotal role in shaping the development of efficient operational protocols for SIT. Furthermore, as the country remains steadfast in its commitment to eliminating malaria, these innovative approaches offer a promising trajectory forward and establish a robust framework for orchestrating the SIT operational phase.

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LIST OF ABBREVIATIONS/NOMENCLATURE

Abbreviation	Description
%	Percent
±	Plus or minus
®	Trademark
°C	Degrees Celsius
µm	micrometre
ACTs	Artemisinin-based combination therapies
ANO IPCL1	*Anopheles arabiensis* genetic sexing strain developed at the Insect Pest Control Laboratory (division of the Food and Agriculture Organization/International Atomic Energy Agency)
ANOVA	Analysis of variance
AW-IPM	Area-wide integrated pest management
BDMI	Botha De Meillon Insectary
Bp	Basepairs
CI	Confidence interval
cm	Centimetre
Cm^3	Cubic centimetre
Corp	Cooperation
DDT	Diethyl diphenyl trichloroethane
df	Degrees of freedom
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
EMS	Ethyl methanesulfonate
e.g.	For example
***et al*.**	And others
Etc.	Et cetera
F	Filial
FAO	Food and Agricultural Organisation

GAMA	*Anopheles arabiensis* strain derived from the genetic sexing strain, ANO IPCL1
γ	Gamma penetrating electromagnetic radiation
GMK	*Anopheles arabiensis* strain derived from GAMA strain and wild-type
GSS	Genetic sexing strain
Gy	Gray
IAEA	International Atomic Energy Agency
IBM	International Business Machines
IGS	Intergenic spacers
IPCL	Insect Pest Control Laboratory
IRS	Indoor residual spraying
IVM	Integrated vector management
h	hour
KZN	KwaZulu-Natal
L1	First instar larvae
Mg	Milligrams
mℓ	Millilitre
$MgCl_2$	Magnesium chloride
MMR	Mark-release-recapture
N	Total number
n	Sample size
NICD	National Institute for Communicable Diseases
NGS	Next-generation sequencing
P	Probability level
PCR	Polymerase Chain Reaction
ppm	Parts per million
R^2	Square of the sample correlation coefficient in regression analysis
Rdl	Resistance to dieldrin
s.l.	Sensu lato

s.s.	Sensu stricto
SD	Standard Deviation
SE	Standard error
SINE	Short interspersed repeated DNA sequences
SIT	Sterile Insect Technique
SOP	Standard Operating Procedure
sp. n.	species nova (new species)
Spp	Species
SPSS	Statistical Package for Social Sciences
tsl	Temperature sensitive lethality
UAV	unmanned Aerial Vehicle
VRL	Vector Reference Laboratory
WHO	World Health Organization
WRIM	Wits Research Institute for Malaria
χ^2	Chi square

CHAPTER 1: Literature review

1.1. General overview

Malaria stands as one of the most dangerous infectious disease worldwide and persist despite continuous efforts to manage the disease. Both incidence and mortality rates are now higher than before the onset of the COVID-19 pandemic (WHO, 2023). This challenge is exacerbated by the escalating impact of climate change and other factors, posing a threat to the progress made in combating the disease (WHO, 2023). The World Health Organization (WHO) malaria report for 2022 revealed a significant surge in global malaria cases, reaching approximately 249 million, a 5 million increase compared to 2021 (WHO, 2023). The global death toll from the disease was estimated at 608 000, marking a nearly 6 % rise since 2019. Of particular concern is the sustained high burden of the disease in Africa, where the region accounted for 94 % of global malaria cases and 95 % of all malaria-related deaths in 2022. Notably, around 78 % of these fatalities occurred in children under the age of five years old.

The disease can have health and socioeconomic consequences (Sachs and Malaney, 2002; Carter *et al*., 2005; Andrade *et al*., 2022). These include persistent developmental abnormalities, reduced appetite, restricted playing and social interaction due to cerebral malaria brain damage. In addition, missed work or school days due to illness and increased risk of death from severe anaemia due to repeated infections or low birth weight from infected pregnant mothers are common. Other factors such as high healthcare spending, loss of income, premature deaths, poverty, poor public health systems, urbanisation, climatic changes, human migration, and effective malaria vectors exacerbate the malaria burden in severely affected countries (Sachs and Malaney, 2002; Caminade *et al*., 2014).

The parasites belonging to the Order: Haemospororida, Family: Plasmodiidae, Genus: *Plasmodium*, and encompass over 100 species capable of infecting various animal species, including reptiles, birds, and mammals (Boundenga *et al*., 2016, 2017). These parasites transmit malaria to their vertebrate hosts through Anopheles mosquitoes (Cox, 2010). Among them, only five species—*Plasmodium falciparum* (Welch), *P. vivax* (Grassi and Feletti), *P. ovale* (Curtisi) and *P. ovale* (Wallikeri), *P. knowlesi* (Sinton and Mulligan), and P. *malariae* (Feletti and Grassi)—significantly affect humans (WHO, 2023). Notably, *P. falciparum* stands out as the most prevalent among these species, responsible for the majority of severe malaria cases and deaths in sub-Saharan Africa (Snow *et al*., 2005; Hay *et al*., 2010; Bhatt *et al*., 2015).

Following closely is *P. vivax*, the primary cause of relapsing malaria, inducing acute and persistent symptoms through blood and liver infections, particularly in countries outside sub-Saharan Africa (Phyo *et al*., 2023; WHO, 2023). *Plasmodium ovale*, *P. malariae*, and *P. knowlesi* are comparatively less prevalent.

The pathogenicity of *P. falciparum* stems from its capacity to manipulate its host's physiology during the blood feeding stages of its growth (Buffet *et al*., 2011). Once infecting humans, the parasite feeds on haemoglobin, develops, and divides within erythrocytes, modifying its host cells to cling to blood vessel walls. This results in common symptoms such as cyclical fever and shivering, joint pains, vomiting, headache, and general body pain. In severe malaria cases, convulsions and or kidney failure can occur, resulting in severe acute anaemia complications, transient or permanent neurological effects, and death (Schumacher and Spinelli, 2012).

The combat against malaria is ongoing on a global scale. As per the WHO's recommendations, South Africa is one of 21 countries committed to eliminating malaria (WHO, 2018). Despite these efforts, challenges prevent the achievement of this goal. For example, during the period of 2017/2018, the country faced a notable setback attributed to factors such as persistent residual local transmission and cross-border migration from neighbouring countries, which through the importation of cases fuelled local malaria transmission, leading to the reporting of nearly 20,000 malaria cases (South African Department of Health, 2019; Raman *et al*., 2020). The majority of these cases were concentrated in the endemic north-eastern provinces (South African Department of Health, 2019; Maharaj *et al*., 2019; Tsoka-Gwegweni *et al*., 2020). This necessitated a revision of the country's strategy to align with each of the pillars of the WHO Global Technical Strategy for Malaria (2016-2030) and the establishment of a new Malaria Elimination Strategic Plan 2019-2023 (South African Department of Health, 2019; Moonasar *et al*., 2021). These recommendations included the need to enhance both vector and parasite surveillance, readiness for epidemics, timely responses, health education, and prioritising vector control, which continues to serve as the primary preventive measure and defence strategy. Current vector control strategies rely on insecticides applied inside homes in endemic areas via indoor residual spraying (IRS) (Brooke *et al*., 2013; South African Department of Health, 2019). However, increasing insecticide resistance, outdoor resting vector behaviour, and logistical concerns/changing house structures call for developing new approaches to complement existing strategies (Brooke *et al*., 2015).

The sterile insect technique (SIT) stands as one of the recommended control methods due to its species-specific nature and environmentally friendly characteristics (Dyck *et al*., 2005, 2021; Munhenga *et al*., 2011, 2014, 2016). This approach focuses on a particular vector by repeatedly introducing laboratory-reared sterile insects of the same species into carefully chosen natural habitats where they can mate with wild native populations and elicit unviable progeny (Dyck *et al*., 2005, 2021). As a result, native insects lay non-viable eggs, causing the number of progeny to be reduced. Over time, target populations can be suppressed or eradicated.

However, several aspects of the technology must be optimised before the SIT can be applied against malaria vectors. One such aspect is having a system to eliminate females before male sterilisation and field releases. The lack of an adequate gender sorting method remains the biggest challenge in implementing the SIT against mosquito vectors, mainly for African malaria vectors (Mashatola *et al*., 2018). The imperative removal of adult female mosquitoes from release material is underscored by their role in diseases transmission (Alphey *et al*., 2010; Lees *et al*., 2014). Although both adult males and females fulfil their energy requirements from nectar and other plant juices, only adult female mosquitoes require a blood meal diet for egg production (Clements, 1992; Briegel, 2003). This inherent difference between male and female mosquitoes makes them exclusively contributor of disease transmission. Consequently, the establishment of a gender separation system is crucial in any mosquito SIT program. It is therefore essential that the South African malaria SIT programme have a release strain which has an efficient separating system that can separate males from females before they can launch a mosquito SIT pilot programme.

In light of this, efforts are underway to create a sexing system for the SIT applications against the principal malaria vector, *Anopheles arabiensis* (Patton (Diptera: Culicidae)). These include investigating the use of temperature sensitivity mutations as a gender separation tool and exploring blood-feeding differences between males and females (Mashatola *et al*., 2018).

1.2. Malaria vectors

There are approximately 3 500 mosquito species recorded worldwide (Harbach and Kitching, 2016). Mosquitoes belong to the class Insecta, order Diptera and the family Culicidae

(Clements, 1992; Rueda and Debboun, 2020). Of all mosquitoes, a comparatively smaller number, approximately 537 species, belong to the subfamily Anophelinae. The Anophelinae are further classified into three genera: Bironella, Chagasia and *Anopheles* Meigen (Harbach, 2013). Within the Afrotropical region, specifically sub-Saharan Africa, there are 140 *Anopheles* species, but only twenty are known to transmit malaria to humans (Sinka *et al*., 2012). Most of these are classified into mainly two taxonomic groups, namely the *Anopheles gambiae sensu lato (s.l)* complex (Diptera: Culicidae) and *An. funestus sensu lato (s.l)* group (Diptera: Culicidae) (Gillies and De Meillon, 1968; Gillies and Coetzee, 1987). The use of the terms "complex" and "group" in mosquito taxonomy reflects the diverse levels of morphological and genetic differentiation observed among closely related species (Gillies and De Meillon, 1968). Specifically, "complex" is employed when closely related species exhibit overlapping morphological traits, posing a challenge for differentiation based solely on physical characteristics. In contrast, the designation "group" is commonly employed when related species share specific characteristics but display more noticeable distinctions among themselves compared to species within a "complex". Both the *An. gambiae* complex and *An. funestus* group comprises morphologically identical or similar species that differ in ecology, behaviour, and contribution to malaria transmission (Gillies and De Meillon, 1968; Gillies and Coetzee, 1987; Dia *et al*., 2013; Coetzee, 2020).

The *An. funestus* group consists of at least 13 morphologically similar African species classified into three subgroups based on molecular and morphological descriptions, namely *An. funestus*-, *An. minimus*-, and *An. rivulorum* subgroup (Harbach, 2013; Dia *et al*., 2013). The *An. funestus* subgroup has seven species (*An. funestus sensu stricto* (*s.s.*) (Giles (Diptera: Culicidae)), *An. funestus*-like (Diptera: Culicidae), *An. aruni* (Sobti (Diptera: Culicidae)), *An. confusus* (Evans and Leeson (Diptera: Culicidae)), *An. parensis* (Gillies (Diptera: Culicidae)), *An. vaneedeni* (Gillies and Coetzee (Diptera: Culicidae)), and *An. longipalpis* type C (Theobald (Diptera: Culicidae)), *An. minimus* subgroup has two species (*An. leesoni* (Evans (Diptera: Culicidae)) and *An. longipalpis* type A (Theobald (Diptera: Culicidae)), and *An. rivulorum* sub group consist of four species (*An. rivulorum s.s.* (Leeson (Diptera: Culicidae)*), An. rivulorum*-like (Diptera: Culicidae), *An. brucei* (Service (Diptera: Culicidae)) and *An. fuscivenosus* (Leeson (Diptera: Culicidae)) (Dia *et al*., 2013). The *An. gambiae* complex consists of nine sibling species, the main malaria vectors being *An. gambiae s.s.* (Giles (Diptera: Culicidae)), *An. coluzzii* (Coetzee, Hunt and Wilkerson (Diptera: Culicidae)), and *An. arabiensis* (Patton

(Diptera: Culicidae)), while *An. merus* (Dönitz (Diptera: Culicidae)), *An. melas* (Theobald (Diptera: Culicidae)), and *An. bwambae* (White (Diptera: Culicidae)) are localised minor vectors (Gillies and Coetzee, 1987; Sinka *et al*., 2010; Dorn *et al*., 2011; Coetzee *et al*., 2013a). *Anopheles quadriannulatus* (Theobald (Diptera: Culicidae)) and *An. amharicus* (Coetzee, Hunt and Wilkerson (Diptera: Culicidae)) are not known to transmit malaria (Gillies and Coetzee, 1987; Hunt *et al*., 1998; Coetzee *et al*., 2013a). A recent addition to the *An. gambiae* complex is *An. fontenillei sp.n* (Diptera: Culicidae) (Barrón *et al*., 2019).

1.3. Malaria in South Africa

Malaria prevalence in South Africa is low compared to other sub-Saharan African countries. This is primarily due to its geographical location (Africa's southernmost extreme of malaria distribution in Africa) and historical malaria control interventions. The result is that the current confinement of malaria transmissions is restricted to the northern and north-eastern low-altitude regions of the provinces of Limpopo, Mpumalanga and KwaZulu-Natal that share inter-country borders with Botswana, Eswatini (formerly Swaziland), Mozambique and Zimbabwe (Figure 1.1).

The endemic areas in South Africa are home to approximately 4.3 million people, or 10 % of the total population, constantly at risk of malaria transmission. The primary vector implicated in malaria transmission is *An. arabiensis* (Gillies and Coetzee, 1987; Coetzee *et al*., 2013a; Dandalo *et al.,* 2017a). *Anopheles arabiensis* achieves its main malaria vector status through its variable resting and feeding behaviour (rests both inside and outside of human dwellings, and can feed on both humans and animals (Gillies and De Meillon, 1968; Gilles and Coetzee, 1987), which allows it to evade/be invulnerable to IRS (Brooke *et al*., 2013). However, historically and during major outbreaks, *An. funestus s.s*. has been implicated as a main vector (De Meillon *et al.,* 1977; Hargreaves *et al*., 2000; Braack *et al.,* 2020). *Anopheles merus* only plays a minor role in malaria transmission (Coetzee *et al*., 2013a) and was recently found infected with *P. falciparum* in northern KZN, cementing its potential position in the ongoing residual malaria transmission (Dandalo, 2016). *Anopheles vaneedeni* and *An. parensis* are two other vectors recently identified as potential minor vectors after field-collected samples tested positive for *P. falciparum* (Burke *et al*., 2017, 2019).

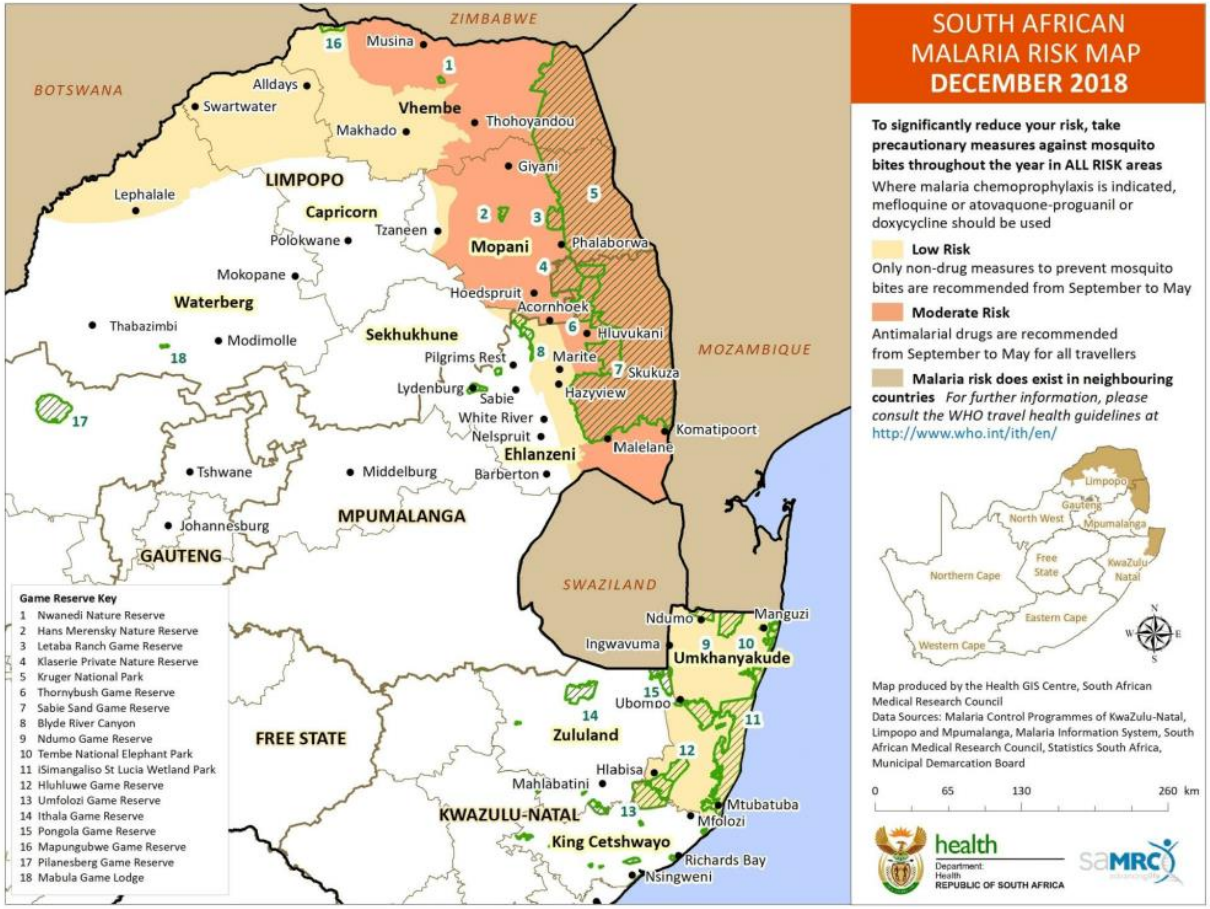


Figure 1.1: A South African malaria risk map depicting malaria-endemic north-eastern low-lying areas (Limpopo, Mpumalanga and KwaZulu-Natal provinces) and their inter-country borders with Botswana, Eswatini, Mozambique and Zimbabwe (https://www.nicd.ac.za/wp-content/uploads/2018/12/south_africa_malaria_risk_dec2018_final.pdf).

1.4. Malaria vector control in South Africa

South Africa has several successful malaria control strategies/initiatives to combat vectors and manage cases (Coetzee *et al*., 2013b). This is primarily because of its competent national and provincial malaria control programmes and relatively well-established scientific, economic, and health infrastructures (Blumberg and Frean, 2007; Moonasar *et al*., 2021). The control approaches include larviciding and IRS targeting vectors and artemisinin-based combination treatments (ACTs) for treatment of confirmed cases (Blumberg *et al*., 2014). The most important of these is the control of malaria vectors through intensive IRS of houses using synthetic pyrethroids to spray modern painted structures or structures made of cement-brick and spraying dichlorodiphenyltrichloroethane (DDT) in traditional mud-walled designs (Tangena *et al*., 2020). In some instances, carbamates or a mosaic of these synthetics is interchangeably used (Brooke *et al*., 2013). These efforts, however, are being jeopardised by an increase in insecticide resistance among target vectors (Hargreaves *et al*., 2000, 2003; Brooke *et al*., 2015; Munhenga *et al*., 2022). This is partly because only five classes of insecticides (organochlorides, pyrethroids, organophosphates, carbamates and neonicotinoids) are approved for vector control in this instance IRS (WHO, 2021; Tangena *et al*., 2020). This limits control options and complicates insecticide resistance management (WHO, 2012). In addition, most of the insecticides used for IRS have a relatively short protection lifespan of approximately two to six months (WHO, 2021). Thus in some cases, people are not protected for the entire malaria season. Furthermore, DDT (organochlorine), a previously widely used and effective insecticide, is discouraged and less used as it is frequently associated with environmental contamination (Eskenazi *et al*., 2009). The logistical challenges of achieving adequate IRS coverage (>95 %) in malaria-affected areas, which are typically characterised by rugged terrain, is another aspect that raises cost-benefit and sustainability concerns (WHO, 2015).

Nonetheless, the South African government had not been dissuaded and had shifted its focus from control to elimination (Elimination 8, 2019; South African Department of Health, 2019). This strategic initiative aims to eradicate locally acquired malaria cases by 2028, building upon the original target of 2023. However, this has thus far not been attained using current control tools exclusively (Raman *et al*., 2016). As a result, complementary malaria control techniques, particularly vector control strategies targeting outdoor resting *An. arabiensis* populations,

linked to residual malaria transmission, are required (Dandalo *et al*., 2017a). The SIT is one such control strategy currently under investigation.

1.5. The sterile insect technique (SIT)

1.5.1. General overview

The SIT concept was independently conceived by three researchers (Serebrovskii, A.S. at Moscow State University USSR, Vanderplank F. L. at Tanganyika (modern-day Tanzania), and Knipling, E.F. of the United States Department of Agriculture) during the 1930s and 1940s (Franz, 2005). They hypothesised that releasing sterile males in more significant numbers than the numbers already present in the natural population (indigenous inhabitants/wild natives) could lead to a reduction in the number of insects in the wild population (Knipling, 1955; reviewed in Klassen *et al*., 2005, 2021). The SIT entails introducing many laboratory-reared male sterile insects into a target population to confer sterility by competing for mating with wild male native populations and eliciting unviable progeny (Knipling, 1955; Alphey *et al*., 2002; Dyck *et al*., 2005, 2021). The strategic applications of the SIT within area-wide integrated pest management can result in suppression, eradication, containment, and prevention (Dyck *et al*., 2005, 2021). A chronological breakdown of the events involved in the SIT is illustrated in Figure 1.2.

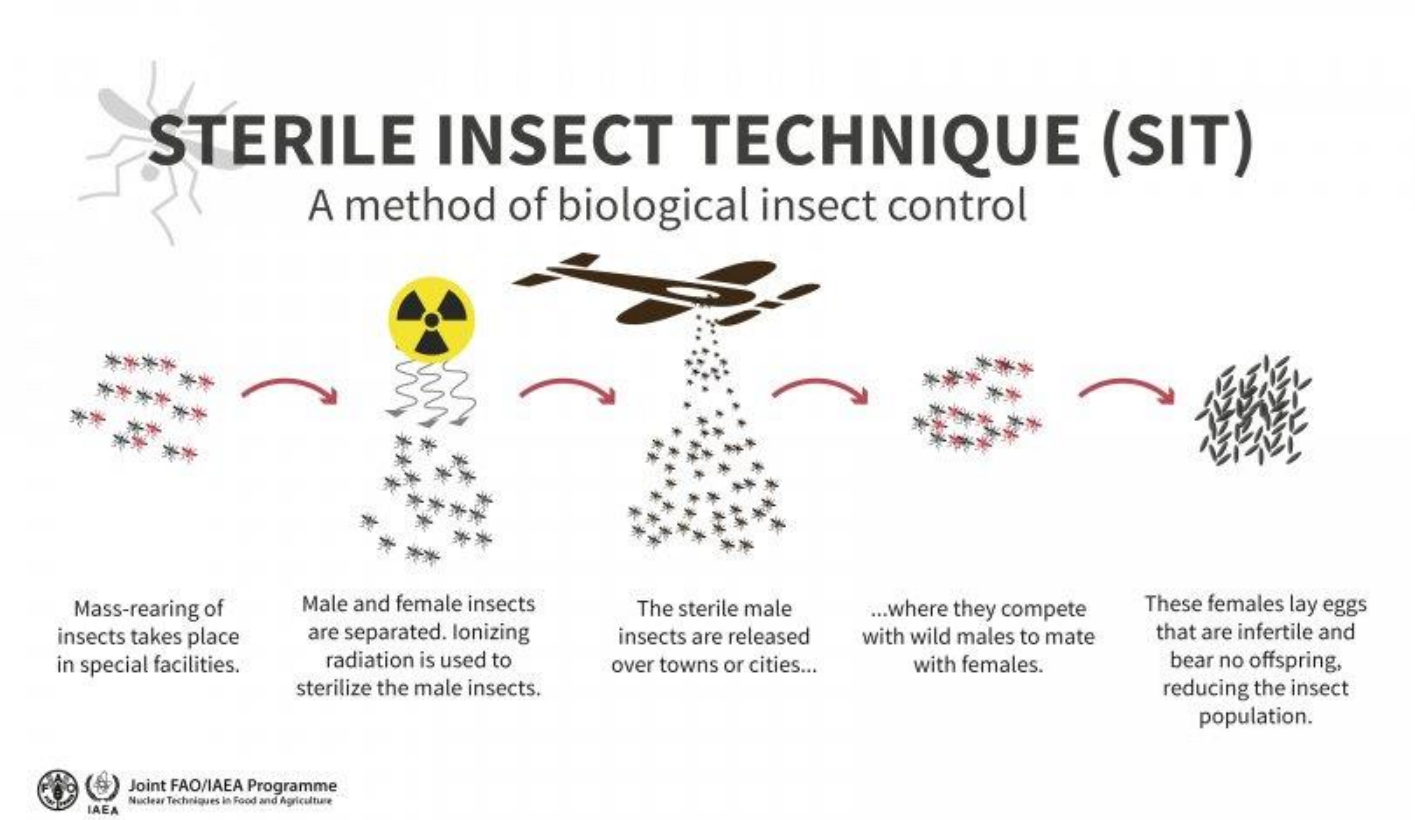


Figure 1.2: A chronological breakdown of the events involved in the SIT, from mass rearing of insects, separation of males and female insects, radiation induced sterilisation to release into a target site where they compete with wild insects for mating, resulting in females laying eggs that do not hatch (https://www.iaea.org/topics/sterile-insect-technique).

1.5.2. Successful SIT programmes

Various successful applications of the SIT as an area-wide integrated pest management (AW-IPM) method for controlling and, on occasion, eradicating major agricultural and human pests have occurred over the years (Benedict and Robinson, 2003; Dyck *et al*., 2005, 2021; Vreysen *et al*., 2021). These encompass insects classified in the Order: Diptera, Family: Tephritidae, such as *Ceratitis capitata* (Mediterranean fruit fly) (Wiedemann) (Rossler *et al*., 2000; Hendrichs et al., 2002; Barnes et al., 2007; Enkerlin, 2005), *Anastrepha ludens* (Mexican fruit fly) (Loew), *Bactrocera dorsalis* (oriental fruit fly) (Hendel), and *Bactrocera cucurbitae* (melon fly) (Coquillett) (Koyama *et al*., 2004) (Dyck *et al*., 2005, 2021). Additionally, other insect species include *Glossina* (tsetse flies) (Wiedemann (Diptera: Glossinidae)) (e.g. *Glossina austeni* (Vreysen *et al*., 2000)), *Cochliomyia* (screwworms) (Townsend (Diptera: Calliphoridae)) (e.g. *Cochliomyia hominivorax* (Lindquist *et al*., 1992; Klassen *et al*., 2005, 2021)), and various *Gynnidomorpha alisman* (moths) (Lepidoptera), such as *Cydia pomonella* (codling moth) (Linnaeus (Lepidoptera: Tortricidae)) (Bloem *et al*., 2007), *Pectinophora gossypiella* (pink bollworm) (Saunders (Lepidoptera: Gelechiidae)), *Thaumatotibia leucotreta* (false codling moth) (Meyrick (Lepidoptera: Tortricidae)), *Cactoblastis cactorum* (cactus moth) (Berg (Lepidoptera: Pyralidae)), and *Teia anartoides* (Australian painted apple moth) (Walker (Lepidoptera: Erebidae)) (Dyck *et al*., 2021). Notably, a few isolated pilot trials involving *Aedes aegypti* (Linnaeus (Diptera: Culicidae)) and *Aedes albopictus* (Skuse (Diptera: Culicidae)) mosquitoes have also been documented (Klassen *et al*., 2005, Chung *et al*., 2018; Iyaloo *et al*., 2020).

In South Africa, the initial SIT efforts occurred almost 20 decades ago against *C. capitata* (Barnes *et al*., 2007; 2015). Subsequently, SIT interventions are being used to control *Thaumatotibia leucotreta* (false codling moths) (Hofmeyr *et al*., 2015) and *Cydia pomonella* (codling moths) (Odendaal *et al*., 2015).

As this study is focused on malaria and only anophelines are responsible for transmitting this disease, a list of anopheline species that have been involved in SIT field investigations to date are shown in Table 1.1. The programmes, however, did not aim to achieve complete eradication but rather to answer specific research questions. These include identifying an appropriate release site, developing species-specific mass rearing procedures, knowledge of the target species ecology and behaviour, developing quality control procedures, and tools to

monitor the development and release of mosquitoes (Benedict and Robinson, 2003; Dyck *et al*., 2005; Dame *et al*., 2009). In South Africa, feasibility studies on the use of the SIT against mosquitoes as a malaria intervention tool have been underway for the past decade (Munhenga *et al*., 2011, 2014, 2016; Dandalo *et al*., 2017a, 2018). Identification of an appropriate release site (Munhenga *et al*., 2014; Dandalo *et al*., 2017a), knowledge, attitude and practices (KAP) study (Manana *et al*., 2018), relative mating suitability between laboratory colonised and native populations (Munhenga *et al*., 2011), population dynamics (Dandalo *et al*., 2017a), vector surveillance (Munhenga *et al*., 2014), species abundance and distribution (Munhenga *et al*., 2014), mating competitiveness (Munhenga *et al*., 2016), sterilisation by irradiation (Dandalo *et al*., 2017b), evaluation of cage rearing conditions (Mashatola *et al*., 2017), development of an insecticide resistance based genetic sexing strain (GSS) (Dandalo *et al*., 2018), estimation of the field population size and dispersal range (Kaiser *et al*., 2021), research looking into using a cultural song to involve the community (Manana *et al*., 2021), are all part of these. However, some critical areas still need to be addressed before the technology can be piloted under open field conditions. These include creating an efficient method for separating males from females during mass production so that only males are released and the development of standard operating procedures for handling and packaging mosquitoes during laboratory production and before transporting the sterile males into a target field site.

Table 1.1: Global list of anopheline SIT field investigations from 1959 to 2021.

Target species	Year	Location	Number released and duration	Method used for sterilisation	Objective	Outcome	Reference
An. albimanus (Wiedemann (Diptera: Culicidae))	1972	El Salvador	4.4 million sterile males released over 22 weeks	Chemo-sterilisation of genetic sexing strain pupae with bisazir	Methods development and population reduction	Induced 100 % sterility in wild population to below detection level after five months	Benedict, 2021; Klassen *et al*., 2005, 2021
	1977–1979	El Salvador	100 million males (genetic sexing strain (MACHO)) released during 1977–1979	Sterilisation with bisazir	Population reduction	Population suppressed	Benedict, 2021; Klassen *et al*., 2005, 2021
An. arabiensis	2008 - 2021	Sudan, South Africa	Variable	Sterilisation with gamma penetrating electromagnetic radiation	Mating between released colony females and wild males. Mark-release recapture	Acceptable male competitiveness and dispersal.	Benedict, 2021; Kaiser *et al*., 2021; Klassen *et al*., 2005, 2021

An. culicifacies (Giles (Diptera: Culicidae))	1979	Pakistan	3 100 males in 1 release	Sterilisation with chromosome translocation	Mating with wild and released colony females	No assortative mating selected during the colonisation process	Baker *et al*., 1980
	1980		7 500 males in 1 week	Chemo-sterilisation	Mating between released colony females and wild males	Poor male competitiveness	Reisen *et al*., 1981
An. gambiae s.l.	1968-1969	Burkina Faso	240 000 over nine weeks	Hybrid male sterility	Population reduction	No significant effect on egg-batch sterility. Dispersal was high, but poor male competitiveness	Davidson *et al*., 1970
An. quadrimaculatus	1959–1960	United States of America	433 600 sterilised pupae released as adult males over 48 weeks at 2 locations	Chemo-sterilisation	Population reduction and semi-sterility	No population decline was achieved due to poor competitiveness	Weidhaas *et al*., 1962
	1962–1963		50 000 colony and wild males	Chemo-sterilisation	Male competitiveness, behaviour, and sterilisation methods	Observed mating between wild males and colony females	Dame *et al*., 1964

1.5.3. Gender separation systems

It is implausible that a mosquito SIT programme will function without a gender separation system. Female mosquitoes, in particular, must be removed from mosquitoes destined for release, owing to their biting nuisance and potential to transmit malaria. As has been demonstrated by Dandalo *et al*. (2017b) and Guissou *et al*. (2020), although females will not lay eggs after irradiation, they are still capable of taking a blood meal and thus can potentially transmit malaria. The release of females can also raise serious ethical concerns. Furthermore, if females are in the release material, males may be unable to disperse or mate with wild females as desired, potentially reducing the efficiency of the SIT programme (Orozco *et al*., 2013; Vreysen *et al*., 2014). Additionally, exclusively rearing males would maximise any facility's capacity, improving the SIT programme's efficiency (Benedict and Robinson, 2003; Vreysen *et al*., 2014). Although gender separation methods for other mosquito SIT species have been developed, none have reached enough throughput to be employed at an operational level in mass-release programmes, especially for *An. arabiensis* (Papathanos *et al*., 2009, 2018; Yamada *et al*., 2012; Gilles *et al*., 2014; Dandalo *et al*., 2018). Below is a summary of some previously developed gender separation strategies for mosquitoes.

1.5.3.1. Morphological, size and developmental differences

In general, male and female pupae of most insects have phenotypic differences, with one gender being larger than the other (Leimar *et al*., 1994). This is known as pupal sexual dimorphism (Moorefield, 1951). This approach has successfully been used for gender separation in various mosquito species, including *Culex quinquefasciatus* (Say (Diptera: Culicidae)) (Krishnamurthy *et al*., 1962) and *Ae. aegypti* (McCray, 1961; Sharma *et al*., 1972). However, similar attempts to use sexual dimorphism in *An. quadrimaculatus* and *An. albimanus* showed that this approach is unreliable (Dame *et al*., 1974). When using this approach, pupae have historically been separated mechanically utilising various methods such as sieves characterised by specific mesh sizes (Sharma *et al*., 1972; Bellini *et al*., 2018) and pupal sex-sorters that utilise protandry and pupal size selection for sex separation (Fay and Morlan, 1959; McCray, 1961; Focks, 1980). One of these is the Fay-Morlan separator, which Fay and Morlan (1959) in which they employed adjustable glass plates to establish a uniformly diminishing gap for larval removal and pupal size selection. While this method proved highly effective in gender separation in *Cx. Pipiens* Linnaeus however, its efficiency was notably lower for *An. albimanus* (Dame *et al*., 1974). In a 1972 sterile male release study in El Salvador, used

the Fay-Morlan separator to mechanically separate approximately 170 000 males getting an average of 86 % male purity. This was primarily due to contamination from small sized female pupae.

As a results of great success in gender seperation in Culicines and Aedines subsequent enhancements were made to the original design of the Fay-Morlan separator (Focks, 1980), including recent automation for improved continuous operational needs (Wolbaki, Guangzhou, China) (Zheng *et al*., 2019). The general disadvantage of the Fay-Morlan separator is that mechanical separation at the pupal stage may compromise the quality of the reared males (Morán-Aceves *et al*., 2021). In addition, the fact that separation only occurs at a late developmental stage limits the applicability of this method, particularly for large-scale production, as it is not cost-effective. These methods are more efficient in species with significant differences in pupal size, so this concept cannot be replicated in anophelines, particularly in *An. arabiensis* (Dame *et al*., 1974; Mashatola *et al*., 2018).

Another device, utilising precisely positioned plates over a sluiceway to exploit the fast developmental time of males compared to females (protandry), has been utilised for *Aedes* (McCray, 1961; Bellini *et al*., 2018) and *Cx. pipiens* (Gerberg *et al*., 1969). However, a challenge emerged as the pupal cephalothorax size increased on successive days of pupation, leading to a reduction in discrimination. Similar difficulties were encountered in *An. arabiensis*, where pupal cephalothorax sizes showed significant overlap, rendering gender separation impossible (Mashatola *et al*., 2018). Additionally, the drawback of protandry is that while it is known that differences in development time are influenced by rearing temperature and larval diet (Tun-Lin *et al*., 2000), no standard method exists to guarantee specific developmental periods between genders. As a result, protandry as a gender separation strategy for *An. arabiensis* appears unlikely (Mashatola *et al*., 2018).

1.5.3.2. Behavioural differences between sexes

Mosquitoes exhibit several behavioural differences between genders. Swarming and blood-feeding/seeking behaviour are examples of these. Adult males almost entirely form swarms to attract females for mating, especially at dusk and dawn (Clements, 1999). Based on this understanding, induced swarming can allow for rapid and efficient separation of adult males and females. For example, as mosquitoes mate in swarms in large numbers that are almost

entirely made up of males, a large number of mixed male and female adult *An. arabiensis* were introduced into large laboratory cages and a suction pump was used to remove swarming males during their peak mating activity at dusk and dawn (Papathanos *et al*., 2009). However, this strategy failed, mainly because there are gaps in research on the chemical cues responsible for luring male or female mosquitoes to swarms (Mozūraitis *et al*., 2020; Poda *et al*., 2022). Furthermore, most swarming studies have been limited to small-scale operations (e.g., Mozūraitis *et al*., 2020) and are not ready for mass-scale production.

Another behavioural strategy for separating sexes at the adult stage takes advantage of the knowledge that only adult females require a blood meal, allowing females to be isolated from males or even eliminated (Clements, 1999). A toxicant added to a blood meal can cause female elimination. Spinosad (Miles, 2003), boric acid (Müller *et al*., 2010), dieldrin (Yamada *et al*., 2012, 2013a) and even household detergents have been tested as blood toxicants (Van Emden *et al*., 1974). Malathion, which was used against *An. albimanus,* as part of the SIT programme in El Salvador and achieved 95 % female elimination, has been by far the most successful toxicant (Lowe *et al*., 1981). Unfortunately, male mortality occurred due to tarsal contact with blood droplets released by females during feeding to thermo-regulate or concentrate erythrocytes (Lahondère and Lazzari, 2012). However, the high female elimination demonstrated potential for application, mainly if alternative toxicants are used. Yamada *et al*. (2013a) investigated various toxins, and ivermectin showed great potential by eliminating all females within four days while not affecting male mortality. Males were not affected because ivermectin toxicity does not occur through tarsal contact but instead directly targets the mosquito nervous system and muscle performance by inhibiting neurotransmission via glutamate-gated chloride channels after oral ingestion (Yates and Wolstenholme, 2004; Moreno *et al*., 2010). Though this toxin was used by Yamada *et al*. (2013a) at Seibersdorf, Austria, it has never been used before to eradicate female *An. arabiensis* in the South African laboratory environment.

1.5.3.3. Genetic sex separation techniques

These techniques involve manipulating/disrupting/interfering with the genetics of the target insect to achieve efficient gender separation (Papathanos *et al*., 2009, 2018; Gilles *et al*., 2014). Typically, a genetic marker that codes for a specific trait is identified, and the allele that

provides the positive selection is linked to the chromosome that determines gender (Franz, 2005). This category includes both transgenic approaches and traditional genetic techniques.

(a) Transgenic approaches

Transgenic sexing methods involve inserting foreign deoxyribonucleic acid (DNA) into the genome of another organism to induce lethality of a specific gender or convert sexual orientation (Papathanos *et al*., 2009, 2018; Nolan *et al*., 2011; Gilles *et al*., 2014). The technology has shown potential as a basis for gender separation in various pests or vector species, in some cases leading to the development of transgenic sexing strains (Papathanos *et al*., 2009; Gilles *et al*., 2014). These include work in *Anopheles* to link the fluorescent protein marker expression to gender (Catterucia *et al*., 2005; Ntoyi *et al*., 2022). In addition, transgenic embryonic sexing systems, which use conditional lethal systems to eliminate females during early embryonic development, have been developed in some agricultural pests such as *Anastrepha suspensa* (Loew (Diptera: Tephritidae)) (Schetelig *et al*., 2012), *Bombyx mori* (silkworm) (Linnaeus (Lepidoptera: Bombycidae) (Tan *et al*., 2013), *Lucilia cuprina* (Australian sheep blow fly) (Wiedemann (Diptera: Calliphoridae) (Yan and Scott, 2015; 2020). Advancements in genome modification tools suggest that transgenic sexing methods can be transferred and adapted between species, including *Anopheles*. The tools include transposable elements (Grossman *et al*., 2001), site-specific recombination systems (Haghighat-Khah *et al*., 2015), ribonucleic acid interference (RNAi) approaches (Wiltshire and Duman-Scheel, 2020), transcription activator-like effector nuclease (TALEN) (Smidler *et al*., 2013), and clustered regulatory interspaced short palindromic repeats (CRISPR)/CRISPR associated protein 9 (Cas9) (Liu *et al*., 2019). Nevertheless, there has been apprehensions regarding the immediate use of transgenic sexing methods for African malaria vectors, especially *An. arabiensis* (Mashatola *et al*., 2018). This hesitation stems from the perceived technical complexity and the challenges associated with public acceptance of releasing genetically modified mosquitoes. Additionally, the stringent regulatory approvals required for such interventions further contribute to the uncertainty surrounding the immediate adoption of transgenic sexing methods in the context of malaria control in Africa (Papathanos *et al*., 2009, 2018; Ntoyi *et al*., 2022). Recently, these concerns have been eased by developments in new, easy-to-use genome modification tools and expanded community engagement (Pare Toe *et al*., 2021, 2022). The South African SIT programme explores this route by investigating the possibility of introgressing an *An. arabiensis* strain bearing the entire Y chromosome from

An. gambiae s.l. and a transgenic red fluorescent marker into a South African genetic background (Ntoyi *et al*., 2022). Although the strain is promising, it still requires assessment and validation in a mass-rearing setting and a risk assessment analysis to address any concerns related to its potential release in ecosystems. Before this strain can be considered, regulatory approvals for open field releases are also necessary. As a result, efforts to develop alternative gender separation approaches are continuing.

(b) Classic genetic techniques

The use of classical genetics techniques has proven to be the most promising. These methods are based on the easy integration of a selectable gene to a gender-determining chromosome, in most cases males only, and the subsequent elimination of females (McDonald, 1971; Seawright *et al*., 1978; Franz, 2002). Success of this approach on various insects has been reported, including in anophelines, by primarily the successful development of a GSS based on insecticide resistance using classical genetics (Seawright *et al*., 1978; Yamada *et al*., 2012; Dandalo *et al*., 2018). The presence of selectable mutations that can be linked to gender via chromosomal translocations is required for the successful construction of any new GSS using classical genetics (Franz, 2005). Most previously established *Anopheles* GSS relied on insecticide resistance as a selectable marker, including *An. gambiae* (Curtis, 1976), *An. albimanus* (Kaiser *et al*., 1978; Seawright *et al*., 1978), *An. stephensi* (Robinson, 1986), *An. quadrimaculatus* (Kim *et al*., 1987) and *An. arabiensis* (Curtis, 1978; Lines and Curtis, 1985; Yamada *et al*., 2012; Dandalo *et al*., 2018). Insecticide resistance was the preferred choice as a model because it is mono-factorial and dominant or semi-dominant, and insecticide resistance mutations are common (Davidson and Jackson, 1961; Hemingway, 1992). However, increased environmental concerns and the possibility of insecticide contamination of the mass-rearing system, combined with maintenance difficulties, resulted in the extinction of these strains in the laboratory.

The use of insecticide resistance as a selectable marker for GSS development was revived by Yamada *et al*. (2012) through using dieldrin to separate *An. arabiensis* males from females. Dandalo (2016) followed the same approach and successfully introgressed the male dieldrin resistance into an *An. arabiensis* strain producing a sexing strain with South African genetic background. This strain shows potential. However, it faces some practical challenges, including the prohibition of using dieldrin enacted in the 1970s (De Kom and Dewan, 2007),

adherence of dieldrin to treatment containers and residual retention of treated eggs through to the adult stage, and potential contamination of other rearing colonies by dieldrin use (Yamada *et al*., 2013b). Furthermore, this strain has low levels of fertility (30 %) and male adult recovery (Dandalo *et al*., 2018). All the above challenges were experienced recently during South Africa's pilot releases of sterile males using a dieldrin based genetic sexing strain (Mashatola, unpublished results).

Despite these challenges, the success use of classical genetics techniques has prompted additional research into other alternative selectable markers that are not insecticide based and have high fertility levels. Temperature-sensitive lethality (*tsl*) mutations (gene variants that allow the normal function of an organism at certain temperatures but lethality at other temperatures) are one type of conditional mutation (Suzuki *et al*., 1967; Nguyen *et al*., 2021). The most successful *tsl*-based GSS is the *C. capitata* strain, which has been used on a large scale for a long time (Caceres, 2002). *Tsl* mutations are commonly used in conjunction with morphological markers to map/trace their fate (e.g., white pupae (*wp*) in *C. capitata*) (Caceres, 2002; Franz, 2005; Nguyen *et al*., 2021). Mutagens such as the ethylating agent ethyl methanesulfonate (EMS) (which commonly involves random point mutations, low levels of chromosomal breaks, and lethal effects) can be used to induce *tsl* mutations and any other associated phenotypic markers (Sega, 1984). Examples of insect species where *tsl* mutations have been used include *Drosophila melanogaster* (Meigen (Diptera: Drosophilidae)) (Suzuki, 1969), *Cx. tritaeniorhynchus* (Giles (Diptera: Culicidae)) (Sakai and Baker; 1974), *An. quadrimaculatus* (Mitchell and Seawright, 1989), *An. gambiae s.l.* (Benedict *et al*., 1996), and *An. arabiensis* (Ndo *et al*., 2018).

Furthermore, despite the importance of the *tsl* marker or other morphological colour markers, GSS has not been developed in many SIT-targeted species because naturally occurring mutant isolation is a random process and inducing these mutations artificially is a labour-intensive exercise (Mashatola *et al*., 2018). Additionally, developing a GSS in one species may not be directly transferable to another. Nonetheless, there are many advantages to any GSS development. These include but are not limited to, more thorough and consistent elimination of females, savings of large amounts of money during production by eliminating females in the early life stages, and increased plasticity of release methods, such as releasing pupae rather than adults (Franz, 2005). Because of the high prevalence of *tsl* mutations in all

organisms in nature, a temperature-sensitive GSS can be potentially used in anophelines. Furthermore, mutagens can induce *tsl* (Sakai and Baker; 1974; Franz, 2005). The *tsl*/*wp* GSS discovered in *C. capitata* is an excellent example (Franz *et al*., 2021).

1.5.4. Mosquito handling and packaging procedures before transport to a field site

The development of standard operating procedures (SOPs) for handling, packaging and transport of sterile male mosquitoes to intended field sites is a critical requirement in SIT programmes (FAO/IAEA, 2018; Guo *et al*., 2022). Several studies have investigated and developed some guidelines in this regard (Culbert *et al*., 2017, 2018, 2019; Chung *et al*., 2018; Iyaloo *et al*., 2020; Zhang *et al*., 2020). However, actual universal SOPs that can be applied across the board remain outstanding. The challenge is that the processes involved in handling, packaging and transport, such as rearing conditions, sexing method, sterilisation conditions, temperature, and compaction, among others (Benedict and Robinson, 2003; Dyck *et al*., 2005), have a significant impact on the quality of sterile males. This particularly affects the ability of males going through these various processes to mate successfully and competitively with native males for female mates (Calkins and Parker, 2005; Pagabeleguem *et al*., 2015; Parker *et al*., 2021).

Lessons on handling and transportation of insects for SIT releases can be drawn from previous large-scale SIT programmes that have been successfully operating over the past several years, can serve as guides for studies involving mosquitoes (Blomefield *et al*., 2011; Nepgen *et al*., 2015; Pagabeleguem *et al*., 2015; Seck *et al*., 2015; Boersma and Carpenter, 2016; Dominiak and Fanson, 2021). These operations overall consider the optimal conditions of the entire processes involved in handling, packaging and transport based on whether ground or aerial release will be performed and if pupae or adults will be transported (Enkerlin, 2007; FAO/IAEA, 2017a, 2018).

Historically, ground releases using automobiles have been a standard choice in SIT studies (Enkerlin, 2007; Bjeliš *et al*., 2013). However, recently aerial releases have been used, including field performance tests of sterile male mosquitoes released from an unmanned aerial vehicle (Bouyer *et al*., 2020a). Each release method has its own technical and operational challenges (Enkerlin, 2007; FAO/IAEA, 2018). Ground release, for example, is not well suited to the AW-IPM programme because sterile male releases must adhere to the

existing road system and thus cannot uniformly cover the area to be treated. Aerial releases, typically used in large-scale settings, are hampered by legal restrictions on aircraft types employed in various scenarios (Bouyer *et al*., 2020a; Culbert, 2020; Faraji *et al*., 2021). As a result, it is recommended that ground release methods be used when regulations limit the availability or possibility of using aircraft, legislation, costs, or in small suppression trials (FAO/IAEA, 2017a, 2018).

Regarding pupae and adult transportation, the choice depends mainly on whether a system to separate sexes, especially in programmes such as mosquitoes SIT where females are usually disease vectors are available and the two developmental stages are more resilient or resistant to laboratory handling processes and transport conditions (FAO/IAEA, 2018). Among the laboratory handling processes to consider is the operational logistics required at the release site, specifically, which package container and at what density the insects would be packaged (FAO/IAEA, 2018; Ernawan *et al*., 2022; Sasmita *et al*., 2022).

Regardless of the stage of transportation (pupae or adult), and regardless of whether releases are performed from the ground or aerial, releasing large numbers of insects involves several steps. These include rearing the insects under standard laboratory conditions, restricting their movement by chilling them at low temperatures, packaging the compacted release material at appropriate densities in their release containers, transporting them, and finally releasing them from the same containers (FAO/IAEA, 2018; Tussey *et al*., 2023). Chilling can reduce the physical damage imposed on the insects due to high compaction densities when in transit (Culbert *et al*., 2017). As a result, besides deciding transportation methods, maintaining a cold chain/chilling conditions from the rearing facility to the release site is a crucial consideration in mosquito handling and transport. The optimal temperature for immobilising mosquito pupae and adults as well as the maximum time that chilling can be applied before affecting survival or performance, differ between different species (e.g. *An. arabiensis* (Culbert *et al*., 2017), *Ae. aegypti* and *Ae. albopictus* (Chung *et al*., 2018; Iyaloo *et al*., 2020; Zhang *et al*., 2020).Thus, for the South African mosquito SIT project this will first need to be optimised.

Another factor to consider is the packaging conditions, i.e. packing densities and packaging container type. For example, in most operations involving *C. capitata*, pupae are transported in a shipping box measuring 74 × 34 × 34 cm^3, that is made of double-walled corrugated

cardboard with a top and bottom full overlap (Enkerlin, 2007; Zavala-López *et al*., 2017). The cupboard is lined inside with additional layers of a 46 cm long central compartment corrugated cardboard. The nine bags of pupae are arranged lengthwise in three layers of three bags each, separated by spacers. Cooling units are placed at either end, and the temperature is maintained at 16 °C to 20 °C. In Australia, 2-liter bags are packed in cardboard cartons. In Argentina, a box with dimensions 42.5 × 33 × 27 cm^3 is used (Zavala-López *et al*., 2017; FAO/IAEA/USDA, 2019). The boxes are sealed with carton staples and two bands of fiber-reinforced plastic adhesive tape. For sterile *T. leucotreta*, a small cardboard box at 4 °C to 8 °C was used during transboundary shipment (Boersma and Carpenter, 2016). The moths were enclosed in Styrofoam containers with cooling units for a 72-hour cool chain, crucial for preserving their non-activity and flight ability. For *C. pomonella*, sterile adults were packed in petri dishes and placed in a polyurethane cooler box with icepacks, within a cardboard box. This was then air freighted at near 0 °C for 67 to 89 hours (h), with minimal detrimental effects on their quality (Blomefield *et al*., 2011). For *G. palpalis gambiensis*, irradiated pupae were packed in petri dishes or carton boxes and shipped in insulated boxes with phase change material packs i.e. substances that can release or absorb a significant amount of energy during a phase transition while maintaining a relatively constant temperature (Noël *et al*., 2022). The *G. palpalis gambiensis* pupae were transported as chilled either at 8 °C to 10 °C before emergence or at ambient temperatures (20 °C to 22 °C). In a specific case, eight phase change material packs were used to maintain a 10 °C temperature for up to four days during the shipment of irradiated pupae from Bobo-Dioulasso to Dakar (Pagabeleguem *et al*., 2015).

The few studies conducted on mosquito handling, packaging and transportation have been performed on a small scale, or as part of the phase I studies (Bouyer *et al*., 2020b). For example, during a pilot semi-field cage trial, the released sterile *An. arabiensis* males in Sudan, were transported in reusable paper coffee cups (50 sterile males per cup) from Khartoum to Dongola, situated approximately 400 km south (Helinksi *et al*., 2008). These males were first transported aerially using a small aeroplane and then by ground using an automobile. A mortality rate of less than 6 % across three experiments was observed (Helinksi *et al*., 2008). No chilling or compaction was performed except for a moistened towel meant to create favourable humidity. Upon arrival at the field site, the males remained sexually competitive and lived longer (Helinksi *et al*., 2008). In another recent study on male *An. arabiensis*, Culbert *et al*. (2017) found that adults could be immobilised and stored at 6 °C without compromising

their reproductive fitness. For transport, chilling conditions of 4 °C to 10 °C for no more than 24 h were also recommended (Culbert *et al*., 2017). The study also suggested that if insects are compacted in release device, some chilling and ventilation should be provided (Culbert *et al*., 2017). When designing the release device, it is essential to take into account the sensitivity to low temperatures, the effects of compaction, relative humidity, and the necessity of proper ventilation of the insects contained in it. Similar studies conducted on *Ae. aegypti* also demonstrated that males contained inside a release device could endure temperatures ranging from 7 °C to 28 °C for up to 24 h with minimal impact on their survival (Chung *et al*., 2018). Additionally, the study revealed that higher levels of compaction resulted in damage that is more physical to mosquitoes, ultimately affecting their competitiveness when vying for females alongside native males (Chung *et al*., 2018). On the other hand, lower levels of compaction increased mortality. Another study by Zhang *et al*. (2020) showed that exposure to temperatures between 5 °C and 10 °C for less than 3 h was optimal for handling *Ae. aegypti* males before release. These handling conditions had no adverse effects on survival rate, longevity, or mating competitiveness. The study also reported that transporting adults at temperatures ranging from 8 °C to 12 °C (Zhang *et al*., 2020) do not affect the survival of adult males. In another study on *Ae. albopictus* by Iyaloo *et al*. (2020), it was reported that storage at 5 °C for 2 h had minimal effect on the survival and competitiveness of males. The same study showed that sterile, chilled and compacted adult males could be transported to a field site and successfully perform their mating purpose. In Brazil, a 1.8 litre clear plastic cylindrical container was employed to transport 500 to 1000 transgenic male *Ae. aegypti* (Carvalho *et al*., 2015). In Thailand, a plastic release container with sugar solution was utilised to transport 10 000 to 25 000 irradiated Wolbachia-infected *Ae. aegypti* males (Kittayapong *et al*., 2019). In Guangzhou, China, a release bucket with a large hole on top covered with mesh gauze was employed to transport approximately 1000 irradiated Wolbachia-infected *Ae. albopictus* males (Zheng *et al*., 2019). In Italy, Bellini *et al*. (2013) transported irradiated active pupal *Ae. albopictus* mosquitoes for one to two hours to a release station using petri dishes placed in thermally insulated plastic containers. Despite the positive outcomes of these suppression programmes, the impact of transportation and packing density has not been thoroughly investigated, as distances between irradiation facilities and target areas are typically short. For extended transport durations, it is preferable to transport male specimens in chilled packages to immobilise them, minimising losses in both quantity and quality of sterile males, while ensuring cost-effectiveness. However, studies have indicated that chilling and

compaction, to some extent, can influence the quality of adult male *Ae. aegypti* (Chung *et al*., 2018; Culbert *et al*., 2018; Culbert *et al*., 2019), *Ae. albopictus* (Zhang *et al*., 2020; Iyaloo *et al*., 2020), and *An. arabiensis* (Culbert *et al*., 2017). Though helpful, not all these studies could develop SOPs that can be universally applied to all mosquito species. Their findings, however, provide a valuable guide for future research involving the handling, packaging and transport of mosquitoes.

Ground releases of sterile adult mosquitoes would seem to be the most feasible in South Africa, based on the background of small-scale releases and the legislative and regulatory factors associated with aerial releases (Munhenga, unpublished data). Thus, to successfully conduct pilot field studies in South Africa, it is critical to determine optimal conditions for handling and packaging a local *An. arabiensis* laboratory strain before ground transport and release.

1.6. Justification of the study

South Africa had aimed to eliminate malaria within its borders (i.e., no local malaria transmission) by 2023 (Elimination 8, 2019). The main factor preventing this was persistent residual transmissions being presumably sustained by *An. arabiensis* (Brooke *et al*., 2013; Dandalo *et al*., 2017a). The behavioural plasticity of *An. arabiensis* (i.e., it rests indoors and outdoors and feeds on both humans and animals (Gillies and De Meillon, 1968; Gilles and Coetzee, 1987)), making the outdoor biting population of this species out of reach to the current vector management strategies. In addition, although the observed insecticide resistance in this vector has not yet reached a level where it has an impact on IRS operations, it is only a matter of time until this happens (Munhenga *et al*., 2022). As a result, additional vector control strategies to supplement the existing IRS were suggested. The SIT is among vector control approaches suggested. Investigations of this technology have been going on for over ten years. A wide range of research and numerous milestones on the feasibility and possibility of applying this technique in South Africa have been achieved (Dandalo *et al*., 2017a, 2018; Kaiser *et al*., 2021; Manana *et al*., 2021; Mashatola *et al*., 2017, 2018; Munhenga *et al*., 2011, 2014, 2016, 2022). However, two critical steps remain before the operational application of the SIT. The first is creating an efficient gender separation system to obtain only males for sterilisation before mass release (Dandalo *et al*., 2018; Mashatola *et al*., 2018). This is because none of the currently available gender separation strategies being used in other

insects is readily applicable or transferrable to *An. arabiensis* (Papathanos *et al*., 2009, 2018; Gilles *et al*., 2014, Mashatola *et al*., 2018). The use of traditional genetic approaches to develop an *An. arabiensis* GSS has been suggested and successfully achieved by Dandalo *et al*. (2018). However, the GSS is confronted with several environmental issues and low productivity, which significantly influence cost and efficiency in mass rearing (Dandalo *et al*., 2018). Because of this, new alternative methods for separating *An. arabiensis* males from females are required. In this study, the use of *tsl* or other morphological markers was targeted for the development of an *An. arabiensis* GSS. This would pave the way for isolating orthologous genes in *An. arabiensis* and/or inducing similar mutations that could be used to develop GSS. This is the separation of sexes at an earlier developmental stage, for example, during embryogenesis, and with high genetic stability during mass rearing. The second critical step required before the operational application of the SIT is the development of an SOP to handle and package mosquitoes during sterilisation and transportation to the field site. Mosquito handling from production until field release affects the survival and quality of the sterile males. This has a knock effect on their mating competitiveness. In turn, this can render the SIT programme ineffective. It is thus essential that the South African SIT programme develop its mosquito handling and packaging SOPs.

1.7. Aims and objectives

This study aimed to develop additional systems to separate males and females prior to male sterilisation and release, as well as optimise the handling and transportation requirements for *An. arabiensis* sterile males for the South African SIT programme. This was achieved by addressing the following objectives:

1) To optimise and acclimatise adult *An. arabiensis* females to an artificial membrane feeding method that can replace live animals for routine feeding and performing of blood toxicant experiments.
2) To use the toxicant ivermectin to eliminate blood-feeding *An. arabiensis* females.
3) To determine the maximum threshold temperature (restrictive) for screening of *tsl* in an *An. arabiensis* laboratory colony.
4) To use EMS to induce *tsl* and morphological body variations in *An. arabiensis* and develop GSSs.
5) To use an already established *An. arabiensis* temperature-sensitive strain from Cameroon to develop a *tsl*-based GSS with a South African genomic background.

6) To investigate and establish the optimal temperature and holding (packaging) conditions for small-scale pilot releases transportation of *An. arabiensis*.

CHAPTER 2: Optimisation and acclimatisation of adult *An. arabiensis* females to an artificial membrane feeding method.

2.1. Introduction

Blood-feeding insects, particularly mosquitoes of the *Anopheles* genus, are crucial subjects of study due to their role in the transmission of vector-borne diseases, notably malaria (Benedict *et al*., 2009; Ayesha *et al*., 2023). The need for blood meals in mosquito research and rearing has historically relied on live animals; however, ethical, logistical, and cost considerations have prompted a paradigm shift towards alternative methods (Kasap *et al*., 2003; Deng *et al*., 2012; Ndebele and Musesengwa, 2012; Harrington *et al*., 2020). A noteworthy development in this evolution is the utilisation of artificial membrane feeding systems, which offer precise control over blood meal composition while addressing ethical concerns associated with live animals (Baughman *et al*., 2017; Lauwereyns, 2018).

Artificial membrane feeding systems offer a promising alternative to the use of live animals for feeding as they can facilitate controlled experiments and efficient insectary operations (Cosgrove *et al*., 1994; Kasap *et al*., 2003; Ooi *et al*., 2005). In practical terms, the utilisation of live animals for feeding typically involves preparatory steps such as removing hair or feathers, applying restraint, and administering anaesthesia, thereby introducing complexities into the process (Bailey *et al*., 1978; Foster, 1980). In contrast, artificial membrane feeding systems do not require these and have demonstrated efficacy in maintaining mosquito colonies, investigating vector competence, and testing interventions (Sattabongkot *et al*., 2003; Damiens *et al*., 2013; Ross *et al*., 2019; Timinao *et al*., 2021). Notable successes include the adaptation/optimisation of various *Anopheles* species to artificial membrane feeding (Vallejo *et al*., 2016; Ayesha *et al*., 2023), as well as in studies involving the interactions of toxicants or pathogens (Costa-da-Silva *et al*., 2013), showcasing the versatility of such systems.

While artificial membrane feeding systems offer valuable alternatives in mosquito research and rearing, addressing the challenges associated with species-specific variations, formulation complexities, acclimatisation nuances, and economic considerations is paramount (Suresh *et al*., 2024). A multidisciplinary approach, combining entomology, physiology, and engineering expertise, is essential to navigate these challenges and unlock the full potential of artificial blood feeding systems. Against this background, it is critical to optimise adaptation to artificial

blood feeding systems to maximise the potential of such approaches. This can be achieved through selection of an appropriate system. In addition, a consideration of the specific biology and behaviour of the mosquito species under investigation is essential when choosing an artificial blood feeding systems. Furthermore, factors such as feeding success rates, cost-effectiveness, and ease of use need careful consideration (Timinao *et al*., 2021; Suresh *et al*., 2024).

The acclimatisation process from live animal-based blood meals to artificial membrane feeding is pivotal for successful adoption. This gradual transition allows mosquitoes to adjust to the novel artificial feeding method, ensuring sustained feeding success rates and reproductive fitness (Ross *et al*., 2019). A thorough understanding of the intricacies of acclimatisation is imperative for the broader adoption of artificial membrane feeding in mosquito research and rearing.

There are several artificial membrane feeding systems available (Novak *et al*., 1991; Cosgrove *et al*., 1994; Deng *et al*., 2012; Luo, 2014; Faber *et al*., 2022). However, none of these can be applied universally to all hematophagous insects. Artificial membrane feeding systems are necessary, mainly to laboratories with limited financial budgets or those moving towards a mass-rearing scale, such as SIT programmes. The main challenge seems to be what type of membrane (that can mimic the skin of vertebrate animals), device/feeding system (to control and maintain blood temperature similar to live animals), or blood source (to satisfy the specific preference of each species) should be used (Ooi *et al*., 2005; Damiens *et al*., 2013; Dias *et al*., 2018, 2021). It is, therefore, important to use an inexpensive, convenient, and effective artificial membrane blood-feeding method that may also consider animal welfare (Faber *et al*., 2022).

In terms of membranes, the primary aim is to have a membrane that does not interfere with factors associated with insect maintenance in the laboratory, such as feeding success, fecundity, and egg-hatching rate. It is critical that the membrane is affordable and easy to use. To date, various membranes have been used, ranging from natural animal skins (e.g., hens (Bishop and Gilchrist, 1946), pig intestine (Costanzo *et al*., 2015), sheep intestines (Novak *et al*., 1991), and bovine collagen (Deng *et al*., 2012) and artificial substances (e.g., collagen (Dias *et al*., 2018, 2021), Parafilm® (Deng *et al*., 2012; Dias *et al*., 2018, 2021), latex condoms (Novak

et al., 1991; Phasomkusolsil *et al*., 2013; Dias *et al*., 2018, 2021), silicone (Novak *et al*., 1991; Dias *et al*., 2018, 2021; Ribeiro *et al*., 2023).

Feeding systems for the provision of blood is another aspect that needs consideration. Although various types have been developed and used (e.g., the Glytube system of artificial blood feeding for mosquitoes (Costa-da-Silva *et al*., 2013, de Almeida Costa *et al*., 2020), novel multiple membrane blood-feeding system (Luo, 2014); and polytetrafluoroethylene-based membrane for blood-feeding (Siria *et al*., 2018). The Hemotek® artificial blood feeding system appears to be a viable option (Damiens *et al*., 2013). This is particularly true for facilities that can afford and are moving toward a mass rearing scale (Mamai *et al*., 2017). This system employs an electric heating element to maintain the blood meal at desired high temperatures (Cosgrove *et al*., 1994).

Concerning blood meal sources, studies have shown that various animals blood sources can be used (e.g., human and mice blood (Pina and Fonseca, 1999), rabbits (Novak *et al*., 1991), bovines (Mamai *et al*., 2017), chicken (Bishop and Gilchrist, 1946), sheep (Phasomkusolsil *et al*., 2013), and horses (Costanzo *et al*., 2015). One of the most used methods is anaesthetised adult mice/guinea pigs (Novak *et al*., 1991, Hunt *et al*., 2005) and rabbits (Phasomkusolsil *et al*., 2013). In certain instances, where the preferred blood meal source of a species is known efforts could be made to provide such sources, e.g., *An. arabiensis*, prefers cattle blood (Tirados *et al*., 2006), provision of cattle blood will be ideal in this instance.

The primary objective of this chapter was to explore/investigate a suitable and cost-effective alternative method for providing blood meals to adult *An. arabiensis* females. The goal was to replace the current approach of using conventional use of live animals with an artificial blood feeding system.

2.2. Material and methods

2.2.1. Biological material and rearing procedure

An *An. arabiensis* laboratory strain, acronymed KWAG, colonised in 2005 from material collected from Mamfene, KwaZulu-Natal, South Africa was used in this study (Mouatcho *et al*., 2009). The colony was maintained in the Botha De Meillon Insectary (BDMI) at the National Institute for Communicable Diseases (NICD), Vector Control Reference Laboratory (VCRL),

Johannesburg, South Africa (26°7’57.62’’E, 28°6’56.603’’S). The BDMI was physically separated from areas used for general staff traffic and offices. It comprised eight separate rooms without windows, ventilated through air ducts equipped with high-efficiency particulate air filters (mesh size 100 µm) to prevent adult mosquitoes from escaping. Access to the facility was restricted to authorised personnel, with entrance via a double-door vestibule designed to prevent flying mosquitoes from escaping simultaneously. The insectary maintained controlled conditions of 27 ± 1 °C room temperature, 70 ± 10 % relative humidity, and a 12:12 h photoperiod, including 45-minute transitions of dusk and dawn each cycle (11 h ~ 250 lux full light, 11 h darkness, and 45 minutes of dawn and dusk transitions) (Hunt *et al*., 2005; Sheppard *et al*., 2017). All rearing and colony maintenance procedures followed those described by Hunt *et al*. (2005), with some modifications (see appendix 1).

2.2.2. Description of live and artificial membrane feeding system and feeding procedure

2.2.2.1. Description of live and artificial membrane feeding system

At the time of this study, live anaesthetised guinea pigs were the standard blood feeding method for mosquito colonies at the BDMI (Ref: W-CJ-150911-1, see appendix 2). These guinea pigs were housed at the NICD in standard laboratory cages, maintained at a temperature range of 21 ± 2 °C with a 12-hour light/dark cycle. The cages contained sawdust, commercial rabbit pellets, and ad-lib water supplemented with vitamin C. Cage cleaning occurred three times per week, and efforts were made to maintain a quiet environment to minimise stress on the guinea pigs. All anaesthesia injections and veterinary procedures, including the shaving of a 3.7 to 4 cm diameter patch of fur on the abdomen, were performed 10 to 20 minutes prior to mosquito blood feeding by trained veterinarians registered with the South African Veterinary Council.

For artificial feeding, the Hemotek® membrane feeding system for blood-sucking insects (Hemotek, Product Code: SP6W1-3, Blackburn, United Kingdom, http://hemotek.co.uk/) was utilised. This system comprises a power unit, multiple heating plates, aluminium reservoirs (3.7 cm diameter × 1.3 cm thickness with a total reservoir capacity of 3 mℓ) and O-shaped black rubber rings (Figure 2.1). Operating electronically via the power unit, the device synchronously regulates the temperatures of the multiple heating plates, facilitating the simultaneous comparison of different membranes (Cosgrove *et al*., 1994).

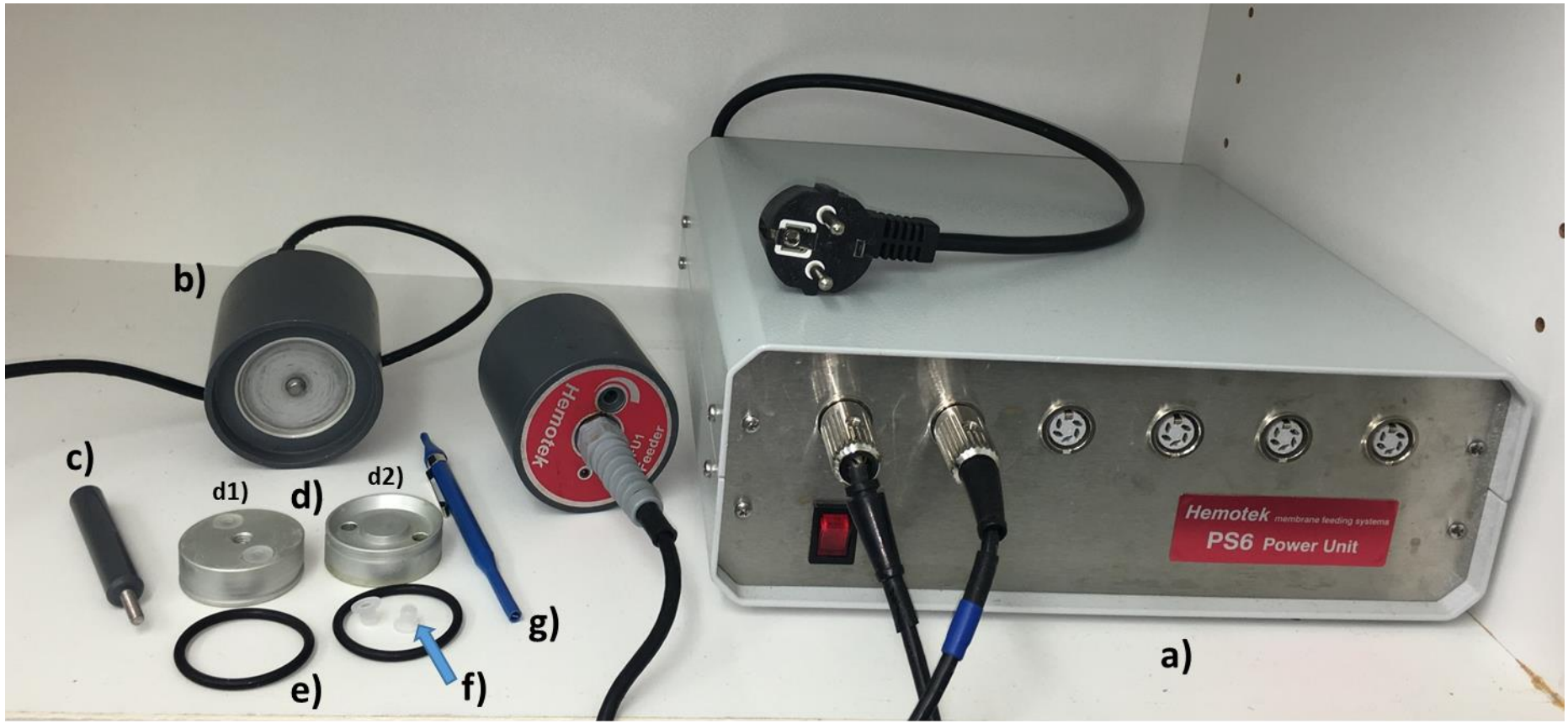


Figure 2.1: The Hemotek® membrane feeding system used during blood feeding optimisation and acclimatisation of a laboratory *An. arabiensis* colony: a) PS6 Power Unit (to accommodate six heating plates simultaneously), b) heating plate, c) plug remover, d) aluminium reservoir (d1 is the flat bottom, d2 is the well top where blood goes in), e) O-ring, f) plug, and g) blue temperature setting device.

2.2.2.2. Blood feeding procedure

The live animal feeding procedure involved placing an shaved and anaesthetised guinea pig on top of a BugDorm-1® insect rearing cage (BugDorm, Model: DP1000, MegaView Science Co., Ltd., https://shop.bugdorm.com/). The BugDorm-1® insect rearing cage was made mainly of polyethylene netting and polypropylene plastic material. The cage's net weight was 1.1 g with dimensions of 30 cm length × 30 cm width × 30 cm height. It featured clear panels on the bottom, front, and top, along with mesh panels on the back, left, and right sides. The mesh size measured 32 cm × 32 cm with an 800 µm aperture. Notably, the front panel included a single sleeve opening measuring 15 cm in diameter and 40 cm in length. During blood feeding procedures, the cage was tilted to the side, positioning the left mesh panel on top to accommodate a guinea pig lying on its shaven abdomen side.

The artificial membrane feeding procedure involved fully enclosing a reservoir with a single layer of one of three different types of membranes. These were collagen membrane (Edicol edible collagen membrane, dimensions: 580 mm wide × 30 m length, thickness: 10 to 20 µm), Parafilm-M® (Parafilm-M®, www.parafilm.com, dimensions: 101.6 mm wide × 100 m length, thickness: 10 to 20 µm), and hog casing (purchased from Jay Jay Butchery, Lyndhurst discount centre, Johannesburg, dimensions: 28 mm wide × 20 m length, thickness: 10 to 20 µm) (Figure 2.2).

a) b) c)

Figure 2.2: Artificial membranes used in this study during blood feeding optimisation of a laboratory *An. arabiensis* colony: (a) Collagen membrane (dimensions: 580 mm wide × 30 m length, thickness: 10 to 20 µm), (b) Parafilm-M® (dimensions: 101.6 mm wide × 100 m length,

thickness: 10 to 20 µm) and (c) Hog casing (dimensions: 28 mm wide × 20 m length, thickness: 10 to 20 µm).

The membranes were trimmed to fit the size needed to cover the reservoir adequately and then firmly secured using a rubber O-shaped ring (Figure 2.3). Before application, the hog casing, which had been stored in brine, was rinsed briefly with tap water for approximately 30 seconds. Furthermore, the hog casing was slit longitudinally to produce a single layer. Parafilm-M® was manually stretched evenly by hand to create a single layer, while the collagen membrane was utilised in its original state, with the textured surface oriented outward. Frozen defibrinated cattle blood, 3 mℓ in volume, was thawed at room temperature for 2 h before been injected into the reservoir using a pipette. A plug was then inserted to seal the reservoir, preventing any spillage or leakage of the blood. Following this, the reservoir was attached to a heating plate that had been pre-warmed to a temperature range of 37 °C to 39 °C, corresponding to temperatures experienced by most live vertebrates that mosquitoes feed on (Cosgrove *et al*., 1994; de Boer *et al*., 2022). The heating plate with the reservoir attached was placed on top of the netted side of a BugDorm-1® cage, allowing mosquitoes to access the blood through the membrane using their proboscis.

The cattle blood utilised in this study was sourced from a local abattoir, Karan Beef (Pty) Ltd, located in Balfour, Mpumalanga, South Africa. The blood collection process involved manual defibrination, which included mixing fresh blood with glass beads in a 5 ℓ container to prevent clotting or coagulation. Subsequently, the blood was transferred into 50 mℓ falcon tubes and placed in cooler boxes containing ice for transportation back to the NICD. Upon arrival at the laboratory, the blood samples were stored in a refrigerator set at -20 °C and were utilised within a period of three months or until fully depleted.

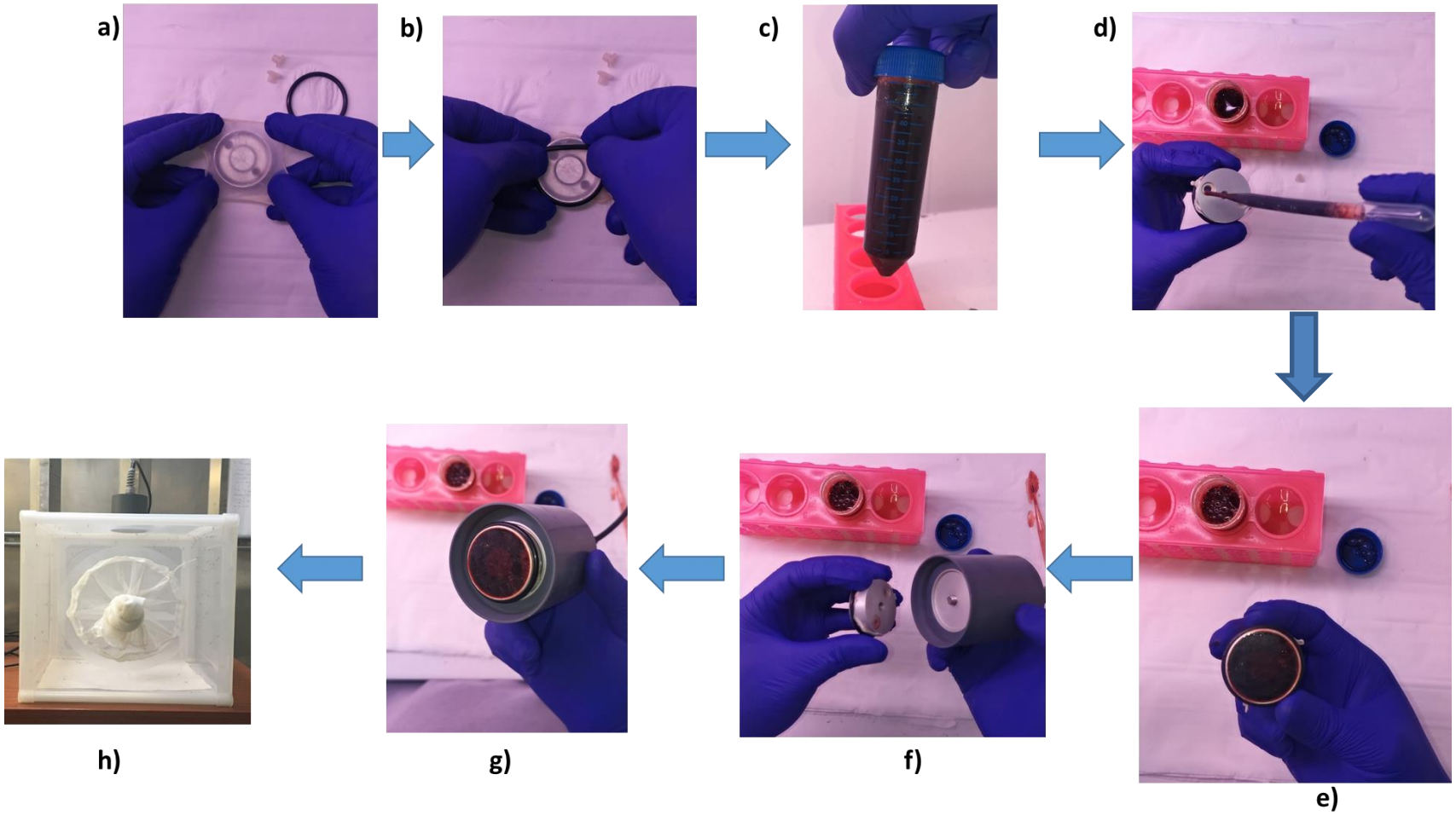


Figure 2.3: Artificial membrane feeding procedure. (a) membrane placed over to top side of reservoir, (b) membrane secured onto an aluminium reservoir using O-ring, (c) defibrinated, thawed cattle blood in a 50 mℓ falcon tube, (d) blood inserted into an aluminium reservoir using a pipette, (e) blood inside an aluminium reservoir, (f) aluminium reservoir with blood inside before attachment to a heating plate (pre-warmed to 37 °C) and (g) after it is attached to a heating plate, (h) heating plate with aluminium reservoir containing blood placed on top of the netted side of a BugDorm-1® insect rearing cage.

2.2.3. Comparison of feeding on live guinea pig vs artificial membrane feeding methods (optimisation).

The experimental design for comparing feeding on a live guinea pig versus artificial membrane feeding methods (optimisation) involved introducing an equal ratio of newly emerged adults (300♂:300♀) into a BugDorm-1® cage and allowed three to four days to mate. After the mating period, females were randomly selected and introduced into new BugDorm-1® cages in batches of 50 per cage. A mouth aspirator constructed with a transparent glass or polycarbonate plastic tube measuring 30 cm in length and having an outer diameter of 2 cm was used to suck mosquitoes. It also includes a latex rubber tube measuring 20 cm in length with a 2 cm outer diameter. These two components are separated by a 50 µm nylon mesh screen. Four separate cages were set up concurrently: cage 1 (control) involved direct natural skin-feeding on a guinea pig, cage 2 consisted of collagen membrane, cage 3 utilised Parafilm® membrane while cage 4 employed hog casing membrane (Figure 2.4).

During the feeding process, blood feeding on a guinea pig lasted 10 minutes following the protocol by Hunt *et al*. (2005), while Damiens *et al*. (2013) conducted artificial membrane feeding for 2 hours. Subsequently, the number of females with visible blood meal in their abdomen (engorged females) was counted and recorded for each cage to determine the mean percentage of females with visible blood meal in their abdomen, which served as the measure of blood-feeding success. Unfed females were removed from the cages, and each cage was provided with a 10 % sugar water solution soaked on cotton wool.

Two days after the first blood meal, only females that had blood-fed in the first feeding were provided a second blood meal. Then, all live female mosquitoes in each cage were counted three days after the second blood meal, and a plastic container spray painted black on the outside (henceforth referred to as egg plate (appendix 1, Figure S1.3)) and filled inside with 150 distilled water was placed inside each cage overnight to serve as an oviposition site. The egg plates were removed from the cages the following day and the total number of eggs laid from each cage counted using an optical binocular magnifying lens (Donegam Optical Company, Lenexa, Kansas 66219, USA). The number of females alive divided this total number of eggs per cage on the day the egg plates were placed inside the cage. This was used to determine fecundity. Additionally, a subsample of 10 females was removed from each cage post-oviposition, and each female was dissected under a microscope to observe the presence

of spermatozoa in their spermatheca (MR4, 2015), which was used to determine insemination success. The absence of sperm indicated a lack of mating.

All eggs from each cage were monitored for hatching once daily for 14 consecutive days. This was done by visually observing for hatchlings inside the egg plates, removing them with a plastic pipette into a new plastic bowl. The total number of hatchlings per egg plate was recorded and the mean percentage of larvae that hatched used to determine fertility. The experiments were repeated three times (i.e., three biological repeats). A summary diagram of the experimental design for comparing direct skin feeding (live guinea pig) and artificial membrane feeding using three different membranes (collagen, Parafilm-M® and hog casing) is shown in Figure 2.4.

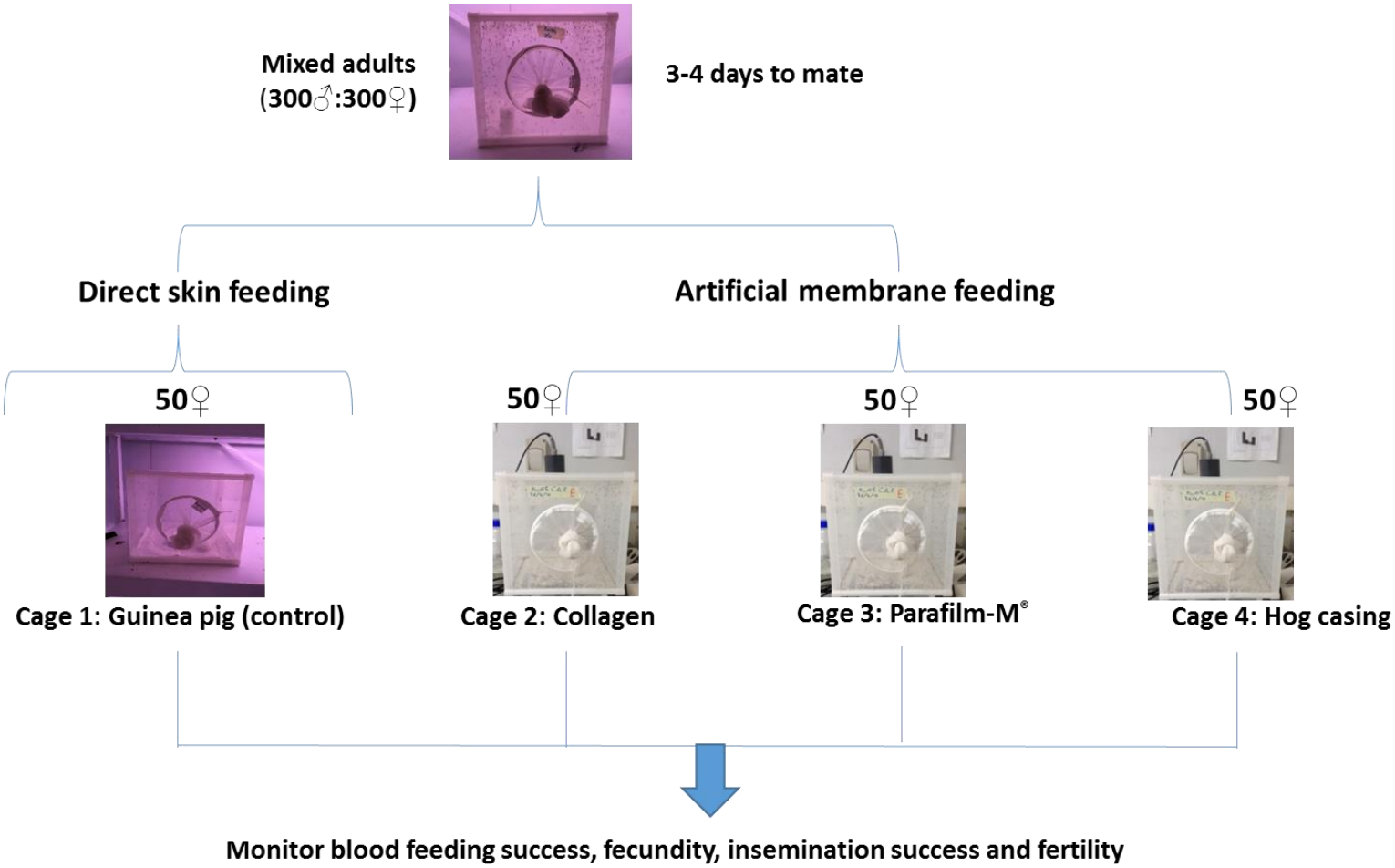


Figure 2.4: Experimental design used for comparison of direct skin feeding (live guinea pig) and artificial membrane feeding using three different membranes (collagen, Parafilm-M® and hog casing). Mixed adults were allowed three to four days to mate, blood fed and monitored for blood-feeding success (mean percentage of females with visible blood meal in their abdomen), fecundity (the mean number of eggs laid from each cage), insemination success (mean percentage of females with sperm in their spermathecal) and fertility (total number of hatchlings per egg batch).

2.2.4. Impact of blood feeding on the reproductive biology of *An. arabiensis* females using hog casing membranes over six successive generations (acclimatisation).

After completing the initial comparison of feeding methods (optimisation), the study investigated the potential for acclimatisation to artificial membrane feeding methods, focusing specifically on the hog casing membrane due to its favourable attributes. The experimental design involved successive generations (F_0, F_3, and F_6) of *An. arabiensis* mosquitoes being exposed to artificial membrane feeding using hog casing.

In each generation, newly emerged adults (100♂:100♀) were introduced into BugDorm-1® cages and allowed to mate for three to four days. Females (50♀) from the selected generation were removed and introduced into a new BugDorm-1® cage. These were provided with two blood meals over a five-day period through artificial membrane feeding. Their reproductive parameters (blood-feeding success, fecundity, insemination success, and fertility) were recorded. These parameters were not measured for the F_1, F_2, F_3 and F_4 generations. Progeny from each generation were reared to adulthood using standard methods and subjected to the same artificial feeding regimen, with data recorded at specific intervals.

The acclimatisation process was assessed by comparing the recorded parameters (blood-feeding success, fecundity, insemination success, and fertility) between generations (F_0, F_3, and F_6). This comparison aimed to determine whether there were any changes indicative of adaptation to the new feeding regimen over successive generations. Each experiment was replicated three times as detailed in the experimental design outlined in Figure 2.5.

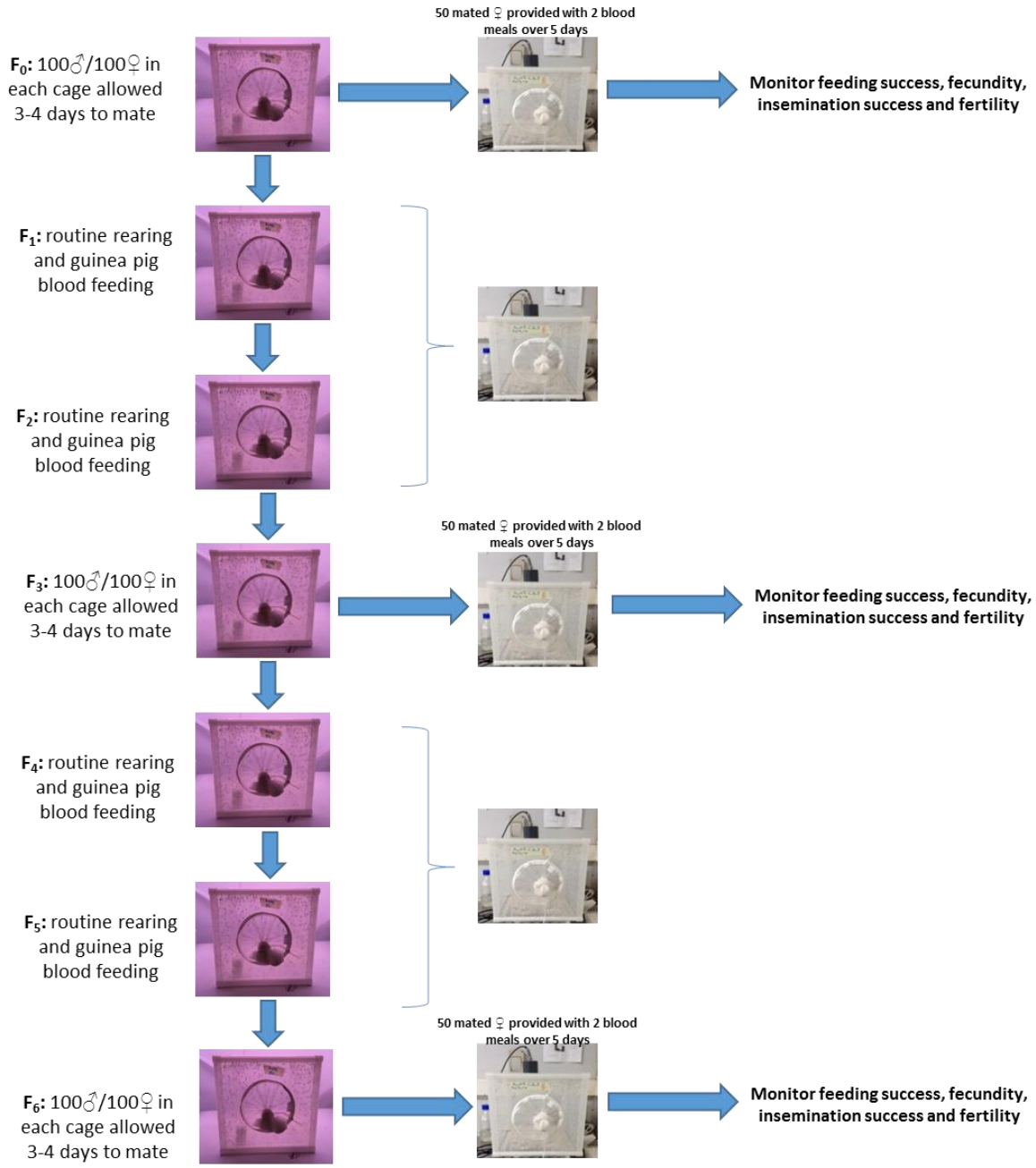


Figure 2.5: Experimental design for acclimatisation of adult *An. arabiensis* females to artificial membrane blood-feeding method. The acclimatisation process was assessed by comparing the recorded parameters (blood-feeding success, fecundity, insemination success, and fertility) between generations (F_0, F_3, and F_6). These parameters were not measured for the F_1, F_2, F_3 and F_4 generations.

2.2.5. Data analysis

All data were recorded and entered into Microsoft Excel (version 2016, Microsoft Corporation, Redmond, WA). Data on both the comparison of natural skin *vs* artificial membrane and selection of artificial feeding method per generation was recorded and summarised over three biological repeats. The data was statistically analysed using the Statistical Package for the Social Sciences (SPSS) (IBM Corp, 2015) software. First, the data was checked for normal distribution (homogeneity) using the Shapiro-Wilk test. The mean percentage of females with visible blood meal in their abdomen (blood-feeding success), the mean number of eggs laid from each cage (fecundity), the mean percentage of females with sperm in their spermatheca (insemination success), and the mean percentage of larvae that hatched (fertility) were compared between feeding method and filial generations with the data analysed using a one-way analysis of variance (ANOVA) where the data was normally distributed or the non-parametric Kruskal–Wallis test where the data was not normally distributed. In all cases, a p-value of less than 0.05 ($p < 0.05$) was used to indicate statistical significance.

2.3. Results

2.3.1. Comparison of feeding on live guinea pig vs artificial membrane feeding methods (optimisation).

The results on comparison of feeding on a live guinea pig vs artificial membrane feeding methods are shown in Table 2.1. Hog casing (74.4% ± 6.3) and guinea pig feeding (71.3% ± 9.9) showed the highest blood-feeding success (± SD). This was followed by collagen membrane (60.7% ± 8.2), and Parafilm® (24.8% ± 9.6) respectively (Table 2.1). A significant statistical difference between blood-feeding success and feeding method was found, with subsequent Bonferroni *post-hoc* test showing a similar feeding success in guinea pig, collagen and hog casing but all being different from Parafilm® (one-way ANOVA, $F_{(3,10)} = 11.43$; $p = 0.001$).

Fecundity, varied across the feeding methods, with the highest average fecundity (± SD) observed in mosquitoes fed on guinea pigs (12.5 ± 5.6 eggs per female) and the lowest in those fed on Parafilm® (3.2 ± 1.3 eggs per female). However, statistical analysis did not reveal a significant difference in fecundity between the various feeding methods (one-way ANOVA, $F_{(3,10)} = 2.71$; $p = 0.0914$) (Table 2.1).

Insemination success was consistently high across guinea pigs, collagen, and Parafilm®, with rates exceeding 96%. Hog casing showed a perfect insemination success rate of 100%.

Importantly, statistical analysis did not detect a significant difference in insemination success among the different feeding methods (one-way ANOVA, $F_{(3,10)} = 0.50$; $p = 0.7368$).

Fertility (± SD) ranged as follows: hog casing (92.2% ± 6.4), collagen (91.5% ± 6.4), guinea pigs (87.4% ± 12.6), and Parafilm® (76.7% ± 6.5). However, these differences were not statistically significant (one-way ANOVA, $F_{(3,10)} = 1.22$; $p = 0.361$).

Table 2.1: Selected life history traits (blood-feeding success, fecundity, insemination success and fertility) of *An. arabiensis* mosquitoes that were fed from different feeding methods (live animal vs artificial membrane).

Feeding method		Blood feeding success (%) ± SD (95% CI)	Fecundity ± SD (95% CI)	Insemination success (%) ± SD (95% CI)	Fertility (%) ± SD (95% CI)
Live animal	**Guinea pig abdomen**	71.3 ± 9.9 (61. 4 – 81.2)[a]	12.5 ± 5.6 (6.9 – 18.1)[a]	96.7 ± 3.3 (93.4 – 100)[a]	87.4 ± 12.6 (74.4 – 100)[a]
Artificial membrane	**Collagen**	60.7 ± 8.2 (52.5 – 68.9)[a]	5.1 ± 2.8 (2.3 – 7.9)[a]	96.7 ± 3.3 (93.4 – 100)[a]	91.5 ± 6.5 (85.0 – 98.0)[a]
	Parafilm®	24.8 ± 9.6 (15.2 – 34.4)[b]	3.2 ± 1.3 (1.9 – 4.5)[a]	96.7 ± 3.3 (93.4 – 100)[a]	76.7 ± 6.5 (84.1 – 80.9)[a]
	Hog casing	74.4 ± 6.3 (68.4 – 90.7)[a]	8.8 ± 6.5 (2.3–15.3)[a]	100 ± 0[a]	92.2 ± 6.4 (85.8 – 98.6)[a]

Numbers within columns are mean ± SD (95% CI). Different superscript letters in a column show significant differences ($p < 0.05$).

2.3.2. Impact of feeding through hog casing for 6 generations on the reproductive biology of *An. arabiensis* females (acclimatisation).

The results regarding feeding success, fecundity, insemination success, and fertility following the selection of the colony for artificial membrane feeding are presented in Table 2.2. The mean blood-feeding success (± SD) exhibited a progression, with values of 62.6% ± 3.7 in the F_0 generation, 82.4% ± 1.7 in the F_3 generation, and 94.9% ± 1.4 in the F_6 generation. Statistical analysis, did not reveal a significant difference in the average feeding success between the generations (one-way ANOVA, $F_{(3,10)} = 1.70$; $p = 0.2593$). In terms of fecundity (± SD), the F_0 generation had 8.8 eggs per female ± 6.5, while the F_3 and F_6 generations showed 3.5 eggs per female ± 1.0 and 13.3 eggs per female ± 1.8, respectively. Although there was a marginal difference, statistical analysis did not find a significant variance in fecundity across these three generations (one-way ANOVA, $F_{(2,6)} = 4.67$, $p = 0.0599$). Insemination success (± SD) displayed a trend of 93.3% ± 6.5 in F_0, 96.7% ± 2.8 in F_3, and 100% in F_6, with no statistically significant difference detected (one-way ANOVA, $F_{(3,10)} = 0.60$; $p = 0.5787$). Fertility (± SD), assessed as 92.2% ± 6.5 in F_0, 88.9% ± 10.5 in F_3, and 95.3% ± 1.7 in F_6, also showed no significant difference between these generations (one-way ANOVA, $F_{(2,6)} = 0.59$, $p = 0.5829$).

Table 2.2: Mean number of selected life history traits (blood feeding success, fecundity, insemination success and fertility) of different generations of adult laboratory-reared *An. arabiensis* females' blood-fed artificially using hog casing.

Generation	Blood feeding success (%) ± SD (95% CI)	Fecundity ± SD (95% CI)	Insemination success (%) ± SD (95% CI)	Fertility (%) ± SD (95% CI)
F_0	62.6 ± 3.7 (58.9 – 66.3)[a]	8.8 ± 6.5 (2.3 – 15.3)[a]	93.3 ± 6.5 (86.8 – 100)[a]	92.2 ± 6.5 (85.7 – 98.7)[a]
F_3	82.4 ± 1.7 (80.7 – 84.1)[a]	3.5 ± 1.0 (2.5 – 6.0)[a]	96.7 ± 2.8 (93.9 – 99.5)[a]	88.9 ± 10.5 (78.4 – 99.4)[a]
F_6	94.9 ± 1.4 (93.5 – 96.3)[a]	13.3 ± 1.8 (11.5 – 15.1)[a]	100 ± 0[a]	95.3 ± 1.7 (93.6 – 97.0)[a]

Numbers within columns are mean ± SD (95% CI). Different superscript letters in a column show significant differences ($p < 0.05$)

2.4. Discussion

This study aimed to address a common blood-feeding challenge faced by insectary facilities worldwide concerning the maintenance of mosquito colonies, particularly *Anopheles* species (Dias *et al*., 2018; Ayesha *et al*., 2023). Through a comparative analysis, the study sought to identify a cost-effective and efficient method for providing blood meals to an *An. arabiensis* laboratory colony (KWAG), aiming to replace the traditional use of live animals. Different feeding techniques, including direct use of live guinea pigs and indirect artificial methods using collagen membrane, hog casing, and Parafilm®, were compared. Key parameters such as blood-feeding success, fecundity, insemination success, and fertility were assessed to determine the suitability of each method for maintaining KWAG. Furthermore, the colony was tested for gradual transition (acclimatisation) to artificial membrane feeding using hog casing.

2.4.1. Comparison of feeding on live guinea pig vs artificial membrane feeding methods (optimisation).

The results from the comparison of feeding methods between live guinea pigs and artificial membrane feeding indicated that mosquitoes feeding through hog casing and guinea pig had comparable blood-feeding success rates. The lowest blood-feeding success was observed when females were fed using the artificial blood-feeding system through Parafilm-M® membrane. Furthermore, feeding success was also accompanied with good fecundity and fertility. This result suggest that hog casing could serve as a feasible alternative to the traditional method involving live animals. This finding is significant, considering the ethical concerns and logistical challenges associated with using live animals in laboratory settings.

However, it is important to note that blood-feeding success can be influenced by various factors, such as the species of the mosquito, whether the mosquito was reared in a colony or collected in the wild and the adaption level of the colony (Timinao *et al*., 2021). Furthermore, blood-feeding success is dependent on the experimental conditions under which the feeding is carried out and this includes the starvation prior to blood-feeding, the parameters of starvation (such as water access), the membrane type utilised, the feeding rate duration, the age of the mosquito, blood source and the amount of blood in the feeder (Timinao *et al*., 2021; Melgarejo-Colmenares *et al*., 2022, 2023). In this study, efforts were made to standardise most of these factors. For example, the choice of a blood meal source is critical for efficient rearing and cattle blood, preferred blood meal for *An. arabiensis* (Meza *et al*., 2019; Mlacha

et al., 2020), and was chosen due to its cost-effectiveness and readily availability from local abattoirs. The other factors were difficult to make direct comparison, for example cattle vs guinea pig or defibrinated vs fresh blood. However, as the focus of this study was on comparison of different membranes, discussion is limited to findings of this study.

Several studies concerning membrane feeding have been conducted with a range of parameters and these resulted in variations in the success of feeding (Dias *et al*., 2018, 2021; Ross *et al*., 2019; Timinao *et al*., 2021). The high feeding success demonstrated by hog casing and collagen membranes natural surface texture of hog casing. The observed high feeding success exhibited by hog casing is consistent with prior studies suggesting that its natural surface texture, which facilitates piercing and access to blood, can enhance feeding success and subsequent reproductive fitness (Novak *et al*., 1991; Lyski *et al*., 2011). The collagen membrane on the other hand, with its textured gripping surface on the side from which mosquitoes probe through, also produced similar results to hog casing. This is because mosquitoes utilise their proboscis to feed, enabling them to pierce through clothes or skin, even from hairy animals or through the netting of the BugDorm-1® cage. In addition, both hog casing and collagen membranes are generally easier to handle, provide results that are more consistent, are cost-effective, and readily available through local suppliers. Collagen membranes are particularly suitable for sustainable or mass-rearing settings, as they are routinely used at the IPCL/FAO/IAEA for blood feeding with the Hemotek® system (Damiens *et al*., 2013; FAO/IAEA, 2017b; Mamai *et al*., 2017). In contrast, Parafilm-M® membrane demonstrated a low feeding success rate of 24.8%, which contradicts the findings reported by Dias *et al*. (2018). In their study, Dias *et al*. (2018) observed high feeding success rates of 98% for *Ae. aegypti* and 96% for *An. aquasalis* when using the Parafilm-M® membrane. However, they noted a lower feeding success rate of 48% for *Cx. quinquefasciatus*. The observed differences in efficiency could be attributed to how Parafilm-M® is prepared, particularly in terms of stretching or distending (Dias *et al*., 2018, 2021). Parafilm-M® can very easily rupture when stretched above over three times its original size, making artificial feeding difficult in a laboratory routine (Cosgrove et al., 1994; Luo et al., 2014). It is essential for the membrane to be sufficiently thin for easy perforation by mosquitoes, yet durable enough to prevent blood leakage (Dias *et al*., 2018, 2021). Although attempts were made to stretch the Parafilm-M® membrane uniformly during this study, standardisation of distension may have been lacking, making it challenging females to pierce and feed through it, thus explaining the low feeding

success. This is supported by the observation that females congregated around the feeder and attempted to pierce the Parafilm-M® membrane unsuccessfully. Another notable drawback of Parafilm-M® is its brittleness after prolonged exposure to 38 °C (temperature of the heated plate mimicking a live animal), which can lead to ruptures. This issue was not observed with collagen membranes and hog casing.

The observed variability in fecundity among feeding methods in this study underscores the importance of considering the overall reproductive fitness of mosquitoes when selecting feeding protocols. While guinea pig feeding resulted in the highest fecundity, hog casing and collagen membrane methods also exhibited relatively high fecundity, indicating their potential suitability for maintaining mosquito colonies. This was expected as research has shown that direct skin feeding on live animals is more useful than indirect membrane feeding using artificial methods (Kasap *et al*., 2003; Deng *et al*., 2012). For example, the colony used in this study has been selected for guinea pig feeding since 2005 (Hunt *et al*., 2005; Mouatcho *et al*., 2009). In terms of membrane type, the results of this study are similar to other researchers who found low fecundity using Parafilm-M® membrane on *Ae. aegypti* and *Ae. albopictus* (Lyski *et al*., 2011; Luo, 2014) but different to those obtained from several artificial blood feeding studies with different objectives on *An. stephensi* (Nasirian and Ladonni, 2006), and *An. sacharovi* (Kasap *et al*., 2003). It is hypothesised that differences in fecundity could be due to blood type (Takken and Verhulst, 2013; de Swart *et al*., 2023). The composition of blood, including factors like protein content, amino acids, and hormonal levels, can vary depending on the blood type of the host. This variation in blood composition may affect the physiological processes within the mosquito, including egg development and fecundity. Overall, although direct comparison of these different blood sources was not accounted for in statistical analysis, the consensus is that high blood feeding success correlated with high fecundity as has been the case in other studies (Hurd *et al*., 1995; Phasomkusolsil *et al*., 2013).

In terms of insemination success, this study found consistently high insemination success rates for across all feeding methods, indicating that blood-feeding method does not compromise the mating efficiency of mosquitoes. This is a crucial aspect as successful mating is essential for generating viable offspring (Chambers and Klowden, 2001; Klowden and Russell, 2004; Sawadogo *et al*., 2013). Also especially considering the next measured parameter, fertility. Mosquitoes can lay eggs after a blood meal, which bodes well for fecundity, but for eggs to

hatch there needs to have been mating that occurred (Chambers and Klowden, 2001; Klowden and Russell, 2004). Given that females were checked for insemination success only after they had matured (three to four days of mating) and undergone blood feeding, the results of this study are not surprising even though they contrast with earlier research on insemination rates in non-blood-fed mosquitoes, where approximately one-fifth of *An. gambiae s.s.* were reported to be unmated at six days old (Charlwood and Jones, 1979). This could be because the *An. arabiensis* colony used in this study is accustomed to colonisation and has no difficulty mating in this environment (Mouatcho *et al*., 2009; Benedict *et al*., 2009). Also, unlike previous studies that found insemination to be influenced by factors such as age, size, mating duration, and to be crucial for egg laying (Chambers and Klowden, 2001; Klowden and Russell, 2004; Sawadogo *et al*., 2013), this was not the case in our study. The mosquitoes used were of the same age, provided with the same rearing conditions, mating period, and egg-laying opportunity. Therefore, the fact that the blood-feeding method did not affect insemination success in this study is well founded. Moreover, these findings suggest that the choice of feeding method does not significantly influence the ability of female mosquitoes to successfully mate with males. This is a crucial result as it indicates that the choice of feeding method can be based on practical considerations without compromising the insemination success of mosquitoes.

Furthermore, any blood feeding method need not only result in high feeding success, insemination success and fecundity, but also the eggs need to be fertile to perpetuate the next generation. As a result, it was necessary to investigate the fertility of resultant eggs from females fed on the various feeding methods. Relatively high fertility was recorded throughout all the treatments affirming egg viability. These findings are similar to several studies where different blood feeding methods were assessed and there was also no significant difference in fertility (Kasap *et al*., 2003; Ooi *et al*., 2005; Pothikasikorn *et al*., 2010 Lyski *et al*., 2011; Luo, 2014). The comparably high fertility found between the routine guinea pigs and all other artificial feeding methods indicates the substantial viability of the eggs and the reliability of the both these methods for colony maintenance. However, further investigation into the long-term effects of different feeding methods on mosquito fitness parameters could provide valuable insights into their suitability for routine laboratory mosquito production.

Overall, the findings from the studies above suggest that hog casing and collagen feeding methods offer comparable outcomes to guinea pigs in terms of blood-feeding success, fecundity, insemination success, and fertility. However, because of availability, practicality and the small scale of subsequent experiments for acclimatisation of the colony for artificial membrane, hog casing was used.

2.4.2. Impact of feeding through hog casing for 6 generations on the reproductive biology of *An. arabiensis* females (acclimatisation).

The progressive increase in blood-feeding success observed across generations suggests an improvement in the adaptation of mosquitoes to the artificial membrane feeding setup. The consistent increase from the F_0 to the F_6 generation indicates a potential adaptation among the mosquito population, leading to more efficient feeding behaviour over time. Although there was no statistically significant difference in feeding success among these generations the gradual increase in proportion of females that took a blood meal as the generation increases suggests that mosquitoes can adapt to and efficiently utilise artificial membrane feeding methods, ultimately improving blood feeding rates. This adaptation is a promising, as it suggests that colonies can transition from live animal feeding to more ethically and logistically convenient artificial membrane feeding while maintaining fertility across successive generations. Insemination success also remained consistently high throughout these generations. Moreover, while there were variations in fecundity across generations, statistical analysis did not reveal significant differences. This indicates that while there might be fluctuations in reproductive output across generations, the overall fecundity remains relatively consistent. Moreover, the slight variance observed in fecundity could be attributed to various factors, such as genetic variability within the mosquito populations, environmental conditions, and feeding protocol adjustments over time, as documented in Ross *et al*. (2019). Thus, overall, these findings support the feasibility of transitioning mosquito colonies from live animal feeding to artificial membrane feeding using hog casing, which is ethically and logistically advantageous, particularly in the context of mosquito research and control programmes.

However, it is important to highlight the importance of considering multiple factors, including blood-feeding success, fecundity, and ethical considerations, when selecting feeding methods for mosquito breeding. Future studies could explore additional factors influencing feeding

success and fecundity, such as diet composition, feeding frequency, and environmental conditions, to further optimise the artificial membrane feeding protocol for mosquito colonisation (Nasirian *et al*., 2006). Thus, investigating the addition of phago-stimulants (compounds detected by insect chemoreceptors that stimulate insects to imbibe their meals to full engorgement) such as natural odour ligands from human skin (Ghaninia *et al*., 2008), L-lactic acid (Sanz *et al*., 2008; Ayesha *et al*., 2023) as well as adenosine triphosphate (ATP) (Galun, 1967) may improve blood feeding success. Additionally, investigating the genetic and physiological mechanisms underlying the observed adaptations in feeding behaviour could provide valuable insights into mosquito biology and vector-host interactions. Importantly, the current results of this study pave a way for investigating toxicants or pathogens that can be added into these artificial feeding methods without worrying about affecting live animals (see next chapter 3).

2.5. Conclusion

Artificial blood-feeding methods using a Hemotek® system with hog casing or collagen membrane can serve as a viable alternative to the traditional direct skin blood-feeding approach involving live guinea pigs, with no significant compromise in reproductive fitness. Furthermore, there were indications of acclimatisation to hog casing membrane feeding, suggesting that it is feasible to gradually select a mosquito colony to adopt indirect blood-feeding methods over time. Building on these findings, it is recommended that artificial blood-feeding techniques be considered as a replacement for direct animal blood-feeding practices. This transition has already been effectively implemented at the BDMI, where all mosquito colonies have successfully transitioned from guinea pig blood feeding to Hemotek® blood-feeding system.

CHAPTER 3: Use of the toxicant, ivermectin, to eliminate blood-feeding *An. arabiensis* females.

3.1. Introduction

As part of efforts towards malaria elimination, there are ongoing investigations into complementary vector control strategies such as the SIT (Munhenga *et al*., 2011, 2014, 2016; Dandalo *et al*., 2017a, 2018). This technique involves a systematic area-wide release of sterile insects that mate with wild ones, resulting in no offspring (Dyck *et al*., 2021). Over time, targeted populations can be suppressed or eliminated. However, because female mosquitoes, unlike male mosquitoes, can transmit disease (Clements, 1992), they must be removed from the mass production process before sterilisation and field releases (Papathanos *et al*., 2009; Gilles *et al*., 2014; Mashatola *et al*., 2018). As a result, mosquito SIT programmes' success will depend on the exclusive release of sterile males, which will be impossible on a large scale without effective methods to separate males from females (Mashatola *et al*., 2018).

Various gender separation strategies for different stages of mosquitoes are currently being tried to successfully establish a sexing mechanism (Papathanos *et al*., 2009; Gilles *et al*., 2014; Mashatola *et al*., 2018). The most commonly used methods include genetic sexing, molecular, mechanical, and behavioural approaches. For example, though sex separation using genetic and molecular techniques are most suitable and preferred for large-scale settings, but their development and application may only become available in the long term (Papathanos *et al*., 2009, 2018; Gilles *et al*., 2014). In comparison, mechanical and behavioural sexing tools are more suitable for small-scale feasibility studies. Mechanical tools were considered suitable for small-scale (Papathanos *et al*., 2009, 2018; Gilles *et al*., 2014), until the automated, mechanical sex separation of *Aedes* mosquitoes in China (Zheng *et al*., 2019) which has paved way for large-scale application.). In *An. arabiensis*, attempts to establish mechanical approaches have already been tried and, due to a lack of significant differences in pupal sizes, were excluded as a possible sexing strategy (Mashatola *et al*., 2018). Because of this, behavioural approaches were considered as a primary focus by researchers (Mashatola *et al*., 2018).

Behavioural methods such as swarming and delayed female emergence have been investigated and excluded as possible gender separation methods in anophelines (Papathanos *et al*., 2009, 2018; Gilles *et al*., 2014). As a result, the exploitation of the haematophagous behaviour of adult female mosquitoes was suggested (Papathanos *et al*., 2009). Previous

studies have shown that blood can be spiked with insecticides (e.g., malathion, dieldrin) or other toxicants (e.g., ivermectin, spinosad) to kill females during blood feeding, allowing males to be separated from females (Lowe *et al*., 1981; Yamada *et al*., 2013a). Of these blood-spiking toxicants, the macrocyclic lactone extract, ivermectin, showed the most significant potential (Yamada *et al*., 2013a). Its mode of toxicity directly targets the mosquito nervous system and muscle performance by inhibiting neurotransmission via glutamate-gated chloride channels after oral ingestion, makes ivermectin the best candidate (Yates and Wolstenholme, 2004; Moreno *et al*., 2010; Yamada *et al*., 2013a).

However, even though behavioural sex separation by spiking blood with ivermectin has been investigated in other studies involving mosquitoes such as *An. arabiensis* (Yamada *et al*., 2013a) and both *Ae. aegypti* and *Ae. albopictus* (Gunathilaka *et al*., 2019), no attempt has been made to assess the efficacy of these methods for the South African mosquito SIT programme. This chapter aimed to assess the efficacy of ivermectin in eliminating females as well as investigate the ability of males to successfully mate (reproductive fitness) following inadvertent exposure to ivermectin.

3.2. Material and methods

3.2.1. Mosquito strain

This study used the same *An. arabiensis* laboratory strain (KWAG) described in 2.2.1.

3.2.2. Determining the optimal concentration of ivermectin to kill adult *An. arabiensis* after a single blood meal.

3.2.2.1. Preparation of ivermectin

A 10 mg/ℓ (equivalent to 10 parts per million (ppm)) stock solution of ivermectin was prepared by dissolving powdered ivermectin formulation (MK-933, CAS No. 70288-86-7) in absolute ethanol (CAS No.: 9005-79-2). Both solutions were procured from Sigma-Aldrich (St. Louis, MO). A 500 mℓ laboratory glass bottle, with GL45 Screw Cap was used for mixing the solutions (SKU: TC4304, https://worldofscience.co.za/). The ivermectin stock solution was serially diluted in distilled water to produce three final concentrations of 2.5, 7.5 and 10 ppm. All solutions were kept chilled in a refrigerator (Labex, South Africa) at 4 °C until used for spiking blood.

3.2.2.2. Experimental design.

The experimental design involved introducing cohorts of 100 newly emerged adult females and 50 males (100♀:50♂) into four different BugDorm-1® cages (Figure 3.1). This 2:1 ratio was used to increase the chance of ending up with at least 50 adult females that could be blood-fed and used as described below. Three cages served as treatments, where each was provided with 3 mℓ of defibrinated, thawed cattle blood (> 3 months old) containing ivermectin concentrations of 2.5, 7.5 and 10 ppm, respectively. The fourth cage served as a control cage, and mosquitoes were fed with 3 mℓ of unspiked (no ivermectin added) cattle blood. Blood feeding was performed an hour after the dusk light cycle of the insectary (i.e., in the dark) using the Hemotek® membrane feeding system through a hog casing membrane (chapter 2). Each cage was provided with a single blood meal through a Hemotek® membrane feeding system on the first day after emergence for a duration of 2 h. After blood feeding, only 50 randomly selected females that contained blood in their abdomen (both partial and fully engorged) were allowed to remain in the cage while the other females were removed using a mouth aspirator. Thus leaving 50 males and 50 blood-fed females in each cage. A 10 % sugar water solution soaked in cotton wool placed in 100 mℓ urine jars was placed into each cage to sustain the adults. Over the next five days, dead adult males and females were removed from the cages, and the number of adults killed was recorded daily by gender. All experiments were repeated three times using mosquitoes from the same strain (i.e., biological repeats).

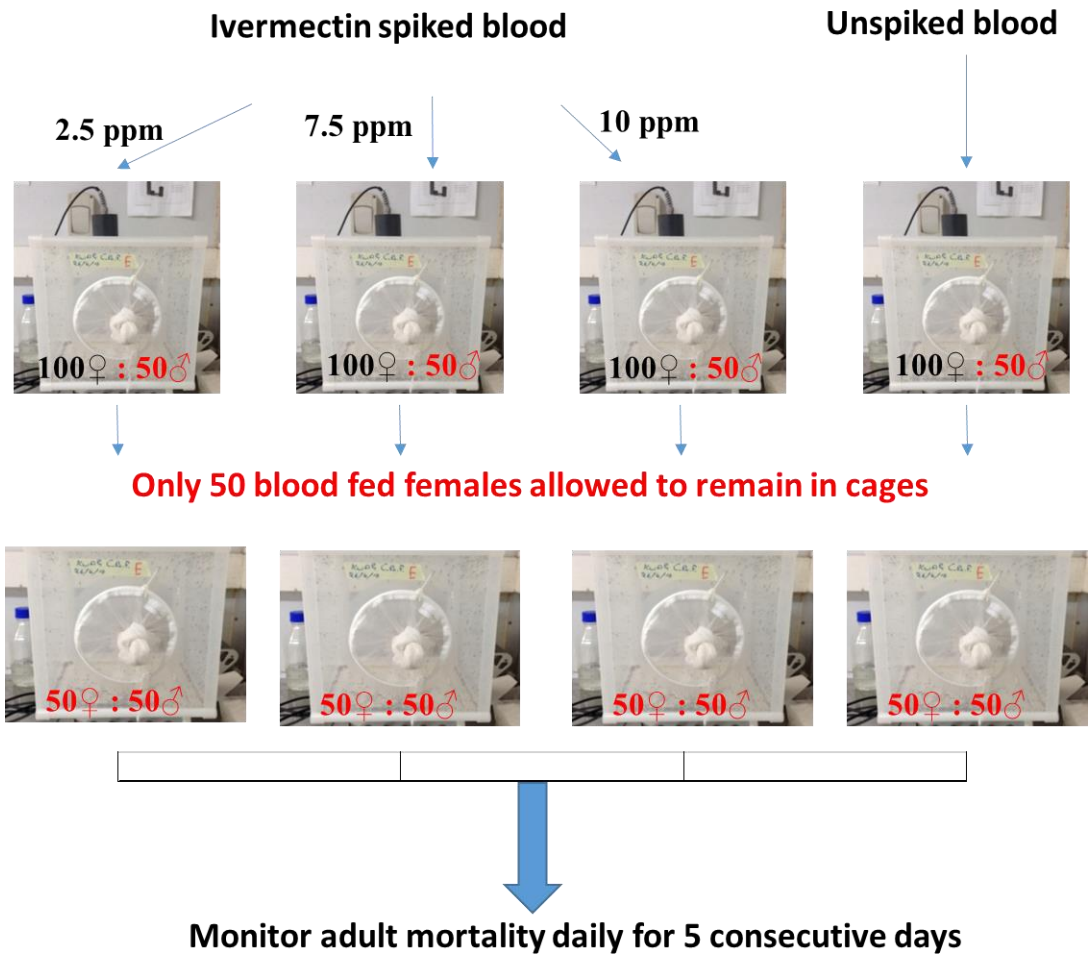


Figure 3.1: Experimental set-up for determining the optimal concentration of ivermectin spiked in the blood, which can kill adult *An. arabiensis*.

3.2.3. Determining the effectiveness of the optimal ivermectin concentration to eliminate female *An. arabiensis*.

Once the optimal ivermectin dose (7.5 ppm) was determined as detailed in 3.2.2., a single BugDorm-1® cage was stocked with an equal number of newly emerged male and female adults (100♂:100♀) (Figure 3.2). A day after, this cage was provided with a 3 mℓ cattle blood meal spiked with the optimal concentration of ivermectin determined in 3.2.2 (7.5 ppm). Blood feeding was performed once daily during the dark light cycle for 2 h until all the females were eliminated. The control for this experiment consisted of a BugDorm-1® cage containing the same number of newly emerged male and female adults (100♂:100♀). The females were fed daily on 3 mℓ of unspiked cattle blood (without ivermectin). In both cages (control and treatment), adult mortality was recorded daily by sex, counting and removing dead mosquitoes from each cage. The number of days taken to achieve 100% female mortality and the number of males remaining on that day were also recorded.

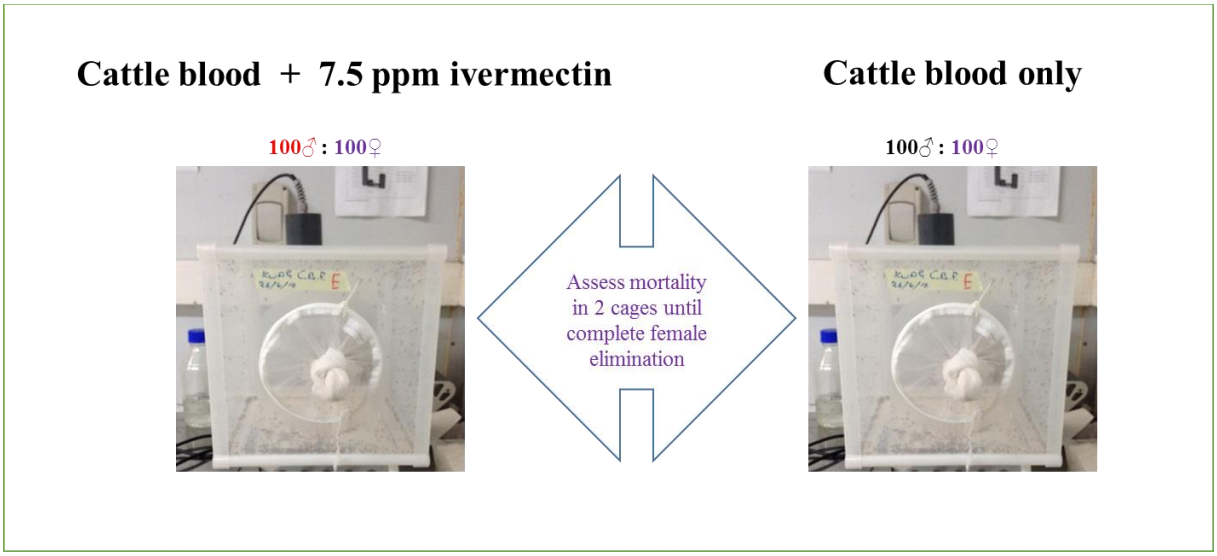


Figure 3.2: Determining the optimal ivermectin concentration's effectiveness (time) to eliminate female *An. arabiensis*.

3.2.4. Assessing the reproductive fitness of males present in a cage when females were fed blood spiked with 7.5 ppm of ivermectin

In this objective, the impact of female elimination using the blood spiking on male reproductive fitness was evaluated by setting up a number of cages concurrently. The first cage (cage 1) contained 100 newly emerged male adults and 100 newly emerged females (i.e., n = 200). Females from this cage were fed with ivermectin-spiked blood as described in 3.2.3 to eliminate all females (Figure 3.3a). Cage 2 contained the same number of adults as cage 1 and was fed on unspiked cattle blood (Figure 3.3a). Cage 3 (fed only sugar water and containing males only, n = 200) and cage 4 (fed unspiked cattle blood (similar to cage 2 control and containing females only, n = 200) were prepared concurrently with cages 1 and 2 to ensure that adult ages between all four cages are similar in terms of age and generation (Figure 3.3b). Cage 3 (males only) was sustained exclusively on a 10% sugar solution, while cage 4 (females only) was provided with both a 10% sugar solution and unspiked 3 mℓ similar to cage 2. Subsequently, males from cage 1 (n = 50), cage 2 (n = 50) and cage 3 (n = 50) were transferred to new respective cages (called treatment cages, untreated control cage and sugar control cage (Figure 3.3c)). Fifty virgin females from cage 4 were transferred into each treatment cage, untreated control cage and sugar control cage. Mosquitoes in all cages were allowed to mate for three consecutive days, after which 20 females were randomly removed from each cage and their spermatheca dissected to check for the presence of spermatozoa. The number of females with sperm in their spermatheca were counted and recorded (this data was used to

determine the insemination rate as a proxy of reproductive fitness). These experiments were repeated on three different occasions, each time using new mosquitoes.

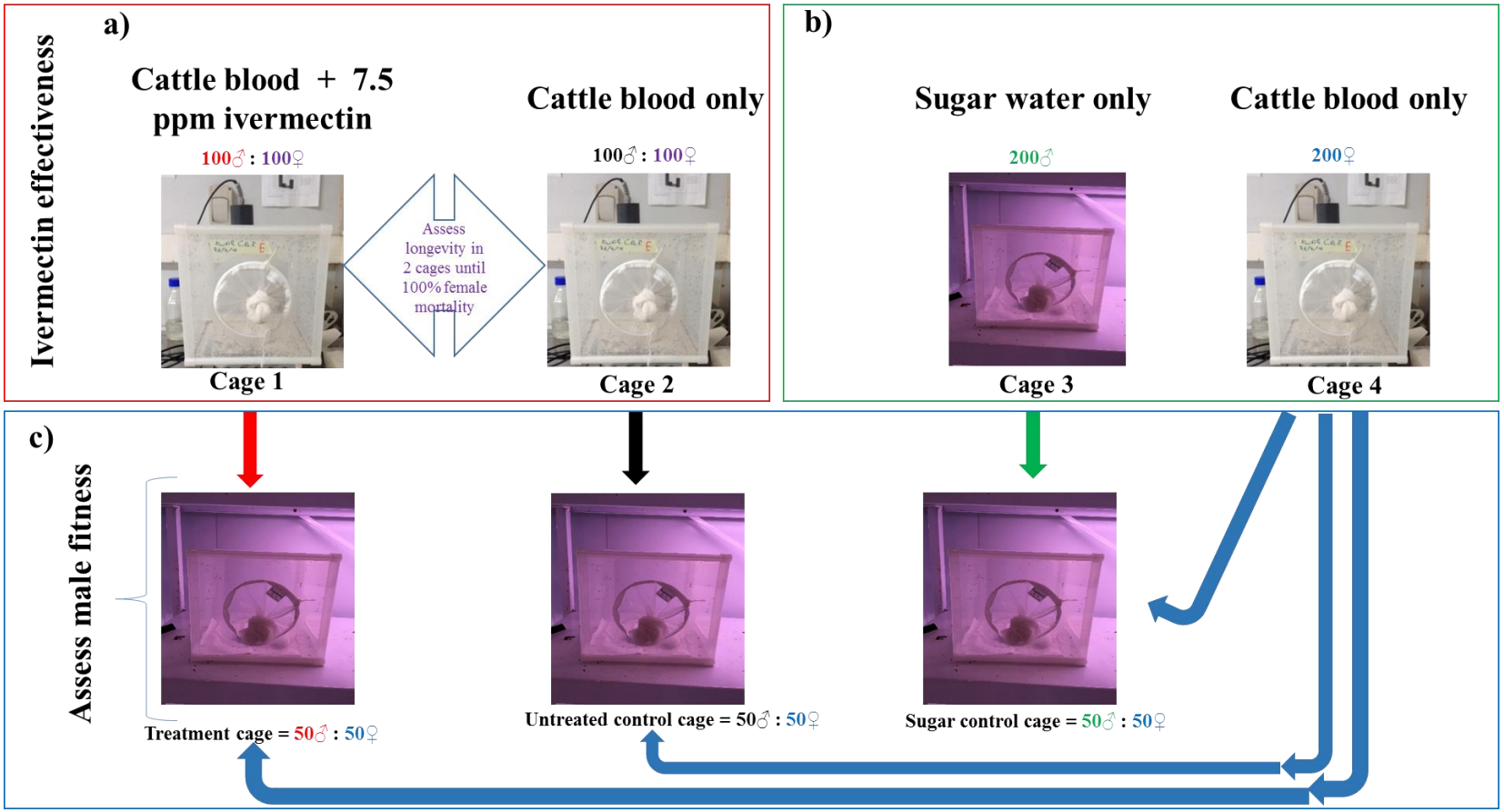


Figure 3.3: Experimental set-up for assessing the reproductive fitness of the male survivors post female elimination by verifying their mating ability. a) cage 1 contained a mix of adult males and females that were exposed to ivermectin spiked blood until all females were eliminated, cage 2 contained a mix of adult males and females that were exposed to unspiked blood; b) cage 3 (containing males only) and cage 4 (containing female only) were respectively provided with sugar water only and cattle blood only, c) treatment cage, untreated control cage and sugar control cage each contained an equal number of females from cage 4 and males from cage 1, 2 and 3 respectively.

3.2.3. Data analysis

Data was entered into Microsoft Excel (version 2016, Microsoft Corporation, Redmond, WA) and imported into the SPSS (IBM Corp, 2015) software to analyse and generate figures. The Shapiro-Wilk test was used to determine if the data was normally distributed. The number of dead males or females was recorded daily for optimal ivermectin concentration investigations. The data were summarised as the rate of males or females dying per day (daily mortality rate), determined as the number of females that died per day (n) over the total number of females at risk/exposed over three replicates (N) (i.e., n/N)). In addition, the proportion of males or females dying in treatment compared to control per day was determined. A Cox proportional hazard regression analysis was utilised to compute the hazard ratio, representing the daily risk of death attributed to the treatment, as well as the overall relative risk (RR) of mortality concerning the predictor variable (ivermectin concentration) and the outcome (female mortality). Additionally, a Cox proportional hazard regression model was employed to investigate whether male mortality was linked to ivermectin treatment following the complete elimination of females. Survival curves were established through the Kaplan-Meier survival analysis and a log-rank test to determine whether there is a difference in female or male survival times between the ivermectin treated and untreated groups. To assess reproductive fitness, data on the proportion of females with sperm in their spermatheca (indicating induced insemination by males) was aggregated per replicate to calculate an average value per treatment, which was then compared by Pearson Chi-Square test.

3.3. Results

3.3.1. Determining the optimal concentration of ivermectin to kill adult *An. arabiensis* after a single blood meal.

3.3.1.1. Female mortality

The mean female daily mortality rate in the cages fed with the lowest ivermectin concentration of 2.5 ppm was 38% on day one, 21.3% on day two, 4% on day three, 0.67% on day four and 1.3% on day five (Table 3.1). In 7.5 ppm, female mortality was 51.3%, 15.3%, 4.7%, 3.3% and 0.7% on days one to five, respectively. The last concentration, 10 ppm, showed an average female mortality rate of 56.7% on day one, 18.7% on day two, 6% on day three and 2% on days four and five. The average female mortality in control was 3.3%, 0.67%, 3.3%, 5.3% and 2.7% on days one to five, respectively. The proportional rate of female death varied daily across days, with the highest risk of death (hazard ratio) recorded on day four from 2.5 ppm (HR = 3.165) (Table 3.1).

Table 3.1: Daily mortality rates and hazard ratio of adult females after feeding (ingestion) on blood spiked with different ivermectin concentrations (0 (control), 2.5 ppm, 7.5 ppm and 10 ppm).

Time period (day)	Ivermectin (ppm)	N	n	Daily mortality rate (%)	Hazard ratio
1	0 (control)	150	5	3.3	
	2.5	150	57	38.0	0.088
	7.5	150	77	51.3	0.065
	10	150	85	56.7	0.059
2	0 (control)	145	1	0.7	
	2.5	93	32	34.4	0.020
	7.5	73	23	31.5	0.0219
	10	65	28	43.1	0.016
3	0 (control)	144	5	3.5	
	2.5	61	6	9.8	0.353
	7.5	50	7	14.0	0.248
	10	37	9	24.3	0.143
4	0 (control)	139	8	5.8	
	2.5	55	1	1.8	3.166
	7.5	43	5	11.6	0.495
	10	28	3	10.7	0.537
5	0 (control)	131	4	3.1	
	2.5	54	2	3.7	0.824
	7.5	38	1	2.6	1.160
	10	25	3	12.0	0.255
Cumulative mortality	0 (control)	150	23	15.3	
	2.5	150	98	65.3	
	7.5	150	113	75.3	
	10	150	128	85.3	

Note: values are N = total number of females at risk/exposed over three replicates, n = number of females that died per day, daily mortality rate (calculated as number died during time period/number of females at risk (i.e., n/N), Hazard ratio = proportion of the rate of females dying per day in treatment compared to control.

Overall, the relative risk of female death after five days was shown to be reduced by a factor of 0.453 (54.7%) (i.e., RR = 0.453 represents a 54.7% reduction in the risk of dying after five days) (Table 3.2). Females fed on the highest ivermectin concentrations (10 ppm and 7.5 ppm) showed a similar relative risk of death (Exp(B) = 1.004 (10 ppm) and 0.977 (7.5 ppm)). Statistically, the female death hazards were significantly different from 2.5 ppm (p = 0.001) but similar between 7.5 ppm (p = 0.864) and 10 ppm (p = 0.973).

Table 3.2: Relative risk of adult females after feeding (ingestion) on blood spiked with different ivermectin concentrations (0 (control), 2.5 ppm, 7.5 ppm and 10 ppm).

Ivermectin	B	SE	Wald	df	Sig.	Exp(B)	95% CI for Exp(B)	
							Lower	Upper
0 ppm			12.504	3	0.006			
2.5 ppm	-0.791	0.233	11.501	1	0.001	.453	0.287	0.716
7.5 ppm	-0.023	0.134	0.029	1	0.864	0.977	0.751	1.272
10 ppm	0.004	0.129	0.001	1	0.973	1.004	0.780	1.294

B: represents the coefficient estimates or regression coefficients, SE: Standard Error, which measures the variability or precision of the coefficient estimate, **Wald:** refers to the Wald statistic, which is used in Wald tests for hypothesis testing in logistic regression models, **df:** Degrees of Freedom, which represent the number of independent values or observations in the model, **Sig.:** Significance level, indicating the probability of observing the data given that the null hypothesis is true, **Exp(B):** Exponential of the regression coefficient, interpreted as the odds ratio or relative risk, **95% CI for Exp(B):** 95% Confidence Interval for the odds ratio or relative risk, indicating the range of values within which the true population parameter is likely to fall with 95% confidence.

3.3.1.2. Male mortality

The mean daily male mortality rates in the cages fed with 2.5 ppm were 3.3% on day one, 4.0% on day two, 0.7% on day three, 0.7% on day four and 2.0% on day five (Table 3.3). In the other concentrations, the highest male mortality in 7.5 ppm was 3.3% on day four while in 10 ppm the highest mean male mortality rate (10%) was observed on day two. Overall, male mortality in the treated cohorts was lowest in 7.5 ppm ivermectin (5.3% ± 5.0), followed by 2.5 ppm (11.3% ± 9.5), and with the highest observed in 10 ppm (20.0% ± 6.0). The proportional rate of male death varied daily. On day one and two, 7.5 ppm showed the lowest risk of death (HR = 0.666 and 0.993 respectively) (Table 3.4). Overall, the relative risk of male death after 5 days was highest at 10 ppm (Exp(B) = 0.535), followed by 7.5 ppm (Exp(B) = 0.380) and 2.5 ppm (Exp(B) = 0.046) (Table 3.4). Statistically, the male death hazards were significantly different in all concentrations ($p < 0.05$).

Table 3.3: Daily mortality rates and hazard ratio of adult males after exposure to blood spiked with different ivermectin concentrations (0 (control), 2.5 ppm, 7.5 ppm and 10 ppm).

Time period (day)	Ivermectin (ppm)	N	n	Daily mortality rate (%)	Hazard ratio
1	0 (control)	150	2	1.3	
	2.5 ppm	150	5	3.3	0.400
	7.5 ppm	150	3	2.0	0.667
	10 ppm	150	5	3.3	0.400
2	0 (control)	148	3	2.0	
	2.5 ppm	145	11	7.6	0.267
	7.5 ppm	147	3	2.0	0.993
	10 ppm	145	20	13.8	0.147
3	0 (control)	145	7	4.8	
	2.5 ppm	134	13	9.7	0.498
	7.5 ppm	144	5	3.5	1.390
	10 ppm	125	22	17.6	0.274
4	0 (control)	138	8	5.8	
	2.5 ppm	121	14	11.6	0.501
	7.5 ppm	139	6	4.3	1.343
	10 ppm	103	26	25.2	0.229
5	0 (control)	130	10	7.7	
	2.5 ppm	107	17	15.9	0.484
	7.5 ppm	133	8	6.0	1.279
	10 ppm	77	30	39.0	0.197
Cumulative mortality	0 (control)	130	10	7.7	
	2.5 ppm	107	17	15.9	
	7.5 ppm	133	8	6.0	
	10 ppm	77	30	39.0	

Note: values are N = total number of males at risk/exposed over three replicates, n = number of males that died per day, rate of males dying (calculated as number died during time period/number of males at risk (i.e., n/N), Hazard ratio = proportion of the rate of males dying in treatment compared to control.

Table 3.4: Relative ratio of adult males after exposure to blood spiked with different ivermectin concentrations (0 (control), 2.5 ppm, 7.5 ppm and 10 ppm).

Ivermectin	B	SE	Wald	df	Sig.	Exp(B)	95% CI for Exp(B)	
							Lower	Upper
0 ppm			226.892	3	0			
2.5 ppm	-3.084	0.22	196.075	1	0	0.046	0.03	0.07
7.5 ppm	-0.969	0.123	62.339	1	0	0.38	0.298	0.483
10 ppm	-0.625	0.117	28.645	1	0	0.535	0.426	0.673

B: represents the coefficient estimates or regression coefficients, **SE:** Standard Error, which measures the variability or precision of the coefficient estimate, **Wald:** refers to the Wald statistic, which is used in Wald tests for hypothesis testing in logistic regression models, **df:** Degrees of Freedom, which represent the number of independent values or observations in the model, **Sig.:** Significance level, indicating the probability of observing the data given that the null hypothesis is true, **Exp(B):** Exponential of the regression coefficient, interpreted as the odds ratio or relative risk, **95% CI for Exp(B):** 95% Confidence Interval for the odds ratio or relative risk, indicating the range of values within which the true population parameter is likely to fall with 95% confidence.

3.3.2. Determining the effectiveness of the optimal ivermectin concentration to eliminate female *An. arabiensis*.

3.3.2.1. Female survival

A Kaplan-Meier survivorship curve for adult *An. arabiensis* females fed on cattle blood spiked with ivermectin are shown in Figure 3.4. Females fed on unspiked cattle blood (controls) had the highest survival rate of 38 days, while females exposed to 7.5 ppm ivermectin had a survival rate of 11 days. The median survival time (days) ± SE (95% CI) of females in cages blood-fed on ivermectin-spiked blood was 2.0 ± 0.66 (1.87 – 2.13), compared to 11.0 ± 0.33 (10.36 – 11.64) in cages fed on unspiked blood. A log-rank (Mantel-Cox) comparison of survival rates between these two groups revealed a significant difference ($\chi^2 = 413.0$, df = 1, $p < 0.05$).

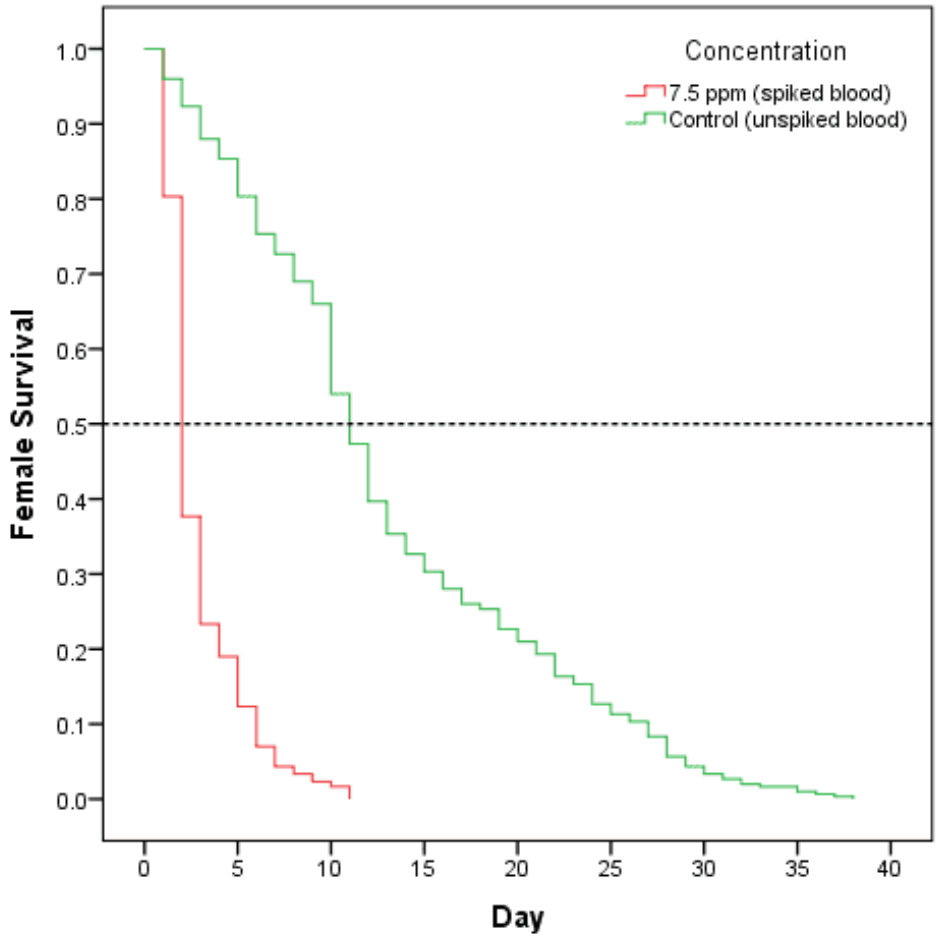


Figure 3.4: Kaplan-Meier survivorship curves for adult *An. arabiensis* females fed with cattle blood spiked with 7.5 ppm ivermectin (red curve) and those blood-fed on unspiked cattle blood (control, green curve).

3.3.2.2. Male survival

During the period it took to eliminate females completely, adult males from both the ivermectin exposed cages and non-exposed cages showed similar survival (cumulative mean male mortality ± SE (95% CI) was 21.0% ± 3.0 (8.1 – 33.9) in the ivermectin treated cages and 20.7% ± 3.8 (4.1 – 37.2)) (Figure 3.5). A Cox proportional hazard regression analysis showed no statistically significant difference in adult male mortality between the two groups ($\chi^2 = 7.33$, df = 1, $p > 0.05$).

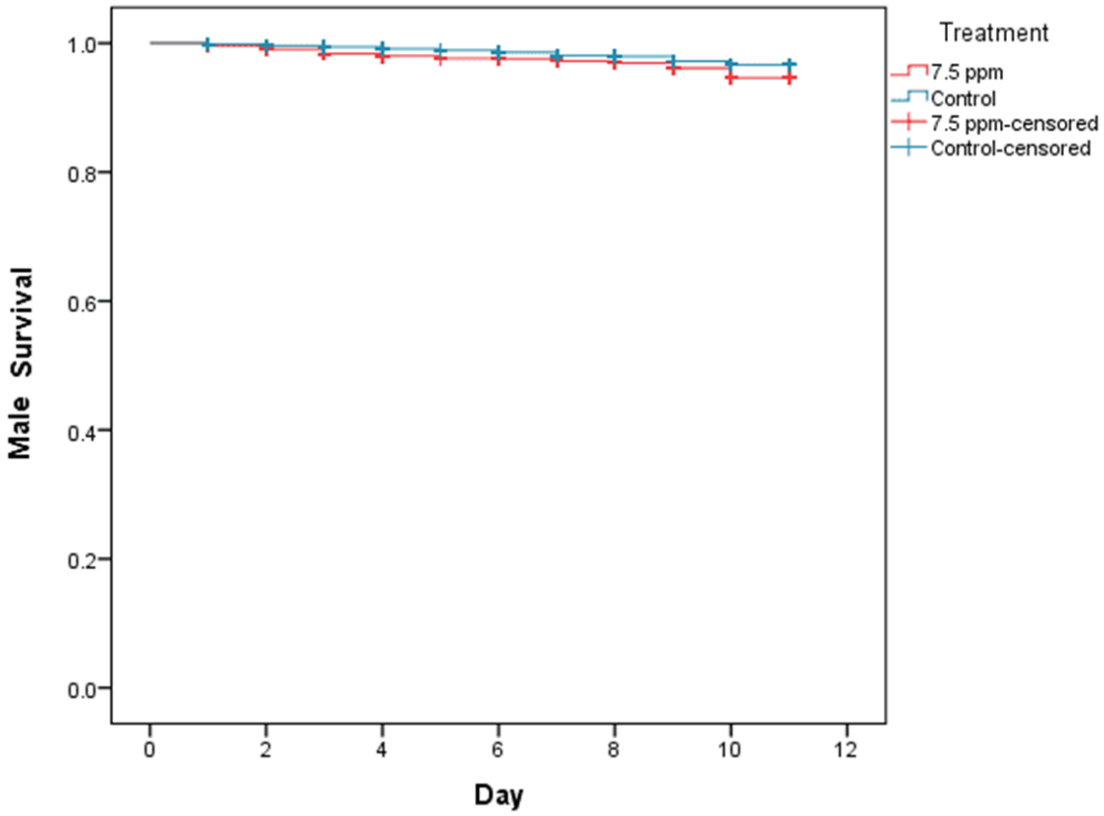


Figure 3.5: Kaplan-Meier survivorship curves for adult *An. arabiensis* males exposed to cattle blood spiked with 7.5 ppm ivermectin (red curve) vs unspiked cattle blood i.e. control (blue curve).

3.3.3. Assessing the reproductive fitness of males present in a cage where females were fed blood spiked with 7.5 ppm of ivermectin.

The proportion of females with sperm in their spermatheca was above 90% for all tested cages (i.e., females drawn from cages pre-exposed to either cattle blood spiked with 7.5 ppm ivermectin (treatment), unspiked cattle blood (control 1) and only sugar water (control 2)) (Figure 3.6). Analysis between the three groups showed no statistical difference in the proportion of females mated or unmated (Pearson Chi-Square, $\chi^2 = 1.189$, df = 2, $p = 0.552$).

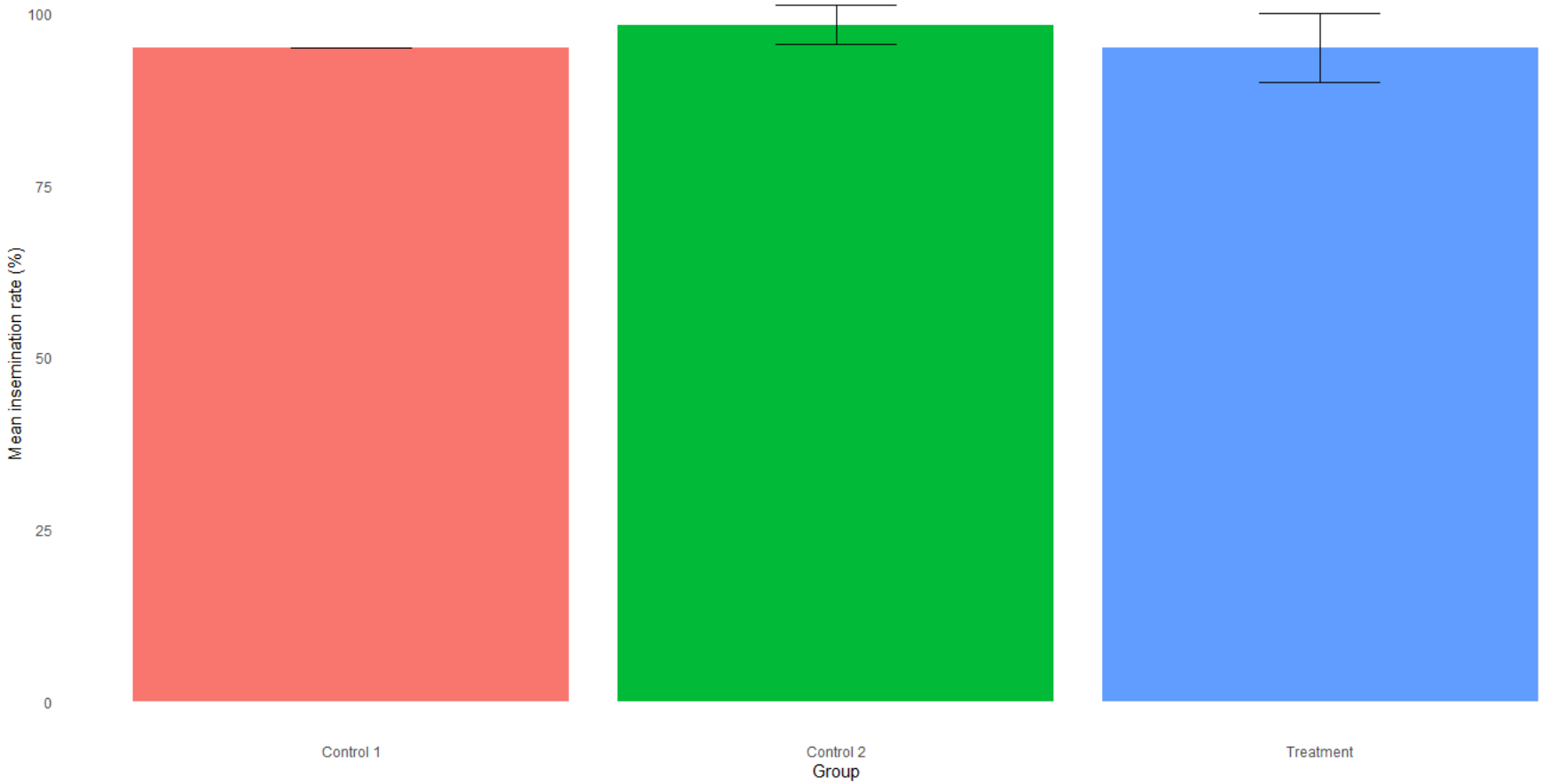


Figure 3.6: Proportion of females with sperm in their spermatheca (insemination rate) after mating with (a) males from the cage where females fed on unspiked-cattle blood (control 1), (b) males from the cage that was fed exclusively on sugar water solution (control 2) and (c) males from the ivermectin-treated cage (Treatment).

3.4. Discussion

This study aimed to determine if the exclusive blood-feeding behaviour of female mosquitoes could be explored as a sex-separation approach for SIT applications through spiking blood meals with ivermectin. Results showed variable but effective female mortality across all tested ivermectin concentrations (2.5, 7.5, and 10 ppm) over five consecutive days of blood feeding with the ivermectin spiked blood. However, none of the concentrations resulted in 100% female mortality over the five-day monitoring period. This could possibly because some of the females did not feed on the blood or are resistant to ivermectin. The hazard ratios and relative risk showed variations between the control and treatments. Subsequent experiments that tested the effectiveness of ivermectin-spiked blood meals provided daily until 100% female mortality was achieved showed that 7.5 ppm ivermectin eliminated females within 11 days without negatively impacting male survival. The median survival time of females fed with 7.5 ppm ivermectin spiked blood was two days with an average lifespan of 11 days, compared to the 11 days' median survival time and 38 days' lifespan in females fed on non-intoxicated blood. This was in contrast with Yamada *et al*. (2013a), who used 7.5 ppm ivermectin to spike blood meals and achieved 100% female elimination by day four without significantly affecting males. The findings of this study also differed from those of Gunathilaka *et al*. (2019), who reported that a blood meal spiked with 8 ppm ivermectin was the most effective method of achieving 100% female elimination for two Aedine species, however, their ivermectin was a mixed formulation with spinosad.

It is thus suspected that other factors may be involved in inducing ivermectin mosquitocidal lethality. These factors could include mosquito age at the time of feeding, ivermectin formulation, feeding method (route of administration) and species or possibly even strains of the same species/individual genetic variation (Canga *et al*., 2009; Kobylinski *et al*., 2014; Chaccour *et al*., 2015). For example, in this study, spiked blood meals were provided from day one, and it was observed that newly emerged female mosquitoes were hesitant to feed on blood in general (personal observation). This could be because virgin female mosquitoes in some species do not readily feed on blood but do so once inseminated (Charlwood *et al*., 2003). This finding contrasts with results from another study, which suggested that female *Ae. aegypti* mosquitoes can attain sexual receptivity as early as one day after emergence. (Ramírez-Sánchez *et al*., 2023). However, they are capable of blood ingestion much earlier, even before mating. This suggests that blood feeding can commence shortly after emergence

and may not necessarily be dependent on mating as a prerequisite. It was also observed in the current study that some females that had ingested low blood volume/semi-engorged did not die as quickly as those that had ingested higher volumes. The assumption is that when ivermectin-fed females survived early, they were only exposed to lower or sub-lethal ivermectin concentrations, resulting in definite delayed female mortality (Sagna *et al*., 2023).

Another possible explanation for the differences in female mortality observed in this study versus other similar studies is the difference in ivermectin formulations used in each study and their pharmacokinetics (Lo *et al*., 1985; Chaccour *et al*., 2017; Smit *et al*., 2019). A veterinary medicine formulation of 1% ivermectin v/v (e.g., Verbamec®, Ivotec®) has been commonly used to spike blood meals (Yamada *et al*., 2013a; Gunathilaka *et al*., 2019). The formulation comprises 40% formal glycerol and 60% propylene glycol as solvents, making ivermectin water-soluble and stable. The ivermectin formulation MK-933, which is in powder form, was used in this study. This formulation had to first be dissolved in a solvent (absolute ethanol) to stabilise and make the toxicant water-soluble. The same approach as the one used in this study (ivermectin formulation MK-933) was used by Focks *et al*. (1991). In contrast, when ivermectin was dissolved in Dimethyl sulfoxide in a study by Hadlett *et al*. (2021), feeding success was not hampered, except that a higher concentration of ivermectin was required per dose to kill females. While not conclusively proven, it is speculated that the utilisation of ethanol in dissolving the ivermectin formulation MK-933 might yield reduced stability compared to pre-made formulations like Verbamec® (Ali *et al*., 2011; Chaccour *et al*., 2015; Das *et al*., 2020). If true, this raises concerns about the quality and efficacy of the MK-933 formulation, as solubility and stability are crucial factors in ensuring that the active ingredient (ivermectin, in this case) is uniformly distributed and effective in killing mosquitoes. It is a hypothesis of this study that adding a solvent such as ethanol to the blood meal potentially reduce its palatability to mosquitoes, which could result in decreased feeding success. In addition, further investigations are required to substantiate any adverse impact of ethanol on female mosquito mortality. To enhance the efficacy of ivermectin in eradicating females and prolonging its mosquitocidal impact, there is an opportunity to create novel formulations explicitly tailored for spiking preserved blood during artificial feeding. These formulations should prioritise stability, prolonged effectiveness, and be purpose-built for this application, potentially allowing the incorporation of multiple lethal agents into a single blood

meal, thus heightening its lethality (Gunathilaka *et al*., 2019; Chaccour *et al*., 2018; Pooda *et al*., 2023).

Furthermore, the findings show that while ivermectin treatment primarily eliminates females, male survival and reproductive fitness were unaffected. The mode of action of ivermectin is through oral ingestion, not through tarsal contact; therefore, the chances of males being affected are minimal because they do not blood-feed (Yamada *et al*., 2013a). Occasionally though, males appear to be prone to premature death. Various independent factors, such as age, size, overcrowding, lack of sugar, and regular blood feeding can influence male adult natural mortality (Dawes *et al*., 2009). In this study, regular blood-feeding, the delicate nature of male mosquitoes, lack of adequate resting sites and the damage and stress caused by aspirating into test cages, toxic ivermectin vapours and males feeding on blood droplets released by females during feeding to reduce thermal stress may have contributed to the observed mortality (Lahondère and Lazzari, 2012). These suspicions are, however, unproven and would require further studies.

Because males can mate multiple times and potentially diminish their sperm quantity in the process (Howell and Knols, 2009), it was essential to determine their reproductive fitness following ivermectin exposure. Results showed no significant statistical difference in the number of females mated whether the males were from ivermectin-exposed cages or unexposed control cages. Males take approximately two days to become sexually mature (spermatogenesis begins in the late pupal stage) and natural insemination rates are low in three-day-old males and peak after seven days (Chambers and Klowden, 2001), therefore, the findings of the current study suggest using ivermectin during this period/eliminating females while males are sexually maturing is optimal.

Adding ivermectin to blood to eliminate blood-feeding females can be helpful in small-scale settings and areas or species where alternatives are lacking, particularly a system for early-stage sex separation (Yamada *et al*., 2013a). As a result, using this strategy exclusively in an SIT programme is not operationally feasible. Some optimisation or standardisation studies into aspects such as waiting for females to be older (three to five days old), starving them of sugar before blood feeding, extending blood-feeding for several hours, or feeding multiple times per day, can be taken to improve feeding success will have to be performed to enhance the

efficiency of sex separation (Yamada *et al*., 2013a). Furthermore, because the approach is dependent on the females' blood-feeding behavioural traits, which can never be controlled and may vary over time or in response to other external circumstances such as prior mating status, hunger, genetic, etc., the method's reliability will not always be 100% effective, especially if it cannot guarantee full blood-feeding by all females in the cage as early as possible (Yamada *et al*., 2013a).

Thus, complete reliance on ivermectin blood spiking as a sex separation strategy is not recommended. Other sexing strategies, such as GSSs, may be worth investigating because they are highly efficient and can be adapted for many mosquito species that currently lack sexing methods (Mashatola *et al*., 2018). This includes research into *tsl* mutations as potential selective markers for the development of GSSs. So far, headway has been made in determining the minimum (permissible) and maximum (restrictive) temperatures at which an *An. arabiensis* laboratory colony survives (i.e., determining the thermal limits/thermosensitive range prior to screening for *tsl* in individuals) (Mashatola *et al*., 2018).

3.5. Conclusion

Provision of blood meals spiked with 7.5 ppm of ivermectin to females is a potential practical way of separating female *An. arabiensis* from males in a production line during laboratory mass rearing for SIT application purposes. However, because this approach relies on females feeding on spiked blood, it does not eliminate all females. Therefore, gender separation using ivermectin blood spiking can be considered as a secondary female elimination phase to eliminate inadvertent females who might have evaded the initial female elimination stage.

CHAPTER 4: Determining the maximum (restrictive) temperature threshold for subsequent screening of *tsl* in an *An. arabiensis* laboratory colony.

4.1. Introduction

The development of a dependable and efficient gender separation strategy is vital for applying the SIT against disease vectors such as *An. arabiensis* (Mashatola *et al*., 2018). The use of GSSs derived from classical genetics, such as Mendelian inheritance, is a promising approach (Bourtzis *et al*., 2018). This is especially true for insects whose sex is determined by XY systems. In such cases, two major genetic alterations must be used to generate GSSs (Franz, 2005; Augustinos *et al*., 2020; Franz *et al*., 2021). The first is identifying a mutation that can be used as a selectable marker (a visible phenotype and/or conditional lethal effect) (Papathanos *et al*., 2009, 2018). The second involves moving the wild-type selectable marker allele to a sex-determining genetic locus, specifically the Y chromosome, resulting in conditions that kill females but promote male production (Franz, 2005; Franz *et al*., 2021).

The most notable and successful GSS used operationally in SIT programmes is the one generated by random mutagenesis and based on a *tsl* mutation. This has been successfully developed in the Mediterranean fruit fly, *C. capitata* (Franz, 2005). In this strain, individuals with *tsl* mutant alleles express the mutant phenotype, i.e., susceptible to high temperatures. However, individuals with wild-type alleles can survive under these temperatures (Robinson, 1999; Franz, 2005). An added advantage of the *C. capitata* GSS is that the *tsl* mutation is closely linked to a *wp* mutation; both mutations are localised on the right arm of chromosome 5, where when *wp*/*tsl* GSS embryos are exposed to a temperature of 34 °C for 24 h, only the males survive (Franz *et al*., 2021). This is because the wild-type copy of the *tsl*-*wp* allele was translocated to the Y chromosome, thus allowing the elimination of females and the eventual release of males only in SIT programmes. Additionally, the *wp* mutation in females allows easy differentiation from male brown pupae (wild-type colour), especially after temperature treatment (Robinson and Van Heemert, 1982). Thus making it easy to preserve the integrity of this genetic sexing system and limit contamination.

The principle used in developing a GSS in *C. capitata* using *tsl* mutations has been adopted in in *A. ludens* (Zepeda-Cisneros *et al*., 2014), *Ae. aegypti* (Koskinioti *et al*., 2021), and *Drosophila melanogaster* (Nguyen *et al*., 2021). T*sl* mutations occur randomly in nature in almost all organisms therefore, it might be possible to develop a *tsl*-based GSS in mosquitoes using the

same approach (Gilles *et al*., 2014; Mashatola *et al*., 2018). However, because of genetic barrier between mosquitoes and *C. capitata* it is highly unlikely that direct copying/transferring of the homologous *C. capitata tsl* mutation to mosquitoes will be possible (FAO/IAEA, 2023). Therefore development of a *tsl* based GSS in mosquitoes has to begin with isolation of these mutations from mosquitoes.

Tsl mutations can be expressed at specific developmental stages depending on the nature of the mutation and its associated temperature sensitivity (Franz, 2005; Ndo *et al*., 2018). This is because the specific developmental stage at which *tsl* mutations are expressed can vary and is determined by the characteristics of the mutation itself (Franz, 2005). Some *tsl* mutations may result in lethality during embryonic development (egg stage), preventing the affected individuals from hatching (Franz *et al*., 2021). Others may cause mortality during larval, pupal, or adult stages. The choice of developmental stage for inducing the temperature-sensitive phenotype depends on the objectives of the research and the specific *tsl* mutations being examined. Studying *tsl* mutations during the egg, larval, and pupal stages of mosquito development is a strategic choice driven by practicality, early intervention potential, and ethical considerations (Franz, 2005; Papathanos *et al*., 2009, 2018; Mashatola *et al*., 2018; Franz *et al*., 2021). The contained/restricted space in which aquatic stages are kept/reared offer controlled environments for precise manipulation of temperature conditions, allowing researchers to induce and study the temperature-sensitive phenotype efficiently (Lyons *et al*., 2012). In addition, investigating *tsl* mutations at these stages provides insights into their stage-specific effects on development, survival, and sex determination, as well as shedding light on the biology of mosquitoes (Mashatola *et al*., 2018). Moreover, working with aquatic stages reduces environmental variability compared to adult mosquitoes, contributing to more controlled experiments (Lyons *et al*., 2012).

Most studies that have investigated the effects of temperature on mosquito aquatic stages have involved a controlled experimental setup (Christiansen-Jucht *et al*., 2015; Lyons *et al*., 2012; Agyekum *et al*., 2021). These include climatic chambers, water baths, etc. In settings with fewer resources, water baths offer a cost-effective and user-friendly solution. They are compact and space-efficient, making them suitable for smaller laboratories, and are accessible to researchers without specialised equipment expertise. While not as precise as high-end equipment, water baths can maintain relatively stable temperatures for experiments with

comparable precision needs, making them ideal for routine studies or initial screenings of *tsl* mutations (Mashatola *et al*., 2018). The energy efficiency and versatility of water baths in various laboratory tasks further enhance their appeal.

There is limited information on the minimum and maximum developmental temperature thresholds for *Anopheles* mosquitoes needed to screen for *tsl* mutations. Such information is useful in determining the minimum and maximum temperature thresholds needed for segregating mutants and non-mutants when developing a *tsl* based GSS (Mashatola *et al*., 2018). Generally, the minimum developmental temperature threshold signifies the lowest temperature at which the mosquito can complete its life cycle, encompassing egg hatching, larval development, pupation, and adult emergence. Typically, around 10 °C for *An. arabiensis*, below this threshold, developmental processes are notably slowed or halted entirely (Lyons *et al*., 2012). Conversely, the maximum developmental temperature threshold denotes the upper limit beyond which development becomes unsustainable due to thermal stress. Typically, around 40 °C for *An. arabiensis*, temperatures above this threshold may disrupt developmental stages, resulting in decreased survival and impaired reproductive fitness (Lyons *et al*., 2012). It is hypothesised that determining these temperature thresholds, particularly sublethal temperatures and upper thermal limits, could aid in identifying *tsl* mutations and evaluating the potential application of temperature sensitivity for segregating males and females.

This chapter establishes the maximum (restrictive) temperatures at which an *An. arabiensis* laboratory colony can thrive, thereby identifying a thermosensitive range for screening *tsl* mutations (Mashatola *et al*., 2018). The choice between higher and lower temperatures for screening *tsl* mutations was based on the genetic makeup of *An. arabiensis*, the nature of the targeted *tsl* mutations, the desired outcomes of the screening process and largely, previous successful work in *C. capitata* (Mashatola *et al*., 2018; Franz *et al*., 2021).

4.2. Materials and methods

4.2.1. Mosquito strain, rearing and maintenance

The *An. arabiensis* Dongola strain (available from the International Atomic Energy Agency (IAEA), Insect Pest Control Laboratory (IPCL) of the FAO/IAEA Agriculture and Biotechnology Laboratories, Seibersdorf, Austria) was used in this study. The strain was colonised in 2004

from specimens collected near Sudan and some of the material was transferred to IPCL. The Dongola strain at IPCL, which was used during this study, was maintained and reared as reported by the IAEA guidelines for standardised mass-rearing of *Anopheles* mosquitoes, volume 1.0 (FAO/IAEA, 2017b).

4.2.2. Construction of experimental equipment

Five stainless steel water baths were used (Product code: FBATH26, Bibby Scientific Ltd., Stone, Staffordshire, ST15 0SA, UK), each equipped with its own analogue thermoregulator (Product code: FTE10ADC, Bibby Scientific Ltd., Stone, Staffordshire, ST15 0SA, UK). The water baths have internal dimensions of 505 mm in length, 300 mm in width, and 200 mm in depth. Within each water bath, a single unit stainless steel rack was installed, featuring four legs (each leg = 190 mm height and 5 cm diameter) welded to a flat stainless steel sheet. The flat sheet, measuring 500 mm in length, 290 mm in width, and 2 mm in height, was drilled with nine (3 × 3) evenly spaced holes, each 8 cm in diameter. These holes accommodated plastic holding cups (https://www.ikea.com/at/en/), measuring 8.5 cm in top rim diameter, 5 cm in bottom rim diameter, and 6 cm in height, which were suspended from the steel rack. The holding cups were perforated with three or four 5 cm diameter holes around the cup's circumference and one 5 cm diameter hole at the bottom. Additionally, 100 mℓ plastic urine jars (57 mm diameter × 76 mm height, https://www.sarstedt.com/) had their base removed and covered with a 50 µm nylon mesh (https://www.amazon.com/), secured in place with a hot glue gun (Product code: PKP 18 E, Bosch Global, Stuttgart, Germany). These urine jars were used to contain the biological material so that they allow direct contact with 38 ℓ of distilled water in the water bath. A schematic diagram showing the construction of the temperature screening equipment material is shown in Figure 4.1.

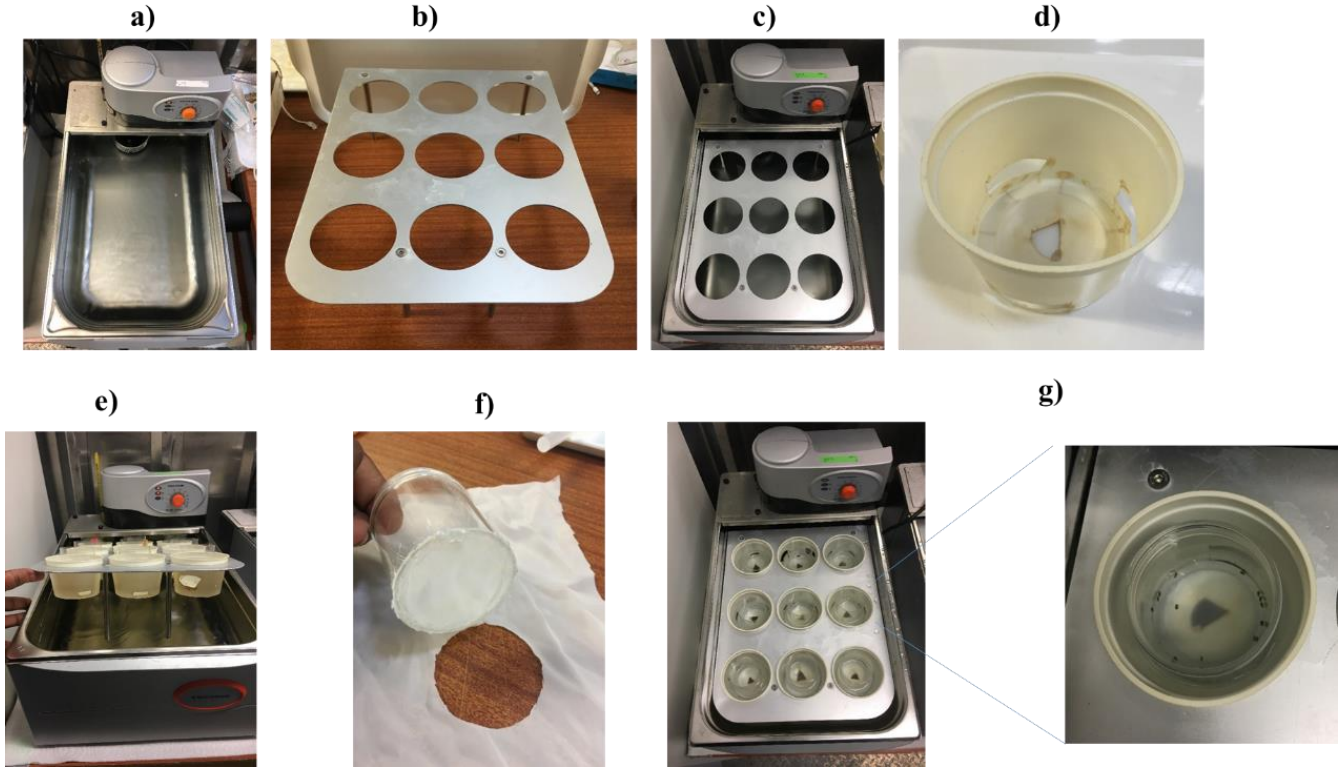


Figure 4.1: A schematic diagram showing the construction of temperature screening equipment. a) water bath, b) single unit stainless steel rack with four legs attached to a flat stainless steel sheet. The sheet is perforated with nine evenly spaced holes, each measuring 8 cm in diameter, c) the steel rack fitted inside a water bath, d) plastic holding cup measuring 8.5 cm in top rim diameter, 5 cm in bottom rim diameter, and 6 cm in height. These cups are perforated with three or four holes of at least 5 cm diameter around the circumference and one 5 cm diameter hole at the bottom, e) plastic holding cups suspended on the steel rack inside the water bath, f) plastic urine jar (57 mm diameter × 76 mm height) with base removed and covered with a 50 µm nylon mesh, g) water bath containing plastic holding cups and urine jars containing biological material, with a zoomed-in view provided.

4.2.3. Experimental design

Various mosquito aquatic developmental stages were used, including eggs (<12 h after oviposition), first instar larvae (L1) (<6 h post-hatching), and pupae (<6 h after pupation from larvae)) from the Dongola laboratory strain. Each developmental stage was placed in separate urine jars, with 100 eggs or 30 L1 or 30 pupae per urine jar. These were subsequently subjected to constant temperatures of 27°C, 39°C, 41°C, 43°C and 45°C for durations of 1 h, 2 h, 3 h, 6 h and 12 h. The temperature conditions were selected based on previous studies on mosquito development that have reported the optimal temperatures for aquatic stages ranging between 22°C to 28°C (Bayoh, 2001; Bayoh and Lindsay, 2004; Lyons *et al*., 2012), while the restrictive temperature threshold for development typically ranges from 39°C to 46°C (Lactin *et al*., 1995; Raghavendra *et al*., 2010a, 2010b; Lyons *et al*., 2012). Two control conditions were established for comparison in the experiment. Control 1 involved a water bath set at a temperature of 27°C, while control 2 involved a plastic cup (dimensions: length and width both = 11 cm and height = 7 cm) filled with 200 mℓ of distilled water and placed under standard insectary conditions (27 ± 1°C room temperature, 70 ± 10% relative humidity, and a 12:12 h photoperiod, including 45-minute each of transitions of dusk and dawn cycles).

The biological material was simultaneously exposed to each respective temperature and per the designated developmental stage and duration of exposure. After each exposure, the biological material from each urine jar was transferred to new corresponding plastic cups (dimensions: length and width both = 11 cm and height = 7 cm) filled with 200 mℓ distilled water under standard insectary conditions.

Several parameters were observed for each developmental life stages: for eggs (number of eggs hatched, or egg hatch rates, over 14 consecutive days), for L1 larvae (number of L1 that died 24 h post-exposure (larval mortality)), and for pupae (number of pupae that died 24 h post-exposure (pupal mortality)). The experimental setup was repeated three times on different days. The schematic diagram below (Figure 4.2) summarises the protocol used to determine the thermosensitive range or a temperature used for screening *tsl* individuals from the *An. arabiensis* Dongola laboratory colony.

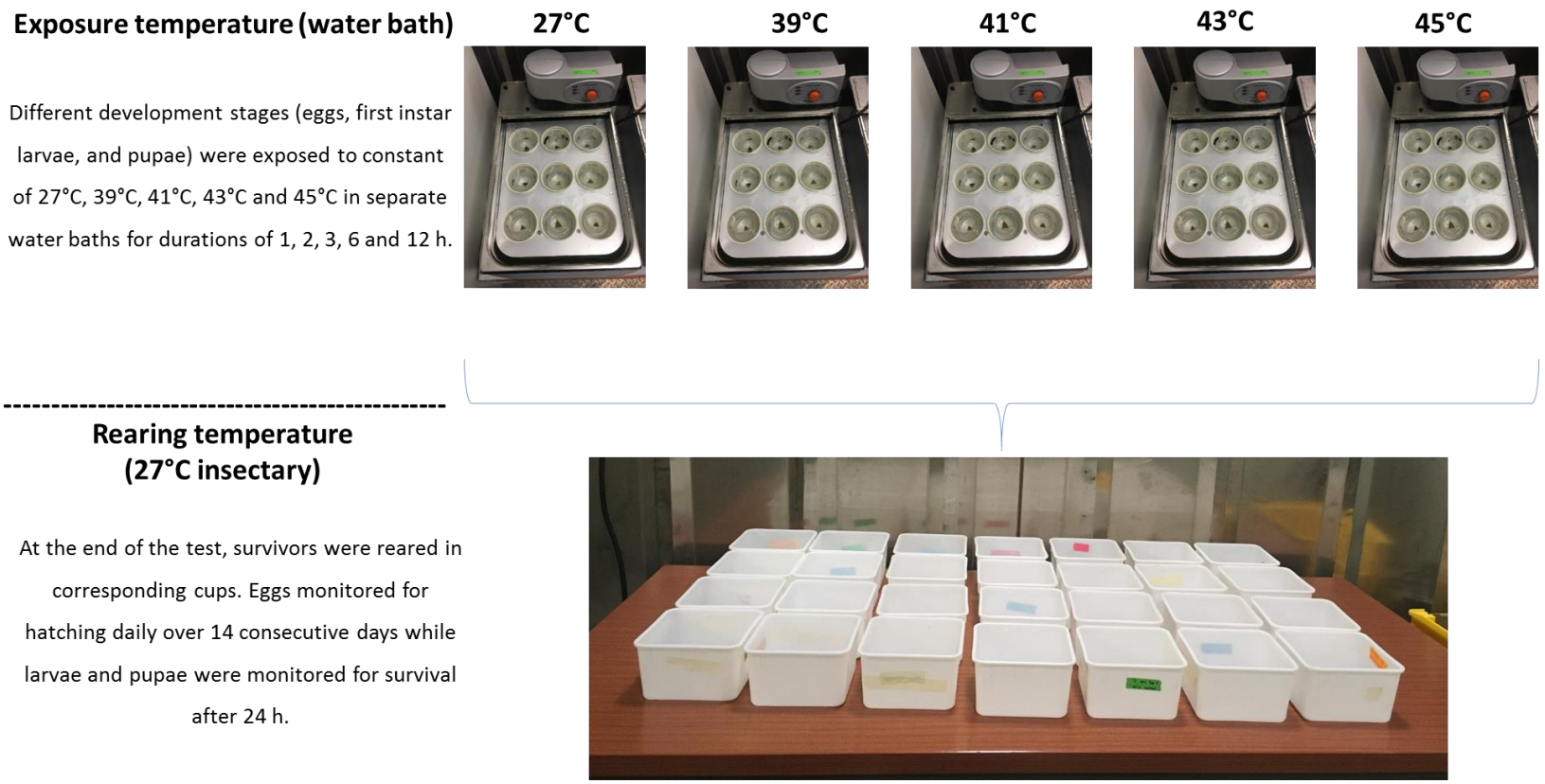


Figure 4.2: A schematic diagram showing the protocol for determining the maximum temperature at which *An. arabiensis* Dongola laboratory colony survives.

4.2.3. Data management and statistical analysis

All data were recorded manually on paper sheets before being electronically transferred to Microsoft Excel 2016 and analysed using IBM SPSS (IBM Corp, 2015) and R software (R Development Core Team, 2003). In all cases, differences between compared groups were considered statistically significant when $p < 0.05$. The effects of temperature and duration of exposure on each respective response variable (i.e., egg hatch rates, larval mortality and pupal mortality) were checked for interaction using a two-way ANOVA where temperature and duration of exposure are the independent variables, while egg hatch rates, larval mortality and pupal mortality were the dependent variables. The thermosensitive range or a temperature used for screening *tsl* individuals, taking the duration of exposure into consideration, was determined by analysing the interaction effect between temperature and duration of exposure using two way-ANOVA and subsequently post-hoc tests. Furthermore, a reliable measure of the developmental stages' ability to endure exposure to a specific temperature was assessed by determining the time at which no more than 50% egg hatch occurred and the time required to induce 50% larval or pupal mortality. This measure, known as the lethal time to 50% mortality (LT_{50}), was calculated using the probit regression analysis (Andrewartha, 2012).

4.3. Results

4.3.1. Egg hatch rate

The average egg hatch rates (mean ± SD) at 27°C (insectary) and 27°C (water bath) remained consistent, ranging from 73.3% ± 7.6 to 81.1% ± 5.1, regardless of the duration of exposure (Figure 4.3). However, at 39°C, the mean egg hatch rate was 60.8% ± 7.1 after 1 h of exposure, but it progressively declined with longer durations to reach 22.2% ± 4.9 after 12 h. Similarly, at 41°C, the hatching rate was 37.8% ± 3.6 after 1 h, 19.1% ± 4.4 after 2 h, 10.2% ± 4.8 after 3 h, and 3.2% ± 1.7 after 6 h (Figure 4.3). Interestingly, no eggs hatched after being exposed to 41°C for 12 h, as well as at 43°C and 45°C, regardless of the duration of exposure.

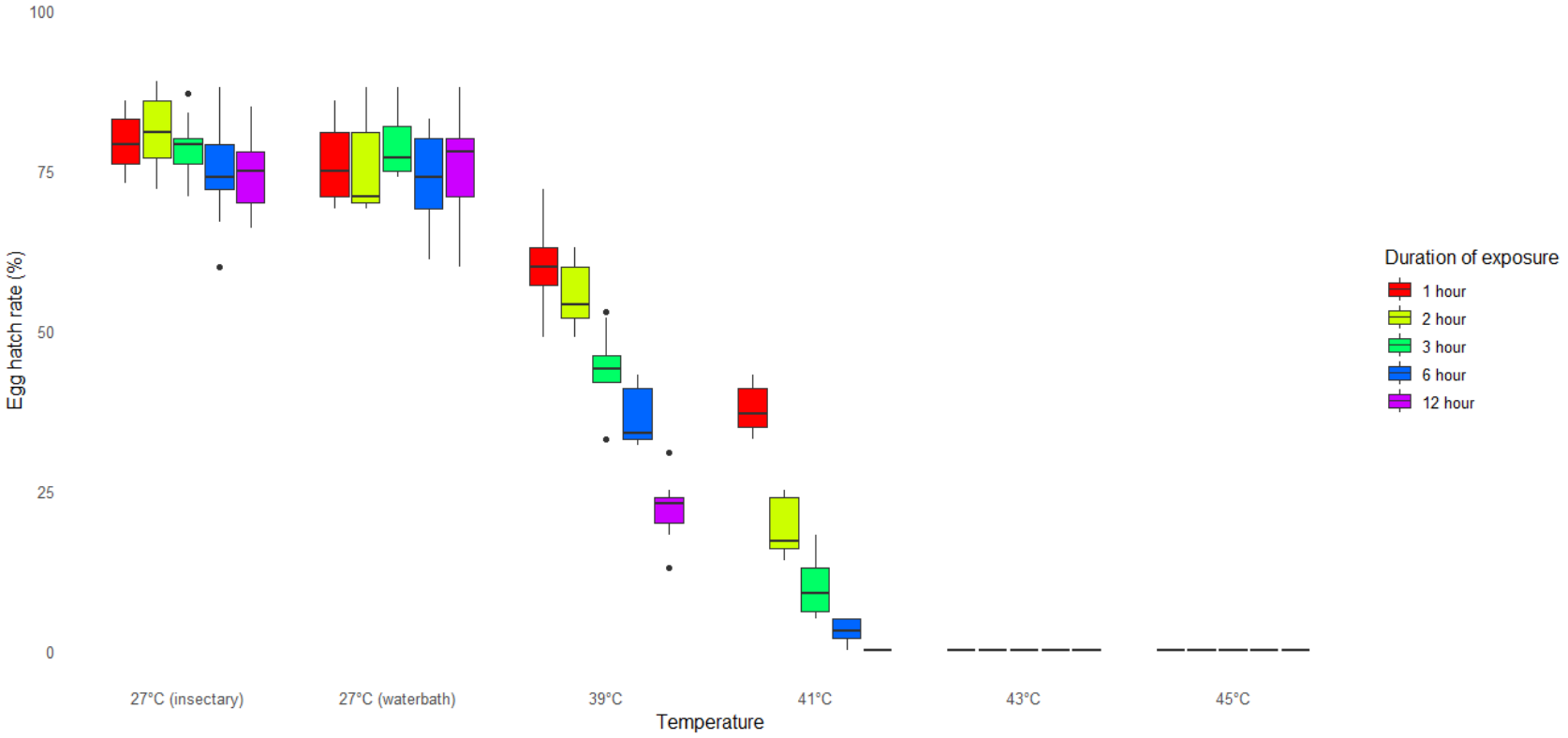


Figure 4.3: The mean percentage of laboratory-reared *An. arabiensis* eggs (Dongola) that hatched following exposure to different constant temperatures of 27°C (insectary), 27°C (water bath), 39°C, 41°C, 43°C and 45°C for durations of 1 h, 2 h, 3 h, 6 h and 12 h.

A two-way ANOVA performed to analyse the effect of temperature and duration of exposure on egg hatching showed a statistically significant in effect of temperature ($F_{(5, 270)} = 2771.95$, $p < 0.05$), duration of exposure ($F_{(4, 270)} = 76.0$, $p < 0.05$) and their interaction (two-way ANOVA: $F_{(20, 270)} = 25.81$, $p < 0.05$) on egg hatching (Table 4.1). Tukey's post-hoc tests were performed to compare different levels of temperature and duration of exposure and to determine which specific combinations are significantly different in terms of their effects on egg hatch rates (Table 4.2). The difference in egg hatch rates between 27°C (water bath) and 27°C (insectary) was not statistically significant ($p = 0.529$). Egg hatch rates significantly decreased at 39°C compared to 27°C (insectary) ($p < 0.001$). Similarly, at 41°C, 43°C, and 45°C, egg hatch rates were significantly lower compared to both 27°C (insectary) and 27°C (water bath) ($p < 0.001$). The duration of exposure had a significant impact on egg hatch rates ($p < 0.001$). Egg hatch rates decreased significantly as the duration of exposure increased, with the largest difference observed between 1 h and 12 h ($p < 0.001$). The differences in egg hatch rates between 1 h and 2 h, as well as between 1 h and 3 h, were statistically significant ($p = 0.045$ and $p < 0.001$, respectively). However, the difference between 2 h and 3 h was not statistically significant ($p = 0.062$).

Table 4.1: Two-way ANOVA showing the effects of temperature, duration of exposure and their interactions on egg hatching.

Source	SS	df	MS	F	p
Corrected Model	308,930.03	29	10,652.76	506.2	<.001
Intercept	336,444.3	1	336,444.3	15,987.32	<.001
Temperature	291,671.14	5	58,334.23	2,771.95	<.001
Duration of exposure	6,397.72	4	1,599.43	76	<.001
Temperature x Duration of exposure	10,861.17	20	543.06	25.81	<.001
Error	5,050.67	240	21.04		
Total	650,425	270			
Corrected total variation	313,980.7	269			

*SS represents the sum of squares, df represents degrees of freedom, MS represents mean squares, F represents F-statistic, and p indicates statistical significance at $p < 0.05$.

The mean temperature at which no more than 50% egg hatching was observed was approximately 39.9°C for 120.2 minutes (Table 4.2).

Table 4.2: Thermal tolerance of *An. arabiensis* Dongola eggs, first instar larvae (L1) and pupae exposed to different temperatures and duration of exposure.

Developmental stage exposed	Temperature (°C)	LT_{50} (in minutes) (lower bound – upper bound)	χ^2 (df)
Eggs	39.9°C	120.2 (102.2 – 137.1)	142.3 (29)
First instar larvae (L1)	41°C	76.4 (102.2 – 137.1)	121.2 (29)
Pupae	43°C	63.1 (52.0 – 74.4)	56.8 (29)

LT_{50}-Lethal time in minutes at which no more than 50% egg hatch occurred, χ^2 (df) – chi square value of heterogeneity (degrees of freedom).

4.3.2. Larval mortality

The average larval (L1) mortality (mean ± SD) at 27°C (insectary) and 27°C (water bath) was consistently low, ranging from 0.3% ± 1.2 to 0.9% ± 1.7, regardless of the duration of exposure (Figure 4.4). However, when subjected to 39°C, the larval (L1) mortality exhibited a wider range, starting at 1.9% ± 2.4 after 1 h of exposure and escalating to 12.6% ± 2.8 after 12 h. Larval (L1) mortality at 41°C displayed a significant increase, ranging from 13.3% ± 3.7 after 1 h exposure to 57.8% ± 6.2 after 12 h exposure. The temperature of 43°C induced even higher larval (L1) mortality rates, with a mortality rate of 82.9% ± 8.7 after 1 h of exposure, intensifying to 95.9% ± 4.9 after 2 h, and peaking at 97.0% ± 3.5 after 3 h. Notably, complete larval mortality was observed at 43°C after 6 h of exposure and at 45°C across all exposure durations.

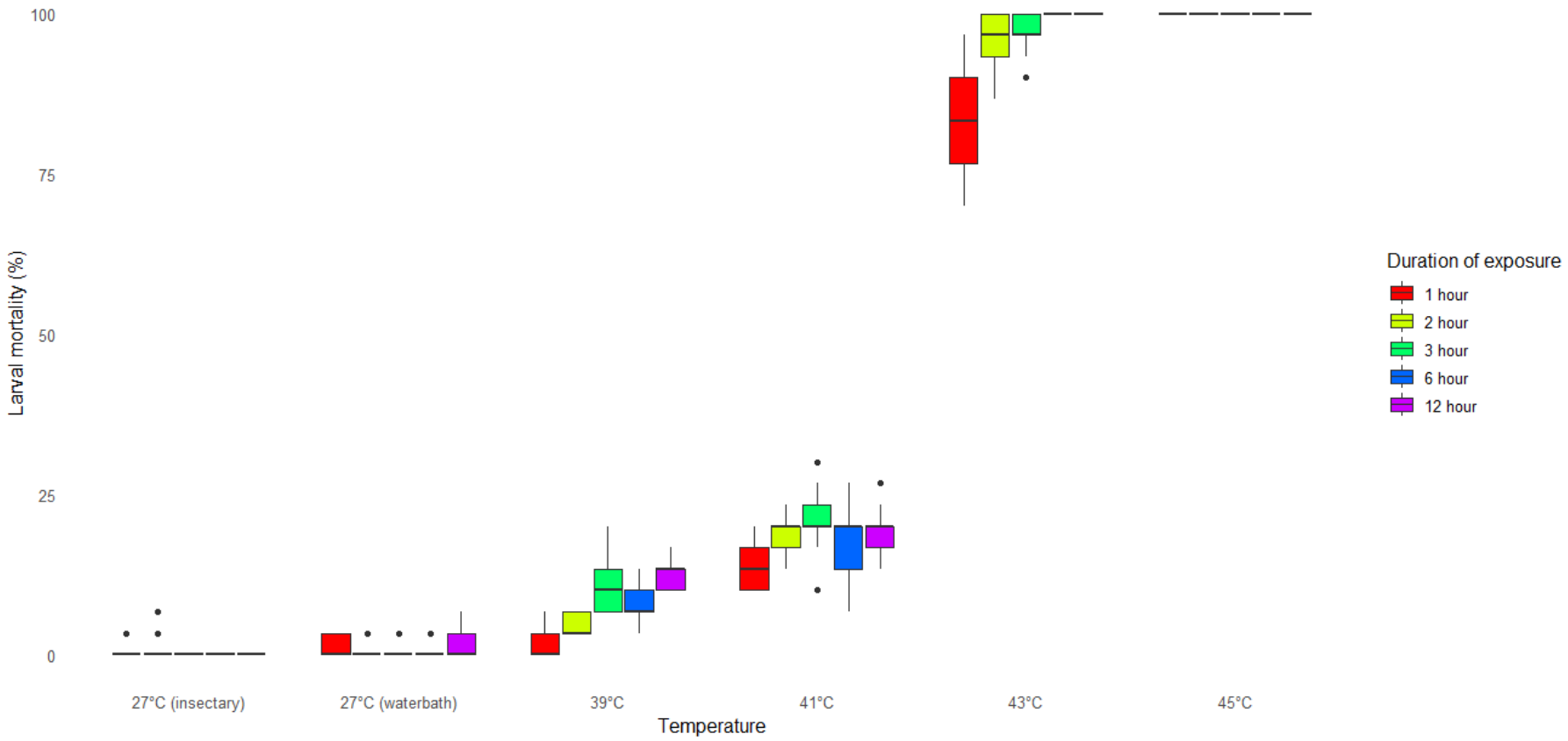


Figure 4.4: Larval mortality (%) of laboratory-reared *An. arabiensis* L1 exposed to different but constant temperatures of 27°C (insectary), 27°C (water bath), 39°C, 41°C, 43°C and 45°C for durations of 1 h, 2 h, 3 h, 6 h and 12 h.

Following analysis, both temperature (two-way ANOVA: $F_{(5, 270)} = 10339.02$, $p < 0.05$) and duration of exposure (two-way ANOVA: $F_{(4, 270)} = 26.96$, $p < 0.05$) and their interaction (two-way ANOVA: $F_{(20, 270)} = 9.08$, $p < 0.05$) showed a statistically significant effect on larval (L1) mortality (Table 4.3).. In addition, L1 mortality at a temperature of 39°C was significantly different from 27°C (insectary), with a $p = 0.0367$. The temperatures of 41°C, 43°C, and 45°C were all significantly different from 27°C (insectary) ($p < 0.05$). The duration of exposure for 12 h was significantly different from 1 h, 2 h, 3 h, and 6 h ($p < 0.05$) (Table 4.3).

Table 4.3: Two-way ANOVA showing the effects of temperature, duration of exposure and their interactions on larval (L1) mortality.

Source	**SS**	**df**	**MS**	**F**	**p**
Corrected Model	508,079.88	29	17,520	1,792.57	<.001
Intercept	369,630	1	369,630	37,818.99	<.001
Temperature	505,250.25	5	101,050.05	10,339.02	<.001
Duration of exposure	1,054.16	4	263.54	26.96	<.001
Temperature x Duration of exposure	1,775.47	20	88.77	9.08	<.001
Error	2,345.68	240	9.77		
Total	880,055.56	270			
Corrected total variation	313,980.7	269			

*SS represents sum of squares, df represents degrees of freedom, MS represents mean squares, F represents F-statistic, and p indicates statistical significance at $p < 0.05$.

The mean temperature at which 50% larval (L1) mortality occurred was approximately 41°C for 76.4 minutes (~1 h and 17 minutes) (Table 4.2).

4.3.3. Pupal mortality

At temperatures of 27°C, whether in the insectary or a water bath, pupal mortality remained low, indicating that this temperature did not induce significant pupal mortality. However, when temperatures were raised to 39°C and above, pupal mortality increased significantly. For instance, at 41°C, pupal mortality ranged from 1.9% ± 2.9 after 1 h to 47.4% ± 5.2 after 12 h. The most notable pupal mortality occurred at 43°C, with mortality rates reaching 88.9% ± 7.1 after 3 h. Complete pupal mortality was observed at 43°C from 6 h onwards, as well as at 45°C for all durations of exposure (Figure 4.5).

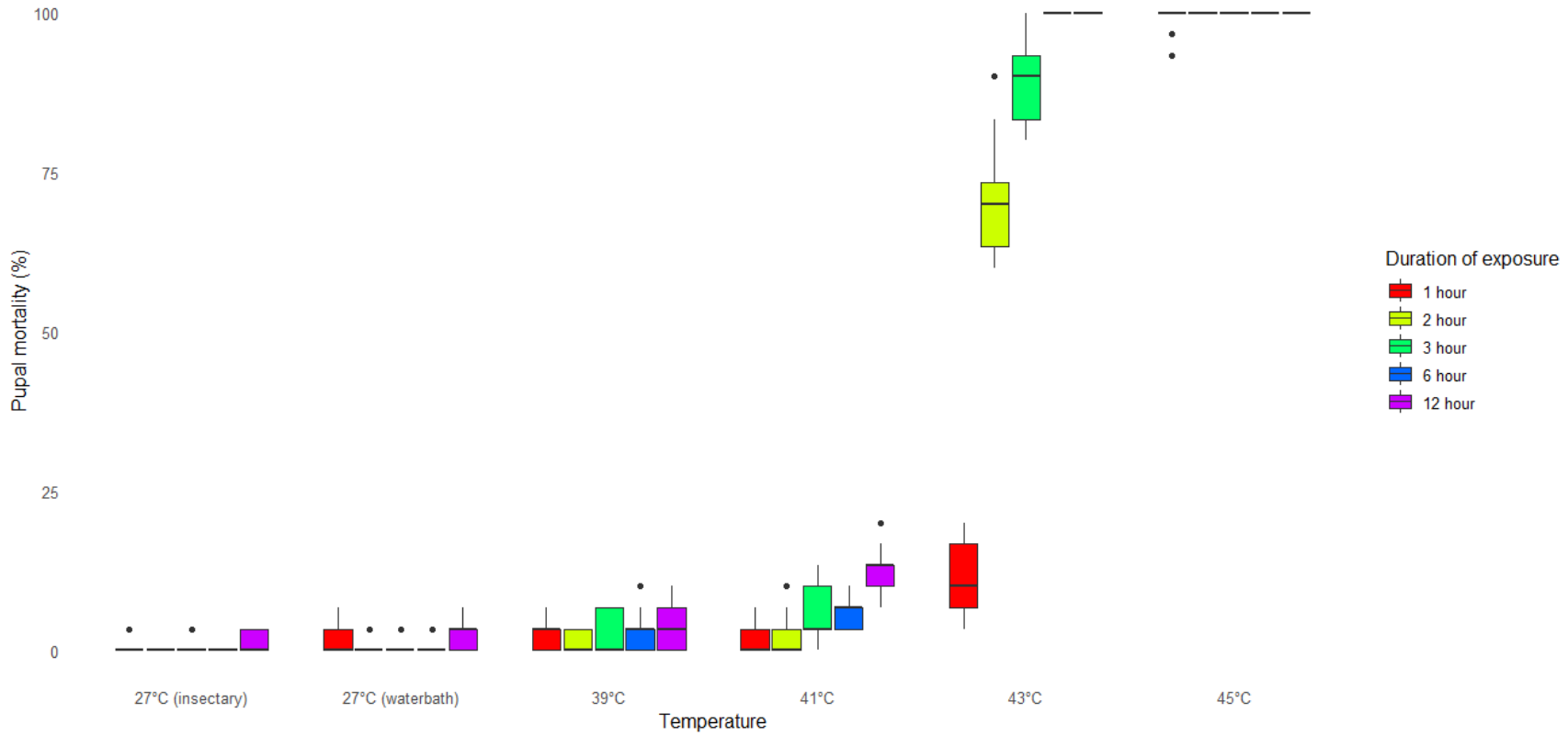


Figure 4.5: Pupal mortality (%) of laboratory-reared *An. arabiensis* pupae exposed to different but constant temperatures of 27°C (insectary), 27°C (water bath), 39°C, 41°C, 43°C and 45°C for durations of 1 h, 2 h, 3 h, 6 h and 12 h.

A statistically significant effect on pupal mortality was shown by both temperature ($F_{(5, 270)}$ = 7.054, $p < 0.05$) and duration of exposure ($F_{(4, 270)}$ = 209.4, $p < 0.05$) and their interaction (two-way ANOVA: $F_{(20, 270)}$ = 87.0, $p < 0.05$) (Table 4.4). Tukey's post-hoc test was then used to determine the permissive and restrictive temperatures and durations of exposure which show the significant differences in pupal mortality. The permissive temperature appeared to be 27°C (insectary) and 27°C (water bath) at all exposure durations, as both result in the lowest pupal mortality, and there were no significant differences when compared to the other temperatures. The test temperatures of 39°C, 41°C, 43°C, and 45°C, resulted in significantly higher pupal mortality compared to 27°C (insectary) and 27°C (water bath). In addition, at 12 h duration of exposure, significantly higher pupal mortality was consistently observed as compared to the lower duration of exposures across various temperatures.

Table 4.4: Two-way ANOVA showing the effects of temperature, duration of exposure and their interactions on pupal mortality

Source	SS	df	MS	F	p
Corrected Model	482,858.6	29	16,650.3	1,305.17	<.001
Intercept	314,390.78	1	314,390.78	24,644.18	<.001
Temperature	449,974.65	5	89,994.93	7,054.44	<.001
Duration of exposure	10,685.35	4	2,671.34	209.4	<.001
Temperature x Duration of exposure	22,198.6	20	1,109.93	87	<.001
Error	3,061.73	240	12.76		
Total	800,311.11	270			
Corrected total variation	485,920.33	269			

*SS represents sum of squares, df represents degrees of freedom, MS represents mean squares, F represents F-statistic, and p indicates statistical significance at $p < 0.05$.

The mean LT_{50} pupal mortality was 43°C for 63.1 minutes (~1 h and 5 minutes) (Table 4.2).

4.4. Discussion

The aim of this study was to establish the maximum restrictive temperatures and duration of exposure at which an *An. arabiensis* laboratory colony (Dongola) can thrive as part of the long-term objective to use these conditions to screen for *tsl* mutations and explore the potential

use of temperature sensitivity as a tool for separating males and females. Investigations showed that the critical temperature thresholds for eggs, first instar larvae (L1) and pupae varied based on exposure duration and developmental stage. Specifically, critical temperature thresholds for egg hatch rates, wherein 50% of the eggs hatch, were determined to be 39.9°C for 120 minutes (2 h). For first instar larvae, a restrictive temperature threshold of approximately 41°C was observed within a relatively short duration of about 77 minutes (1 h and 17 minutes). Similarly, pupae exhibited a mean lethal temperature associated with 50% pupal mortality at around 43°C after approximately 65 minutes (1 hour and 5 minutes) of exposure.

The findings of the current study highlight the significant impact of temperature, duration of exposure, and their interaction on egg hatch rates, larval mortality, and pupal mortality. Generally, higher temperatures and longer exposure times tend to result in lower egg hatch rates, increased larval mortality, and heightened pupal mortality (Christiansen-Jucht *et al*., 2015; Lyons *et al*., 2012; Agyekum *et al*., 2021; Sivan *et al*., 2021). Moreover, these results align with prior research. For instance, in *Cx. quinquefasciatus* eggs exposed to 39°C for 24 h completely halted egg hatching (Rayah and Groun, 1984). Huang *et al*. (2006) discovered noteworthy mortality rates in eggs of *An. gambiae s.l.* obtained from the wild when subjected to brief heating ranging between 42°C to 44°C. At 45°C after 10 minutes, minimal egg hatching was observed, and no eggs hatched at temperatures beyond this threshold. Similarly, investigations on eggs from a laboratory *An. gambiae s.s.* strain (Kisumu) exhibited a similar pattern, albeit with a slight decrease in temperatures by 1°C (Huang *et al*., 2006). It was noted that in general egg mortality increased with time as temperatures exceeded 40°C.

When it comes to larvae, the majority of studies on temperature effects have focused on late instar stages rather than the first instar larvae (L1). For instance, research on late third instar larvae of *An. culicifacies* showed that larvae were able to survive at 37°C for up to 360 minutes of exposure but reached 100% mortality within 240 minutes of exposure at 41°C, with larvae typically succumbing within 60 minutes at 45°C (Raghavendra *et al*., 2010a). Similar results were reported for *An. stephensi* whereby third instar larvae died within 30 minutes at 45°C (Raghavendra *et al*., 2010b) while *Ae. aegypti* third instar larvae exhibited a higher survival rate at 39°C for up to 420 minutes compared to temperatures of 27°C and 37°C (Sivan *et al*., 2021). Studies by McDonald *et al*. (1980) and Muirhead-Thomson (1940) demonstrated 100%

larval mortality in *An. minimus* exposed to 40°C. In another study, fourth-instar larvae of *An. stephensi* and *Ae. aegypti* typically perished within 90 minutes at 43°C (Patil *et al*., 1996).

The contrasts in thermal resistance between eggs, larvae and pupae observed in this study is not a new phenomenon. Studies by Rocca *et al*. (2009) have reported that pupae exhibit greater resilience to thermal stress compared to larvae. This trend is not unique to mosquitoes as *Drosophila* pupae have also been observed to possess higher thermo-tolerance than larvae or eggs (Krebs and Loeschcke, 1995). The ecological rationale behind this difference becomes apparent when considering the behaviour and mobility of larvae versus pupae. While both mosquito larvae and pupae are mobile, larvae tend to move more actively within the water column, whereas pupae typically remain at the water surface and only dive when disturbed (Clements, 1992).

These observations underscore the varying temperature sensitivities across different developmental stages, highlighting species-specific responses to temperature. Notably, earlier stages tend to exhibit heightened temperature sensitivity compared to later developmental stages, a trend also observed in *C. capitata* (Franz, 2005). These insights into the role of temperature in insect development have wide-ranging implications. They not only inform *tsl* screenings and the development of sex separation techniques but also offer valuable guidance for designing strategies aimed at controlling mosquito populations and, by extension, insect populations in general.

The findings of this study provide knowledge on the temperature required for *tsl* screenings or maintenance of different developmental stages to improve production and possibly avoid damage to individuals carrying *tsl* mutations. This is more so because a protein product of a *tsl* gene is suspected of working generally at permissive temperatures but not at restrictive temperatures (Franz, 2005). However, as temperature sensitivity/thermal limits may vary depending on properties such as strain, age, sex, and acclimatisation (Patil *et al*., 1996; Rocca *et al*., 2009; Lyons *et al*., 2012), among other factors, the findings cannot be generalised for application to all mosquitoes.

Overall, the method employed in this study represents a significant step toward standardising *tsl* screening in mosquitoes since it involves direct contact of biological specimens with water

(without a barrier) and is cost-effective. In addition, these findings can serve as a foundation for future studies that aim to locate and characterise *tsl* mutations in mosquito populations. In fact, the findings of this study were applied in the next chapter when using EMS to induce conditional mutations (*tsl* and morphological body variations) in *An. arabiensis*.

4.5. Conclusion

This study has established that the temperature sensitivity of the Dongola *An. arabiensis* laboratory strain varies depending on the duration of exposure and developmental stage. Specifically, this study found that critical temperature thresholds for egg hatch rates/at which 50% of the eggs hatch was 39.9°C for a 2 h exposure. For first instar larvae (L1), the restrictive temperature threshold was approximately 41°C within a relatively short duration of about 1 hour and 17 minutes. Similarly, for pupae, the mean lethal temperature associated with 50% pupal mortality occurs at around 43°C for approximately 1 h and 5 minutes of exposure.

CHAPTER 5: Use of EMS to induce conditional mutations in *An. arabiensis* for purposes of developing GSSs.

5.1. Introduction

Development of *An. arabiensis* GSSs based on *tsl* or morphological variation are vital for SIT programmes involving mosquitoes (Mashatola *et al*., 2018). This is because SIT involves the release of sterilised male mosquitoes into the wild population, where they mate with wild females (Dyck *et al*., 2005, 2021). Since the females do not produce viable offspring due to mating with sterile males, the targeted mosquito population declines over time. However, the operational success of SIT depends on the effective release of only sterile males (Orozco *et al*., 2013; Vargas-Teran *et al*., 2021), as female mosquitoes can transmit diseases like malaria (Meibalan, and Marti, 2017).

Notable examples where GSSs have been successfully developed and are being used in large-scale operational SIT programs is in *C. capitata* (Caceres, 2002) and *A. ludens* (Quintero-Fong *et al*., 2018). These GSSs are designed to produce males under specific conditions or carry markers that enable the visual identification of males and females during the rearing process (Rössler, 1979). One of the most promising approaches for developing GSSs involves conditional mutations, such as *tsl* mutations (Franz, 2005, Franz *et al*., 2021). These mutations cause lethality in mosquitoes at specific temperature ranges but are harmless at other temperatures, for example in *C. capitata* (Caceres, 2002), *A. ludens* (Quintero-Fong *et al*., 2018) and *An. arabiensis* (Ndo *et al*., 2018). By exploiting these mutations, researchers can induce male-only production by subjecting mosquito developmental stages to temperature conditions that favour male development (Mashatola *et al*., 2018).

However, the development of GSSs based on *tsl* mutations is a complex process that requires the identification of additional mutations to act as moprphological markers that are stable and reliable (Franz, 2005; Nguyen *et al*., 2021). For example, previous efforts in *C. capitata* have revealed challenges with using markers like *tsl* mutations and the *wp* mutation on their own in GSSs due to issues of genetic instability over time (Franz, 2005, Franz *et al*., 2021). Thus, efforts were initiated over 40 decades ago to isolate additional selectable markers for *C. capitata* that can be used in conjunction/trace each other (Rössler, 1979). This led to development and isolation of two different strains of *C. capitata* that each contain the *wp* selectable marker or the *tsl* gene located on autosome 5 (Franz *et al*., 1996, 2021). The process

involved treating male individuals with EMS, which induces random mutations in the DNA (Franz *et al*., 1996). After treatment, the mutated males were crossed with wild-type females. The resulting offspring inherited the mutated genes from their fathers. Subsequently, the pupae from these crosses were subjected to different temperature regimes during development. The goal was to identify individuals carrying mutations that rendered them sensitive to specific temperature ranges. Those individuals exhibiting lethality or sterility at certain temperatures were selected as potential candidates for the *tsl* strain. Once promising candidates were identified, further breeding and selection processes were conducted to establish a stable *tsl* strain. This involved repeated backcrossing and screening for individuals exhibiting the desired temperature-sensitive phenotype. Through iterative cycles of mutation induction, selection, and breeding, a stable *tsl* strain that happened to be accompanied by a T(Y-5) autosome translocation, which pseudo-links the wild-type allele of the aforementioned selectable markers to the male sex was eventually established. (Franz, 2005). In these strains, incubating eggs aged 24 h old at 34 °C for 12 h – 24 h eliminated all females (homozygous for *tsl*), resulting in the development and release of males only (heterozygous for *tsl*). The *wp* mutation is a visual tracker of contamination female elimination after heat treatment or recombination (Franz, 2002; Augustinos *et al*., 2017).

Although *tsl*-based GSSs have been successfully implemented in *C. capitata* (Augustinos *et al*., 2017; Franz, 2005, Franz *et al*., 2021), their adaptation and utilisation for mosquitoes, particularly *Anopheles* species, remain an ongoing endeavour (Mashatola *et al*., 2018). Mosquitoes exhibit distinct temperature requirements and developmental patterns compared to *C. capitata*. Hence, subsequent to the research conducted in Chapter 4 aimed at establishing the temperature thresholds/conditions that can be used to screen for *tsl* mutations in an *An. arabiensis* laboratory colony, further investigations were warranted to induce and identify appropriate *tsl* mutations, along with other selectable morphological or phenotypic markers (Mashatola *et al*., 2018). Against this background, this chapter focused on the application of EMS to induce *tsl* mutations and morphological body/colour variations (those that can be visualised with the naked eye) in *An. arabiensis* with the aim to ultimately use these mutations to create a GSS.

5.2. Methods

5.2.1. Mosquito strain and rearing

The *An. arabiensis* strain, KWAG, described in 2.2.1, was used in this study. Rearing was performed as described by Hunt *et al*. (2005) and detailed in appendix 1.

5.2.2. Experimental design

This experiment involved sex-separating approximately 400 pupae based on genitalia observation under a compound microscope (Model BX53F, Olympus, Tokyo, Japan) as described in MR4, (2015). After separation, 200 male and 200 female pupae were placed in separate plastic cups (12 cm diameter and 7 cm height) filled with 250 mℓ of distilled water. The plastic cups with pupae were each introduced into their own BugDorm-1® cage for adult emergence overnight. Upon emergence into adults, unmated males were removed from the cages by using a mouth aspirator and divided equally into two separate BugDorm-1® cages (100 males per cage). The first cage (treatment) was provided with a 10 % sugar solution spiked with an EMS dose of 0.05 % that was soaked on cotton wool (HGRA2805, Lasec, South Africa) (see appendix 4). The second cage (control) was provided with a plain 10 % sugar solution (no EMS). Adult males in both cages were allowed to feed on the respective solutions cages for 24 h. Concurrently, unmated adult females were allowed to remain in their BugDorm-1® cage and fed on a plain 10 % sugar solution, also for 24 h. Subsequently, all sugar solutions were removed from the cages before females were introduced into the treatment and control cages in a 1:1 ratio. A new plain 10 % sugar solution was placed in each cage. The mixed adults were left in cages to mate over three consecutive days. On the fourth day, a cattle blood meal was provided to the females of each cage for 2 h through artificial feeding method as described in chapter 2. The mixed adults were again left in cages with just a plain 10 % sugar solution on day five and day six. A second blood meal was provided as described above to each mixed cage on seventh day). On day 10, plastic cups spray-painted black on the outside (egg plates (see appendix 1, Figure S1.3) were placed at the bottom of each cage for females to lay eggs overnight. The resultant eggs from each cage (F_1 generation progeny) were introduced into corresponding plastic larval rearing trays (41 cm length × 30 cm width × 8 cm height, Model: DP1101_6P, MegaView Science Co., Ltd., https://shop.bugdorm.com/) and reared to adulthood as described in appendix 1 and Hunt *et al*., (2005). The resulting sibling adults (both males and females) from each treatment/control (P_0) were introduced into their own corresponding new BugDorm-1® cages. These were allowed to mate (inbreeding) per

cage, blood feed and lay eggs (now F_1) the same as their parents. The F_1 progeny were also allowed to bear F_2 and F_3 progeny following the same inbreeding and rearing method.

The F_3 adults were placed in a BugDorm-1® cage, provided a plain 10 % sugar solution soaked on cotton wool, allowed to mate and also provided two blood meals over a five-day period. On day 10, gravid F_3 females (n = 20) were randomly removed from each cage and placed individually into a glass oviposition vials (11 mm radius and 50 mm height (GLSC2V5M32, Lasec, South Africa)) lined inside with a damp filter paper (lot: 20-104, Munktell, Sweden) cut into a conical shape of about 5 cm either side as described by Choi *et al*. (2014). The top of the lid was removed and replaced with a 50 µm nylon mesh. A cotton wool ball soaked with a 10 % sugar solution was placed on top of each oviposition vial. The oviposition vials were checked once daily for eggs on the damp filter paper and dead females for 10 consecutive days. If eggs were found, the total number of eggs laid by each female was counted and recorded. The individual adult females in each oviposition vial were regarded as "iso-family". Thus, eggs from each iso-family were placed in their own 350 mℓ foam cups (SKU: 11230 Westpark stores, South Africa) filled with 100 mℓ – 150 mℓ of distilled water and allowed to hatch. Upon hatching, L1 were counted, and the total number of L1 over two to three days was determined (studies have shown that the majority of (>80 %) of egg hatching in *Anopheles* occurs within two to three days (Clements, 1992; Kaiser *et al*., 2014)). Half of the L1 that hatched over this period from each iso-family were removed and heat-shocked at 41 °C for 3 h to screen for temperature sensitivity, while the other half served as negative control kept under standard insectary conditions. The rationale behind selecting the specific temperature and duration of exposure utilised in this study was influenced by the previous work of Ndo *et al*. (2018). Their research had previously achieved the successful isolation of a temperature-sensitive lethal strain through the use of EMS and exposing L1 to a temperature of 41 °C for a duration of 3 h in a water bath. Given the successful outcomes of their protocol, it was deemed appropriate to adopt and replicate these conditions in the present study as a basis for experimentation and comparison. Thus, after exposure to heat shock, the remaining larvae were transferred into corresponding larval rearing bowls, returned to standard insectary conditions and larval mortality was recorded after 24 h. In all cases, a control with larvae from iso-families that were never exposed to EMS was also set up, with half of the hatched L1 exposed to heat and the other half not exposed to heat. Families with larval mortality of 50 % or above after 24 h were suspected of carrying *tsl* and therefore selected and reared to the next generation (families

with larval mortality below 50 % after 24 h were discarded as they were deemed not to have any *tsl* mutation). The larvae and pupae of the selected iso-families were visually observed for any distinct morphological markers that deviate from the wild-type, and the numbers were recorded. Iso-families showing a sex bias favouring males (>50 %) were also selected as possibly containing a chromosomal translocation, optimistically a *tsl* mutation. A schematic diagram of the experimental protocol described in this section is summarised in Figure 5.1.

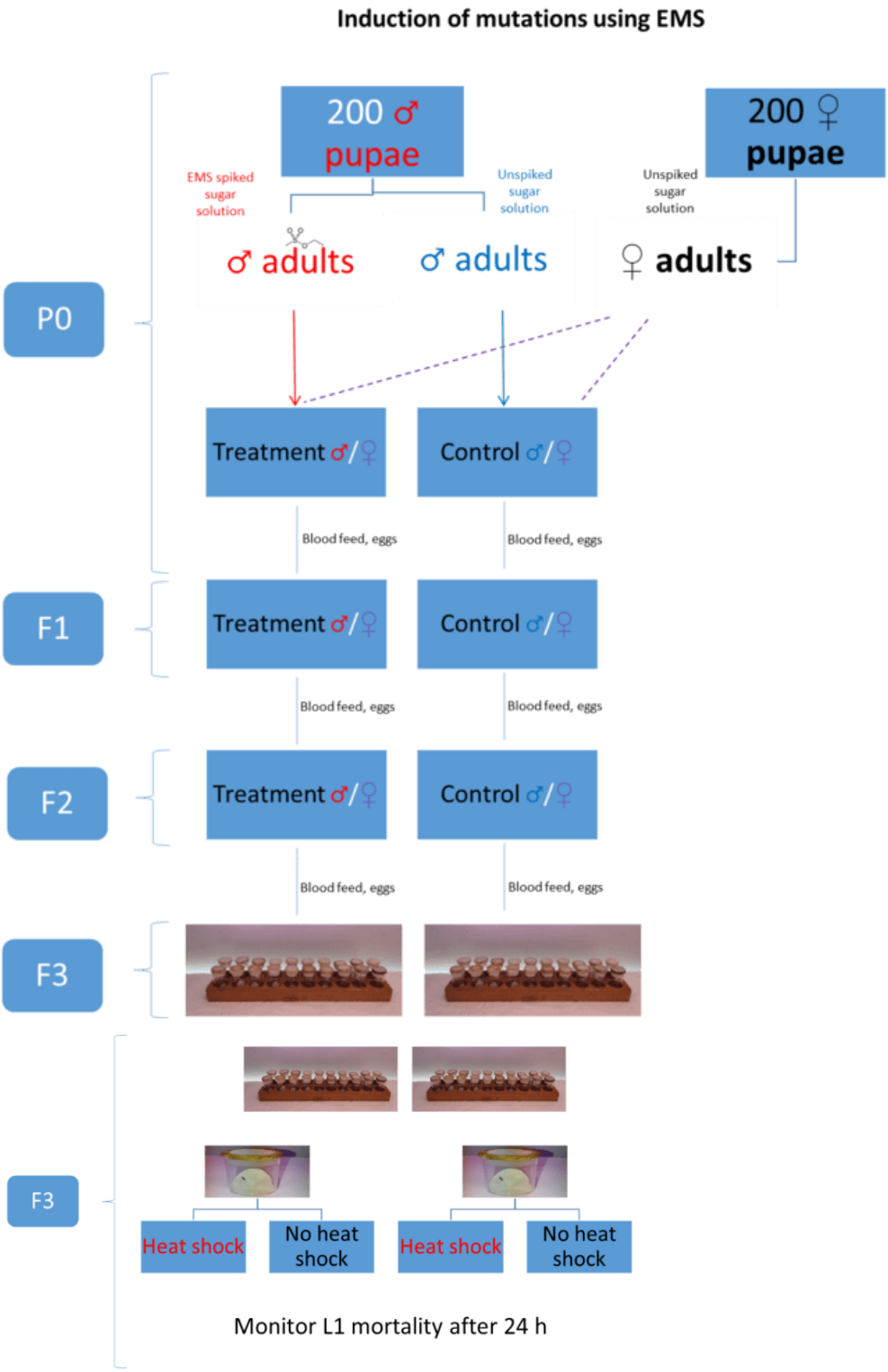


Figure 5.1: Schematic diagram of the experimental protocol used to induce conditional mutations in *An. arabiensis* male mosquitoes using EMS and screening for iso-families that can contain *tsl* alleles (protocol adopted and modified from Ndo *et al*. (2018)).

5.2.3. Data management and statistical analysis

All data were recorded manually on paper sheets before being electronically transferred to Microsoft Excel 2016 and analysed using IBM SPSS (IBM Corp, 2015) and DATAtab software (DATAtab, 2022). In all cases, differences between compared groups were considered statistically significant when $p < 0.05$. Data on the induction of conditional mutations in *An. arabiensis* male mosquitoes using EMS and screening for iso-families with the potential to contain *tsl* alleles were summarised as descriptive statistics (frequencies and percentages). The number of mutations generated from individual parental female mothers (iso-families) was recorded in the F_3 and F_4 populations and an association between EMS-treated and non-treated was determined using a chi-square test.

5.3. Results

A total of 120 iso-families were utilised at F_3 generation, with half of them subjected to EMS treatment (n = 60), while the other half served as a control group with no EMS treatment (n = 60). Only 60.8% of these iso-families, encompassing both the EMS-treated group (n = 37) and the control group (n = 36), laid eggs (Table 5.1). When examining the number of eggs laid per female (fecundity), it was found that the EMS-treated group exhibited a lower mean (± SD) of 46.3 ± 18.2 eggs laid per female compared to the control group's higher mean of 80.2 ± 30.6 eggs laid per female. The observed difference in fecundity between the treatment and control groups was statistically significant (Independent t-test; $t = -8.088$, $df = 59.187$; $p < 0.05$).

Further analysis revealed that out of the 37 iso-families that laid eggs in the treatment cohort, only 26 iso-families had eggs that successfully hatched, with an average hatching rate (± SD) of 17.9% ± 7.6. In contrast, all 36 iso-families in the control group had their eggs hatch at a significantly higher rate of 61.8% ± 27.6.

Table 5.1: The percentage of iso-families that laid eggs, the mean eggs laid per female and the total number of eggs that hatched from iso-families that mated with males fed with either 0.05% EMS or control.

Treatment	% iso-families that laid eggs (n)	Mean number of eggs laid per female ± SD	% iso-families in which eggs hatched (n)	Total number of eggs hatched (%) ± SD
(0.05% EMS)	50.7 (37)[a]	46.3 ± 18.2[a]	70.3 (26)	17.9 ± 7.6[a]
Control (0 EMS)	49.3 (36)[a]	80.2 ± 30.6[b]	100 (36)	61.8 ± 27.6[b]

Different superscript letters within a column indicate significant difference at 0.05 significance level. n = sample size; SD = standard deviation

Following the examination of larvae from 26 hatched iso-families in the treatment group and 36 in the control group, a total of 13 iso-families from the treatment group and 18 from the control group were subjected to heat shock as part of temperature/*tsl* screening and the monitoring of phenotypic variants. Among these iso-families, only nine from the treatment group displayed larval mortality greater than 50 %, indicating potential carriers of tsl mutations. Notably, two iso-families exhibited 100 % L1 larval mortality after exposure to heat shock and were therefore selected as promising *tsl* carriers (Table 5.2). In contrast, none of the L1 from the control group exhibited mortality exceeding 50 % after exposure to heat shock.

Simultaneously, morphological variations, specifically larval and pupal body colour variations were investigated in all iso-families that produced progeny (94 from the treatment group and 480 from the control group). Only 58.5 % (n = 55) of the iso-families from the treatment group pupated, in contrast to 81.7 % (n = 392) of the larvae from the control group. Throughout this observation, a range of morphological variations was noted in both the EMS-treated and control groups. Some of the most remarkable variations included white larvae and pupae (n = 2), variations in the larval collar and dorsum (n = 6), pupae with white-eye colouration (n = 1), and pupae displaying variable intensity of body colouration (both in larvae and pupae) (Figure

5.2). Notably, the majority of conditional morphological variations observed in both larvae and pupae were related to variations in green body colouration.

Table 5.2: Nine selected iso-families that recorded L1 mortality >50 % following heat shock and their corresponding controls.

Assay	Iso-family ID	Total L1 exposed to heat shock	Total L1 died after heat shock	Mortality (%) 24 h after heat shock	Total L1 placed under insectary conditions (not exposed to heat shock)	Total L1 died under insectary conditions	Mortality (%) 24 h under insectary conditions
Treatment	TR1F11	16	10	62.5	15	1	6.67
Treatment	TR1F14	15	9	60	14	0	0
Treatment	TR2F9	2	2	100	1	0	0
Treatment	TR2F10	13	12	92.31	12	0	0
Treatment	TR2F13	1	1	100	1	1	100
Treatment	TR2F15	16	13	81.25	16	0	0
Treatment	TR2F19	7	5	71.43	7	0	0
Treatment	TR3F11	9	6	66.67	9	0	0
Treatment	TR3F13	1	1	100	1	1	100
Control	CR1F11	56	0	0	56	0	0

Control	CR1F14	28	0	0	28	0	0
Control	CR2F1	54	0	0	53	0	0
Control	CR2F9	26	0	0	26	0	0
Control	CR2F11	20	0	0	21	0	0
Control	CR2F14	44	0	0	42	0	0
Control	CR2F16	22	0	0	23	0	0
Control	CR3F12	34	0	0	33	0	0
Control	CR3F13	16	0	0	16	0	0

Iso-family coding: T = treatment, R1 = replicate 1, F = female number, C = control.

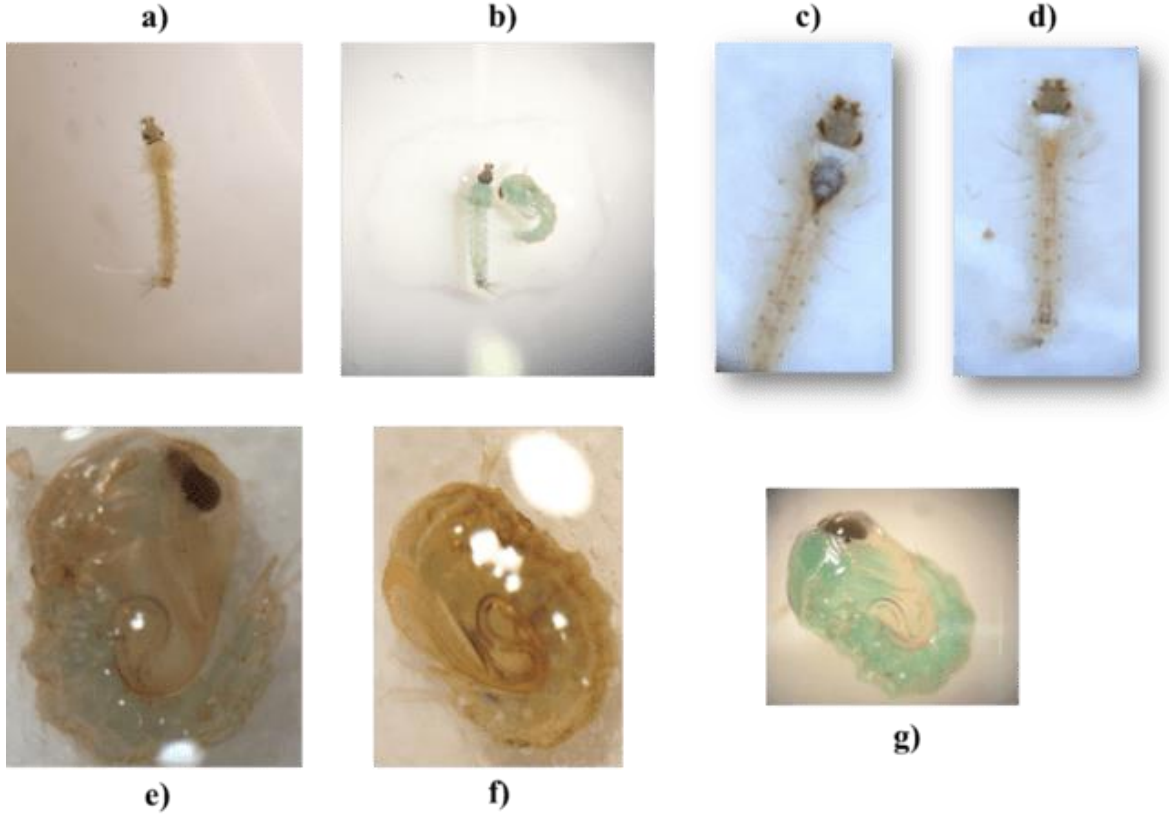


Figure 5.2: Morphological variations observed in larval and pupal stages of *An. arabiensis*. (a) wild-type larva, (b) green larva and green pupa, (c) and (d) larvae with collar and dorsum variation, (e) pupa with low/faded intensity green colour (f) pupa with white eye colour and (g) pupa with high intensity green colour.

Adult emergence from pupae was 92.7% (n = 51) in the treatment iso-families and 91.8% (n = 360) in the control. A slight sex ratio favouring males was observed in only three treatment iso-families. These were selected as possibly containing both *tsl* and a chromosomal translocation, and the progeny inbred to bear the next generation. However, none showed L1 mortality >50% after heat shock nor morphological markers and were thus discarded (Table 5.3).

Table 5.3: A summary of F_4 iso-female families that were subsequently selected as possibly containing *tsl* and chromosomal translocation.

Iso-family_ID	Number of adults	Male	Female	Total eggs laid	Total L1	Total L1 exposed to heat shock	Total L1 died after heat shock	Mortality (%) 24 h after heat shock	Total L1 placed under insectary conditions (not exposed to heat shock)	Total L1 died under insectary conditions	Mortality % under insectary conditions	Total L1 died after 24 mh	Iso-line selected for further rearing if (L1 mortality >50%) or discarded if (L1 <50%)
TR1F11	12	8	4	74	51	26	7	26.92	25	0	0	7	Discarded
TR1F14	15	9	6	42	28	14	3	21.43	14	0	0	3	Discarded
TR2F10	9	4	5	121	107	54	19	35.19	53	2	3.77	21	Discarded
TR2F15	8	5	3	118	88	44	21	47.73	44	0	0	21	Discarded
TR2F19	7	3	4	217	42	21	5	23.81	21	0	0	5	Discarded
CR1F11	96	44	42	*	*	30	2	6.67	30	0	0	2	Discarded
CR1F14	41	19	22	*	*	30	1	3.33	30	0	0	1	Discarded
CR2F1	37	16	21	*	*	30	4	13.33	30	2	6.67	6	Discarded
CR2F9	22	11	11	*	*	30	2	6.67	30	1	3.33	3	Discarded
CR2F16	26	10	16	*	*	30	2	6.67	30	0	0	2	Discarded

Iso-family coding: T = treatment, R1 = replicate 1, F = female number, C = control, *total eggs laid and *total L1s not counted in control.

5.4. Discussion.

The use of EMS to induce mutations, particularly *tsl* mutations and morphological variations, in *An. arabiensis* is a promising avenue for the development of GSSs. This approach has been successful in other species (e.g. *C. capitata* and *An. arabiensis*) (Caceres, 2002; Ndo *et al*., 2018). Hence, this work aimed to use EMS to induce *tsl* mutations and morphological body/colour variations as selectable markers that can be used in solace or parallel to develop a GSS.

The study observed differences in fecundity and hatch rates between treated iso-families and control groups. Also, while a greater number of iso-families from the treatment laid eggs as compared to the control group, the control group exhibited higher egg production and better hatch rates. This suggests that while the mutagenesis treatment did not significantly affect the number of females capable of laying eggs, it did however impact the viabilityof the eggs. These findings align with a previous study by Ndo *et al*. (2018), which also reported similar numbers of eggs laid and hatch rates between females mated with EMS mutagenised males and those from the untreated control group. However, the effect of EMS on fecundity and fertility across the treated group was more variable. This indicates that while EMS may not consistently impact the fecundity and fertility of the iso-families, especially at the dose used in this study, it still retains mutagenic properties.

The potential reason behind these findings could be associated with the type of chromosomal damage induced by EMS. This is because EMS is known to induce mutations at loci that regulate various important traits (FAO/ IAEA, 2021). In this context, it is possible that the mutagenesis primarily affected specific genetic loci responsible for traits other than fecundity and fertility. This hypothesis opens the door to further investigations aimed at understanding the precise gene functions affected by EMS-induced mutations and how these mutations influence mosquito biology. Thus, understanding that EMS mutagenesis might not always significantly compromise male mosquito reproductive capacity allows researchers to focus on other traits, such as temperature sensitivity or morphological variations, when developing targeted mutational screenings. This knowledge contributes to the ongoing efforts to refine and enhance the efficiency of *tsl* screening and mosquito control strategies.

Subsequent temperature/*tsl* screening and monitoring of phenotypic variants showed that only nine iso-families from the treatment group exhibited larval mortality exceeding 50 %. Among these, two iso-families experienced 100 % L1 mortality after exposure to heat shock, indicating the potential presence of *tsl* mutations. In contrast, none of the L1 from the control group exhibited mortality exceeding 50 % post-exposure. Despite these efforts, the study did not manage to isolate any *tsl* mutations. This could be due to a combination of factors, including their rarity, insufficient sample size, limited understanding of the specific mutation, and the complexity of genetic interactions involved (Tan *et al*., 2009). These can hinder targeted screening efforts. Further research with a focus on these factors may be necessary to successfully isolate *tsl* mutations in *An. arabiensis*.

In addition, to enhance the chances of locating *tsl* mutations, more advanced molecular and cytogenetic techniques can be employed. These may include whole genome sequencing, DNA hybridisation, and restriction enzyme digestion methods (Cotton, 1993; Mahdieh and Rabbani, 2013). However, it is important to note that these advanced techniques can be costly and resource-intensive.

The study also observed several morphological variations, including green larvae and green pupae, as well as white-eye larvae and white-eye pupae. These variations have been reported in previous studies (Benedict *et al*., 1996; Seawright *et al*., 1979). The presence of green larvae and green pupae in both the EMS-treated and control groups raises questions about whether these mutations are induced by EMS or occur naturally.

The green pupae mutation (*gp*) has previously been associated with green pigmentation in the fat body of late fourth instar larvae and young pupae (Warren *et al*., 1975; Seawright *et al*., 1979). However, it is important to recognise that the development of such colour variations might involve multiple mutations or different genes interacting to produce the observed morphological changes (Stearns, 2010). This complexity in the genetic basis of colour variations is not uncommon in organisms.

To gain a deeper understanding of these morphological variations and their genetic underpinnings, further molecular analyses should be conducted. These analyses can help identify the specific genes or genetic changes responsible for body colour variations in mutant

larvae, pupae, and adult mosquitoes. Such insights could provide valuable information for both basic genetic research and potential applications in mosquito control and GSS development.

Indeed, it is crucial to continue investigating other traits beyond those screened in this study, particularly traits that might have lethal or developmental effects. Although not specifically addressed in this study, there are potentially valuable mutations that deserve further exploration. One notable example is the "slow development" (*sd*) gene identified in *C. capitata* which has a close relationship with the *tsl* gene (Meza *et al*., 2020; Porras *et al*., 2020). This *sd* gene has been of interest because it influences the development rate of individuals. Understanding such mutations, which affect the timing of development, can have implications for the mass rearing and production of insects for various purposes, including SIT programmes. Therefore, future research efforts could consider exploring these additional mutations and their effects on development, as they might offer valuable insights and tools for mosquito control and GSS development.

5.5. Conclusion

Despite encountering challenges in inducing *tsl* mutations and isolating morphological variations, the groundwork laid by previous research indicates the potential for innovative mosquito control strategies using genetic approaches. Also, while further research and refinement are needed to overcome the challenges encountered in this study, the potential of *tsl*-based GSSs in mosquito control remains promising.

CHAPTER 6: Use of an already established *An. arabiensis* temperature-sensitive strain from Cameroon to develop a *tsl*-based GSS with a South African genetic background

6.1. Introduction

The failure to successfully isolate a *tsl* strain or develop a *tsl*–based GSS for the South African SIT programme has continued to hamper the potential application of this technique against malaria vectors, in particular *An. arabiensis* (Mashatola *et al*., 2018). However, the successful isolation and characterisation of an *An. arabiensis* strain (henceforth referred to as CMR-*tsl*) containing a conditionally lethal selectable temperature sensitivity mutation provided a significant step towards the development of a sexing system for this species (Ndo *et al*., 2018). This strain's temperature-sensitive phenotype is expressed when L1 is exposed to a temperature of 41°C for 3 h, resulting in complete larval mortality within 24 h post-exposure (Ndo *et al*., 2018). This character provides hope towards the possible linkage of this conditionally lethal selectable genetic marker to a sex chromosome and thus develop a GSS (Curtis *et al*., 1976).

The CMR-*tsl,* which was developed as part of a Coordinated Research Project (CRP, D44003) initiated by the IAEA which brings together research institutions from its developing and developed Member States to collaborate on research projects of common interest (FAO/IAEA, 2019), contains a Cameroonian genetic background and requires integration into a local genetic background of the same species earmarked for release before it could be used in other settings (Ndo *et al*., 2018). In this instance, in South Africa. If a strain containing a foreign genetic background is directly released into a local geographical field site, unintended consequences such as foreign gene introduction, incompatibility in mating, and poor competitiveness between the strain and the native population could occur (Carvalho *et al*., 2020).

Foreign genetic backgrounds can be incorporated into native strains through techniques such as introgressive hybridisation, backcrossing, marker-assisted selection, cytogenetic methods, molecular biology, and genetic engineering (National Research Council, 2004). Using these approaches, it is possible to introduce specific traits or genes from foreign strains into native populations while maintaining the overall genetic integrity of the strain (Dandalo *et al*., 2018; Carvalho *et al*., 2020; Augustinos *et al*., 2022; Misbah-ul-Haq *et al*., 2022). The selection of the

most suitable method depends on aspects such as available resources, and targeted desired traits.

Against this background, the primary objective in this chapter was to create a GSS by utilising a combination of cross-mating and backcrossing between two distinct *An. arabiensis* strains: a temperature-sensitive strain originating from Cameroon and a non-temperature-sensitive strain from South Africa. The ultimate aim was to establish a GSS that carries the genetic background of the local South African strain but incorporates the valuable trait of temperature-sensitive sex determination. This would result in conditions were both males and females from this GSS are viable under permissive temperatures, while under restrictive temperatures females would be selectively eliminated.

6.2. Materials and methods

6.2.1. Mosquito strains

Two laboratory mosquito strains were used in this study. The first, CMR-*tsl,* was established at the Centre for Research in Infectious Diseases, Malaria Research Laboratory, OCEAC, Yaoundé, Cameroon, by continuously feeding male wild-type material from North Cameroon with cotton wool soaked in 10% sugar water solution spiked with 0.05% EMS for 24 h (Ndo *et al*., 2018). The result was the successful isolation of individuals that express complete larval mortality when L1 is exposed to a temperature of 41°C for 3 h. The eggs of this strain were imported from Cameroon into South Africa in 2020 during the height of the COVID-19 pandemic. Upon arrival, the eggs were quarantined and reared in one of the rooms in the BDMI (details in chapter 2) that is specifically designed to house field collected material or material from other countries. The strain is still maintained under quarantined conditions.

The second strain, KWAG, is the same strain described in 2.2.1. It was hypothesised in this study that by crossing KWAG (wild-type) with CMR-*tsl* (carrying the *tsl* mutation), it would be possible to introduce the South African wild-type genetic background into the CMR-*tsl* strain. This is essential for developing a *tsl*-based GSS.

6.2.2. Confirming the temperature sensitivity status and species identity of the CMR-*tsl* strain.

6.2.2.1. Confirming temperature sensitivity status

Larvae of the CMR-*tsl* strain at the L1 stage, aged between 6 h and 24 h, were randomly picked from larval rearing trays (41 cm length × 30 cm width × 8 cm height, Model: DP1101_6P, MegaView Science Co., Ltd., https://shop.bugdorm.com/) using 3 mℓ disposable pasteur pipettes (PFLSBSV136-3R, Lasec, South Africa). These larvae were counted into separate groups of 30 L1 and placed on a 70 mm diameter grade 3hw filter papers (lot: 20-104, Munktell, Sweden) that was folded into a conical and suspended within a 175 mℓ foam cup (SKU: 12743, West park stores, South Africa) (Figure 6.1). Each batch of 30 L1 was then placed into a 36 ℓ capacity thermostatic water bath (Model: 103, Labotec, South Africa) and exposed to different temperatures of either 27 °C (water bath) and 41 °C for a duration of 3 h. After the specified treatment, the L1 were removed and transferred per cup into new corresponding 350 mℓ foam cups (SKU: 11230 West park stores, South Africa) filled with 100 mℓ – 150 mℓ of distilled water. Another batch of 3 × 30 L1 was kept in a 350 mℓ foam cup (SKU: 11230 West park stores, South Africa) at the standard insectary ambient temperature (no water bath) as a control 27 °C (insectary). Larvae were not provided with any food during this period. Larval mortality was recorded after 24 h. The tests were repeated on three different occasions using larvae from various egg batches or generations. The water bath was pre-set to the experimental temperatures for 1 h before exposing the larvae. A schematic diagram showing the confirming of temperature sensitivity status described in this section is summarised in Figure 6.1.

Figure 6.1: A schematic diagram showing the construction of temperature screening equipment. a) A 36 ℓ capacity, thermostatic controlled water bath filled with 34 ℓ distilled water, covered with a Plexiglas with holes to support plastic cup, plastic cups suspended, zoomed view of cup and folded filter paper.

6.2.2.2. Strain species identity confirmation

For species specific identity of the CMR-*tsl* and and KWAG strains, 7 newly emerged (> 2 h old) adults were randomly selected from cages and placed into net covered coffee cups using a mouth aspirator. The cups were placed in a -70 °C Blizzard™ vertical ultra-low freezer (NU-99728JE, Lasec, South Africa) for 10 minutes to kill the adults. Then, each adult was exposed to the *An. gambiae* complex PCR as per Scott *et al*. (1993). Each PCR reaction contained one leg or wing from individual mosquito specimen and 12.5 µℓ of a PCR master mix containing 1.25 mM 10× PCR reaction buffer; 200 µM of dNTPs; 25 mM MgCl2; 0.16 µM of each of the primers for *An. gambiae s.l.*, *An. merus*, *An. arabiensis* and universal primers and 0.08 µM for *An. quadriannulatus* species A; 4.9 µℓ of deionised water and 0.5 units of Thermus aquaticus (Taq) DNA polymerase enzyme, sourced from Inqaba biotec™, South Africa. The primer sequence used were ME primer (5'TGACCAACCCACTCCCTTGA3') that anneals to both *An. merus* and *An. melas*, GA primer (5'CTGGTTTGGTCGGCACGTTT3') that anneals specifically to *An. gambiae s.l*, AR primer (5'AAGTGTCCTTCTCCATCCTA3') that anneals to *An. arabiensis*, QD primer (5'CAGACCAAGATGGTTAGTAT3') that anneals to *An. quadriannulatus* species A and UN primer (5'GTGTGCCCCTTCCTCGATGT3') that anneals to the same position of ribosomal DNA (rDNA) in all five species. To release the template DNA, the microcentrifuge tubes were placed in a centrifuge (Eppendorf® 5301 concentrator, Eppendorf, Hamburg, Germany) and centrifuged at 13 000 revolutions per minute for 10 seconds. Positive control specimens derived from well-known laboratory strains of *An. arabiensis*, *An. merus*, *An. quadriannulatus* species A and *An. gambiae s.l.* and negative control, consisting of the reaction mixture only (no DNA) were also included. Each reaction mixture was placed individually in a 96-well T100™ Thermal Cycler (Bio-Rad, Hercules, California, USA) and subjected to the following PCR conditions: 30 cycles of 94 °C denaturation for 30 seconds, 50 °C annealing for 30 seconds, 72 °C extension for 30 seconds, and a final auto extension step of 5 minutes at 72 °C. Following the PCR reaction, 10 µℓ of each amplified product was loaded into individual wells of a 2.5 % agarose gel, stained with ethidium bromide. A 1 kilobase molecular marker (O'Gene rulerTM, Fermentas Life Sciences) was also added to the first and last wells of each gel. The gel was then submerged in 1× Tris-acetate-EDTA (TAE) buffer and electrophoresed at 100 Volts for 90 minutes or until bromophenol blue was 3 cm from the point of origin to allow for proper separation of amplicons. A ChemiDoc XRS+ Gel Imaging System (Bio-Rad, Hercules, California, USA) was used to generate the gel image. The amplified fragment sizes are expected to be 464

bp for *An. merus*/*An. melas*, 390 bp for *An. gambiae s.l.*, 315 bp for to *An. arabiensis* and 153 bp for *An. quadriannulatus* species A. A schematic diagram showing the species identity confirmation protocol described in this section is summarised in Figure 6.2.

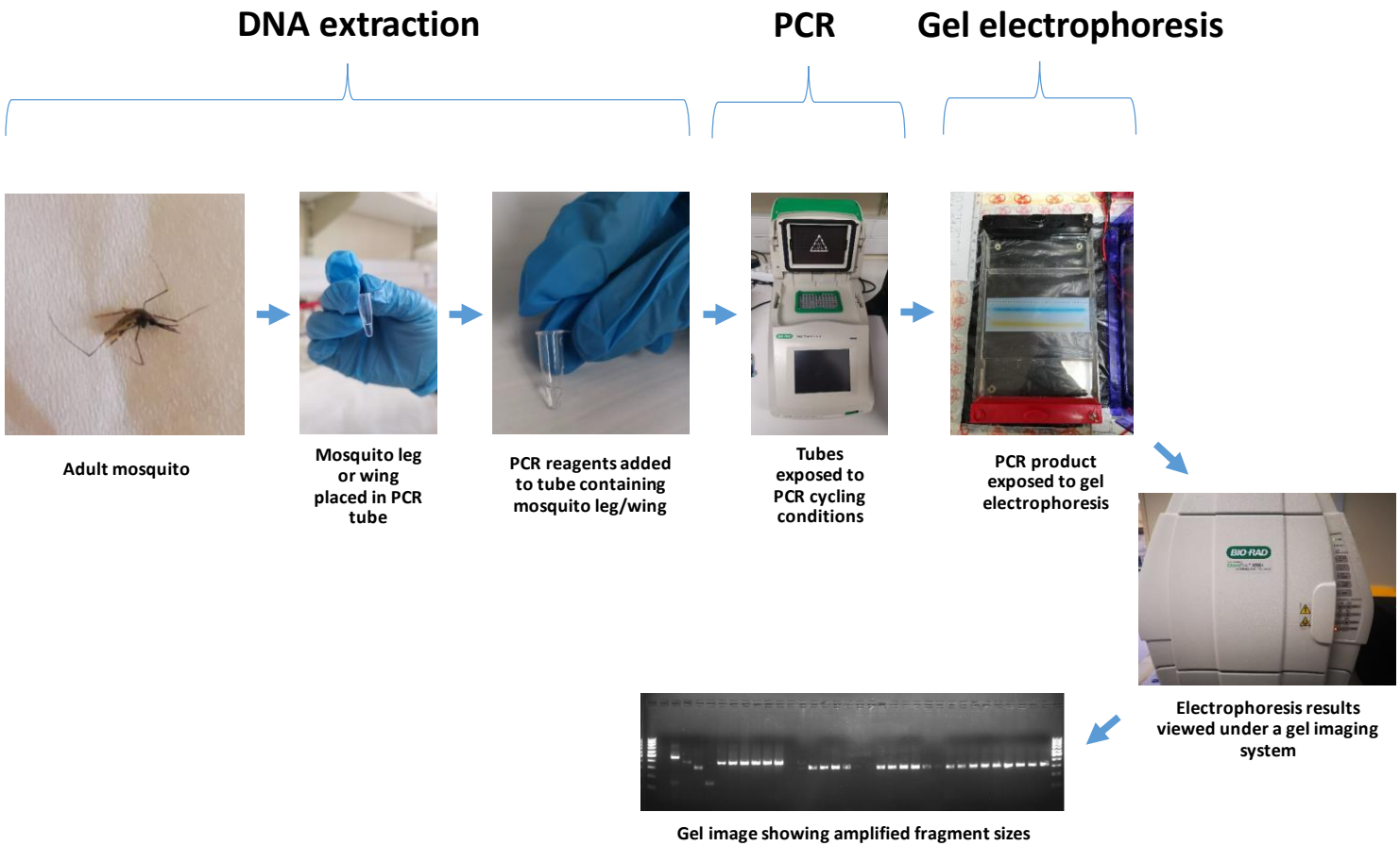


Figure 6.2: A schematic representation of steps followed to identify specimens to species level using PCR (species identity confirmation protocol).

6.2.2.3. PCR differentiation of samples that amplified as *An. gambiae s.l.* into species specificity (i.e., *An. gambiae sensu stricto* (*s.s.*) or *An. coluzzii*).

To add more certainty to the species identity, an additional PCR was performed to differentiate the *An. gambiae s.l.* samples into *An. gambiae s.s.* or *An. coluzzii* species (formerly known respectively as *An. gambiae* S molecular form and *An. gambiae* M molecular form) (Coetzee *et al*., 2013a). The extracted DNA from each sample of the CMR-*tsl* strain was added to a reaction tube containing 1.25 mM 10× PCR buffer; 200 µM of dNTPs; 0.16 µM of each of the primers R3 (5'GAATTCTAGGGAGCTCCAG3'), R5 (5'CCAATCCGAGCTGATAGCGC3'), Mopint (5'GCCCCTTCCTCGATGCAT3') and B/S int (5'ACCAAGATGGTTCGTTGC3'); 25 µℓ PCR grade water and 0.5 units of Taq polymerase enzyme (Inqaba biotec™, South Africa). For each set of samples, a negative extraction control (no DNA) was included and positive controls from known M and S species colonies kept in the BDMI. The PCR cycling conditions were as follows: Ten minutes at 94 °C, followed by 25 cycles of 94 °C for 30 seconds, 62 °C for 30 seconds, and 72 °C for 30 seconds, with a final 7-minute extension at 72 °C. The samples were held at 8 °C until removed. Each reaction mixture was subjected to agarose gel electrophoresis, and a gel image was generated as described above. The amplified fragment sizes are expected to be 367 bp for *An. coluzzii* and 257 bp and 110 bp for *An. gambiae s.s*. These were compared to a 1 kilobase molecular marker.

6.2.3. Cross mating CMR-*tsl* with the South African local strain, KWAG

Cross-mating experiments were pursued further in an effort to establish genetic compatibility, and the potential for hybridisation between the CMR-*tsl* strain and the South African local KWAG strain. Four mating combinations involving these two strains were set up in individual 15 cm length × 15 cm width × 15 cm height BugDorm-4® adult insect holding cages (Model: 4E1515, MegaView Science Co., Ltd., https://shop.bugdorm.com/) (Figure 6.3.a). The cages are constructed using fiberglass poles that interconnect at the corners, forming a cube-shaped frame. A mesh cage, made of polyester netting with a mesh size of 680µm, is threaded onto these poles to create an enclosed environment. The front panel features an 18 cm diameter sleeve opening, providing access for replacing food and adding or removing insects, while a thin strip across the ceiling allows for the suspension of objects like feeders. Each cage contained 50♂:50♀ newly emerged (> 2 h old) virgin adults of the following cross matings: cross 1: CMR-*tsl* ♂ × KWAG ♀, cross 2: CMR-*tsl* ♀ × KWAG ♂, cross 3: CMR-*tsl* ♀ × CMR-*tsl* ♂and cross 4: KWAG ♀ × KWAG ♂ (Figure 6.3.a). The crosses were provided with a 10 % sugar water

solution soaked on cotton wool that was contained in a urine jar. The adults were then allowed to mate in their respective cages for three days, after which females were given two blood meals over five days (fourth and seventh day). A plastic egg plate (appendix 1, Figure S1.3) filled with 100 mℓ of distilled water was placed in each cage for adult females to lay eggs overnight. The following day, the number of eggs laid per cage was counted, and fecundity was determined by dividing the number of eggs laid over the number of females alive the day egg plates were placed into cages. on the same day, eggs were placed in 350 mℓ polystyrene cups filled with 150 mℓ of distilled water, and egg hatch rates (fertility) were monitored daily by counting and removing L1 into corresponding plastic larval rearing trays (41 cm length × 30 cm width × 8 cm height, Model: DP1101_6P, https://shop.bugdorm.com/) filled with 2 ℓ of distilled water for 14 consecutive days. The larvae were fed once daily with a standard 1 % IAEA *Anopheles* diet at a rate equivalent to 1 mg/larva/day until they reached the pupal stage (FAO/IAEA, 2017b). A sub-sample of resulting F_1 adult males (n = 10, aged three to five days old) from each of the above crosses had their reproductive tracts (testes and ejaculatory ducts) dissected and microscopically examined to confirm the presence or absence of sperm as per Huho *et al*. (2006). This was done to determine male sterility (i.e., fertile (presence of sperm) *vs* infertile (absence of sperm)). In addition, PCR species identify of F_1 progeny was performed as described in section 6.2.2.2. The mating crosses were repeated on three separate occasions, each time using parents from different generations. A schematic diagram showing the cross mating of CMR-*tsl* strain with the South African local strain, KWAG is summarised in Figure 6.3.

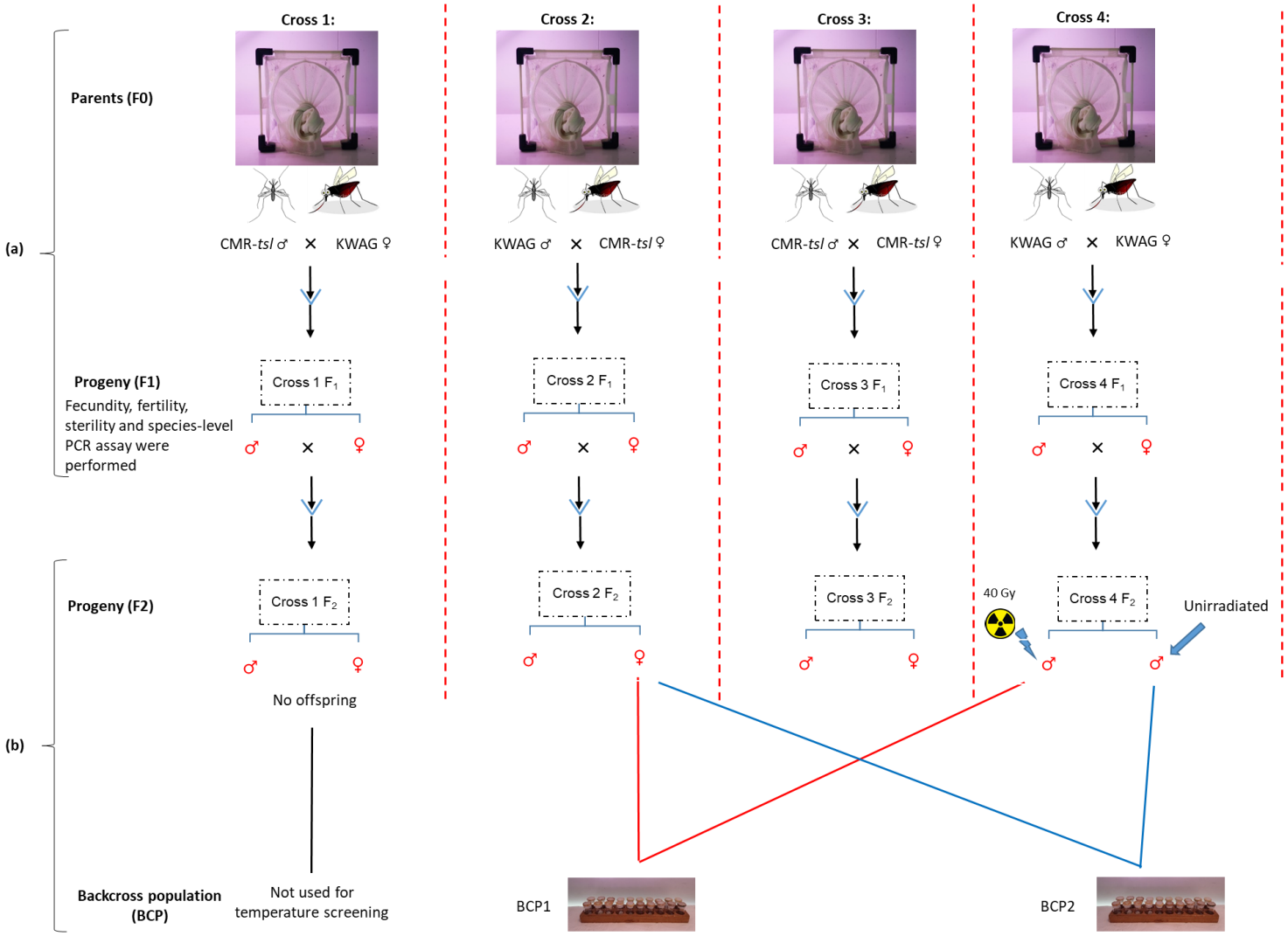


Figure 6.3: A schematic diagram showing (a) the mating scheme used to cross CMR-*tsl* and KWAG. Parameters such as fecundity, fertility, sterility and species-level PCR assay were performed in the F_1 progeny, (b) backcross population generated from crossing irradiated F_2 males with virgin CMR-*tsl* females (BCP1) and unirradiated F_2 males with virgin CMR-*tsl* females (BCP2).

6.2.4. Development of a temperature sensitive GSS through an irradiation-induced reciprocal Y-autosome translocation

F_1 adults from the CMR-*tsl* and KWAG strains were allowed to inbreed per cross and produced F_2 progeny. There was no progeny from cross 1. Because of this, only KWAG F_2 ♂ resulting from cross 4 were irradiated with 40 Gy (at pupal stage aged 20 – 24 h old) using a cobalt-60 gamma irradiator (see appendix 4). The pupae were placed in Bugdorm cages to emerge overnight. The emerging irradiated adult males were crossed with virgin, unirradiated females resulting from cross 2 F_2 progeny (Figure 6.3.b). Unirradiated KWAG F_2 adult males resulting from cross 4 were crossed with virgin, unirradiated adult females resulting from cross 2 to serve as the control. These backcross populations (BCP) were termed BCP1 and BCP2, respectively. A 10 % sugar water solution soaked on cotton wool contained in a urine jar was placed in each cage. Adults were allowed to mate in their own cages for three to four days. After the mating period, females from each cage were blood-fed on the fourth day. Females that did not blood-feed were removed from each cage. A second blood meal was provided on the seventh day to the remaining females that had successfully taken the first blood meal. Twenty females from each mating cross that had been blood-fed twice were randomly selected and tubed individually in glass oviposition vials lined inside with a damp filter paper as described by Choi *et al*. (2014) and also detailed in section 5.2.2. Cotton wool balls soaked with a 10 % sugar solution were placed on top of each oviposition vial. The glass oviposition vials were checked once daily for eggs and dead females for 10 consecutive days. If eggs were found, the total number of eggs laid by each female was counted and recorded. Eggs were placed in 350 mℓ polystyrene cups filled with 150 mℓ of distilled water and allowed to hatch into larvae. First instar larvae (L1 (aged 20 – 24 h)) from each female/isofamily were counted. The total larvae from each iso-family were subsequently split into two batches. One batch of the larvae (L1) were exposed to a thermostatically controlled water bath set at 41 °C for 3 h, while the other batch remained unexposed and were kept under standard insectary conditions. From the exposed larvae, if families showed total larval mortality (100 %) post temperature exposure, the other half that was unexposed but from the same family were isolated as potentially carrying the *tsl* mutation/thermo-sensitive and reared to adulthood. Unmated adult males resulting from these iso-families were backcrossed with parental thermos-sensitive virgin females from the CMR-*tsl* strain. The same procedure whereby L1 (aged 20 – 24 h) resulting from each iso-family were exposed to either 41 °C for 3 h or unexposed under standard insectary conditions was followed, and families showing total

larval mortality (100 %) post temperature exposure were selected. In addition, families presenting a sex-ratio distortion 100 % in favour of males were suspected of carrying a Y-autosome translocation, conferring heat tolerance to the male mosquitoes.

6.2.5. Data interpretation and statistical analysis

All data were manually recorded daily on data sheets before being electronically transferred to Microsoft Excel 2016. IBM SPSS (IBM Corp, 2015) and DATAtab software (DATAtab, 2022) were used for data analysis and generating figures, with a p-value of less than 0.05 considered significant at 95 % CI. Data on the generation of F_1 hybrids were summarised as follows: fecundity as the mean number of eggs per female of females that were alive the day egg plates were placed in cages, fertility as the number of hatched eggs divided by the total number of eggs, sterility scored as either fertile or infertile, while species-specific PCR identification was scored against standard amplicon sizes. To establish differences between crosses, fecundity and fertility were analysed using a one-way ANOVA, while sterility and species-specific PCR data were summarised as descriptive statistics. The selection of families suspected of carrying *tsl* mutation was based on the families showing total larval mortality (100 %) post temperature exposure and a 100 % sex ratio biased towards males.

6.3. Results

6.3.1. Confirming the temperature sensitivity status and species identity of the CMR-*tsl* strain.

6.3.1.1. Confirming the temperature sensitivity status

The temperature sensitivity of the CMR-*tsl* and KWAG strains, as evaluated by the mortality rates of L1 recorded 24 h post-exposure to different temperature conditions is displayed in Figure 6.4. At 27 °C (insectary), there were no observed larval mortalities for either strains 24 h post-exposure. In contrast, at 27 °C (water bath), CMR-*tsl* displayed minimal larval mortality, averaging at 1.11 %, while KWAG showed no larval mortalities. When the L1 were subjected to a temperature of 41 °C for 3 h, the mortality rate exhibited a stark contrast. For the CMR-*tsl* strain, the mortality rate reached 100 % (total larval mortality) 24 h post-exposure. On the other hand, the KWAG strain exhibited a considerably lower mortality rate of 12.2 % under the same conditions.

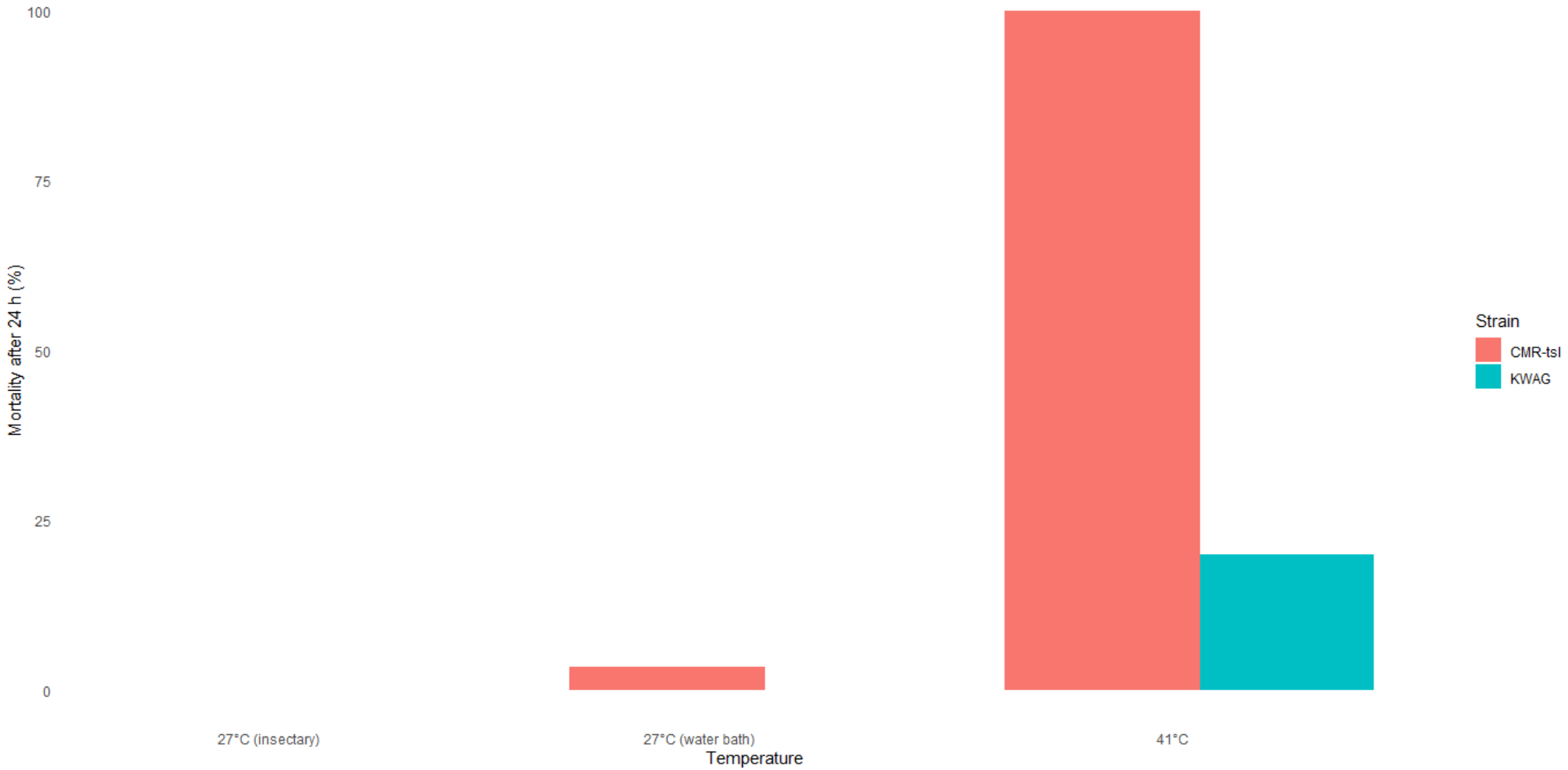


Figure 6.4: Mortality rates of CMR-*tsl* and KWAG strain L1 recorded 24 h post-exposure to different temperature conditions.

6.3.1.2. Confirming the species identity of the CMR-*tsl* strain

Of the 21 CMR-*tsl* F_0 (parents) samples assayed using species-specific PCR, including repeats of specimens that did not initially amplify, all were identified as *An. gambiae s.l.* As this was repeated on three different occasions, each time using seven different samples, figure 6.5 below shows species-specific PCR results of the CMR-*tsl* and KWAG strains for one of the repeats.

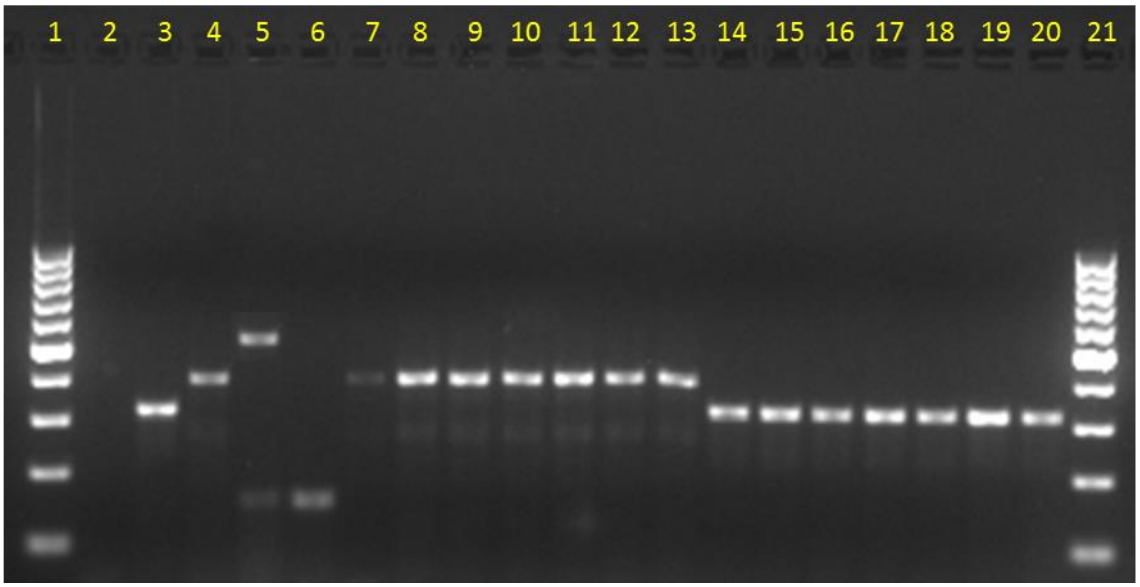


Figure 6.5: Species-specific PCR results of the CMR-*tsl* and KWAG strains. Lane 1 and 21: 1 kilobase molecular weight marker; lane 2: negative control; lane 3: *An. arabiensis* positive control; lane 4: *An. gambiae s.l.* positive control; lane 5: *An. merus* positive control; lane 6: *An. quadriannulatus* species A positive control; lanes 7 – 13: *An. gambiae s.l.*; lanes 14 – 20: *An. arabiensis*.

As the CMR-*tsl* strain, originally thought to be *An. arabiensis*, proved to be *An. gambiae s.l.* (Figure 6.5), further differentiation of the *An. gambiae s.l.* revealed that all the PCR assayed CMR-*tsl* samples specimens were *An. coluzzii* (Figure 6.6).

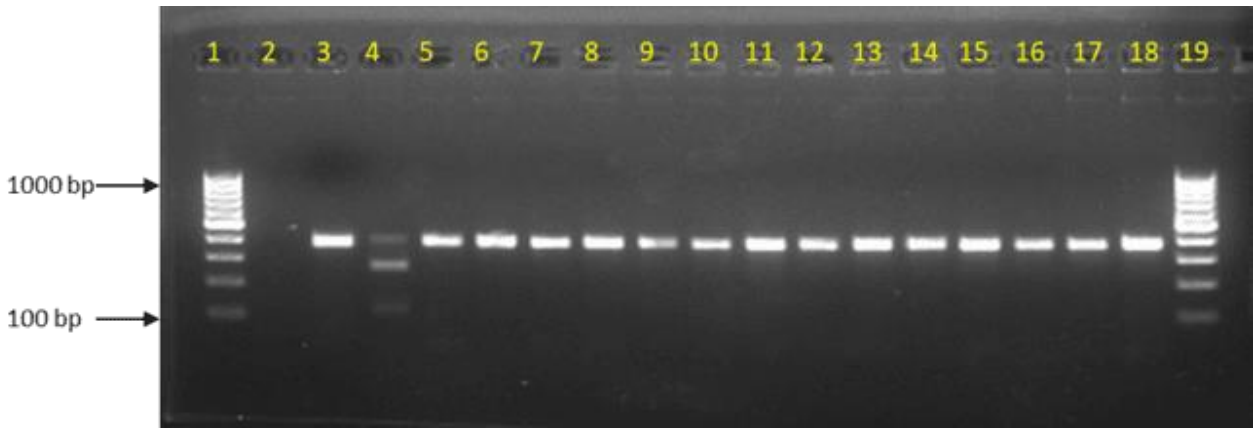


Figure 6.6: Molecular differentiation of the CMR-*tsl* strain into *An. gambiae s.s.* or *An. coluzzii* species (formerly known respectively as *An. gambiae* S molecular form and *An. gambiae* M molecular form. Lane 1 and 19: 1 kilobase molecular weight marker; lane 2: negative control; lane 3: *An. coluzzii* positive control; lane 4: *An. gambiae s.s.* positive control; lane 5 – 18: *An. coluzzii*.

6.3.2. Cross mating CMR-*tsl* with the South African local strain, KWAG

6.3.2.1. Fecundity

The mean number of eggs laid (fecundity) (± SD) by each of the crosses between CMR-*tsl* and KWAG were as follows: cross 1: CMR-*tsl* ♂ × KWAG ♀ = 81.46 ± 5.91 eggs per female, cross 2: CMR-*tsl* ♀ × KWAG ♂ = 77.42 ± 4.12 eggs per female, cross 3: CMR-*tsl* ♀ × CMR-*tsl* ♂ = 76.63 ± 8.21 eggs per female and cross 4: KWAG ♀ × KWAG ♂ = 80.22 ± 6.21 eggs per female (Figure 6.7). Statistically, no significant difference in fecundity was found between the crosses (one-way ANOVA: df = 3, F = 1.4, P = 0.312).

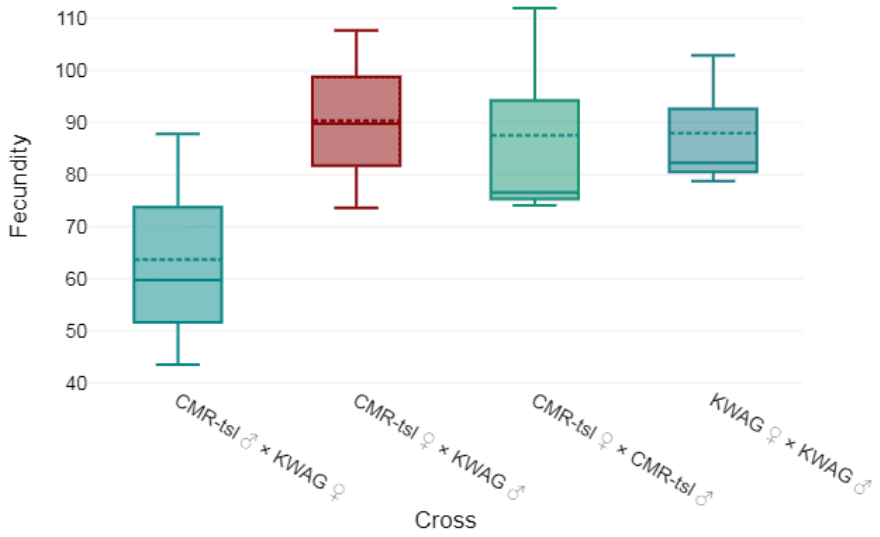


Figure 6.7: Mean number of eggs laid per female (fecundity) from various cross combinations between CMR-*tsl* and KWAG strains.

6.3.2.2. Fertility (egg hatch rate)

The mean egg hatch rates (± SD) of the crosses between CMR-*tsl* and KWAG were as follows: cross 1: CMR-*tsl* ♂ × KWAG ♀ = 73.23 % ± 3.41, cross 2: CMR-*tsl* ♀ × KWAG ♂ = 76.50 % ± 5.40, cross 3: CMR-*tsl* ♀ × CMR-*tsl* ♂ = 77.37 % ± 2.89 and cross 4: KWAG ♀ × KWAG ♂ = 78.67 % ± 11.06 (Figure 6.8). Statistical interpretation showed no significant difference in egg hatch rates between the crosses (one-way ANOVA: df = 3, F = 0.38, P = 0.773).

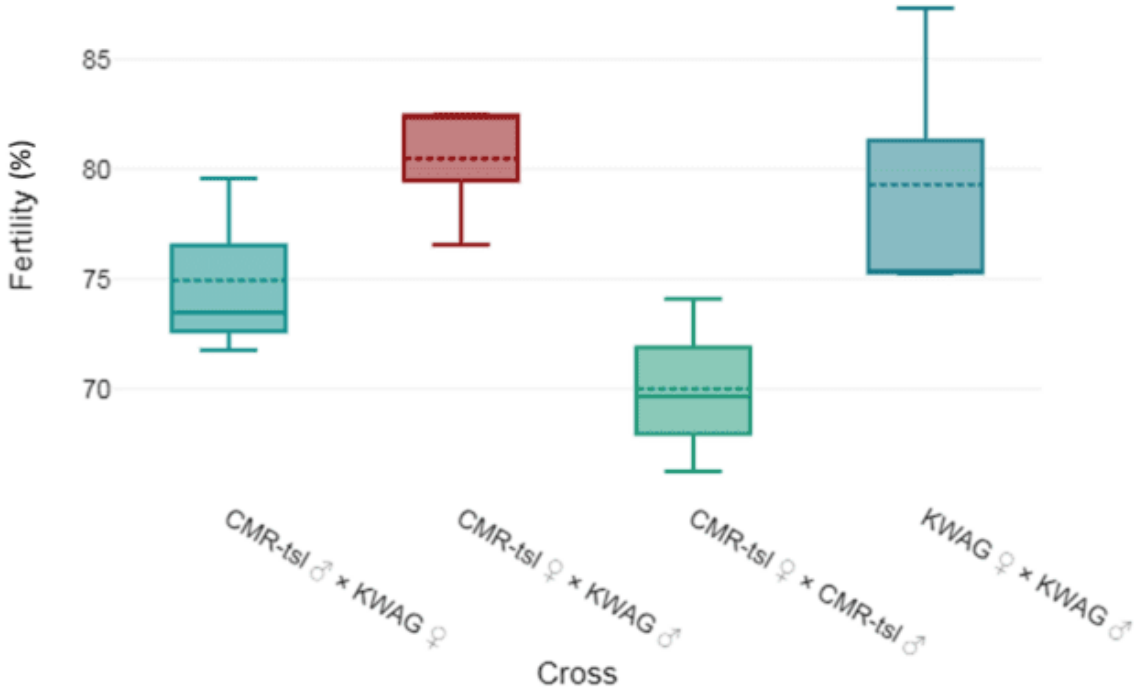


Figure 6.8: Mean fertility (egg hatch rates) of the crosses between CMR-*tsl* and KWAG strain**.**

6.3.2.3. Sterility of F_1 hybrid males.

The sterility of F_1 males (± SD) resulting from cross 1 (i.e., CMR-*tsl* ♂ × KWAG ♀) was categorised infertile based on the fact that no mature spermatozoa were observed in 90 % ± 2.3 of the 30 reproductive tracts dissected. The rest were damaged or unclear under a microscope and thus regarded as undetermined. In F_1 males resulting from cross 2 (CMR-*tsl* ♀ × KWAG ♂), mature spermatozoa were found in 86.7 % ± 3.1 of the reproductive tracts. These were thus regarded as fertile. Similarly, normal reproductive tract morphology and the presence of mature spermatozoa (i.e., fertile) was observed in F_1 males resulting from the two controls (i.e., cross 3: (96.7 % ± 2.9) and cross 4: (90 % ± 2.3).

6.3.2.4. Species PCR results of F_1 offspring

Results from cross 1 F_1 males exhibited an *An. arabiensis* background (single band of 315 bp on gel) while F_1 males from cross 2 showed an *An. gambiae s.l.* background (single band of 390 bp) (from both cross 1 and cross 2 expressed double bands on gel image (both 315 bp and 390 bp, suggesting they are hybrids). In the two controls (cross 3 and cross 4), both F_1 male and female adults exhibited

expected pure breed results (i.e., single band of 390 bp (*An. gambiae s.l.*) for cross 3 and single band of 315 bp (*An. arabiensis*) for cross 4) (Figure 6.9).

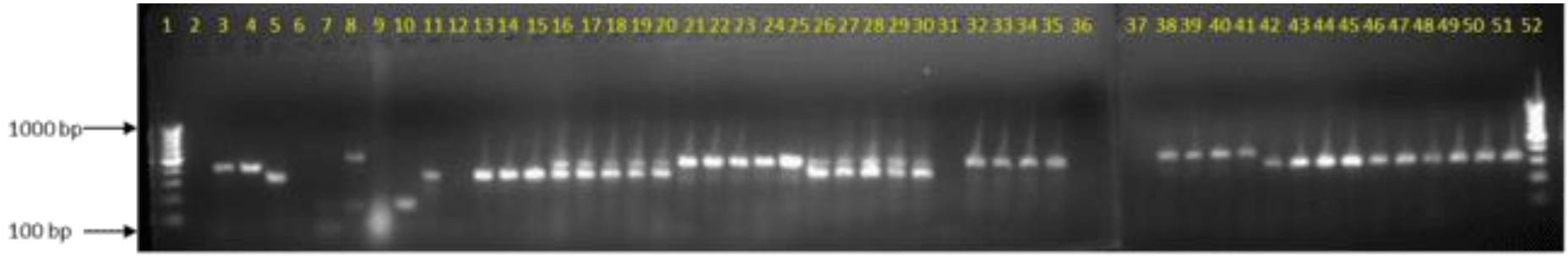


Figure 6.9: Species-specific PCR results of F_1 progeny resulting from crosses between CMR-*tsl* × KWAG. Lane 1 and 52: 1 kilobase molecular weight marker; lane 2: negative control; lane 3 and 4: *An. gambiae s.l.* positive control; lane 5: *An. arabiensis* positive control; lanes 6, 7, 9, 12, 31, 36 and 37: no amplicon; lane 8: *An. merus* positive control; lane 10: *An. quadrannulatus* positive control; lanes 11, 13 – 15: *An. arabiensis* (cross 1 F_1 ♂); lanes 16 – 20: hybrid (cross 1 F_1 ♀); lanes 21 – 25: *An. gambiae s.l.* (cross 2 F_1 ♂); lanes 26 – 30: hybrid (cross 2 F_1 ♀); lanes 32 – 35: *An. gambiae s.l.* (cross 3 F_1 both ♂ and ♀); lanes 38 – 41: *An. gambiae s.l.* (cross 3 F_1 both ♂ and ♀); lanes 42 – 51: *An. arabiensis* (cross 4 F_1 both ♂ and ♀). *note that two gels were merged in order to show the all the results in one plane (space between lane 36 and 37 show where the gels were merged).

6.3.3. Development of a temperature sensitive GSS through an irradiation-induced reciprocal Y-autosome translocation.

One hundred and twenty iso-families resulting from the two BCPs (60 from BCP1 and 60 from BCP2) were screened for thermosensitivity. Of these, 35 iso-families from BCP1 laid eggs that hatched *vs* 43 iso-families from BCP2. Following temperature screening of the L1 from each iso-families whose eggs hatched, only eight families from BCP1 showed L1 mortality of 100 % after heat shock of half of the progeny. The other half of non-heat shocked male adults from these eight families were each backcrossed with virgin parental CMR-*tsl* females. Unfortunately, none of these backcrosses showed 100 % thermosensitivity and no complete sex bias towards males (Table 6.1).

Table 6.1: L1 mortality after heat shock and number of adults per sex of offspring from BCP1 F_1 males crossed with CMR-*tsl* females.

Iso-family	Iso-family no.	Number of eggs laid	Number of L1 hatched	Total L1 died 24h after heat shock	Mortality (%) after heat shock	no. adults emerged	no. adult males	no. adult females	Decision*
BCP1 F_1 ♂ × CMR-*tsl* ♀	1	180	138	91	66	37	25	12	Discarded
BCP1 F_1 ♂ × CMR-*tsl* ♀	2	251	140	39	28	20	12	8	Discarded
BCP1 F_1 ♂ × CMR-*tsl* ♀	3	165	148	112	76	54	32	22	Discarded
BCP1 F_1 ♂ × CMR-*tsl* ♀	4	203	139	88	63	29	16	13	Discarded
BCP1 F_1 ♂ × CMR-*tsl* ♀	5	170	128	71	55	47	22	25	Discarded
BCP1 F_1 ♂ × CMR-*tsl* ♀	6	319	227	129	57	31	12	19	Discarded
BCP1 F_1 ♂ × CMR-*tsl* ♀	7	264	125	101	81	39	20	19	Discarded
BCP1 F_1 ♂ × CMR-*tsl* ♀	8	112	69	53	77	33	16	17	Discarded

*Iso-family selected or discarded if L1 mortality or sex ratio post heat shock = 100 % in favour of males**.**

6.4. Discussion

The aim of this study was to use the already established Cameroon temperature-sensitive strain to develop a *tsl*-based GSS with a South African genetic background. However, despite significant efforts, these yielded unsuccessful results. Several reasons are suspected to have contributed to this failure as discussed below.

The initial suspected cause is the discrepancy in species identification. This stems from the fact that the CMR-*tsl* strain was initially classified as *An. arabiensis* by Ndo *et al*. (2018) based on short interspersed repeated DNA sequences (SINE) diagnostic assays (Santolamazza *et al*., 2008). However, upon arrival at the BDMI, the CMR-*tsl* strain was identified as *An. gambiae s.l.*, specifically *An. coluzzii* using the rDNA PCR method outlined by Scott *et al*. (1993). Both the rDNA and SINE PCR methods rely on detecting X chromosome intergenic spacers (IGS) (Scott *et al*., 1993; Santolamazza *et al*., 2008). These methods frequently yield associated results because IGS are closely situated in a region of the X chromosome with low recombination rates. In light of the discrepancy in species identification results for the CMR-*tsl* strain, it is hypothesised that other factors, such as pre-zygotic or post-zygotic mating barriers, or both, may have been breached, facilitating the crossover of X chromosome markers crucial for species discrimination, as noted by Touré *et al*. (1998), Tripet *et al*. (2001) as well as Lanzaro and Tripet, (2003). Pre-zygotic mating barriers encompass various mechanisms preventing fertilisation, such as mating seasons, habitat isolation, mechanical hindrances, gamete incompatibility, and behavioural distinctions. Post-zygotic mating barriers pertain to situations where homogametic hybrid females (XX) remain fertile while heterogametic hybrid males (XY) become sterile, as elucidated by Haldane (1922) and Schilthuizen *et al*. (2011). Previous studies have indicated that hybrids of *An. gambiae s.l.* and *An. arabiensis* occur infrequently (0.02% – 0.76%) in regions where these species coexist (Temu *et al*., 1997; Toure *et al*., 1998; Weetman *et al*., 2014). Nonetheless, it is generally accepted that gene flow or introgression between species can transpire, including instances of multiple inseminations in field-collected *An. gambiae s.l.* females, as revealed by DNA analysis of transferred sperm (Yuval and Fritz, 1994; Tripet *et al*., 2001, 2005), resulting in morphologically indistinguishable offspring. With regards to the CMR-*tsl* strain, it is important to note that the original species identification was conducted on a parental female, which, even though molecularly identified as *An. arabiensis*, may have circumvented pre-zygotic barriers (e.g., carrying fertile sperm from a male *An. coluzzii*) or post-zygotic barriers (e.g.,

bypassing Haldane's rule by producing eligible progeny, as reported in other studies (Della Torre *et al*., 1997), although this was not confirmed in this study. Consequently, progeny from such parental females could have given rise to viable hybrid offspring which if mated with a sibling species can propagate the line. Given that, to the best of our knowledge, no further species PCR identification was conducted beyond the field-collected parental females, the detection of hybrids would have been impossible. To validate these hypotheses, additional studies can be undertaken in the original mosquito collection area to explore the extent of species mixing in sympatric regions and the mating specificity prevailing within the swarms. Although these investigations fall outside this study's objectives, their outcomes could enhance our comprehension of the mechanisms and importance of both pre- and post-zygotic barriers among species within the *An. gambiae* complex.

Another alternative explanation for the species PCR results is that contamination (mix up of biological material such as eggs) occurred in the insectary either prior to the CMR-*tsl* strain's arrival at the Cameroon lab or upon arrival at BDMI, where strains were housed in the same room as other strains. This is because the strain exchange occurred during the height of the COVID-19 pandemic, when laboratory staffing, courier services, and other resources were limited.

The temperature sensitivity trait of the CMR-*tsl* strain was confirmed by subjecting L1 to a temperature of 41 °C for 3 h, as previously reported (Ndo *et al*., 2018). Thus, although the CMR-*tsl* used in this study was identified as *An. coluzzi* rather than *An. arabiensis*, we proceeded to investigate the potential introgression of genetic material from the Cameroonian strain into a local South African strain through mating crosses. The data from the parental mating crosses revealed general variability in fecundity, fertility, sterility, and species-specific identity depending on the specific mating combinations and sexes involved. Surprisingly, there were no significant differences in fecundity and fertility observed between the parental crosses. This finding was unexpected, given that mating crosses can potentially lead to gene flow or chromosomal translocations, which in other insects have been known to negatively impact fertility due to meiotic segregation, resulting in both genetically balanced and unbalanced individuals (Slotman *et al*., 2004. 2005; Lee *et al*., 2013). Unbalanced individuals are typically unviable and perish as embryos (Deitz, 2017). However, some level of introgression-induced sterility was observed. The results indicated that hybrid F_1 males from

cross 1 were infertile, whereas F_1 males from cross 2 were fertile. This was confirmed when these F_1 hybrids were subjected to inbreeding, resulting in no offspring. This suggests that while there was no direct post-F_1 gene flow in cross 1, gene flow continued in cross 2. It is this phenomenon that further raises the hypothesis that Haldane's rule may have been violated, allowing gene flow between species through an unidentified pathway (Okerere, 1980). Furthermore, the observed sterility implies that some form of introgression or fitness cost was triggered by cross-mating of these sibling species (Slotman *et al*., 2004. 2005; Lee *et al*., 2013). PCR-based species-specific detection or identification of F_1 hybrids clearly demonstrated the presence of introgressive hybridisation between *An. coluzzii* and *An. arabiensis*. This was supported by the fact that only F_1 males from cross 1 had an *An. arabiensis* background, while F_1 males from cross 2 had an *An. gambiae s.l.* (*An. coluzzii*) background. In both cross 3 and cross 4 (control groups), male and female adults exhibited the expected results, i.e., *An. gambiae s.l.* (*An. coluzzii*) for cross 3 and *An. arabiensis* for cross 4. Given the initial species identification indicating that the CMR-*tsl* strain was *An. gambiae s.l.*, specifically *An. coluzzii*, it is suspected that these results occurred because the nucleotide sequences in the ribosomal DNA intergenic spacers are inherited from parental females, thus only being expressed in F_1 females, as described by Scott *et al*. (1993). These results further suggest that the prevalence of natural hybrids and their detection after the parental female stage may be underestimated (Vicente *et al*., 2017). Ultimately, to confirm hybrids and avoid potential issues with specificity, Sanger sequencing of single-plex species-specific IGS PCR products for representative hybrid samples followed by subsequent sequencing and phylogenetic analysis of other gene fragments can be employed, as outlined by Scott *et al*. (1993), Temu *et al*. (1997) and Santolamazza *et al*. (2008). However, it is worth noting that implementing this approach on a day-to-day basis may present practical challenges such as shortage of laboratory workers or expertise, although it is essential for accurate hybrid detection and understanding the dynamics of introgression.

Subsequent backcrosses aimed at utilising the temperature sensitivity phenotype as a selectable marker for developing a GSS by linking the wild-type allele to the Y chromosome through irradiation-induced reciprocal Y-autosome translocation were performed. The objective was to create a unique mosquito strain with distinctive genetic traits whereby males are capable of surviving at certain temperatures while females are selectively eliminated under the same conditions (i.e., link the wild-type allele to the Y chromosome, resulting in

males with temperature resistance and females with temperature sensitivity). However, because cross 1 yielded no offspring, subsequent backcrosses employed females from cross 2, mated with irradiated males from cross 4 (referred to as BCP1) and unirradiated males from cross 4 (referred to as BCP2). Despite thorough screening for families exhibiting the temperature sensitivity phenotype, none were identified in the screened BCPs. it is suspected that the potential hybridisation events or selective forces favouring *tsl* fixation (such as ecological factors or the original temperature sensitivity properties of the strains) may have played a role. Hybridisation can facilitate the transfer of mutations between species, as observed in studies by Hedrick *et al*. (2013). Additionally, it is hypothesised that due to the absence of a morphological marker to trace *tsl*, recombination events may have contributed to the absence of detectable temperature-sensitive individuals. Thus, it is plausible that the *tsl* allele acquired by the CMR-*tsl* strain lacks fixation or occurs at a low frequency due to a potentially limited adaptive advantage in the recipient KWAG strain (Abbott *et al*., 2013).

It is essential to acknowledge that the hypotheses presented here are unverified and would require further research to establish their validity. In future, advanced genome engineering techniques like CRISPR/Cas9 could be explored to develop GSSs through targeted mutagenesis (Chaverra-Rodriguez *et al*., 2018). This approach entails the selection of appropriate candidates from existing temperature-sensitive mutations, the introduction of a corresponding mutation into the genome of the target pest species using CRISPR/Cas9, and then relocating a dominant wild-type allele to the male-specific Y chromosome to rescue males or relocating a dominant mutant allele to the female-specific X chromosome to induce conditional lethality in females (Gamez *et al*., 2020). This includes the creation of transgenic strains, which is another viable alternative (Ntoyi *et al*., 2022).

Furthermore, the research conducted in this study in the pursuit of sex separation strategies is closely aligned with another significant challenge faced by the South African SIT project. This challenge relates to identifying the optimal temperature ranges and conditions for immobilising adult sterile males during laboratory packaging, handling, and transport to release sites (see next chapter 7).

6.5. Conclusion

This study did not successfully develop a GSS based on the temperature-sensitivity in *An. arabiensis*. However, investigating introgression between *An. coluzzii* and *An. arabiensis*, the cross-mating experiments yielded clear evidence of introgressive hybridisation between these closely related sibling species. This finding further advances our understanding of the adaptive and practical implications of genetic introgression among sibling species within the *Anopheles* genus.

CHAPTER 7: Development of optimal handling, packaging and transport conditions of irradiated-sterilised *An. arabiensis* adult male mosquitoes

7.1. Introduction

South Africa has made significant progress in investigating the feasibility of the SIT as a possible complementary control strategy against the primary malaria vector, *An. arabiensis* (Munhenga *et al*., 2011, 2014, 2016; Dandalo *et al*., 2017a, 2017b, 2018; Manana *et al*., 2018, 2021; Mashatola *et al*., 2017; 2018; Kaiser *et al*., 2021). This has placed the technique at a point where small-scale pilot trials are possible. The success of an SIT field trial requires a number of steps such as mass rearing, sex separation, sterilisation, packaging, transportation, release and monitoring (Benedict and Robinson, 2003; Dyck *et al*., 2005, 2021).

While substantial progress has been made in optimising some of these procedures, a critical aspect that remains to be fully addressed is the handling and packaging of mosquitoes during laboratory production and their subsequent transport (Gomez *et al*., 2022; Tussey *et al*., 2023). A key consideration in mosquito handling and packaging is the impact of temperature/chilling (Culbert *et al*., 2017; Chung *et al*., 2018; Iyaloo *et al*., 2020; Zhang *et al*., 2020). Mosquito activity is greatly affected by temperature, with lower temperatures often immobilising them (Culbert *et al*., 2017). Therefore, recommendations have been made to conduct mosquito handling and packaging under chilled conditions, which makes the process more manageable and reduces physical damage to the insects (Culbert *et al*., 2017; Tussey *et al*., 2023). Against this, there is a need to be aware of the critical lethal lower temperature limit, below which the mosquitoes will die when exposed. Additionally, compaction which is employed to efficiently package large quantities of mosquitoes into confined spaces, is especially important when large numbers of mosquitoes are released weekly (Tussey *et al*., 2023).

However, the chilling and compaction processes may have a negative impact on the sterile male mosquitoes intended for release (Calkins and Parker, 2005; Parker *et al*., 2021). These include factors such as reduced survival, impaired mating competence, decreased flight ability, poor quality and altered reproductive fitness of the males (Culbert, 2020). The effects of these procedures on mosquito quality and fitness need thorough investigation to ensure that they do not compromise the effectiveness of the SIT strategy.

This chapter aimed to establish an optimal temperature range that can be used to chill/immobilise adult sterile males during handling in the laboratory. Furthermore, the study aimed to determine the optimal packaging/compaction conditions that can be used to transport adult sterile males.

7.2. Methods

7.2.1. Mosquito strains and maintenance

The dieldrin based GSS, acronymed GMK was used in this study. The strain was developed through a series of cross-mating of various *An. arabiensis* laboratory strains (Dandalo *et al*., 2018). This included cross-mating of the Sudanese *An. arabiensis* GSS ANO IPCL1 males (carrying the resistance to dieldrin (*rdl*) gene on the Y-chromosome) (Yamada *et al*., 2012) with the South African *An. arabiensis* females collected from Malahlapanga in the Kruger National park to generate a GSS acronymed GAMA (Munhenga *et al*., 2016). The resultant GAMA offspring were backcrossed with KWAG (*rdl*-susceptible genotype) to produce GMK males carrying dieldrin resistance on the Y-chromosome with a genetic background representing the South African population and housed at the BDMI under standard insectary conditions (Dandalo *et al*., 2018). Biological material was reared following protocols stated in Hunt *et al*. (2005).

GMK third/fourth instar larvae were exposed to 0.1 ppm dieldrin solution for 2 h to kill females and retain males prior to chilling and compaction experiments (Dandalo *et al*., 2018). Male pupae (20 – 22 h old) were transferred to a cobalt-60 gamma irradiation source, and a dose of 75 Gy was administered to pupae (see Munhenga *et al*., (2016) and appendix 4). After irradiation, pupae were placed in corresponding small plastic cups containing 100 mℓ of distilled water. The cups were placed in Bugdorm-1® adult insect rearing cages, and pupae were left to emerge overnight. Successfully emerged adult males were used in temperature and compaction tests.

7.2.2. Determining the optimal temperature that can be used to immobilise adult sterile males

7.2.2.1. Determining the time taken for adults to recover (i.e., for all adults to resume flight) following immobilisation at different temperatures

Batches of 30 newly emerged adult sterile male mosquitoes were aspirated into transparent cylindrical plastic cups (5 cm base diameter 12 cm length 8 cm top diameter, West park stores, South Africa) covered with a fabric net (Product ID: 9902056133316, Makro, South Africa) that was secured with an elastic rubber band (SKU: 951282B2, Waltons, South Africa) (Figure 7.1). The plastic cups were placed inside a 25 ℓ climate chamber (Jeio Tech, model: TH3-ME-025, South Korea) set at temperatures of either 0°C, 2°C, 4°C, 6°C, 8°C, 10°C, 12°C or 27 ± 1°C (control) for 20 minutes (reasonable time required to efficiently knock down mosquitoes operationally or practically (Culbert *et al*., 2017; Culbert, 2020)). The relative humidity was set constantly at 70% ± 10%. In each case, the time until all adults were knocked out were recorded. After 20 minutes of exposure, the plastic cups with adult male mosquitoes were removed from the climatic chamber and introduced into a Bugdorm-4® adult insect holding cage placed under standard insectary rearing conditions. The time taken for adults to recover (i.e., for all adults to resume flight) was recorded.

Figure 7.1: Equipment used for determining the optimal temperature that can be used to immobilise adult sterile males: A 25 ℓ climate chamber (Jeio Tech, model: TH3-ME-025, South Korea) with a transparent cylindrical plastic cup covered with an elastic band secured fabric net inside which mosquitoes were aspirated and through visual observation knockdown time recorded.

7.2.2.2. Determining the survival of adult sterile males following immobilisation to different temperatures.

For this part, only adults that recovered following immobilisation (7.2.2.1) at temperatures between 4°C and 10°C, as well the control was used to determine how long they can survive after 20 minutes of exposure at different temperatures (2°C, 4°C, 6°C, 8°C, 10°C,) in the climatic chamber. Twenty adults from each temperature were monitored for survival by removing and counting dead adults in each cage daily for 14 consecutive days. Monitoring was limited to 14 days as previously suggested by Culbert *et al*. (2017) who at the time postulated that survival of male adults after release is unlikely to extend beyond this time frame.

7.2.3. Determining the optimal packaging/compaction conditions that can be used to transport adult sterile males

To enhance efficiency, it may be necessary to transport adult male mosquitoes using various containers and packing densities, depending on resource availability and the scale of releases. Consequently, the impact of compaction density and packaging chambers on adult male mosquito mortality and longevity were evaluated in three distinct chambers (as illustrated in Figure 7.2). The first chamber was the cubic Bugdorm-1® cage, with dimensions of 30 cm in length, 30 cm in width, and 30 cm in height, resulting in a volume of 27 000 cm^3. However, this larger Bugdorm-1® cage was included as a control as it had been previously utilised in other research studies. The second chamber utilised was a plastic petri dish (SKU PLAS-HP0005, Lasec, South Africa) with dimensions of 15 mm in height and 90 mm in diameter, providing a volume of 60 cm^3. The third chamber employed was a non-pyrogenic disposable syringe (SKU: RLSMED-SYRCT50, https://labequipsupply.co.za/) measuring 155 mm in height and 27 mm in diameter, offering an equivalent volume of 60 cm^3.

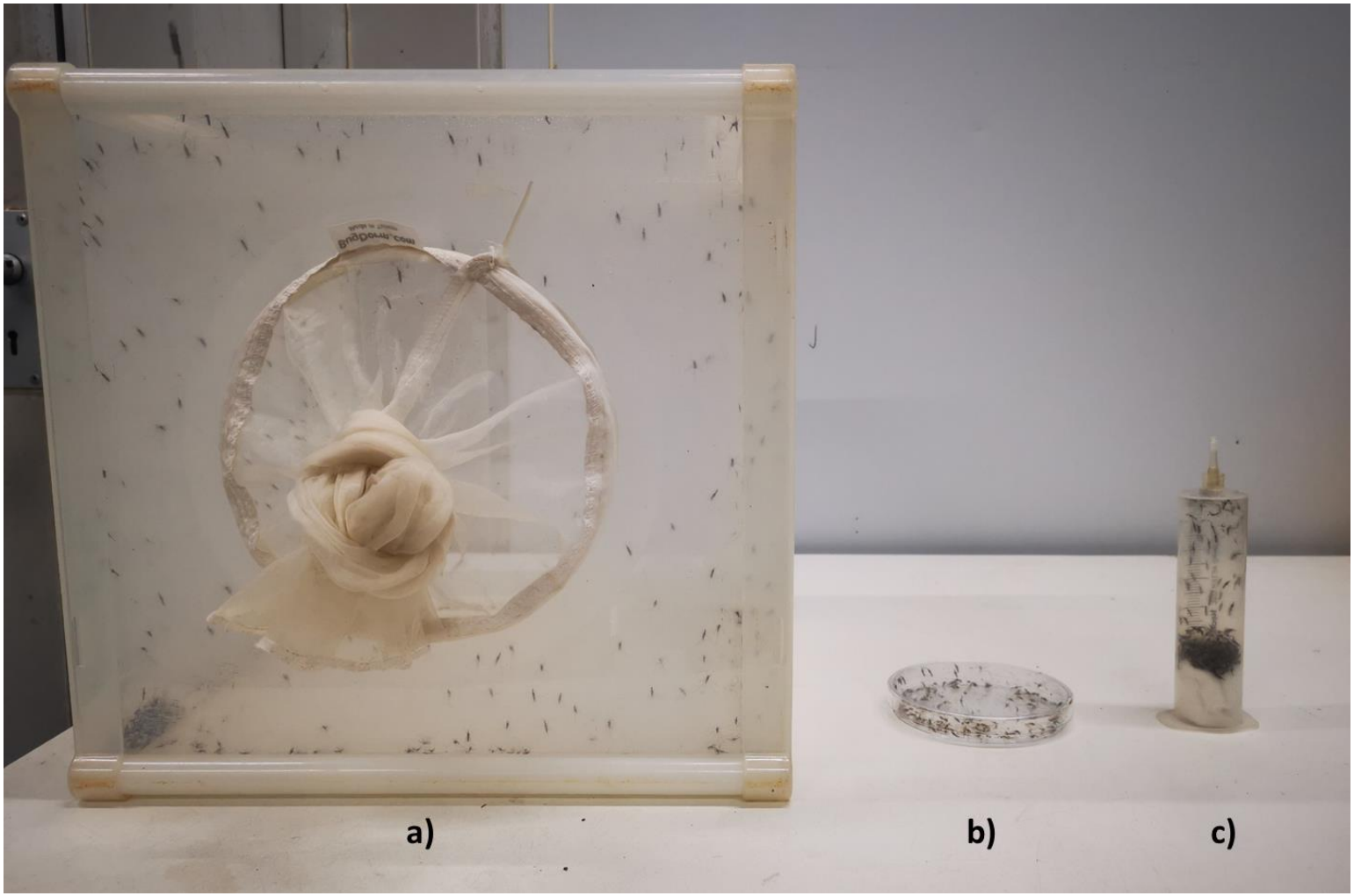


Figure 7.2: Three distinct chambers used in this study. a) Bugdorm-1® cage (30 cm in length, 30 cm in width, and 30 cm in height), b) plastic petri dish (15 mm in height and 90 mm in diameter) and c) non-pyrogenic disposable syringe (155 mm in height and 27 mm in diameter).

The experimental protocol involved rendering adult male mosquitoes held in standard rearing Bugdorm-1® cages immobile in a built in walk-in cold room maintained at temperatures between 4°C and 8°C. This immobility facilitated accurate weight measurements of the adult male mosquitoes using an analytical balance (United Scientific Equipment Pty Ltd, Singapore; model: Kern ABT 120-5DM/Adam PW124). The adult male mosquitoes (held in a Bugdorm-1® cage) were counted as singles and divided into groups of 1000, 2500 and 5000. This was done to estimate the number of adults by weight, other than by counting individually as weighing is quicker for operational purposes. Subsequently, each group of 1000, 2500, and 5000 adult males were introduced into their own compaction chamber. The average weight of the adult males per group (± SD) was as follows: 12.72 g ± 0.42 for 1000 adults, 31.81 g ± 0.56 for 2500 adults, and 63.81 g ± 0.74 for 5000 adults. These varying weights led to distinct densities per chamber: 212.03 males/cm³ for 1000 adult males, 1325.21 males/cm³ for 2500 adult males, and 5317.50 males/cm³ for 5000 adult males, both in the petri dish and syringe (Table 7.1). In contrast, the Bugdorm-1® cage exhibited densities of 0.037 males/cm³ for 1000 adult males, 0.093 males/cm³ for 2500 adult males, and 0.185 males/cm³ for 5000 adult males, reflecting the significantly larger volume of the cage (Table 7.1).

Table 7.1: Measurements of densities per chamber.

Chamber	Density (males/cm³) - 1000 adult males	Density (males/cm³) - 2500 adult males	Density (males/cm³) - 5000 adult males
Petri dish	212.03	1325.21	5317.5
Syringe	212.03	1325.21	5317.5
Bugdorm-1® cage	0.037	0.093	0.185

Subsequently, the chambers containing the adult males were left undisturbed within the cold room set at 4°C for a duration of 8 h (chilled), mirroring the time required for transportation from the mass rearing facility to the field release site. Following the 8 h exposure to cold temperatures, the adult males from each chamber were respectively transferred to new Bugdorm-1® cages. These cages were then placed under standard insectary conditions of 27 ± 1°C room temperature, 70 ± 10% relative humidity, and a 12:12 h photoperiod, including 45-minute transitions of dusk and dawn each cycle. After 24 h, the number of deceased or

moribund males, characterised by their inability to fly were removed from the Bugdorm-1® cages and meticulously counted. Additionally, a subsample of 100 live adult males were randomly selected from each chamber and introduced into new Bugdorm-1® cages and kept under standard insectary conditions. These individuals were subjected to continuous monitoring by counting and recording the number of dead adults daily until all died. This was done to assess their longevity. The experiments were replicated three times, with each replication utilising a new distinct cohort of mosquitoes of the same age.

7.2.4. Data analysis

Data and statistical analyses were conducted using the IBM SPSS software (IBM Corp, 2015). One-way ANOVA and Bonferroni post-hoc test was performed to compare the mean time to complete knockout and mean time to complete recovery of adult GMK sterile males after confirming the normal distribution of the data using the Shapiro-Wilk test. The longevity after immobilisation and after compaction was compared using the Log-rank (Mantel-Cox) test with the Kaplan–Meier method used to generate the survival curves. Compaction mortality after 24 h was compared between compaction chamber and number of adults per chamber using two-way ANOVA and Bonferroni post-hoc test. In all cases, a p-value of less than 0.05 was used to indicate significance at 95% CI.

7.3. Results

7.3.1. Determining the optimal temperature that can be used to immobilise adult sterile males

7.3.1.1. Determining the time taken for adults to recover (i.e., for all adults to resume flight) following immobilisation at different temperatures

Complete knock out (immobilisation) of adult males was observed within the 20 minutes' exposure. The time until all adults were knocked down varied across temperatures, with mean durations (min ± SE) ranging from 0.90 ± 0.28 at 0°C, 2.57 ± 0.35 minutes at 2°C, 2.44 ± 0.74 minutes at 4°C, 3.28 ± 0.54 minutes at 6°C, 5.13 ± 0.86 minutes at 8°C, and 5.74 ± 0.18 minutes at 10°C (Figure 7.3). No mosquitoes were knocked down from 12°C and 27 ± 1°C within the 20 minutes' exposure. Statistical comparison showed a difference in knock down time across temperatures (one-way ANOVA: $df = 5$, $F = 25.71$, $p < 0.05$).

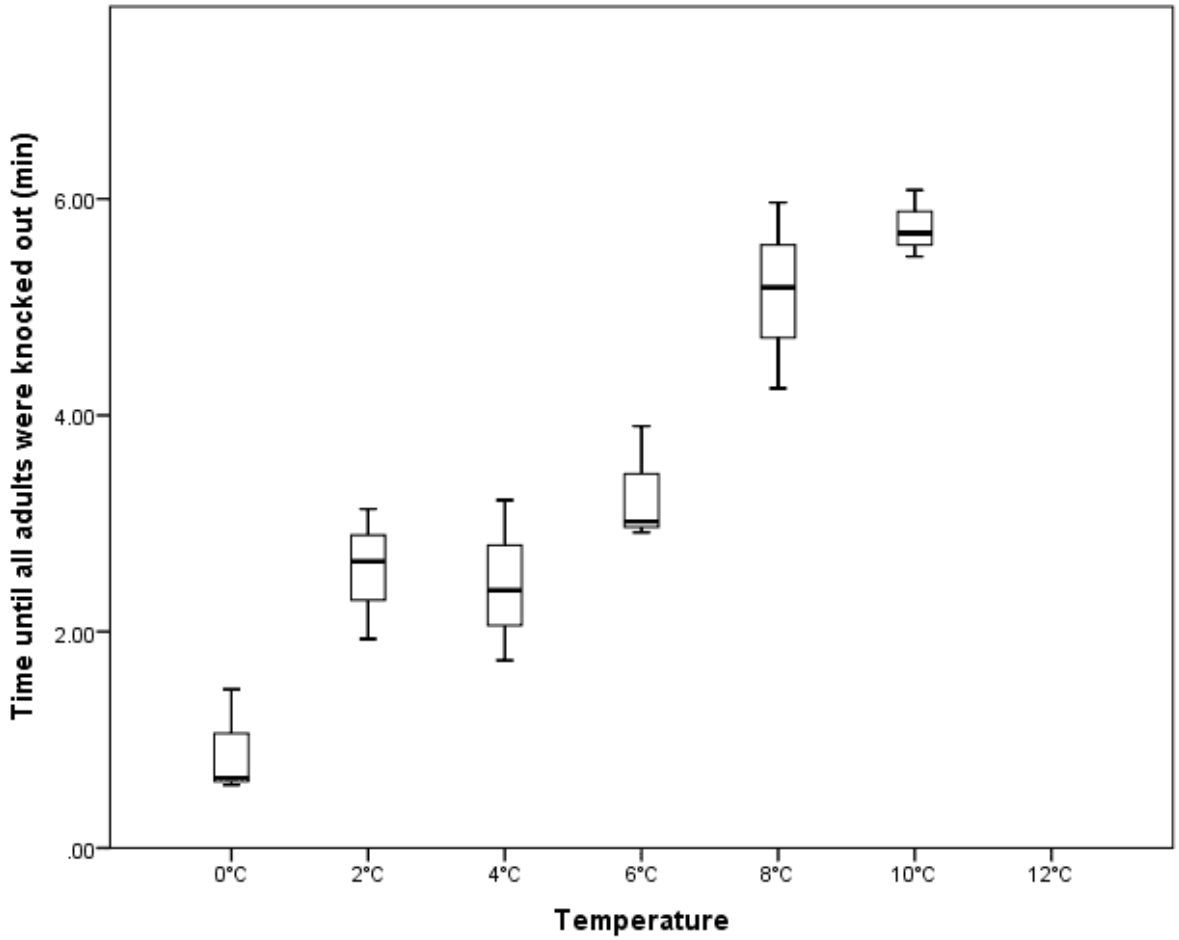


Figure 7.3: Mean (± SE) times (minutes) it took adult males to be knocked out (immobilisation) following their exposure to different temperatures (range 0°C – 12°C) for 20 minutes.

Subsequent multiple comparisons analysis using the Bonferroni method to compare the mean differences between various temperature conditions showed that the mean time until all adults were knocked out at 0°C was similar to 2°C (Table 7.2). When comparing 0°C to higher temperatures (4°C, 6°C, 8°C, 10°C), there were significant differences, indicating that as the temperature increased, the time until knockout significantly decreased. Similarly, comparisons between 2°C and higher temperatures showed significant differences, except for the comparison with 4°C.

Table 7.2: Multiple comparisons analysis using the Bonferroni method to compare the mean differences between various temperature conditions.

(I) Temperature	(J) Temperature	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound

0°C	2°C	-1.67222	.50428	.092	-3.5123	.1678
	4°C	-1.54444	.50428	.148	-3.3845	.2956
	6°C	-2.37778*	.50428	.008	-4.2178	-.5377
	8°C	-4.23333*	.50428	.000	-6.0734	-2.3933
	10°C	-4.84444*	.50428	.000	-6.6845	-3.0044
2°C	0°C	1.67222	.50428	.092	-.1678	3.5123
	4°C	.12778	.50428	1.000	-1.7123	1.9678
	6°C	-.70556	.50428	1.000	-2.5456	1.1345
	8°C	-2.56111*	.50428	.004	-4.4012	-.7210
	10°C	-3.17222*	.50428	.001	-5.0123	-1.3322
4°C	0°C	1.54444	.50428	.148	-.2956	3.3845
	2°C	-.12778	.50428	1.000	-1.9678	1.7123
	6°C	-.83333	.50428	1.000	-2.6734	1.0067
	8°C	-2.68889*	.50428	.003	-4.5290	-.8488
	10°C	-3.30000*	.50428	.000	-5.1401	-1.4599
6°C	0°C	2.37778*	.50428	.008	.5377	4.2178
	2°C	.70556	.50428	1.000	-1.1345	2.5456
	4°C	.83333	.50428	1.000	-1.0067	2.6734
	8°C	-1.85556*	.50428	.047	-3.6956	-.0155
	10°C	-2.46667*	.50428	.006	-4.3067	-.6266
8°C	0°C	4.23333*	.50428	.000	2.3933	6.0734
	2°C	2.56111*	.50428	.004	.7210	4.4012
	4°C	2.68889*	.50428	.003	.8488	4.5290
	6°C	1.85556*	.50428	.047	.0155	3.6956
	10°C	-.61111	.50428	1.000	-2.4512	1.2290
10°C	0°C	4.84444*	.50428	.000	3.0044	6.6845
	2°C	3.17222*	.50428	.001	1.3322	5.0123
	4°C	3.30000*	.50428	.000	1.4599	5.1401
	6°C	2.46667*	.50428	.006	.6266	4.3067
	8°C	.61111	.50428	1.000	-1.2290	2.4512

* The mean difference is significant at the 0.05 level.

Recovery (flight resumption) of adult males post exposure was observed from all temperatures (Figure 7.4). However, the average times it took adult males to recover also differed depending on temperature (one-way ANOVA: df = 6, F = 22.93, $p < 0.05$).

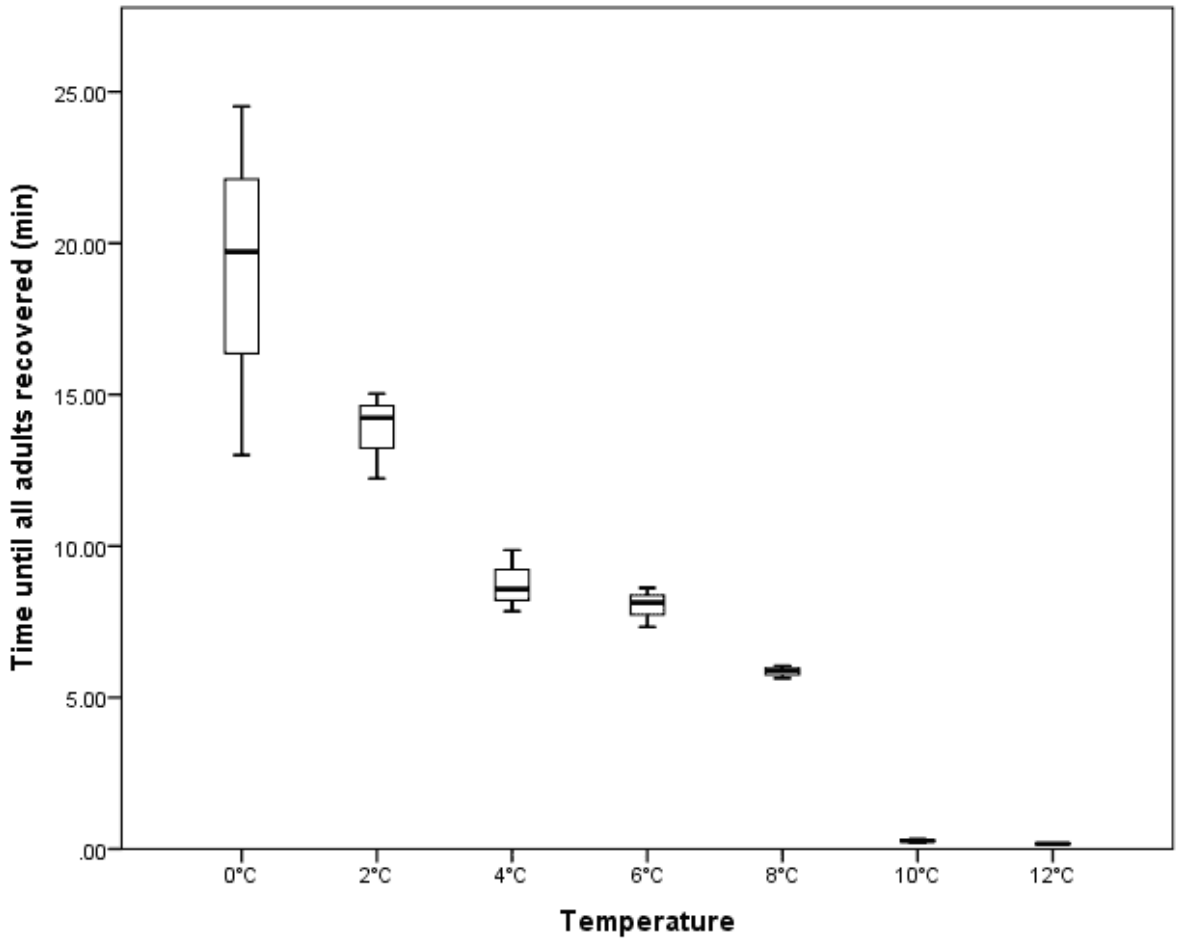


Figure 7.4: Mean (± SE) times (minutes) to adult males' recovery (resume flight) following exposure to different temperatures (range 0°C – 12°C) for 20 minutes.

Multiple comparisons analysis for the time until all adults recovered (in minutes) at different temperatures using the Bonferroni method showed that at 0°C, there was no significant difference in mean recovery time compared to 2°C, with a mean difference of approximately 5.24 minutes (Table 7.3). However, when comparing 0°C to higher temperatures (4°C, 6°C, 8°C, 10°C, 12°C), significant differences were observed, indicating that as the temperature increased, the recovery time significantly decreased. Similarly, comparisons between 2°C and higher temperatures showed significant differences except for the comparison with 4°C. At

4°C, the mean recovery time was significantly different from 10°C and 12°C. At 6°C, the mean recovery time was significantly different from all higher temperatures (8°C, 10°C, 12°C). At 8°C, the mean recovery time was significantly different from 12°C. At 10°C, the mean recovery time was significantly different from 12°C. Overall, as the temperature increased, the time until all adults recovered significantly decreased.

Table 7.3: Multiple comparisons analysis of the recovery times for adults at varying temperatures.

(I) Temperature	(J) Temperature	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
0°C	2°C	5.24444	1.94947	.389	-2.0859	12.5748
	4°C	10.31111*	1.94947	.003	2.9808	17.6414
	6°C	11.05000*	1.94947	.002	3.7197	18.3803
	8°C	13.22222*	1.94947	.000	5.8919	20.5525
	10°C	18.80556*	1.94947	.000	11.4752	26.1359
	12°C	18.90278*	2.17957	.000	10.7072	27.0983
2°C	0°C	-5.24444	1.94947	.389	-12.5748	2.0859
	4°C	5.06667	1.94947	.463	-2.2637	12.3970
	6°C	5.80556	1.94947	.224	-1.5248	13.1359
	8°C	7.97778*	1.94947	.027	.6475	15.3081
	10°C	13.56111*	1.94947	.000	6.2308	20.8914
	12°C	13.65833*	2.17957	.001	5.4628	21.8539
4°C	0°C	-10.31111*	1.94947	.003	-17.6414	-2.9808
	2°C	-5.06667	1.94947	.463	-12.3970	2.2637
	6°C	.73889	1.94947	1.000	-6.5914	8.0692
	8°C	2.91111	1.94947	1.000	-4.4192	10.2414
	10°C	8.49444*	1.94947	.016	1.1641	15.8248
	12°C	8.59167*	2.17957	.035	.3961	16.7872
6°C	0°C	-11.05000*	1.94947	.002	-18.3803	-3.7197
	2°C	-5.80556	1.94947	.224	-13.1359	1.5248
	4°C	-.73889	1.94947	1.000	-8.0692	6.5914

	8°C	2.17222	1.94947	1.000	-5.1581	9.5025
	10°C	7.75556*	1.94947	.033	.4252	15.0859
	12°C	7.85278	2.17957	.067	-.3428	16.0483
8°C	0°C	-13.22222*	1.94947	.000	-20.5525	-5.8919
	2°C	-7.97778*	1.94947	.027	-15.3081	-.6475
	4°C	-2.91111	1.94947	1.000	-10.2414	4.4192
	6°C	-2.17222	1.94947	1.000	-9.5025	5.1581
	10°C	5.58333	1.94947	.279	-1.7470	12.9137
	12°C	5.68056	2.17957	.457	-2.5150	13.8761
10°C	0°C	-18.80556*	1.94947	.000	-26.1359	-11.4752
	2°C	-13.56111*	1.94947	.000	-20.8914	-6.2308
	4°C	-8.49444*	1.94947	.016	-15.8248	-1.1641
	6°C	-7.75556*	1.94947	.033	-15.0859	-.4252
	8°C	-5.58333	1.94947	.279	-12.9137	1.7470
	12°C	.09722	2.17957	1.000	-8.0983	8.2928
12°C	0°C	-18.90278*	2.17957	.000	-27.0983	-10.7072
	2°C	-13.65833*	2.17957	.001	-21.8539	-5.4628
	4°C	-8.59167*	2.17957	.035	-16.7872	-.3961
	6°C	-7.85278	2.17957	.067	-16.0483	.3428
	8°C	-5.68056	2.17957	.457	-13.8761	2.5150
	10°C	-.09722	2.17957	1.000	-8.2928	8.0983

* The mean difference is significant at the 0.05 level.

7.3.1.2. Determining the survival of adult sterile males following immobilisation to different temperatures

Survival of adult sterile males over 14 consecutive days following their 20 minutes' exposure to different temperatures (range 4°C – 10°C) is shown in Figure 7.5. The mean survival of adult sterile males (days ± SE) following 20 minutes' exposure to different temperatures was lower in males exposed to 4°C (10.467 ± 0.550) and highest in 10°C (11.65 ± 0.49). Control (27 ± 1°C) showed similar mean survival as 10°C (11.93 ± 0.49). However, overall statistical comparison showed no difference in survival between the various temperatures over 14 consecutive days (Log Rank (Mantel-Cox): $\chi^2 = 5.85$, df = 4, p = 0.210).

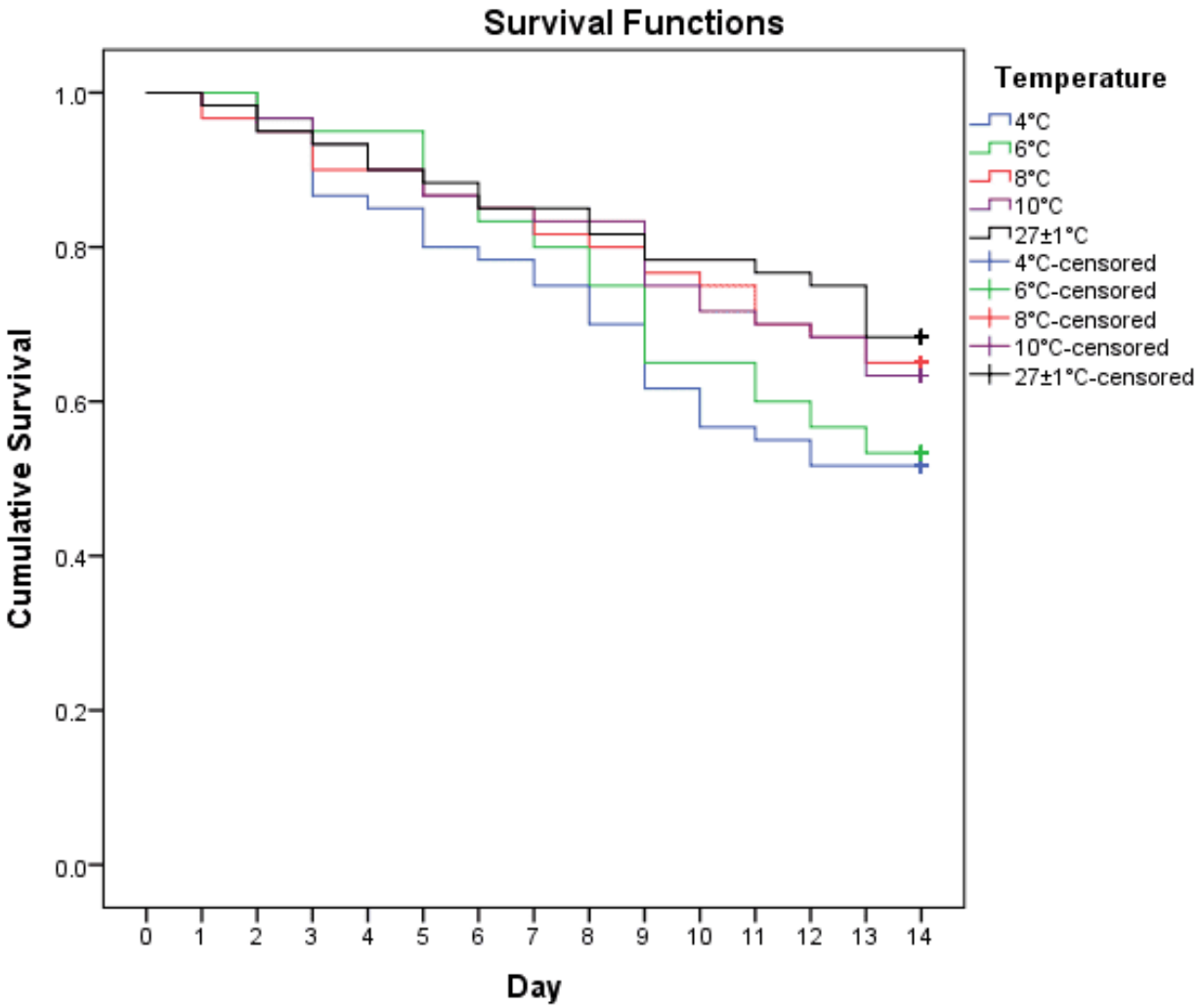


Figure 7.5: Cumulative survival of adult males monitored over 14 consecutive days post 20 minutes exposure to constant temperatures of 4°C to 10°C and corresponding control exposed at (27 ± 1°C).

7.3.2. Determining the optimal packaging/compaction conditions that can be used to transport adult sterile males

The mean mortality (± SD) of adults that died 24 h following compaction in different chambers for 8 h is shown in Table 7.4. In the petri dish chamber, the mean mortality after 24 h ± SD per 1000 adult males was 16.97% ± 4.95 at a density of 212.03 adult males/cm³, 19.40% ± 5.08 with 2500 adult males at a density of 1325.21males/cm³, and 36.67% ± 6.28 with 5000 adult males at a density of 5317.5 males/cm³. Conversely, in the syringe chamber, under equivalent conditions as the petri dish, the mean mortality ± SD were 16.07% ± 3.71, 29.55% ± 3.35, and 44.45% ± 4.33 respectively. In the Bugdorm-1® chamber, the mean mortality ± SD were notably lower, with 6.47% ± 1.80 for 1000 adult males at a density of 0.037 males/cm³, 12.19% ± 3.39 for 2500 adult males at a density of 0.093 males/cm³, and 18.05% ± 3.42 for 5000 adult males at a density of 0.185 males/cm³.

Table 7.4: Mean mortality of adult sterile males 24 h after being compacted in different chambers for 8 h.

Compaction chamber	Number of adult males per chamber (Density)	Mean mortality (%) ± SD
Petri dish	1000 (212.03 males/cm³)	16.97 ± 4.95
Petri dish	2500 (1325.21 males/cm³)	19.40 ± 5.08
Petri dish	5000 (5317.5 males/cm³)	36.67 ± 6.28
Syringe	1000 (212.03 males/cm³)	16.07 ± 3.71
Syringe	2500 (1325.21 males/cm³)	29.55 ± 3.35
Syringe	5000 (5317.5 males/cm³)	44.45 ± 4.33
Bugdorm-1®	1000 (0.037 males/cm³)	6.47 ± 1.80
Bugdorm-1®	2500 (0.093 males/cm³)	12.19 ± 3.39
Bugdorm-1®	5000 (0.185 males/cm³)	18.05 ± 3.42

SD represents standard deviation.

Statistical analysis using a two-way ANOVA to assess which groups have significantly different means in terms of mortality after 24 h showed a significant effect on adult mortality from both compaction chamber ($F_{(2, 4)} = 100.35$, $p < 0.05$) and number of adult mosquitoes per chamber ($F_{(2, 8)} = 96.39$, $p < 0.05$) but not their interaction (two-way ANOVA: $F_{(2, 8)} = 2.39$, $p > 0.05$) (Table 7.5). Extensive pairwise comparisons using the Bonferroni method have revealed noteworthy differences among various combinations of compaction chamber types and number of adults per chamber (density). The key findings included statistically significant differences in mortality between Bugdorm: 1000 and syringe: 2500 (p = 0.0053), Bugdorm: 1000 and petri dish: 5000 (p = 0.0159), Bugdorm: 1000 and syringe: 5000 (p = 0.0247), petri dish: 1000 and syringe: 2500 (p = 0.0091), syringe: 2500 and Bugdorm: 5000 (p = 0.0097), and syringe: 2500 and petri dish: 5000 (p = 0.0293).

Table 7.5: Two-way ANOVA showing the effects of compaction chamber, number of adults per chamber and their interactions on mortality of adult sterile males 24 h following compaction in different chambers for 8 h.

Source	**SS**	**df**	**MS**	**F**	**p**
Corrected Model	25,696.3	8	3,212.04	385.44	<.001
Intercept	5,778.7	1	5,778.7	693.44	<.001
Compaction chamber	5,279.63	2	2,639.81	316.78	<.005
Number of adults per chamber	9,468.52	2	4,734.26	568.11	<.005
Compaction chamber x Number of adults per chamber	10,948.15	4	2,737.04	328.44	>.005
Error	150	18	8.33		
Total	31,625	27			
Corrected total variation	25,846.3	26			

*SS represents the sum of squares, df represents degrees of freedom, MS represents mean squares, F represents F-statistic, and p indicates statistical significance at $p < 0.05$.

Subsequent investigation into the longevity/survival rate of adult sterile males following their compaction in different chambers at different numbers of adults per chamber showed variation (Figure 7.6). The median survival when the number of adults per chamber was 1000, 2500 and 5000 was 30.0 ± 1.03, 33.0 ± 0.96 and 30.0 ± 0.87 days for Bugdorm-1® cages, 18.0 ± 1.52, 16.0 ± 1.21 and 16.0 ± 0.94 for syringe and 29.0 ± 0.74, 30.0 ± 1.180 and 19.0 ± 0.959 for petri dish respectively. Statistically, when compaction chambers were compared, Bugdorm-1® cages showed the longest longevity, followed by petri dish and syringe, respectively (Log Rank (Mantel-Cox): $\chi^2 = 124.009$, df = 2, $p < 0.05$). In terms of the number of adults per chamber, males stored at 1000 per chamber survived significantly longer than those stored at 2500 and 5000 (Log Rank (Mantel-Cox): $\chi^2 = 515.76$, df = 2, $p < 0.05$). Overall, Bugdorm-1® cages containing 1000 adult males showed the highest longevity.

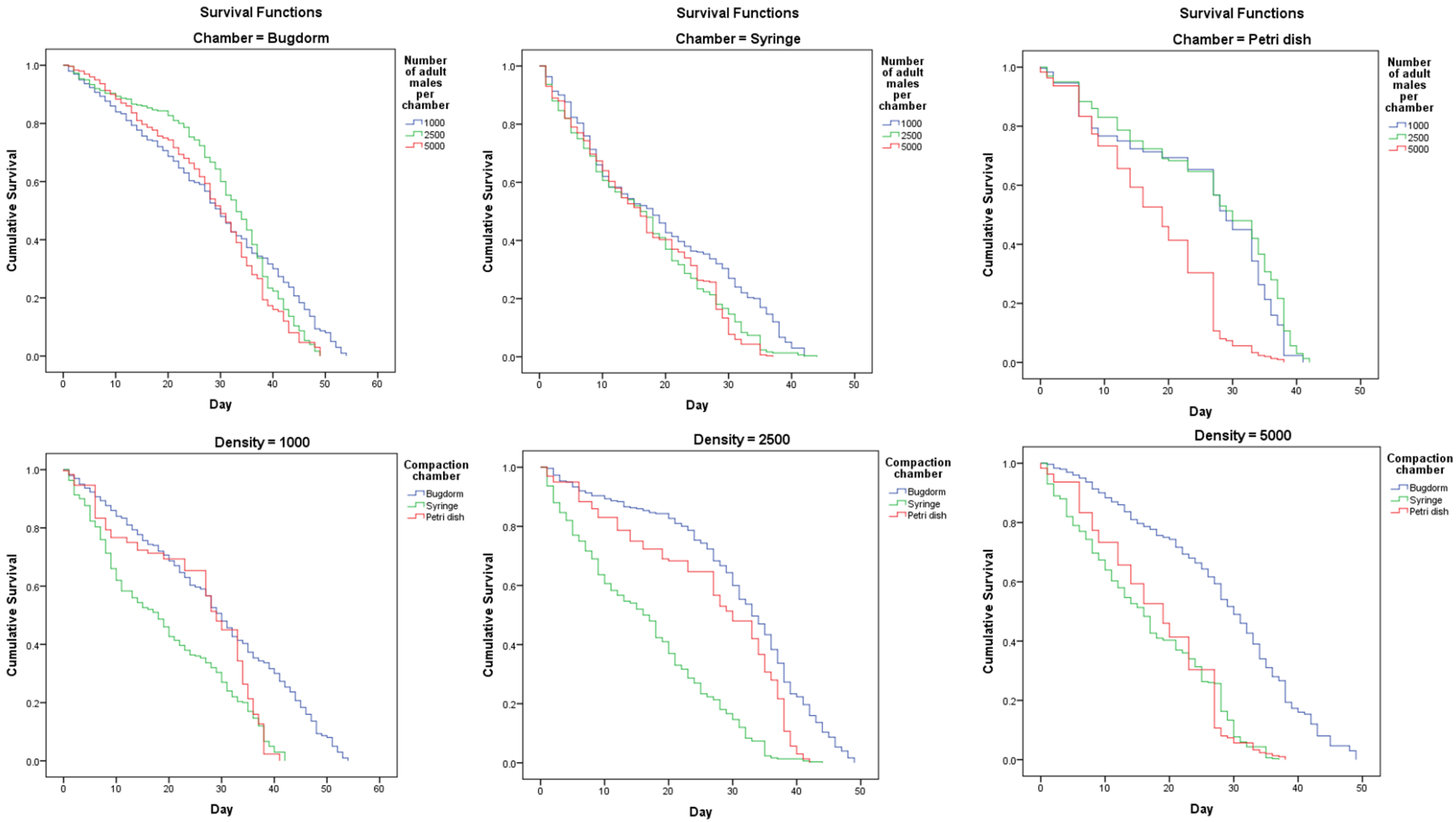


Figure 7.6: Cumulative Survival of adult sterile males following their compaction in different chambers at different numbers of adults per chamber.

7.4. Discussion

The development of SOPs for handling, packaging, and transporting sterile male mosquitoes is of utmost importance to ensure the effectiveness of SIT programmes (Ernawan *et al*., 2022). In line with these principles, the current study aimed to identify the optimal temperature range for immobilising adult sterile males during laboratory handling and to determine optimal packaging and compaction settings that can be used during releases.

Results showed that complete immobilisation occurred within 20 minutes of exposure to temperatures ranging from 0°C to 10°C, with the quickest immobilisation achieved at 0°C (approximately 0.90 minutes). Additionally, temperatures between 4°C to 8°C resulted in mosquito knockdown with rapid knockdown and recovery time, without affecting survival or longevity. These findings align with previous research. For instance, a study on *An. arabiensis* by Culbert *et al*. (2017) found that only 0°C had an impact on male survival, consistent with similar observations in *Ae. aegypti* by Culbert *et al*. (2018). Furthermore, *An. arabiensis* could be immobilised at temperatures ranging from 4°C to 10°C for up to 24 h without significant adverse impacts (Culbert *et al*., 2017). Other studies have also reported that temperatures between 7°C to 10°C during transportation can maintain the survival of *Ae. aegypti* and *Ae. albopictus*. Zhang *et al*. (2020) noted that *Ae. albopictus* males incubated for up to 3 h at 5°C showed no adverse effects on survival compared to the control, while survival decreased at 10°C after 60 minutes of incubation. Tussey *et al*. (2023) observed no significant difference in survival among *Ae. aegypti* males chilled at 2°C to 6°C for up to 16 h. However, chilling had a notable effect on the performance of sterile *Ae. aegypti* mosquitoes, with more frequent and longer chilling times resulting in stronger adverse effects, as observed by Sánchez-Aldana-Sánchez *et al*. (2023). Furthermore, the findings of the current study are also close to those resulting from the temperature treatments used to maintain *C. capita* (Zavala-López *et al*., 2017) and *G. palpalis gambiensis* (Pagabeleguem *et al*., 2015) for long-distance transportation (around 10°C).

Rendering mosquitoes immobile, especially by using low temperatures (chilling, or cold treatment), holds significant potential for improved efficiency in an operational SIT programme. For example, the temperature ranges of 4°C to 8°C, as well as the rapid knockdown and recovery time observed in this study not only allow for more efficient handling

of immobilised mosquitoes without compromising their survival but also provide tangible practical benefits during actual operations. It is also notable that the rapid recovery offers significant advantage when considering the post-release behaviour of immobilised male mosquitoes. Shorter recovery times are advantageous, as they reduce the period during which males are vulnerable on the ground during release and before they can fly again, decreasing the risk of predation and other forms of mortality (Somda *et al*., 2022). Furthermore, being able to immobilise mosquitoes for 20 minutes or longer offers flexibility in timing the handling and transportation and release of mosquitoes, as chilled specimens can be irradiated under chilled conditions (Bimbilé Somda *et al*., 2022; Yamada *et al*., 2022) before deployment to the field. This flexibility can allow researchers in the SIT programme to coordinate release schedules with optimal environmental conditions or operational logistics, enhancing the success of programme. Furthermore, chilling helps preserve the quality and viability of mosquitoes during storage, ensuring that they maintain their reproductive potential, genetic integrity, and physiological characteristics (Culbert *et al*., 2017). Immobilisation/chilling also enables the safe handling and transportation of live mosquitoes over long distances (reference). By immobilising mosquitoes at low temperatures, their metabolic activity is reduced, minimising the risk of physical damage, escape, or disease transmission during transit (Culbert *et al*., 2017). These are essential for maintaining the efficacy of SIT programmes.

Compaction is also an essential aspect of packaging, especially with regards to space efficiency (Culbert, 2020; Ernawan *et al*., 2022). Thus, finding the optimal number of adult sterile males that can be packaged per chamber/container without affecting their quality is vital. In the current study, three different chambers and densities were investigated, with results showing the highest mortality rates when 5000 adult males were confined in a syringe (density: 5317.5 males/cm^3), whereas the lowest mortality rates occurred when 1000 adult males were accommodated in a 30 × 30 × 30 cm^3 Bugdorm-1® cage (density: 0.037 males/cm^3). Furthermore, longer longevity was observed when a lower number of adults was used per chamber, regardless of the compaction chamber. In general, lower numbers of adults in each chamber resulted in longer longevity, regardless of the compaction chamber used. In comparison to other studies, Culbert *et al*. (2018) found that a combination of chilling, compaction, irradiation did significantly reduce survival of *Ae. aegypti*. Also, Ernawan *et al*.

(2022) found significant decreases in survival in *Ae. aegypti* adult males compacted to densities over 40 males/cm^3 for durations greater than 3 h at 7°C. Similarly, low *Ae. aegypti* male survival was observed by Chung *et al*. (2018) at 40 males/cm^3. In contrast, Gomez *et al*. (2022) found that a combination of chilling to 7°C and a compaction density of 100 adult males/cm^3 did not reduce male *Ae. aegypti* ability to mate with females compared to control groups even with 24 h of compaction. Iyaloo *et al*. (2020) found that 2000 sterile adult males per Bugdorm cage (27 000 cm^3) is the optimal density at which *Ae. albopictus* could be stored and transported to a field site, with minimal effects of the latter's survival. Interestingly in this study, both the petri dish chamber and the syringe chamber had similar trends in mortality rates, with no significant difference in their performance in terms of minimising adult mortality during compaction. This result contrasts with the findings reported by Zhang *et al*. (2020), who observed that the shape of the container could indeed influence mortality and the distribution of adults within it. Certain container shapes may allow for more even distribution, reducing the risk of overcrowding or uneven compaction. Excessive mosquitoes in a chamber can result in overcrowding and physical damage, while too few mosquitoes can lead to excess space, potentially causing physical interactions, friction, and damage during transportation (Culbert *et al*. 2017). These considerations are especially critical for large-scale releases, where logistical challenges related to vehicle space and handling efficiency come into play.

The findings of the current study played a crucial role in the recent South African mosquito SIT pilot release trials. Adult mosquitoes were immobilised in a walk-in cold room set at temperatures between 4°C and 8°C for easy handling. Subsequently, the immobilised adults were compacted into 50 mℓ Falcon tubes, equivalent to syringes, with a range of 1000 to 5000 adult males per tube. The adults were then irradiated using a Rad Source 2400 X-ray irradiator, delivering a dose of 75 Gy to immobile adult males. Following irradiation, adult males were marked with fluorescent powder (sales@bastionpaint.co.za) inside the cold room within three to five minutes. After marking, the mosquitoes were transferred back into BugDorm-1® cages for recovery, where they were provided with a 10% sugar water solution soaked on a cotton ball. The cages were then placed in a mobile trailer maintained at ambient temperature and transported to the release site, which was six to eight hours away. Through this, 30 000 to 50

000 sterile marked makes were successfully transported and released as part of a pilot SIT study (Mashatola, unpublished results).

By integrating chilling into the handling, irradiation, marking protocols in SIT programmes, stakeholders can enhance the efficiency, safety, and effectiveness of mosquito control efforts, ultimately contributing to the reduction of mosquito-borne disease transmission and the protection of public health. Although Bugdorm-1® cage worked for the South African SIT pilot releases, next time when releasing millions of mosquitoes per week space efficiency will be vital. Thus further studies on small compaction chambers is suggested. The studies should include investigations on competitiveness in both laboratory and field, as well as flight ability as a proxy for fitness (Culbert *et al*. 2019). Additionally, none of these previous studies assessed the sexual competitiveness of sterilised adult males after chilling, compaction, and irradiation treatments. Measuring how well-sterilised males can compete with fertile males for access to females is necessary for assessing the production quality of sterile males.

7.5. Conclusion

This work has established an optimal knockdown temperature range of 4°C – 8°C for 20 minutes that can be used to chill/immobilise adult sterile males during handling in the laboratory without any immediate impact on longevity. In general, lower numbers of adults in each chamber resulted in longer longevity, regardless of the compaction chamber used. These findings were useful during recent pilot releases conducted by the South African SIT programme, in which 30 000 – 50 000 sterile adults were packaged at 1000 adult males in a Bugdorm-1® cage per week and transported to a field site 6 – 8 h away.

CHAPTER 8: Final Discussion and Conclusions

8.1. Introduction

Malaria has undoubtedly remained a global public health concern, responsible for millions of cases and thousands of fatalities annually (WHO, 2022). Sub-Saharan Africa accounts for more than 80% of all illnesses and 90% of deaths, primarily among pregnant women and children under five years of age. South Africa, one of the impacted countries, had set a mandate to eliminate malaria this year (i.e., no local transmission by 2023) (Elimination 8, 2019). However, despite tremendous progress in malaria case reduction over the years due to the consistent deployment of IRS as a vector control intervention and case management measures, elimination has not been achieved (Raman *et al*., 2016). The use of IRS of dwellings with insecticides is at the forefront of vector control (Brooke *et al*., 2013, 2015; Brooke, 2022). However, despite these efforts, it is clear that more resources and actions are required to achieve the malaria elimination objective. This includes the incorporation of additional vector control mechanisms to supplement IRS. One such technique is the SIT targeting the primary malaria vector *An. arabiensis* (Dyck *et al*., 2021). In South Africa, investigations into the SIT are currently at an advanced development level, with various technical aspects of the technology refined to support the ongoing small-scale pilot field trial (Munhenga *et al*., 2011, 2014, 2016).

However, the complete operation or application of the SIT for *An. arabiensis* control is hindered by the lack of an efficient operational sex separation strategy (Mashatola *et al*., 2018) and optimal handling and packaging of *An. arabiensis* sterile males during laboratory production and transport to the field (Culbert *et al*., 2017). Regarding the former, it is very critical to have an efficient sex separation mechanism to exclude female mosquitoes from the production line before irradiation and field releases (Mashatola *et al*., 2018). This is because, unlike males, females are capable of biting, blood feeding and transmitting the *Plasmodium* parasites that cause the malaria disease (Clements, 1992). The current methods for separating *An. arabiensis* sexes are proving inefficient in supporting the operational application of the current small-scale pilot trials carried out by the South African SIT project. For example, the GSS developed by Dandalo *et al*. (2018), acronymed “GMK”, exhibits low productivity and inherited sterility, possibly due to the Y-autosome chromosomal rearrangement of the dieldrin resistance gene (Willhoeft and Franz, 1996). Thus, this study aimed to develop additional systems to separate males and females before male sterilisation and release.

8.2. Work covered in this thesis

Investigations into the potential use of the toxicant ivermectin to eliminate females via artificial membrane blood-feeding were part of developing additional ways to separate males and females (Yamada *et al*., 2013a). As a precursor to blood toxicant trials, adult females were optimised and acclimatised to an artificial membrane feeding system with the objective of replacing live guinea pigs during routine mosquito blood feeding. Indirect blood-feeding with a Hemotek® feeding system using cattle blood through hog casing or collagen membrane is equally as effective as direct feeding with live guinea pigs. It can be proposed that indirect blood-feeding procedures be utilised to replace direct blood-feeding with animals without affecting mosquito colony maintenance.

Optimisation of artificial blood-feeding systems allowed experiments using blood-spiked ivermectin as a female elimination strategy to be conducted. In these experiments ivermectin eliminated >90% of fed females within the first two days. The downturn of this approach as a sexing strategy is that it took 7.5 ppm of ivermectin up to 11 days to eliminate the remaining females. This was because most females were uninterested in blood feeding, underlining the behavioural challenges associated with utilising this strategy. It can therefore be concluded that ivermectin should not be solely depended upon as the exclusive sex separation strategy. However, it may be useful as a supplementary approach to eliminate any residual females left after a primary method such as the GSS using the dieldrin-resistance as selection marker.

The inefficiency of spiking blood with ivermectin as a female elimination approach led to the development of alternative -sex separation procedures. Ideally, a good sexing system should target the embryonic and early developmental stages so that rearing costs can be minimised (Franz, 2005). Against this background, traditional genetic techniques have been used to generate GSSs and thus conditionally produce males only. The most widely used of these is the GSS for the medfly *C. capitata*, which was produced using the mutagen EMS. This system relies on two selectable markers: the *wp* gene and the *tsl* genes (Franz *et al*., 1994, 2020, 2021). Homozygous females carrying the recessive mutation do not survive when exposed to elevated temperatures (34 °C for 24 h), while hemizygous males are capable of surviving (Caceres, 2002; Franz, 2005; Franz *et al*., 2021). This distinction may arise from the fact that a

protein product of a *tsl* gene operates effectively at permissive temperatures but not under restrictive temperature conditions. The pupal colour is a random mutation whereby males emerge from brown pupae while females emerge from white pupae, providing a technique for confirming female elimination after high-temperature treatment (Caceres, 2002; Franz, 2005; Franz *et al*., 2021).

In *Anopheles* species, GSSs have been produced based on the Y-autosome translocation of an insecticide-resistant mutation and none based on *tsl* mutation (Papathanos *et al*., 2009; Gilles *et al*., 2014; Yamada *et al*., 2012; Dandalo *et al*., 2018). The work performed in this thesis attempted to emulate the approach used in developing the *C. capitata* GSS. The first attempted to determine the minimum (permissible) and maximum (restrictive) temperatures at which an *An. arabiensis* laboratory colony survives (determining the thermal limits/thermosensitive range) before screening for *tsl* in individuals. Results showed that the temperature that can be used for *tsl* screenings at the egg stage is 39 °C for 1 h, 41 °C for 76.4 minutes (~1 h and 17 minutes) for first instar larvae and 43 °C for 63.1 minutes (~1 h and 5 minutes) for pupae. Furthermore, it was found that temperature sensitivity varies based on exposure duration and developmental stage. These findings were applied in EMS experiments to induce conditional mutations (*tsl* and morphological body variations) in *An. arabiensis* towards the development of *tsl*-based GSSs. These attempts were, unfortunately, unsuccessful. Furthermore, morphological body abnormalities were observed, no success was achieved in isolating or recovering the mutations in later generations. However, findings from this work were shared through an IAEA-initiated CRP in which EMS was successfully utilised to isolate and characterise an *An. arabiensis tsl* strain (Ndo *et al*., 2018).

Subsequently, attempts were made to use this strain (CMR-*tsl*) to develop a *tsl*-based GSS containing a South African genetic background. Unfortunately, the CMR-*tsl* was found to be *An. coluzzii* instead of *An. arabiensis* following molecular species identification. Cross-mating experiments clearly showed the presence of introgressive hybridisation between *An. coluzzii* and *An. arabiensis*. Experiments were continued to prove the principle of developing a GSS by linking the wild-type *tsl* allele to the sex-determining Y chromosome. However, this work failed to develop an *An. arabiensis tsl*-based GSS.

8.3. Recommendation for future research

Based on the results of the work in this thesis, it is recommended that indirect blood-feeding techniques be considered as a replacement for direct animal blood-feeding practices. Notably, this transition has already been effectively implemented at the BDMI, where all mosquito colonies have successfully transitioned from guinea pig blood-feeding to Hemotek® blood-feeding system. It is also recommended that other sexing strategies, such as GSSs, be investigated as complete reliance on ivermectin blood spiking as a sex separation strategy is not recommended. Data on the temperature range for survival in an *An. arabiensis* laboratory colony, serves as a foundation for future studies that aim to locate and characterise *tsl* mutations in mosquito populations.

Furthermore, it is recommended that the use of EMS to generate conditional mutations continue, but that methods of application and screening be refined. For example, to produce sex-linked recessive lethal mutations, utilise vapour inhalation rather than direct ingestion (Marec, 1990). It also recommended to expand the mutational screening to include other life stages or body parts, for example eye colour in adults (Koskinioti *et al*., 2021). Furthermore, to improve recovery of induced mutations in subsequent progeny, the number of parental material treated in the beginning could be increased. Moreover, the failure to develop a *tsl*-based GSS using the described methods in this study calls for exploration of other alternative approaches to develop sexing strains, especially methods that would reduce financial costs and time for research and development, allowing SIT programmes to be more readily developed and implemented (FAO/IAEA, 2023).

Among these are transgenic approaches that have been successfully used to develop various *An. arabiensis* fluorescent marked strains for the South African SIT programme. One is an *An. arabiensis* strain that has been introgressed into a South African genetic background (KWAG) and carries the entire Y chromosome from *An. gambiae* (AY-2), including a transgenic red fluorescent marker (Ntoyi *et al*., 2022). The created fluorescent strain (acronym KWAG-AY2) was evaluated for its potential to be a reliable sexing system. The results of this evaluation demonstrated the strain's genetic stability and a high level of efficiency in sexing. Importantly, over three rounds of introgression crosses, no recombination events involving the fluorescent marker were observed. In terms of hatching rates and larval development, KWAG-AY2

exhibited superior performance compared to both AY-2 and KWAG strains. Fecundity amongst the strains showed no significant difference. In fight ability tests (a proxy for mating competitiveness), KWAG-AY2 males outperformed the reference strains. These findings suggest that the newly developed KWAG-AY2 fluorescent strain has the potential for use as a sexing system. However, it is crucial to note that before its operational use in South Africa's SIT programme, comprehensive assessments are required. These assessments should include an evaluation of its large-scale rearing efficiency, determination of the optimal sterility irradiation dose-response, examination of the effect of irradiation on male mating competitiveness, assessment of mating compatibility with the wild-type KWAG strain, and finally, field trials conducted under open field conditions using mark-release-recapture (MRR) studies (Benedict and Robinson, 2003; Dame *et al*., 2009; Dyck *et al*., 2021). Moreover, a risk assessment analysis is required to address any concerns about the potential release of this generated strain in ecosystems and to get the appropriate regulatory licenses for open field releases (Bourtzis *et al*., 2016). However, while waiting for approval/in parallel, other methods to create transgenic strains but release non-transgenic strains are being developed. In fact, KWAG was used again to successfully develop two more *An. arabiensis* fluorescent-based transgenic sexing strains (unpublished data). The fluorescence in one strain is tied to the X-chromosome, whereas the fluorescence in the other is linked to the Y-chromosome. Cross-mating of these sexing strains will allow for the sorting and release of non-transgenic males (Lutrat *et al*., 2022). The next steps on these strains will be similar to those of any newly developed strain (e.g., KWAG-AY2). Nonetheless, the progress made towards establishment of these strains has provided the South African SIT with a strong foundation for planning the operational phase and successful completion of the current small-scale pilot trials, especially a move from sex separation methods that included manual aspiration of adults and use of the insecticide dieldrin.

There are other recent breakthroughs that should be examined as alternate techniques to developing sexing strains and possibly overcome some of the hurdles observed by the other methods. Next-generation sequencing (NGS) technologies, novel cytogenetics and chromosome manipulation approaches, new tools for modifying genotypes, and advancements in RNAi for pest management are examples of these (Meccariello *et al*., 2019; Chen *et al*., 2022). Using these methods, genes that are suitable as selectable markers for the

development of GSS in *An. arabiensis* can be identified and characterised; embryonic microinjections can be performed to induce mutations that will be used in the development of non-transgenic GSS; and any developed mutant line(s) can be genetically and molecularly characterised, as well as compared for genetic stability, reproductive and physiological properties.

Another goal of this research was to improve the South African SIT programme by optimising the handling and packing conditions for *An. arabiensis* sterile males (Culbert *et al*., 2017, 2018, 2019). This is because these parameters can alter the quality of sterile males and, as such, must be evaluated in order to develop standard protocols for the SIT's efficacy against mosquitoes (Pagabeleguem *et al*., 2015; Parker *et al*., 2021). This study determined an appropriate temperature range (4 °C – 8 °C for up to 20 minutes) that can be utilised to chill/immobilise adult sterile males during laboratory handling without affecting survival/longevity. Furthermore, the study revealed the optimal packaging/compaction conditions (1000 adult males in a Bugdorm-1® cage) for transporting adult sterile males, particularly during small scale pilot trials. These two parameters do not fully cover the topic of handling, transporting, and releasing sterile male mosquitoes, but they were useful during recent small scale pilot trials by the South African SIT programme, in which 30 000 to 50 000 adult sterile males were packaged and transported to a field site 6 h to 8 h away. These advancements have laid a solid foundation for planning the SIT operational phase. It is hoped that the SOP developed from this work will be of great value in other SIT programmes working with *An. arabiensis* or any other SIT programme against the main vectors of mosquito-borne diseases. Future studies should focus on optimised conditions and practical protocols for pupal vs adult irradiation and releases, walking vs vehicle ground releases vs aerial releases with drones or unmanned aerial vehicle (UAV) for example (Bouyer *et al*., 2020a; Guo *et al*., 2022). Also, quality and fitness parameters should be measured, including mating competitiveness of the sterile males against wild males, instead of focusing only on survivorship and longevity parameters (Calkins and Parker, 2005; Parker *et al*., 2021).

With increase in the geographical range in insecticide resistant populations (Ranson and Lissenden, 2016; Suh *et al*., 2023), several malaria vectors have been reported to have been selected for resistance to almost all classes recommended for vector control (Wiebe *et al*.,

2017). This is further complicated by invasive species such as *An. stephensi* (Sinka *et al*., 2020) and climate change change resulting in favourable conditions that support mosquito disease vectors (Caldwell *et al*., 2021; Colón-González *et al*., 2021). Changes in vector behaviour, i.e. vector behavioural plasticity is an additional factor complicating malaria vector control (Gatton *et al*., 2013; Kreppel *et al*., 2020). All these factors motivate the need for complementary vector control tools such as SIT to be investigated for potential application.

REFERENCES

Chapter 1

Alphey, L., 2002. **Re-engineering the sterile insect technique.** *Insect Biochemistry and Molecular Biology*, 32(10), pp.1243-1247.

Alphey, L., Benedict, M., Bellini, R., Clark, G.G., Dame, D.A., Service, M.W. and Dobson, S.L., 2010. **Sterile-insect methods for control of mosquito-borne diseases: an analysis.** *Vector-Borne and Zoonotic Diseases*, 10(3), pp.295-311.

Andrade, M.V., Noronha, K., Diniz, B.P., Guedes, G., Carvalho, L.R., Silva, V.A., Calazans, J.A., Santos, A.S., Silva, D.N. and Castro, M.C., 2022. **The economic burden of malaria: a systematic review.** *Malaria Journal*, *21*(1), p.283.

Ashley, E.A., Phyo, A.P., Carrara, V.I., Tun, K.M., Nosten, F., Smithuis, F. and White, N.J., 2019. ***Plasmodium vivax* relapse rates following Plasmodium falciparum malaria reflect previous transmission intensity.** *The Journal of infectious diseases*, 220(1), pp.100-104.

Barnes, B., Rosenberg, S., Arnolds, L. and Johnson, J., 2007. **Production and quality assurance in the SIT Africa Mediterranean fruit fly (Diptera: Tephritidae) rearing facility in South Africa.** *Florida Entomologist*, 90(1), pp.41-52.

Barnes, B.N., Hofmeyr, J.H., Groenewald, S., Conlong, D.E. and Wohlfarter, M., 2015. **The sterile insect technique in agricultural crops in South Africa: a metamorphosis…. but will it fly?.** *African Entomology*, 23(1), pp.1-18.

Barrón, M.G., Paupy, C., Rahola, N., Akone-Ella, O., Ngangue, M.F., Wilson-Bahun, T.A., Pombi, M., Kengne, P., Costantini, C., Simard, F. and González, J., 2019. **A new species in the major malaria vector complex sheds light on reticulated species evolution.** *Scientific Reports*, *9*(1), p.14753.

Bellini, R., Medici, A., Puggioli, A., Balestrino, F. and Carrieri, M., 2013. **Pilot field trials with *Aedes albopictus* irradiated sterile males in Italian urban areas.** *Journal of Medical Entomology*, 50(2), pp.317-325.

Bellini, R., Puggioli, A., Balestrino, F., Carrieri, M. and Urbanelli, S., 2018. **Exploring protandry and pupal size selection for *Aedes albopictus* sex separation.** *Parasites and Vectors*, 11, pp.65-71.

Benedict, M.Q. and Chang, H., 1996. **Rapid isolation of anopheline mosquito eye-colour mutants, based on larval colour change.** *Medical and Veterinary Entomology*, 10(1), pp.93-96.

Benedict, M.Q. and Robinson, A.S., 2003. **The first releases of transgenic mosquitoes: an argument for the sterile insect technique.** *Trends in Parasitology*, 19(8), pp.349-355.

Bjeliš, M., Radunić, D. and Bulić, P., 2013. **Pre-and post-release quality of sterile *Ceratitis capitata* males released by an improved automated ground release machine.** *Journal of Applied Entomology*, 137, pp.154-162.

Bloem, S., Carpenter, J., McCluskey, A., Fugger, R., Arthur, S. and Wood, S., 2007. **Suppression of the codling moth Cydia pomonella in British Columbia, Canada using an area-wide integrated approach with an SIT components.** In *Area-wide control of insect pests: from research to field implementation* (pp. 591-601). Dordrecht: Springer Netherlands.

Blomefield, T., Carpenter, J.E. and Vreysen, M.J.B., 2011. **Quality of mass-reared codling moth (Lepidoptera: Tortricidae) after long-distance transportation: 1. Logistics of shipping procedures and quality parameters as measured in the laboratory.** *Journal of Economic Entomology*, 104(3), pp.814-822.

Blumberg, L. and Frean, J., 2007. **Malaria control in South Africa-challenges and successes.** *South African Medical Journal*, 97(11), pp.1193-1197.

Blumberg, L., Frean, J. and Moonasar, D., 2014. **Successfully controlling malaria in South Africa.** *South African Medical Journal*, 104(3), pp.224-227.

Boersma, N. and Carpenter, J.E., 2016. **Influence of holding temperature and irradiation on field performance of mass-reared *Thaumatotibia leucotreta* (Lepidoptera: Tortricidae).** *Florida Entomologist*, 99(sp1), pp.215-221.

Boundenga, L., Makanga, B., Ollomo, B., Gilabert, A., Rougeron, V., Mve-Ondo, B., Arnathau, C., Durand, P., Moukodoum, N.D., Okouga, A.P. and Delicat-Loembet, L., 2016. **Haemosporidian parasites of antelopes and other vertebrates from Gabon, Central Africa.** *PLoS One*, 11(2), p.e0148958.

Boundenga, L., Perkins, S.L., Ollomo, B., Rougeron, V., Leroy, E.M., Renaud, F. and Prugnolle, F., 2017. **Haemosporidian parasites of reptiles and birds from Gabon, Central Africa.** *Journal of Parasitology*, 103(4), pp.330-337.

Bouyer, J., Culbert, N.J., Dicko, A.H., Pacheco, M.G., Virginio, J., Pedrosa, M.C., Garziera, L., Pinto, A.M., Klaptocz, A., Germann, J. and Wallner, T., 2020a. **Field performance of sterile male mosquitoes released from an uncrewed aerial vehicle.** *Science Robotics*, 5(43).

Bouyer, J., Yamada, H., Pereira, R., Bourtzis, K. and Vreysen, M.J., 2020b. **Phased conditional approach for mosquito management using sterile insect technique.** *Trends in Parasitology*, 36(4), pp.325-336.

Braack, L., Bornman, R., Kruger, T., Dahan-Moss, Y., Gilbert, A., Kaiser, M., Oliver, S.V., Cornel, A.J., Lee, Y., Norris, D.E. and Coetzee, M., 2020. **Malaria vectors and vector surveillance in Limpopo province (South Africa): 1927 to 2018.** *International Journal of Environmental Research and Public Health*, *17*(11), p.4125.

Briegel, H., 2003. **Physiological bases of mosquito ecology.** *Journal of Vector Ecology: Journal of the Society for Vector Ecology*, 28(1), pp.1-11.

Brooke, B., Koekemoer, I., Kruger, P., Urbach, J., Misiani, E. and Coetzee, M., 2013. **Malaria vector control in South Africa.** *South African Medical Journal*, 103(10), pp.784-788.

Brooke, B.D., Robertson, L., Kaiser, M.L., Raswiswi, E., Munhenga, G., Venter, N., Wood, O.R. and Koekemoer, L.L., 2015. **Insecticide resistance in the malaria vector *Anopheles arabiensis* in Mamfene, KwaZulu-Natal.** *South African Journal of Science*, 111(11-12), pp.1-3.

Buffet, P.A., Safeukui, I., Deplaine, G., Brousse, V., Prendki, V., Thellier, M., Turner, G.D. and Mercereau-Puijalon, O., 2011. **The pathogenesis of *Plasmodium falciparum* malaria in humans: insights from splenic physiology.** *The Journal of the American Society of Hematology*, 117(2), pp.381-392.

Burke, A., Dahan-Moss, Y., Duncan, F., Qwabe, B., Coetzee, M., Koekemoer, L. and Brooke, B., 2019. ***Anopheles parensis* contributes to residual malaria transmission in South Africa.** *Malaria Journal*, 18(1), pp.1-7.

Burke, A., Dandalo, L., Munhenga, G., Dahan-Moss, Y., Mbokazi, F., Ngxongo, S., Coetzee, M., Koekemoer, L. and Brooke, B., 2017. **A new malaria vector mosquito in South Africa.** *Scientific Reports*, 7(1), pp.1-5.

Caceres, C., 2002. **Mass rearing of temperature sensitive genetic sexing strains in the Mediterranean fruit fly (*Ceratitis capitata*).** *Genetica*, 116(1), pp.107-116.

Calkins, C.O. and Parker, A.G., 2005. **Sterile insect quality.** In Sterile insect technique (pp. 269-296). *Springer, Dordrecht*.

Caminade, C., Kovats, S., Rocklov, J., Tompkins, A.M., Morse, A.P., Colón-González, F.J., Stenlund, H., Martens, P. and Lloyd, S.J., 2014. **Impact of climate change on global malaria distribution.** *Proceedings of the National Academy of Sciences*, 111(9), pp.3286-3291.

Carter, J.A., Ross, A.J., Neville, B.G., Obiero, E., Katana, K., Mung'ala-Odera, V., Lees, J.A. and Newton, C.R., 2005. **Developmental impairments following severe falciparum malaria in children.** *Tropical Medicine and International Health*, 10(1), pp.3-10.

Carvalho, D.O., McKemey, A.R., Garziera, L., Lacroix, R., Donnelly, C.A., Alphey, L., Malavasi, A. and Capurro, M.L., 2015. **Suppression of a field population of *Aedes aegypti* in Brazil by sustained release of transgenic male mosquitoes.** *PLoS Neglected Tropical Diseases*, *9*(7), p.e0003864.

Catteruccia, F., Benton, J.P. and Crisanti, A., 2005. **An *Anopheles* transgenic sexing strain for vector control.** *Nature Biotechnology*, 23(11), pp.1414-1417.

Chung, H.N., Rodriguez, S.D., Gonzales, K.K., Vulcan, J., Cordova, J.J., Mitra, S., Adams, C.G., Moses-Gonzales, N., Tam, N., Cluck, J.W. and Attardo, G.M., 2018. **Toward implementation of mosquito sterile insect technique: the effect of storage conditions on survival of male *Aedes aegypti* mosquitoes (Diptera: Culicidae) during transport.** *Journal of Insect Science*, 18(6), p.2.

Clements, A.N., 1992. **The biology of mosquitoes. Volume 1: development, nutrition and reproduction.** *Chapman and Hall*.

Clements, A.N., 1999. **The biology of mosquitoes.** *Volume 2: Sensory Reception and Behaviour*. CABI publishing.

Coetzee, M., 2020. **Key to the females of Afrotropical *Anopheles* mosquitoes (Diptera: Culicidae).** *Malaria Journal*, 19(1), pp.1-20.

Coetzee, M., Hunt, R.H., Wilkerson, R., Della Torre, A., Coulibaly, M.B. and Besansky, N.J., 2013a. ***Anopheles coluzzii* and *Anopheles amharicus*, new members of the *Anopheles gambiae* complex.** *Zootaxa*, 3619(3), pp.246-274.

Coetzee, M., Kruger, P., Hunt, R.H., Durrheim, D.N., Urbach, J. and Hansford, C.F., 2013b. **Malaria in South Africa: 110 years of learning to control the disease.** *South African Medical Journal*, 103(10), pp.770-778.

Cox, F.E., 2010. **History of the discovery of the malaria parasites and their vectors.** *Parasites and Vectors*, 3, pp.1-9.

Culbert, N.J., 2020. **Improving the handling, transport and release of sterile male mosquitoes as part of an area-wide integrated pest management strategy.** The University of Liverpool (United Kingdom). https://agritrop.cirad.fr/596806/

Culbert, N.J., Lees, R.S., Vreysen, M.J., Darby, A.C. and Gilles, J.R., 2017. **Optimised conditions for handling and transport of male *Anopheles arabiensis*: effects of low temperature, compaction, and ventilation on male quality.** *Entomologia Experimentalis et Applicata*, 164(3), pp.276-283.

Culbert, N.J., Maiga, H., Somda, N.S.B., Gilles, J.R.L., Bouyer, J. and Mamai, W., 2018. **Longevity of mass-reared, irradiated and packed male *Anopheles arabiensis* and *Aedes aegypti* under simulated environmental field conditions.** *Parasites and Vectors*, 11(1), pp.1-8.

Culbert, N.J., Somda, N.S.B., Hamidou, M., Soma, D.D., Caravantes, S., Wallner, T., Wadaka, M., Yamada, H. and Bouyer, J., 2020. **A rapid quality control test to foster the development of the sterile insect technique against *Anopheles arabiensis.*** *Malaria Journal*, 19(1), pp.1-10.

Curtis, C.F., 1978. **Genetic sex separation in *Anopheles arabiensis* and the production of sterile hybrids.** *Bulletin of the World Health Organization*, 56(3), p.453.

Curtis, C.F., Akiyama, J. and Davidson, G., 1976. **A genetic sexing system in *Anopheles gambiae* species A.**

Dame, D.A., Curtis, C.F., Benedict, M.Q., Robinson, A.S. and Knols, B.G., 2009. **Historical applications of induced sterilisation in field populations of mosquitoes.** *Malaria Journal*, 8(2), pp.1-10.

Dame, D.A., Lofgren, C.S., Ford, H.R., Boston, M.D., Baldwin, K.F. and Jeffery, G.M., 1974. **Release of chemosterilized males for the control of *Anopheles albimanus* in El Salvador II. Methods of rearing, sterilization, and distribution.** *American Journal of Tropical Medicine of Hygiene*, *23*(2), pp.282-287.

Dandalo, L.C., 2016. **Characterization of a local genetic sexing strain as well as a wild population of *Anopheles arabiensis* from KwaZulu-Natal, South Africa.** PhD Thesis. Johannesburg: University of the Witwatersrand. https://wiredspace.wits.ac.za/handle/10539/45.

Dandalo, L.C., Brooke, B.D., Munhenga, G., Lobb, L.N., Zikhali, J., Ngxongo, S.P., Zikhali, P.M., Msimang, S., Wood, O.R., Mofokeng, M. and Misiani, E., 2017a. **Population dynamics and *Plasmodium falciparum* (Haemosporida: Plasmodiidae) infectivity rates for the malaria vector *Anopheles arabiensis* (Diptera: Culicidae) at Mamfene, KwaZulu-Natal, South Africa.** *Journal of Medical Entomology*, 54(6), pp.1758-1766.

Dandalo, L.C., Kemp, A., Koekemoer, L.L. and Munhenga, G., 2017b. **Effect of ionising (gamma) radiation on female *Anopheles arabiensis*.** *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 111(1), pp.38-40.

Dandalo, L.C., Munhenga, G., Kaiser, M.L. and Koekemoer, L.L., 2018. **Development of a genetic sexing strain of *Anopheles arabiensis* for KwaZulu-Natal, South Africa.** *Medical and Veterinary Entomology*, 32(1), pp.61-69.

Davidson, G. and Jackson, C.E., 1961. **Insecticide resistance in mosquitoes.** *Nature*, 190(4773), pp.364-365.

De Meillon, B., Van Eeden, G.J., Coetzee, L., Coetzee, M., Meiswinkel, R., Du Toit, C.L.N. and Hansford, C.F., 1977. **Observations on a species of the *Anopheles funestus* subgroup, a suspected exophilic vector of malaria parasites in north-eastern Transvaal,** *South Africa. Mosquito News*, 37(4), pp.657-61.

Dia, I., Guelbeogo, M.W. and Ayala, D., 2013. **Advances and Perspectives in the Study of the Malaria Mosquito *Anopheles funestus*. *Anopheles* mosquitoes**-*New Insights into Malaria Vectors*, 10, p.55389.

Dominiak, B.C. and Fanson, B., 2021. **Transport from production facility to release locations caused a decline in quality of sterile Queensland fruit fly received for SIT application.** *Entomologia Experimentalis et Applicata*, 169(11), pp.1020-1026.

Dyck, V.A., Hendrichs, J. and Robinson, A.S., 2005. **Sterile insect technique: principles and practice in area-wide integrated pest management** (p. 1216). *Taylor and Francis*.

Dyck, V.A., Hendrichs, J. and Robinson, A.S., 2021. **Sterile insect technique: principles and practice in area-wide integrated pest management** (p. 1216). *Taylor and Francis*.

Elimination 8, 2019. **Southern African Democratic Community.** *Annual Report*.

Enkerlin, W. ed., 2007. **Guidance for packing, shipping, holding and release of sterile flies in area-wide fruit fly control programmes (No. 190).** *Food and Agriculture Org*..

Ernawan, B., Anggraeni, T., Yusmalinar, S., Sasmita, H.I., Fitrianto, N. and Ahmad, I., 2022. **Assessment of compaction, temperature, and duration factors for packaging and transporting of sterile male *Aedes aegypti* (Diptera: Culicidae) under laboratory conditions.** *Insects*, 13(9), p.847.

Eskenazi, B., Chevrier, J., Rosas, L.G., Anderson, H.A., Bornman, M.S., Bouwman, H., Chen, A., Cohn, B.A., De Jager, C., Henshel, D.S. and Leipzig, F., 2009. **The Pine River statement: human health consequences of DDT use.** *Environmental Health Perspectives*, *117*(9), pp.1359-1367.

FAO/IAEA. 2017. **Guideline for packing, shipping, holding and release of sterile flies in area-wide fruit fly control programmes**, Second edition, by Zavala-López J.L. and Enkerlin W.R. (eds.). Rome, Italy. 140 pp.

FAO/IAEA. 2018. **Third Research Co-ordination meeting, Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture, Co-ordinated Research Project on Mosquito Handling, Transport, Release and Male Trapping Methods. 12 – 16 Nov 2018. Juazeiro, Bahia, Brazil.** http://www-naweb.iaea.org/nafa/ipc/crp/RCM3-Mosquito-handlingtransport.pdf. Accessed 23 Nov 2021.

FAO/IAEA/United States Department of Agriculture (USDA). 2019. **Product Quality Control for Sterile Mass-Reared and Released Tephritid Fruit Flies.** Version 7.0. IAEA, Vienna. Accessed 25 December 2023, https://www.iaea.org/resources/manual/product-quality-control-for-sterile-mass-reared-andreleased-tephritid-fruit-flies-version-70.

Faraji, A., Haas-Stapleton, E., Sorensen, B., Scholl, M., Goodman, G., Buettner, J., Schon, S., Lefkow, N., Lewis, C., Fritz, B. and Hoffman, C., 2021. **Toys or tools? Utilization of unmanned aerial systems in mosquito and vector control programs.** *Journal of Economic Entomology*, 114(5), pp.1896-1909.

Fay, R.W. and Morlan, H., 1959. **A mechanical device for separating the developmental stages, sexes and species of mosquitoes.** *Mosquito News*, *19*(3), pp.144-147.

Focks, D.A., 1980. **An improved separator for the developmental stages, sexes, and species of mosquitoes (Diptera: Culicidae).** *Journal of Medical Entomology*, *17*(6), pp.567-568.

Franz, G., 2002. **Recombination between homologous autosomes in medfly (*Ceratitis capitata*) males: type-1 recombination and the implications for the stability of genetic sexing strains.** *Genetica*, 116(1), pp.73-84.

Franz, G., 2005. **Genetic sexing strains in Mediterranean fruit fly, an example for other species amenable to large-scale rearing for the sterile insect technique.** In Sterile Insect Technique (pp. 427-451). *Springer, Dordrecht*.

Franz, G., Kerremans, P., Rendon, P. and Hendrichs, J., 2021. **Development and application of genetic sexing systems for the Mediterranean fruit fly based on a temperature sensitive lethal.** In *Fruit Fly Pests* (pp. 185-191). CRC Press.

Gerberg, E.J., Hopkins, T.M. and Gentry, J.W., 1969. **Mass rearing of *Culex pipiens L*.** *Mosquito News*, *29*(3).

Gilles, J.R., Schetelig, M.F., Scolari, F., Marec, F., Capurro, M.L., Franz, G. and Bourtzis, K., 2014. **Towards mosquito sterile insect technique programmes: exploring genetic, molecular, mechanical and behavioural methods of sex separation in mosquitoes.** *Acta Tropica*, 132, pp.S178-S187.

Gillies, M.T. and Coetzee, M., 1987. **A supplement to the Anophelinae of Africa South of the Sahara.** *Publication of the South African Institute of Medical Research*, 55, pp.1-143.

Gillies, M.T. and De Meillon, B., 1968. **The Anophelinae of Africa south of the Sahara (Ethiopian zoogeographical region).** *The Anophelinae of Africa South of the Sahara (Ethiopian Zoogeographical Region).*

Grossman, G.L., Rafferty, C.S., Clayton, J.R., Stevens, T.K., Mukabayire, O. and Benedict, M.Q., 2001. **Germline transformation of the malaria vector, *Anopheles gambiae*, with the piggyBac transposable element.** *Insect Molecular Biology*, 10(6), pp.597-604.

Guissou, E., Poda, S., de Sales Hien, D.F., Yerbanga, S.R., Da, D.F., Cohuet, A., Fournet, F., Roux, O., Maiga, H., Diabaté, A. and Gilles, J., 2020. **Effect of irradiation on the survival and susceptibility of female *Anopheles arabiensis* to natural isolates of *Plasmodium falciparum*.** *Parasites and Vectors*, 13, pp.1-11.

Guo, J., Zheng, X., Zhang, D. and Wu, Y., 2022. **Current Status of Mosquito Handling, Transporting and Releasing in Frame of the Sterile Insect Technique.** *Insects*, *13*(6), p.532.

Haghighat-Khah, R.E., Scaife, S., Martins, S., St John, O., Matzen, K.J., Morrison, N. and Alphey, L., 2015. **Site-specific cassette exchange systems in the *Aedes aegypti* mosquito and the *Plutella xylostella* moth.** *PLoS One*, *10*(4), p.e0121097.

Harbach, R.E. and Kitching, I.J., 2016. **The phylogeny of Anophelinae revisited: inferences about the origin and classification of Anopheles (Diptera: Culicidae).** *Zoologica Scripta*, 45(1), pp.34-47.

Harbach, R.E., 2013. **The phylogeny and classification of Anopheles. In *Anopheles* mosquitoes-New insights into malaria vectors.** *IntechOpen*.

Hargreaves, K., Koekemoer, L.L., Brooke, B.D., Hunt, R.H., Mthembu, J. and Coetzee, M., 2000. ***Anopheles funestus* resistant to pyrethroid insecticides in South Africa.** *Medical and Veterinary Entomology*, 14(2), pp.181-189.

Hargreaves, K., Koekemoer, L.L., Brooke, B.D., Hunt, R.H., Mthembu, J. and Coetzee, M., 2000. ***Anopheles funestus* resistant to pyrethroid insecticides in South Africa.** *Medical and Veterinary Entomology*, 14(2), pp.181-189.

Harrison, G., 1978. **Mosquitoes, malaria and man: a history of the hostilities since 1880** (pp. xii+-314).

Hay, S.I., Okiro, E.A., Gething, P.W., Patil, A.P., Tatem, A.J., Guerra, C.A. and Snow, R.W., 2010. **Estimating the global clinical burden of *Plasmodium falciparum* malaria in 2007.** *PLoS Medicine*, *7*(6), p.e1000290.

Helinski, M.E., Hassan, M.A.M., El-Motasim, W.M., Malcolm, C.A., Knols, B.G. and El-Sayed, B., 2008. **Towards a sterile insect technique field release of Anopheles arabiensis mosquitoes in Sudan: irradiation, transportation, and field cage experimentation.** *Malaria Journal*, 7(1), pp.1-10.

Hemingway, J., 1992. **Genetics of insecticide resistance in mosquito vectors of disease.** *Parasitology Today*, 8(9), pp.296-298.

Hendrichs, J., Robinson, A.S., Cayol, J.P. and Enkerlin, W., 2002. **Medfly area wide sterile insect technique programmes for prevention, suppression or eradication: the importance of mating behavior studies.** *Florida Entomologist*, 85(1), pp.1-13.

Hofmeyr, J.H., Hofmeyr, M., Carpenter, J.E., Bloem, S., Slabbert, J.P. and Groenewald, S.S., 2015. **Development of the sterile insect technique to suppress false codling moth *Thaumatotibia leucotreta* (Lepidoptera: Tortricidae) in citrus fruit: research to implementation (Part 1).** *African Entomology*, 23(1), pp.180-186.

Hunt, R.H., Coetzee, M. and Fettene, M., 1998. **The *Anopheles gambiae* complex: a new species from Ethiopia.** *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 92(2), pp.231-235.

Iyaloo, D.P., Facknath, S. and Bheecarry, A., 2020. **Investigating the effects of low temperature and compaction on the quality of adult radio-sterilised *Aedes albopictus* (Diptera: Culicidae) males in view of their optimal transport to the pilot sterile release site in Mauritius.** *International Journal of Tropical Insect Science*, 40(1), pp.53-62.

Kaiser, M.L., Wood, O.R., Damiens, D., Brooke, B.D., Koekemoer, L.L. and Munhenga, G., 2021. **Estimates of the population size and dispersal range of *Anopheles arabiensis* in Northern KwaZulu-Natal, South Africa: implications for a planned pilot programme to release sterile male mosquitoes.** *Parasites and Vectors*, 14(1), pp.1-18.

Kaiser, P.E., Seawright, J.A., Dame, D.A. and Joslyn, D.J., 1978. **Development of a genetic sexing system for *Anopheles albimanus*.** *Journal of Economic Entomology*, 71(5), pp.766-771.

Kim, S.S., Seawright, J.A. and Kaiser, P.E., 1987. **A genetic sexing strain of *Anopheles quadrimaculatus*, species A.** *Journal of the American Mosquito Control Association*, 3(1), pp.50-53.

Kittayapong, P., Ninphanomchai, S., Limohpasmanee, W., Chansang, C., Chansang, U. and Mongkalangoon, P., 2019. **Combined sterile insect technique and incompatible insect technique: The first proof-of-concept to suppress *Aedes aegypti* vector populations in semi-rural settings in Thailand.** PLoS neglected tropical diseases, 13(10), p.e0007771.

Klassen, W., Curtis, C.F. and Hendrichs, J., 2021. **History of the sterile insect technique.** In *Sterile Insect Technique* (pp. 1-44). CRC Press.

Klassen, W., Curtis, C.F., Dyck, V.A., Hendrichs, J. and Robinson, A.S., 2005. **History of the sterile insect technique. Sterile Insect Technique: Principles and Practice in Area-Wide Integrated Pest Management.** *Dordrecht, The Netherlandfs: Springer*, pp.1-34.

Knipling, E.F., 1955. **Possibilities of insect control or eradication through the use of sexually sterile males.** *Journal of Economic Entomology*, 48(4), pp.459-462.

Knipling, E.F., 1959. **Sterile-Male Method of Population Control: Successful with some insects, the method may also be effective when applied to other noxious animals.** *Science*, 130(3380), pp.902-904.

Koyama, J., Kakinohana, H. and Miyatake, T., 2004. **Eradication of the melon fly, *Bactrocera cucurbitae*, in Japan: importance of behavior, ecology, genetics, and evolution.** *Annual Reviews in Entomology*, *49*(1), pp.331-349.

Krishnamurthy, B.S., Ray, S.N. and Joshi, G.C., 1962. **A note on preliminary field studies of the use of irradiated males for reduction of *C. fatigans* Wied populations.** *Indian Journal of Malariology*, (4).

Lahondère, C. and Lazzari, C.R., 2012. **Mosquitoes cool down during blood feeding to avoid overheating.** *Current Biology*, 22(1), pp.40-45.

Lees, R.S., Knols, B., Bellini, R., Benedict, M.Q., Bheecarry, A., Bossin, H.C., Chadee, D.D., Charlwood, J., Dabire, R.K., Djogbenou, L. and Egyir-Yawson, A., 2014. **Improving our knowledge of male mosquito biology in relation to genetic control programmes.** *Acta Tropica*, 132, pp.S2-S11.

Leimar, O., Karlsson, B. and Wiklund, C., 1994. **Unpredictable food and sexual size dimorphism in insects.** *Proceedings of the Royal Society of London. Series B: Biological Sciences*, 258(1352), pp.121-125.

Lindquist, D.A., Abusowa, M. and Hall, M.J.R., 1992. **The New World screwworm fly in Libya: a review of its introduction and eradication.** *Medical and Veterinary Entomology*, *6*(1), pp.2-8.

Lines, J.D. and Curtis, C.F., 1985. **Genetic sexing systems in *Anopheles arabiensis* Patton (Diptera: Culicidae).** *Journal of Economic Entomology*, 78(4), pp.848-851.

Liu, T., Yang, W.Q., Xie, Y.G., Liu, P.W., Xie, L.H., Lin, F., Li, C.Y., Gu, J.B., Wu, K., Yan, G.Y. and Chen, X.G., 2019. **Construction of an efficient genomic editing system with CRISPR/Cas9 in the vector mosquito *Aedes albopictus*.** *Insect Science*, *26*(6), pp.1045-1054.

Lowe, R.E., Fowler, J.E.F., Bailey, D.L., Dame, D.A. and Savage, K.E., 1981. **Separation of sexes of adult *Anopheles albimanus* by feeding of insecticide-laden blood.** *Mosquito News*, 41(4), pp.634-638.

Maharaj, R., Seocharan, I., Qwabe, B., Mkhabela, M., Kissoon, S. and Lakan, V., 2019. **Decadal epidemiology of malaria in KwaZulu-Natal, a province in South Africa targeting elimination.** *Malaria Journal*, 18, pp.1-7.

Manana, P.N., Jewett, S., Zikhali, J., Dlamini, D., Mabaso, N., Mlambo, Z., Ngobese, R. and Munhenga, G., 2021. **"Maskandi experience": exploring the use of a cultural song for community engagement in preparation for a pilot Sterile Insect Technique release programme for malaria vector control in KwaZulu-Natal Province, South Africa 2019.** *Malaria Journal*, 20(1), pp.1-11.

Manana, P.N., Kuonza, L., Musekiwa, A., Mpangane, H.D. and Koekemoer, L.L., 2018. **Knowledge, attitudes and practices on malaria transmission in Mamfene, KwaZulu-Natal Province, South Africa 2015.** *BMC Public Health*, 18(1), pp.1-7.

Martín-Park, A., Che-Mendoza, A., Contreras-Perera, Y., Pérez-Carrillo, S., Puerta-Guardo, H., Villegas-Chim, J., Guillermo-May, G., Medina-Barreiro, A., Delfín-González, H., Méndez-Vales, R. and Vázquez-Narvaez, S., 2022. **Pilot trial using mass field-releases of sterile males produced with the incompatible and sterile insect techniques as part of**

integrated *Aedes aegypti* control in Mexico. *PLoS Neglected Tropical Diseases*, 16(4), p.e0010324.

Mashatola, T., Munhenga, G. and Koekemoer, L.L., 2017. **Evaluating different cages for rearing *Anopheles arabiensis* (Diptera: Culicidae) under laboratory conditions.** *African Entomology*, 25(2), pp.534-539.

Mashatola, T., Ndo, C., Koekemoer, L.L., Dandalo, L.C., Wood, O.R., Malakoane, L., Poumachu, Y., Lobb, L.N., Kaiser, M., Bourtzis, K. and Munhenga, G., 2018. **A review on the progress of sex-separation techniques for sterile insect technique applications against *Anopheles arabiensis*.** *Parasites and Vectors*, 11(2), pp.127-137.

McCray Jr, E.M., 1961. **A mechanical device for the rapid sexing of *Aedes aegypti* pupae.** *Journal of Economic Entomology*, 54(4).

McDonald, I.C., 1971. **A male-producing strain of the house fly.** *Science*, 172(3982), pp.489-489.

Miles, M., 2003. **The effects of spinosad, a naturally derived insect control agent to the honeybee.** *Bulletin of Insectology*, 56, pp.119-124.

Mitchell, S.E. and Seawright, J.A., 1989. **EMS-induced mutations in Anopheles quadrimaculatus (Say), species A.** *Journal of Heredity*, 80(1), pp.58-61.

Moonasar, D.M., Davies, C., Balawanth, R., Misiani, E., Shandukani, M.B., Raman, J. and Pillay, Y.G., 2021. **Progress, challenges and priorities for malaria elimination in South Africa.** *Transactions of the Royal Society of South Africa*, pp.1-12.

Moorefield, H.H., 1951. **Sexual dimorphism in mosquito pupae.** *Mosquito News*, 11, pp.175-177.

Morán-Aceves, B.M., Marina, C.F., Dor, A., Liedo, P. and Toledo, J., 2021. **Sex separation of *Aedes* spp. mosquitoes for sterile insect technique application: a review.** *Entomologia Experimentalis et Applicata*, 169(10), pp.918-927.

Moreno, Y., Nabhan, J.F., Solomon, J., Mackenzie, C.D. and Geary, T.G., 2010. **Ivermectin disrupts the function of the excretory-secretory apparatus in microfilariae of *Brugia malayi*.** *Proceedings of the National Academy of Sciences*, 107(46), pp.20120-20125.

Mozūraitis, R., Hajkazemian, M., Zawada, J.W., Szymczak, J., Palsson, K., Sekar, V., Biryukova, I., Friedländer, M.R., Koekemoer, L.L., Baird, J.K. and Borg-Karlson, A.K., 2020. **Male swarming aggregation pheromones increase female attraction and mating success**

among multiple African malaria vector mosquito species. *Nature Ecology and Evolution*, 4(10), pp.1395-1401.

Müller, G.C., Junnila, A., Qualls, W., Revay, E.E., Kline, D.L., Allan, S., Schlein, Y. and Xue, R., 2010. **Control of *Culex quinquefasciatus* in a storm drain system in Florida using attractive toxic sugar baits.** *Medical and Veterinary Entomology*, 24(4), pp.346-351.

Munhenga, G., Brooke, B.D., Chirwa, T.F., Hunt, R.H., Coetzee, M., Govender, D. and Koekemoer, L.L., 2011. **Evaluating the potential of the sterile insect technique for malaria control: relative fitness and mating compatibility between laboratory colonised and a wild population of *Anopheles arabiensis* from the Kruger National Park, South Africa.** *Parasites and Vectors*, 4(1), pp.1-11.

Munhenga, G., Brooke, B.D., Gilles, J.R., Slabbert, K., Kemp, A., Dandalo, L.C., Wood, O.R., Lobb, L.N., Govender, D., Renke, M. and Koekemoer, L.L., 2016. **Mating competitiveness of sterile genetic sexing strain males (GAMA) under laboratory and semi-field conditions: steps towards the use of the Sterile Insect Technique to control the major malaria vector *Anopheles arabiensis* in South Africa.** *Parasites and Vectors*, 9(1), pp.1-12.

Munhenga, G., Brooke, B.D., Spillings, B., Essop, L., Hunt, R.H., Midzi, S., Govender, D., Braack, L. and Koekemoer, L.L., 2014**. Field study site selection, species abundance and monthly distribution of anopheline mosquitoes in the northern Kruger National Park, South Africa.** *Malaria Journal*, 13(1), pp.1-11.

Munhenga, G., Oliver, S.V., Lobb, L.N., Mazarire, T.T., Sekgele, W., Mashatola, T., Mabaso, N., Dlamini, D.M., Zulu, M., Molestane, F. and Letinic, B.D., 2022. **Malaria risk and receptivity: Continuing development of insecticide resistance in the major malaria vector *Anopheles arabiensis* in northern KwaZulu-Natal, South Africa.** *South African Journal of Science*, *118*(3-4), pp.1-7.

Ndo, C., Poumachu, Y., Metitsi, D., Awono-Ambene, H.P., Tchuinkam, T., Gilles, J.L.R. and Bourtzis, K., 2018. **Isolation and characterization of a temperature-sensitive lethal strain of *Anopheles arabiensis* for SIT-based application.** *Parasites and Vectors*, 11(2), pp.97-105.

Nepgen, E.S., Hill, M.P. and Moore, S.D., 2015. **The effect of long-distance transportation on the fitness of irradiated false codling Moth (Lepidoptera: Tortricidae) for use in a sterile insect release program.** *Journal of Economic Entomology*, 108(6), pp.2610-2619.

Nguyen, T.N., Choo, A. and Baxter, S.W., 2021. **Lessons from Drosophila: engineering genetic sexing strains with temperature-sensitive lethality for sterile insect technique applications.** *Insects*, 12(3), p.243.

Noël, J.A., Kahwaji, S., Desgrosseilliers, L., Groulx, D. and White, M.A., 2022. **Phase change materials.** *In Storing Energy* (pp. 503-535). Elsevier.

Nolan, T., Papathanos, P., Windbichler, N., Magnusson, K., Benton, J., Catteruccia, F. and Crisanti, A., 2011. **Developing transgenic *Anopheles* mosquitoes for the sterile insect technique.** *Genetica*, 139, pp.33-39.

Ntoyi, N.L., Mashatola, T., Bouyer, J., Kraupa, C., Maiga, H., Mamai, W., Bimbile-Somda, N.S., Wallner, T., Carvalho, D.O., Munhenga, G. and Yamada, H., 2022. **Life-history traits of a fluorescent *Anopheles arabiensis* genetic sexing strain introgressed into South African genomic background.** *Malaria Journal*, *21*(1), pp.1-12.

Odendaal, D., Addison, M.F. and Malan, A.P., 2015. **Control of codling moth (*Cydia pomonella*) (Lepidoptera: Tortricidae) in South Africa with special emphasis on using entomopathogenic nematodes.** *African Entomology*, 23(2), pp.259-274.

Oliva, C.F., Benedict, M.Q., Collins, C.M., Baldet, T., Bellini, R., Bossin, H., Bouyer, J., Corbel, V., Facchinelli, L., Fouque, F. and Geier, M., 2021. **Sterile Insect Technique (SIT) against *Aedes* species mosquitoes: A roadmap and good practice framework for designing, implementing and evaluating pilot field trials.** *Insects*, 12(3), p.191.

Orozco, D., Hernández, M.R., Meza, J.S. and Quintero, J.L., 2013. **Do sterile females affect the sexual performance of sterile males of *Anastrepha ludens* (Diptera: Tephritidae)?**. *Journal of Applied Entomology*, 137(5), pp.321-326.

Pagabeleguem, S., Seck, M.T., Sall, B., Vreysen, M.J., Gimonneau, G., Fall, A.G., Bassene, M., Sidibé, I., Rayaissé, J.B., Belem, A.M. and Bouyer, J., 2015. **Long distance transport of irradiated male *Glossina palpalis gambiensis* pupae and its impact on sterile male yield.** *Parasites and Vectors*, 8(1), pp.1-9.

Papathanos, P.A., Bossin, H.C., Benedict, M.Q., Catteruccia, F., Malcolm, C.A., Alphey, L. and Crisanti, A., 2009. **Sex separation strategies: past experience and new approaches.** *Malaria Journal*, 8(2), pp.1-8.

Papathanos, P.A., Bourtzis, K., Tripet, F., Bossin, H., Virginio, J.F., Capurro, M.L., Pedrosa, M.C., Guindo, A., Sylla, L., Coulibaly, M.B. and Yao, F.A., 2018. **A perspective on the need and current status of efficient sex separation methods for mosquito genetic control.** *Parasites and Vectors*, 11(2), pp.165-171.

Pare Toe, L., Barry, N., Ky, A.D., Kekele, S., Meda, W., Bayala, K., Drabo, M., Thizy, D. and Diabate, A., 2021. **Small-scale release of non-gene drive mosquitoes in Burkina Faso: from engagement implementation to assessment, a learning journey.** *Malaria Journal*, 20(1), pp.1-18.

Pare Toe, L., Barry, N., Ky, A.D., Kekele, S., Meda, W.I., Bayala, K., Drabo, M., Thizy, D., Majorin, O., Coche, I. and Epopa, P.S., 2022. **A multi-disciplinary approach for building a common understanding of genetic engineering for malaria control in Burkina Faso.** *Humanities and Social Sciences Communications*, 9(1), pp.1-8.

Parker, A.G., Vreysen, M.J.B., Bouyer, J. and Calkins, C.O., 2021. **Sterile insect quality control/assurance.** *In Sterile insect technique* (pp. 399-440). CRC Press.

Poda, S.B., Nignan, C., Gnankiné, O., Dabiré, R.K., Diabaté, A. and Roux, O., 2019. **Sex aggregation and species segregation cues in swarming mosquitoes: role of ground visual markers.** *Parasites and Vectors*, 12, pp.1-15.

Raman, J., Gast, L., Balawanth, R., Tessema, S., Brooke, B., Maharaj, R., Munhenga, G., Tshikae, P., Lakan, V., Mwamba, T. and Makowa, H., 2020. **High levels of imported asymptomatic malaria but limited local transmission in KwaZulu-Natal, a South African malaria-endemic province nearing malaria elimination.** *Malaria Journal*, 19(1), pp.1-13.

Raman, J., Morris, N., Frean, J., Brooke, B., Blumberg, L., Kruger, P., Mabusa, A., Raswiswi, E., Shandukani, B., Misani, E. and Groepe, M.A., 2016. **Reviewing South Africa's malaria elimination strategy (2012–2018): progress, challenges and priorities.** *Malaria Journal*, 15(1), pp.1-11.

Robinson, A.S., 1986. **Genetic sexing in *Anopheles stephensi* using dieldrin resistance.** cabidigitallibrary.org, pp.93-95.

Rossler, Y., Ravins, E. and Gomes, P.J., 2000. **Sterile insect technique (SIT) in the near east—a transboundary bridge for development and peace.** *Crop Protection*, *19*(8-10), pp.733-738.

Sachs, J. and Malaney, P., 2002. **The economic and social burden of malaria.** *Nature*, 415(6872), pp.680-685.

Sakai, R.K. and Baker, R.H., 1974. **Induction of heat-sensitive lethals in *Culex tritaeniorhynchus* by ethyl methanesulfonate.** *Mosquito News*, 34(4), pp.420-424.

Sasmita, H.I., Ernawan, B., Sadar, M., Nasution, I.A., Indarwatmi, M., Tu, W.C. and Neoh, K.B., 2022. **Assessment of packing density and transportation effect on sterilized pupae and adult *Aedes aegypti* (Diptera: Culicidae) in non-chilled conditions.** *Acta Tropica*, 226, p.106243.

Schetelig, M.F. and Handler, A.M., 2012. **A transgenic embryonic sexing system for *Anastrepha suspensa* (Diptera: Tephritidae).** *Insect Biochemistry and Molecular Biology*, 42(10), pp.790-795.

Schumacher, R.F. and Spinelli, E., 2012. **Malaria in children.** *Mediterranean Journal of Hematology and Infectious Diseases*, 4(1).

Seawright, J.A., Kaiser, P.E., Dame, D.A. and Lofgren, C.S., 1978. **Genetic method for the preferential elimination of females of *Anopheles albimanus*.** *Science*, 200(4347), pp.1303-1304.

Seck, M.T., Pagabeleguem, S., Bassene, M.D., Fall, A.G., Diouf, T.A., Sall, B., Vreysen, M.J., Rayaisse, J.B., Takac, P., Sidibe, I. and Parker, A.G., 2015. **Quality of sterile male tsetse after long distance transport as chilled, irradiated pupae.** *PLoS Neglected Tropical Diseases*, 9(11), p.e0004229.

Sega, G.A., 1984. **A review of the genetic effects of ethyl methanesulfonate.** *Mutation Research/Reviews in Genetic Toxicology*, 134(2-3), pp.113-142.

Sharma, V.P., Patterson, R.S. and Ford, H.R., 1972. **A device for the rapid separation of male and female mosquito pupae.** *Bulletin of the World Health Organization*, *47*(3), p.429.

Sinka, M.E., Bangs, M.J., Manguin, S., Coetzee, M., Mbogo, C.M., Hemingway, J., Patil, A.P., Temperley, W.H., Gething, P.W., Kabaria, C.W. and Okara, R.M., 2010. **The dominant *Anopheles* vectors of human malaria in Africa, Europe and the Middle East: occurrence data, distribution maps and bionomic précis.** *Parasites and Vectors*, 3(1), pp.1-34.

Smidler, A.L., Terenzi, O., Soichot, J., Levashina, E.A. and Marois, E., 2013. **Targeted mutagenesis in the malaria mosquito using TALE nucleases.** *PloS one*, *8*(8), p.e74511.

Snow, R.W., Guerra, C.A., Noor, A.M., Myint, H.Y. and Hay, S.I., 2005. **The global distribution of clinical episodes of *Plasmodium falciparum* malaria.** *Nature*, *434*(7030), pp.214-217.

South Africa National Department of Health. 2019. **Malaria Elimination Strategic Plan for South Africa 2019–2023.** *Pretoria: South African National Department of Health*.

South Africa National Department of Health. 2019. **Malaria Elimination Strategic Plan for South Africa 2019–2023.** *Pretoria: South African National Department of Health*.

Suzuki, D.T., 1970. **Temperature-Sensitive Mutations in *Drosophila melanogaster*: Conditional lethality is a useful feature of mutations used in a variety of analyses in higher organisms.** *Science*, 170(3959), pp.695-706.

Tan, A., Fu, G., Jin, L., Guo, Q., Li, Z., Niu, B., Meng, Z., Morrison, N.I., Alphey, L. and Huang, Y., 2013. **Transgene-based, female-specific lethality system for genetic sexing of the silkworm, *Bombyx mori*.** *Proceedings of the National Academy of Sciences*, 110(17), pp.6766-6770.

Tangena, J.A.A., Hendriks, C.M., Devine, M., Tammaro, M., Trett, A.E., Williams, I., DePina, A.J., Sisay, A., Herizo, R., Kafy, H.T. and Chizema, E., 2020. **Indoor residual spraying for malaria control in sub-Saharan Africa 1997 to 2017: an adjusted retrospective analysis.** *Malaria Journal*, *19*(1), pp.1-15.

Tsoka-Gwegweni, J.M., 2022. **Status of malaria and its implications for elimination in an endemic province of South Africa: retrospective analysis.** *Pan African Medical Journal*, 41(1).

Tun-Lin, W., Burkot, T.R. and Kay, B.H., 2000. **Effects of temperature and larval diet on development rates and survival of the dengue vector *Aedes aegypti* in north Queensland, Australia.** *Medical and Veterinary Entomology*, 14(1), pp.31-37.

Tussey, D.A., Morreale, R., Carvalho, D.O., Stenhouse, S., Lloyd, A.M., Hoel, D.F. and Hahn, D.A., 2023. **Developing methods for chilling, compacting, and sterilizing adult *Aedes aegypti* (Diptera: Culicidae) and comparing mating competitiveness between males sterilized as adults versus pupae for sterile male release.** *Journal of Medical Entomology*, p.tjad079.

Van Emden, H.M., Kroon, C.C.M., Schoeman, E.N. and Van Seventer, H.A., 1974. **The toxicity of some detergents tested on *Aedes aegypti L., Lebistes reticulatus* Peters, and *Biomphalaria glabrata* (Say).** *Environmental Pollution (1970)*, 6(4), pp.297-308.

Vreysen, M.J., Abd-Alla, A.M., Bourtzis, K., Bouyer, J., Caceres, C., de Beer, C., Oliveira Carvalho, D., Maiga, H., Mamai, W., Nikolouli, K. and Yamada, H., 2021. **The insect pest control laboratory of the joint FAO/IAEA programme: ten years (2010–2020) of research and development, achievements and challenges in support of the sterile insect technique.** *Insects*, 12(4), p.346.

Vreysen, M.J., Saleh, K., Mramba, F., Parker, A., Feldmann, U., Dyck, V.A., Msangi, A. and Bouyer, J., 2014. **Sterile insects to enhance agricultural development: the case of sustainable tsetse eradication on Unguja Island, Zanzibar, using an area-wide integrated pest management approach.** *PLoS Neglected Tropical Diseases*, 8(5), p.e2857.

Wiltshire, R.M. and Duman-Scheel, M., 2020. **Advances in oral RNAi for disease vector mosquito research and control.** *Current Opinion in Insect Science*, *40*, pp.18-23.

World Health Organization (WHO), 2018. **Update on the E-2020 Initiative of 21 Malaria-Eliminating Countries. World Health Organization.** *World Health Organization*.

World Health Organization (WHO), 2023. **World malaria report 2023.** https://www.who.int/teams/global-malaria-programme/reports/world-malaria-report-2023 (accessed 24 December 2023).

World Health Organization, 2012. **Global plan for insecticide resistance management in malaria vectors.** *World Health Organization***.**

World Health Organization, 2015. **Indoor residual spraying: an operational manual for indoor residual spraying (IRS) for malaria transmission control and elimination.** *World Health Organization.*

World Health Organization, 2021. **Global insecticide use for vector-borne disease control: a 10-year assessment (2010–2019).** *World Health Organization*.

Yamada, H., Benedict, M.Q., Malcolm, C.A., Oliva, C.F., Soliban, S.M. and Gilles, J.R., 2012. **Genetic sex separation of the malaria vector, *Anopheles arabiensis*, by exposing eggs to dieldrin.** *Malaria Journal*, 11(1), pp.1-11.

Yamada, H., Soliban, S.M., Vreysen, M.J., Chadee, D.D. and Gilles, J.R., 2013. **Eliminating female *Anopheles arabiensis* by spiking blood meals with toxicants as a sex separation method in the context of the sterile insect technique.** *Parasites and Vectors*, 6, pp.1-10.

Yamada, H., Vreysen, M.J., Bourtzis, K., Tschirk, W., Chadee, D.D. and Gilles, J.R., 2015. **The *Anopheles arabiensis* genetic sexing strain ANO IPCL1 and its application potential for the sterile insect technique in integrated vector management programmes.** *Acta Tropica*, 142, pp.138-144.

Yan, Y. and Scott, M.J., 2015. **A transgenic embryonic sexing system for the Australian sheep blow fly *Lucilia cuprina*.** *Scientific Reports*, 5(1), pp.1-12.

Yan, Y., Williamson, M.E., Davis, R.J., Andere, A.A., Picard, C.J. and Scott, M.J., 2020. **Improved transgenic sexing strains for genetic control of the Australian sheep blow fly *Lucilia cuprina* using embryo-specific gene promoters.** *Molecular Genetics and Genomics*, 295, pp.287-298.

Yates, D.M. and Wolstenholme, A.J., 2004. **An ivermectin-sensitive glutamate-gated chloride channel subunit from *Dirofilaria immitis*.** *International Journal for Parasitology*, 34(9), pp.1075-1081.

Zavala-López, J.L. and Enkerlin, W.R., 2017. **Guideline for packing, shipping, holding and release of sterile flies in area-wide fruit fly control programmes (No. Ed. 2).** *Food and Agriculture Organization of the United Nations (FAO).* Rome, Italy. 140 pp.

Zhang, D., Xi, Z., Li, Y., Wang, X., Yamada, H., Qiu, J., Liang, Y., Zhang, M., Wu, Y. and Zheng, X., 2020. **Toward implementation of combined incompatible and sterile insect techniques for mosquito control: Optimized chilling conditions for handling *Aedes albopictus* male adults prior to release.** *PLOS Neglected Tropical Diseases*, 14(9), p.e0008561.

Zheng, X., Zhang, D., Li, Y., Yang, C., Wu, Y., Liang, X., Liang, Y., Pan, X., Hu, L., Sun, Q. and Wang, X., 2019. **Incompatible and sterile insect techniques combined eliminate mosquitoes.** *Nature*, 572(7767), pp.56-61.

Chapter 2

Ayesha, S.A., Christian, R. and Koekemoer, L., 2023. **Optimisation of adult Anopheles funestus blood-feeding on an artificial membrane feeding system.** *African Entomology*, 31.

Bailey, D.L., Dame, D.A., Munroe, W.L. and Thomas, J.A., 1978. **Colony maintenance of *Anopheles albimanus* Wiedemann by feeding preserved blood through natural membrane.** *Mosquito News*, 38, pp.403-408.

Baughman, T., Peterson, C., Ortega, C., Preston, S.R., Paton, C., Williams, J., Guy, A., Omodei, G., Johnson, B., Williams, H. and O'Neill, S.L., 2017. **A highly stable blood meal alternative for rearing *Aedes* and Anopheles mosquitoes.** *PLOS Neglected Tropical Diseases*, 11(12), p.e0006142.

Benedict, M.Q., Knols, B.G., Bossin, H.C., Howell, P.I., Mialhe, E., Caceres, C. and Robinson, A.S., 2009. **Colonisation and mass rearing: learning from others.** *Malaria Journal*, 8, pp.1-11.

Bishop, A. and Gilchrist, B.M., 1946. **Experiments upon the feeding of *Aedes aegypti* through animal membranes with a view to applying this method to the chemotherapy of malaria.** *Parasitology*, *37*(1-2), pp.85-100.

Chambers, G.M. and Klowden, M.J., 2001. **Age of *Anopheles gambiae* Giles male mosquitoes at time of mating influences female oviposition.** *Journal of Vector Ecology: Journal of the Society for Vector Ecology*, 26(2), pp.196-201.

Charlwood, J.D. and Jones, M.D.R., 1979. **Mating behaviour in the mosquito, *Anopheles gambiae s. 1*. save: I. Close range and contact behaviour.** *Physiological Entomology*, *4*(2), pp.111-120.

Cosgrove, J.B., Wood, R.J., Petrić, D., Evans, D.T. and Abbott, R.H., 1994. **A convenient mosquito membrane feeding system.** *Journal of the American Mosquito Control Association*, 10(3), pp.434-436.

Costa-da-Silva, A.L., Navarrete, F.R., Salvador, F.S., Karina-Costa, M., Ioshino, R.S., Azevedo, D.S., Rocha, D.R., Romano, C.M. and Capurro, M.L., 2013. **Glytube: a conical tube and parafilm M-based method as a simplified device to artificially blood-feed the dengue vector mosquito, *Aedes aegypti*.** *PloS One*, *8*(1), p.e53816.

Costanzo, K.S., Schelble, S., Jerz, K. and Keenan, M., 2015. **The effect of photoperiod on life history and blood-feeding activity in *Aedes albopictus* and *Aedes aegypti* (Diptera: Culicidae).** *Journal of Vector Ecology*, *40*(1), pp.164-171.

Damiens, D., Soliban, S.M., Balestrino, F., Alsir, R., Vreysen, M.J.B. and Gilles, J.R.L., 2013. **Different blood and sugar feeding regimes affect the productivity of *Anopheles arabiensis* colonies (Diptera: Culicidae).** *Journal of Medical Entomology*, 50(2), pp.336-343.

de Almeida Costa, K., Garcia Rosário, I.N., Eiras, Á.E. and Da Silva, I.M., 2020. **Artificial bloodfeeder glytube: evaluating different types of membranes and blood sources for feeding *Aedes aegypti* and *Aedes albopictus*.** *Journal of the American Mosquito Control Association*, 36(4), pp.233-239.

de Boer, H.D., Korteweg, F.J., Wagenaar, L.S. and Smid-Nanninga, H., 2022. **Maintenance of normothermia.** In The ERAS® *Society Handbook for Obstetrics and Gynecology* (pp. 97-106). Academic Press.

de Swart, M.M., Balvers, C., Verhulst, N.O. and Koenraadt, C.J., 2023. **Effects of host blood on mosquito reproduction.** *Trends in Parasitology*, 39(7).

Deng, L., Koou, S.Y., Png, A.B., Ng, L.C. and Lam-Phua, S.G., 2012. **A novel mosquito feeding system for routine blood-feeding of *Aedes aegypti* and *Aedes albopictus*.** *Tropical Biomed*, 29(1), pp.169-74.

Dias, L.D.S., Bauzer, L.G.S.D.R. and Lima, J.B.P., 2018. **Artificial blood feeding for Culicidae colony maintenance in laboratories: does the blood source condition matter?.** *Revista do Instituto de Medicina Tropical de São Paulo*, 60.

Dias, L.S., Caldeira, J.C., Bauzer, L.G. and Lima, J.B., 2021. **Assessment of synthetic membranes for artificial blood feeding of Culicidae.** *Insects*, 12(1), p.15.

Faber, P.A., Dorai, A.J. and Chown, S.L., 2022. **A standardised low-cost membrane blood-feeder for *Aedes aegypti* made using common laboratory materials.** *PeerJ*, 10, p.e14247.

FAO/IAEA, 2017. **Guidelines for Standardised Mass Rearing of *Anopheles* Mosquitoes**—Version 1.0., p. 44.

Foster, W.A., 1980. **Colonization and maintenance of mosquitoes in the laboratory.** In *Pathology, Vector Studies, and Culture* (pp. 103-151). Academic Press.

Galun, R.A.C.H.E.L., 1967. **Feeding stimuli and artificial feeding.** *Bulletin of the World Health Organization*, 36(4), p.590.

Ghaninia, M., Larsson, M., Hansson, B.S. and Ignell, R., 2008. **Natural odor ligands for olfactory receptor neurons of the female mosquito *Aedes aegypti*: use of gas chromatography-linked single sensillum recordings.** *Journal of Experimental Biology*, 211(18), pp.3020-3027.

Harrington, L.C., Foy, B.D. and Bangs, M.J., 2020. **Considerations for human blood-feeding and arthropod exposure in vector biology research: an essential tool for investigations and disease control.** *Vector-Borne and Zoonotic Diseases*, 20(11), pp.807-816.

Hunt, R.H., Brooke, B.D., Pillay, C., Koekemoer, L.L. and Coetzee, M., 2005. **Laboratory selection for and characteristics of pyrethroid resistance in the malaria vector *Anopheles funestus*.** *Medical and Veterinary Entomology*, 19(3), pp.271-275.

Hurd, H., Hogg, J.C. and Renshaw, M., 1995. **Interactions between blood feeding, fecundity and infection in mosquitoes.** *Parasitology Today*, 11(11), pp.411-416.

IBM Corp, 2015. **IBM SPSS Statistics for Windows, Version 23.0.** *Armonk, NY: IBM Corp*.

Kasap, H., Alptekin, D., Kasap, M., Güzel, A.I. and Lüleyap, U., 2003. **Artificial blood feeding of *Anopheles sacharovi* on a membrane apparatus.** *Journal of the American Mosquito Control Association*, 19(4), pp.367-370.

Klowden, M.J. and Russell, R.C., 2004. **Mating affects egg maturation in *Anopheles gambiae* Giles (Diptera: Culicidae).** *Journal of Vector Ecology*, 29, pp.135-139.

Lauwereyns, J., 2018. **Rethinking the three R's in animal research: Replacement, reduction, refinement.** *Springer*.

Luo, Y.P., 2014. **A novel multiple membrane blood-feeding system for investigating and maintaining *Aedes aegypti* and *Aedes albopictus* mosquitoes.** *Journal of Vector Ecology*, *39*(2), pp.271-277.

Lyski, Z.L., Saredy, J.J., Ciano, K.A., Stem, J. and Bowers, D.F., 2011. **Blood feeding position increases success of recalcitrant mosquitoes.** *Vector-Borne and Zoonotic Diseases*, *11*(8), pp.1165-1171.

Malaria Research and Reference Reagent Resource (MR4) Center--MR4, 2015. **Methods in Anopheles Research.** https://www.beiresources.org/Portals/2/VectorResources/2016%20Methods%20i%20Anopheles%20Research%20full%20manual.pdf.

Mamai, W., Bimbile-Somda, N.S., Maiga, H., Juarez, J.G., Muosa, Z.A., Ali, A.B., Lees, R.S. and Gilles, J.R., 2017. **Optimization of mosquito egg production under mass rearing setting: effects of cage volume, blood meal source and adult population density for the malaria vector, *Anopheles arabiensis*.** *Malaria Journal*, 16(1), pp.1-10.

Melgarejo-Colmenares, K., Cardo, M.V. and Vezzani, D., 2022. **Blood feeding habits of mosquitoes: hardly a bite in South America.** *Parasitology Research*, 121(7), pp.1829-1852.

Melgarejo-Colmenares, K., Vezzani, D., Gallego, A. and Cardo, M.V., 2023. **Blood meal sources of mosquitoes (Diptera: Culicidae) in domestic and open green environments from two urbanisations of temperate Argentina.** *Bulletin of Entomological Research*, pp.1-11.

Meza, F.C., Kreppel, K.S., Maliti, D.F., Mlwale, A.T., Mirzai, N., Killeen, G.F., Ferguson, H.M. and Govella, N.J., 2019. **Mosquito electrocuting traps for directly measuring biting rates and host-preferences of *Anopheles arabiensis* and *Anopheles funestus* outdoors.** Malaria journal, 18(1), pp.1-11.

Mlacha, Y.P., Chaki, P.P., Muhili, A., Massue, D.J., Tanner, M., Majambere, S., Killen, G.F. and Govella, N.J., 2020. **Reduced human-biting preferences of the African malaria vectors *Anopheles arabiensis* and *Anopheles gambiae* in an urban context: controlled, competitive host-preference experiments in Tanzania.** *Malaria Journal*, 19(1), pp.1-8.

Mouatcho, J.C., Munhenga, G., Hargreaves, K., Brooke, B.D., Coetzee, M. and Koekemoer, L.L., 2009. **Pyrethroid resistance in a major African malaria vector *Anopheles arabiensis* from Mamfene, northern KwaZulu-Natal, South Africa.** *South African Journal of Science*, 105(3-4), pp.127-131.

Nasirian, H. and Ladonni, H., 2006. **Artificial blood feeding of *Anopheles stephensi* on a membrane apparatus with human whole blood.** *Journal of the American Mosquito Control Association*, 22(1), pp.54-56.

Ndebele, P. and Musesengwa, R., 2012. **Ethical dilemmas in malaria vector research in Africa: making the difficult choice between mosquito, science and humans.** *Malawi Medical Journal*, 24(3), pp.65-68.

Novak, M.G., Berry, W.J. and Rowley, W.A., 1991. **Comparison of four membranes for artificially blood feeding mosquitoes.** *Journal of the American Mosquito Control Association*, 7(2), pp.327-329.

Ooi, C.P., Ahmad, R., Ismail, Z. and Chua, W.S., 2005. **Effectiveness of several locally available membranes used for artificial feeding of *Aedes albopictus* Skuse.** *Tropical biomedicine*, *22*(1), pp.69-71.

Phasomkusolsil, S., Tawong, J., Monkanna, N., Pantuwatana, K., Damdangdee, N., Khongtak, W., Kertmanee, Y., Evans, B.P. and Schuster, A.L., 2013. **Maintenance of mosquito vectors: effects of blood source on feeding, survival, fecundity, and egg hatching rates.** *Journal of Vector Ecology*, *38*(1), pp.38-45.

Pina, I.G. and da Fonseca, A.H., 1999. **Comportamento de *Aedes aegypti* L., 1762 (Diptera: Culicidae) alimentados artificialmente com sangue de diferentes espécies de doadores.** *Revista de Patologia Tropical/Journal of Tropical Pathology*, 28(1).

Pothikasikorn, J., Boonplueang, R., Suebsaeng, C., Khaengraeng, R. and Chareonviriyaphap, T., 2010. **Feeding response of *Aedes aegypti* and *Anopheles dirus* (Diptera: Culicidae) using out-of-date human blood in a membrane feeding apparatus.** *Journal of Vector Ecology*, 35(1), pp.149-155.

Ribeiro, C.C.D.U., Cordeiro, M.D., Cepeda, P.B., Valim, J.R.A., Fonseca, A.H. and de Azevedo Baêta, B., 2023. **Artificial feeding of *Ornithodoros rostratus* using a silicone membrane system.** *Parasitology Research*, *122*(5), pp.1213-1219.

Ross, P.A., Lau, M.J. and Hoffmann, A.A., 2019. **Does membrane feeding compromise the quality of Aedes aegypti mosquitoes?.** *PLoS One*, 14(11), p.e0224268.

Sanz, G., Thomas-Danguin, T., Hamdani, E.H., Le Poupon, C., Briand, L., Pernollet, J.C., Guichard, E. and Tromelin, A., 2008. **Relationships between molecular structure and perceived odor quality of ligands for a human olfactory receptor.** *Chemical Senses*, 33(7), pp.639-653.

Sattabongkot, J., Maneechai, N., Phunkitchar, V., Eikarat, N., Khuntirat, B., Sirichaisinthop, J., Burge, R. and Coleman, R.E., 2003. **Comparison of artificial membrane feeding with direct skin feeding to estimate the infectiousness of *Plasmodiun vivax* gametocyte carriers to mosquitoes.** *American Journal of Tropical Medicine and Hygiene*, 69(5), p.529.

Sawadogo, S.P., Costantini, C., Pennetier, C., Diabaté, A., Gibson, G. and Dabiré, R.K., 2013. **Differences in timing of mating swarms in sympatric populations of *Anopheles coluzzii* and *Anopheles gambiae ss* (formerly *An. gambiae* M and S molecular forms) in Burkina Faso, West Africa.** *Parasites and Vectors*, *6*(1), pp.1-14.

Sheppard, A.D., Rund, S.S., George, G.F., Clark, E., Acri, D.J. and Duffield, G.E., 2017. **Light manipulation of mosquito behaviour: acute and sustained photic suppression of biting activity in the *Anopheles gambiae* malaria mosquito.** *Parasites and Vectors*, 10(1), pp.1-14.

Siria, D.J., Batista, E.P., Opiyo, M.A., Melo, E.F., Sumaye, R.D., Ngowo, H.S., Eiras, A.E. and Okumu, F.O., 2018. **Evaluation of a simple polytetrafluoroethylene (PTFE)-based membrane for blood-feeding of malaria and dengue fever vectors in the laboratory.** *Parasites and Vectors*, 11, pp.1-10.

Suresh, Y., Azil, A.H. and Abdullah, S.R., 2024. **A scoping review on the use of different blood sources and components in the artificial membrane feeding system and its effects on blood-feeding and fecundity rate of *Aedes aegypti*.** *Plos one*, 19(1), p.e0295961.

Takken, W. and Verhulst, N.O., 2013. **Host preferences of blood-feeding mosquitoes.** *Annual Review of Entomology*, 58, pp.433-453.

Timinao, L., Vinit, R., Katusele, M., Schofield, L., Burkot, T.R. and Karl, S., 2021. **Optimization of the feeding rate of *Anopheles farauti ss* colony mosquitoes in direct membrane feeding assays.** *Parasites and Vectors*, 14(1), p.356.

Tirados, I., Costantini, C., Gibson, G. and Torr, S.J., 2006. **Blood-feeding behaviour of the malarial mosquito *Anopheles arabiensis*: implications for vector control.** *Medical and Veterinary Entomology*, 20(4), pp.425-437.

Vallejo, A.F., Rubiano, K., Amado, A., Krystosik, A.R., Herrera, S. and Arevalo-Herrera, M., 2016. **Optimization of a membrane feeding assay for *Plasmodium vivax* infection in *Anopheles albimanus*.** *PLoS Neglected Tropical Diseases*, 10(6), p.e0004807.

Chapter 3

Ali, M., Alam, S., Ahmad, S., Dinda, A.K. and Ahmad, F.J., 2011. **Determination of ivermectin stability by high-performance thin-layer chromatography.** *International Journal of Drug Development and Research*, 3, pp.240-247.

Canga, A.G., Prieto, A.M.S., Liébana, M.J.D., Martínez, N.F., Vega, M.S. and Vieitez, J.J.G., 2009. **The pharmacokinetics and metabolism of ivermectin in domestic animal species.** *The Veterinary Journal*, *179*(1), pp.25-37.

Chaccour, C., Barrio, Á.I., Royo, A.G.G., Urbistondo, D.M., Slater, H., Hammann, F. and Del Pozo, J.L., 2015. **Screening for an ivermectin slow-release formulation suitable for malaria vector control.** *Malaria Journal*, 14(1), pp.1-9.

Chaccour, C., Hammann, F. and Rabinovich, N.R., 2017. **Ivermectin to reduce malaria transmission I. Pharmacokinetic and pharmacodynamic considerations regarding efficacy and safety.** *Malaria Journal*, *16*(1), pp.1-16.

Chaccour, C.J., Ngha'bi, K., Abizanda, G., Irigoyen Barrio, A., Aldaz, A., Okumu, F., Slater, H., Del Pozo, J.L. and Killeen, G., 2018. **Targeting cattle for malaria elimination: marked reduction of *Anopheles arabiensis* survival for over six months using a slow-release ivermectin implant formulation.** *Parasites and Vectors*, 11, pp.1-9.

Chambers, G.M. and Klowden, M.J., 2001. **Age of *Anopheles gambiae* Giles male mosquitoes at time of mating influences female oviposition.** *Journal of Vector Ecology: Journal of the Society for Vector Ecology*, 26(2), pp.196-201.

Charlwood, J.D., Thompson, R. and Madsen, H., 2003. **Observations on the swarming and mating behaviour of *Anopheles funestus* from southern Mozambique.** *Malaria Journal*, *2*(1), pp.1-10.

Clements, A.N., 1992. **The biology of mosquitoes. Volume 1: development, nutrition and reproduction.** *Chapman and Hall*.

Dandalo, L.C., Brooke, B.D., Munhenga, G., Lobb, L.N., Zikhali, J., Ngxongo, S.P., Zikhali, P.M., Msimang, S., Wood, O.R., Mofokeng, M. and Misiani, E., 2017a. **Population dynamics and *Plasmodium falciparum* (Haemosporida: Plasmodiidae) infectivity rates for the malaria vector *Anopheles arabiensis* (Diptera: Culicidae) at Mamfene, KwaZulu-Natal, South Africa.** *Journal of Medical Entomology*, 54(6), pp.1758-1766.

Dandalo, L.C., Munhenga, G., Kaiser, M.L. and Koekemoer, L.L., 2018. **Development of a genetic sexing strain of *Anopheles arabiensis* for KwaZulu-Natal, South Africa.** *Medical and Veterinary Entomology*, 32(1), pp.61-69.

Das, S., Lee, S.H., Chia, V.D., Chow, P.S., Macbeath, C., Liu, Y. and Shlieout, G., 2020. **Development of microemulsion based topical ivermectin formulations: Pre-formulation and formulation studies.** *Colloids and Surfaces B: Biointerfaces*, 189, p.110823.

Dawes, E.J., Churcher, T.S., Zhuang, S., Sinden, R.E. and Basáñez, M.G., 2009. ***Anopheles* mortality is both age-and Plasmodium-density dependent: implications for malaria transmission.** *Malaria Journal*, *8*(1), pp.1-16.

Dyck, V.A., Hendrichs, J. and Robinson, A.S., 2021. **Sterile insect technique: principles and practice in area-wide integrated pest management** (p. 1216). *Taylor and Francis*.

Focks, D.A., McLaughlin, R.E. and Linda, S.B., 1991. **Effects of ivermectin (MK-933) on the reproductive rate of *Aedes aegypti* (Diptera: Culicidae).** *Journal of Medical Entomology*, 28(4), pp.501-505.

Gilles, J.R., Schetelig, M.F., Scolari, F., Marec, F., Capurro, M.L., Franz, G. and Bourtzis, K., 2014. **Towards mosquito sterile insect technique programmes: exploring genetic, molecular, mechanical and behavioural methods of sex separation in mosquitoes.** *Acta Tropica*, 132, pp.S178-S187.

Gunathilaka, N., Ranathunge, T., Udayanga, L. and Abeyewickreme, W., 2017**. Efficacy of blood sources and artificial blood feeding methods in rearing of *Aedes aegypti* (Diptera: Culicidae) for sterile insect technique and incompatible insect technique approaches in Sri Lanka.** *BioMed Research International*, 2017.

Hadlett, M., Nagi, S.C., Sarkar, M., Paine, M.J. and Weetman, D., 2021. **High concentrations of membrane-fed ivermectin are required for substantial lethal and sublethal impacts on *Aedes aegypti*.** *Parasites and Vectors*, *14*(1), pp.1-8.

Howell, P.I. and Knols, B.G., 2009. **Male mating biology.** *Malaria Journal*, *8*(2), pp.1-10.

Kobylinski, K.C., Alout, H., Foy, B.D., Clements, A., Adisakwattana, P., Swierczewski, B.E. and Richardson, J.H., 2014. **Rationale for the coadministration of albendazole and ivermectin**

to humans for malaria parasite transmission control. *The American Journal of Tropical Medicine and Hygiene*, *91*(4), p.655.

Lahondère, C. and Lazzari, C.R., 2012. **Mosquitoes cool down during blood feeding to avoid overheating.** *Current Biology*, 22(1), pp.40-45.

Lo, P.K.A., Fink, D.W., Williams, J.B. and Blodinger, J., 1985. **Pharmacokinetic studies of ivermectin: effects of formulation.** *Veterinary Research Communications*, 9(1), pp.251-268.

Lowe, R.E., Fowler, J.E.F., Bailey, D.L., Dame, D.A. and Savage, K.E., 1981. **Separation of sexes of adult *Anopheles albimanus* by feeding of insecticide-laden blood.** *Mosquito News*, 41(4), pp.634-638.

Mashatola, T., Ndo, C., Koekemoer, L.L., Dandalo, L.C., Wood, O.R., Malakoane, L., Poumachu, Y., Lobb, L.N., Kaiser, M., Bourtzis, K. and Munhenga, G., 2018. **A review on the progress of sex-separation techniques for sterile insect technique applications against *Anopheles arabiensis*.** *Parasites and Vectors*, 11(2), pp.127-137.

Moreno, Y., Nabhan, J.F., Solomon, J., Mackenzie, C.D. and Geary, T.G., 2010. **Ivermectin disrupts the function of the excretory-secretory apparatus in microfilariae of *Brugia malayi*.** *Proceedings of the National Academy of Sciences*, 107(46), pp.20120-20125.

Munhenga, G., Brooke, B.D., Chirwa, T.F., Hunt, R.H., Coetzee, M., Govender, D. and Koekemoer, L.L., 2011. **Evaluating the potential of the sterile insect technique for malaria control: relative fitness and mating compatibility between laboratory colonised and a wild population of *Anopheles arabiensis* from the Kruger National Park, South Africa.** *Parasites and Vectors*, 4(1), pp.1-11.

Munhenga, G., Brooke, B.D., Gilles, J.R., Slabbert, K., Kemp, A., Dandalo, L.C., Wood, O.R., Lobb, L.N., Govender, D., Renke, M. and Koekemoer, L.L., 2016. **Mating competitiveness of sterile genetic sexing strain males (GAMA) under laboratory and semi-field conditions: steps towards the use of the Sterile Insect Technique to control the major malaria vector *Anopheles arabiensis* in South Africa.** *Parasites and Vectors*, 9(1), pp.1-12.

Munhenga, G., Brooke, B.D., Spillings, B., Essop, L., Hunt, R.H., Midzi, S., Govender, D., Braack, L. and Koekemoer, L.L., 2014**. Field study site selection, species abundance and monthly**

distribution of anopheline mosquitoes in the northern Kruger National Park, South Africa. *Malaria Journal*, 13(1), pp.1-11.

Papathanos, P.A., Bossin, H.C., Benedict, M.Q., Catteruccia, F., Malcolm, C.A., Alphey, L. and Crisanti, A., 2009. **Sex separation strategies: past experience and new approaches.** *Malaria Journal*, 8(2), pp.1-8.

Papathanos, P.A., Bourtzis, K., Tripet, F., Bossin, H., Virginio, J.F., Capurro, M.L., Pedrosa, M.C., Guindo, A., Sylla, L., Coulibaly, M.B. and Yao, F.A., 2018. **A perspective on the need and current status of efficient sex separation methods for mosquito genetic control.** *Parasites and Vectors*, 11(2), pp.165-171.

Pooda, S.H., Moiroux, N., Porciani, A., Courjaud, A.L., Roberge, C., Gaudriault, G., Sidibé, I., Belem, A.M.G., Rayaissé, J.B., Dabiré, R.K. and Mouline, K., 2023. **Proof-of-concept study for a long-acting formulation of ivermectin injected in cattle as a complementary malaria vector control tool.** *Parasites and Vectors*, *16*(1), pp.1-12.

Ramírez-Sánchez, L.F., Hernández, B.J., Guzmán, P.A., Alfonso-Parra, C. and Avila, F.W., 2023. **The effects of female age on blood-feeding, insemination, sperm storage, and fertility in the dengue vector mosquito *Aedes aegypti* (Diptera: Culicidae).** *Journal of Insect Physiology*, 150, p.104570.

Sagna, A.B., Zéla, L., Ouedraogo, C.O.W., Pooda, S.H., Porciani, A., Furnival-Adams, J., Lado, P., Somé, A.F., Pennetier, C., Chaccour, C.J. and Dabiré, R.K., 2023. **Ivermectin as a novel malaria control tool: Getting ahead of the resistance curse.** *Acta Tropica*, 245, p.106973.

Smit, M.R., Ochomo, E.O., Aljayyoussi, G., Kwambai, T.K., Abong'o, B.O., Bousema, T., Waterhouse, D., Bayoh, N.M., Gimnig, J.E., Samuels, A.M. and Desai, M.R., 2019. **Human direct skin feeding versus membrane feeding to assess the mosquitocidal efficacy of high-dose ivermectin (IVERMAL Trial).** *Clinical Infectious Diseases*, *69*(7), pp.1112-1119.

Yamada, H., Soliban, S.M., Vreysen, M.J., Chadee, D.D. and Gilles, J.R., 2013. **Eliminating female *Anopheles arabiensis* by spiking blood meals with toxicants as a sex separation method in the context of the sterile insect technique.** *Parasites and Vectors*, 6, pp.1-10.

Yates, D.M. and Wolstenholme, A.J., 2004. **An ivermectin-sensitive glutamate-gated chloride channel subunit from *Dirofilaria immitis*.** *International Journal for Parasitology*, 34(9), pp.1075-1081.

Zheng, X., Zhang, D., Li, Y., Yang, C., Wu, Y., Liang, X., Liang, Y., Pan, X., Hu, L., Sun, Q. and Wang, X., 2019. **Incompatible and sterile insect techniques combined eliminate mosquitoes.** *Nature*, 572(7767), pp.56-61.

Chapter 4

Agyekum, T.P., Arko-Mensah, J., Botwe, P.K., Hogarh, J.N., Issah, I., Dwomoh, D., Billah, M.K., Dadzie, S.K., Robins, T.G. and Fobil, J.N., 2022. **Effects of Elevated Temperatures on the Growth and Development of Adult *Anopheles gambiae* (*sl*) (Diptera: Culicidae) Mosquitoes.** *Journal of Medical Entomology*, 59(4), pp.1413-1420.

Andrewartha, H.G., 2012. **Introduction to the Study of Animal Populations: Reprint edition.** *Springer Science and Business Media*.

Augustinos, A.A., Misbah-ul-Haq, M., Carvalho, D.O., de la Fuente, L.D., Koskinioti, P. and Bourtzis, K., 2020. **Irradiation induced inversions suppress recombination between the M locus and morphological markers in *Aedes aegypti*.** *BMC Genetics*, 21(2), pp.1-10.

Bayoh, M.N. and Lindsay, S.W., 2003. **Effect of temperature on the development of the aquatic stages of *Anopheles gambiae sensu stricto* (Diptera: Culicidae).** *Bulletin of Entomological Research*, 93(5), pp.375-381.

Bayoh, M.N., 2001. **Studies on the development and survival of *Anopheles gambiae sensu stricto* at various temperatures and relative humidities** (Doctoral dissertation, Durham University).

Bourtzis, K. and Tu, Z.J., 2018. **Joint FAO/IAEA Coordinated Research Project on "Exploring genetic, molecular, mechanical and behavioural methods of sex separation in mosquitoes"–an introduction.** *Parasites and Vectors*, *11*, pp.1-3.

Bourtzis, K., Lees, R.S., Hendrichs, J. and Vreysen, M.J., 2016. **More than one rabbit out of the hat: radiation, transgenic and symbiont-based approaches for sustainable management of mosquito and tsetse fly populations.** *Acta Tropica*, *157*, pp.115-130.

Christiansen-Jucht, C.D., Parham, P.E., Saddler, A., Koella, J.C. and Basáñez, M.G., 2015. **Larval and adult environmental temperatures influence the adult reproductive traits of *Anopheles gambiae ss*.** *Parasites and Vectors*, 8(1), pp.1-12.

Clements, A.N., 1992. **The biology of mosquitoes. Volume 1: development, nutrition and reproduction.** *Chapman and Hall*.

FAO/IAEA, 2017. **Guidelines for Standardised Mass Rearing of *Anopheles* Mosquitoes**—Version 1.0., p. 44.

FAO/IAEA, 2023. **IAEA CRP third research coordination meeting: Generic Approach for the Development of Genetic Sexing Strains for SIT Applications (D44003).** https://www.iaea.org/sites/default/files/23/08/d44003_crp_rcm_3_report_for_website_final_20230713.pdf. Accessed 23 December 2023.

Franz, G., 2005. **Genetic sexing strains in Mediterranean fruit fly, an example for other species amenable to large-scale rearing for the sterile insect technique.** In Sterile Insect Technique (pp. 427-451). *Springer, Dordrecht*.

Franz, G., Bourtzis, K. and Cáceres, C., 2021b. **Practical and operational genetic sexing systems based on classical genetic approaches in fruit flies, an example for other species amenable to large-scale rearing for the sterile insect technique.** *In Sterile Insect Technique* (pp. 575-604). CRC Press.

Franz, G., Kerremans, P., Rendon, P. and Hendrichs, J., 2021a. **Development and application of genetic sexing systems for the Mediterranean fruit fly based on a temperature sensitive lethal.** In *Fruit Fly Pests* (pp. 185-191). CRC Press.

Gilles, J.R., Schetelig, M.F., Scolari, F., Marec, F., Capurro, M.L., Franz, G. and Bourtzis, K., 2014. **Towards mosquito sterile insect technique programmes: exploring genetic, molecular, mechanical and behavioural methods of sex separation in mosquitoes.** *Acta Tropica*, 132, pp.S178-S187.

Huang, J., Walker, E.D., Vulule, J. and Miller, J.R., 2006. **Daily temperature profiles in and around Western Kenyan larval habitats of *Anopheles gambiae* as related to egg mortality.** *Malaria Journal*, 5, pp.1-9.

Koskinioti, P., Augustinos, A.A., Carvalho, D.O., Misbah-ul-Haq, M., Pillwax, G., de la Fuente, L.D., Salvador-Herranz, G., Herrero, R.A. and Bourtzis, K., 2021. **Genetic sexing strains for the population suppression of the mosquito vector *Aedes aegypti*.** *Philosophical Transactions of the Royal Society B*, 376(1818), p.20190808.

Krebs, R.A. and Loeschcke, V., 1995. **Resistance to thermal stress in preadult *Drosophila buzzatii*: variation among populations and changes in relative resistance across life stages.** *Biological Journal of the Linnean Society*, 56(4), pp.517-531.

Lactin, D.J., Holliday, N.J., Johnson, D.L. and Craigen, R., 1995. **Improved rate model of temperature-dependent development by arthropods.** *Environmental Entomology*, 24(1), pp.68-75.

Lyons, C.L., Coetzee, M., Terblanche, J.S. and Chown, S.L., 2012. **Thermal limits of wild and laboratory strains of two African malaria vector species, *Anopheles arabiensis* and *Anopheles funestus*.** *Malaria Journal*, 11(1), pp.1-14.

Mashatola, T., Ndo, C., Koekemoer, L.L., Dandalo, L.C., Wood, O.R., Malakoane, L., Poumachu, Y., Lobb, L.N., Kaiser, M., Bourtzis, K. and Munhenga, G., 2018. **A review on the progress of sex-separation techniques for sterile insect technique applications against *Anopheles arabiensis*.** *Parasites and Vectors*, 11(2), pp.127-137.

McDonald, G., McLaren, I.W., Shelden, G.P. and Smith, I.R., 1980. **The effect of temperature on the population growth potential of *Culex annulirostris* Skuse (Diptera: Culicidae).** *Australian Journal of Ecology*, 5(4), pp.379-384.

Ndo, C., Poumachu, Y., Metitsi, D., Awono-Ambene, H.P., Tchuinkam, T., Gilles, J.L.R. and Bourtzis, K., 2018. **Isolation and characterization of a temperature-sensitive lethal strain of *Anopheles arabiensis* for SIT-based application.** *Parasites and Vectors*, 11(2), pp.97-105.

Nguyen, T.N., Choo, A. and Baxter, S.W., 2021. **Lessons from Drosophila: engineering genetic sexing strains with temperature-sensitive lethality for sterile insect technique applications.** *Insects*, 12(3), p.243.

Papathanos, P.A., Bossin, H.C., Benedict, M.Q., Catteruccia, F., Malcolm, C.A., Alphey, L. and Crisanti, A., 2009. **Sex separation strategies: past experience and new approaches.** *Malaria Journal*, 8(2), pp.1-8.

Papathanos, P.A., Bourtzis, K., Tripet, F., Bossin, H., Virginio, J.F., Capurro, M.L., Pedrosa, M.C., Guindo, A., Sylla, L., Coulibaly, M.B. and Yao, F.A., 2018. **A perspective on the need and current status of efficient sex separation methods for mosquito genetic control.** *Parasites and Vectors*, 11(2), pp.165-171.

Patil, N.S., Lole, K.S. and Deobagkar, D.N., 1996. **Adaptive larval thermotolerance and induced cross-tolerance to propoxur insecticide in mosquitoes *Anopheles stephensi* and *Aedes aegypti*.** *Medical and Veterinary Entomology*, 10(3), pp.277-282.

R Development Core Team 2008; https://www.R-project.org/)

Raghavendra, K., Barik, T.K. and Adak, T., 2010b. **Development of larval thermotolerance and its impact on adult susceptibility to malathion insecticide and Plasmodium vivax infection in *Anopheles stephensi*.** *Parasitology Research*, 107, pp.1291-1297.

Raghavendra, K., Barik, T.K. and Swain, V., 2010a. **Studies on the impact of thermal stress on survival and development of adaptive thermotolerance in immature stages of *Anopheles culicifacies*.** *Journal of Ecobiotechnology*, 2(5).

Rayah, E.A.E. and Groun, N.A.A., 1984. **Effect of temperature on hatching eggs and embryonic survival in the mosquito *Culex quinquefasciatus***, 349-351.

Robinson, A.S. and Van Heemert, C., 1982. ***Ceratitis capitata*—a suitable case for genetic sexing.** *Genetica*, *58*, pp.229-237.

Robinson, A.S., 1999. **Genetic sexing strains in the medfly, *Ceratitis capitata*: development, mass rearing and field application.** *Trends in Entomology*, *2*, pp.81-104.

Rocca, K.A., Gray, E.M., Costantini, C. and Besansky, N.J., 2009. **2La chromosomal inversion enhances thermal tolerance of *Anopheles gambiae* larvae.** *Malaria Journal*, 8, pp.1-7.

Sivan, A., Shriram, A.N., Vanamail, P. and Sugunan, A.P., 2021. **Thermotolerance and acclimation in the immature stages of *aedes aegypti* (L) (Diptera: Culicidae) to simulated thermal stress.** *International Journal of Tropical Insect Science*, 41, pp.333-344.

Zepeda-Cisneros, C.S., Hernández, J.S.M., García-Martínez, V., Ibañez-Palacios, J., Zacharopoulou, A. and Franz, G., 2014. **Development, genetic and cytogenetic analyses of genetic sexing strains of the Mexican fruit fly, *Anastrepha ludens* Loew (Diptera: Tephritidae).** *BMC Genetics*, 15(2), pp.1-11.

Chapter 5

Augustinos, A.A., Targovska, A., Cancio-Martinez, E., Schorn, E., Franz, G., Cáceres, C., Zacharopoulou, A. and Bourtzis, K., 2017. ***Ceratitis capitata*** **genetic sexing strains: laboratory evaluation of strains from mass-rearing facilities worldwide.** *Entomologia Experimentalis et Applicata*, 164(3), pp.305-317.

Benedict, M.Q., Besansky, N.J., Chang, H., Mukabayire, O. and Collins, F.H., 1996. **Mutations in the *Anopheles gambiae* pink-eye and white genes define distinct, tightly linked eye-color loci.** *Journal of Heredity*, 87(1), pp.48-53.

Caceres, C., 2002. **Mass rearing of temperature sensitive genetic sexing strains in the Mediterranean fruit fly (*Ceratitis capitata*).** *Genetica*, 116(1), pp.107-116.

Choi, K.S., Christian, R., Nardini, L., Wood, O.R., Agubuzo, E., Muleba, M., Munyati, S., Makuwaza, A., Koekemoer, L.L., Brooke, B.D. and Hunt, R.H., 2014. **Insecticide resistance and role in malaria transmission of *Anopheles funestus* populations from Zambia and Zimbabwe.** *Parasites and Vectors*, 7(1), pp.1-8.

Clements, A.N., 1992. **The biology of mosquitoes. Volume 1: development, nutrition and reproduction.** *Chapman and Hall*.

Cotton, R.G.H., 1993. **Current methods of mutation detection.** *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis*, *285*(1), pp.125-144.

DATAtab, 2022. **DATAtab: Online Statistics Calculator.** DATAtab e.U. Graz, Austria. URL https://datatab.net.

Dyck, V.A., Hendrichs, J. and Robinson, A.S., 2005. **Sterile insect technique: principles and practice in area-wide integrated pest management** (p. 1216). *Taylor and Francis*.

Dyck, V.A., Hendrichs, J. and Robinson, A.S., 2021. **Sterile insect technique: principles and practice in area-wide integrated pest management** (p. 1216). *Taylor and Francis*.

FAO/IAEA, 2021. **Mutation induction**. Available at: https://www.iaea.org/topics/mutation-induction.

Franz, G., 2002. **Recombination between homologous autosomes in medfly (*Ceratitis capitata*) males: type-1 recombination and the implications for the stability of genetic sexing strains.** *Genetica*, 116(1), pp.73-84.

Franz, G., Bourtzis, K. and Cáceres, C., 2021b. **Practical and operational genetic sexing systems based on classical genetic approaches in fruit flies, an example for other species amenable to large-scale rearing for the sterile insect technique.** *In Sterile Insect Technique* (pp. 575-604). CRC Press.

Franz, G., Kerremans, P., Rendon, P. and Hendrichs, J., 2021a. **Development and application of genetic sexing systems for the Mediterranean fruit fly based on a temperature sensitive lethal.** In *Fruit Fly Pests* (pp. 185-191). CRC Press.

IBM Corp, 2015. **IBM SPSS Statistics for Windows, Version 23.0.** *Armonk, NY: IBM Corp*.

Kaiser, M.L., Duncan, F.D. and Brooke, B.D., 2014. **Embryonic development and rates of metabolic activity in early and late hatching eggs of the major malaria vector *Anopheles gambiae*.** *Plos one*, 9(12), p.e114381.

Mahdieh, N. and Rabbani, B., 2013. **An overview of mutation detection methods in genetic disorders.** *Iranian Journal of Pediatrics*, *23*(4), p.375.

Mashatola, T., Ndo, C., Koekemoer, L.L., Dandalo, L.C., Wood, O.R., Malakoane, L., Poumachu, Y., Lobb, L.N., Kaiser, M., Bourtzis, K. and Munhenga, G., 2018. **A review on the progress of sex-separation techniques for sterile insect technique applications against *Anopheles arabiensis*.** *Parasites and Vectors*, 11(2), pp.127-137.

Meibalan, E. and Marti, M., 2017. **Biology of malaria transmission.** *Cold Spring Harbor Perspectives in Medicine*, 7(3), p.a025452.

Meza, J.S., Bourtzis, K., Zacharopoulou, A., Gariou-Papalexiou, A. and Cáceres, C., 2020. **Development and characterization of a pupal-colour based genetic sexing strain of *Anastrepha fraterculus sp. 1* (Diptera: Tephritidae).** *BMC Genetics*, 21(2), pp.1-9.

Ndo, C., Poumachu, Y., Metitsi, D., Awono-Ambene, H.P., Tchuinkam, T., Gilles, J.L.R. and Bourtzis, K., 2018. **Isolation and characterization of a temperature-sensitive lethal strain of *Anopheles arabiensis* for SIT-based application.** *Parasites and Vectors*, 11(2), pp.97-105.

Nguyen, T.N., Choo, A. and Baxter, S.W., 2021. **Lessons from Drosophila: engineering genetic sexing strains with temperature-sensitive lethality for sterile insect technique applications.** *Insects*, 12(3), p.243.

Orozco, D., Meza, J.S., Zepeda, S., Solís, E. and Quintero-Fong, J.L., 2013. **Tapachula-7, a new genetic sexing strain of the Mexican fruit fly (Diptera: Tephritidae): sexual compatibility and competitiveness.** *Journal of Economic Entomology*, 106(2), pp.735-741.

Porras, M.F., Meza, J.S., Rajotte, E.G., Bourtzis, K. and Cáceres, C., 2020. **Improving the phenotypic properties of the *Ceratitis capitata* (Diptera: Tephritidae) temperature-sensitive lethal genetic sexing strain in support of sterile insect technique applications.** *Journal of Economic Entomology*, *113*(6), pp.2688-2694.

Quintero-Fong, L., Luis, J.H., Montoya, P. and Orozco-Dávila, D., 2018. **In situ sexual competition between sterile males of the genetic sexing Tapachula-7 strain and wild *Anastrepha ludens* flies.** *Crop Protection*, 106, pp.1-5.

Rössler, Y., 1979. **Automated sexing of *Ceratitis capitata* [Dip.: Tephritidae]: the development of strains with inherited, sex-limited pupal color dimorphism.** *Entomophaga*, 24(4), pp.411-416.

Seawright, J.A., Childress, L.V. and Benedict, M.Q., 1979. **Genetics of green larva, a recessive mutant on chromosome 2 in *Anopheles albimanus*.** *Mosquito News*, 39, pp.55-58.

Stearns, F.W., 2010. **One hundred years of pleiotropy: a retrospective.** *Genetics*, 186(3), pp.767-773.

Tan, G., Chen, M., Foote, C. and Tan, C., 2009. **Temperature-sensitive mutations made easy: generating conditional mutations by using temperature-sensitive inteins that function within different temperature ranges.** *Genetics*, *183*(1), pp.13-22.

Vargas-Terán, M., Spradbery, J.P., Hofmann, H.C. and Tweddle, N.E., 2021. **Impact of screwworm eradication programmes using the sterile insect technique.** *In Sterile insect technique* (pp. 949-978). CRC Press.

Warren, M., Richardson, B.B. and Collins, W.E., 1975. **Pupal pleomorphism in a strain of *Anopheles albimanus* from El Salvador [Insect pests].** *Mosquito News*.

Chapter 6

Abbott, R., Albach, D., Ansell, S., Arntzen, J.W., Baird, S.J., Bierne, N., Boughman, J., Brelsford, A., Buerkle, C.A., Buggs, R. and Butlin, R.K., 2013. **Hybridization and speciation.** *Journal of Evolutionary Biology*, 26(2), pp.229-246.

Augustinos, A.A., Nikolouli, K., Duran de la Fuente, L., Misbah-ul-Haq, M., Carvalho, D.O. and Bourtzis, K., 2022. **Introgression of the *Aedes aegypti* red-eye genetic sexing strains into different genomic backgrounds for sterile insect technique applications.** *Frontiers in Bioengineering and Biotechnology*, 10, p.821428.

Carvalho, D.O., Torres-Monzon, J.A., Koskinioti, P., Dilrukshi Wijegunawardana, N.A., Liang, X., Pillwax, G., Xi, Z. and Bourtzis, K., 2020. ***Aedes aegypti* lines for combined sterile insect technique and incompatible insect technique applications: the importance of host genomic background**. *Entomologia Experimentalis et Applicata*, *168*(6-7), pp.560-572.

Chaverra-Rodriguez, D., Macias, V.M., Hughes, G.L., Pujhari, S., Suzuki, Y., Peterson, D.R., Kim, D., McKeand, S. and Rasgon, J.L., 2018. **Targeted delivery of CRISPR-Cas9 ribonucleoprotein into arthropod ovaries for heritable germline gene editing.** *Nature Communications*, 9(1), p.3008.

Choi, K.S., Christian, R., Nardini, L., Wood, O.R., Agubuzo, E., Muleba, M., Munyati, S., Makuwaza, A., Koekemoer, L.L., Brooke, B.D. and Hunt, R.H., 2014. **Insecticide resistance and role in malaria transmission of *Anopheles funestus* populations from Zambia and Zimbabwe.** *Parasites and Vectors*, 7(1), pp.1-8.

Coetzee, M., Hunt, R.H., Wilkerson, R., Della Torre, A., Coulibaly, M.B. and Besansky, N.J., 2013. ***Anopheles coluzzii* and *Anopheles amharicus*, new members of the *Anopheles gambiae* complex.** *Zootaxa*, 3619(3), pp.246-274.

Curtis, C., 1976. **A genetic sexing system in *Anopheles gambiae* species A.** Mosquito News.

Curtis, C.F., 1978. **Genetic sex separation in *Anopheles arabiensis* and the production of sterile hybrids.** *Bulletin of the World Health Organization*, 56(3), p.453.

Dandalo, L.C., Munhenga, G., Kaiser, M.L. and Koekemoer, L.L., 2018. **Development of a genetic sexing strain of *Anopheles arabiensis* for KwaZulu-Natal, South Africa.** *Medical and Veterinary Entomology*, 32(1), pp.61-69.

DATAtab, 2022. **DATAtab: Online Statistics Calculator.** DATAtab e.U. Graz, Austria. URL https://datatab.net.

Deitz, K.C., 2017. **Population Genomics, Hybrid Sterility, & Gene Expression in the *Anopheles gambiae* Species Complex** (Doctoral dissertation).

Della Torre, A., Merzagora, L., Powell, J.R. and Coluzzi, M., 1997. **Selective introgression of paracentric inversions between two sibling species of the *Anopheles gambiae* complex.** *Genetics*, 146(1), pp.239-244.

FAO/IAEA, 2017. **Guidelines for Standardised Mass Rearing of *Anopheles* Mosquitoes**—Version 1.0., p. 44.

FAO/IAEA, 2019. **New CRP: Generic Approach for the Development of Genetic Sexing Strains for SIT Applications (D44003).** https://www.iaea.org/newscenter/news/new-crp-generic-approach-for-the-development-of-genetic-sexing-strains-for-sit-applications-d44003.

Gamez, S., 2020. **Improving genetic control strategies for insect vector control.** University of California, San Diego.

Haldane, J.B., 1922. **Sex ratio and unisexual sterility in hybrid animals.** *Journal of Genetics*, 12, pp.101-109.

Hedrick, P.W., 2013. **Adaptive introgression in animals: examples and comparison to new mutation and standing variation as sources of adaptive variation.** *Molecular Ecology*, 22(18), pp.4606-4618.

Huho, B.J., Ng'habi, K.R., Killeen, G.F., Nkwengulila, G., Knols, B.G. and Ferguson, H.M., 2006. **A reliable morphological method to assess the age of male *Anopheles gambiae*.** *Malaria Journal*, *5*(1), pp.1-11.

IBM Corp, 2015. **IBM SPSS Statistics for Windows, Version 23.0.** *Armonk, NY: IBM Corp*.

Lanzaro, G.C. and Tripet, F., 2003. **Gene flow among populations of *Anopheles gambiae*: a critical review.** *Ecological Aspects for Application of Genetically Modified Mosquitoes*, 109, p.132.

Lee, Y., Marsden, C.D., Norris, L.C., Collier, T.C., Main, B.J., Fofana, A., Cornel, A.J. and Lanzaro, G.C., 2013. **Spatiotemporal dynamics of gene flow and hybrid fitness between the M**

and S forms of the malaria mosquito, *Anopheles gambiae*. *Proceedings of the National Academy of Sciences*, 110(49), pp.19854-19859.

Mashatola, T., Ndo, C., Koekemoer, L.L., Dandalo, L.C., Wood, O.R., Malakoane, L., Poumachu, Y., Lobb, L.N., Kaiser, M., Bourtzis, K. and Munhenga, G., 2018. **A review on the progress of sex-separation techniques for sterile insect technique applications against *Anopheles arabiensis*.** *Parasites and Vectors*, 11(2), pp.127-137.

Misbah-ul-Haq, M., Carvalho, D.O., Duran De La Fuente, L., Augustinos, A.A. and Bourtzis, K., 2022. Genetic Stability and Fitness of Aedes Aegypti Red-Eye Genetic Sexing Strains with Pakistani Genomic Background for Sterile Insect Technique Applications. Frontiers in Bioengineering and Biotechnology, 10, p.871703.

National Research Council, 2004. **Methods and Mechanisms for Genetic Manipulation of Plants.** *Animals, and Microorganisms*.

Ndo, C., Poumachu, Y., Metitsi, D., Awono-Ambene, H.P., Tchuinkam, T., Gilles, J.L.R. and Bourtzis, K., 2018. **Isolation and characterization of a temperature-sensitive lethal strain of *Anopheles arabiensis* for SIT-based application.** *Parasites and Vectors*, 11(2), pp.97-105.

Ntoyi, N.L., Mashatola, T., Bouyer, J., Kraupa, C., Maiga, H., Mamai, W., Bimbile-Somda, N.S., Wallner, T., Carvalho, D.O., Munhenga, G. and Yamada, H., 2022. **Life-history traits of a fluorescent *Anopheles arabiensis* genetic sexing strain introgressed into South African genomic background.** *Malaria Journal*, *21*(1), pp.1-12.

Santolamazza, F., Mancini, E., Simard, F., Qi, Y., Tu, Z. and della Torre, A., 2008. **Insertion polymorphisms of SINE200 retrotransposons within speciation islands of *Anopheles gambiae* molecular forms.** *Malaria Journal*, 7(1), pp.1-10.

Schilthuizen, M., Giesbers, M.C.W.G. and Beukeboom, L.W., 2011. **Haldane's rule in the 21st century.** *Heredity*, 107(2), pp.95-102.

Scott, J.A., Brogdon, W.G. and Collins, F.H., 1993. **Identification of single specimens of the Anopheles gambiae complex by the polymerase chain reaction.** *The American journal of Tropical Medicine and Hygiene*, 49(4), pp.520-529.

Slotman, M., Torre, A.D. and Powell, J.R., 2004. **The genetics of inviability and male sterility in hybrids between *Anopheles gambiae* and *An. arabiensis*.** *Genetics*, 167(1), pp.275-287.

Slotman, M., Torre, A.D. and Powell, J.R., 2005. **Female sterility in hybrids between *Anopheles gambiae* and *An. arabiensis*, and the causes of Haldane's rule.** *Evolution*, 59(5), pp.1016-1026.

Temu, E.A., Hunt, R.H., Coetzee, M., Minjas, J.N. and Shiff, C.J., 1997. **Detection of hybrids in natural populations of the *Anopheles gambiae* complex by the rDNA-based, PCR method.** *Annals of Tropical Medicine and Parasitology*, 91(8), pp.963-965.

Touré, Y.T., Petrarca, V., Traore, S.F., Coulibaly, A., Maiga, H.M., Sankare, O., Sow, M., Di Deco, M.A. and Coluzzi, M., 1998. **The distribution and inversion polymorphism of chromosomally recognized taxa of the *Anopheles gambiae* complex in Mali, West Africa.** *Parassitologia*, 40(4), pp.477-511.

Tripet, F., Dolo, G. and Lanzaro, G.C., 2005. **Multilevel analyses of genetic differentiation in Anopheles gambiae ss reveal patterns of gene flow important for malaria-fighting mosquito projects.** *Genetics*, 169(1), pp.313-324.

Tripet, F., Toure, Y.T., Taylor, C.E., Norris, D.E., Dolo, G. and Lanzaro, G.C., 2001. **DNA analysis of transferred sperm reveals significant levels of gene flow between molecular forms of *Anopheles gambiae*.** *Molecular Ecology*, 10(7), pp.1725-1732.

Vicente, J.L., Clarkson, C.S., Caputo, B., Gomes, B., Pombi, M., Sousa, C.A., Antao, T., Dinis, J., Bottà, G., Mancini, E. and Petrarca, V., 2017. **Massive introgression drives species radiation at the range limit of *Anopheles gambiae*.** *Scientific Reports*, 7(1), p.46451.

Weetman, D., Steen, K., Rippon, E.J., Mawejje, H.D., Donnelly, M.J. and Wilding, C.S., 2014. **Contemporary gene flow between wild *An. gambiae ss* and *An. arabiensis*.** *Parasites and Vectors*, 7, pp.1-9.

Yuval, B. and Fritz, G.N., 1994. **Multiple mating in female mosquitoes—evidence from a field population of *Anopheles freeborni* (Diptera: Culicidae).** *Bulletin of Entomological Research*, 84(1), pp.137-139.

Benedict, M.Q. and Robinson, A.S., 2003. **The first releases of transgenic mosquitoes: an argument for the sterile insect technique.** *Trends in Parasitology*, 19(8), pp.349-355.

Bimbilé Somda, N.S., Yamada, H., Kraupa, C., Mamai, W., Maiga, H., Kotla, S.S., Wallner, T., Martina, C. and Bouyer, J., 2022. **Response of male adult *Aedes* mosquitoes to gamma radiation in different nitrogen environments.** *Frontiers in Bioengineering and Biotechnology*, 10, p.942654.

Calkins, C.O. and Parker, A.G., 2005. **Sterile insect quality.** In Sterile insect technique (pp. 269-296). *Springer, Dordrecht*.

Chung, H.N., Rodriguez, S.D., Gonzales, K.K., Vulcan, J., Cordova, J.J., Mitra, S., Adams, C.G., Moses-Gonzales, N., Tam, N., Cluck, J.W. and Attardo, G.M., 2018. **Toward implementation of mosquito sterile insect technique: the effect of storage conditions on survival of male *Aedes aegypti* mosquitoes (Diptera: Culicidae) during transport.** *Journal of Insect Science*, 18(6), p.2.

Culbert, N.J., 2020. **Improving the handling, transport and release of sterile male mosquitoes as part of an area-wide integrated pest management strategy.** The University of Liverpool (United Kingdom). https://agritrop.cirad.fr/596806/

Culbert, N.J., Maiga, H., Somda, N.S.B., Gilles, J.R.L., Bouyer, J. and Mamai, W., 2018a. **Longevity of mass-reared, irradiated and packed male *Anopheles arabiensis* and *Aedes aegypti* under simulated environmental field conditions.** *Parasites and Vectors*, 11(1), pp.1-8.

Culbert, N.J., Balestrino, F., Dor, A., Herranz, G.S., Yamada, H., Wallner, T. and Bouyer, J., 2018b. **A rapid quality control test to foster the development of genetic control in mosquitoes.** *Scientific Reports*, 8(1), p.16179.

Culbert, N.J., Lees, R.S., Vreysen, M.J., Darby, A.C. and Gilles, J.R., 2017. **Optimised conditions for handling and transport of male *Anopheles arabiensis*: effects of low temperature, compaction, and ventilation on male quality.** *Entomologia Experimentalis et Applicata*, 164(3), pp.276-283.

Dandalo, L.C., Brooke, B.D., Munhenga, G., Lobb, L.N., Zikhali, J., Ngxongo, S.P., Zikhali, P.M., Msimang, S., Wood, O.R., Mofokeng, M. and Misiani, E., 2017a. **Population dynamics and *Plasmodium falciparum* (Haemosporida: Plasmodiidae) infectivity rates for the malaria**

vector *Anopheles arabiensis* (Diptera: Culicidae) at Mamfene, KwaZulu-Natal, South Africa. *Journal of Medical Entomology*, 54(6), pp.1758-1766.

Dandalo, L.C., Kemp, A., Koekemoer, L.L. and Munhenga, G., 2017b. **Effect of ionising (gamma) radiation on female *Anopheles arabiensis*.** *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 111(1), pp.38-40.

Dandalo, L.C., Munhenga, G., Kaiser, M.L. and Koekemoer, L.L., 2018. **Development of a genetic sexing strain of *Anopheles arabiensis* for KwaZulu-Natal, South Africa.** *Medical and Veterinary Entomology*, 32(1), pp.61-69.

Dyck, V.A., Hendrichs, J. and Robinson, A.S., 2005. **Sterile insect technique: principles and practice in area-wide integrated pest management** (p. 1216). *Taylor and Francis*.

Dyck, V.A., Hendrichs, J. and Robinson, A.S., 2021. **Sterile insect technique: principles and practice in area-wide integrated pest management** (p. 1216). *Taylor and Francis*.

Ernawan, B., Anggraeni, T., Yusmalinar, S., Sasmita, H.I., Fitrianto, N. and Ahmad, I., 2022. **Assessment of compaction, temperature, and duration factors for packaging and transporting of sterile male *Aedes aegypti* (Diptera: Culicidae) under laboratory conditions.** *Insects*, 13(9), p.847.

Gómez, M., Macedo, A.T., Pedrosa, M.C., Hohana, F., Barros, V., Pires, B., Barbosa, L., Brito, M., Garziera, L., Argilés-Herrero, R. and Virginio, J.F., 2022. **Exploring conditions for handling packing and shipping Aedes aegypti Males to support an SIT field project in Brazil.** *Insects*, 13(10), p.871.

Hunt, R.H., Brooke, B.D., Pillay, C., Koekemoer, L.L. and Coetzee, M., 2005. **Laboratory selection for and characteristics of pyrethroid resistance in the malaria vector *Anopheles funestus*.** *Medical and Veterinary Entomology*, 19(3), pp.271-275.

Iyaloo, D.P., Facknath, S. and Bheecarry, A., 2020. **Investigating the effects of low temperature and compaction on the quality of adult radio-sterilised *Aedes albopictus* (Diptera: Culicidae) males in view of their optimal transport to the pilot sterile release site in Mauritius.** *International Journal of Tropical Insect Science*, 40(1), pp.53-62.

Kaiser, M.L., Wood, O.R., Damiens, D., Brooke, B.D., Koekemoer, L.L. and Munhenga, G., 2021. **Estimates of the population size and dispersal range of *Anopheles arabiensis* in**

Northern KwaZulu-Natal, South Africa: implications for a planned pilot programme to release sterile male mosquitoes. *Parasites and Vectors*, 14(1), pp.1-18.

Manana, P.N., Jewett, S., Zikhali, J., Dlamini, D., Mabaso, N., Mlambo, Z., Ngobese, R. and Munhenga, G., 2021. **"Maskandi experience": exploring the use of a cultural song for community engagement in preparation for a pilot Sterile Insect Technique release programme for malaria vector control in KwaZulu-Natal Province, South Africa 2019.** *Malaria Journal*, 20(1), pp.1-11.

Manana, P.N., Kuonza, L., Musekiwa, A., Mpangane, H.D. and Koekemoer, L.L., 2018. **Knowledge, attitudes and practices on malaria transmission in Mamfene, KwaZulu-Natal Province, South Africa 2015.** *BMC Public Health*, 18(1), pp.1-7.

Mashatola, T., Munhenga, G. and Koekemoer, L.L., 2017. **Evaluating different cages for rearing *Anopheles arabiensis* (Diptera: Culicidae) under laboratory conditions.** *African Entomology*, 25(2), pp.534-539.

Mashatola, T., Ndo, C., Koekemoer, L.L., Dandalo, L.C., Wood, O.R., Malakoane, L., Poumachu, Y., Lobb, L.N., Kaiser, M., Bourtzis, K. and Munhenga, G., 2018. **A review on the progress of sex-separation techniques for sterile insect technique applications against *Anopheles arabiensis*.** *Parasites and Vectors*, 11(2), pp.127-137.

Munhenga, G., Brooke, B.D., Chirwa, T.F., Hunt, R.H., Coetzee, M., Govender, D. and Koekemoer, L.L., 2011. **Evaluating the potential of the sterile insect technique for malaria control: relative fitness and mating compatibility between laboratory colonised and a wild population of *Anopheles arabiensis* from the Kruger National Park, South Africa.** *Parasites and Vectors*, 4(1), pp.1-11.

Munhenga, G., Brooke, B.D., Gilles, J.R., Slabbert, K., Kemp, A., Dandalo, L.C., Wood, O.R., Lobb, L.N., Govender, D., Renke, M. and Koekemoer, L.L., 2016. **Mating competitiveness of sterile genetic sexing strain males (GAMA) under laboratory and semi-field conditions: steps towards the use of the Sterile Insect Technique to control the major malaria vector *Anopheles arabiensis* in South Africa.** *Parasites and Vectors*, 9(1), pp.1-12.

Munhenga, G., Brooke, B.D., Spillings, B., Essop, L., Hunt, R.H., Midzi, S., Govender, D., Braack, L. and Koekemoer, L.L., 2014**. Field study site selection, species abundance and monthly**

distribution of anopheline mosquitoes in the northern Kruger National Park, South Africa. *Malaria Journal*, 13(1), pp.1-11.

Pagabeleguem, S., Seck, M.T., Sall, B., Vreysen, M.J., Gimonneau, G., Fall, A.G., Bassene, M., Sidibé, I., Rayaissé, J.B., Belem, A.M. and Bouyer, J., 2015. **Long distance transport of irradiated male *Glossina palpalis gambiensis* pupae and its impact on sterile male yield.** *Parasites and Vectors*, 8(1), pp.1-9.

Parker, A.G., Vreysen, M.J.B., Bouyer, J. and Calkins, C.O., 2021. **Sterile insect quality control/assurance.** *In Sterile insect technique* (pp. 399-440). CRC Press.

Sánchez-Aldana-Sánchez, G.A., Liedo, P., Bond, J.G. and Dor, A., 2023. **Release of sterile *Aedes aegypti* mosquitoes: chilling effect on mass-reared males survival and escape ability and on irradiated males sexual competitiveness.** *Scientific Reports*, 13(1), p.3797.

Somda, N.S.B., Maïga, H., Mamai, W., Bakhoum, T., Wallner, T., Poda, S.B., Yamada, H. and Bouyer, J., 2022. **Adult mosquito predation and potential impact on the sterile insect technique.** *Scientific Reports*, 12.

Tussey, D.A., Morreale, R., Carvalho, D.O., Stenhouse, S., Lloyd, A.M., Hoel, D.F. and Hahn, D.A., 2023. **Developing methods for chilling, compacting, and sterilizing adult *Aedes aegypti* (Diptera: Culicidae) and comparing mating competitiveness between males sterilized as adults versus pupae for sterile male release.** *Journal of Medical Entomology*, p.tjad079.

Yamada, H., Maiga, H., Kraupa, C., Mamai, W., Bimbilé Somda, N.S., Abrahim, A., Wallner, T. and Bouyer, J., 2022. **Effects of chilling and anoxia on the irradiation dose-response in adult *Aedes* mosquitoes.** *Frontiers in Bioengineering and Biotechnology*, 10, p.856780.

Zavala-López, J.L. and Enkerlin, W.R., 2017. **Guideline for packing, shipping, holding and release of sterile flies in area-wide fruit fly control programmes (No. Ed. 2).** *Food and Agriculture Organization of the United Nations (FAO).* Rome, Italy. 140 pp.

Zhang, D., Xi, Z., Li, Y., Wang, X., Yamada, H., Qiu, J., Liang, Y., Zhang, M., Wu, Y. and Zheng, X., 2020. **Toward implementation of combined incompatible and sterile insect techniques for mosquito control: Optimized chilling conditions for handling *Aedes albopictus* male adults prior to release.** *PLOS Neglected Tropical Diseases*, 14(9), p.e0008561.

Chapter 8

Benedict, M.Q. and Robinson, A.S., 2003. **The first releases of transgenic mosquitoes: an argument for the sterile insect technique.** *Trends in Parasitology*, 19(8), pp.349-355.

Bourtzis, K., Lees, R.S., Hendrichs, J. and Vreysen, M.J., 2016. **More than one rabbit out of the hat: radiation, transgenic and symbiont-based approaches for sustainable management of mosquito and tsetse fly populations.** *Acta Tropica*, 157, pp.115-130.

Bouyer, J., Culbert, N.J., Dicko, A.H., Pacheco, M.G., Virginio, J., Pedrosa, M.C., Garziera, L., Pinto, A.M., Klaptocz, A., Germann, J. and Wallner, T., 2020a. **Field performance of sterile male mosquitoes released from an uncrewed aerial vehicle.** *Science Robotics*, 5(43).

Brooke, B., Koekemoer, I., Kruger, P., Urbach, J., Misiani, E. and Coetzee, M., 2013. **Malaria vector control in South Africa.** *South African Medical Journal*, 103(10), pp.784-788.

Brooke, B.D., 2022. **Malaria vector surveillance and control in an elimination setting in South Africa.** *Tropical Medicine and Infectious Disease*, 7(11), p.391.

Brooke, B.D., Robertson, L., Kaiser, M.L., Raswiswi, E., Munhenga, G., Venter, N., Wood, O.R. and Koekemoer, L.L., 2015. **Insecticide resistance in the malaria vector *Anopheles arabiensis* in Mamfene, KwaZulu-Natal.** *South African Journal of Science*, 111(11-12), pp.1-3.

Caceres, C., 2002. **Mass rearing of temperature sensitive genetic sexing strains in the Mediterranean fruit fly (*Ceratitis capitata*).** *Genetica*, 116(1), pp.107-116.

Caldwell, J.M., LaBeaud, A.D., Lambin, E.F., Stewart-Ibarra, A.M., Ndenga, B.A., Mutuku, F.M., Krystosik, A.R., Ayala, E.B., Anyamba, A., Borbor-Cordova, M.J. and Damoah, R., 2021. **Climate predicts geographic and temporal variation in mosquito-borne disease dynamics on two continents.** *Nature Communications*, 12(1), p.1233.

Calkins, C.O. and Parker, A.G., 2005. **Sterile insect quality. Sterile insect technique: principles and practice in area-wide integrated pest management**, pp.269-296. *Dordrecht: Springer Netherlands*.

Colón-González, F.J., Sewe, M.O., Tompkins, A.M., Sjödin, H., Casallas, A., Rocklöv, J., Caminade, C. and Lowe, R., 2021. **Projecting the risk of mosquito-borne diseases in a warmer and more populated world: a multi-model, multi-scenario intercomparison modelling study.** *The Lancet Planetary Health*, *5*(7), pp.e404-e414.

Culbert, N.J., Lees, R.S., Vreysen, M.J., Darby, A.C. and Gilles, J.R., 2017. **Optimised conditions for handling and transport of male *Anopheles arabiensis*: effects of low temperature,**

compaction, and ventilation on male quality. *Entomologia Experimentalis et Applicata*, 164(3), pp.276-283.

Culbert, N.J., Maiga, H., Somda, N.S.B., Gilles, J.R.L., Bouyer, J. and Mamai, W., 2018. **Longevity of mass-reared, irradiated and packed male *Anopheles arabiensis* and *Aedes aegypti* under simulated environmental field conditions.** *Parasites and Vectors*, 11(1), pp.1-8.

Culbert, N.J., Somda, N.S.B., Hamidou, M., Soma, D.D., Caravantes, S., Wallner, T., Wadaka, M., Yamada, H. and Bouyer, J., 2020. **A rapid quality control test to foster the development of the sterile insect technique against *Anopheles arabiensis.*** *Malaria Journal*, 19(1), pp.1-10.

Dame, D.A., Curtis, C.F., Benedict, M.Q., Robinson, A.S. and Knols, B.G., 2009. **Historical applications of induced sterilisation in field populations of mosquitoes.** *Malaria Journal*, 8(2), pp.1-10.

Dandalo, L.C., Munhenga, G., Kaiser, M.L. and Koekemoer, L.L., 2018. **Development of a genetic sexing strain of *Anopheles arabiensis* for KwaZulu-Natal, South Africa.** *Medical and Veterinary Entomology*, 32(1), pp.61-69.

Dyck, V.A., Hendrichs, J. and Robinson, A.S., 2021. **Sterile insect technique: principles and practice in area-wide integrated pest management** (p. 1216). *Taylor and Francis*.

Elimination 8, 2019. **Southern African Democratic Community.** *Annual Report*.

FAO/IAEA, 2023. **IAEA CRP third research coordination meeting: Generic Approach for the Development of Genetic Sexing Strains for SIT Applications (D44003).** https://www.iaea.org/sites/default/files/23/08/d44003_crp_rcm_3_report_for_website_final_20230713.pdf. Accessed 23 December 2023.

Franz, G., 2005. **Genetic sexing strains in Mediterranean fruit fly, an example for other species amenable to large-scale rearing for the sterile insect technique.** In Sterile Insect Technique (pp. 427-451). *Springer, Dordrecht*.

Franz, G., Kerremans, P., Rendon, P. and Hendrichs, J., 2021. **Development and application of genetic sexing systems for the Mediterranean fruit fly based on a temperature sensitive lethal.** In *Fruit Fly Pests* (pp. 185-191). CRC Press.

Gatton, M.L., Chitnis, N., Churcher, T., Donnelly, M.J., Ghani, A.C., Godfray, H.C.J., Gould, F., Hastings, I., Marshall, J., Ranson, H. and Rowland, M., 2013. **The importance of mosquito behavioural adaptations to malaria control in Africa.** *Evolution: International Journal of Organic Evolution*, 67(4), pp.1218-1230.

Gilles, J.R., Schetelig, M.F., Scolari, F., Marec, F., Capurro, M.L., Franz, G. and Bourtzis, K., 2014. **Towards mosquito sterile insect technique programmes: exploring genetic, molecular, mechanical and behavioural methods of sex separation in mosquitoes.** *Acta Tropica*, 132, pp.S178-S187.

Guo, J., Zheng, X., Zhang, D. and Wu, Y., 2022. **Current Status of Mosquito Handling, Transporting and Releasing in Frame of the Sterile Insect Technique.** *Insects*, *13*(6), p.532.

Koskinioti, P., Augustinos, A.A., Carvalho, D.O., Misbah-ul-Haq, M., Pillwax, G., de la Fuente, L.D., Salvador-Herranz, G., Herrero, R.A. and Bourtzis, K., 2021. **Genetic sexing strains for the population suppression of the mosquito vector *Aedes aegypti*.** *Philosophical Transactions of the Royal Society B*, 376(1818), p.20190808.

Kreppel, K.S., Viana, M., Main, B.J., Johnson, P.C.D., Govella, N.J., Lee, Y., Maliti, D., Meza, F.C., Lanzaro, G.C. and Ferguson, H.M., 2020. **Emergence of behavioural avoidance strategies of malaria vectors in areas of high LLIN coverage in Tanzania.** *Scientific Reports*, 10(1), pp.1-11.

Lutrat, C., Burckbuchler, M., Olmo, R.P., Beugnon, R., Fontaine, A., Akbari, O.S., Argilés-Herrero, R., Baldet, T., Bouyer, J. and Marois, E., 2023. **Combining two genetic sexing strains allows sorting of non-transgenic males for *Aedes* genetic control.** *Communications Biology*, 6(1), p.646.

Marec, F., 1990. **Genetic control of pest Lepidoptera: induction of sex-linked recessive lethal mutations in *Ephestia kuehniella* (*Pyralidae*)**. *Acta Entomologica Bohemoslovaca*, 87(6), pp.445-458.

Mashatola, T., Ndo, C., Koekemoer, L.L., Dandalo, L.C., Wood, O.R., Malakoane, L., Poumachu, Y., Lobb, L.N., Kaiser, M., Bourtzis, K. and Munhenga, G., 2018. **A review on the progress of sex-separation techniques for sterile insect technique applications against *Anopheles arabiensis*.** *Parasites and Vectors*, 11(2), pp.127-137.

Meccariello, A., Salvemini, M., Primo, P., Hall, B., Koskinioti, P., Dalíková, M., Gravina, A., Gucciardino, M.A., Forlenza, F., Gregoriou, M.E. and Ippolito, D., 2019. **Maleness-on-the-Y (MoY) orchestrates male sex determination in major agricultural fruit fly pests.** *Science*, 365(6460), pp.1457-1460.

Munhenga, G., Brooke, B.D., Chirwa, T.F., Hunt, R.H., Coetzee, M., Govender, D. and Koekemoer, L.L., 2011. **Evaluating the potential of the sterile insect technique for malaria control: relative fitness and mating compatibility between laboratory**

colonised and a wild population of *Anopheles arabiensis* from the Kruger National Park, South Africa. *Parasites and Vectors*, 4(1), pp.1-11.

Munhenga, G., Brooke, B.D., Gilles, J.R., Slabbert, K., Kemp, A., Dandalo, L.C., Wood, O.R., Lobb, L.N., Govender, D., Renke, M. and Koekemoer, L.L., 2016. **Mating competitiveness of sterile genetic sexing strain males (GAMA) under laboratory and semi-field conditions: steps towards the use of the Sterile Insect Technique to control the major malaria vector *Anopheles arabiensis* in South Africa.** *Parasites and Vectors*, 9(1), pp.1-12.

Munhenga, G., Brooke, B.D., Spillings, B., Essop, L., Hunt, R.H., Midzi, S., Govender, D., Braack, L. and Koekemoer, L.L., 2014**. Field study site selection, species abundance and monthly distribution of anopheline mosquitoes in the northern Kruger National Park, South Africa.** *Malaria Journal*, 13(1), pp.1-11.

Ndo, C., Poumachu, Y., Metitsi, D., Awono-Ambene, H.P., Tchuinkam, T., Gilles, J.L.R. and Bourtzis, K., 2018. **Isolation and characterization of a temperature-sensitive lethal strain of *Anopheles arabiensis* for SIT-based application.** *Parasites and Vectors*, 11(2), pp.97-105.

Ntoyi, N.L., Mashatola, T., Bouyer, J., Kraupa, C., Maiga, H., Mamai, W., Bimbile-Somda, N.S., Wallner, T., Carvalho, D.O., Munhenga, G. and Yamada, H., 2022. **Life-history traits of a fluorescent *Anopheles arabiensis* genetic sexing strain introgressed into South African genomic background.** *Malaria Journal*, *21*(1), pp.1-12.

Pagabeleguem, S., Seck, M.T., Sall, B., Vreysen, M.J., Gimonneau, G., Fall, A.G., Bassene, M., Sidibé, I., Rayaissé, J.B., Belem, A.M. and Bouyer, J., 2015. **Long distance transport of irradiated male *Glossina palpalis gambiensis* pupae and its impact on sterile male yield.** *Parasites and Vectors*, 8(1), pp.1-9.

Papathanos, P.A., Bossin, H.C., Benedict, M.Q., Catteruccia, F., Malcolm, C.A., Alphey, L. and Crisanti, A., 2009. **Sex separation strategies: past experience and new approaches.** *Malaria Journal*, 8(2), pp.1-8.

Papathanos, P.A., Bourtzis, K., Tripet, F., Bossin, H., Virginio, J.F., Capurro, M.L., Pedrosa, M.C., Guindo, A., Sylla, L., Coulibaly, M.B. and Yao, F.A., 2018. **A perspective on the need and current status of efficient sex separation methods for mosquito genetic control.** *Parasites and Vectors*, 11(2), pp.165-171.

Parker, A.G., Vreysen, M.J.B., Bouyer, J. and Calkins, C.O., 2021. **Sterile insect quality control/assurance.** *In Sterile insect technique* (pp. 399-440). CRC Press.

Raman, J., Morris, N., Frean, J., Brooke, B., Blumberg, L., Kruger, P., Mabusa, A., Raswiswi, E., Shandukani, B., Misani, E. and Groepe, M.A., 2016. **Reviewing South Africa's malaria elimination strategy (2012–2018): progress, challenges and priorities.** *Malaria Journal*, 15(1), pp.1-11.

Ranson, H. and Lissenden, N., 2016. **Insecticide resistance in African Anopheles mosquitoes: a worsening situation that needs urgent action to maintain malaria control.** *Trends in Parasitology*, *32*(3), pp.187-196.

Sinka, M.E., Bangs, M.J., Manguin, S., Coetzee, M., Mbogo, C.M., Hemingway, J., Patil, A.P., Temperley, W.H., Gething, P.W., Kabaria, C.W. and Okara, R.M., 2010. **The dominant *Anopheles* vectors of human malaria in Africa, Europe and the Middle East: occurrence data, distribution maps and bionomic précis.** *Parasites and Vectors*, 3(1), pp.1-34.

Suh, P.F., Elanga-Ndille, E., Tchouakui, M., Sandeu, M.M., Tagne, D., Wondji, C. and Ndo, C., 2023. **Impact of insecticide resistance on malaria vector competence: a literature review.** *Malaria Journal*, *22*(1), pp.1-11.

Wiebe, A., Longbottom, J., Gleave, K., Shearer, F.M., Sinka, M.E., Massey, N.C., Cameron, E., Bhatt, S., Gething, P.W., Hemingway, J. and Smith, D.L., 2017. **Geographical distributions of African malaria vector sibling species and evidence for insecticide resistance.** *Malaria Journal*, *16*(1), pp.1-10.

Willhoeft, U. and Franz, G., 1996. **Identification of the sex-determining region of the *Ceratitis capitata* Y chromosome by deletion mapping.** *Genetics*, 144(2), pp.737-745.

World Health Organization (WHO), 2023. **World malaria report 2023.** https://www.who.int/teams/global-malaria-programme/reports/world-malaria-report-2023 (accessed 24 December 2023).

Yamada, H., Benedict, M.Q., Malcolm, C.A., Oliva, C.F., Soliban, S.M. and Gilles, J.R., 2012. **Genetic sex separation of the malaria vector, *Anopheles arabiensis*, by exposing eggs to dieldrin.** *Malaria Journal*, 11(1), pp.1-11.

Yamada, H., Soliban, S.M., Vreysen, M.J., Chadee, D.D. and Gilles, J.R., 2013. **Eliminating female *Anopheles arabiensis* by spiking blood meals with toxicants as a sex separation method in the context of the sterile insect technique.** *Parasites and Vectors*, 6, pp.1-10.

APPENDICES

Appendix 1: Protocol for routine laboratory colony rearing and maintenance as per Hunt *et al*. (2005), with some modifications.

Introduction

This protocol describes routine rearing and maintenance of *Anopheles* mosquito colonies at the Botha De Meillon Insectary (BDMI), National Institute for Communicable Diseases (NICD), Vector Control Reference Laboratory (VCRL), Johannesburg, South Africa. The insectary is under controlled standard conditions of 27 ± 1°C room temperature, 70 ± 10% relative humidity, 12:12 h photoperiod of light and dark interrupted by 45 minutes of dusk and dawn each cycle.

Materials needed

Plastic rearing trays (41 cm length × 30 cm width × 8 cm height) and plastic tubs (38 cm length × 32 cm width × 14 cm height), mosquito nets to cover the trays, distilled water for aquatic life stages, larval food (dog biscuits and yeast), mosquito cages to hold adults, 10% sterile sugar solution to sustain adults, sucking tubes to collect adults, guinea pigs to feed females so that they produce eggs, filter paper, cotton wool,

Procedure

Larval and pupal rearing

First, second and third instar larvae are transferred to plastic rearing trays filled with about 2.5 ℓ of distilled water (Figure S1.1). Larvae are fed twice daily with a mixture of brewers' yeast ((Vital Health Foods, South Africa) and finely ground dog biscuits (West's Beeno® Traditional Crunchy Biscuit Treats, Martin and Martin, South Africa) prepared at a ratio of 1:3. The dog biscuits are grinded using a planetary ball mill PM100 (Retsch® GMBH, Haan, Germany). The final particle size of the ingredient powder is between 50 and 150 µm. The powdered larval diet mixture is transferred into glass vials and passed through a mesh sieve to feed the larvae. Food quantity is adjusted according to the larval stage and number of larvae in bowl, roughly 0.2 – 0.8 mg per larvae per day/two shakes per feed two times a day. The pupae are allowed to emerge to adults for the next 2 – 3 days. Food is given every day to the larvae/pupae by carefully removing the net to avoid escaping of adults.

Prior to pupation, fourth instar larvae were transferred into 5 ℓ plastic tubs/containers containing 2.5 ℓ of distilled water and covered with meshed nets to prevent adult mosquitoes from escaping after eclosion.

Figure S1. 1: First to third larval stages reared in 41 × 30 × 8 cm^3 plastic rearing trays filled with about 2.5 ℓ distilled water b) fourth instar larvae and pupae reared in 38 × 32 × 14 cm^3 plastic tubs containing 2.5 ℓ of distilled water and covered with meshed nets to prevent adult mosquitoes from escaping after eclosion.

Preparation of cages for holding adult mosquitoes:

Post adult emergence, a mouth aspirator (made of separate glass tube and rubber tube (each 30 cm length × 1 cm diameter) that are connected and separated by a gauze to prevent mosquitoes from been swallowed) is used to transfer adults into a BugDorm-1® adult rearing cages (Figure S1.2). A 10% sugar solution, made by dissolving 100 g of sugar in 1 ℓ of distilled water is soaked on cotton balls, placed in urine jars and made available in each cage to sustain the adult mosquitoes. The mosquitoes are left to mate for 3 – 5 days. Sugar solution changed twice a week.

Figure S1. 2: A white polypropylene Bugdorm-1 cage used to house adult mosquitoes.

Blood feeding

Adult female mosquitoes are fed on anaesthetised guinea pigs (Ethics Clearance REF:W-CJ-100510-1, appendix 2), 3 – 5 days after emergence, thereafter twice weekly or twice in 5 days. All the blood feeding takes place during the induced night cycle in the insectary between 12:30 and 14:00. The guinea pigs are maintained in an animal husbandry at the National Health Laboratory Services and were shaven on one side of their abdomen for mosquitoes to easily access and feed. Blood feeding is done by placing the exposed abdomen of the unconscious guinea pig on top of the mesh side of the cage. For easy feeding in the Bugdorm-1® cage, the cover panels were displaced to the sides to allow one mesh panel to be on top. Feeding on the guinea pigs is done for 10 minutes in each cage.

Oviposition

Two days after the second blood meal, a plastic container (27 cm length × 16 cm width × 6.5 cm height) spray painted dark on the outside (egg plate) is placed inside each cage. The plate is filled with 100 mℓ of distilled water and left overnight in the cages (Figure S1.3). The following day eggs are removed from the cups and transferred to a small filter paper wrapped in a conical shape is put in a small, empty beaker. The eggs are washed/rinsed with 10%

formalin twice and distilled water once before been transferred into a small plastic larval rearing bowl containing 100 mℓ of distilled water and left to hatch. The cycle was repeated continuously.

Figure S1. 3**:** A dark painted egg plate that served as an oviposition site and a plastic bowl containing 150 mℓ of distilled water for egg harvesting.

Reference

Hunt, R.H., Brooke, B.D., Pillay, C., Koekemoer, L.L. and Coetzee, M., 2005. **Laboratory selection for and characteristics of pyrethroid resistance in the malaria vector *Anopheles funestus*.** *Medical and Veterinary Entomology*, 19(3), pp.271-275.

Appendix 2: Ethics waiver for research on mosquitoes

ETHICS WAIVER

Human Research Ethics Committee (Medical)

Research Office Secretariat: Senate House Room SH10005, 10th floor Tel +27 (0)11-717-1252
Medical School Secretariat: Tobias Health Sciences Building, 2nd floor Tel +27 (0)11-717-2700
Private Bag 3, Wits 2050, www.wits.ac.za Fax +27 (0)11-717-1255

Ref: W-CJ-150911-1 11/09/2015

TO WHOM IT MAY CONCERN

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

Investigator: **Prof Maureen Coetzee.**

Project title: **Research on mosquitos.**

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

UNIVERSITY OF THE WITWATERSRAND
2015 -09- 11

Professor Peter Cleaton-Jones

Chair: Human Research Ethics Committee (Medical)

Copy – HREC (Medical) Secretariat : Zanele Ndlovu

Appendix 3: Waiver from the Animal Research Ethics Committee of the University of the Witwatersrand

ANIMAL RESEARCH ETHICS COMMITTEE

Registration number: AREC-101210-002

Date: July 1, 2019

Certificate reference: 20190701-6O
Category: O
Re: Waiver from the Animal Research Ethics Committee of the University of the Witwatersrand

Student: Thabo Mashatola
Student number: 0500939P
Degree: PhD Candidate
Study Title: "Development of *Anopheles arabiensis* gender separation tools for the Sterile Insect Technique malaria control strategy in South Africa.".
Department: Wits Research Institute for Malaria; laboratory
Supervisor: Dr Givemore Munhenga and Prof Lizette Koekemoer
Email: givemorem@nicd.ac.za
Address: National Institute for Communicable Diseases, 1 Modderfontein Road, Sandringham, Johannesburg
Tel: 011-885-5413

Full clearance certificate from the AREC of the University of the Witwatersrand is NOT required.
Reason: This study uses mosquitoes (invertebrates).
Comment/Notes: Waiver issued.

Please contact me should you require further information.

GP Candy

Geoffrey Candy PhD
Chair : Animal Research Ethics Committee

Geoffrey P Candy PhD
Professor, Department of Surgery, Faculty of Health Sciences, University of the Witwatersrand, 7 York Road Parktown 2193, Johannesburg; Tel: 27-11-717-2574; Email: geoffrey.candy@wits.ac.za

Appendix 4: Use of EMS and γ to induce morphological variations in *An. arabiensis* larvae.

Introduction

The development of GSSs in programmes such as the SIT requires a visible or conditional lethal mutation as a selectable marker and its linkage (wild-type allele) to the male-determining locus (Franz, 2005). Males in the resulting strain exhibit the wild-type phenotype, whereas females have the recessive allele of the selectable marker and exhibit the mutant phenotype.

Several markers, including insecticide resistance, *tsl*, and morphological variations, have previously been used to develop GSSs in other insects (Franz, 2005; Zepeda-Cisneros *et al*., 2014; Meza *et al*., 2020). Variations in morphology has received the least attention in Anophelines, despite being reported to be stable and evident early in development, with full penetrance and expressivity, and in some cases already sex linked (Koskinioti *et al*., 2021). If used, the wild type gene can be linked to the male sex chromosome through chromosomal translocations, resulting in females that are different from the males. This will allow easy visual separation of sexes, for example, using sorting machines.

Phenotypic mutants that are expressed during early aquatic stages, such as larval stage can be useful in development of GSS. Several of such markers have been reported in mosquitoes, these include red stripe phenotype, which was apparent in *An. arabiensis* larvae (Mason, 1967), black larvae mutation in *An. stephensi* (Malcolm and Mali, 1986), green larvae in *Ae. aegypti* (Mukiama, 1985), etc. These morphological variations are commonly observed in nature and often occur spontaneously. However, their yield/expression and genetic diversity can be increased by using mutagenic agents.

Various mutagenic agents exist, including physical agents, chemical mutagens, biological agents, transgenic technologies, and so on (Ling and Robinson, 1997). All these mutagenic agents vary in effect or mode of action. Biological agents and transgenic technologies are still in their early stages of practical/operational/regular use. In addition, these methods are faced with obstacles of regulatory restrictions, public acceptance, and sustainability (Papathanos *et al*., 2018). Furthermore, in low-income countries, the skills and resources required to operate such complex technology are not always available or affordable. Hence, in this study the focus was directed at physical agents and chemicals mutagens that were accessible.

The use of physical agents and chemicals to induce mutations has been extensively demonstrated in various organisms, particularly plants (FAO/IAEA, 2021). Physical agents typically cause chromosomal rearrangements (deletions, translocations, and chromosomal aberrations) whereas chemical mutagens induce single nucleotide polymorphisms (SNPs) (FAO/IAEA, 2021). The most common physical and chemical mutagens are ionising-radiation and EMS respectively (FAO/IAEA, 2021). These mutagens vary in potency/degree of genetic diversity mutation. Various factors influence this, including the type of tissue or life stage of the biological material treated, the geographical origin of strains, the dosage and duration of treatment, and so on (LaChance and Graham, 1984; Bakri *et al*., 2005; FAO/IAEA, 2021). Against this background, this study aimed to determine the effectiveness of different γ irradiation and EMS doses in inducing morphological variations in *An. arabiensis* larvae.

Methods

Mosquito strain and rearing

The *An. arabiensis* strain, KWAG, described in 2.2.1 was used in this study. Rearing was performed as described in Hunt *et al*. (2005) and appendix 1.

Effect of various EMS and irradiation doses on selected reproductive traits

γ mutagenesis

(a) Pupal collection and irradiation procedure

Pupae were collected from rearing trays in the morning (8 a.m.) and then repeatedly at every 2 – 3 h intervals until the afternoon (4 – 6 p.m.). The pupae were placed in separate cups as per age (collection time) and enclosed in corresponding BugDorm-4® cages overnight. All pupae were separated into males and females under a microscope based on genital morphology the following day. Female pupae were placed in BugDorm-1® cages to emerge into adults. Male pupae (aged 20 – 22 h old) were placed in a doughnut-shaped irradiation jig containing 100 – 200 mℓ of distilled water (Figure S4.1) and subjected to seven different irradiation doses, including the control (0, 20, 25, 30, 35, 40, 45, and 50 Gy). A cobalt-60 (^{60}Co) γ irradiator (Gammacell220, MDS Nordion, Ottawa, Canada) located at the BDMI, NICD, Johannesburg; South Africa was used to administer a dose rate of 23.15 Gy/min. Dose mapping was previously performed on the irradiator (Dandalo, 2016; Munhenga *et al*., 2016). Post irradiation, pupae were placed in small plastic cups containing 100 mℓ of distilled water and placed in BugDorm-4® cages for pupae to emerge into adults and the following parameters measured as described below.

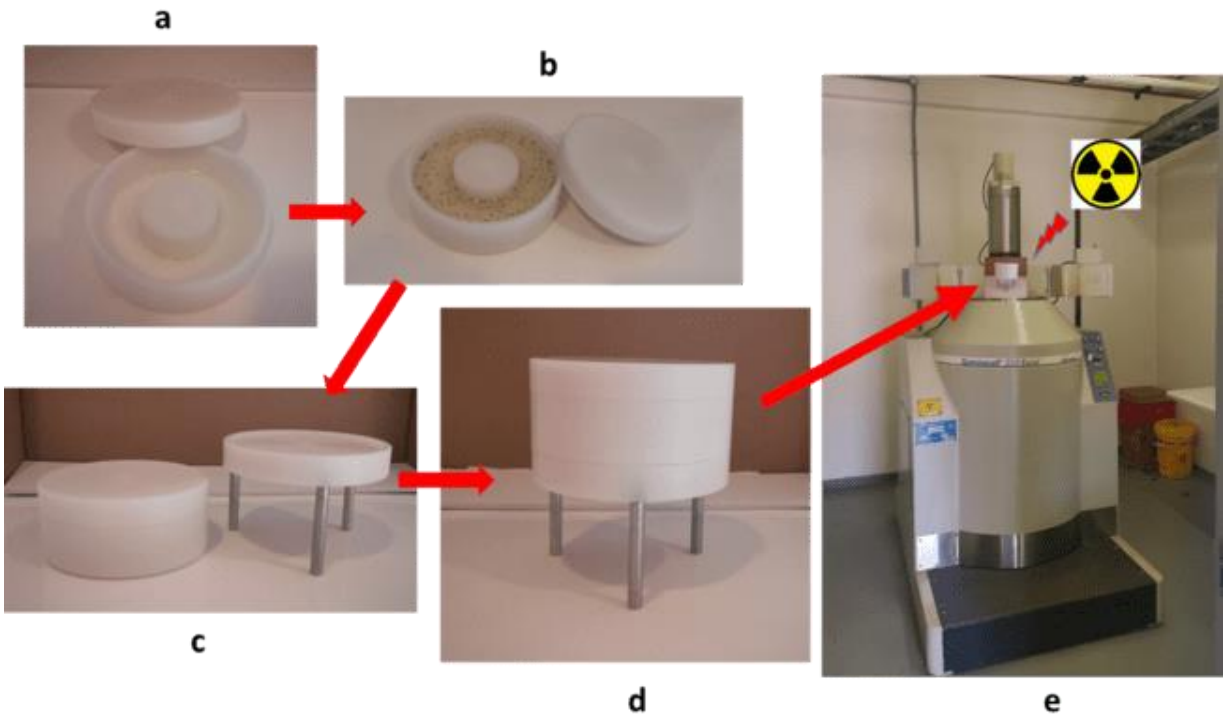


Figure S4. 1**:** Irradiation procedure a) the nylon phantom irradiation canister, b) pupae inside irradiation jig suspended in 100 – 200 mℓ of distilled water, c) closed irradiation canister and tripod stand, d) irradiation canister on top of tripod stand, e) irradiation canister containing male pupae placed into the cobalt-60 (^{60}Co) γ irradiator.

(b) Measured parameters

Pupal emergence

Fifty of the irradiated male pupae were placed per dose in small polystyrene cups containing 100 mℓ of distilled water. The cups were placed in corresponding BugDorm-4® cages for pupae to emerge. The same number of unirradiated pupae were introduced into another cage to serve as control. Thirty-six to 48 h after irradiation treatment, the number of dead pupae (not moving when gently poked with a pipette) or semi-emerged adults (not flying) were recorded as non-emerged while the number of successfully emerged adults (flying) were recorded as emerged.

Fecundity

The effect of irradiation on fecundity was determined by placing 50 unmated, unirradiated adult females of the same age in new 15 × 15 × 15 cm^3 BugDorm-4® cages with an equal number of unmated, irradiated adult males**.** A 10% sugar solution was placed in all cages

throughout. The adults were allowed to mate for three to five days after which a blood meal, directly through the shaven abdomen of a live anaesthetised guinea pig, was provided to females of each cage for a period of 10 minutes each. Immediately post blood feeding, unfed females were removed from each cage. The remaining females were offered a second blood meal two to four days after the first. Two days after the second blood meal, 20 fed females from each cage were randomly selected and transferred into individual glass vials (11 mm radius × 50 mm height) lined on the inside with a damp filter paper and covered on top with a mesh lid (Choi *et al*., 2014). Each glass vial was provisioned with a 10% sugar solution soaked on a cotton wool ball placed on top of the netted lid. Irradiated males were considered F_0 while the resulting progeny from the cross between these irradiated F_0 males and untreated F_0 females were defined as F_1. Glass vials were checked once daily (mornings) for eggs (F_1 generation) and dead females. This was performed for 10 consecutive days. If eggs were observed, an optical Glass Binocular Magnifier (Donegam Optical Company, Lenexa, Kansas 66219 USA) was used to count the total number of eggs laid by each female. All females, either dead before laying eggs or laid eggs or still alive by day 10, were dissected and checked for mating success/insemination rates by observing for the presence/absence of spermatozoa in their spermatheca. In addition, the ovaries of all females were dissected, and the presence/absence of un-laid eggs retained in ovaries was recorded.

Fertility

Eggs laid by individual females were transferred from glass vials into corresponding 350 mℓ polystyrene cups filled with 150 mℓ of distilled water and allowed to hatch. Upon hatching, larvae were counted and removed into new plastic cups daily for 14 consecutive days.

EMS mutagenesis

(a) Preparation of EMS solutions

A colourless EMS liquid from Sigma-Aldrich (catalogue No. M0880) was used in this study. The EMS liquid was added directly to a 10% sugar-water solution (w/v) to create different test doses.

(b) Effect of EMS-Induced Mutagenesis in adults:

Pupae were collected and separated by gender (Harbach and Knight, 1980). Male pupae were transferred into different cups containing 100 mℓ distilled water. Each cup was placed in a corresponding cage furnished with a 10% sugar solution spiked with either 0.025%, 0.050%,

0.075%, 0.100%, 0.250%, or 0.500% EMS. The EMS-spiked sugar solution was soaked on cotton wool balls and placed in a urine jar. A cage containing the same number of males was provisioned with plain 10% sugar solution and served as control. The males were allowed continuous feeding and tarsal contact with EMS-spiked sugar solution for 24 hours before the solutions were removed and adult male mortality recorded. Dead males were removed from each cage. Females (n = 100) of the same age as males were added to each cage. A new 10% sugar solution without EMS was placed in each cage. Adults were allowed two – three days to feed on the new 10% sugar solution and mate before males were removed from cages and blood meals provided to females twice over a five-day period. Two days after the second blood meal, the number of females alive in each cage were counted before a plastic egg bowl filled with distilled water was placed in each cage overnight. The following day, the egg bowls were removed from the cages and eggs counted to determine fecundity (i.e., calculated by dividing the number of eggs laid overnight by the number of females alive when egg bowls were placed into the cages). After counting, eggs were placed into dose corresponding cups and monitored for hatching by counting and removing newly hatched L1 into new trays daily for 14 consecutive days. Screening of F_1 larvae for mutations was performed as described in the irradiation mutagenesis section above.

Screening F_1 larvae resulting from mutagen treated parents for larval morphological mutations in comparison to the wild-type

Progeny from each F_1 iso-family were screened visually using a magnifying glass once daily for any morphological variations to the wild-type. Where variations were observed, an OLYMPUS SZ61 (12× magnification) stereomicroscope was used to confirm the variations and captured photos through a SC30 digital camera (OLYMPUS, Tokyo, Japan). The experiments were repeated at least two times, each time using different parents.

Data management and statistical analysis

The effect of the mutagens on pupal emergence/adult male mortality, fertility and fecundity was determined using ANOVA followed by the Bonferronni pairwise mean comparison. Furthermore, as per the formula suggested by Gaul, (1964), the mutagenic frequency of each mutagen was calculated as the number of larvae with morphological variations over the total number of larvae that hatched from eggs.

$$Mutagenic\ frequency = \frac{Number\ of\ larvae\ with\ morphological\ variations}{Total\ number\ of\ larvae} \times 100$$

All statistical analyses were performed using SPSS (IBM Corp, 2015) and DATAtab (DATAtab, 2022) statistical softwares, with differences between compared groups considered statistically significant when $p < 0.05$.

Results

Effect of various EMS and irradiation doses on selected reproductive traits

Irradiation treatment

Pupal emergence

The mean number (± SD) of adults that emerged from pupae was lowest in 50 Gy (88.67% ± 5.77) and highest in 25 Gy (94.67% ± 4.16) (Figure S4.2). There was no significant difference between the irradiation dose and emergence (one-way ANOVA; $F = 0.8$, $p = 0.602$).

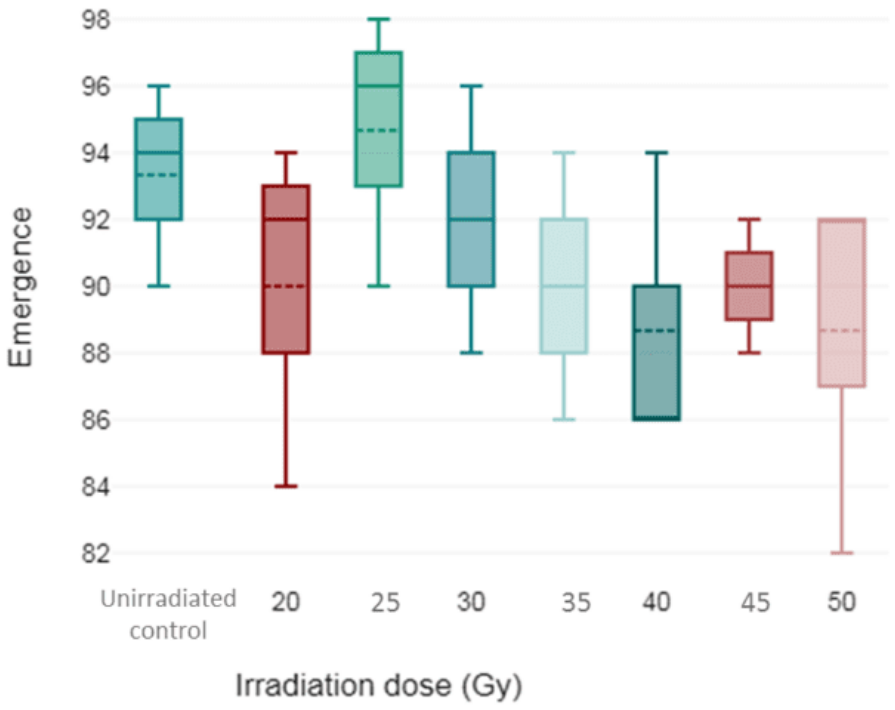


Figure S4. 2: Emergence (%) of pupae into adults following irradiation with different doses.

Fecundity

The total number (± SD) of eggs laid per female were the lowest at 50 Gy (55.48% ± 21.43) and highest in 0 Gy (92.78% ± 20.38) (Figure S4.3). Statistically this showed a significant difference when compared across irradiation doses, especially the control (one-way ANOVA, $F = 5.34$, $p = 0.001$). This was confirmed by a Bonferroni post-hoc test which revealed that the pairwise

group comparisons of 0 - 20, 0 - 25, 0 - 30, 0 - 35, 0 - 40 and 0 - 50 have a p-value less than 0.05.

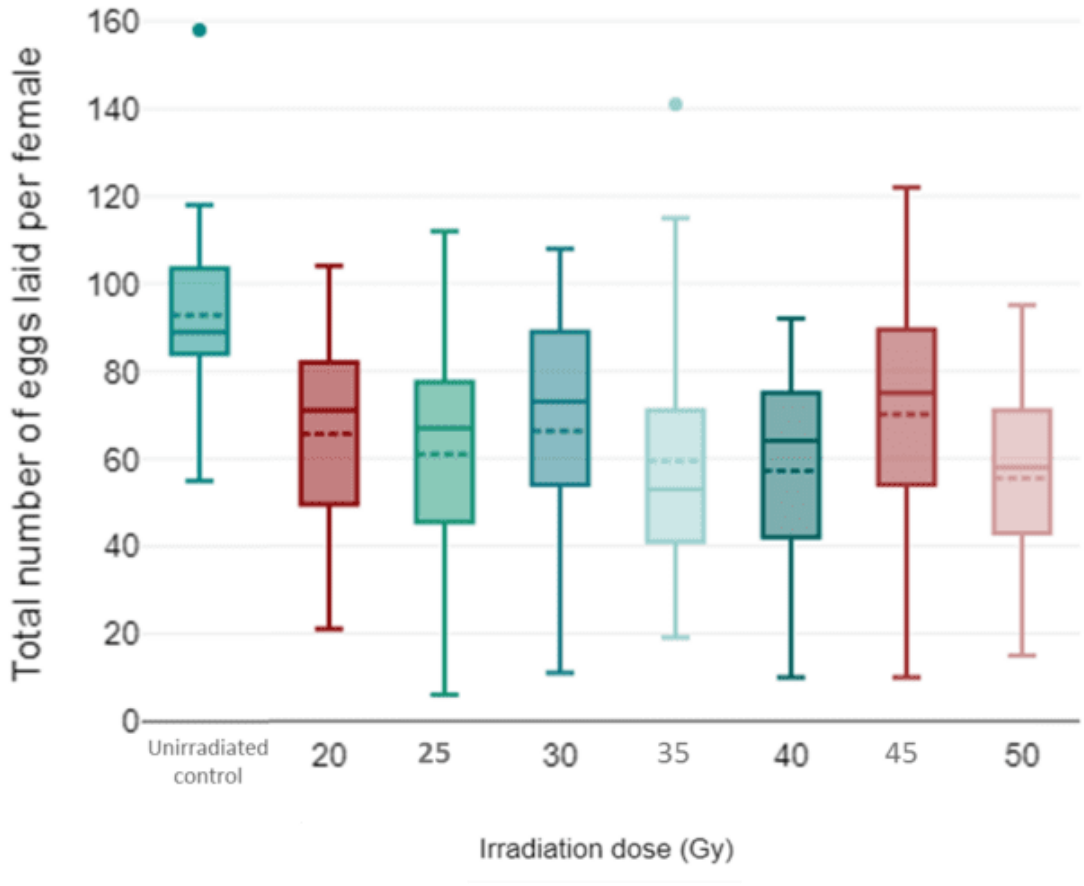


Figure S4. 3**:** Fecundity (number of eggs laid per female) of females mated with males irradiated at different doses and their corresponding control. *dots show outliers.

Fertility

The percentage of eggs that hatched (± SD) decreased as the irradiation dose increased, with the highest observed in 0 Gy (66.74% ± 11.5) and the lowest in 50 Gy (12.7% ± 10.48) (Figure S4.4). A statistical test showed a significant difference in the percentage of eggs that hatched depending on irradiation dose (one-way ANOVA, F = 32.97, p = 0.001). A Bonferroni post-hoc test revealed the differences to be between 0 and all the other doses, 20 and 30, 35, 40, 45, 50; 25 and 35, 40, 45, 50; 30 and 40, 45, 50; and between 35 and 50 Gy.

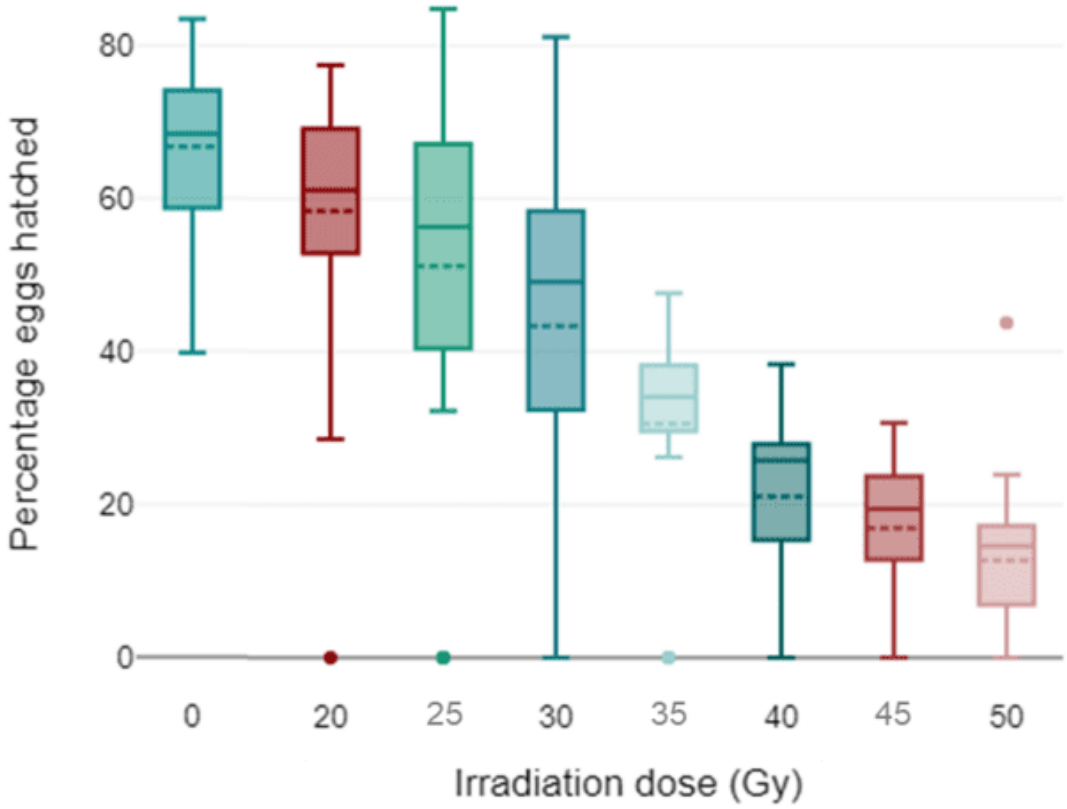


Figure S4. 4**:** Fertility (percentage of eggs that hatched) from females mated with males irradiated at different dosages and corresponding controls. *dot shows outliers.

EMS treatment

Adult male mortality

Adult male mortality rates of males 24 h (± SD) post EMS treatment showed an increase in mortality as the doses increased. The lowest mortality was observed in control (no EMS): 4.67% ± 3.06 and highest in 0.500%: 92.0% ± 3.33. (Figure S4.5). A one-way ANOVA showed a significant difference between EMS dose and male mortality after 24 h ($F = 162.29$, $p = 0.001$), with a Bonferroni post-hoc test revealing the groups with significant pairwise differences to be 0 and all the other doses, 0.025 and 0.075, 0.1, 0.25, and 0.5, 0.05 and 0.1, 0.25, and 0.5, 0.075 and 0.1, 0.25, and 0.5.

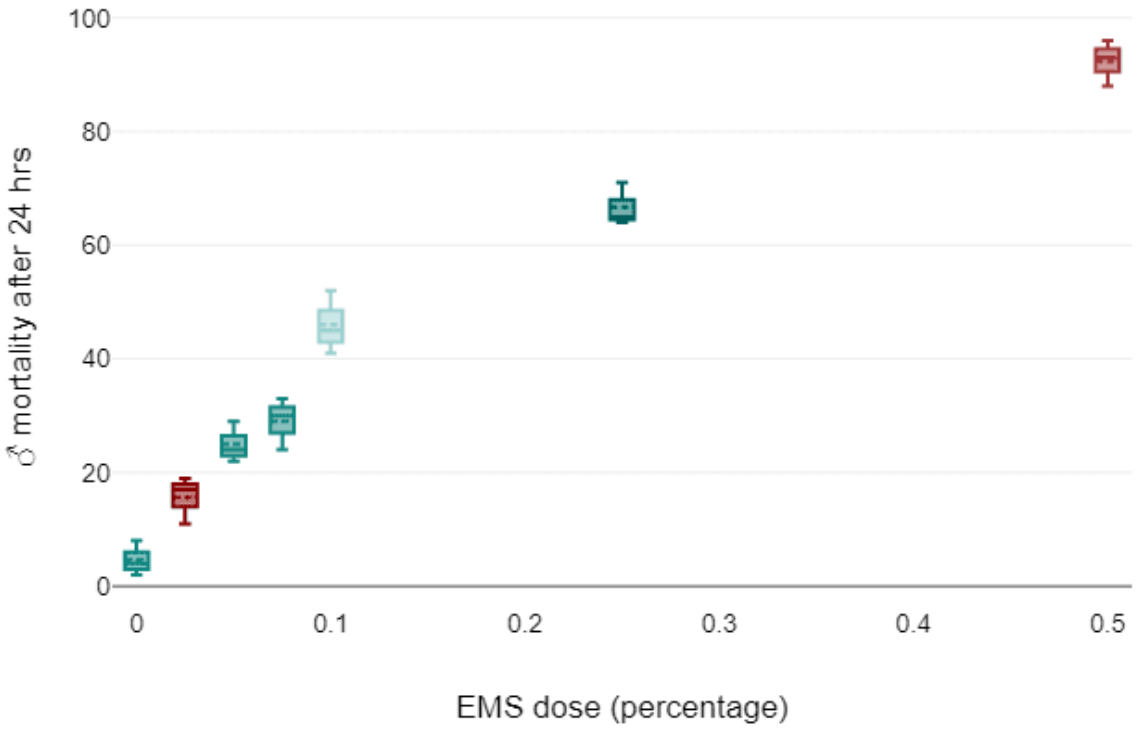


Figure S4. 5**:** Mortality (mean %) of adult males' 24 h post feeding overnight on different doses of EMS.

Fecundity

Fecundity decreased as the doses increased. The highest fecundity (± SD) was observed in control (no EMS): 66.74% ± 11.5 and lowest in 0.500%: 12.7% ± 10.48 (Figure S4.6). There was a significant difference between EMS dose and fecundity (one-way ANOVA, F = 61.84, p = 0.001). A Bonferroni post-hoc test revealed that the pairwise group comparisons of 0 and all other doses; 0.025 and 0.075 to 0.5; 0.05 and 0.075 to 0.5; 0.075 and 0.5; 0.1 and 0.5; and 0.25 and 0.5 are each significantly different from each other.

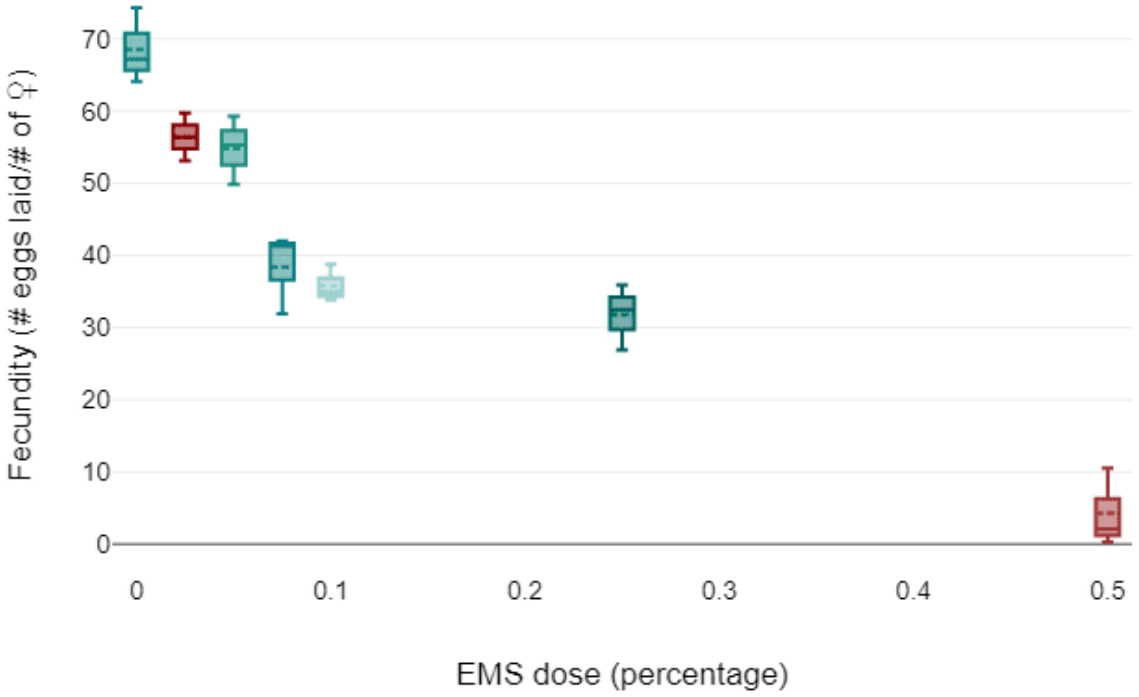


Figure S4. 6**:** Fecundity of females mated with males that fed on different doses of EMS and their corresponding.

Fertility

Fertility decreased as the doses increased. The highest fertility (± SD) was observed in control (no EMS): 68.9% ± 8.02 and lowest in 0.500% where none of the eggs hatched (Figure S4.7). A statistical test showed a significant difference between EMS dose and fertility (one-way ANOVA, F = 19.96, p = 0.001). A pairwise group comparisons with a Bonferroni post-hoc test revealed differences to be between 0 and 0.1, 0.25, and 0.5; 0.025 and 0.25 and 0.5; 0.05 and 0.25 and 0.5; 0.075 and 0.25 and 0.5; and 0.1 and 0.5.

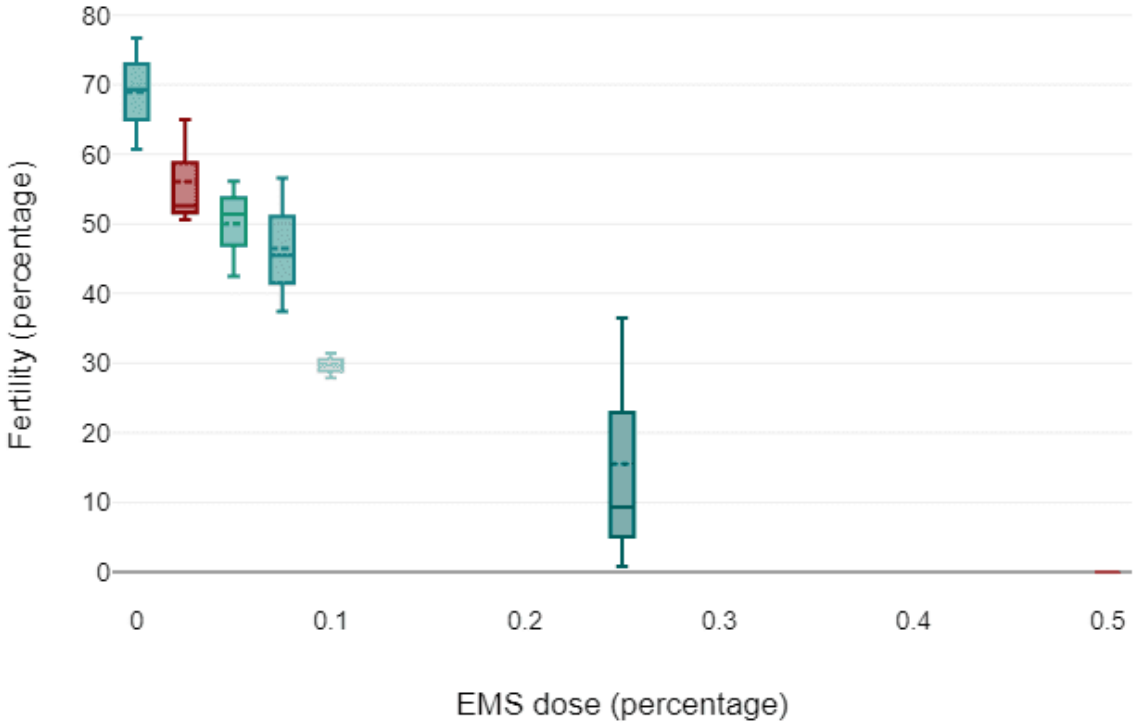


Figure S4. 7**:** Fertility of females mated with males that fed on different doses of EMS and their corresponding.

Screening F_1 larvae resulting from mutagen treated parents for larval morphological mutations in comparison to the wild-type

A number of morphological variation in F_1 larval progenies were observed in both γ and EMS treatments (Figure S4.8). The frequency of larval morphological mutants (± SD) induced by both γ and EMS showed a linear increase with increase in dose. These ranged from 1.39% ± 0.48 to 4.20% ± 0.60 in γ (Figure S4.9.a) and 0.08% ± 0.1 to 10.71% ± 3.67 in EMS treatments (Figure S4.9.b). There was a significant difference in the frequency of larval morphological variations among both γ (one-way ANOVA, F = 72.96, p = 0.001) and EMS doses (one-way ANOVA, F = 16.32, p = 0.001).

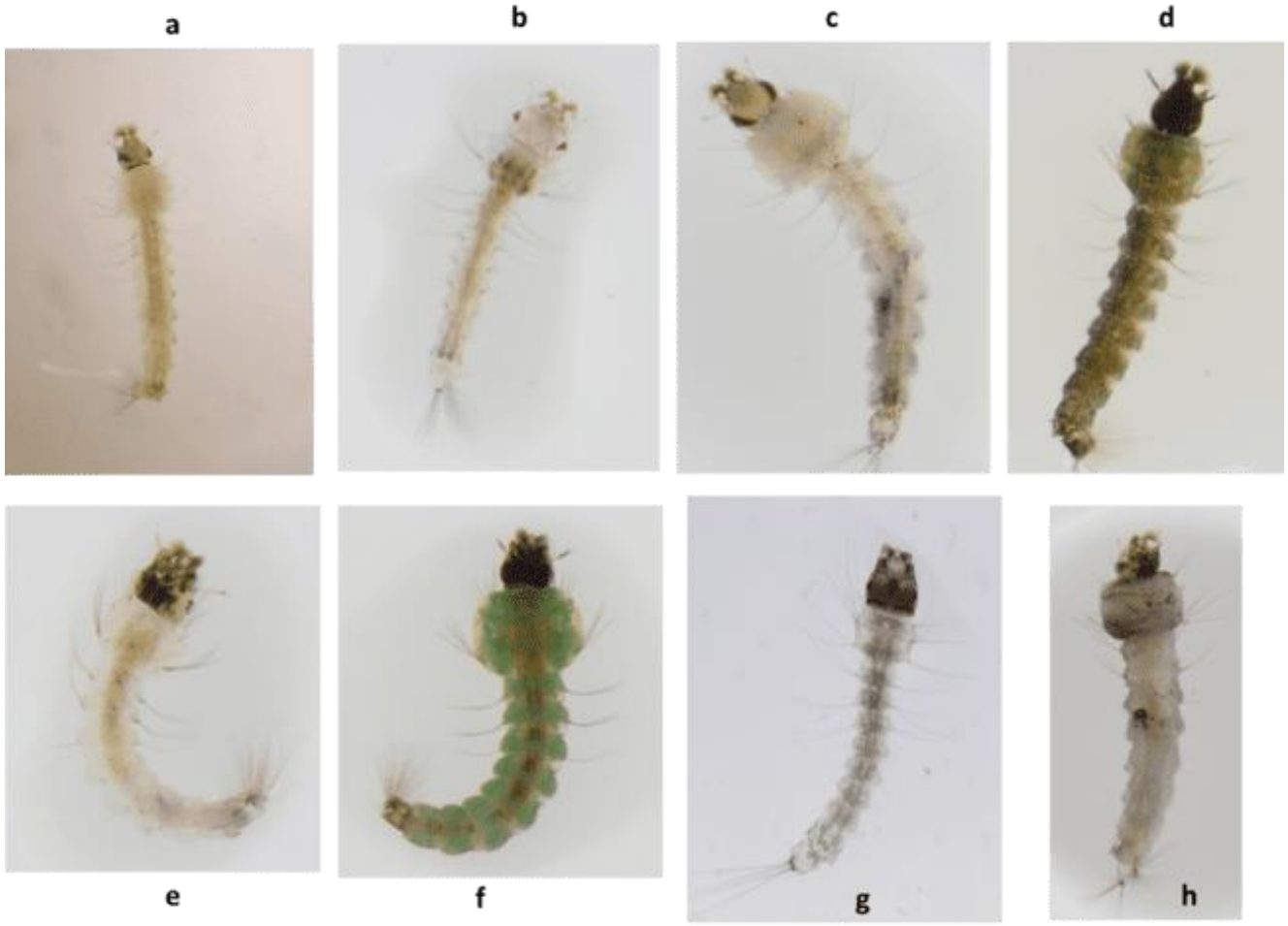
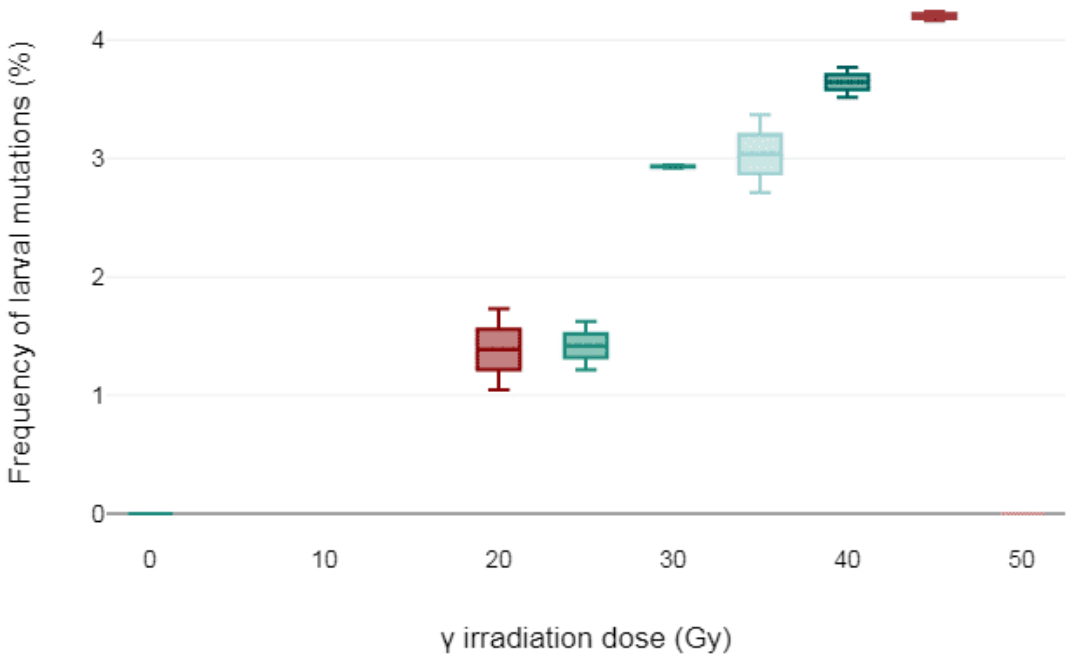
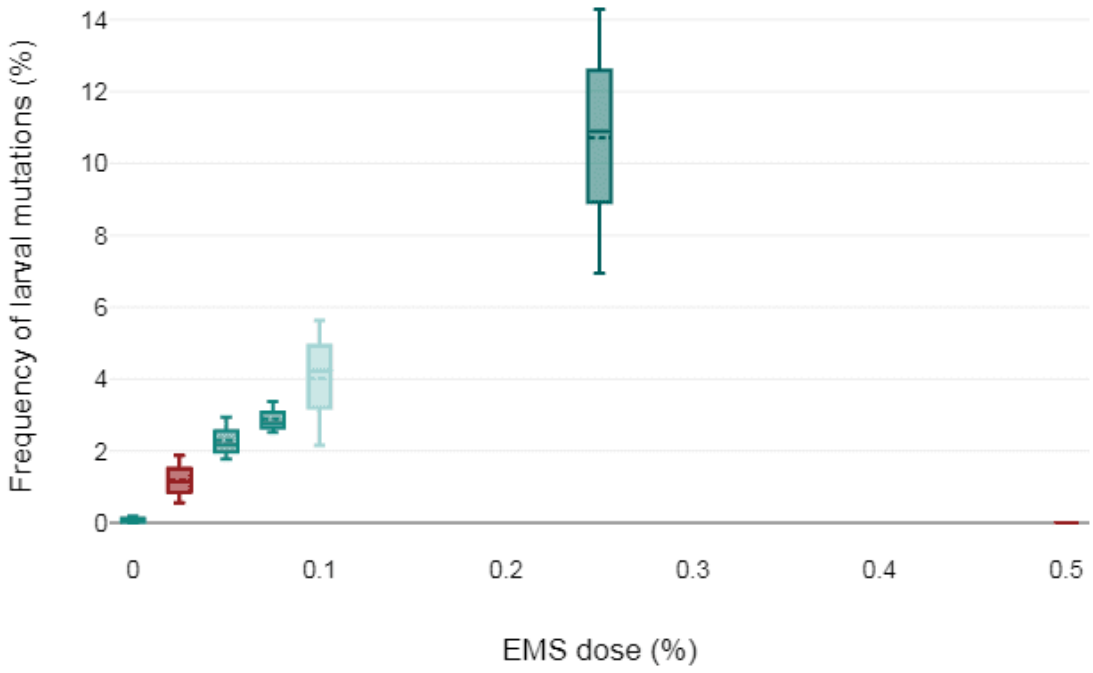


Figure S4. 8**:** Selected morphological variations observed in F_1 third/fourth instar larvae whose parents were exposed to different doses of either EMS or γ. (a) wild-type, (b) - (h) various larval variations to the wild-type.



(a)



(b)

Figure S4. 9**:** Frequency of morphological mutants induced in F_1 generation of *An. arabiensis* larvae following treatment with various doses of **(a)** γ and **(b)** EMS.

Discussion

The aim of this study was to use both γ and EMS to induce conditional mutations and phenotypically screen larval progeny for morphological variations in comparison to the wild-type. Two approaches were used to achieve this goal. The first involved determination of the effect of various EMS and γ doses on selected reproductive traits. The second was to screen F_1 larvae resulting from mutagen treated parents for larval morphological mutations in comparison to the wild-type.

Irradiation had no effect on the emergence of adults from pupae treated with different doses. Other studies have reported similar results. For example, both Helinski *et al*., (2006) and Munhenga *et al*., (2016) reported no adverse effects on pupae in *An. arabiensis* at doses as high as 100 Gy. In contrast, Bhuyan and Barik, (2016) reported similar results in *Ae. aegypti* exposed to doses up to 40 Gy. In terms of fecundity of the females mated with irradiated males, this was also similar for all irradiation doses compared to the control. Other studies have also reported similar outcomes. For example, *Ae. aegypti* females that mated with sterile males continued to lay eggs even after males had been exposed to doses in the range 10 Gy to 300 Gy (Shetty *et al*., 2016). Similarly, outcomes were reported in *An. arabiensis* females that mated with males that had been exposed to 25 Gy to 100 Gy (Helinski *et al*., 2006). Fertility on the other hand showed significant difference between the irradiation doses. In previous studies on *An. arabiensis*, complete infertility was observed in doses of 70 Gy and 90 Gy (Helinski *et al*., 2006; Munhenga *et al*., 2016). Similar findings were reported by Shetty *et al*., (2016) in *Ae. aegypti* whereby egg fertility was reduced following exposure of males to doses of 20 Gy to 50 Gy.

Concerning EMS treatments, mortality of adults fed EMS increased as EMS dose increased. On the contrary, fecundity and fertility decreased as EMS doses increased. Similar trends have been reported in studies such as those by Rodriguez (1985) who reported that treatment with EMS did not significantly reduce egg production, percentage hatchability, or total productivity when administered to *Ae. aegypti.* In a more recent study, Ndo *et al*. (2018) reported that the number of eggs laid and their hatch rate were similar between females that mated with males mutagenised with 0.05% EMS and those that were untreated.

In terms of screening F_1 larvae resulting from mutagen treated parents for larval

morphological mutations in comparison to the wild-type, several larval morphological mutations were recovered from both γ and EMS treated mosquitoes. These ranged from larvae with body colour that is white, black, green, etc, a clear contrast to the wild-type which has a brown/beige colour. Most of these mutations were observed as the dose of the mutagen increased. For example, γ (% ± SD) showed a larval mutagenic frequency that ranged from 1.39 ± 0.48 at 20 Gy to 4.2 ± 0.06 at 45 Gy. No eggs hatched at 50 Gy. In the EMS treatment, there was also a trend of increment in mutagenic frequency as the doses increased. The majority of the mutants were observed at 0.250% EMS dose (10.71 ± 3.67%). Most of the treated material did not survive at 0.50%. In general, EMS induced a higher mutagenic frequency of larval morphological mutations than γ at the doses tested in this study. Based on these results, it is recommended that EMS in the doses of up to 0.250% be used for mass scale mutagenesis of *An. arabiensis*, depending on the mutagenic traits of interest.

Conclusion

The effect of EMS and γ on selected reproductive traits varies. It is also possible to screen F_1 larvae resulting from mutagen treated parents for larval morphological mutations. EMS induced a higher mutagenic frequency of larval morphological mutations than γ at the doses tested in this study. As such, it is recommended that EMS in the doses of up to 0.250% be used for mass scale mutagenesis of *An. arabiensis*. In fact, this was tested in this in this thesis by using EMS to induce conditional mutations and screening for iso-families with the potential to contain *tsl* alleles (see chapter 5).

References

Bakri, A., Heather, N., Hendrichs, J. and Ferris, I., 2005. **Fifty years of radiation biology in entomology: lessons learned from IDIDAS.** *Annals of the Entomological Society of America*, 98(1), pp.1-12.

Bhuyan, K.B. and Barik, T.K., 2016**. Impact of high dose of gamma radiation on field collected *Aedes aegypti*.** *Journal of Mosquito Research*, *6*.

Choi, K.S., Christian, R., Nardini, L., Wood, O.R., Agubuzo, E., Muleba, M., Munyati, S., Makuwaza, A., Koekemoer, L.L., Brooke, B.D. and Hunt, R.H., 2014. **Insecticide resistance and role in malaria transmission of *Anopheles funestus* populations from Zambia and Zimbabwe.** *Parasites and Vectors*, 7(1), pp.1-8.

Dandalo, L.C., 2016. **Characterization of a local genetic sexing strain as well as a wild population of *Anopheles arabiensis* from KwaZulu-Natal, South Africa.** PhD Thesis. Johannesburg: University of the Witwatersrand. https://wiredspace.wits.ac.za/handle/10539/45.

FAO/IAEA, 2021. **Mutation induction**. Available at: https://www.iaea.org/topics/mutation-induction.

Franz, G., 2005. **Genetic sexing strains in Mediterranean fruit fly, an example for other species amenable to large-scale rearing for the sterile insect technique.** In Sterile Insect Technique (pp. 427-451). *Springer, Dordrecht*.

Gaul, H., 1964. **Mutations in plant breeding.** *Radiation Botany*, *4*(3), pp.155-232.

Harbach, R.E. and Knight, K.L., 1980. **Taxonomists' glossary of mosquito anatomy**. *Plexus Publishing Inc*..

Helinski, M.E., Parker, A.G. and Knols, B.G., 2006. **Radiation-induced sterility for pupal and adult stages of the malaria mosquito *Anopheles arabiensis*.** *Malaria Journal*, *5*, pp.1-10.

Hunt, R.H., Brooke, B.D., Pillay, C., Koekemoer, L.L. and Coetzee, M., 2005. **Laboratory selection for and characteristics of pyrethroid resistance in the malaria vector *Anopheles funestus*.** *Medical and Veterinary Entomology*, 19(3), pp.271-275.

Koskinioti, P., Augustinos, A.A., Carvalho, D.O., Misbah-ul-Haq, M., Pillwax, G., de la Fuente, L.D., Salvador-Herranz, G., Herrero, R.A. and Bourtzis, K., 2021. **Genetic sexing strains for the population suppression of the mosquito vector *Aedes aegypti*.** *Philosophical Transactions of the Royal Society B*, 376(1818), p.20190808.

LaChance, L.E. and Graham, C.K., 1984. **Insect radiosensitivity: dose curves and dose-fractionation studies of dominant lethal mutations in the mature sperm of 4 insect species.** *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis*, 127(1), pp.49-59.

Ling, M.M. and Robinson, B.H., 1997. **Approaches to DNA mutagenesis: an overview.** *Analytical Biochemistry*, 254(2), pp.157-178.

Malcolm, C.A. and Mali, P., 1986. **Genetic sexing of *Anopheles stephensi* with the larval morphological mutant Bl.** *Genetica*, 70(1), pp.37-42.

Mason, G.F., 1967. **Genetic studies on mutations in species A and B of the *Anopheles gambiae* complex.** *Genetics Research*, 10(3), pp.205-217.

Meza, J.S., Bourtzis, K., Zacharopoulou, A., Gariou-Papalexiou, A. and Cáceres, C., 2020. **Development and characterization of a pupal-colour based genetic sexing strain of *Anastrepha fraterculus sp. 1* (Diptera: Tephritidae).** *BMC Genetics*, 21(2), pp.1-9.

Mukiama, T.K., 1985. **Y-autosome genetic sexing strain of *Anopheles albimanus* (Diptera: Culicidae).** *International Journal of Tropical Insect Science*, 6(6), pp.649-652.

Munhenga, G., Brooke, B.D., Gilles, J.R., Slabbert, K., Kemp, A., Dandalo, L.C., Wood, O.R., Lobb, L.N., Govender, D., Renke, M. and Koekemoer, L.L., 2016. **Mating competitiveness of sterile genetic sexing strain males (GAMA) under laboratory and semi-field conditions: steps towards the use of the Sterile Insect Technique to control the major malaria vector *Anopheles arabiensis* in South Africa.** *Parasites and Vectors*, 9(1), pp.1-12.

Ndo, C., Poumachu, Y., Metitsi, D., Awono-Ambene, H.P., Tchuinkam, T., Gilles, J.L.R. and Bourtzis, K., 2018. **Isolation and characterization of a temperature-sensitive lethal strain of *Anopheles arabiensis* for SIT-based application.** *Parasites and Vectors*, 11(2), pp.97-105.

Papathanos, P.A., Bourtzis, K., Tripet, F., Bossin, H., Virginio, J.F., Capurro, M.L., Pedrosa, M.C., Guindo, A., Sylla, L., Coulibaly, M.B. and Yao, F.A., 2018. **A perspective on the need and current status of efficient sex separation methods for mosquito genetic control.** *Parasites and Vectors*, 11(2), pp.165-171.

Rodriguez, P.H., 1985. **Ethyl-methanesulfonate-induced changes in filarial susceptibility in *Aedes aegypti* (Diptera: Culicidae).** *Journal of Medical Entomology*, *22*(4), pp.366-369.

Shetty, V., Shetty, N.J., Harini, B.P., Ananthanarayana, S.R., Jha, S.K. and Chaubey, R.C., 2016. **Effect of gamma radiation on life history traits of *Aedes aegypti* (L.).** *Parasite Epidemiology and Control*, 1(2), pp.26-35.

Zepeda-Cisneros, C.S., Hernández, J.S.M., García-Martínez, V., Ibañez-Palacios, J., Zacharopoulou, A. and Franz, G., 2014. **Development, genetic and cytogenetic analyses of genetic sexing strains of the Mexican fruit fly, *Anastrepha ludens* Loew (Diptera: Tephritidae).** *BMC Genetics*, 15(2), pp.1-11.

Appendix 5: Plagiarism declaration

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FACULTY OF HEALTH SCIENCES

PLAGIARISM DECLARATION TO BE SIGNED BY ALL HIGHER DEGREE STUDENTS

SENATE PLAGIARISM POLICY: APPENDIX ONE

I Thabo Mashatola (Student number: 0500939P) am a student registered for the degree of CMID 9000A - PHD THESIS in the academic year 2023 .

I hereby declare the following:

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

Signature:  Date: 12 October 2023

Appendix 6: Sex separation techniques publication

Mashatola et al. Parasites & Vectors 2018, 11(Suppl 2):646
https://doi.org/10.1186/s13071-018-3219-4

Parasites & Vectors

REVIEW Open Access

A review on the progress of sex-separation techniques for sterile insect technique applications against *Anopheles arabiensis*

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Abstract

The feasibility of the sterile insect technique (SIT) as a malaria vector control strategy against *Anopheles arabiensis* has been under investigation over the past decade. One of the critical steps required for the application of this technique to mosquito control is the availability of an efficient and effective sex-separation system. Sex-separation systems eliminate female mosquitoes from the production line prior to irradiation and field release of sterile males. This is necessary because female mosquitoes can transmit pathogens such as malaria and, therefore, their release must be prevented. Sex separation also increases the efficiency of an SIT programme. Various sex-separation strategies have been explored including the exploitation of developmental and behavioural differences between male and female mosquitoes, and genetic approaches. Most of these are however species-specific and are not indicated for the major African malaria vectors such as *An. arabiensis*. As there is currently no reliable sex-separation method for *An. arabiensis*, various strategies were explored in an attempt to develop a robust system that can be applied on a mass-rearing scale. The progress and challenges faced during the development of a sexing system for future pilot and/or large-scale SIT release programmes against *An. arabiensis* are reviewed here. Three methods of sex separation were examined. The first is the use of pupal size for gender prediction. The second is the elimination of blood-feeding adult females through the addition of an endectocide to a blood meal source. The third is the establishment of a genetic sexing strain (GSS) carrying an insecticide resistance selectable marker (dieldrin-resistance *rdl* gene and/or other GABA receptor antagonists that can be used as alternative insecticides to dieldrin) or a temperature-sensitive lethal marker.

Keywords: *Anopheles arabiensis*, sterile insect technique, sex-separation, genetic sexing strain

Background

Malaria is a major global health problem responsible for approximately 216 million cases and 445,000 deaths worldwide in 2016 alone [1]. An estimated 80% of malaria-related deaths occur in sub-Saharan Africa, particularly in pregnant women and children below the age of five years. By affecting health at the personal and community levels, malaria also has a direct impact on national economies, education and social development [1, 2].

Various vector control interventions such as larval source management, distribution of insecticide-treated bednets (ITNs) and indoor residual spraying (IRS) of formulated insecticides have contributed significantly towards suppression of the global malaria burden [3–5]. However, the burgeoning incidence of insecticide resistance in target vector populations is threatening control efforts [6–8], as is the diversity of malaria vector species assemblages which often include outdoor-resting and feeding components that are less susceptible to control by indoor applications of insecticide [9, 10]. To address these challenges, supplementary vector control interventions that are effective, environmentally friendly and

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economical are required. Numerous technologies with potential such as transgenics including gene drive, *Wolbachia*-based methods and the sterile insect technique (SIT) are currently under development or are undergoing open field-testing and validation [11–20].

The SIT has been successfully applied as an area-wide integrated vector management (AW-IVM) method against several insect pests [21, 22]. This technique involves the serial release of laboratory mass-produced sterile insects, usually males, at a ratio that effectively inundates a target wild population. This forces the majority of females to mate with sterile males, substantially reducing their fecundity, and resulting in population suppression [21].

Attempts to use the SIT against mosquitoes dates back six decades [23]. Since then substantial research, answering key questions on mosquitoes and the SIT, has been conducted [11]. For the African malaria vector *An. arabiensis* several research activities are underway in Reunion Island, Sudan and South Africa. Data on the following aspects has been generated: (i) colonization and rearing conditions [24, 25], and (ii) male sterility induction through irradiation and related longevity, mating competitiveness, mating compatibility [24, 26–30], and means of transportation to release localities [24, 26]. In addition to these, field study site selection, and assessments of the monthly species and genetic abundances, and variability of wild populations have been undertaken [31–33]. A study on the technical and social perspectives of using the SIT to control malaria has been conducted [34], including the recent knowledge, attitude and practices (KAP) survey performed in South Africa in an area selected for pilot SIT releases [35]. Before the SIT can be applied at an operational level, there is one critical aspect remaining. This is the development of a robust sex separation system to exclusively obtain males prior to releases [36, 37].

An efficient sex separation system is essential for any mosquito SIT-based control programme for several reasons. The most important reason is that released females have the potential to transmit malaria or other disease pathogens [38], thus putting the human population at risk. Also, the co-release of laboratory females can interfere with the frequency at which sterile males mate with wild females, thus decreasing the effectiveness of the SIT programme. This has been demonstrated in the Mediterranean fruit fly (medfly) where, by releasing males only, the effectiveness of the SIT programme was increased [39, 40].

Several sex separation methods have been developed and implemented in SIT programmes, including mosquito programmes [41, 42]. However, most of these methods are species-specific and are not indicated for *An. arabiensis* or, if applicable, have never been tested at an operational level [42]. Investigations and the development of various robust sexing strategies that can be applied on a mass scale are therefore being conducted in an attempt to address this shortfall. The progress, challenges and lessons learned from these investigations, and future prospects, are reported here.

Use of pupal size for gender prediction

In many insect species, female pupae are usually larger than their male counterparts [43]. This phenomenon, known as sexual size dimorphism, is a trait that has been exploited for mechanical separation means, with numerous mechanical devices developed for mosquitoes. Examples include standard sieves, the Fay-Morlan glass plate and the McCray adjustable opening separation system that have been used to separate male and female pupae in *Aedes aegypti*, *Ae. albopictus* and *Culex quinquefasciatus* [44–47]. Attempts to use sexual size dimorphism in *An. albimanus* were without success during the 1972 sterile male release study in El Salvador [48]. It was observed that *An. albimanus* male and female pupal sizes overlap substantially, thus making efficient sexing by size difficult. To our knowledge, this assumption has never been tested in other anophelines.

Recently, pupal size dimorphism was investigated in a South African *An. arabiensis* strain. Pupal cephalothorax sizes of larvae reared on a standard larval diet were measured and recorded by gender. Cephalothorax size in males averaged 3.64 mm while those of females averaged 3.66 mm. These are not significantly different, as has also been noted in *An. albimanus*. These data are consistent with other reports that pupal size sexual dimorphism is not apparent in anophelines [49]. Compounding this, the results also illustrated a greater variation in pupal size in males compared to females, making the use of sieve and Fay-Morlan glass plate sex-separation technically challenging and even less reliable. It was consequently concluded that this method of sex-separation is not viable for *An. arabiensis*.

Addition of toxicants to blood meal sources to eliminate blood-feeding females

As only female mosquitoes take blood meals, this auto-segregation is a highly alluring feature that might be exploited by adding a toxicant to a blood meal (blood-spiking) so as to eliminate blood-feeding females. This approach was used in an SIT programme in El Salvador against *An. albimanus* by adding malathion to a citrated bovine blood meal. The result was a 95% elimination of females and 25% male mortality [50]. During this study, adults had to be kept in cages for several days to allow females to mate prior to blood-feeding. It is important to note that although females can take blood meals prior to mating [51], feeding rates, and therefore

the efficacy of this selection method, would more likely improve should the blood meal be presented post mating. This extended storing of mosquitoes in cages is problematic because it creates a bottleneck during mass rearing and male sperm are potentially depleted during holding, negatively impacting their mating potential post release [52]. Another concern raised during the El Salvador trial was a high level of male mortality. The reason for high male mortality was assumed to be tarsal contact of males with malathion from blood excreted by females during feeding [53]. This led to investigations of other toxicants such as boric acid, household detergent and dieldrin [54]. Unfortunately, experiments with these also resulted in high male mortality [54]. Subsequently, spinosad and ivermectin, which do not rely on tarsal contact for their mode of toxicity, were tested [54]. Both spinosad and ivermectin eliminated all females from a mixed sex population of *An. arabiensis* within 4 days without causing significant male mortality. At higher concentrations, both toxins eliminated all females within twelve hours but at a significantly high male mortality cost.

Recently, attempts were made to optimize and adapt this strategy as a sex separation method in a South African *An. arabiensis* strain using ivermectin only. During these experiments, difficulties arose relating to blood-feeding success. The South African *An. arabiensis* strain is routinely maintained on blood from anaesthetized guinea pigs and thus did not readily feed on blood spiked with ivermectin using artificial membrane feeders. An investigation of artificial membrane feeding systems and the adaptability of this strain to feed on this system was therefore required. Direct feeding from live guinea pig skin was compared to Hemotek membrane blood-feeding using three different membranes (collagen, pig intestine casing and Parafilm-M). The following parameters were measured to aid in selection of the optimal membrane: feeding success, fecundity and fertility (egg hatch rates). Feeding success averaged 79% in both collagen and pig intestine casing and only 15% in Parafilm-M (unpublished data). Fecundity of females ranged between 23-46 eggs laid per female and there was no difference in fecundity between the three membranes tested (unpublished data). Eggs oviposited by these females hatched at rates ranging from 76% to 92%. This difference was however not statistically significant (unpublished data). Based on these results and considering other factors such as membrane price, thickness and local availability/production level, pig intestine casing was chosen as the optimal membrane and henceforth used to acclimatize the colony to membrane feeding. This paved the way to do baseline studies to explore the use of ivermectin as a blood toxicant to eliminate *An. arabiensis* females for male-only releases.

Subsequent experiments on the use of blood spiked with ivermectin to eliminate females in the South African strain showed that this approach could eliminate females, albeit at a low female elimination rate compared to that reported in Yamada et al. [54]. About 90% of females were eliminated on a single meal after five consecutive days post feeding, while the remainder were eliminated within 10 days. The difference in female elimination rates observed between this study and that of Yamada et al. [54] could be due to several reasons, one of them being the difference in ivermectin formulation. The South African study used ivermectin MK-933 (Sigma, CAS No. 70288-86-7) while Yamada et al. [54] used 1% ivermectin (Virbamec, Virbac Oesterreich GmbH, Vienna, Austria), a formulation that is similar to the one used in veterinary medicine or in mass drug administration studies in vertebrates to kill mosquitoes [55–57]. Another possibility to consider is that the South African *An. arabiensis* strain has an intrinsic metabolic insecticide resistance mechanism in place [58]. If placed under insecticide selective pressure, the strain has potential to trigger this resistant genotype. It is therefore conceivable that the metabolic detoxification system in this strain, although it is phenotypically insecticide susceptible, could inadvertently have reduced its susceptibility to ivermectin, making female elimination by the spiked blood less effective. Further investigations would be required to test these hypotheses.

Another concern observed during the South African ivermectin optimization trial was a high level of male mortality (21%) when spiked blood meals were presented serially to enhance female elimination. This was however not surprising as similar results have been reported [54] whereby increased tarsal contact with insecticide from blood excreted by females is the likely cause of male mortality. A high rate of male loss prior to releases would adversely affect production efficiency during large-scale SIT operations.

An additional disadvantage of using blood-spiking to eliminate females is the effect on the reproductive capacity of males. The potential loss of male mating capacity as a result of potential exposure to ivermectin was investigated. This was achieved by mating the males that survived post 100% female elimination with untreated virgin females and then dissecting their spermathecae to determine insemination rates. High insemination rates (> 95%) were recorded, with no statistically significant difference found in comparison to virgin males and females that had not been previously exposed to ivermectin, suggesting that ivermectin treatment did not negatively impact male fitness (unpublished data).

Although these results show that ivermectin can be used as a blood toxicant for sex separation, factors such as the inability to reliably guarantee complete elimination of females within a short period have to be addressed before this method can be applied on a mass-rearing scale. Possible improvements could involve

improving blood-feeding success, particularly of females that opt not to feed or ingest sufficient quantities of spiked blood. This can be achieved by the addition of phago-stimulants (e.g. adenosine triphosphate (ATP) [59], L-lactic acid [60], or natural odor ligands from human skin [61]) and other attractants into or near the blood. Another possible approach, given that this method is based on auto-segregation of sexes based solely on blood-feeding behaviour, may be to use any nonblood-based stimulus to attract females away from males followed by electrification to kill the females. Additionally, the use of artificial diets [62, 63] can be investigated as they would allow manipulation of ingredients and possible improvements in the efficacy of toxicants. Investigation into artificial diets would contribute positively to both routine colony maintenance and blood-spiking studies.

Development of a genetic sexing strain (GSS)

Genetic sexing strains (GSSs) are based on the linkage of a selectable marker to a sex-determining chromosome [64]. GSSs have been developed in many insect species, particularly in major agricultural pests such as the medfly *Ceratitis capitata*, the Mexican fruit fly *Anastrepha ludens*, the Oriental fruit fly *Bactrocera dorsalis* and the melon fruit fly *Zeugodacus* (*Bactrocera*) *cucurbitae* [65–69]. In all of these insects, the construction of a GSS involved a mutation that can be used as a selectable marker for sex separation and a Y-autosome translocation linking the inheritance of the wild type allele to the Y chromosome. This results in females carrying the mutant allele and males hemizygous for the wild type. Currently, only the *C. capitata* GSSs are being used at an operational level for mass-rearing [70–72].

The usefulness of Y-autosome translocation based GSSs may be hindered by genetic instability resulting from pre-meiotic recombination in the parental male [73] and/or the survival of genetically unbalanced individuals resulting from simultaneous segregation of non-homologous centromeres (adjacent-1 segregation) during meiosis in the parental males [71]. Recombination events can occur between the translocation break point and the selectable marker, which may result in the accumulation of recombinants and the collapse of the genetic sexing character of the strain [72]. Reduction and/or elimination of recombination can be achieved by the selection of a Y-autosome translocation where the autosomal breakpoint is close to the selectable marker or by the induction of a chromosomal inversion that includes the region of the translocation breakpoint and the selectable markers [71]. In addition, a Filter Rearing System (FRS) can be adopted to remove recombinants that might possibly accumulate during mass-rearing [71, 74, 75]. This would guarantee that the genetic purity of the mass-reared GSS is kept intact. However, a FRS can better work in conjunction with a visible mutation.

Several genetic/selectable markers exist in anophelines [76–84]. However, most of them have not been assessed in respect to their potential use in the construction of a GSS. The most promising or stable selectable markers successfully used to date for the development of GSSs in insects include insecticide resistance [85] as well as color- and temperature-sensitive lethal mutations (*tsl*) [71]. GSS based on insecticide resistance markers have already been developed in *An. arabiensis* while a *tsl*-based GSS is currently under development [36, 83, 85].

Insecticide resistance based GSS

Several GSSs based on insecticide resistance markers have been developed in a number of anophelines, including *An. gambiae* (s.s.) [64], *An. albimanus* [86], *An. stephensi* [87], *An. quadrimaculatus* [88] and *An. arabiensis* [89, 90]. Of these, only *An. albimanus* has been used on a mass-rearing scale [91]. The drawback of using insecticides as selectable markers for sex separation during mass-rearing for SIT applications is the negative impact to the environment caused by accidental release of insecticides during treatment to eliminate females, risk of insecticide contaminating a mass-rearing colony, genetic instability of the marker and maintenance difficulties due to inherent sterility caused by chromosomal translocations [92].

Owing to a scarcity of other alternative selectable markers, the organochloride insecticide, dieldrin, was recently used to develop an *An. arabiensis* GSS ANO IPCL1 strain that allowed separation of males from females [85]. Life history characteristics such as egg hatch rates, development, longevity, female elimination reliability, radiation sensitivity and mating competitiveness were evaluated as part of efforts to establish a strain that can be useful in mass rearing [93–95]. Apart from high semi-sterility (73%) in males from the ANO IPCL1 strain, no differences in life history traits were found when compared to a dieldrin susceptible strain [94]. Tests on the genetic stability of the strain showed recombination rates to be as low as ~0.4%, lower than previously reported [96], making it favorable for mass-rearing. An added advantage that was observed when using dieldrin to separate males from females was the possible synergistic effect of the dieldrin whereby irradiated male pupae from dieldrin treated eggs continued to produce sperm in the first week of adult life, while adult males that had only been irradiated as pupae without the dieldrin treatment ceased to produce sperm. The advantage of this is that, from a sterile male release perspective, these males are expected to maintain their mating vigour post-release [51]. This led to the hypothesis that dieldrin treatment might have a protective

effect on the germinal cells of *An. arabiensis* against radiation [96]. Despite all these advantages, the use of the strain is threatened by environmental concerns. Dieldrin use for field research and applications has been prohibited since the 1970's. In addition, recent studies showed that dieldrin adhered to treatment containers and treated eggs retained residual dieldrin until adulthood following absorption through the chorion [97], and the overuse of dieldrin could aggravate the ever-present risk of potential contamination of other non-targeted colonies in the laboratory [95]. Lastly, the stability of this strain requires further verification as the low recombination rates observed in Yamada et al. [96] were based on phenotypic expression of the insecticide selectable marker rather than genetic monitoring.

Considerations need to be taken relating to the genetic background of a GSS and its mating compatibility with mosquitoes of a different geographic location for SIT purposes. Until recently, the only available *An. arabiensis* GSS was the ANO IPCL1 that comprised a Sudanese genetic background and as such may not be directly used for SIT releases in other countries. The introduction of an exogenous mosquito strain may face difficulties, such as possible mating incompatibility, that will dramatically affect the efficiency of SIT applications, regulatory approval, ethical concerns of releasing mosquitoes from a different geographical region and public acceptance. The South African SIT programme addressed these concerns by introgressing the dieldrin resistant gene from GSS ANO IPCL1 males into a locally colonized *An. arabiensis* wild-type strain (acronym KWAG), therefore maintaining a locally representative genetic background in the resultant new GSS strain (acronym GMK) [36].

The strain initially showed a reduction in egg hatch rates following repeated treatment with dieldrin at each generation (about 19.2%), which then improved to 30% with successive backcrossing [37]. The dieldrin resistance marker of GMK has been stable over 10 successive generations [37]. Tests for the presence or absence of the resistance to dieldrin (*Rdl*) mutation showed that 100% of the males were hemizygous for the resistant allele and 100% of the females were homozygous for the susceptible allele [37]. A high mating competitiveness against wild-type males when in competition for wild females was also observed in GMK males [37]. Additionally, the effect of irradiation on GMK females was investigated and compared to unirradiated females showing a negative effect of irradiation on female adult emergence [98]. However, GMK females were still capable of blood-feeding and demonstrated no difference in longevity post-irradiation. This result illustrates the importance for sex separation in mosquito SIT programmes because these females could potentially transmit pathogens during blood-feeding. Additionally, GMK is showing similar problems as observed in GSS ANO IPCL1, such as low productivity, dieldrin adherence to containers and absorption through the chorion. Due to these problems investigations of alternative, more environmentally acceptable, insecticides are taking place.

Theoretically, insecticides with a similar mode of action to dieldrin, i.e. targeting the γ-aminobutyric acid (GABA) receptor, should be able to be used as substitutes to dieldrin [99]. Several insecticides (lindane, picrotoxin, isoxoxale) which target the GABA receptor were tested. Treating third and fourth instar GMK larvae with lindane and picrotoxin eliminated 90% of the females showing that any insecticide that solely targets the GABA site can act as an alternative to dieldrin. Further investigations are currently ongoing to exploit plant based/organic alternatives (unpublished data).

Temperature-sensitive lethal based GSS

Temperature sensitive lethal mutations have been used successfully as sexing system selectable markers for *C. capitata* where females that are homozygous for the recessive mutation die when exposed to high temperatures, while hemizygous males survive under the same conditions [71, 100]. This is probably because the *tsl* mutation alters the function of an essential protein at different temperatures, the practical upshot of which is that at regular rearing temperatures protein function is maintained, but at higher temperature restrictive protein function is lost [100]. Other than the financial savings in not using insecticides and avoiding environmental or equipment contamination, *tsl*-based GSS also has the added advantage of the removal of females at a very early developmental stage (during embryonic stage). This translates to rearing cost reduction [71].

Initiatives are currently underway to develop a *tsl*-based GSS in *An. arabiensis* [42, 83]. Progress made thus far includes the successful isolation and characterization of an *An. arabiensis tsl* [83] following similar methods as [101]. During these efforts, wild-type male mosquitoes originating from North Cameroon were provided with a sugar solution spiked with 0.05M of ethyl methanesulfonate (EMS), for 24 hours [83]. Mutant male mosquitoes were crossed with virgin wild-type females and third generation (F3) progeny were heat-shocked at 41°C for 3 hours to screen for *tsl*. The established *tsl* strain showed similar life history traits (fertility, larval development time and adults' emergence) compared to the wild-type strain, and can be maintained at the same rearing temperature, i.e. 26 ± 1°C, as the wild-type strain. Preliminary genetic analysis suggests that the *tsl* phenotype is due to a recessive allele located on an autosome [83].

The successful establishment of the *An. arabiensis tsl* strain is a valuable tool towards the development of a

GSS for SIT applications against this species. Future research will focus on the characterization of the temperature-sensitivity range, the induction of a Y-autosome translocation to link the wild-type allele to the Y chromosome as well as the identification and characterization of the *tsl* gene and its potential use for novel approaches to develop a GSS for this species.

The characterization of the temperature-sensitive period is important as the temperature sensitivity status of strains differs depending on the developmental stage, duration of exposure and their insecticide resistance status [102–110]. Tests on the temperature sensitivity range (permissive and restrictive) should be performed to aid in establishing optimal rearing conditions, particularly under mass rearing settings. This can be achieved by exposing various developmental stages (from embryos to adults) to different temperatures and time ranges with an emphasis on the embryonic stage as this is the most practical stage [71].

The induction of a Y-autosome translocation will enable establishment of families where males are wild-type (temperature resistant) and females are mutant (temperature sensitive). Irradiation can be used to translocate alleles to the male determining Y chromosome [111, 112]. This can be followed by determination of the inheritance pattern to confirm that *tsl* is indeed inherited in a sex-specific manner.

Isolation of a traceable selectable marker linked to the *tsl* will allow for tracking of recombinants in mass-rearing systems [71]. In a *tsl*-based GSS, it would almost be impossible to detect and remove the recombinants without a traceable selectable marker [71]. A selectable marker that is close to the *tsl* locus would most likely be a good candidate, especially in the case of a visible selectable marker, as has been the case with the medfly VIENNA 8 GSS which carries a visible *white pupae* (*wp*) marker closely linked to the *tsl* on chromosome 5 [72].

There are several *tsl* loci in a genome; however, traceable phenotypic selectable markers such as *Rdl* may possibly be used in conjunction with *tsl* in a *tsl*-based GSS (Fig. 1). This hypothesis was drawn against reported findings in *Drosophila melanogaster* which showed *Rdl* and *tsl* to occur at the same locus [113, 114], leading to the hypothesis that this might also be true in other insects, including mosquitoes. Exploiting the *Rdl* locus in *An. arabiensis* may be promising because it has already been proven to induce conditional lethality [36, 85], both dominant and semi-dominant *Rdl* alleles are known [115], which allows for easy discrimination of homozygous susceptible and heterozygous resistant larvae and/or adults using a diagnostic dose of dieldrin. The mechanism of resistance is also known to be due to a single amino acid substitution in the target site [116], and the loci can easily be detected using polymerase chain reaction (PCR) [117].

Since multiple insecticide resistance and cross-resistance [118–123] together with trade-offs between temperature and insecticide resistance have been reported in various

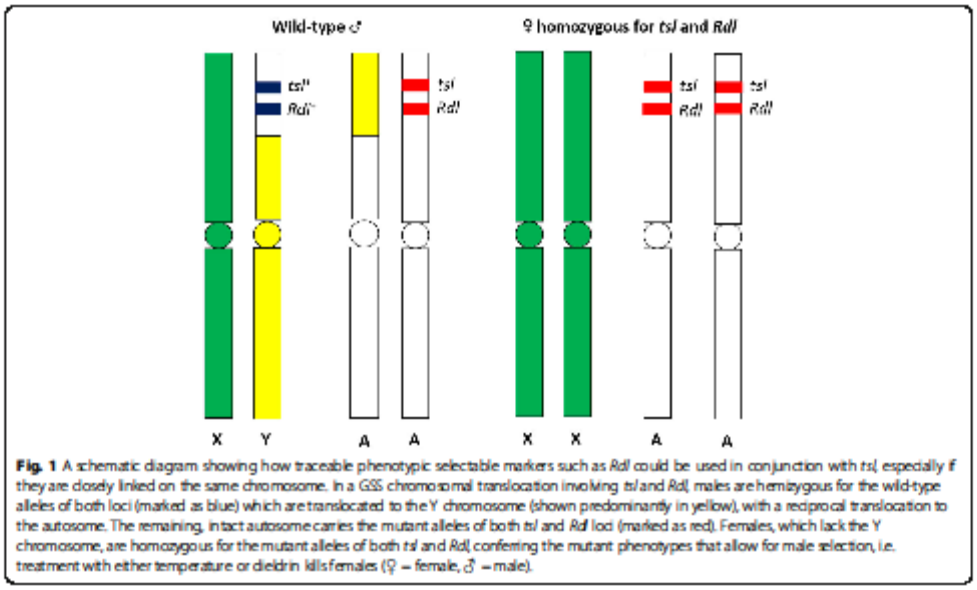

Fig. 1 A schematic diagram showing how traceable phenotypic selectable markers such as *Rdl* could be used in conjunction with *tsl*, especially if they are closely linked on the same chromosome. In a GSS chromosomal translocation involving *tsl* and *Rdl*, males are hemizygous for the wild-type alleles of both loci (marked as blue) which are translocated to the Y chromosome (shown predominantly in yellow), with a reciprocal translocation to the autosome. The remaining, intact autosome carries the mutant alleles of both *tsl* and *Rdl* loci (marked as red). Females, which lack the Y chromosome, are homozygous for the mutant alleles of both *tsl* and *Rdl*, conferring the mutant phenotypes that allow for male selection, i.e. treatment with either temperature or dieldrin kills females (♀ – female, ♂ – male).

studies [102, 105, 106, 108, 110], it can be hypothesized that these markers possibly behave in a similar fashion as *Rdl* and also could occur at the same loci as *tsl*. Therefore, investigations into insecticide resistance markers should be extended to include other target sites, including the knock-down resistance gene (*kdr*) [124–126], the acetylcholinesterase gene (*ace-1*^{R}) mutation [127–130] and molecular gene markers [131–133]. Alternatively, EMS can be used to induce other mutations as has been previously reported for three new *An. quadrimaculatus* mutants: (1) *rose eye* (*ro*), (2) *short antenna* (*Sa*) and (3) *melanotic* (*Mel*) [134–136]. The advantage of this approach is that a new GSS based on color can also be established.

Additionally, as the efficiency of a *tsl*-based GSS relies on the genetic composition of the sex chromosomes, knowledge on the cytogenetics of the target species will also be necessary. Fortunately, detailed reports exist on the *Anopheles* Y chromosome, which constitute essential components necessary for sex separation [137–139]. Cytogenetics can aid in the determination of the origin and size of the translocated segment, and localization of the translocation break-points [140–142], or map the extent of inversions introduced to reduce recombination [143, 144]. To maintain stability and avoid high recombination rates, a chromosomal inversion, ideally covering the region of translocation break-point, the *tsl*, and any other selectable markers, could also be integrated in the GSS. Inversions are known suppressors of genetic recombination through a positive heterotic system. In fact, maintenance and stability of dieldrin resistance in *An. gambiae* is associated with a paracentric inversion, 2La [145] and this could greatly improve the genetic stability of the GSS [71, 72, 143]. This has been shown in the medfly VIENNA 8 GSS [71]. Cytogenetic analyses such as karyotype examination of mitotic and meiotic chromosomes [146] and salivary gland polytene chromosomes [147] can be performed to map mutations and identify Y-autosome breakpoints in the resultant Y-autosome translocations/GSS. Subsequent investigations into life history traits together with genetic characterization needed prior to the strain's use in large-scale operational SIT programmes [71].

GSSs can also be developed using novel molecular-based approaches including, among others, conditional lethal systems, RNAi approaches or by using transgenic strains with integrated sex-specific fluorescent markers [41, 148, 149].

Conclusions

The development of a sex separation strategy using pupal sexual dimorphism is not applicable to *An. arabiensis*. The use of ivermectin to eliminate females shows great potential, but currently cannot be relied upon as a sole sex-separation strategy and would require further investigation on formulations and effectiveness if added to an artificial blood-meal. The practicality of using ivermectin in mass-rearing will also need to be tested. Alternatively, research can be focused on exploiting female blood-seeking behaviour without using blood at all but simply as a segregation tool. Furthermore, male behaviour though not fully explored in this review has potential. It has been suggested that sex-specific male swarming behaviour in anophelines could be exploited to develop efficient sex separation strategy. The success achieved in developing a GSS containing a South African genetic background and positive attributes such as accelerated development of aquatic stages and high survival rates at all life stages has provided encouragement for the application of this GSS in the local SIT pilot studies, unless a suitable alternative to dieldrin-based sex-separation can be found. Further investigations and optimization of treatment procedures and monitoring of the genetic stability of the strain are still required. The promising results shown by some alternate insecticides may provide additional options and solutions to the difficulties faced by the current dieldrin-based GSS. The successful establishment and characterization of an *An. arabiensis tsl* strain offers possibilities into development of a new GSS. In its current form, the *tsl* strain cannot be directly applied as a GSS and would require the characterization of the temperature-sensitivity range, inheritance linkage of the wild-type allele to the sex determining Y chromosome and isolation of a visible selectable marker closely linked to *tsl*. If the linkage of *tsl* and *Rdl* in Drosophila also exists in *An. arabiensis*, this would allow the easier monitoring of the *tsl* marker until a better, ideally visible, marker linked to *tsl* is isolated. This can be achieved through the screening of laboratory and natural populations for spontaneous mutations or EMS and/or irradiation-induced mutagenesis. Finally, knowledge on the cytogenetics of the target species will be necessary and subsequent investigations into life history traits together with genetic characterization are warranted prior to the strain being used in large-scale operational SIT programmes.

Abbreviations

ACT: Artemisinin-based combination therapy; ATP: Adenosine triphosphate; EMS: Ethyl methanesulfonate; GABA: γ-aminobutyric acid; GSS: Genetic sexing strain; IRS: Indoor residual spraying; ITNs: Insecticide-treated bednets; *Rdl*: Resistance to dieldrin; SIT: Sterile insect technique; *tsl*: Temperature-sensitive lethal

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Availability of data and materials
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Authors' contributions
TM carried out most of the laboratory work and wrote the first and subsequent drafts of the manuscript. CN was involved in *tsl* work and contributed to the subsequent writing of the manuscript. LLK conceived the project, was involved in project implementation and contributed to the writing of the manuscript. LCD helped with development of GSS strain and provided comments on the manuscript. ORW helped with alternative to dieldrin experimental work and provided comments on the manuscript. LM was involved in alternative to dieldrin experimental work and provided comments on the manuscript. YP was involved *tsl* work and provided comments on the final draft. LNL was involved in GSS strain stability experiments and provided comments on the final draft. MK was involved in GSS and alternative to dieldrin experimental work and contributed to the writing of the manuscript. KB coordinated the activities related to the CRP project "Exploring genetic, molecular, mechanical and behavioural methods of sex separation in mosquitoes" and contributed to the final version of the manuscript. GM conceived the project, oversaw its implementation, helped in the interpretation of results, and contributed to the subsequent writing of the manuscript. All authors read and approved the final manuscript.

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References

1. WHO. World Malaria Report 2017. WHO Global Malaria Programme. World Health Organisation. 2017. http://www.who.int/malaria/publications/world_malaria_report/en/index.html. Accessed 23 Mar 2018.
2. Sachs J, Malaney P. The economic and social burden of malaria. Nature. 2002;415:680.
3. Beier JC, Keating J, Githure JI, Macdonald MB, Impoinvil DE, Novak RJ. Integrated vector management for malaria control. Malar J. 2008;7:S4.
4. Chanda E, Masaninga F, Coleman M, Sikaala C, Katebe C, MacDonald M, Baboo KS, Govere J, Mpanga L. Integrated vector management: the Zambian experience. Malar J. 2008;7:164.
5. Fillinger U, Lindsay SW. Larval source management for malaria control in Africa: myths and reality. Malar J. 2011;10:353.
6. Ranson H, N'Guessan R, Lines J, Moiroux N, Nkuni Z, Corbel V. Pyrethroid resistance in African anopheline mosquitoes: what are the implications for malaria control? Trends Parasitol. 2011;27:91–8.
7. Brooke B, Koekemer L, Kruger P, Urbach J, Mbalati E, Coetzee M. Malaria vector control in South Africa. S Afr Med J. 2013;103:784–8.
8. Haldar K, Bhattacharjee S, Safeukui I. Drug resistance in *Plasmodium*. Nat Rev Microbiol. 2018;16(3):156–70.
9. Gillies MT, de Meillon B. The Anophelinae of Africa South of the Sahara (Ethiopian zoogeographical region). Johannesburg: Publ S Afr Inst Med Res. 1968;541–343.
10. Gillies MT, Coetzee M. A supplement to the Anophelinae of Africa South of the Sahara (Afrotropical region), Johannesburg, South Africa. Publ S Afr Inst Med Res. 1987;55:1–143.
11. Benedict MQ, Robinson AS. The first releases of transgenic mosquitoes: an argument for the sterile insect technique. Trends Parasitol. 2003;19:349–55.
12. James AA. Gene drive systems in mosquitoes: rules of the road. Trends Parasitol. 2005;21:64–7.
13. Bouyer J, Lefrançois T. Boosting the sterile insect technique to control mosquitoes. Trends Parasitol. 2014;30:271–3.
14. Alphey L, Benedict M, Bellini R, Clark GG, Dame DA, Service MW, Dobson SL. Sterile-insect methods for control of mosquito-borne diseases: an analysis. Vector Borne Zoonotic Dis. 2010;10:295–311.
15. Lees RS, Gilles JR, Hendrichs J, Vreysen MJ, Bourtzis K. Back to the future: the sterile insect technique against mosquito disease vectors. Curr Opin Insect Sci. 2015;10:156–62.
16. Zhang D, Zheng X, Xi Z, Bourtzis K, Gilles JR. Combining the sterile insect technique with the incompatible insect technique: impact of Wolbachia infection on the fitness of triple and double infected strains of *Aedes albopictus*. PLoS One. 2015;10:e0121126.
17. Zhang D, Lees RS, Xi Z, Gilles JR, Bourtzis K. Combining the sterile insect technique with Wolbachia-based approaches: II-a safer approach to *Aedes albopictus* population suppression programmes, designed to minimize the consequences of inadvertent female release. PLoS One. 2015;10:e0135194.
18. Zhang D, Lees RS, Xi Z, Bourtzis K, Gilles JR. Combining the Sterile Insect Technique with the Incompatible Insect Technique: III-robust mating competitiveness of irradiated triple *Wolbachia*-infected *Aedes albopictus* males under semi-field conditions. PLOS One. 2016;11:e0151864.
19. Bourtzis K, Lees RS, Hendrichs J, Vreysen MJ. More than one rabbit out of the hat: radiation, transgenic and symbiont-based approaches for sustainable management of mosquito and tsetse fly populations. Acta Trop. 2016;157:115–30.
20. Eckhoff PA, Wenger EA, Godfray HC, Burt A. Impact of mosquito gene drive on malaria elimination in a computational model with explicit spatial and temporal dynamics. Proc Natl Acad Sci USA. 2017;114:E255–64.
21. Dyck VA, Hendrichs J, Robinson AS. Sterile Insect Technique - principles and practice in area-wide integrated pest management. Dordrecht: Springer; 2005.
22. Vreysen MJB, Robinson AS, Hendrichs J, editors. Area-Wide Control of Insect Pests: From Research to Field Implementation. Dordrecht: Springer; 2007.
23. Morlan HB, McCray EM Jr, Kilpatrick JW. Field-tests with sexually sterile males for control of *Aedes aegypti*. Mosq News. 1962;22:295–300.
24. Hassan MM, El-Motasim WM, Ahmed RT, El-Sayed BB. Prolonged colonisation, irradiation, and transportation do not impede mating vigour

and competitiveness of male *Anopheles arabiensis* mosquitoes under semi-field conditions in northern Sudan. Malar World J. 2010;1:2.
25. Mashatola T, Munhenga G, Koekemoer LL. Evaluating different cages for rearing *Anopheles arabiensis* (Diptera: Culicidae) under laboratory conditions. Afr Entomol. 2017;25:534–9.
26. Helinski ME, Malcolm CA, El-Motasim WM, Malcolm CA, Knols BG, El-Sayed B. Towards a sterile insect technique field release of *Anopheles arabiensis* mosquitoes in Sudan: irradiation, transportation, and field cage experimentation. Malar J. 2008;7:65.
27. Munhenga G, Brooke BD, Chirwa TF, Hunt RH, Govender D, Coetzee M, et al. Evaluating the potential of the sterile insect technique for malaria control: relative fitness and mating compatibility between laboratory colonized and a wild population of *Anopheles arabiensis* from the Kruger National Park, South Africa. Parasit Vectors. 2011;4:208.
28. Oliva CF, Jacquet M, Gilles J, Lemperiere G, Maquart PO, Quilici S, et al. The sterile insect technique for controlling populations of *Aedes albopictus* (Diptera: Culicidae) on Reunion Island: mating vigour of sterilized males. PLoS One. 2012;7:e49414.
29. Oliva CF, Maier M-J, Gilles J, Jacquet M, Lemperiere G, Quilici S, Vreysen MJ, Schooneman F, Chadee DD, Boyer S. Effects of irradiation, presence of females, and sugar supply on the longevity of sterile males *Aedes albopictus* (Skuse) under semi-field conditions on Reunion Island. Acta Trop. 2013;125:287–93.
30. Agnew TB, Damiens D, Alsharif B, Ahmed A, Salih EH, Ahmed FT, Diabaté A, Lees RS, Gilles JR, El Sayed BB. Participation of irradiated *Anopheles arabiensis* males in swarms following field release in Sudan. Malar J. 2014;13:484.
31. Munhenga G, Brooke BD, Spillings B, Esrap L, Hunt RH, Midzi S, et al. Sterile Insect Technique feasibility study in northern Kruger National Park, South Africa: field study site selection, species abundance and monthly distribution of anopheline mosquitoes. Malar J. 2014;13:27.
32. Azrag RS, Ibrahim K, Malcolm C, El Rayah E, El-Sayed B. Laboratory rearing of *Anopheles arabiensis*: impact on genetic variability and implications for Sterile Insect Technique (SIT) based mosquito control in northern Sudan. Malar J. 2016;15:432.
33. Munhenga G, Brooke BD, Gilles JR, Slabbert K, Kemp A, Dandalo LC, et al. Mating competitiveness of sterile genetic sexing strain males (GAMA) under laboratory and semi-field conditions: steps towards the use of the Sterile Insect Technique to control the major malaria vector *Anopheles arabiensis* in South Africa. Parasit Vectors. 2016;9:122.
34. Oliva CF, Vreysen MJ, Dupé S, Lees RS, Gilles JR, Gouagna LC, Chiem R. Current status and future challenges for controlling malaria with the sterile insect technique: technical and social perspectives. Acta Trop. 2014;132:S130–9.
35. Manana PN, Kuonza L, Musekiwa A, Mpangane HD, Koekemoer LL. Knowledge, attitudes and practices on malaria transmission in Mamfene, KwaZulu-Natal Province, South Africa 2015. BMC Public Health. 2018;18:41.
36. Dandalo LC, Munhenga G, Kaiser ML, Koekemoer LL. Development of a genetic sexing strain of *Anopheles arabiensis* for KwaZulu-Natal. South Africa Med Vet Entomol. 2018;32:61–9.
37. Dandalo LC. Characterization of a local genetic sexing strain as well as a wild population of Anopheles arabiensis from KwaZulu-Natal, South Africa. In: PhD Thesis. Johannesburg: University of the Witwatersrand; 2016.
38. Clements AN. The biology of mosquitoes development, nutrition and reproduction vol. 1. CAB International.: Wallingford; 1992.
39. Hendrichs J, Franz G, Rendón P. Increased effectiveness and applicability of the Sterile Insect Technique through male-only release for control of Mediterranean fruit-flies during fruiting seasons. J Appl Entomol. 1995;119:371–7.
40. Rendón P, McInnis D, Lance D, Stewart J. Medfly (Diptera: Tephritidae) genetic sexing: large-scale field comparison of males-only and bisexual sterile fly releases in Guatemala. J Econ Entomol. 2004;97:1547–53.
41. Papathanos PA, Bossin HC, Benedict MQ, Catteruccia F, Malcolm CA, Alphey L, Crisanti A. Sex separation strategies: past experience and new approaches. Malar J. 2009;8:S5.
42. Gilles JR, Schetelig MF, Scolari F, Marec F, Capurro ML, Franz G, Bourtzis K. Towards mosquito sterile insect technique programmes: exploring genetic, molecular, mechanical and behavioural methods of sex separation in mosquitoes. Acta Trop. 2014;132:S178–87.
43. Leimar O, Karlsson B, Wiklund C. Unpredictable food and sexual size dimorphism in insects. Proc R Soc Lond B. 1994;258:121–5.
44. McCray EM Jr. A mechanical device for the rapid sexing of *Aedes aegypti* pupae. J Econ Entomol. 1961;54.
45. Sharma VP, Patterson RS, Ford HR. A device for the rapid separation of male and female mosquito pupae. Bull World Health Organ. 1972;47:429.
46. Focks DA. An improved separator for the developmental stages, sexes and species of mosquitoes (Diptera, Culicidae). J Med Entomol. 1980;17:567–8.
47. Medici A, Carrieri M, Scholte EJ, Maccagnani B, Dindo ML, Bellini R. Studies on *Aedes albopictus* larval mass-rearing optimization. J Econ Entomol. 2011;104:266–73.
48. Dame DA, Lofgren CS, Ford HR, Boston MD, Baldwin KF, Jeffery GM. Release of chemosterilized males for the control of *Anopheles albimanus* in El Salvador. Am J Trop Med Hyg. 1974;23:282–7.
49. Zacarés M, Salvador-Herranz G, Almenar D, Tur C, Argilés R, Bourtzis K, Bossin H, Pla I. Exploring the potential of size distribution analysis for the sex sorting of different vector mosquito species. Parasit Vectors (this special issue).
50. Lowe RE, Fowler JEF, Bailey DL, Dame DA, Savage KE. Separation of sexes of adult *Anopheles albimanus* by feeding of insecticide-laden blood. Mosq News. 1981;41:634–8.
51. Charlwood JD, Pinto J, Sousa CA, Ferreira C, Petrarca V, Rosario Vle. 'A mate or a meal' - Pre-gravid behaviour of female *Anopheles gambiae* from the Islands of Sao Tome and Principe, West Africa Malar J. 2003;2:9–10.
52. Damiens D, Vreysen MJB, Gilles JRL. *Anopheles arabiensis* sperm production after genetic manipulation, dieldrin treatment, and irradiation. J Med Entomol. 2013;50:314–6.
53. Lahondère C, Lazzari CR. Mosquitoes cool down during blood feeding to avoid overheating. Curr Biol. 2012;22:40–5.
54. Yamada H, Soliban SM, Vreysen MJ, Chadee DD, Gilles JR. Eliminating female *Anopheles arabiensis* by spiking blood meals with toxicants as a sex separation method in the context of the sterile insect technique. Parasit Vectors. 2013;6:197.
55. Chaccour C, Barrio Al, Royo AG, Urbistondo DM, Slater H, Hammann F, Del Pozo JL. Screening for an ivermectin slow-release formulation suitable for malaria vector control. Malar J. 2015;14:102.
56. Chaccour C, Abizanda G, Irigoyen A, Del Pozo JL. Pilot study of a slow-release ivermectin formulation for malaria control in a pig model. Antimicrob Agents Chemother. 2017;61:e02104–16.
57. Chaccour CJ, Ngha'bi K, Abizanda G, Barrio AI, Aldaz A, Okumu F, Slater H, Del Pozo JL, Killeen G. Targeting cattle for malaria elimination: marked reduction of *Anopheles arabiensis* survival for over six months using a slow-release ivermectin implant formulation. Parasit Vectors. 2018;11:287.
58. Mouatcho JC, Munhenga G, Hargreaves K, Brooke BD, Coetzee M, Koekemoer LL. Pyrethroid resistance in a major African malaria vector, *Anopheles arabiensis*, from Mamfene, northern KwaZulu/Natal. S Afr J Sci. 2009;105:127–31.
59. Galun RA. Feeding stimuli and artificial feeding. Bull World Health Organ. 1967;36:590.
60. Davis EE, Sokolove PG. Lactic acid-sensitive receptors on the antennae of the mosquito, *Aedes aegypti*. J Comp Physiol. 1976;105:43–4.
61. Ghaninia M, Larsson M, Hansson BS, Ignell R. Natural odor ligands for olfactory receptor neurons of the female mosquito *Aedes aegypti*: use of gas chromatography-linked single sensillum recordings. J Exp Biol. 2008;211:3000–7.
62. Gonzales KK, Hansen IA. Artificial diets for mosquitoes. Int J Environ Res Public Health. 2016;13:1267.
63. Pitts RJ. A blood-free protein meal supporting oogenesis in the Asian tiger mosquito, *Aedes albopictus* (Skuse). J Insect Physiol. 2014;64:41–6.
64. Curtis CF, Akiyama J, Davidson G. A genetic sexing system in *Anopheles gambiae* species A. Mosq News. 1976;36:492–8.
65. McCombs SD, Saul SH. Translocation-based genetic sexing system for the oriental fruit fly (Diptera: Tephritidae) based on pupal color dimorphism. Ann Entomol Soc Am. 1995;88:659–68.
66. Robinson AS. Genetic sexing strains in medfly, *Ceratitis capitata*, sterile insect technique programmes. Genetica. 2002;116:5–13.
67. McInnis DO, Tam S, Lim R, Komatsu J, Kurashima R, Albrecht C. Development of a pupal color-based genetic sexing strain of the melon fly, *Bactrocera cucurbitae* (Coquillett)(Diptera: Tephritidae). Ann Entomol Soc Am. 2004;97:1026–33.
68. Zepeda-Cisneros CS, Hernández JS, García-Martínez V, Ibañez-Palacios J, Zacharopoulou A, Franz G. Development, genetic and cytogenetic analyses of genetic sexing strains of the Mexican fruit fly, *Anastrepha ludens* Loew (Diptera: Tephritidae). BMC Genetics. 2014;15:51.
69. Zacharopoulou A, Augustinos AA, Drosopoulou E, Tsoumani KT, Gariou-Papalexiou A, Franz G, Mathiopoulos KD, Bourtzis K, Mavragani-Tsipidou P. A review of more than 30 years of cytogenetic studies of Tephritidae in support of sterile insect technique and global trade. Entomol Exp Appl. 2013;148:204–25.

70. Caceres C. Mass rearing of temperature sensitive genetic sexing strains in the Mediterranean fruit fly (*Ceratitis capitata*). Genetica. 2002;116:107–16.
71. Franz G. Genetic sexing strains in Mediterranean fruit fly, an example for other species amenable to large-scale rearing for the sterile insect technique. In: Dyck VA, Hendrichs J, Robinson AS, editors. Sterile Insect Technique-principles and practice in area-wide integrated pest management. Dordrecht: Springer; 2005.
72. Augustinos AA, Targovska A, Cancio-Martinez E, Schorn E, Franz G, Cáceres C, Zacharopoulou A, Bourtzis K. *Ceratitis capitata* genetic sexing strains: laboratory evaluation of strains from mass-rearing facilities worldwide. Entomol Exp Appl. 2011;164:305–17.
73. Franz G. Recombination between homologous autosomes in medfly (*Ceratitis capitata*) males type-1 recombination and the implications for the stability of genetic sexing strains. Genetica. 2002;116:73–84.
74. Cáceres C, Fisher K, Rendón P. Mass rearing of the medfly Temperature Sensitive Lethal genetic sexing strain in Guatemala. In: Area-wide control of fruit flies and other insect pests. Joint proceedings of the International conference on area-wide control of insect pests, and the Fifth International Symposium on Fruit Flies of Economic Importance, Penang, Malaysia, 2000.
75. Fisher K, Caceres C. A filter rearing system for mass reared genetic sexing strains of Mediterranean fruit fly (Diptera: Tephritidae). In: Area-wide control of fruit flies and other insect pests. Joint proceedings of the International conference on area-wide control of insect pests, and the Fifth International Symposium on Fruit Flies of Economic Importance, Penang, Malaysia, 2000.
76. Seawright JA, Childress LV, Benedict MQ. Genetics of green larva, a recessive mutant on chromosome 2 in *Anopheles albimanus* Wiedemann. Mosq News. 1979.
77. Dubash C, Sakai RK, Baker RH. Two new body color mutants in the mosquito: the genetics of yellow and green in *Anopheles culicifacies*. J Hered. 1982;73:340–4.
78. Mitchell SE, Seawright JA. A red stripe mutant and its relationship in an allelic series in *Anopheles quadrimaculatus*. J Hered. 1984;75:421–2.
79. Mukiama TK. Y-autosome genetic sexing strain of *Anopheles albimanus* (Diptera: Culicidae). Int J Trop Insect Sci. 1985;6:649–52.
80. Malcolm CA, Mali P. Genetic sexing of *Anopheles stephensi* with the larval morphological mutant BI. Genetica. 1986;70:37–42.
81. Benedict MQ, Besansky NJ, Chang H, Mukabayire O, Collins FH. Mutations in the *Anopheles gambiae* pink-eye and white genes define distinct, tightly linked eye-color loci. J Hered. 1996;87:48–53.
82. Benedict MQ, McNitt LM, Collins FH. Genetic traits of the mosquito *Anopheles gambiae*: red stripe, frizzled, and homochromy1. J Hered. 2003;94:227–35.
83. Ndo C, Poumachu Y, Mettsl D, Awono-Ambene H, Tchuinkam T, Gilles JLR, Bourtzis K. Isolation and characterization of a temperature-sensitive lethal strain of *Anopheles arabiensis*. Parasit Vectors. (this special issue).
84. Seawright JA, Kaiser PE, Dame DA, Lofgren CS. Genetic method for the preferential elimination of females of *Anopheles albimanus*. Science. 1978; 200:1303–4.
85. Yamada H, Benedict MQ, Malcolm CA, Oliva CF, Soliban SM, Gilles JR. Genetic sex separation of the malaria vector, *Anopheles arabiensis*, by exposing eggs to dieldrin. Malar J. 2012;11:208.
86. Kaiser PE, Seawright JA, Dame DA, Joslyn DJ. Development of a genetic sexing system for *Anopheles albimanus*. J Econ Entomol. 1978;71:766–1.
87. Robinson AS. Genetic sexing in *Anopheles stephensi* using dieldrin resistance. J Am Mosq Control Assoc. 1986;2:93–5.
88. Kim SS, Seawright JA, Kaiser PE. A genetic sexing strain of *Anopheles quadrimaculatus*, species A. J Am Mosq Control Assoc. 1987;3:50–3.
89. Curtis CF. Genetic sex separation in *Anopheles arabiensis* and the production of sterile hybrids. Bull World Health Organ. 1978;56:453–4.
90. Lines JD, Curtis CF. Genetic sexing systems in *Anopheles arabiensis* Patton (Diptera: Culicidae). J Econ Entomol. 1985;78:848–1.
91. Klassen W, Curtis CF. History of the sterile insect technique. In: Dyck VA, Hendrichs J, Robinson AS, editors. Sterile Insect Technique Principles and Practice in Area-Wide Integrated Pest Management. Dordrecht: Springer; 2005.
92. Calkins CO, Parker AG. Sterile insect quality. In: Dyck VA, Hendrichs J, Robinson AS, editors. Sterile Insect Technique Principles and Practice in Area-Wide Integrated Pest Management. Dordrecht: Springer; 2005.
93. Ndo C, Yamada H, Damiens DD, N'do S, Seballos G, Gilles JRL. X-ray sterilization of the *Anopheles arabiensis* genetic sexing strain "ANO IPCL1" at pupal and adult stages. Acta Trop. 2014;131:124–8.
94. Oliva CF, Benedict MQ, Soliban SM, Lemperiere G, Balestrino F, Gilles JR. Comparisons of life-history characteristics of a genetic sexing strain with laboratory strains of *Anopheles arabiensis* (Diptera: Culicidae) from northern Sudan. J Med Entomol. 2014;49:1045–51.
95. Yamada H, Vreysen MJB, Gilles JRL, Munhenga G, Damiens DD. The effect of genetic manipulation, dieldrin treatment and irradiation on the mating competitiveness of male *Anopheles arabiensis* in field cages. Malar J. 2014;13:318.
96. Yamada H, Vreysen MJ, Bourtzis K, Tschirk W, Chadee DD, Gilles JR. The *Anopheles arabiensis* genetic sexing strain ANO IPCL1 and its application potential for the sterile insect technique in integrated vector management programmes. Acta Trop. 2015;142:138–44.
97. Yamada H, Jandric Z, Chhem-Kieth S, Vreysen MJ, Rathor MN, Gilles JR, Carnavan A. *Anopheles arabiensis* egg treatment with dieldrin for sex separation leaves residues in male adult mosquitoes that can bioaccumulate in goldfish (*Carassius auratus auratus*). Environ Toxicol Chem. 2013;32:2786–91.
98. Dandalo LC, Kemp A, Koekemoer LL, Munhenga G. Effect of ionising (gamma) radiation on female *Anopheles arabiensis*. Trans R Soc Trop Med Hyg. 2017;111:38–40.
99. Salgado VL, Schnatterer S, Holmes KA. Ligandgated chloride channel antagonists (fiprole s). In: Kraemer W, Schirmer U, editors. Modern crop protection compounds. Wiley-VCH Verlag GmbH & Co, Weinheim: Germany; 2007. p. 1048–69.
100. Kerremans P, Franz G. Use of a temperature-sensitive lethal mutation strain of medfly (*Ceratitis capitata*) for the suppression of pest populations. Theor Appl Genet. 1995;90:511–8.
101. Sakai RK, Baker RH. Induction of heat-sensitive lethals in *Culex tritaeniorhynchus* by ethyl methanesulfonate. Mosq News. 1974;34:420–4.
102. Patil NS, Lole KS, Deobagkar DN. Adaptive larval thermotolerance and induced cross-tolerance to propoxur insecticide in mosquitoes *Anopheles stephensi* and *Aedes aegypti*. Med Vet Entomol. 1996;10:277–82.
103. Bayoh MN, Lindsay SW. Effect of temperature on the development of the aquatic stages of *Anopheles gambiae sensu stricto* (Diptera: Culicidae). Bull Entomol Res. 2003;93:375–81.
104. Bayoh MN, Lindsay SW. Temperature-related duration of aquatic stages of the Afrotropical malaria vector mosquito *Anopheles gambiae* in the laboratory. Med Vet Entomol. 2004;18:174–9.
105. Swain V, Seth RK, Mohanty SS, Raghavendra K. Effect of temperature on development, eclosion, longevity and survivorship of malathion-resistant and malathion-susceptible strain of *Culex quinquefasciatus*. Parasitol Res. 2008;103:299–3.
106. Swain V, Seth RK, Raghavendra K, Mohanty SS. Impact of temperature on susceptible and resistant strains of *Culex quinquefasciatus* to synthetic pyrethroids. Acta Trop. 2009;111:203–7.
107. Raghavendra K, Barik TK, Swain V. Studies on the impact of thermal stress on survival and development of adaptive thermotolerance in immature stages of *Anopheles culicifacies*. J Ecobiotechnol. 2010;2(5):25–30.
108. Muturi EJ, Lampman R, Costanzo K, Alto BW. Effect of temperature and insecticide stress on life-history traits of *Culex restuans* and *Aedes albopictus* (Diptera: Culicidae). J Med Entomol. 2011;48:243–50.
109. Christiansen-Jucht C, Parham PE, Saddler A, Koella JC, Basáñez MG. Temperature during larval development and adult maintenance influences the survival of *Anopheles gambiae* s.s. Parasit Vectors. 2014;7:489.
110. Oliver SV, Brooke BD. The effect of elevated temperatures on the life history and insecticide resistance phenotype of the major malaria vector *Anopheles arabiensis* (Diptera: Culicidae). Malar J. 2017;16:73.
111. Rabbani MG, Kitzmiller B. Chromosomal translocations in *Anopheles albimanus* Wiedemann. Entomol Exp Appl. 1972;11:277–85.
112. Rabbani MG, Seawright JA. Use of Y-autosome translocations in assigning the stripe locus to chromosome 3 in the mosquito *Anopheles albimanus*. Ann Entomol Soc Am. 1976;69:266–8.
113. Ffrench-constant RH, Steichen JC, Ode PJ. Cyclodiene insecticide resistance in *Drosophila melanogaster* (Meigen) is associated with a temperature-sensitive phenotype. Pest Biochem Physiol. 1993;46:73–7.
114. Steichen JC, Rocheleau TA, Aronstein K, Roush RT. A single-amino acid substitution in a gamma-aminobutyric acid subtype A receptor locus is associated with cyclodiene insecticide resistance in Drosophila populations. Proc Natl Acad Sci USA. 1993;90:1957–61.
115. Davidson G. Insecticide resistance in *Anopheles gambiae* Giles: a case of simple Mendelian inheritance. Nature. 1956;178:861–3.
116. Du W, Awolola TS, Howell PI, Koekemoer LL, Brooke BD, Benedict MQ, et al. Independent mutations in the *Rdl* locus confer dieldrin resistance to *Anopheles gambiae* and *An. arabiensis*. Insect Mol Biol. 2005;14:179–83.

117. Wilkins EE, Howell PI, Benedict MQ. IMP PCR primers detect single nucleotide polymorphisms for *Anopheles gambiae* species identification, Mopti and Savanna rDNA types, and resistance to dieldrin in *Anopheles arabiensis*. Malar J. 2006;5:125–31.
118. Koekemoer LL, Spillings BL, Christian RN, Lo TC, Kaiser ML, Norton RA, et al. Multiple insecticide resistance in *Anopheles gambiae* (Diptera: Culicidae) from pointe Noire, Republic of the Congo. Vector Borne Zoonotic Dis. 2011;11:1193–200.
119. Djouaka R, Riveron JM, Yessoufou A, Tchigossou G, Akoton R, Irving H, et al. Multiple insecticide resistance in an infected population of the malaria vector *Anopheles funestus* in Benin. Parasit Vectors. 2016;9:453.
120. Djouaka RJ, Atoyebi SM, Tchigossou GM, Riveron JM, Irving H, Akoton R, et al. Evidence of a multiple insecticide resistance in the malaria vector *Anopheles funestus* in South West Nigeria. Malar J. 2016;15:565.
121. Menze BD, Riveron JM, Ibrahim SS, Irving H, Antonio-Nkondjio C, Awono-Ambene PH, Wondji CS. Multiple insecticide resistance in the malaria vector *Anopheles funestus* from northern Cameroon is mediated by metabolic resistance alongside potential target site insensitivity mutations. PLoS One. 2016;11:e0161261.
122. Riveron JM, Osae M, Egyir-Yawson A, Irving H, Ibrahim SS, Wondji CS. Multiple insecticide resistance in the major malaria vector *Anopheles funestus* in southern Ghana: implications for malaria control. Parasit Vectors. 2016;9:504.
123. Ibrahim SS, Ndula M, Riveron JM, Irving H, Wondji CS. The P450 CYP6Z1 confers carbamate/pyrethroid cross-resistance in a major African malaria vector beside a novel carbamate-insensitive N485I acetylcholinesterase-1 mutation. Mol Ecol. 2016;25:3436–52.
124. Reimer L, Fondjo E, Patchoké S, Diallo B, Lee Y, Ng A, et al. Relationship between *kdr* mutation and resistance to pyrethroid and DDT insecticides in natural populations of *Anopheles gambiae*. J Med Entomol. 2008;45:260–6.
125. Martinez-Torres D, Chandre F, Williamson MS, Darriet F, Berge JB, Devonshire AL, et al. Molecular characterization of pyrethroid knockdown resistance (*kdr*) in the major malaria vector *Anopheles gambiae* s.s. Insect Mol Biol. 1998;7:179–84.
126. Ranson H, Jensen B, Vulule JM, Wang X, Hemingway J, Collins FH. Identification of point mutation in the voltage-gated sodium channel gene of Kenyan *Anopheles gambiae* associated with resistance to DDT and pyrethroids. Insect Mol Biol. 2000;9:491–7.
127. Weill M, Malcolm C, Chandre F, Mogensen K, Berthomieu A, Marquine M, Raymond M. The unique mutation in *ace-1* giving high insecticide resistance is easily detectable in mosquito vectors. Insect Mol Biol. 2004;13:1–7.
128. Djogbénou L, Weill M, Hougard JM, Raymond M, Akogbeto M, Chandre F. Characterization of insensitive acetylcholinesterase (*ace-1*^{R}) in *Anopheles gambiae* (Diptera: Culicidae): resistance levels and dominance. J Med Entomol. 2007;44:805–10.
129. Djogbenou L, Chandre F, Berthomieu A, Dabire R, Koffi A, Alout H, Weill M. Evidence of introgression of the *ace-1*^{R} mutation and of the *ace-1* duplication in West African *Anopheles gambiae* s. s. PLoS One. 2008;3:e2172.
130. Bass C, Nikou D, Vontas J, Williamson MS, Field LM. Development of high-throughput real-time PCR assays for the identification of insensitive acetylcholinesterase (*ace-1*^{R}) in *Anopheles gambiae*. Pest Biochem Physiol. 2010;96:80–5.
131. Muriithenga G, Koekemoer LL. Differential expression of cytochrome P450 genes in a laboratory selected *Anopheles arabiensis* colony. Afr J Biotechnol. 2011;10:12741–6.
132. Nardini L, Christian RN, Coetzer N, Ranson H, Coetzee M, Koekemoer LL. Detoxification enzymes associated with insecticide resistance in laboratory strains of *Anopheles arabiensis* of different geographic origin. Parasit Vectors. 2012;5:113.
133. Nardini L, Hunt RH, Dahan-Moss YL, Christie N, Christian RN, Coetzee M, Koekemoer LL. Malaria vectors in the Democratic Republic of the Congo: the mechanisms that confer insecticide resistance in *Anopheles gambiae* and *Anopheles funestus*. Malar J. 2017;16:448.
134. Sakai RK, Ainsley RW, Baker RH. The inheritance of rose eye, a sex linked mutant in the malaria vector *Anopheles culicifacies*. Can J Genet Cytol. 1977; 19:633–6.
135. Sakai RK, Chaudhry ME, Baker RH. An EMS-induced mutant, rose eye, in *Culex quinquefasciatus*. J Hered. 1980;71:136–9.
136. Mitchell SE, Seawright JA. EMS-induced mutations in *Anopheles quandrimaculatus* (Say), species A. J Hered. 1989;80:58–1.
137. Hall AB, Qi Y, Timoshevskiy V, Sharakhova MV, Sharakhov IV, Tu Z. Six novel Y chromosome genes in *Anopheles* mosquitoes discovered by independently sequencing males and females. BMC Genomics. 2013;14:273.
138. Bernardini F, Galizi R, Menichelli M, Papathanos PA, Dritsou V, Marois E, Crisanti A, Windbichler N. Site-specific genetic engineering of the *Anopheles gambiae* Y chromosome. Proc Natl Acad Sci USA. 2014;111:7600–5.
139. Hall AB, Papathanos PA, Sharma A, Cheng C, Akbari OS, Assour L, Bergman NH, Cagnetti A, Crisanti A, Dottorini T, Fiorentini E. Radical remodelling of the Y chromosome in a recent radiation of malaria mosquitoes. Proc Natl Acad Sci USA. 2016;201525164.
140. Marec F, Traut W. Sex chromosome pairing and sex chromatin bodies in W-Z translocation strains of *Ephestia kuehniella* (Lepidoptera). Genome. 1994; 37:426–35.
141. Cladera JL, Delprat MA. Genetic and cytological mapping of a" Y–2" translocation in the Mediterranean fruit fly *Ceratitis capitata*. Genome. 1995; 38:1091–7.
142. Delprat MA, Stolar CE, Manso FC, Cladera JL. Genetic stability of sexing strains based on the locus *sw* of *Ceratitis Capitata*. Genetica. 2002;116:85–5.
143. Franz G, Gencheva E, Kerremans P. Improved stability of genetic sex-separation strains for the Mediterranean fruit fly, *Ceratitis capitata*. Genome. 1994;37:72–2.
144. Stratikopoulos EE, Augustinos AA, Petalas YG, Vrahatis MN, Mintzas A, Mathiopoulos KD, Zacharopoulou A. An integrated genetic and cytogenetic map for the Mediterranean fruit fly, *Ceratitis capitata*, based on microsatellite and morphological markers. Genetica. 2008;133:147–57.
145. Brooke BD, Hunt RH, Chandre F, Carnevale P, Coetzee M. Stable chromosomal inversion polymorphisms and insecticide resistance in the malaria vector mosquito *Anopheles gambiae* (Diptera: Culicidae). J Med Entomol. 2002;39:568–73.
146. French WL. Preparation of mosquito chromosomes. Mosq News. 1962;22: 377–83.
147. Cornel A. *An. gambiae sl.* salivary gland chromosome preparation. In Methods in Anopheles Research Manual. 2015.
148. Papathanos PA, Bourtzis K, Tripet F, Bossin H, Virginio JF, Capurro ML, Pedrosa MC, Guindo A, Sylla L, Coulibaly MB, Yao FA, Epopa PA, Diabate A. A perspective on the need and current status of efficient sex separation methods for mosquito genetic control. Parasit Vectors. (this special issue).
149. Bernardini F, Haghighat-Khah RE, Galizi R, Hammond AM, Nolan T, Crisanti A. Molecular tools and genetic markers for the generation of transgenic sexing strains in Anopheline mosquitoes. Parasit Vectors. (this special issue).

Appendix 7: Fluorescent strain publication

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Life-history traits of a fluorescent *Anopheles arabiensis* genetic sexing strain introgressed into South African genomic background

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Abstract

Background: South Africa has set a mandate to eliminate local malaria transmission by 2023. In pursuit of this objective a Sterile Insect Technique programme targeting the main vector *Anopheles arabiensis* is currently under development. Significant progress has been made towards operationalizing the technology. However, one of the main limitations being faced is the absence of an efficient genetic sexing system. This study is an assessment of an *An. arabiensis* (AY-2) strain carrying the full Y chromosome from *Anopheles gambiae*, including a transgenic red fluorescent marker, being introgressed into a South African genetic background as a potential tool for a reliable sexing system.

Methods: Adult, virgin males from the *An. arabiensis* AY-2 strain were outcrossed to virgin females from the South African, Kwazulu-Natal *An. arabiensis* (KWAG strain) over three generations. *Anopheles arabiensis* AY-2 fluorescent males were sorted as first instar larvae (L1) using the Complex Object Parametric Analyzer and Sorter (COPAS) and later screened as pupae to verify the sex. Life history traits of the novel hybrid KWAG-AY2 strain were compared to the original fluorescent AY-2 strain, the South African wild-type KWAG strain and a standard laboratory *An. arabiensis* (Dongola reference strain).

Results: The genetic stability of the sex-linked fluorescent marker and the integrity and high level of sexing efficiency of the system were confirmed. No recombination events in respect to the fluorescent marker were detected over three rounds of introgression crosses. KWAG-AY2 had higher hatch rates and survival of L1 to pupae and L1 to adult than the founding strains. AY-2 showed faster development time of immature stages and larger adult body size, but lower larval survival rates. Adult KWAG males had significantly higher survival rates. There was no significant difference between the strains in fecundity and proportion of males. KWAG-AY2 males performed better than reference strains in flight ability tests.

Conclusion: The life history traits of KWAG-AY2, its rearing efficiency under laboratory conditions, the preservation of the sex-linked fluorescence and perfect sexing efficiency after three rounds of introgression crosses, indicate that it has potential for mass rearing. The potential risks and benefits associated to the use of this strain within the Sterile Insect Technique programme in South Africa are discussed.

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Keywords: Malaria, Vector control, COPAS

Background

South Africa is one of 21 countries that had set a mandate to eliminate malaria by 2020 as per the World Health Organization's (WHO) recommendations [1]. This was seen as promising as South Africa's malaria case numbers have remained constant at around 10,000 cases per annum. However, in 2017, the country experienced a major setback with a spike of almost 20,000 cases, mostly reported in the endemic north-eastern provinces [1]. This called for increased efforts towards vector and parasite surveillance, epidemic preparedness and response, health promotion and vector control as recommended by the WHO. Vector control remains the backbone of malaria strategies in South Africa. Current vector control methods rely on the use of insecticides through indoor residual spraying (IRS) inside dwellings in endemic areas [2]. However, due to increasing insecticide resistance, logistical challenges of implementing IRS under low transmission settings, changing of house structures into modern ones that are unsuitable for IRS application coupled with change in vector behaviour (majority of transmission is now occurring outdoors) [3], calls have been raised for development of new approaches that are more reliable, environment friendly and sustainable to compliment IRS.

Several genetic control methods have been developed aiming at the control of major insect pest and disease vector species, mainly through population suppression strategies, including human disease vectors, such as *Aedes* and *Anopheles* mosquitoes [4]. These include, the sterile insect technique (SIT), the release of insects carrying dominant lethals (RIDL), the incompatible insect technique (IIT), among others [5, 6]. However, the main obstacle towards the implementation of such genetic control population suppression programmes has been the lack of an effective and efficient sex sorting method, particularly for *Anopheles* species, that can guarantee the release of males only [7]. The sterile insect technique (SIT) is one of the proposed approaches because of its species specificity, environmental friendliness and proven success in the suppression or eradication of various major agricultural and animal pests [8, 9]. This technique targets a specific vector population through repeated releases of sterile males of the same species into selected natural environments in order for them to mate with wild females [8, 10]. The result is females laying non-viable eggs. Over time, the number of progeny in target populations is reduced, eventually leading to suppression or even eradication. It is crucial to exclude females from release material as females may contribute to disease transmission through their bites. Males on the contrary cannot contribute to pathogen transmission as they do not bite. It is, therefore, vital that a reliable sex separation system is available prior to deployment of a genetic control method as an additional malaria vector control strategy in South Africa [7, 11].

Some genetic control projects against insect pests and disease vectors have a sex separation system in place, however, none are readily applicable or transferrable between organisms and species [12–15]. With specific reference to mosquito programmes, the main malaria vector in South Africa, *Anopheles arabiensis* remains without an efficient sex separation method [7, 16]. Several methods have been attempted to eliminate females but unfortunately none has been fully developed to be used at an operational level. The available approaches include spiking blood with toxicants to eliminate females and development of a genetic sexing strain (GSS) using classical genetic approaches, reviewed in [7]. To date, the GSS "ANO IPCL1" based on dieldrin resistance developed by Yamada et al. [17], and introgressed into the South African genetic background to create "GMK" by Dandalo et al. [18] has been the only potentially usable genetic sexing strain of *An. arabiensis*. However, the use of dieldrin, a banned insecticide, to achieve sex separation presents challenges. Not only is there the problem of potential harm to the personnel that would need to work with the insecticide on a daily basis, but it has also been found that the dieldrin residues can accumulate in the environment [19]. In addition to this, the strain shows a highly reduced natural fertility rate due to the translocation of the *rdl* (dieldrin resistant) gene, making the strain only 27% fertile [20]. This, coupled with possibilities of recombination, makes it challenging and uneconomic to use in a mass rearing setting [21].

Efforts are being made to develop alternative GSSs using classical genetic approaches and lethal temperature sensitivity mutations and colour-based selectable markers similar to the ones successfully used for the construction of *Ceratitis capitata* VIENNA 8 GSS which is currently used for SIT applications worldwide [8, 22]. Although GSSs are available for some mosquito species, unfortunately, no advanced, ready-to-use sexing systems are currently available for any mosquito species that could be used in an operational programme [23]. A number of alternative sex separation systems are being considered [24–26]. One of the promising methods under development is the sex-specific expression of fluorescent markers at an early developmental stage, ideally

embryonic or first instar larval [24, 27, 28]. Such methods can be used to produce a male-only population very early in development, either by conditionally killing the females or by removing them using sex-specific differential expression of fluorescent transgenic markers [24, 29, 30]. Sex separation in early development minimizes rearing costs and facilitates the handling and processing of male-only based releases. In respect to mosquitoes, one of the initial sex-specific strains based on a fluorescent marker was the transgenic sexing line of *Anopheles gambiae*. In this strain, male mosquitoes express enhanced green fluorescent protein (EGFP), under the control of β2-tubulin promoter, thus allowing separation from females at the 4th larval instar stage by using a complex parametric analyser and sorter (COPAS) flow cytometry machine [24]. This system was subsequently improved by Magnussen et al. [31], and the EGFP expression could be detected as early as the 1st instar larval stage. This led to further developments in which the COPAS machine was optimized such that a high-throughput sorting of as much as 20,000 first instar larvae could be achieved in a half hour [32]. Subsequently, Bernardini et al. established a site-specific genetic engineering of the Y chromosome for *An. gambiae* [33] and through this, they developed a hybrid *An. arabiensis* strain carrying the complete Y chromosome of *An. gambiae*, including a red fluorescence protein (DsRed), and potentially other small autosomal genomic regions [34]. This strain, named AY-2, expresses DsRed, in male larvae in the optic lobe and extends down the ventral nerve cord [34].

The use of the one genetic sexing system for several geographic locations is a common practice in population control programmes against agricultural pests. For example, the VIENNA 8 GSS is being used in SIT applications against *Ceratitis capitata* populations worldwide [8]. However, such a practice is not acceptable for the control of mosquito vector populations because of potential inherent differences in vectorial capacity between same species from different geographical locations. If the hybrid transgenic *An. arabiensis* AY-2 strain, which is of foreign (Sudanese (Dongola)) genetic background, is to be considered for the SIT in South Africa, several steps need to be completed, including its introgression into a local genetic background, and basic parameters need to be evaluated. The introgression is necessary to avoid unintended negative impacts such as mating incompatibility and poor competitiveness between the fluorescent strain and the native population. The aim of this study was to start the introgression of the fluorescent marker, which resides on an *An. gambiae* Y chromosome, into a local *An. arabiensis* South African strain and the assessment of the newly developing strain (KWAG-AY2) for its rearing efficiency, fitness, and genetic stability (i.e. sexing reliability), as well as the preservation of the fluorescence intensity and the related sorting efficiency during the introgression process.

Methods

Mosquito strains

Four *An. arabiensis* strains, kept at the Insect Pest Control Laboratory (IPCL) of the Joint FAO/IAEA Centre of Nuclear Techniques in Food and Agriculture, Seibersdorf, Austria, were used in this study. The first, Dongola (MRA-856) was acquired from the Northern state of Sudan in 2005 and has since been maintained at the IPCL [20]. The Dongola strain has been used to demonstrate successful mass rearing [35]. The second strain, AY-2 described above was developed from Dongola to express an *An. gambiae* Y-linked red fluorescent protein (DsRed) marker [34]. The third, KWAG was acquired from Kwa-Zulu natal in South Africa and has been under colony since 2005 in the Botha De Meillon Insectary (BDMI), Vector Control Reference Laboratory (VCRL) Johannesburg, South Africa [36]. KWAG originates from Mamfene in Kwa-Zulu Natal, an area earmarked for South Africa's mosquito SIT pilot studies and is thus a representative of the wild-type population. The fourth, KWAG-AY2, the test strain, was developed by crossing AY-2 males with KWAG females over 3 generations in order to introgress the *An. gambiae* Y chromosome with the DsRed fluorescent marker into the local *An. arabiensis* and, at the same time, achieve about 87.5% replacement of the Sudanese (Dongola) by the South African genomic background.

Rearing methods

All strains were reared in a climate-controlled room at 27 ± 1 °C and 70% ± 10% RH and a 12:12 h light and dark cycle that includes a 1 h dusk-dawn period. Larvae were reared in a $40 \times 80 \times 8$ cm^3 tray at a density of ≥ 1000 larvae in 1 L deionized water and were fed a standard IAEA 1% liquid diet mix of bovine liver powder, Tuna meal and Vitamin mix [35, 37]. Pupae were collected in plastic sample cups of 120 mL capacity and placed in $30 \times 30 \times 30$ cm plastic Bugdorm cages (MegaView Science Co. Ltd., Taichung, Taiwan) and the resultant adults were provided with a constant supply of 5% sucrose solution. Females were provided with defibrinated, defrosted bovine blood twice weekly. Oviposition cups were prepared using filter paper placed on a damp sponge inside a 250 mL round plastic bowl with black lining around the inner perimeter of the bowl.

Sorting of AY-2 larval populations and introgression of AY-2 into KWAG genetic background

The fluorescence of AY-2 is detectable as early as the first instar under a fluorescent microscope. Initial sorting of

AY-2 was done under a fluorescent microscope (Leica MZ16FA, Leica Microsystems GmbH, Wetzlar, Germany), based on the fluorescence on the optic lobe extending down the ventral nerve cord. These were reared to pupation and the sex confirmed as male under a stereomicroscope. KWAG pupae were also gender separated under a stereomicroscope and only females retained. Female pupae were allowed to emerge in individual tubes to confirm sex at adult stage as an extra measure to ensure virginity. AY-2 males were mated with KWAG females for the first round of introgression crosses. The hybrid strain ("KWAG-AY2 (cross 1)") was then amplified for three consecutive generations before crossing the KWAG-AY2 (1) fluorescent males with KWAG females as described above. The resulting strain was named KWAG-AY2 (2) and is composed respectively of about 25% AY-2 and 75% KWAG genetic background. Progeny resulting from KWAG-AY2 (2) were screened for fluorescence under the fluorescent microscope at varying stages. The hybrid strain ("KWAG-AY2(2)") was then amplified for three consecutive generations before crossing the KWAG-AY2(2) fluorescent males with KWAG females. The resulting strain was named KWAG-AY2 (3) and is composed respectively of about 12.5% AY-2 and 87.5% KWAG genetic background. As the effects of the introgression progress on the life history traits are not known, it was decided to check the strain's stability and preservation of the fluorescence, and potentially acquired biological parameters (87.5% introgressed) before continuing with the next 4 rounds of introgression crosses to obtain an *An. arabiensis* strain that contains > 99% South African genetic background with the exception of males that will be carrying an *An. gambiae* Y chromosome since this sex chromosome is strictly paternally inherited. All subsequent life history trait experiments were completed using the KWAG-AY2 (3), henceforth referred to as simply KWAG-AY2. The Leica Application Suite v4.2 was used to capture images of the fluorescent individuals.

L1 larvae need to be unfed in order to be sorted on the large particle flow cytometry machine. Newly hatched, unfed AY-2 larvae, 24–48 h old, were transferred to the reservoir of the large-particles flow cytometry COPAS SELECT™ instrument (Union Biometrica, Inc., Holliston, MA. USA) equipped with a multiline argon laser (488, 514 nm) and a diode laser (670 nm). Analysis and sorting were performed with the Biosort5281 software equipped with a 488 nm filter. The following acquisition parameters: Green PMT 500, Red PMT 600, Delay 8; Width 6, pure mode selection with super drops were used. This is the most stringent setting, meaning that drops that contain two particles (i.e. larvae), of which one is non-fluorescent (females) will be diverted to the "female/waste" receptacle. Post sorting, fluorescent larvae were reared in trays until pupation, pupae sex separated under a stereomicroscope (Leica MZ16FA, Leica Microsystems GmbH, Wetzlar, Germany). Progeny resulting from KWAG-AY2 (3) were screened for fluorescence under the fluorescent microscope at L1 and pupal stage. In other instances, fluorescence was also confirmed in male adults by observing for fluorescence around the eyes.

Life history traits

Egg hatch rates (fertility)

To assess the egg hatching rates of each strain, newly laid eggs (n = 400–500 from each strain) were collected from their respective cages and placed in corresponding clear plastic cups containing 250 mL of deionized water and a drop of larval diet. The plastic cups were lined on the inside with a strip of filter paper to which the eggs adhere. L1 larvae were removed daily. After 72 h, hatched vs. unhatched eggs were counted under a stereomicroscope and comparisons made between the strains. This was repeated three times for each strain with eggs from different cohorts and three oviposition cycles each time.

Larval development, pupal emergence, and adult sex ratio

One hundred randomly selected L1 hatchlings from each plastic cup setup during the egg hatch rates experiments were transferred to a 600 mL plastic bowl containing 75 mL of deionized water. Larvae were fed the standard 1% IAEA *Anopheles* diet as follows: 2.5 mL on days 1–3 and 4 mL from day 4 until pupation. Larval trays were inspected once daily for pupae which were then removed and recorded. Once pupation occurred, the number of pupae dead or alive were removed and recorded. The number of days taken for L1 to reach pupation was also recorded. Alive pupae were transferred daily to individual 100 mL sample cups containing distilled water and the cups placed in 17.5 × 17.5 × 17.5 cm bugdorm cage (BugDorm-1H; MegaView, Taichung, Taiwan) for adults to emerge. The number of days taken for L1 and pupae to reach adulthood was also recorded. Similarly, the number of pupae that emerged into adults and the sex ratio of adults were recorded. This was replicated three times, each with three technical repetitions with larvae from different egg batches for each strain.

Adult wing lengths

As a proxy for adult size, wing lengths were measured for each strain. Dead adults were collected from cages daily. The right wing was removed from each mosquito, secured onto a sheet of paper using clear tape and the wings were measured by taking images on a microscope camera using Olympus essential v1.9.4 (Olympus Corporation, Shinjuku, Japan). Wings were measured from the distal edge of the alula to the end of the radius vein. The

mean length of each wing was recorded, and comparison made between the sexes and strains. These experiments were also repeated three times.

Fecundity

Twenty-five newly emerged adult females and males (1:1 ratio) were transferred to 17.5 × 17.5 × 17.5 cm Bugdorm cages and provided with 5% sucrose solution continuously. The adults were allowed to mate for five days after which defibrinated, defrosted bovine blood was provided to each cage for 30–40 min over two consecutive days. All the females were transferred individually into sample cups lined on the inside with a damp filter paper and covered on top with a mesh fabric secured by rubber bands. A 5% sucrose solution was provided through a small strip of filter paper placed on top of each sample cup. Each cup was checked for eggs daily and upon oviposition the total number of eggs laid by each female counted under a stereomicroscope. From this, fecundity was determined from the total number of eggs laid by each female. Three replicates were performed. Females that did not lay any eggs were not included in the eggs per female average.

Longevity

Thirty newly emerged males and females (1:1 ratio) from each strain were introduced into 17.5 × 17.5 × 17.5 cm Bugdorm cages, each provided with a 5% sucrose solution continuously. Longevity was monitored daily by removing and counting the dead until no adults remained. A total of two biological repetitions, each with 3 technical replicates were performed for all strains.

Flight ability

A flight test device (FTD) developed by the IPCL initially for *Aedes* mosquitoes and modified for *An. arabiensis* was used as described by Culbert et al. [38]. The flight ability test was performed on 100 adult males of AY2, KWAG and KWAG-AY2, with three replicates each. The males were aspirated into the bottom of the flight ability device. A lure was placed on top of the device and a fan was placed over the lure. The adult mosquitoes were allowed to escape for two hours. Escaped males in the chamber and those remaining at the bottom of the FTD were counted as escaped and not escaped respectively, giving an escape rate.

Statistical analysis

All statistical analyses were performed in R (version 4.0.3) [39] using RStudio (RStudio, Inc. Boston, MA, USA, 2016). Generalized Linear Mixed Models (GLMM, lme4 package) from R were used with the appropriate distribution family. The binomial generalized linear mixed models fit by maximum likelihood (Laplace Approximation) was used, with egg hatch, pupation rate and male flight ability as response variables, strain as fixed effect and the replicate as a random effect.

For the fecundity, A Gaussian linear mixed-effects model was used, with egg number per female as variable, strain as fixed effect and replicate as random effect. Fecundity was analysed with a zero-inflated negative binomial distribution with egg number per female as a response variable, strain (four levels) as fixed factor and replicate as a random factor. Adult wing lengths were analyzed with a Gaussian distribution with strain (four levels) and sex (two levels) as fixed factors and mosquito individuals as a random factor. Data on longevity were expressed as Coxme survival curves. Survival curves were generated using RStudio [39]. A Cox mixed-effects model fit by maximum likelihood with mosquito time to death as response variable, strain (four levels: AY2, KWAG, KWAG-AY2 and DONGOLA) as a fixed factor and replicate as a random factor was used. The full models were checked for overdispersion (using Bolker's function) [40] and for normality and homogeneity of variances on the residuals [41] for validation. The models were simplified using the stepwise removal of terms, followed by likelihood ratio tests (LRTs) when appropriate. Multiple comparisons using the emmeans function (in package emmeans) [42] were performed between the levels where significant differences were found. A P-value of less than 0.05 was used to indicate statistical significance in all cases.

Results

Phenotypic expression of KWAG-AY2

The phenotypic representation of AY2 and KWAG–AY2 is shown in Fig. 1. Approximately 400 larvae were sorted both manually and using the COPAS sorter for AY2, 1472 of KWAG-AY2(1) and 779 KWAG-AY2(2) and over 2500 of KWAG-AY2(3). All sorted larvae were kept to adulthood at which the sex was confirmed. Two females were found in pupae or adults sorted as males but these were not fluorescent. The "pure sort" setting was still able to recover ~ 97% of all males. No recombinants (i.e. no non-fluorescent males, or fluorescent females) were found throughout the entire study in all sorted samples (a combined total of 3679 larvae).

Developmental parameters

The mean egg hatch rates were 67% for DONGOLA, 68.3% for AY-2 while KWAG and KWAG-AY2 were 74.2% and 88.1%, respectively (Fig. 2). Statistical interpretation showed a significant difference between the strains ($p < 0.05$). KWAG hatch rate was significantly higher than AY2 ($p = 0.0179$) and KWAG-AY2 hatch rate was significantly higher than AY2 ($p < 0.001$).

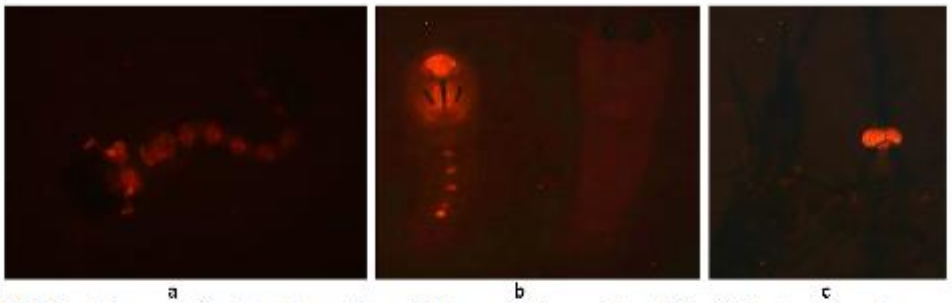

Fig. 1 Phenotypic representation of the sorted populations resulting from reciprocal crosses between AY-2 and KWAG under a fluorescent microscope: **a** DsRed fluorescence in L1, **b** Male pupa showing fluorescence on the ocular nerve extending down the nerve cord, left and non-fluorescent female pupa, right, **C** non fluorescent adult female on the left and visible fluorescence in the eyes of adult male, right

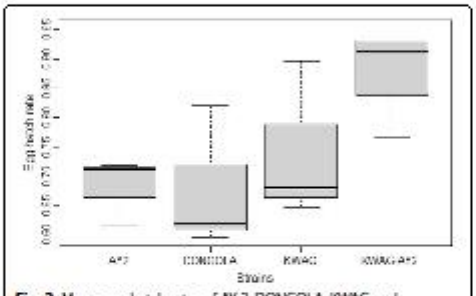

Fig. 2 Mean egg hatch rates of AY-2, DONGOLA, KWAG and KWAG-AY2. Each box shows the upper and lower quartiles, the line inside the box (horizontal) represents the median of the sample, while the line outside the box (vertical) represents variability outside the upper and lower quartiles

Larval development and adult sex ratio

Out of the 100 L1 setup for development, the mean proportion surviving to pupal stage was 58.0% for DONGOLA, 26.7% for AY-2, 44.3% for KWAG and 70.3% for KWAG-AY2 (Table 1). Statistical analysis showed significant differences in proportion of L1 surviving to pupal stage between the strains (Table 1 $p < 0.001$). A Generalized linear mixed model showed that KWAG-AY2 had a significantly higher number of L1s surviving to pupae, whereas AY-2 showed the lowest. The survival rate of L1 to adulthood was significantly different between strains and followed the same pattern as the mean survivorship from L1 to pupae; it was 37.7% for DONGOLA, 19.0% for AY-2, 41.0% for KWAG and 53.3% for KWAG-AY. The proportion of males was as follows: DONGOLA = 60%, AY-2 = 51.7%, KWAG = 49% and KWAG-AY2 = 57.3%. Slight difference in the proportion of males was found only between DONGOLA and KWAG ($z = -1.965$, $p = 0.05$).

There was a significant difference in the rate of pupation between AY2 and Dongola, KWAG and KWAG-AY2 ($p < 0.001$) (Fig. 3).

Adult wing lengths

Generally wing length sizes differed between males and females regardless of strain ($\chi^2 = 74.1$, $df = 1$, $p < 0.001$) (Fig. 4). AY-2 females were larger than the males and females of the other strains ($\chi^2 = 67.3$, $df = 3$, $p < 0.001$). Pairwise analysis showed that AY2 males were not significantly different in body size to females of Dongola, KWAG and KWAG-AY2 ($p > 0.05$).

Table 1 Mean proportion of L1 surviving to pupae, adulthood and proportion of males of four laboratory reared *An. arabiensis* strains. Mean values ± SD, lower–upper 95% CI in brackets

Strain	Proportion surviving from L1 to pupae	Proportion surviving from L1 to adult	Percentage of males
DONGOLA	58.0 ± 23.9 (− 1.4–117.4)^a	37.7 ± 15.0 (0.3–75.0)^a	60.0 ± 4.0 (50.1–69.9)^a
AY-2	26.7 ± 10.4 (0.8–10.4)^b	19.0 ± 5.6 (5.2–32.8)^b	51.7 ± 4.9 (39.4–63.9)^a
KWAG	44.3 ± 15.9 (4.9–83.8)^a	41.0 ± 26.6 (− 25.1–107.2)^a	49.0 ± 9.5 (25.3–72.7)^a
KWAG-AY2	70.3 ± 6.5 (54.2–86.5)^c	53.3 ± 1.5 (49.5–57.1)^c	57.3 ± 6.8 (40.4–74.2)^a

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

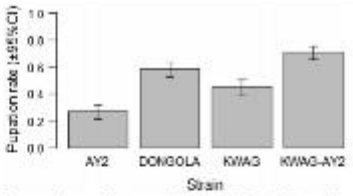

Fig. 3 A comparison of the pupation rate of *Anopheles arabiensis* males from the AY2, DONGOLA, KWAG and KWAG-AY2 strains

Table 2 Mean number of females that laid eggs and total number of eggs laid per female (fecundity) between four laboratory reared *An. arabiensis* strains. Mean values ± SD, lower–upper 95% CI in brackets

Strain	Mean number of eggs laid per female ± SD (Lower–upper 95% CI)
DONGOLA	76.0 ± 28.5 (5.2–146.8)[a]
AY-2	63.3 ± 15.0 (26.1–100.5)[a]
KWAG	58.7 ± 7.4 (40.4–77.0)[a]
KWAG-AY2	70.7 ± 18.1 (25.6–115.8)[a]

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

Fecundity

The mean proportion of females that survived in oviposition tubes and laid eggs was 36.0% for Dongola, 41.3% for AY-2, 34.7% for KWAG and 30.7% for KWAG-AY2. Fecundity (total number of eggs laid by each female) of each strain was 76.0 for DONGOLA, 63.3 for AY-2, 58.7 for KWAG and 70.7 for KWAG -AY2 (Table 2). There was no significant difference in fecundity between the strains ($p > 0.05$).

Longevity

Statistical analysis between the four strains showed significant differences in male survival between the strains ($p < 0.0001$) as well as in in female survival ($p = 0.0075$) as shown in the Coxme survival curves in Fig. 5. Coxme analysis revealed that males have a higher risk of death than females. KWAG males had a significantly higher probability of survival than the males of AY2, Dongola and KWAG-AY2 ($p < 0.0001$).

The median survival time of males in days was as follows: 12.0 for DONGOLA, 9.0 for AY-2, 21.0 for KWAG and 9.0 for KWAG-AY2 while for females it was: 15.0 for DONGOLA, 14.0 for AY-2, 19.0 for KWAG and 10.0 for KWAG-AY2. KWAG females had the highest probability for survival and KWAG-AY2 females had the lowest.

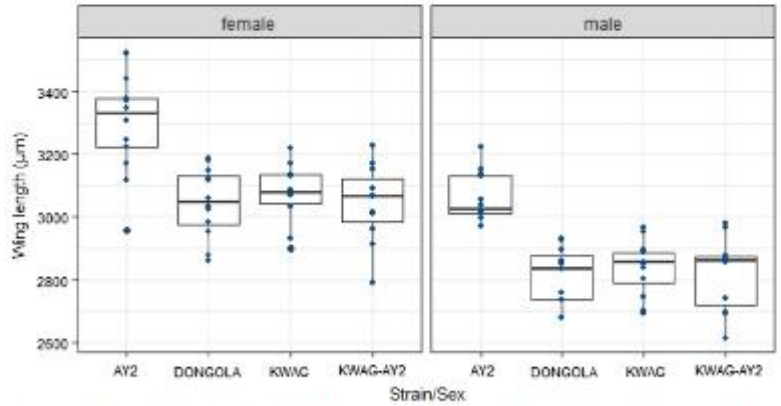

Fig. 4 Box plot of wing length measurements for females (left) and males (right) of the 4 comparative strains. Each box shows the upper and lower quartiles, the line inside the box represents the median of the sample, lines extending from the boxes (whiskers) indicate variability outside the upper and lower quartiles and circles indicate outliers

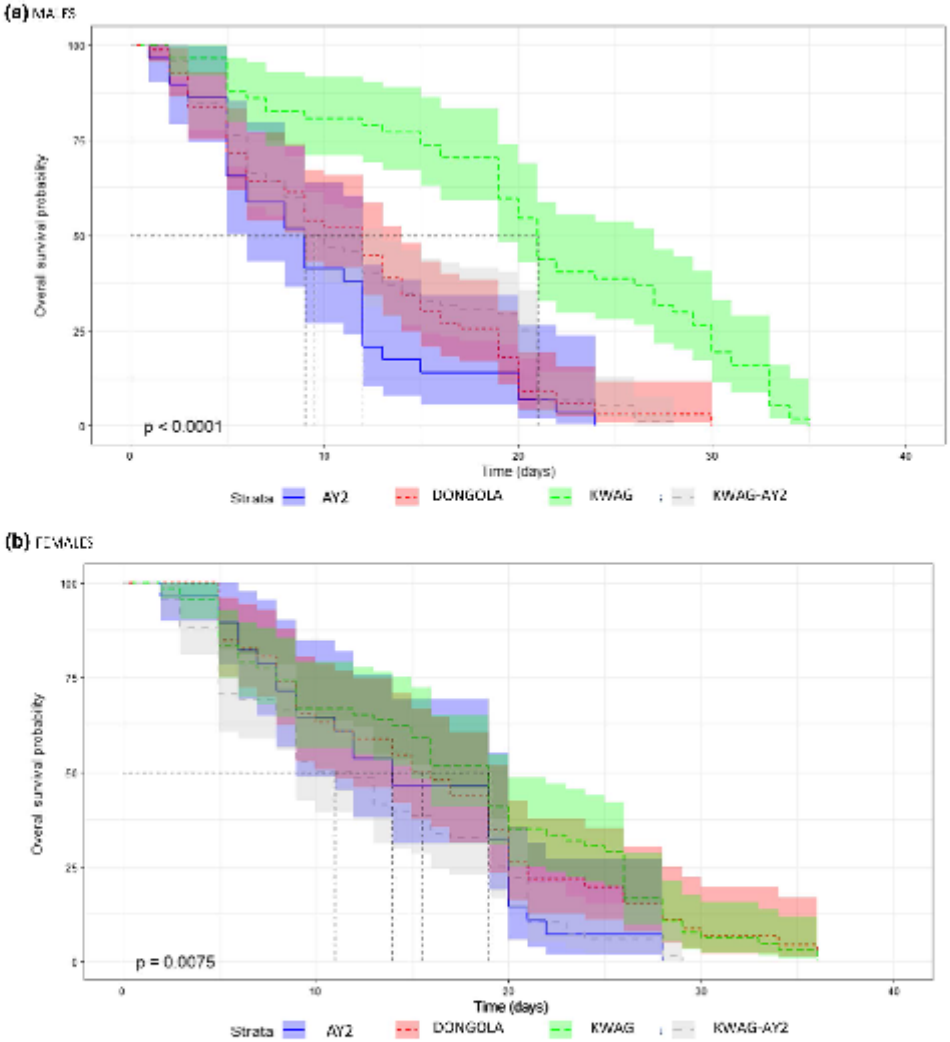

Fig. 5 Coxme survival curves for adult males **a** and females **b** of the four *An. arabiensis* laboratory strains

Flight ability

The difference in the mean escape rate of the KWAG males compared to KWAG-AY2 and AY2 males was significant ($p < 0.0001$) (Fig. 6). The mean escape rates were, 64.7% in AY2, 50.1% in KWAG and 66.5% in KWAG-AY2. Pairwise analysis found no significant difference in the escape rates of AY2 and KWAG-AY2 males ($p > 0.05$).

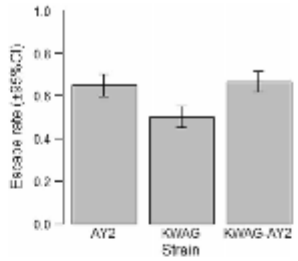

Fig. 6 A comparison of the escape rates of *Anopheles arabiensis* AY2, KWAG and KWAG-AY2 males from the flight ability test

Discussion

The aim of this study was to develop a fluorescent sexing strain with a South African genetic background with potential use during the South African sterile insect technique programme. The first part of this work was to assess the strain's rearing efficiency, its reliability in sex sorting, and to identify any biological factors that may significantly differ from other *An. arabiensis* laboratory strains with a historical record of successful colonisation and the wild type KWAG strain. An *An. arabiensis* KWAG-AY2 sexing strain with a South African genetic background was successfully developed. After three rounds of introgression crosses, the progeny of this strain has approximately 87.5% South African (KWAG) genetic background and with continuous backcrossing a $\geq$ 99.9% local genetic background will be achieved. The fluorescence and its intensity, and linkage to the male sex were maintained and have still been found to be very stable.

Initial activities involved sorting of AY-2 males, which was mostly done manually under the fluorescent microscope prior to the acquisition of the automated COPAS sorter. No recombination was found from either sorting method in both the original AY-2 strain and the newly established KWAG-AY2 strain. The fluorescence was detectable as early as the first instar larvae both under a fluorescent microscope and the COPAS sorter. It is important to note that larvae need to be unfed in order to be sorted in the large particle flow cytometer, as it seems that larval diet components produce some sort of luminescence that interferes with the laser detection of the fluorescence, producing false positives. Debris either from food, eggshells or legs and wings of adult mosquitoes can cause clogs in the system or cause sorting errors if the particles are of similar size and density to the L1 larvae. However, this should not affect the purity of the males sorted for release. For this reason, larvae were sorted in the first 24–48 h post hatch and before feeding. Young larvae can survive for some time by feeding on the yolk of the eggs after hatching without the addition of food. The female elimination efficiency of the COPAS sorter on KWAG-AY2 was very high (99.97% of over 5000 L1 sorted) as opposed to the 97% elimination efficiency of females reported in GMK [18]. The lack of recombinants in KWAG-AY2, in contrast to the 0.4% recombination rate recorded in ANO IPCL1 and likely related strains, is another advantage towards the applicability of this strain as a sex separation option for the South African SIT programme [18]. The fact that no insecticides are used in the sorting of KWAG-AY2 is another great advantage: the GMK GSS relies on the use of dieldrin for sex separation which poses a constant threat to the colony, staff health, and other non-target insects, and the environment, in which dieldrin residues can accumulate when abundant treated males are released over time [19].

In terms of life history traits, KWAG-AY2 compared favourably against other established *An. arabiensis* laboratory strains in most instances. The life history traits that were assessed included egg hatch rates, larval development, pupal emergence, sex ratio, wing length, adult fecundity, longevity, and flight ability. The egg hatch rate of KWAG-AY2 was similar to that of KWAG and significantly higher than for both AY-2 and Dongola. This is expected due to the genetic similarity between the KWAG-base, and DONGOLA-base strains [34]. In contrast, the GMK GSS, developed from KWAG females and males of the original GSS ANO IPCL1, has a high natural sterility due to the chromosomal translocation and possibly exacerbated by introgression into the local genomic background, giving low egg hatch rates of 19.2% [18]. This low productivity makes the GMK GSS uneconomic for mass rearing as a male recovery rate of less than 10% of any egg batch produced during mass rearing renders the production inefficient.

KWAG-AY2 had a significantly higher proportion of L1 surviving to pupae as compared to AY-2, and slightly higher, but not statistically different to KWAG and Dongola. Dongola provides the standard for mass rearing; thus, this characteristic bodes well for the chances of KWAG-AY2 performing well in a mass rearing setting. Each strain was fed the same amount of food and larval density was the same at experimental set up. The optimal feeding regime for Dongola as described in the standardized mass rearing guidelines, may not be ideal for the other tested strains. The rearing water in the AY-2 containers was clear, whereas the water became cloudy in the 2 KWAG-based strains. This suggests that AY-2 requires more diet than the other strains, while the KWAG strains might require less. The early mortality and overall low survival rate in the AY-2 larvae may be due to starvation of some of the larvae and necrophagy by those

remaining (as no dead larvae were seen). This led to a reduced rearing density, and thus more food for the survivors, resulting in faster development times and larger adult body size. Adjusting diet amounts to food per larva would improve the experimental design to compare body size. Also, a feeding regime that yields higher survival of the transgenic AY2 and KWAG-AY2 strains needs to be further investigated as overall the survival of larvae for all the strains used in this comparison were low. However, the importance lies in the result that the KWAG-AY2 adults did not differ in size to the KWAG *wild-type* adults, as compatible body size is important in mating preferences [43].

Marois et al. [32] suggested that when mass rearing transgenic insects, fitness costs may arise by inbreeding, as is seen in most colonized and artificially reared insects [44, 45]. To counteract this and increase the gene pool, it is necessary to apply good colonization and maintenance principles, allowing genetic diversity [23, 46].

It is important to consider the different developmental stage characteristics as they have a bearing on the productivity and quality of males to be released. The faster larval developmental time shown by KWAG-AY2 as compared to the other strains is comparable to results from other studies such as 6.9–7.4 days in GSS ANO IPCL compared to Dongola (7.5–7.8 days) [20]. In this study, it took Dongola on average of 8 days to pupation and 10 days to reach adult emergence. Pupation of KWAG-AY2 took on average 8 days and 9 days to adult emergence compared to GMK which took an average of 9 days to develop to pupae and 12 days to adult emergence.

The percentage of females that laid eggs was lower than what is usually observed in *Aedes* spp. *Aedes* mosquitoes are not averse to being isolated in small containers for individual oviposition, presumably because such an environment is close to that encountered by these species in nature. In contrast, *Anopheles* are generally more fragile, and do not survive well when confined in small spaces and are often observed to prefer withholding eggs rather than laying them in an "unsuitable" place (personal observations). The low fecundity levels recorded is therefore not unusual and was expected in all the four strains, and those that survived and did not lay eggs may be the result of not all females having blood fed or mated, or females withholding eggs. KWAG-AY2 showed high fecundity, and high natural fertility (hatch rate). These characteristics imply that there are no major limitations in the mass rearing of this strain. Taking only those individuals into account that laid eggs, the differences in numbers of eggs laid/female between the four strains were not significant. Under mass rearing conditions, Mamai et al. [47] demonstrated that *Anopheles* females laid an average of 40 eggs per female when 15 000 pupae were placed in mass rearing cages, however, the eggs were quantified *en masse*, and were divided by the initial number of female pupae placed in the cage. In another study, Oka et al. [48] found female *Anopheles* laid an average of 52 eggs per female in a substrate preference experiment. Mosquito fecundity is variable and ranges between 50 and 200 eggs per oviposition have been recorded [49, 50]. Egg counts in this current study are comparable with other *Anopheles* egg counts considering the scale of the experiment.

Longevity of adult males is important for SIT mass rearing and release. Male median survival time was longer for KWAG males than the other strains. KWAG-AY2 (9 days) was not significantly different from Dongola and AY-2 (9 and 12 days respectively). For field releases, males need to survive long enough to find suitable mates and copulate with as many females as possible. Further tests need to be conducted to determine how irradiation and release in semi-field conditions may affect longevity and mating capacity of KWAG-AY2 as compared to the KWAG strain.

The use of flight test cylinders as a routine method of quality control in insect rearing has been shown to effectively demonstrate the quality of flying insects [38, 51]. Released laboratory males are required to find and mate with wild females and this is dependent on their ability to fly. Flight test devices have been adapted for *Anopheles* and used to demonstrate the effects of different treatments on male flight ability, which was correlated to mating propensity and survival [38]. In this study, KWAG-AY2 had an escape rate that was significantly higher than KWAG but similar to the AY2 strain. Further studies of flight ability of KWAG-AY2 after irradiation need to be assessed as males earmarked for release need to be sterile. KWAG-AY2 has performed well when compared to reference strains in terms of egg hatch rates, larval survival and adult size, fecundity and flight ability, compared to the other reference strains and thus is promising for its mass rearing potential. The relative faster developmental rate for KWAG-AY2 observed in this study is also a favourable characteristic from a mass rearing perspective as this could reduce operational costs. Taking all of the rearing parameters into account (fecundity × egg hatch rate × survival to adulthood), the rate of increase was found to be 19.2, 8.2, 17.9 and 33.0 for the *An. arabiensis* strains Dongola, AY2, KWAG and KWAG-AY2 respectively. The success of the KWAG-AY2 strain may have been contributed by heterosis, by crossing the KWAG and AY2 strains.

The use of strains with sex-linked fluorescence coupled with COPAS sorting may represent a valid option to large-scale male-only releases for the South African SIT programme. The sorting speed of COPAS would allow the accurate sorting of almost 2 million male mosquitoes

a week with 8 h shifts per day [32]. The use of COPAS for sorting fluorescent mosquito larvae has proven to be a highly efficient and reliable method for *Anopheles* sex separation [32].

Conclusion

Realistic conclusions on the potential use of KWAG-AY2 can only be drawn after the full introgression of the local genomic background, the evaluation of its rearing efficiency at large scale, irradiation dose–response of the strain, the effect of irradiation on male mating competitiveness, mating compatibility with the wild-type KWAG strain, and then ultimately mark release recapture (MRR) studies in open field conditions to complete the evaluation phases to consider this new strain for larger scale suppression programmes [11, 52, 53]. In addition, a risk assessment analysis is required to address any concerns related to the potential release of this transgenic and hybrid *An. arabiensis* strain in ecosystems as well as to obtain the necessary regulatory approvals for open field releases [26]. Finally, the release of irradiated fluorescent males would allow their easy monitoring in the environment and checking for the absence of introgression of the wild target population of *Anopheles*. However, the development of non-transgenic strains by linking a marker on the Y chromosome using genome editing approaches would be desirable in the future.

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Author contributions
NN carried out the experiments and wrote the first and subsequent drafts of the manuscript. TM, WM, HM, JB and GM carried out data analysis and contributed to the writing of the manuscript. All other co-authors contributed to experimental setup, data collection and sections of the manuscript. HY and JB conceived the project, supervised it and contributed to the subsequent writing of the manuscript. All authors read and approved the final manuscript

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References

1. WHO. 2018 Update on the E-2020 initiative of 21 malaria-eliminating countries report and country briefs. Geneva, World Health Organization
2. Brooke BD, Koekemoer LL, Kruger P, Urbach J, Misiani E, Coetzee M. Malaria vector control in South Africa. S Afr Med J. 2013;103:784–8.
3. Musiime AK, Smith DL, Kilama M, Rek J, Arinaitwe E, Nankabirwa JL, et al. Impact of vector control interventions on malaria transmission intensity, outdoor vector biting rates and *Anopheles* mosquito species composition in Tororo, Uganda Malar J. 2019;18:445.
4. Eappen AG, Sung-Jae C, Jacobs-Lorena M. Towards the genetic control of insect vectors: an overview. Entomol Res. 2007;37:213–20.
5. Lees RS, Gilles J, Hendrichs J, Vreysen MJB, Bourtzis K. Back to the future: the sterile insect Technique against mosquito disease vectors. Curr Opin Insect Sci. 2015;10:156–62.
6. Alphey L, Nimmo D, O'Connell S, Alphey N. Insect population suppression using engineered insects. Adv Exp Med Biol. 2008;627:93–103.
7. Mashatola T, Ndo C, Koekemoer LL, Dandalo LC, Wood OR, Malakoane L, et al. A review on the progress of sex-separation techniques for sterile insect technique applications against *Anopheles* arabiensis. Parasit Vectors. 2018;11:127.
8. Klassen W, Vreysen MJB. Principles of the sterile insect technique: area-wide integrated pest management and the sterile insect technique. In: Dyck VA, Hendrichs J, Robinson AS, editors. Sterile insect technique Principles and practice in area-wide integrated pest management. 2nd ed. Boca Raton: CRC Press; 2021. p. 93–7.
9. Munhenga G, Brooke BD, Chirwa TF, Hunt RH, Coetzee M, Govender D, et al. Evaluating the potential of the sterile insect technique for malaria control: relative fitness and mating compatibility between laboratory colonized and wild population of *Anopheles arabiensis* from the Kruger national Park, South Africa. Parasit Vectors. 2011;4:208.
10. Knipling EF. Possibilities of insect control or eradication through the use of sexually sterile males. J Econ Entomol. 1955;48:459–69.
11. Alphey L, Benedict M, Bellini R, Clark GG, DameService DA, et al. Sterile insect methods for control of mosquito borne diseases an analysis. Vector Borne Zoonotic Dis. 2010;10(3):295–311.
12. Focks DA. An improved separator for the developmental stages, sexes, and species of mosquitoes (*Diptera*: Culicidae). J Med Entomol. 1980;17:567–8.
13. Papathanos PA, Bossin HC, Benedict MQ, Catteruccia F, Malcolm CA, Alphey L, et al. Sex separation strategies: past experience and new approaches. Malar J. 2009;8(Suppl 1):S5.
14. Gilles J, Schetelig M, Scolari F, Marec F, Capurro M, Franz G, et al. Towards mosquito sterile insect technique programmes: exploring genetic, molecular, mechanical and behavioural methods of sex separation in mosquitoes. Acta Trop. 2014;132S:S178–87.
15. Papathanos PA, Bourtzis K, Tripet F, Bossin H, Virginio JF, Capurro ML, et al. A perspective on the need and current status of efficient sex separation methods for mosquito genetic control. Parasit Vectors. 2018;11:165–71.
16. Dandalo LC, Brooke BD, Munhenga G, Lobb LN, Zikhali J, Ngxongo SP, et al. Population dynamics and *Plasmodium falciparum* (Haemosporida: *Plasmodiidae*) infectivity rates for the malaria vector *Anopheles arabiensis* (*Diptera*: Culicidae) at Mamfene, KwaZulu-Natal. J Med Entomol. 2017;54:1758–66.

17. Yamada H, Benedict MQ, Malcolm CA, Oliva CF, Soliban SM, Gilles JRL. Genetic sex separation of the malaria vector, *Anopheles arabiensis*, by exposing eggs to dieldrin. Malar J. 2012;11:208.
18. Dandalo LC, Munhenga G, Kaiser ML, Koekemoer LL. Development of a genetic sexing strain of *Anopheles arabiensis* for KwaZulu-Natal. South Africa Med Vet Entomol. 2018;32:61–9.
19. Yamada H, Jandric Z, Chhem-Kieth S, Vreysen MJB, Rathor MN, Gilles JRL, et al. *Anopheles arabiensis* egg treatment with dieldrin for sex separation leaves residues in male adult mosquitoes that can bioaccumulate in goldfish (*Carassius auratus auratus*). Environ Toxicol Chem. 2013;32:2786–91.
20. Oliva CF, Benedict MQ, Soliban SM, Lemperiere G, Balestrino F, Gilles JR. Comparisons of life-history characteristics of a genetic sexing strain with laboratory strains of *Anopheles arabiensis* (Diptera: Culicidae) from northern Sudan. J Med Entomol. 2012;49:1045–51.
21. Yamada H, Vreysen MJB, Bourtzis K, Tschirk W, Chadee DD, Gilles JRL. The *Anopheles arabiensis* genetic sexing strain ANO IPCL1 and its application potential for the sterile insect technique in integrated vector management programmes. Acta Trop. 2015;142:138–44.
22. Franz G, Bourtzis K, Caceres C. Supportive technologies to improve the sterile insect technique chapter 43. In: Dyck VA, Hendrichs J, Robinson AS, editors. sterile insect technique principles and practices in area wide integrated pest management. Boca Raton: CRC Press; 2021.
23. Lutrat C, Giesbrecht D, Marois E, Whyard S, Baldet T, Bouyer J. Sex sorting for pest control: it's raining men! Trends Parasitol. 2019;35:649–62.
24. Catteruccia F, Benton JP, Crisanti A. An *Anopheles* transgenic sexing strain for vector control. Nat Biotechnol. 2005;23:1414–7.
25. Nolan T, Papathanos P, Windbichler N, Magnusson K, Benton J, Catteruccia F, et al. Developing transgenic *Anopheles* mosquitoes for the sterile insect technique. Genetica. 2011;139:33–9.
26. Bourtzis K, Lees RS, Hendrichs J, Vreysen MJ. More than one rabbit out of the hat: radiation, transgenic and symbiont-based approaches for sustainable management of mosquito and tsetse fly populations. Acta Trop. 2016;157:115–30.
27. Smith RC, Walter MF, Hice RH, O'Brochta DA, Atkinson PW. Testis-specific expression of the β2 tubulin promoter of *Aedes aegypti* and its application as a genetic sex-separation marker. Insect Mol Biol. 2007;16:61–71.
28. Bernardini F, Haghighat-Khah RE, Galizi R, Hammond AM, Nolan T, Crisanti A. Molecular tools and genetic markers for the generation of transgenic sexing strains in anopheline mosquitoes. Parasit Vectors. 2018;11(Suppl 2):660.
29. Thomas DD, Donnelly CA, Wood RJ, Alphey LS. Insect population control using a dominant repressible lethal genetic system. Science. 2000;287:2474–6.
30. Alphey L, Beard CB, Billingsley P, Coetzee M. Malaria control with genetically manipulated insect vectors. Science. 2002;298:119.
31. Magnusson K, Mendes AM, Windbichler N, Papathanos PA, Nolan T, Dottorini T, et al. Transcription regulation of sex-biased genes during ontogeny in the malaria vector *Anopheles gambiae*. PLoS ONE. 2011;6:e21572.
32. Marois E, Scali C, Soichot J, Kappler C, Levashina EA, Catteruccia F. High-throughput sorting of mosquito larvae for laboratory studies and for future vector control interventions. Malar J. 2012;11:302.
33. Bernardini F, Galizi R, Menichelli M, Papathanos PA, Dritsou V, Marois E, et al. Site-specific genetic engineering of the *Anopheles gambiae* Y chromosome. Proc Natl Acad Sci USA. 2014;111:7600–5.
34. Bernardini F, Galizi R, Wunderlich M, Tadarchi C, Kranjc N, Kyrou K, et al. Cross-species Y chromosome function between malaria vectors of the *Anopheles gambiae* species complex. Genetics. 2017;207:729–40.
35. FAO/IAEA. 2017 Guidelines for standardised mass rearing for *Anopheles* mosquitoes. Version 1.0. http://www-naweb.iaea.org/nafa/ipc/public/manuals-ipc.html. Accessed 09 Jan 2021
36. Mouatcho JC, Munhenga G, Hargreaves K, Brooke BD, Coetzee M, Koekemoer LL. Pyrethroid resistance in a major African malaria vector *Anopheles arabiensis* from mamfene, northern KwaZulu-Natal. South Africa S Afr J Sci. 2009;105:127–31.
37. Damiens D, Benedict MQ, Wille M, Gilles JRL. An inexpensive and effective larval diet for *Anopheles arabiensis* (Diptera: Culicidae): eat like a horse, a bird or a fish? J Med Entomol. 2012;49:1001–11.
38. Culbert NJ, Somda NSB, Hamidou M, Sorna DD, Caravantes S, Wallner T, et al. A rapid quality control test to foster the development of the sterile insect technique against *Anopheles arabiensis*. Malar J. 2020;19:44.
39. R Development CORE TEAM. R. 2008 A Language and Environment for Statistical Computing; R Foundation for Statistical Computing: Vienna, Austria. https://cran.r-project.org/doc/manuals/fullrefman.pdf. Accessed on 10 October 2020
40. Bolker, B. GLMM FAQ: 2020 Testing for overdispersion/computing overdispersion factor. https://bbolker.github.io/mixedmodels-misc/glmmFAQ.html#testing-for over dispersion computing-over dispersion-factor Accessed on 10 September 2020
41. Kary M, Hatfield JS. Normality of raw data in general linear models: the most widespread myth in statistics. Bull Ecol Soc Am. 2003;84:92–4.
42. Lenth R. Emmeans: estimated marginal means, aka least-squares means. R Package Version 1.2.3. 2018. https://CRAN.R-project.org/package=emmeans. Accessed on 10 October 2020
43. Sawadogo SP, Diabaté A, Toé HK, Sanon A, Lefèvre T, Baldet T, et al. Effects of age and size on *Anopheles gambiae s s* male mosquito mating success. J Med Entomol. 2013;50:285–93.
44. Briscoe DA. Rapid loss of genetic variation in large captive populations of *Drosophila* flies: Implications for the genetic management of captive populations. Conserv Biol. 1992;6:416–25.
45. Ross PA, Endersby-Harshman NM, Hoffmann AA. A comprehensive assessment of inbreeding and laboratory adaptation in *Aedes aegypti* mosquitoes. Evol Appl. 2019;12:572–86.
46. FAO/IAEA. 2018 Guidelines for colonization of AEDES mosquito species—Version 1.0. 2018. http://www-naweb.iaea.org/nafa/ipc/public/Guidelines-for-colonisation-of-Aedes-mosquito-species-v1.0.final.pdf
47. Mamai W, Bimbile-Somda NS, Maiga H, Juarez JG, Muosa ZAI, Ali AB, et al. Optimization of mosquito egg production under mass rearing setting: effects of cage volume, blood meal source and adult population density for the malaria vector. Anopheles arabiensis Malar J. 2017;16:41.
48. Okal MN, Lindh JM, Torr SJ, Masinde E, Orindi B, Lindsay SW, et al. Analysing the oviposition behaviour of malaria mosquitoes: design considerations for improving two-choice egg count experiments. Malar J. 2015;14:250.
49. Service MW. Mosquitoes (Culicidae) medical insects and arachnids. In: Lane, Crosskey RPRW, editors. Chapman and Hall. Dordrecht: Springer; 1993.
50. Service MW. Medical entomology for students. 2nd ed. Cambridge: Cambridge University Press; 2000.
51. Carpenter JE, Blomefield T, Vreysen MJ. A flight cylinder bioassay as a simple, effective quality control test for *Cydia pomonella*. J Appl Entomol. 2012;136:711–20.
52. Benedict MQ, Robinson AS. The first releases of transgenic mosquitoes: an argument for the sterile insect technique. Trends Parasitol. 2003;19:349–55.
53. Dame DA, Curtis CF, Benedict MQ, Robinson AS, Knols BGJ. Historical applications of induced sterilisation in field populations of mosquitoes. Malar J. 2009;8(Suppl 2):S2.

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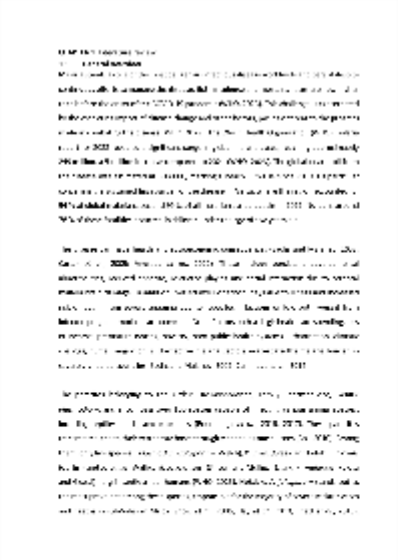

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