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OPTIMISING ADULT HOUSING AND INSECTARY LIGHTING TO INCREASE MATING SUCCESS IN COLONISED *ANOPHELES FUNESTUS*

Paul Calist Mrosso

A dissertation submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Master of Science in Medicine.

Johannesburg, 2024

DECLARATION

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

_____  _____

(Signature of candidate)

On the__**26th**__day of__**July**___**2024**_____in__**Johannesburg**_

DEDICATION

I would like to dedicate this work to my beloved daughter Alicia Paul Mrosso.

PRESENTATIONS ARISING FROM THE STUDY

A. Oral conference presentations

1. **Paul C. Mrosso**, Ashley M. Burke, Halfan Ngowo, Megan Riddin, Bernard W.T. Coetzee, Fredros Okumu and Lizette L. Koekemoer. **Optimising adult mosquitoes housing to increase mating success in colonised *Anopheles funestus.*** 2023. The 9th PAMCA Annual Conference, Addis Ababa, Ethiopia.

   Contribution to presentation:

   P.C Mrosso interpreted and analysed the results that were presented, and prepared the PowerPoint presentation and orally presented during the conference.

2. **Paul C. Mrosso**, Ashley M. Burke, Halfan Ngowo, Megan Riddin, Bernard W.T. Coetzee, Fredros Okumu and Lizette L. Koekemoer. **Adult *Anopheles funestus* from different geographical localities differ in response to similar mating stimuli in captivity. 2023.** The 8th Southern Africa Malaria Research Conference 2023, Pretoria, South Africa.

   Contribution to presentation:

   P.C Mrosso interpreted and analysed the results that were presented, and prepared the PowerPoint presentation and orally presented during the conference.

B. Poster conference presentations

1. **Paul C. Mrosso**, Ashley M. Burke, Halfan Ngowo, Megan Riddin, Fredros Okumu, Lizette L. Koekemoer and Bernard W.T. Coetzee. **Malaria vector *Anopheles funestus* from different geographical locations express differed response to same light environment in captivity.** 2023. The 12th Oppenheimer Research Conference, Midrand, South Africa.

   Contribution to presentation:

   P.C Mrosso interpreted and analysed the results that were presented, and prepared and presented the poster during the conference.

2. **Paul C. Mrosso**, Ashley M. Burke, Halfan Ngowo, Megan Riddin, Fredros Okumu, Lizette L. Koekemoer and Bernard W.T. Coetzee. **Optimising artificial insectary lighting to increase mating success in colonised *Anopheles funestus.*** 2022. The 11th Oppenheimer Research Conference, Midrand, South Africa.

   Contribution to presentation:

   P.C Mrosso interpreted and analysed the results that were presented, and prepared and presented the poster during the conference.

ABSTRACT

In nature, malaria vectors mate in swarms that form at dusk in relation to different ground markers that either affect the amount of light or contrast light at the point of swarm formation. Specific light regimes known to stimulate mating in mosquitoes have been simulated indoors for laboratory rearing of *Anopheles*. These conditions have proven effective for establishing and sustaining colonies of species in the *Anopheles gambiae* complex, but the same has not been achieved with members of the *An. funestus* group. *Anopheles funestus sensus stricto*, a main African malaria vector species in this group, has been difficult to colonise due to insufficient mating success in laboratory cages. This study aimed to optimise adult mosquito housing conditions to increase the mating success of two *An. funestus* strains housed under laboratory conditions. Mating success ratios were compared in different insectaries, cage sizes, and vector densities. The light environment in adult mosquito-holding cages was manipulated with artificial horizons and visual markers to simulate natural swarm cues. Mating success ratios did not differ between insectaries, but I found that cage size and manipulating adult mosquito-rearing cage by covering them with a cloth or placing a light-contrasting marker inside the cages increased mating success of *An. funestus*. Furthermore, the two geographically distinct *An. funestus* strains were affected differently by the same cage manipulation. Covering the top half of rearing cages with black opaque cloth and placing a black light-contrasting marker on the base of the cage significantly increased the mating success of *An. funestus* from Mozambique (FUMOZ strain). Contrastingly, the *An. funestus* from Angola (FANG strain) showed increased mating success in large cage sizes and when the side of the cage was covered in black cloth. The FANG strain had consistently higher mating success ratios compared to the FUMOZ strain. These findings indicate that the mating success ratios of *An. funestus* in the laboratory is likely to be influenced by geographical locations or underlying genetic diversities resulting in differences in the response of colony strain to the same mating-enhancement cues. Hence, adult mosquito housing should be customised when attempting to colonise and mass-produce *An. funestus*.

Keywords: Artificial horizons, cage size, insectary, spermatheca, visual markers, vector density.

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More than anything, I offer this endeavour to the Almighty God for the peace of mind, the strength and good health he bestowed upon me to be able to finish the study and compile the outputs.

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NOMENCLATURE/LIST OF ABBREVIATIONS AND SYMBOLS

%: Percent

°C: Degrees Celsius

±: Plus, or minus

=: Equal sign

<: Less than sign

®: Registered trademark

$\chi 2$: Chi square

An.: *Anopheles*

BDMI: Botha de Meillon insectary

CDC: Centre for Disease Control

CI: Confidence interval

CFL: Compact Fluorescent Lamps

cm: Centimetre

COI: Cytochrome oxidase I

COVID – 19: Coronavirus Disease 2019

DNA: Deoxyribonucleic Acid

E-2025: Elimination 2025

E-8: Elimination Eight

ELF: Environmental Light Field

et al.: et alia

FANG: *Anopheles funestus* Angola strain

FUMOZ: *Anopheles funestus* Mozambique strain

IHI: Ifakara Health Institute

LED: Light-emitting diode

LRT: Likelihood Ratio Tests

N: Total sample size

n: Sample size

NADH: Nicotinamide Adenine Dehydrogenase

NICD: National Institute for Communicable Diseases

NHLS: National Health Laboratory Services

OR: Odds Ratio

P: *Plasmodium*

RFLP: Restriction Fragment Length Polymorphism

SE: Standard Error

SIT: Sterile Insect Technique

s.s.: Sensu stricto

UV: Ultraviolet

VCRU: Vector Control Reference Unit

W: Watts

Wits: University of the Witwatersrand

WHO: World Health Organization

WRIM: Wits Research Institute for Malaria

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CHAPTER ONE

Literature Review

1.1.Background

1.1.1. Malaria burden

Even though malaria infections in humans can be prevented, and the disease treated, it is still the most important parasitic disease with the highest transmission rates and deaths worldwide. The disease is caused by protozoan parasites of specific *Plasmodium* species and is transmitted by female mosquitoes of certain *Anopheles* species (Ross, 1910). In the year 2022, there were more than 249,000,000 recorded cases and 625,000 recorded deaths reported due to malaria worldwide with the World Health Organization (WHO) African region accounting for more than 94% and 96% of the cases and deaths respectively (WHO, 2023). Generally, there was a steep decrease in malaria burden globally between the years 2000 (million to 230 million cases and 897,000 deaths) and 2015 (230 million cases and 577,000 deaths) (WHO, 2023). However, the malaria burden decline rates stalled from 2015 to 2019 with about 230 million malaria cases recorded in 2015 and more than 232 million cases in 2019. Similarly, malaria deaths did not have significant changes in the same period where about 577,000 deaths were recorded in 2015 and more than 568,000 deaths were recorded in 2019 (WHO, 2023). Malaria cases and deaths increased in the year 2020 mainly due to the COVID – 19 global pandemic and its mitigation measure that disrupted essential malaria services. For instance, lockdown and restricted movement affected vector control activities and antimalarial rollout as well as reduced malaria testing and case investigation (WHO, 2023). The lack of significant gains in malaria control since 2015 has been attributed to the reduced efficiency of malaria control interventions especially those that target vectors (WHO, 2023).

Against the disruptions of the COVID – 19 pandemic, many among the E-2025 countries (21 countries identified by WHO that have the potential to eliminate indigenous malaria transmission by 2025), still managed to maintain progress towards malaria elimination (WHO, 2023). Between 2015 and 2020, South Africa, a member of the E-2025 countries, reported two peaks of high resurgence of malaria cases. First in the year 2017 (over 28,000 cases) which was attributed to climate, environmental and socio-economic factors (Njau *et al.*, 2021; Tsoka-Gwegweni, 2022), as well as imported cases from neighbouring countries and other travellers

(Abiodun *et al.*, 2020; Munsense and Tsoka-Gwegweni, 2023). The second was in 2020 which was mainly due to the COVID – 19 global pandemic disruptions. However, South Africa managed a reduction in malaria cases after the pandemic (WHO, 2023).

Active malaria transmission in South Africa takes place mostly along the northern border provinces which include Limpopo, Mpumalanga, and KwaZulu-Natal (Brooke, 2022; Kollipara *et al.*, 2023; Maharaj *et al.*, 2023; Munsense and Tsoka-Gwegweni, 2023). High numbers of malaria cases are reported in the warm and rainy months which start in October and peak in January and February and slow towards May (Tsoka-Gwegweni, 2022; Maharaj *et al.*, 2023). To ensure malaria elimination, South Africa is working closely with the neighbouring countries through the Elimination 8 (E-8) Initiative to reach zero malaria cases in the border zones (Rahman *et al.*, 2021).

1.1.2. Malaria transmission

Malaria transmission is dependent on the feeding behaviour of the female anopheline mosquitoes that are the intermediate hosts of the malaria parasites. The female *Anopheles* mosquito seeks and takes a blood meal purposely for egg production when it is inseminated (Jones, 1968). Pathogenesis of malaria is dependent on the malaria parasite while the *Plasmodium's* life cycle has multiple development stages and involves two hosts: female *Anopheles* mosquito (invertebrate) and warm-blooded vertebrates (Fig. 1.1). There are five species of *Plasmodium* that are known to cause malaria in humans which include *Plasmodium falciparum, P. vivax, P. malariae, P. ovale,* and *P. knowlesi* (Sato, 2021; WHO, 2023). The first four species cause human malaria while *P. knowlesi* is zoonotic causing malaria in humans and macaque monkeys (Sato, 2021). *Plasmodium falciparum* is the most fatal malaria parasite and is responsible for almost all malaria deaths in sub-Saharan Africa (Sato, 2021; WHO, 2023).

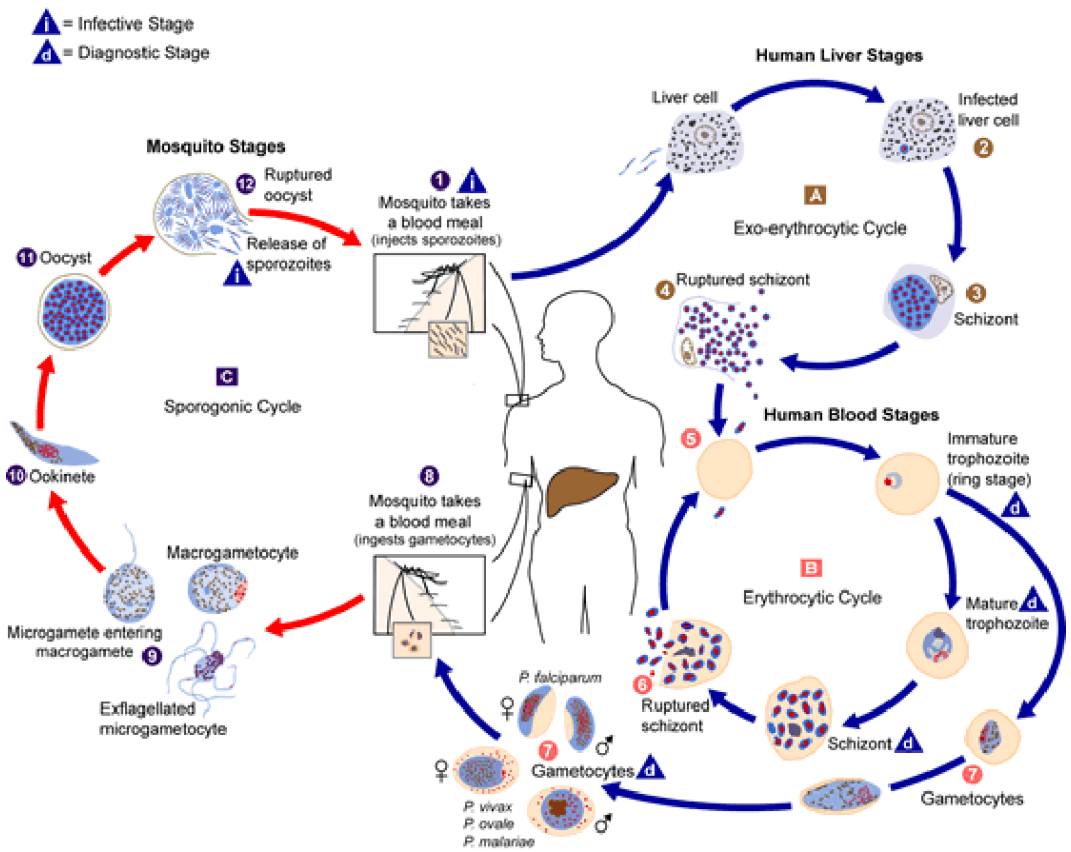

Figure 1.1. The malaria parasite life cycle and malaria transmission path depicting the asexual development of the *Plasmodium* parasite in the warm-blooded host (human) and sexual development in *Anopheles* mosquito. Adapted from and courtesy of the Centre for Disease Control and Prevention (CDC) (http://www.cdc.gov/malaria/about/biology/, Accessed on 12th December 2023

1.1.3. Malaria control

Some countries have managed to eliminate malaria while others are at the pre-elimination stage and most of the sub-Saharan countries are still in the control phase (WHO, 2023). Malaria control involves targeting the malaria parasite through antimalarials (early diagnosis and treatment) and breaking the transmission chain by preventing vector-human contact and reducing vector populations. The main interventions in malaria vector control in Sub-Saharan Africa are insecticide-based that include use of treated bed nets and indoor residual spraying (WHO, 2023). In some settings they are integrated with the management of mosquito breeding sites (Msugupakulya *et al.*, 2023). The insecticide-dependent interventions that target adult mosquitoes have been credited for the gains in malaria control in the past, but their efficiency has been hampered due to the development of resistance within vector populations (Msugupakulya *et al.*, 2023; Odero *et al.*, 2023; Paaijmans and Lobo, 2023; WHO, 2023). New interventions that are less prone to resistance development such as the use of sterile insect

technique (SIT) and gene drive technologies have been proposed for malaria vector control (Kaiser *et al.*, 2021; Wang *et al.*, 2022). These new technologies rely on effective mass rearing and release of modified insects. However, some major malaria vector species such as *An. funestus* cannot be reared at large scales with the current rearing procedures due to their low mating success ratios in rearing facilities.

1.2.Malaria vectors

Anopheles mosquitoes are responsible for human malaria transmission and are considered the most notorious vectors in the world. Globally, there are about 465 known *Anopheles* mosquito species (Sinka *et al.*, 2012; Sinka, 2013; Norris and Norris, 2015). However, only about 100 *Anopheles* species can transmit human malaria with 45 species being described as dominant vectors that contribute to transmission levels of significant public health concern (Sinka *et al.*, 2010; Sinka, 2013; Norris and Norris, 2015). Africa has over 140 recorded *Anopheles* species, of which at least eight species are considered efficient malaria vectors (Gillies and De Meillon, 1968; Sinka *et al.*, 2012; Wiebe *et al.*, 2017). Malaria vector species differ in their vectorial ability to transmit malaria and are categorised as primary or secondary vectors (Gillies and De Meillon, 1968; Coetzee and Koekemoer, 2013; Coetzee, 2020). They differ in vectorial capacity due to several factors such as geographical distribution, insecticide resistance status, biting and resting behaviour, and host preference (Wiebe *et al.*, 2017; Tsegaye *et al.*, 2022).

The main malaria vectors in Africa belong to species within the *An. gambiae* complex and *An. funestus* group (Gillies and De Meillon, 1968; Coetzee and Koekemoer, 2013; Coetzee, 2020; Msugupakulya *et al.*, 2023). A mosquito species complex represents individuals of different species that are morphologically indistinguishable while species groups consist of individuals of different species that are morphologically similar as adults but can be differentiated in their aquatic stages (Coetzee, 2020). The *An. gambiae* complex consists of nine characterised species which include *An. gambiae sensu stricto* (*s.s*), *An. coluzzii*, *An. arabiensis*, *An. amharicus*, *An. bwambae*, *An. merus*, *An. melas*, *An. fontenillei*, and *An. quadriannulatus* (Sinka, 2013; Coetzee, 2020). Among these, *An. gambiae s.s*, *An. coluzzii* and *An. arabiensis* are the most efficient malaria vectors in the sub-Saharan Africa region (Coetzee, 2020). The *Anopheles funestus* group includes at least 13 Afrotropical species which include *An. funestus s.s.*, *An. rivulorum*, *An. longipalpis*, *An. rivulorum-like*, *An. parensis*, *An. funestus-like*, *An. vaneedeni*, *An. leesoni*, *An. fuscivenosus*, *An. aruni*, *An. brucei*, *An. culicifacies*, and *An. confuses* (Gillies and De Meillon, 1968; Harbach, 2004; Sinka *et al.*, 2012; Wiebe *et al.*, 2017; Coetzee, 2020;

Odero *et al.*, 2023). Effective malaria vector control initiatives depend on the proper understanding of the ecology and biology of *Anopheles* mosquitoes.

1.2.1. *Anopheles* mosquito life history in their natural habitats

Field studies have established that *Anopheles* mosquitoes prefer to breed in almost all types of water sources including temporary and permanent sources, ranging from dirty to clear water (Sinka *et al.*, 2010; Nambunga *et al.*, 2020; Zengenene *et al.*, 2020; Debrah *et al.*, 2021). *Anopheles* larvae obtain their food from plankton and neustons formed in their aquatic habitats (Wotton *et al.*, 1997). *Anopheles* mosquitoes emerge from their aquatic habitats after pupation and adult mosquitoes feed on sugar from flower nectar and fruits for energy (Ross, 1910; Gary *et al.*, 2009). Only the female mosquito seeks blood meal for egg development after they are inseminated (Beier, 1996; Sinka *et al.*, 2010; Carvajal-Lago *et al.*, 2021; Omondi *et al.*, 2022). Some primary malaria vectors such as *An. gambiae s.s* (called *An. gambiae* going forward) and *An. funestus s.s* (called *An. funestus* going forward) are anthropophagic meaning they almost exclusively feed on human blood, while others such as *An. arabiensis* and *An. parensis* express opportunistic feeding where they can feed on a range of warm-blooded vertebrates (Sinka *et al.*, 2010; Burke *et al.*, 2019; Zengenene *et al.*, 2020; Carnevale and Manguin, 2021). Adult *Anopheles* mosquitoes are known to prefer resting in cool, dark places and close to human settlements during the day (Odiere *et al.*, 2007; Dandalo *et al.*, 2017). Both male and female *Anopheles* have shown specific preference for resting in dark places such as clay pots, boxes, buckets, and other containers especially in human compounds (Odiere *et al.*, 2007). High densities of *An. funestus, An. gambiae.* and *An. arabiensis* (males and females) have been observed to rest indoors (McCann *et al.*, 2017; Machani *et al.*, 2020; Msugupakulya *et al.*, 2020; Nambunga *et al.*, 2020; Bedasso *et al.*, 2022).

1.2.2. *Anopheles* mating behaviour in their natural environment

Anopheles mosquitoes mating activities are regulated by their circadian clock and are induced by specific changes in the light environment. They naturally mate in swarms that are initiated by male mosquitoes and joined by newly emerged females at the onset of dusk (Charlwood *et al.*, 2003; Charlwood, 2011; Diabaté *et al.*, 2011; Sawadogo *et al.*, 2013; Facchinelli *et al.*, 2015; Kaindoa *et al.*, 2017; Zawada *et al.*, 2018; Kaindoa *et al.*, 2019). The swarms usually start within 10 minutes of the onset of sunset and last between 15 and 30 minutes which coincides with the duration of twilight (Charlwood *et al.*, 2003; Diabate *et al.*, 2003; Sawadogo *et al.*, 2013; Achinko *et al.*, 2016). After swarm initiation by males, females join where males select their mate then the couple leave to copulate out of the swarm. *Anopheles* mosquito

swarms form repeatedly at specific locations and time of day (Sawadogo *et al.*, 2013; Kaindoa *et al.*, 2017; Zawada *et al.*, 2018; Kaindoa *et al.*, 2019).

Mating swarms of members of the *An. gambiae* complex and *An. funestus* group have been observed and detailed in different locations including Tanzania (Harper, 1944; Kaindoa *et al.*, 2019), Zambia (Zawada *et al.*, 2018), Mozambique (Charlwood *et al.*, 2003; Charlwood, 2011), and west African countries (Charlwood and Jones, 1979; Diabate *et al.*, 2003; Diabaté *et al.*, 2011; Sawadogo *et al.*, 2013; Diabate and Tripet, 2015; Achinko *et al.*, 2016). Generally, *An. gambiae* complex species prefer to swarm in open areas over swarm markers such as light-contrasting surfaces and swarms can be stimulated artificially by man-made swarm markers (Diabate *et al.*, 2003; Charlwood, 2011; Kaindoa *et al.*, 2017). On the other hand, *An. funestus* swarms are naturally found close to human dwellings mostly in very close proximity to resting/sleeping houses with no specific ground swarm markers detailed (Harper, 1944; Charlwood *et al.*, 2003; Zawada *et al.*, 2018; Kaindoa *et al.*, 2019).

In a study conducted in Southern Mozambique, *An. funestus* swarms were observed to avoid light-contrast markers and swarms dissolved upon introduction of light-contrast markers (Charlwood *et al.*, 2003). Both male and female *An. funestus* have been observed to rest inside houses (Msugupakulya *et al.*, 2020; Nambunga *et al.*, 2020), and there is evidence from semi-field experiments and field observations that this vector species can mate freely indoors (Nambunga *et al.*, 2021), indicating this important vector can be reared in captivity. Furthermore, Nambunga *et al.* (2021), noticed males and unmated females of *An. funestus* leaving houses before sunset which may suggest their intention of joining outdoor mating swarms induced by the dimming natural light.

1.3.*Anopheles* mosquito colonisation and rearing

Laboratory and semi field rearing of malaria vectors have proven instrumental in understanding the biology and ecology of these species as well as devising vector control interventions (Hunt *et al.*, 2005; Ng'Habi *et al.*, 2010). To improve *Anopheles* mosquito rearing, modifications to mimic the natural photoperiodism of 12:12 hours light:dark cycle including a dusk and dawn period have been made using artificial light in insectaries (Coluzzi, 1964; Hunt *et al.*, 2005; Maramorosch and Mahmood, 2014). Moreover, different designs in shape and size of adult mosquito cages have been designed for holding and rearing adult mosquitoes in insectaries (Coluzzi, 1964; Marchand, 1985; Mashatola *et al.*, 2017). Not all malaria vector species have been easy to house and maintain successfully (Coluzzi, 1964; Gillies and De Meillon, 1968;

Coetzee and Koekemoer, 2013). The rearing of malaria vector species in the *Anopheles gambiae* complex has been well standardised both in semi-field (Ng'Habi *et al.*, 2010; Ng'Habi *et al.*, 2015; Achinko *et al.*, 2016; Niang *et al.*, 2019) and laboratory (Hunt *et al.*, 2005; Maramorosch and Mahmood, 2014) settings. But the same standardisation has not yet been achieved with malaria vectors in the *An. funestus* group.

Research on the life history of *Anopheles* mosquitoes has established their preferred conditions of temperature and humidity in the natural habitat, effective life stage for vector control as well as sampling for colony establishment (Maramorosch and Mahmood, 2014; Vatandoost, 2021). The *Anopheles* life cycle, like other mosquitoes, involves aquatic and terrestrial life stages (Ross, 1910; Coluzzi, 1964; Maramorosch and Mahmood, 2014; Vatandoost, 2021). The egg, larvae, and pupae development account for the aquatic life stage while the adult mosquito is the terrestrial life stage (Ross, 1910; Coluzzi, 1964; Vatandoost, 2021). Generally, *Anopheles* mosquito colony establishment is initiated either by field collection of gravid mosquitoes or by obtaining starter material as eggs, larvae, or adults from an established colony (Service and Oguamah, 1958; Coluzzi, 1964; Maramorosch and Mahmood, 2014). To establish a colony from field collected material, gravid and/or blood-fed female *Anopheles* mosquitoes are collected, characterised morphologically, and placed into individual vials prepared to stimulate oviposition and held overnight to lay eggs (Service and Oguamah, 1958; Hunt *et al.*, 2005; Maramorosch and Mahmood, 2014). Collected unfed females can also be given a blood meal before being separated for egg production. After laying eggs the mosquitoes are further characterised with molecular techniques to ascertain species (Koekemoer *et al.*, 2002; Hunt *et al.*, 2005), and the eggs from species of interest are pooled together for rearing.

1.3.1. *Anopheles* larvae rearing

Mosquito larval fitness including development time from first to fourth instar, body size, and pupation, as well as adult mosquito fitness parameters such as body size, wing length and flight activities are directly proportional to larval-housing conditions and the nutritional quality of larval food (Coluzzi, 1964; Kivuyo *et al.*, 2014; Maramorosch and Mahmood, 2014; Benedict *et al.*, 2020; Vatandoost, 2021; Zengenene *et al.*, 2021; Felamboahangy *et al.*, 2022). Larval food with a good balance of proteins, fatty acids, vitamins, and sugar is preferable for larvae development and adult mosquito physiological and reproductive fitness (Kivuyo *et al.*, 2014; Benedict *et al.*, 2020). Apart from the food content, the physical state of larval food (Kivuyo *et al.*, 2014; Akpodiete *et al.*, 2019; Benedict *et al.*, 2020), feeding ratios and feeding cycles (Coluzzi, 1964; Epopa *et al.*, 2018; Akpodiete *et al.*, 2019; Benedict *et al.*, 2020; Carvajal-Lago

et al., 2021), as well as water quality, level and surface area (Coluzzi, 1964; Ng'Habi *et al.*, 2005; Epopa *et al.*, 2018; Akpodiete *et al.*, 2019; Zengenene *et al.*, 2022), are important parameters for determining the fitness of larvae and emerged adults. Some of the reported and commonly used *Anopheles* larvae food include simple formulations of dried yeast (Service and Oguamah, 1958), yeast and dog biscuits (Coluzzi, 1964; Hunt *et al.*, 2005; Damiens *et al.*, 2014; Ngowo *et al.*, 2021; Zengenene *et al.*, 2021; Felamboahangy *et al.*, 2022; Zengenene *et al.*, 2022), liver powder, fish-food flakes (Damiens *et al.*, 2014; Akpodiete *et al.*, 2019), algae, and cereal powder and pollens (Kivuyo *et al.*, 2014; Benedict *et al.*, 2020).

1.3.2. Adult *Anopheles* rearing

Adult mosquitoes are maintained with sugar feeding that is delivered either through soaked cotton wool or filter paper (Service and Oguamah, 1958; Coluzzi, 1964). Different types of sugar are used for adult mosquito feeding ranging from industrial sugar refined from sugar canes to glucose solution and honey (Coluzzi, 1964; Zanin *et al.*, 2019; Omondi *et al.*, 2022). A 10% sugar or glucose solution is the widely used sugar source for adult mosquito rearing (Coluzzi, 1964; Hunt *et al.*, 2005). Blood meal is essential for egg development after mosquitoes are mated (Beier, 1996; Sinka *et al.*, 2010; Maramorosch and Mahmood, 2014; Carvajal-Lago *et al.*, 2021; Vatandoost, 2021; Omondi *et al.*, 2022). In mosquito rearing facilities blood meal is delivered by either allowing mosquitoes to feed on a live organism (Service and Oguamah, 1958; Coluzzi, 1964; Vatandoost, 2021), or through artificial membrane feeding (Vatandoost, 2021; Felamboahangy *et al.*, 2022). Anthropophilic species such as *An. gambiae* and *An. funestus* have shown to adapted well to non-human blood (Service and Oguamah, 1958; Coluzzi, 1964; Felamboahangy *et al.*, 2022). Live organisms including human (Service and Oguamah, 1958; Verhoek and Takken, 1994; Ngowo *et al.*, 2021; Oliver *et al.*, 2022), chicken (Ngowo *et al.*, 2021), defibrinated cow blood (Felamboahangy *et al.*, 2022), and anaesthetised guinea pig (Service and Oguamah, 1958; Felamboahangy *et al.*, 2022), have been reported to be used in *An. funestus* mosquito rearing.

Adult *Anopheles* mosquito rearing in insectaries and laboratories is done in holding cages that are available in different sizes, shapes, and colours of materials (Coluzzi, 1964; Marchand, 1985; Maramorosch and Mahmood, 2014; Vatandoost, 2021). Adult mosquito cages are generally made of clear fine-mesh net material that allows for light and air penetration inside the cage and for ease of observation (Marchand, 1985; Maramorosch and Mahmood, 2014). The most used adult mosquito holding and rearing cages are the cubic cages that are available as customised or industrial made with either 15 cm sides (small cubic) or 30 cm sides (large

cubic) (Coluzzi, 1964; Maramorosch and Mahmood, 2014; Mashatola *et al.*, 2017; Ngowo *et al.*, 2021; Vatandoost, 2021). However, there are several designs of custom-made cages that range from round bucket cages of varying sizes with a clear fine-meshed top cover to rectangular cages of varying sizes (Coluzzi, 1964; Mashatola *et al.*, 2017; Vatandoost, 2021). The commercial cubic cages are made of clear fine-meshed net side with a nylon base, and some involved one side made of clear nylon. Mosquito cage size and vector density per cage are important determinants of physiological and reproductive fitness (Coluzzi, 1964; Maramorosch and Mahmood, 2014). Adult mosquito density per cage and cage size have been indicated to be important parameters in ensuring physiological and reproductive fitness of malaria vectors in the *An. gambiae* complex (Coluzzi, 1964; Maramorosch and Mahmood, 2014; Mashatola *et al.*, 2017).

1.4.*Anopheles funestus* group

The *An. funestus* species has been classified in the Subgenus Cellia and Myzomyia series along with other species groups like *An. marshallii* group, *An. demeilloni* group (Ross, 1910; Gillies and De Meillon, 1968). Members of the *An. funestus* group are found across all territories of the malaria endemic sub-Saharan Africa region (Sinka *et al.*, 2010; Sinka *et al.*, 2012; Coetzee and Koekemoer, 2013; Odero *et al.*, 2023). Even though their role in malaria transmission was evident since the start of the 20th century (Ross, 1910; Gillies and De Meillon, 1968), the *An. funestus* group remained understudied for a long time owing to lack of efficient methods to characterise species and laboratory rearing (Coetzee and Koekemoer, 2013). However, the group has recently gained more attention due to availability of molecular method to identify and distinguish its member species (Koekemoer *et al.*, 2002; Coetzee and Koekemoer, 2013). The group is believed to play major role in active and residual malaria transmission in sub-Saharan Africa (Coetzee and Koekemoer, 2013). *Anopheles funestus* is the more efficient vector species in the group and is categorised as a primary malaria vector in most regions of sub-Sahara Africa (Gillies and De Meillon, 1968; Hargreaves *et al.*, 2000; Brooke *et al.*, 2001; Hunt *et al.*, 2005; Sinka *et al.*, 2012; Choi *et al.*, 2013; Coetzee and Koekemoer, 2013; Msugupakulya *et al.*, 2023; Odero *et al.*, 2023). Other members of the group including *An. vaneedeni, An. leesoni, An. rivulorum, An. longipalpis,* and *An. parensis* have been found positive for *Plasmodium falciparum* sporozoites and are hence classified as secondary or potential malaria vectors (Temu *et al.*, 2007; Coetzee and Koekemoer, 2013; Burke *et al.*, 2017; Burke *et al.*, 2019; Brooke, 2022). However, species in the *An. funestus* group remain understudied owing to a lack of enough established and sustained colonies. This study focused on *An. funestus*.

1.4.1. *Anopheles funestus*

Anopheles funestus was first described in 1900 in Sierra Leone by Giles and its role in malaria transmission was described by Ross (1910). The species is native to many parts of the sub-Sahara Africa especially the swampy areas (Gillies and De Meillon, 1968; Brooke *et al.*, 2001; Sinka *et al.*, 2012; Coetzee and Koekemoer, 2013; Odero *et al.*, 2023). The female *An. funestus* is an anthropophilic insect meaning that it exclusively (prefers to) blood feed on humans (Beier, 1996; Brooke *et al.*, 2001). *Anopheles funestus* is also highly endophilic, meaning it rests indoors, making it an efficient malaria vector (Brooke *et al.*, 2001; Nambunga *et al.*, 2021). *Anopheles funestus* prefers breading in clear permanent shallow waters and along slow-moving streams with submerged vegetation (Nambunga *et al.*, 2020).

Anopheles funestus expresses high levels of resistance towards the insecticide classes used in vector control across the sub-Saharan Africa region (Brooke *et al.*, 2001; Hunt *et al.*, 2005; Coetzee and Koekemoer, 2013; Odero *et al.*, 2023). However, the expression of insecticide resistance related genes has been observed to differ between *An. funestus* from different geographical locations in Africa (Coetzee and Koekemoer, 2013; Tchouakui *et al.*, 2018; Tchouakui *et al.*, 2021; Menze *et al.*, 2022; Nolden *et al.*, 2022; Odero *et al.*, 2023). Insecticide susceptibility status has been linked to physiological fitness that can impact the life history of the mosquito (Berticat *et al.*, 2002; Platt *et al.*, 2015; Tchouakui *et al.*, 2018; Tchouakui *et al.*, 2021; Menze *et al.*, 2022; Nolden *et al.*, 2022). For example, expression of insecticide resistance genes has been linked to reduced reproductive fitness including reduction of egg production, egg hatching rates and male mating competitiveness in other mosquitoes (Berticat *et al.*, 2002; Platt *et al.*, 2015), as well as in *An. funestus* (Tchouakui *et al.*, 2019; Tchouakui *et al.*, 2021; Menze *et al.*, 2022; Nolden *et al.*, 2022).

Anopheles funestus has been found to express specific population structuring across regions. Different techniques including chromosomal inversion and molecular techniques have grouped *An. funestus* into distinct genetic groups (Choi *et al.*, 2013; Norris and Norris, 2015; Zawada *et al.*, 2018; Odero *et al.*, 2023). Some of the populations have been found to be present in specific geographical regions while others have been found to spread across large territories and coexist (Zawada *et al.*, 2018; Odero *et al.*, 2023). The *An. funestus* population structuring has been observed to follow the natural gene flow barriers such as forests, large lakes, mountain ranges and rift valleys (Koekemoer *et al.*, 2006; Amvongo-Adjia *et al.*, 2020; Kaddumukasa *et al.*, 2020; Odero *et al.*, 2023).

Anopheles funestus is categorised into two cryptic subpopulations: clade I and clade II following mitochondrial DNA analysis and into M-type, W-type and MW-type following genotyping by restriction fragment length polymorphism (RFLP) (Choi *et al.*, 2013; Zawada *et al.*, 2018; Odero *et al.*, 2023). While clade I is widespread across the sub-Saharan Africa territory, clade II has so far been reported only in the southeastern parts of the African continent including Tanzania, Zambia, Mozambique, Madagascar and South Africa (Choi *et al.*, 2013; Zawada *et al.*, 2018). The RFLP genetic segregation follows the same trend as the clade structuring where the M-type is found on the east side of the great Rift valley including Madagascar, Mali, and Kenya (Odero *et al.*, 2023). Like clade I, the W-type is widespread across Africa while the hybrid, MW-type is found on both sides of the Rift Valley including Angola, South Africa, Mozambique, and Malawi (Odero *et al.*, 2023). These genetic segregations are based on phylogenetic studies following the sequencing of the mitochondrial *Cytochrome Oxidase I* (COI) and *Nicotinamide Adenine Dehydrogenase* (NADH) subunit 5 (ND5) (Zawada *et al.*, 2018; Odero *et al.*, 2023). These two clades coexist in the south-eastern regions of the African continent (Zawada *et al.*, 2018; Odero *et al.*, 2023). However, the effect of this genetic diversity within species on the life history of *An. funestus* in captivity have not been examined.

1.4.2. *Anopheles funestus* rearing

The first recorded attempts to colonise *An. funestus* were reported as early as the 1950s where a colony was established from wild collected material (Service and Oguamah, 1958). The first colonisation was achieved in 1955/56 from eggs laid by wild collected female *An. funestus* in Nigeria (Service and Oguamah, 1958). However, the colony succumbed due to low egg production (Service and Oguamah, 1958). Service and Oguamah, (1958) made a second colonisation attempt from wild caught gravid *An. funestus* that were maintained on guinea pig blood and they managed to reach the eighth filial generations. Like the first colony, the second colony was not successfully colonised and sustained (Service and Oguamah, 1958). An attempt to colonise *An. funestus* from wild collected material in Tanzania proved difficult in multiple attempts (Ngowo *et al.*, 2021). The main reason that has been observed to hinder *An. funestus* propagation under captivity has been low mating success and small quantity of eggs (Service and Oguamah, 1958; Ngowo *et al.*, 2021).

To date there has been only two fully established and sustained colonies of *An. funestus* (Hunt *et al.*, 2005), and one that is in the establishment stage (Ngowo *et al.*, 2021). Hunt *et al.* (2005) managed to establish two strains from wild caught *An. funestus*: one from mosquitoes collected

in Angola and the other from mosquitoes collected in Mozambique. The colony from Angola has since been denoted as FANG (F – *funestus*, ANG – Angola) and the one from Mozambique is denoted as FUMOZ (FU – *funestus*, MOZ – Mozambique) (Zengenene *et al.*, 2021). The two fully established strains have been shared with other laboratories and used as models in studies to enhance the understanding of their biology and physiology (Williams *et al.*, 2019; Ngowo *et al.*, 2021; Wondji *et al.*, 2022). The FANG strain is known to be susceptible to insecticide used in malaria vector control while the FUMOZ strain is reported to express metabolic resistance (Brooke *et al.*, 2001; Hunt *et al.*, 2005; Tchouakui *et al.*, 2019; Tchouakui *et al.*, 2021; Menze *et al.*, 2022; Nolden *et al.*, 2022). Consequently, benefiting from these two established *An. funestus* strains, different variables that affect the larval and adult life history of this important vector have been studied and reported (Zengenene *et al.*, 2021; Felamboahangy *et al.*, 2022; Zengenene *et al.*, 2022).

Anopheles funestus have shown to feed and grow well under a variety of larvae food formulations such as pure dried yeast (Service and Oguamah, 1958) and a mixture of crushed dog biscuits and brewer's yeast (Hunt *et al.*, 2005; Ngowo *et al.*, 2021; Zengenene *et al.*, 2021; Zengenene *et al.*, 2022). A 3:1 ratio of finely powdered dog biscuit and brewer's yeast yielding the best feeding reported so far (Hunt *et al.*, 2005; Zengenene *et al.*, 2021; Felamboahangy *et al.*, 2022; Zengenene *et al.*, 2022). Together with adequate nutrient content, feeding amount and cycles, *An. funestus* larvae have been demonstrated to perform better when reared in containers with low-depth levels of distilled water at around 1 cm water depth and at a surface area of about 0.48 larvae/cm^2 (Felamboahangy *et al.*, 2022). *Anopheles funestus* larvae prefer resting around the water-rearing container edge and the addition of anchoring surfaces were found to enhance larvae growth and enable high larval density per rearing container (Zengenene *et al.*, 2022).

It is well established that to achieve sustainable *Anopheles* mosquito strains, rearing parameters should ensure good physiological and reproductive fitness over successive generations (Coluzzi, 1964; Jones *et al.*, 1967; Marchand, 1985; Mashatola *et al.*, 2017). Mosquito fitness parameters such as body size, flight and mating activities, and fecundity are directly proportional to the mosquito's physiological fitness which is determined by feeding and housing at each life cycle stage (Coluzzi, 1964; Ng'Habi *et al.*, 2005; Akpodiete *et al.*, 2019; Zengenene *et al.*, 2022). Furthermore, mosquito flight activities both in captivity and in their natural environment are regulated by specific cues related to feeding, swarming, oviposition and resting (Jobling, 1935; Coluzzi, 1964; Jones *et al.*, 1967). Most of these cues are controlled

by the mosquito olfaction and circadian clock which are regulated by the light environment (Coluzzi, 1964; Jones *et al.*, 1967).

Ecological factors that are known to affect *Anopheles* mosquitoes mating in their natural environment such as onset of dusk and visual markers that initiate swarming have been highlighted for research to optimise the rearing conditions for *An. funestus* group adults (Jones *et al.*, 1967). Visual swarm markers in the form of light-contrasting black plastic surfaces on a big chamber floor together with dimming ceiling light have been reported to influence swarming and mating for *An. gambiae* under semi field settings (Facchinelli *et al.*, 2015). Swarm induction and improved physiological fitness have been achieved both with simulated artificial horizons in laboratory cages in *An. gambiae* and *An. arabiensis* (Marchand, 1985; Mashatola *et al.*, 2017), and light contrast markers, dimming lights and other natural swarm markers in large semi field experiment cages/chambers (Paton *et al.*, 2013; Facchinelli *et al.*, 2015; Niang *et al.*, 2019). Evaluation of simulated conditions on the life history of adult *An. funestus* is limited by the lack of sustained strains of this important vector species. Hence, there is a lack of studies on the parameters that affect adult *An. funestus* mating in captivity such as rearing cage type and size, light properties both in the rearing room and inside the rearing cage as well as vector density per rearing cage. Since these before-mentioned parameters will be investigated in the current study, more detailed background on the mosquito eye, vision and light are provided below.

1.5. *Anopheles* mosquito eye and vision

Anopheles mosquitoes are generally categorised as nocturnal insects meaning they are more active during the night or in dark places (Jones *et al.*, 1967; Sheppard *et al.*, 2017; Baik *et al.*, 2020; Sizemore and Dacks, 2020; Fyie *et al.*, 2021; Rahman *et al.*, 2021; Alonso San Alberto *et al.*, 2022; Au *et al.*, 2022; Liu *et al.*, 2022). However, *Anopheles* mosquitoes do express certain activities in the light environment as they swarm for mating during sunset or dimming lights within rearing facilities (Jones *et al.*, 1967; Charlwood and Jones, 1979; Charlwood *et al.*, 2003; Diabate *et al.*, 2003; Hunt *et al.*, 2005; Facchinelli *et al.*, 2015). *Anopheles* mosquitoes, like other insects, have compound eyes made of ommatidia that contain eight photoreceptors each (Liu *et al.*, 2022). Photoreception and phototransduction processes in mosquito eyes take place in the microvillar rhabdomere that are contained in the photoreceptors (Liu *et al.*, 2022). Microvillar rhabdomeres are lined with opsins that are made of G-protein-coupled receptors and light-sensitive chromophore responsible for initiation of

phototransduction (Baik *et al.*, 2020; Liu *et al.*, 2022). Moreover, mosquitoes express a blue- and ultraviolet (UV)-light-sensitive cryptochrome non-opsin photoreceptor (Baik *et al.*, 2020; Au *et al.*, 2022). *Anopheles* compound eyes express high spectral sensitivity to short-wavelength light which is vital in shaping their physiological adaptations and modulation of important survival and reproduction behaviours (Sheppard *et al.*, 2017; Au *et al.*, 2022; Liu *et al.*, 2022).

Some important visual-based mosquito activities such as feeding, flight, mating, oviposition, and resting are controlled by a light dependent molecular circadian clock (Baik *et al.*, 2020). Prolonged and temporal changes in the light environment have been observed to affect the mosquito circadian clock modulated behaviours meaning the clock is dependent on mosquito vision (Sheppard *et al.*, 2017; Baik *et al.*, 2020). Moreover, the light evoked behaviours differ between male and female *Anopheles* mosquitoes and their circadian clock modulated behaviour differences coincide with light spectrum evoked behaviours (Baik *et al.*, 2020). This means that managing the light environment is crucial when evaluating behavioural related activities such as mating in *Anopheles* mosquitoes.

1.6. Effect of artificial lights on *Anopheles* mosquito behaviour

The use of artificial lights in the human environment and settlements is associated with the changing behaviour of both human and insects' circadian rhythms, including mosquitoes (Barghini and de Medeiros, 2010). The effects of artificial light have been assessed on disease-transmitting insects, particularly the flight behaviour of various mosquito vector species (Sheppard *et al.*, 2017; Fyie *et al.*, 2021; Wilson *et al.*, 2021). The increased use of artificial light, especially at night, is believed to result in either an increase or reduction of mosquito borne disease depending on the type of light and time of day the light is used (Barghini and de Medeiros, 2010; Coetzee *et al.*, 2022). Artificial lights have been demonstrated to regulate attraction, avoidance, feeding, and oviposition in *Anopheles* mosquito species (Jobling, 1935; Jones *et al.*, 1967; Huang *et al.*, 2007; Sheppard *et al.*, 2017; Au *et al.*, 2022). Lights with UV light emissions have been found to attract mosquitoes and have thus been used in mosquito light traps (Wilson *et al.*, 2021). Light type and presence of light-contrasting surfaces that can alter the light environment have been demonstrated to affect the choice of oviposition sites by *Anopheles* mosquitoes (Jobling, 1935; Huang *et al.*, 2007).

Mating is among the activities that are regulated by the circadian clock in *Anopheles* mosquitoes (Maramorosch and Mahmood, 2014). Rearing of malaria vectors has benefited from the use of

artificial light to create the important photoperiods in rearing facilities (Jones *et al.*, 1967; Hunt *et al.*, 2005; Sheppard *et al.*, 2017). Consequently, improved mating of *Anopheles* mosquitoes in rearing insectaries has been achieved by installing light systems that dim at the onset of photophase and scotophase to mimic natural dusk and dawn (Hunt *et al.*, 2005; Sheppard *et al.*, 2017). Some *Anopheles* insectaries do not have dimming cycles (Kivuyo *et al.*, 2014; Ngowo *et al.*, 2021), whereas others have dimming cycles of varying period of time such as 30 minutes (Hunt *et al.*, 2005), to one hour (Sheppard *et al.*, 2017). Dimming in *Anopheles* rearing insectaries has been achieved either by setting lights to switch on/off automatically in a serial order at specific intervals or by installation of systems that will fade/increase light at a specific voltage rate for a specified period (Hunt *et al.*, 2005; Maramorosch and Mahmood, 2014; Sheppard *et al.*, 2017). However, the spectra of the dimming lights and the length of the dimming cycle has not been optimised especially for the mating success ratios of *An. funestus*. In consequence, the type of light used and the general light environment in *Anopheles* mosquito rearing facilities are important parameters that should be researched and optimised for effective establishment and maintenance of malaria vectors.

1.7. Problem Statement

Anopheles funestus remain the major vector responsible for active and malaria transmission in most parts of Africa (Coetzee and Koekemoer, 2013; Msugupakulya *et al.*, 2023; Odero *et al.*, 2023). This malaria vector has been reported to express high levels of insecticide resistance (Coetzee and Koekemoer, 2013; Odero *et al.*, 2023). The development of resistance has called for new interventions such as SIT and use of gene drive technology (Munhenga *et al.*, 2016; Kaiser *et al.*, 2021; Mudziwapasi *et al.*, 2021; Wang *et al.*, 2022). These technologies require optimised conditions to allow mass production of the target species. However, development and optimisation of interventions to control *An. funestus* is complicated without sustained strains of the species from different geographical regions to study. Low mating success in artificial insectary conditions have been reported as one of the reasons *An. funestus* strains have been difficult to establish and maintain in insectaries (Service and Oguamah, 1958; Gillies and De Meillon, 1968; Coetzee and Koekemoer, 2013; Ngowo *et al.*, 2021; Felamboahangy *et al.*, 2022; Odero *et al.*, 2023). Even with the two available colonies of *An. funestus* it takes more than ten mating days to achieve optimum insemination ratios (Zengenene *et al.*, 2021), compared to *An. gambiae* and *An. arabiensis* that takes around five days (Munhenga *et al.*, 2016). This study evaluated parameters around adult *An. funestus* rearing conditions that might impact the mating of this vector in captivity. The parameters that were evaluated are light

conditions in different rearing rooms, cage size and shape, vector density per cage, and artificial manipulation of the rearing cage by covering cages with a black opaque cloth material and by inclusion of a light contrasting visual marker inside the rearing cage.

1.8. Main Objective

To optimise the adult housing and ideal photophase to scotophase insectary conditions for *Anopheles funestus* to enhance mating success.

1.8.1. Specific objectives

i. Evaluate the impact of different insectary lighting regimes: spectra, intensity, and timing on mating success ratios of insectary reared *An. funestus.*

ii. Determine the effect of adult mosquito-rearing cage coverings and visual swarm markers on the mating success ratios of insectary-reared *An. funestus.*

iii. Assess the impact of available adult mosquito rearing cage surface area and vector density on the mating success ratios of insectary-reared *An. funestus* mosquitoes.

iv. Evaluate the impact of dimming duration and globe type on the mating success rate of *An. funestus.*

CHAPTER TWO

The impact of different insectary lighting regimes: spectra, intensity, and timing on mating success ratios of insectary reared *An. funestus*

2.1. Introduction

The discovery of the link between the malaria parasite and mosquitoes led to increased interest in establishing standard facilities for housing and experimenting with *Anopheles* mosquitoes (Ross, 1910; Maramorosch and Mahmood, 2014). Housing conditions that ensure improved physiological fitness in semi-field and laboratory settings have been clearly demonstrated for several *Anopheles* species (Coluzzi, 1964; Marchand, 1985; Verhoek and Takken, 1994; Hunt *et al.*, 2005; Maramorosch and Mahmood, 2014; Vatandoost, 2021). Studies on the life history have revealed that the reproductive fitness of *Anopheles* is determined by physiological fitness, but mating activities follows the circadian rhythms and are stimulated by specific changes in the light environment (Coluzzi, 1964; Jones *et al.*, 1967; Charlwood and Jones, 1979; Maramorosch and Mahmood, 2014). The understanding of the important parameters that affect *Anopheles* life history has necessitated artificial simulation of the necessary conditions for improving the physiological and reproductive fitness of *Anopheles* (Service and Oguamah, 1958; Coluzzi, 1964; Jones *et al.*, 1967; Ng'Habi *et al.*, 2010; Maramorosch and Mahmood, 2014). However, the existing artificial light environment in rearing facilities have not enhance the reproductive fitness of *An. funestus* in the same way as they have for *An. gambiae* and *An. arabiensis*(Coetzee and Koekemoer, 2013; Ngowo *et al.*, 2021; Felamboahangy *et al.*, 2022; Odero *et al.*, 2023).

Together with factors such as age, sex ratios and density, mating success ratios in *Anopheles* mosquitoes is significantly influenced by the properties of the available light (Coluzzi, 1964; Charlwood and Jones, 1979; Maramorosch and Mahmood, 2014). Insects, including mosquitoes, have been reported to express unique responses to light types including specific aspects of light such as spectra and intensity (Coluzzi, 1964; Maramorosch and Mahmood, 2014; Wakefield *et al.*, 2016; Sheppard *et al.*, 2017; Deichmann *et al.*, 2021; Fyie *et al.*, 2021; Liu *et al.*, 2022; Taniyama and Hori, 2022). However, the light environment remains uncharacterised in *Anopheles* insectariums and different light types (including compact fluorescent, light-emitting diodes, incandescent and halogen) are used across facilities and often mixed light types are used in the same room. Even when similar lights are used across insectaries, the light environment may be determined by the available space, wall paint,

positioning of light and presence of objects (Jobling, 1935; Maramorosch and Mahmood, 2014). The fact that the light environment in some insectaries is simulated by artificial lights and the type of lights used are not standardised, it is important to deduce how light environments may vary across insectaries and how they affect the reproductive fitness of *Anopheles*. This chapter focused on comparing the light environment of four insectary rooms and how it influences the mating success of *An. funestus* which has been notoriously difficult to colonise.

2.2. Materials and methods

2.2.1 Study site

Experiments for this chapter were conducted in Room 4B of the Botha de Meillon insectary (BDMI) and Hugh Paterson insectary located at the National Institute for Communicable Diseases (NICD), and in the Maureen Coetzee insectary at the Wits Research Institute for Malaria (WRIM), University of the Witwatersrand, Johannesburg. These insectaries are fully operated on artificially controlled conditions of photoperiods, humidity, temperature, and air circulation.

The BDMI insectary is partitioned into eight main mosquito rearing/experimental rooms (room 1 – 8) each divided into two isolated rooms marked “A or B”. Each individual room has four rows of wall shelves, bench lights (to support working in the insectary) and three ceiling lights to create the required photoperiods. Under normal operational conditions, the insectary climate is automated at 26 ± 2 °C and relative humidity is 70 ± 10%. The BDMI insectary lighting is controlled by two automated light systems. One is set at 12:12 hours light: dark (or day: night) phase cycle using eight light emitting diode (LED) tube lights (F58W/GRO-T8, Germany) installed underneath the middle shelves to simulate day and night cycles and enable human working in the insectary. The second is a set of three 42-watt halogen bulbs (Pick n Pay brand, South Africa) installed on the ceiling which are set to simulate the onset of sunrise and sunset (dimming) and remain lit during the day/light phase. The ceiling lights are set to illuminate gradually 30 minutes before the shelf lights switch on to start the day/light phase and again to dim gradually for 30 minutes from the start of the dark/night phase. For the onset of the dark phase the ceiling lights gradually dim by a decrease of voltage over 30 minutes until the lights go off. Adult cages for the reared strains are placed on the top shelf to be away from the brighter and warm working bench lights but directly exposed to the light emanating from the ceiling lights. Similarly, all experimental cages for this study were held on the top shelves, meaning that they were exposed to the dimming ceiling lights.

The Hugh Paterson insectary has three working rooms and only one room was used for experiments for this study. Under normal operational conditions the temperature in the room is automated at 26±1°C and relative humidity at 70±5%. The room is illuminated by four fluorescent tube lights (F58W/GRO, Germany) installed on the ceiling. For this study, the lights were set to illuminate continuously for the room to be maintained at full photophase for the whole duration of the experiments. This was done to evaluate the impact dark phase deprivation on the mating success of *An. funestus* compared to systems with both light and dark phases. The room has steel shelves stationed around the room and experimental cages were held on the top shelf.

The Maureen Coetzee insectary at the Wits Research Institute for Malaria referred to as the WRIM insectary in this study is set to operate at temperature of 26±1°C and relative humidity of 90±5%. The light environment is controlled by 42-Watt halogen lights (Pick n Pay brand, South Africa) installed on the ceiling and ten 45-Watt mixed-brand bench bar lights. The insectary is set to operate at 12:12 hours light: dark (or day: night) phase cycle with a 45-minute diming transition before and after each phase. The room is fitted with rows of wall shelves and adult mosquito cages for this study were held on the top shelves.

2.2.2 *Anopheles funestus* used in the experiment

Two colonised strains of *An. funestus* were used. Both strains were established from field collected mosquitoes from two geographical locations; one from Mozambique (denoted as FUMOZ) and the other from Angola (denoted as FANG) (Hunt *et al.*, 2005; Zengenene *et al.*, 2021). Both strains are MW-type, however, the FANG strain is clade I while FUMOZ is a mixture of clade I and II (Choi *et al.*, 2013; Coetzee and Koekemoer, 2013). Adult FUMOZ strain were obtained from a colony that was routinely reared in square BugDorm cages (30 x 30 x 30 cm, height x length x width) (MegaView Science Co. Ltd., Taichung, Taiwan). Four sides of the BugDorm cages are made of clear fine mesh while the base of the cage is made of non-porous opaque nylon material and the front of the cage is made of transparent nylon material with a fine meshed white net sleeve for transfers into and out of the cage. Adult FANG strain colony were reared in 20 litre non-transparent white bucket cages with a clear fine mesh top cover and a round side hole covered by a clear fine-meshed sleeve for transferring material into and out of the cage. Both FUMOZ and FANG adult mosquitoes were reared in densities of approximately 1,500 to 2,000 mosquitoes per cage and provided with a 10% sugar solution through a soaked cotton pad that was changed twice a week.

After emerging, adult *An. funestus* were allowed to mate for at least nine days then blood fed twice per week on anaesthetised guinea pigs. Egg plates, which were black plastic bowls (120 mm, 50 mm; diameter, height) containing about 100 ml of distilled water, were used for collecting mosquito eggs after at least two blood feeds and every two nights following blood feeding. Mosquito eggs were washed into a rectangular plastic larval rearing tub (8 cm x 21 cm x 15 cm, height x length x width) filled with distilled water up to 2 cm depth and were loosely covered with a plastic lid to prevent contamination and water loss through evaporation. On hatching, mosquito larvae were fed twice per day on powdered food containing 3:1 ratio of crushed dog biscuits (Beeno®, South Africa, http://www.beeno.co.za) and brewer's yeast (Vital®, Vital Health Foods, South Africa), respectively. Third to fourth instar larvae were transferred into large non-transparent white plastic basins (15 cm x 35 cm x 13 cm, height x length x width) that were covered by a clear fine net for pupation and adult emergence.

2.2.3 Light and climate data

The light regimes were measured during full photophase and at the start of the scotophase in the BDMI and WRIM insectaries and only the full light phase was recorded in the Hugh Paterson insectary. Light intensity and light spectra of each phase were recorded by using the environmental light field (ELF) method which utilises a digital camera and an open-source software for image analysis (Nilsson and Smolka, 2021; Nilsson *et al.*, 2022). A digital camera (Nikon D850) with an 180° lens (Sigma 8 mm F3.5, EX DG Fisheye) was used. The camera was mounted on a tripod and oriented horizontally to take circular images covering all elevation angles in the insectary room at a 90°/-90° vertical line. Each recorded image was taken by rotating the stand for the camera to capture the four cardinal directions of the room. To account for sensor-specific dark-noise, one series of images was taken with the camera shutter closed for calibration. Relative humidity, temperature, and light intensity were recorded by using HOBO Onset MX1104 (USA) data loggers at each insectary room where the experiments were conducted.

2.2.4 Experiment setting

Anopheles funestus FANG and FUMOZ strain pupae were obtained from the colony held at BDMI. For the experiments, a total of 100 *An. funestus* FANG and FUMOZ pupae at 1:1 male: female ratio were sorted (Fig 2.1) using an Olympus dissecting microscope (OLYMPUS SZX7, model SZ2-ILST, Tokyo, Japan), and placed in a plastic bowl (12 cm, 5 cm; diameter, height). These batches of 100 pupae were then each put in separate standard small cubic BugDorm cages 15 x 15 x 15 cm; L x W x and placed in the different insectaries (section 2.2.1 in this dissertation). The pupae bowls were removed from the experiment cage on the third day. The

emerged mosquitoes were provided 10% sugar solution and allowed to mate for 10 days with a change of sugar solution every two days. Four distinct biological replicates of the experiment were conducted using different mosquito generational cohorts and only one cage per biological replicate was set for each treatment. At the end of the experiment (day 10) all surviving females were removed from the cages and spermathecal dissection was conducted (See section 2.2.5).

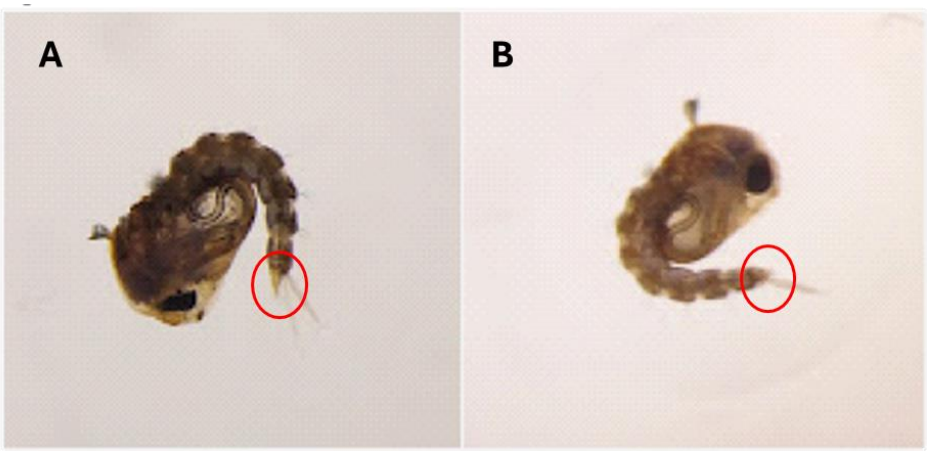

Figure 2.1. *Anopheles* pupae as seen under an Olympus dissecting microscope, **A)** Male pupae with a sharp claw at the end of the last abdominal segment, **B)** Female pupae with no sharp claw at the tip of the last abdominal segment.

2.2.5 Spermathecal dissection

Spermatheca dissections were done as described in the MR4 manual (2015), with some modifications. Mosquitoes were first knocked down in a paper cup covered with a net and placed in a -20 °C freezer for 10 minutes. Then mosquitoes were mounted on a clean microscope slide with a drop of distilled water and the last abdominal segment was removed using a pair of fine forceps under an Olympus stereo microscope (OLYMPUS SZX7, model SZ2-ILST, Tokyo, Japan) to isolate the spermatheca capsule. The mosquito corpses were discarded and the spermatheca capsule was observed under an Olympus light microscope (OLYMPUS BX50, model BX50F4, Tokyo, Japan) with 40X magnification on the Stream Essentials 1.9.4 Olympus soft image solutions. Spermatheca capsules from females with grey content inside were recorded as “mated” while clear spermatheca were scored as “not mated” (Fig. 2.2).



Figure 2. 2 Appearance of *Anopheles* spermatheca under light microscope after dissection, **A)** An inseminated spermatheca with the grey matter (sperm) inside, **B)** Plain un-inseminated spermatheca.

2.2.6 Data analysis

Data were analysed in R statistical software (version 4.2.2), where logistic regression models using the *lme4* package (Bates *et al.*, 2015), were used to estimate the mean proportional mating success for *An. funestus* and compare the odds mating success between FANG and FUNOZ strains within and between insectaries. Mating success ratios were modelled as a binomial variate with insectaries used as fixed effects while replicates as random effects to evaluate if there was any difference in mating success between rearing facilities. The final model was model appeared as 'Mating success ~ insectary + (1|EXP_Replicate)-1, family = binomial(link = "logit"), data =)'. The interaction effect between insectary and strains was only used to differentiate the difference in mating success between the two strains across insectaries. Temperature, relative humidity, and lux data were analysed as mean per hour per day across the experiment period and plotted across a 24-hour scale. Pairwise multiple comparison was performed using Tukey's *post-hoc* test to determine the mating success ratios, temperature, relative humidity, and light intensity differences between the insectaries. Log likelihood ratio tests (LRT) were used to test the significance of insectaries on the mating success ratios. All figures were produced using *ggplot2* package version 3.4.2 in R statistical software (Team, 2013; Wickham, 2016).

2.3. Results

2.3.1 Mating success ratios across insectaries

Out of the four insectaries where experiments were conducted the Hugh Paterson insectary had insufficient mating success ratios for analysis. There were only three observed inseminations

out of 129 dissected females for the FUMOZ strain while the FANG strain had zero inseminations out of 105 female mosquitoes (Table 2.1). Therefore, only three insectaries (BDMI room 2A and room 4B, and the WRIM insectaries) were involved in the final model. Holding adult *An. funestus* in different mosquito rearing insectaries with defined photoperiod to simulate natural lighting regimes did not have significant influence on the mating success ratios of the FUMOZ strain (χ^2 = 0.54, p = 0.76) and FANG strain (χ^2 = 2.45, p = 0.29). Consequently, the mating success of *An. funestus* FANG and FUMOZ did not differ between insectaries when the Hugh Paterson insectary was removed from the analysis (Table 2.1, Fig. 2.3). The mating success of FANG was significantly higher than that of the FUMOZ in the BDMI room 4B and WRIM insectaries (Table 2.1).

Table 2. 1. The estimate proportions of mating success of *An. funestus*, FUMOZ and FANG strains reared in different mosquito rearing insectaries as estimated by the GLMM.

Strain	Insectary	Sample size	% Insemination [95% CI]	OR [95% CI]	P-Values
FUMOZ	Hugh Paterson*	129	2.3		
	BDMI 2A	157	50.0 [37.0 63.0] [a]	1	
	BDMI 4B	177	43.3 [31.5 56.0]	0.76 [0.37, 1.60]	0.48
	WRIM	186	45.2 [33.3 57.6]	0.82 [0.40, 1.71]	0.60
FANG	Hugh Paterson*	105	0		
	BDMI 2A	135	71.2 [55.2 83.3] [b]	1	
	BDMI 4B	125	73.9 [57.5 85.6]	1.14 [0.44, 2.95]	0.78
	WRIM	102	85.4 [68.5 94.0]	2.36 [0.75, 7.46]	0.14

CI – Confidence interval**, OR –** Odd ratios. *The Hugh Paterson insectary data was omitted from the model because of insufficient mating success ratios. [a] Insectary that was used as intercept when comparing mating success ratios for the FUMOZ strain, [b] Insectary that was used as intercept when comparing mating success ratios for the FANG strain.

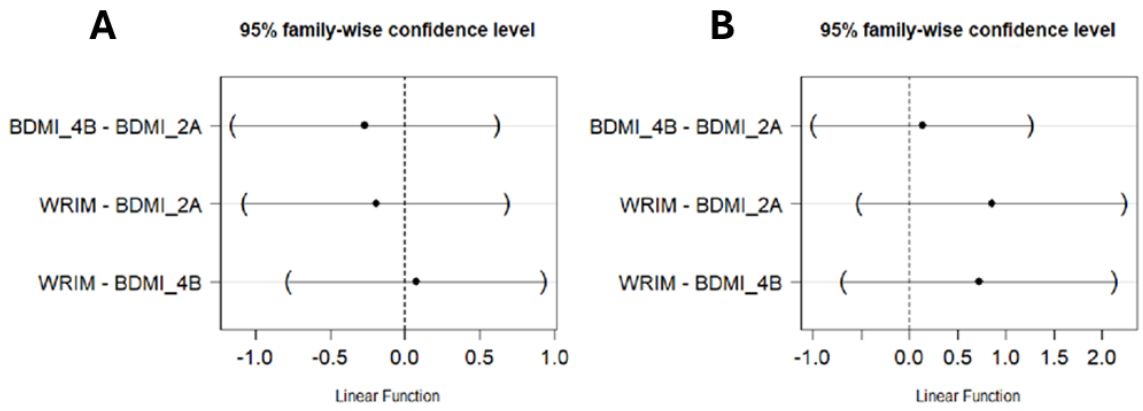

Figure 2.3. Pairwise multiple comparison using Tukey's *post-hoc* test for mating success ratios of *An. funestus* between different mosquito rearing insectaries, A). FANG strain, B). FUMOZ strain. BDMI - Botha de Meillon insectary (Room 2A and room 4B), WRIM - Maureen Coetzee insectary at the Wits Research Institute for Malaria. The dot on the horizontal line represents the mean difference between the two compared groups while the bracketed line represents the measure of precision from the mean (standard error). When the bracketed line crosses the vertical dotted line, it indicates the two groups are not significantly different from each other.

2.3.2 Insectary climate and light conditions

The temperature trends in the BDMI room 2A and BDMI room 4B were similar while the WRIM and Hugh Paterson insectaries had more uniform temperature levels across the 24 hours of the day for the duration of the experiments (Fig. 2.4). The relative humidity was significantly different across the mosquito insectaries used in this study (Fig. 2.5). The BDMI rooms 2A and 4B had the lowest relative humidity while WRIM had the highest relative humidity percentages. The light regime was different across the insectaries. The Hugh Paterson light was fixed at photophase and had the highest light intensity compared to the other insectaries based on the data obtained by the HOBO MX1104 data logger (Fig. 2.6). The light intensity was obtained as a lux unit which is the measure of the total amount of visible light per unit area. The WRIM insectary had the lowest light intensity at full photophase compared to the rest of the insectaries while the BDMI room 2A and 4B had a similar light regime. Photoperiods across the 24 hours of the day were similar in the BDMI rooms 2A and 4B, with similar trends of dimming process at the start of photophase and scotophase respectively. The photoperiods in the WRIM insectary were slightly different at the start of the photophase which had a two stage increase in light intensity until full light while having one stage light decrease during the dimming process at the start of the scotophase.

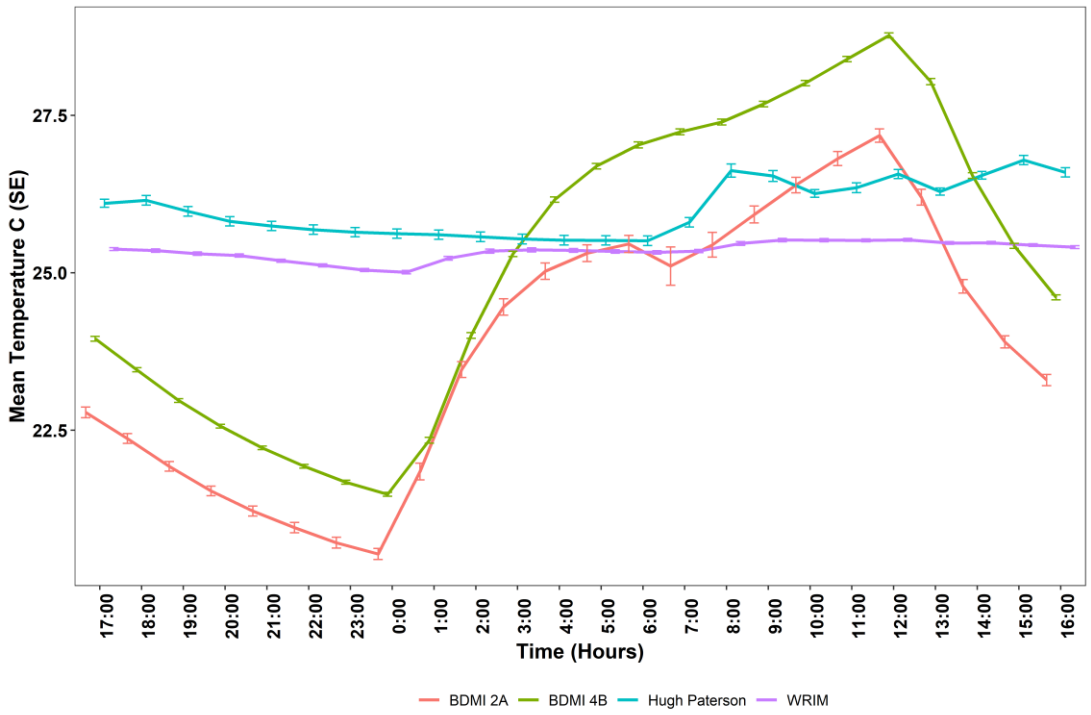

Figure 2.4. The mean hourly temperature in the four experimental rooms during the duration of the insectary comparison studies as recorded by HOBO Onset MX1104 (USA) data loggers.

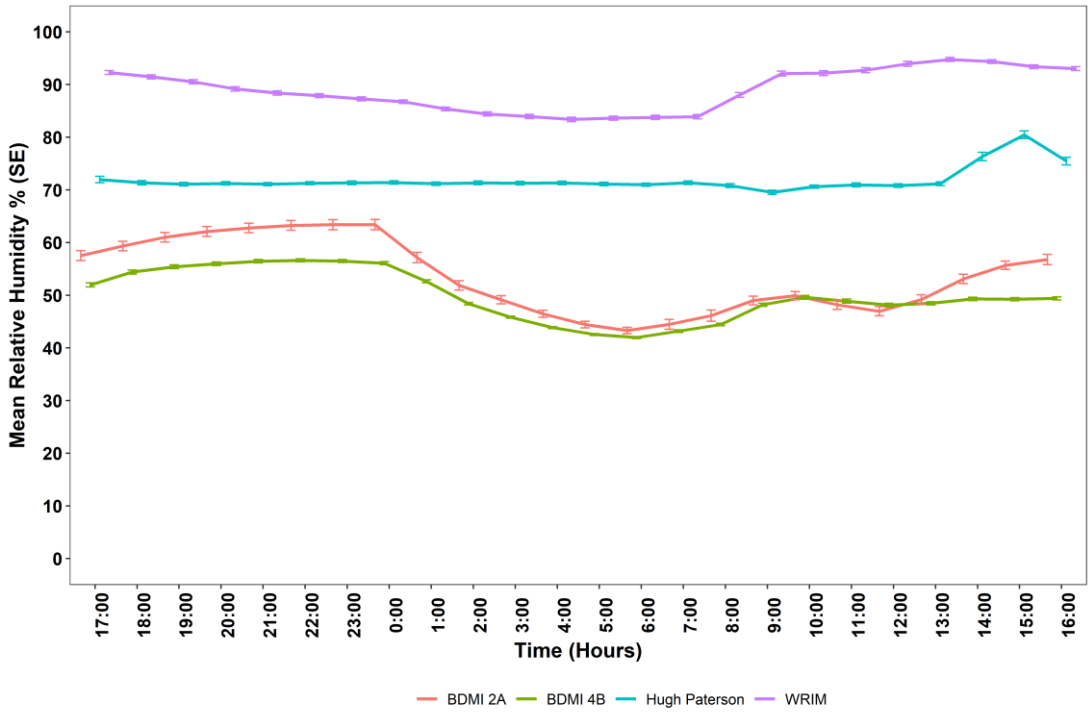

Figure 2.5. The mean hourly relative humidity (%) in the four experimental rooms during the duration of the insectary comparison studies as recorded by HOBO Onset MX1104 (USA) data loggers.

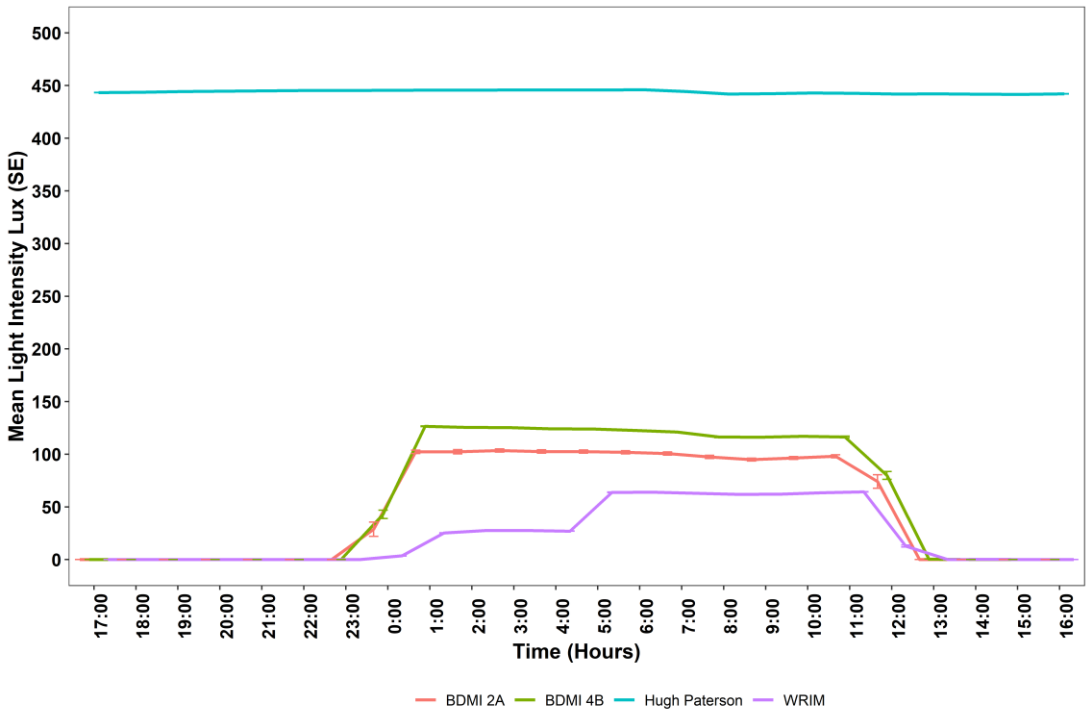

Figure 2.6. The mean hourly light intensity in the four experimental rooms within the different insectaries for the duration of the insectary comparison studies recorded in the Lux unit by HOBO Onset MX1104 (USA) data loggers.

Using the environmental light field (ELF) method, the four insectaries were found to have similar light environments in terms of spectral photon radiance, relative intensity range and relative colour (2.7A-G). For the three insectaries with changing photoperiods there was decrease in spectral photon radiance and slight change in the spectral resolution when the working (bench lights) went off at the start of the dimming process. Light types installed in the four insectaries at the time of this comparisons were recorded; the WRIM insectary was installed with incandescent ceiling lights (45W, Pick n Pay brand, South Africa), and a mix of two compact fluorescent lamps bars (CFL) and eight light-emitting diodes (LED) bars (42W, Pick n Pay brand, South Africa) on the benches. The Hugh Paterson insectary was installed with four ceiling LED bars (F58W/GRO-T8, Germany) with no bench lights. The BDMI room 2A and 4B were both installed with three ceiling halogen bulbs (42W, Pick n Pay brand, South Africa) and four LED bars (F58W/GRO, Germany) on the benches. All lights in the insectary were sourced from regular electronic shops. The light environment in the insectaries differed in intensity and contrast between the full photophase and at the start of the dimming before scotophase (Fig. 2.7A-G). However, the spectra composition of the full photophase and the start of the dimming process were similar based on the ELF method.

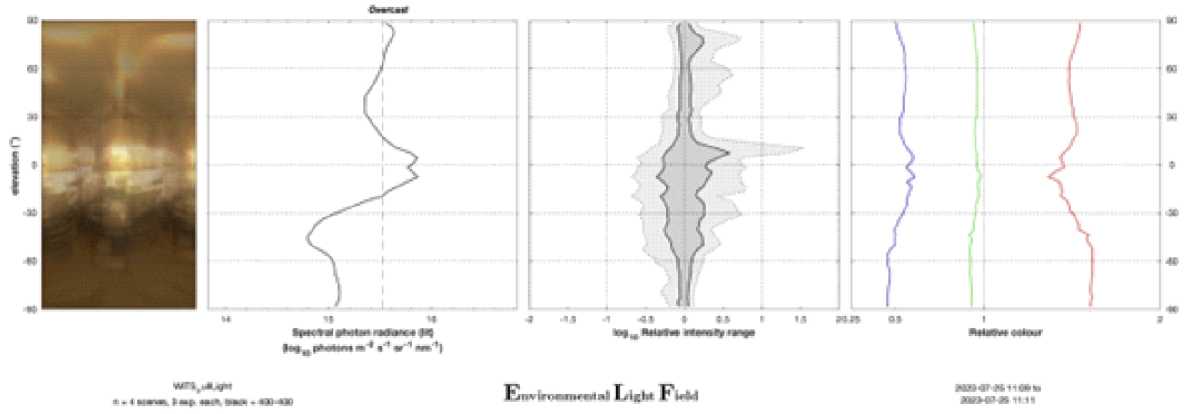

Figure 2.7A. The light environment as recorded by using the environmental light field method at full photophase in the Maureen Coetzee insectary at the Wits Research Institute for Malaria, University of the Witwatersrand.

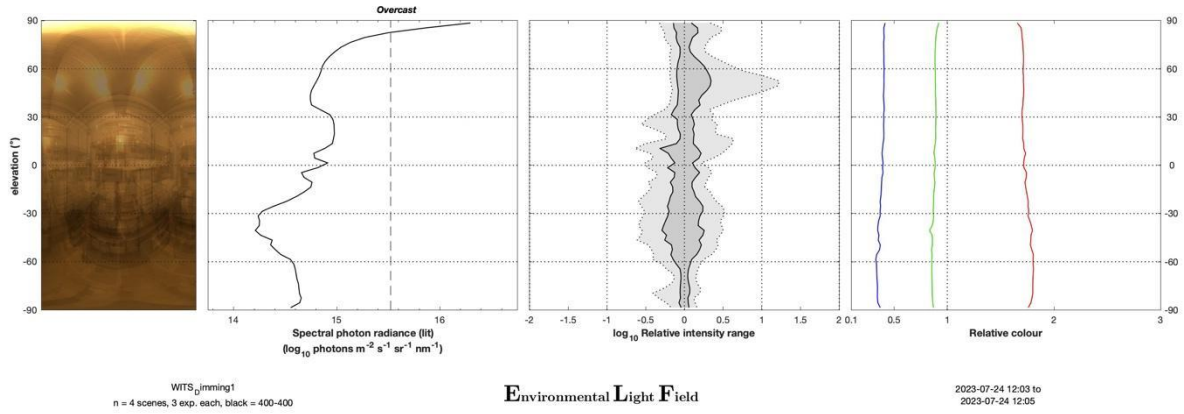

Figure 2.7B. The light environment as recorded by using the environmental light field method at starting of scotophase in the Maureen Coetzee insectary at the Wits Research Institute for Malaria, University of the Witwatersrand.

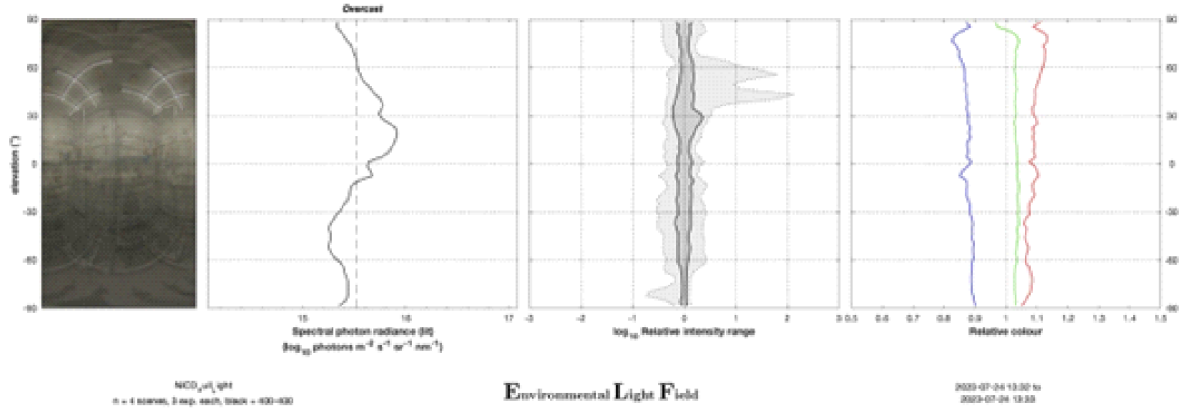

Figure 2.7C. The light environment as recorded by using the environmental light field method at full photophase in the Hugh Paterson insectary at NICD.

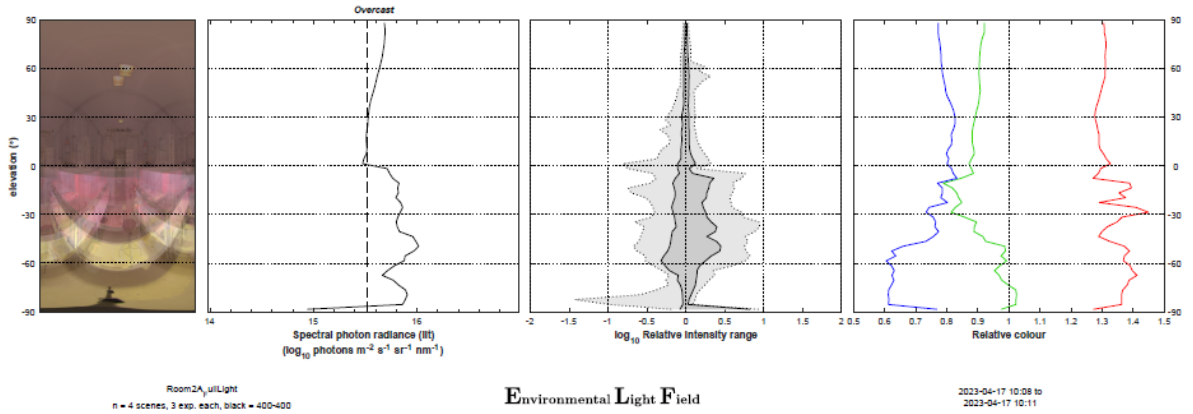

Figure 2.7D. The light environment as recorded by using the environmental light field method at full photophase in the Botha de Meillon insectary (BDMI) Room 2A at National Institute for Communicable Diseases (NICD).

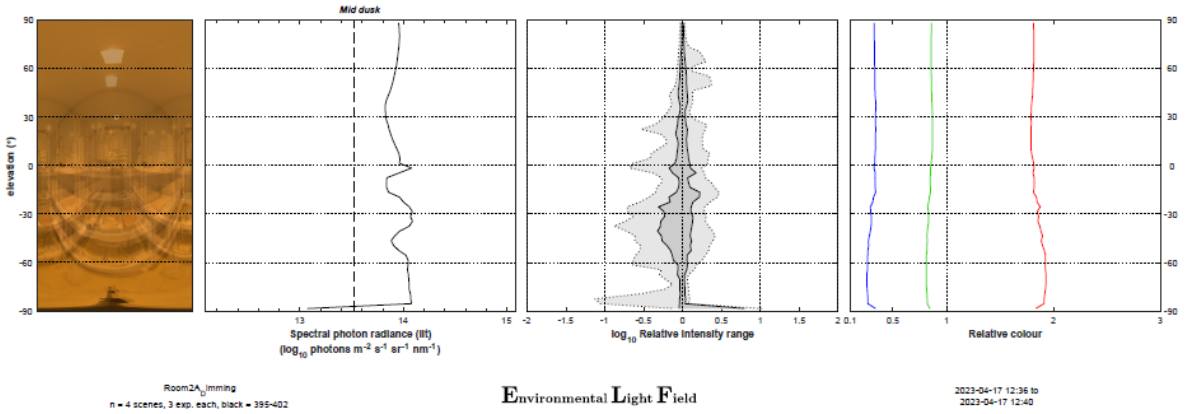

Figure 2.7E. The light environment as recorded by using the environmental light field method at the start of scotophase in the BDMI Room 2A at NICD.

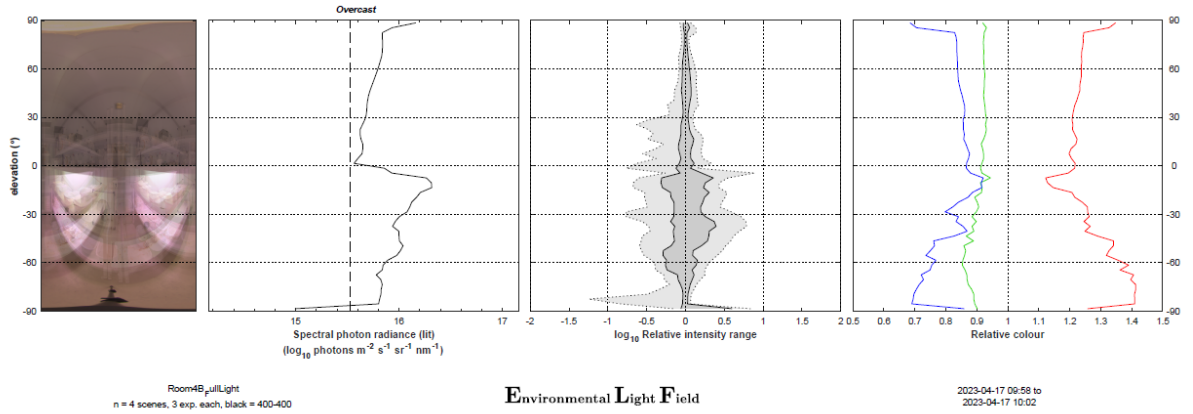

Figure 2.7F. The light environment as recorded by using the environmental light field method at full photophase in the BDMI Room 4B at NICD.

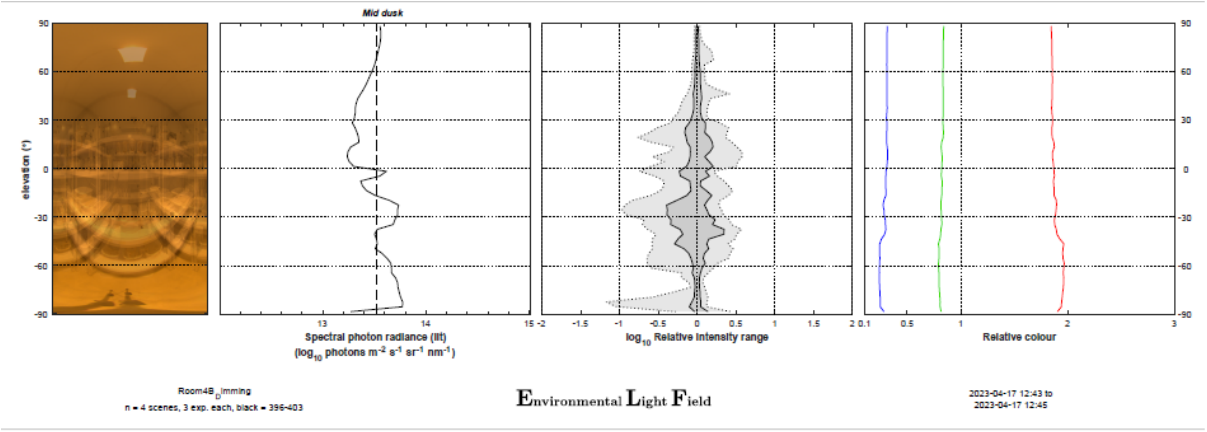

Figure 2.7G. The light environment as recorded by using the environmental light field method at the start of scotophase in the BDMI Room 4B at NICD.

2.4. Discussion

This chapter evaluated the effect of holding adult *An. funestus* in different insectaries taking in account the role of light intensity and light regime on their mating success. The mating success ratios of the two strains of *An. funestus* were similar across the different insectaries with photoperiods set to mimic the natural environment light regimes. However, mating was negatively affected in the insectary where light was constantly held at full photophase. The changing light environments (having dark, light, and diming phases) in the three insectaries might have been the main reason the observed mating success ratios. This observation confirms the fact that mating activities in *Anopheles* mosquitoes are modulated by the circadian clock hence changing photoperiods are essential even for colonies that have been housed for several years (Maramorosch and Mahmood, 2014; Manoukis, 2021; Wang *et al.*, 2021). Genomic studies have revealed that specific changes in the light environment induce expression of genes responsible for regulating pheromones that are excreted by the males to initiate swarming (Mozūraitis *et al.*, 2020) and control circadian clock-based behaviours in *Anopheles* mosquitoes (Rund *et al.*, 2011; Rund *et al.*, 2013; Rahman *et al.*, 2021; Wang *et al.*, 2021; Baeshen, 2022). Therefore, leaving the light fully lit in the Hugh Paterson insectary for the duration of the experiment might have disrupted gene expression hence the circadian clock of the mosquitoes and thus inhibiting their ability to induce mating activities. The mating success ratios of the FANG strain were about two times that of the FUMOZ strain. The observed difference in mating success between the two strains may not have been due to the FANG strain adaptation to the insectary conditions over time since both strains were colonised around the same time (Hunt *et al.*, 2005; Zengenene *et al.*, 2021). However, the observed differences in the mating success ratios between the two strains might be due to other underlying genetic attributes related

to the geographical origin and susceptibility status of the two strains. The two strains differ on their susceptibility status where the FUMOZ strain is known to express insecticide resistance genes, a factor that has been related to reduced reproductive fitness (Hunt *et al.*, 2005; Tchouakui *et al.*, 2018).

Even though the spectral photon radiance were similar across the four insectaries as per the ELF method, the light intensity was observed to differ when compared to the lux recorded by the HOBO MX1104 data logger. The main reason for this might be the mechanism by which the two methods work. The ELF method measures the amount of light available per area taking into account the effect of the surrounding environment features while the lux records the photons directly reaching the lux meter sensor from the source of light (Nilsson and Smolka, 2021; Nilsson *et al.*, 2022). Furthermore, the spectral balance shift at full photophase in the BDMI insectary rooms was distributed lower on the vertical scale than in the WRIM insectary which reflects the actual position of the bench lights in the two facilities. The spectral balance in the Hugh Paterson and at the start of the scotophase was evenly distributed on the vertical scale as only the ceiling lights were on at that time. The spectral balance shift at full photophase observed with the ELF method was due to presence of bench light in all insectaries used except the Hugh Paterson insectary. The ELF method takes a 180-degree image at 90/-90 angle from the camera lens which provides the ability to present spectral distribution on a vertical scale (Nilsson and Smolka, 2021; Nilsson *et al.*, 2022).

Even though there was a noticeable difference in the temperature trends and relative humidity across the three insectaries, these variables did not have noticeable influence on the mating success of the two strains. The two variables, temperature and relative humidity might have influenced the longevity of the strains. However, the strains were only held for 10 days and longevity was not evaluated.

2.5. Recommendations

For these experiments four biological replicates in the three insectaries (WRIM, BDMI2A and BDMI4B) and three biological replicates in the Hugh Paterson insectary were conducted. In this chapter only mating success ratios were evaluated. However, light environment and climate (temperature and relative humidity) were different in the four insectaries. Future studies should consider conducting more biological replicates in different insectaries to ascertain the effect of light environment on the mating success of *An. funestus*. To improve the setting of the experiments, the effect of other variables including the room features that may affect the visual environment, and adult mosquito holding cage condition should be evaluated on the mating

success ratios of *An. funestus*. Since the insectaries were observed to have different levels of relative humidity and temperature trends, studies should be conducted to evaluate other fitness parameters such as fecundity and longevity across different mosquito-rearing conditions. Future work should consider conducting similar experiments across insectaries with similar climate conditions (temperature and humidity) while variating light intensity and photoperiods. This will give more information on the effect of fine differences in light regimes on the mating success ratios of *An. funestus.*

CHAPTER THREE

The effect of adult mosquito cage coverings and inclusion of visual markers on the mating success ratios of *An. funestus*

3.1. Introduction

In their natural habitats, *An. funestus* mate in swarms that are known to be induced by specific changes in the environmental light regime (Charlwood *et al.*, 2003; Zawada *et al.*, 2018; Kaindoa *et al.*, 2019). Still, low mating ratios of *An. funestus* have been the major issue even in facilities where natural environmental light regimes have been simulated by including dimming lights to stimulate swarming related activities. Considering swarming activities for *Anopheles* mosquitoes happen during sunset and at a specific location in nature (Charlwood *et al.*, 2003; Diabate *et al.*, 2003; Achinko *et al.*, 2016; Kaindoa *et al.*, 2017; Zawada *et al.*, 2018; Kaindoa *et al.*, 2019), there are a multitude of environmental features that can affect the light environment in the vicinity that initiate *An. funestus* swarming. Presence of features such as mountains, trees, man-made structures, and the natural process of sunset may affect the angle and direction of sunlight beams resulting in suitable conditions for *An. funestus* to swarm (Fig. 3.1). Surface or ground markers, such as contrasts in soil colour and the presence or absence of objects have been identified underneath *An. funestus* swarms in Mozambique, Zambia and Tanzania (Charlwood *et al.*, 2003; Charlwood, 2011; Zawada *et al.*, 2018; Kaindoa *et al.*, 2019). These ground markers and other feature in the vicinity may have important modifying effects on the sunset light beams that might induce swarming in nature.

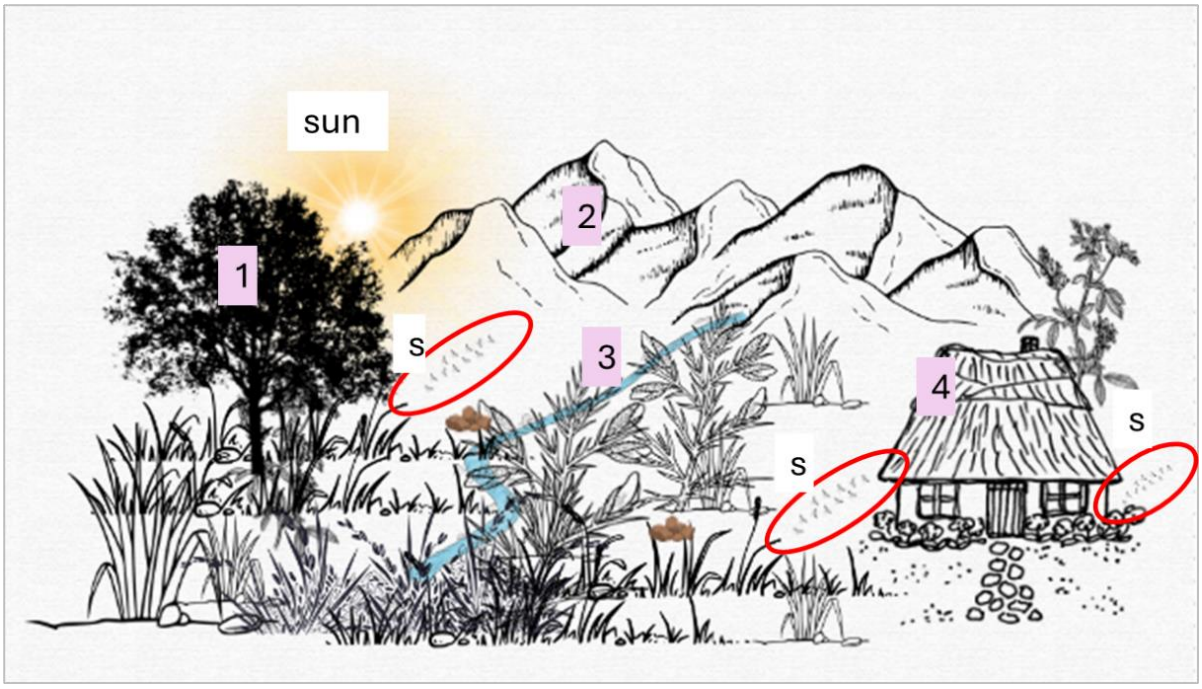

Figure 3.1. Diagrammatic illustration of different features such as **1)** trees, **2)** mountains, **3)** rivers, and **4)** built structures such as houses that may impact the properties of sunset light and hence create areas with conducive light environment for *Anopheles* mosquito swarms (red circles denoted 's'). The diagram is a stereotypical illustration of natural scenarios based on various narratives in the literature created by considering environmental features that may contrast or form horizons against rays of light at sunset.

Swarm induction and improved physiological fitness have been achieved with simulated artificial horizons in laboratory cages in *An. gambiae* and *An. arabiensis* (Marchand, 1985; Mashatola *et al.*, 2017), and light contrasting markers, dimming lights and other natural swarm markers in large semi-field experiment cages/chambers (Paton *et al.*, 2013; Facchinelli *et al.*, 2015; Achinko *et al.*, 2016; Niang *et al.*, 2019). Cage covering with black cloth may have a noticeable effect on the light environment inside rearing cages and has so far demonstrated to improve longevity and mating of some reared *Anopheles* species namely *An. arabiensis* (Mashatola *et al.*, 2017). Evaluation of lighting environment on the life history of adult *An. funestus* has been limited by the lack of sustainable strains of this important vector species that can be maintained and experimented upon in captivity. Establishing conditions that will improve the mating success of *An. funestus* in rearing cages might prove a major tool in enhancing colonisation effectiveness. In this chapter, the impact of artificial light horizons and light contrasting visual markers were evaluated in terms of their effects on the mating success of two *An. funestus* strains from different southern African geographical locations.

3.2. Materials and Methods

3.2.1 Experimental site

Two sets of experiments for this chapter were conducted simultaneously in the BDMI room 4B where the positioning of cages was as described in chapter two, section 2.2.1 in this dissertation (Fig. 3.1). A total of six biological replicates were done for each set of experiments (artificial horizons and visual markers).

3.2.2 The impact of artificial light horizon on mating success of *An. funestus*

The effect of horizon lighting was assessed by comparing the mating success of *An. funestus* females placed into five BugDorm cages (15 x 15 x 15 cm, height x length x width) that were covered with a black opaque cloth to create different planes of horizons: one cage was fully covered, two cages were half-covered with a horizontal split to create top and bottom horizons, one cage was half-covered along vertical plane to create vertical horizon, and one cage was completely uncovered to serve as a standard control (Fig. 3.2). Equal numbers of male and female *An. funestus* pupa (FANG or FUMOZ) at 1:1 ratio (N = 100) were selected, put a single cage for each of the prepared experiment cages, and sustained while allowed to mate over an extended period of 10 days as described in chapter two (section 2.2.4). The experimental cages were maintained and dissections were carried out exactly as previous described in this dissertation chapter two section 2.2.4 and 2.2.5 respectively. A total of six distinct biological replicates of the experiment were conducted using different mosquito generational cohorts and only one cage per biological replicate was set for each treatment.

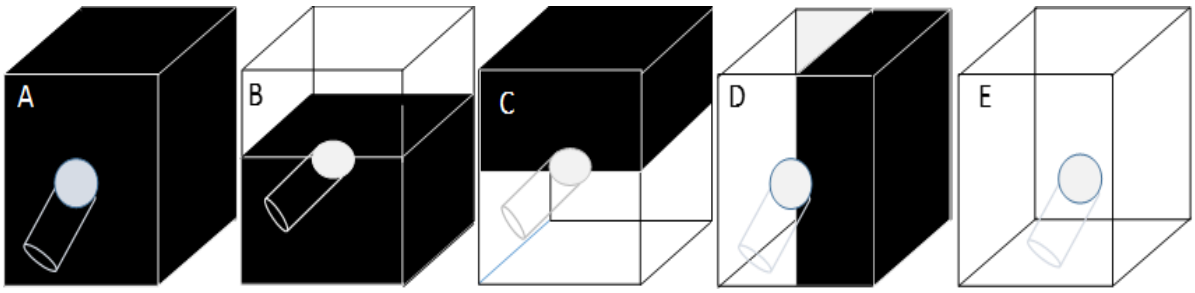

Figure 3.2. Illustration of adult mosquito rearing cage black cloth coverings: **A)** fully covered, **B)** half covered horizontal top horizon, **C)** half covered horizontal bottom horizon, **D)** half right-side covered vertical horizon, and **E)** not covered (control cage).

3.2.3 The impact of swarm markers on mating success of *An. funestus*

A set of four small BugDorm cages (15 cm, 15 cm, 15 cm; width, length, height) were used for this experiment. A black round paper card (7 cm diameter) was fixed on three of the cages as a light contrast visual cue for mosquito swarming within the cages. The black card was fixed at

the centre of the base, left side, and top of the three separate cages respectively, and the fourth cage was set up without a round black paper card to serve as the control (Fig. 3.3). An equal number of male and female *An. funestus* pupa (FANG or FUMOZ) at 1:1 ratio (N = 100) were selected and put into a single cage for each of the prepared adult rearing cages and the experiment was continued as previous described in this dissertation, section 2.2.4 and 2.2.5 respectively. A total of six distinct biological replicates of the experiment were conducted using different mosquito generational cohorts and only one cage per biological replicate was set for each treatment.

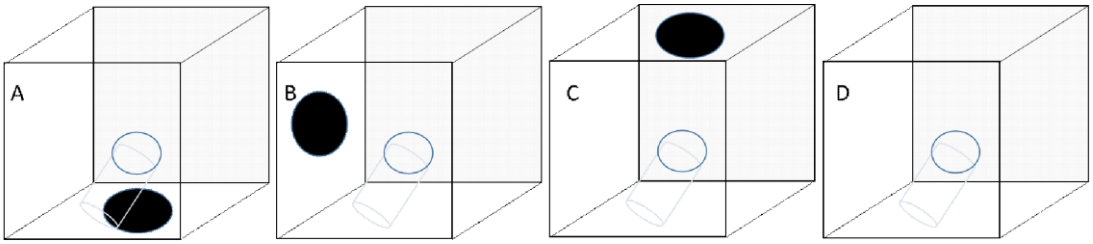

Figure 3.3. Illustration of adult mosquito rearing cages with visual swarm markers: A) swarm marker fixed on the base of the cage, B) swarm marker fixed on one side of the cage, C) swarm marker fixed on the top of the cage, and D) no swarm marker (control cage).

3.2.4 Data analysis

The insemination results were expressed as the proportion of mated mosquitoes as the function of cage modification which were cage covering and inclusion of visual markers. Mean insemination values were estimated per cage modification for both *An. funestus* strains. The mean insemination values for both FUMOZ and FANG strains were estimated using a generalised linear mixed model in R studio (version 4.2.2) with the *lme4* package (version 1.1-32) (Bates *et al.*, 2015). Mating successes were modelled as binomial variate with cage modification used as a fixed categorical independent variable, experimental replicate as the random effect, and the standard cage was set as a reference group. The final model was constructed as Mating success ~ Cage specifications + (1|EXP_Replicate)-1, family = binomial(link = "logit"), data =). Pairwise multiple comparison was performed using Tukey's *post-hoc* test to determine the individual statistically significant differences between the cage modifications. Log likelihood ratio tests (LRT) were used to test the significance of each variable of interest in all models. Data analysis and figure were plotted in R statistical software as indicated previous in section 2.2.6.

3.3 Results

3.3.1 The effect of artificial horizons on mating success of *An. funestus*

Covering adult *An. funestus* rearing cages with a black opaque cloth to create artificial horizons significantly influenced the mating status of FUMOZ ($\chi^2 = 27.6$, $p < 0.001$), and FANG ($\chi^2 = 27.6$, $p < 0.001$) (Table 3.1). The two *An. funestus* strains expressed unique responses to the same cage modification. When compared to the uncovered (control) cage, the FUMOZ females had significantly higher mating success in the top covered cage (48.3%, n = 230). The side covered cage had the highest proportion of mated females for the FANG strain, but significantly higher mating success was observed in the side covered (79.2%, n = 278) and top covered (72.6%, n = 272) cages when compared to the control group (uncovered cage). The fully covered cages recorded the lowest mating success ratio 25.6% (n = 229) and 61.0% (n = 279) for FUMOZ and FANG at $p < 0.05$ and CI = 95% respectively (Table 3.1). Generally, the *An. funestus* FANG strain had higher mating success ratios than the FUMOZ strain across all categories of artificial horizon experiments (Table 3.1).

Following pairwise multiple comparison using Tukey's *post-hoc* test on the FUMOZ experiments, the top covered cage showed significantly higher mating success ratios compared to the uncovered cage (control) ($z = 3.70$, $p = 0.002$), fully covered cage ($z = 4.57$, $p < 0.001$), and bottom covered cage ($z = 3.63$, $p = 0.003$), (Fig. 3.4). However, following pairwise multiple comparison using Tukey's *post-hoc* test across the different artificial horizons, the mating success ratios for FANG strain were significantly higher in the side covered cage compared to the uncovered cage (control) ($z = 2.84$, $p = 0.04$), full covered cage ($z = 4.94$, $p < 0.001$), and side covered cage ($z = 3.63$, $p = 0.003$), (Fig. 3.4). Also, the top covered cage had significantly higher mating success ratios compared to the fully covered cage ($z = 2.90$, $p = 0.03$) but was not significantly different compared to the side covered cage ($z = -1.730$, $p = 0.41$), (Fig. 3.4).

Table 3. 1. The GLMM estimate proportions of mating success of *An. funestus*, FUMOZ and FANG housed in adult rearing cages covered with black opaque cloth to create different artificial horizon planes analysed with the uncovered cage being treated as the control.

Strain	Cage Modification	Sample Size (N)	% Insemination [95% CI]	OR [95% CI]	*P*-Values
FUMOZ	No Cover	252	35.4 [28.7, 42.6]	1	
	Full Cover	229	25.6 [19.7, 32.5]	0.63 [0.42, 0.93]	0.021
	Bottom Cover	240	37.1 [30.2, 44.6]	1.08 [0.74, 1.56]	0.689
	Side Cover	238	31.7 [25.2, 38.9]	0.85 [0.58, 1.24]	0.387
	Top Cover	**230**	**48.3 [40.7, 55.9]**	**1.70 [1.18, 2.46]**	**0.004**
FANG	No Cover	275	64.9 [56.1, 72.8]	1	
	Full Cover	273	61.0 [52.0, 69.3]	0.85 [0.60, 1.20]	0.350
	Bottom Cover	253	67.2 [58.3, 74.9]	1.11 [0.77, 1.59]	0.587
	Side Cover	**278**	**79.2 [71.9, 85.0]**	**2.06 [1.40, 3.02]**	**<0.001**
	Top Cover	**272**	**72.6 [64.6, 79.8]**	**1.45 [1.01, 2.10]**	**0.046**

CI – Confidence interval**, OR –** Odd ratios, bold font indicates statistically significant difference from the control cage.

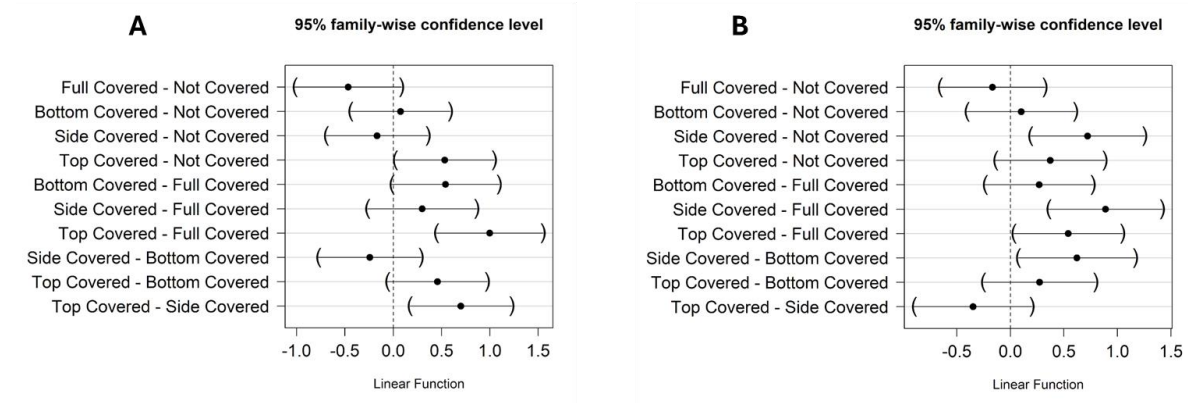

Figure 3.4. Pairwise multiple comparison using Tukey's *post-hoc* test for the mating success ratios of *An. funestus*; **A)** FUMOZ strain, and **B)** FANG strain when compared between different artificial horizons placed on adult mosquito holding cages. The dot on the horizontal line represents the mean difference between the two compared groups while the bracketed line represents the measure of precision from the mean (standard error). When the bracketed line does not cross the vertical dotted line, it indicates the two groups are significantly different from each other.

3.3.2 Impact of light contrast visual markers on mating success of *An. funestus*

Inclusion of artificial light contrast visual markers significantly influenced the mating success ratios of FUMOZ (χ^2 = 18.7, p < 0.001) but did not have significant influence on the mating success ratios of FANG (χ^2 = 1.96, p = 0.58). The adult mosquito holding cage with a light contrast visual marker placed on the base had significantly higher mating success ratios for the FUMOZ strain (49.5%, n = 262) compared to the control cage with no marker. Generally, the mating success ratios were higher for the FANG strain compared to the FUMOZ strain in all categories of light contrast markers (Table 3.2). Following pairwise multiple comparison using Tukey's *post-hoc* test on the FUMOZ experiments, placing the visual marker on the base of the adult rearing cage significantly increased the mating success compared to the cages with the side marker (z = 3.39, p = 0.004), top marker (z = 3.32, p = 0.005) and those without a visual marker (z = 3.69, p = 0.001), (Table 3.2, Fig. 3.5). However, following pairwise multiple comparisons using Tukey's *post-hoc* test, there was no significant difference in mating success ratios between different positioning of light contrast visual markers for the FANG strain (Fig. 3.5).

Table 3. 2. The proportion of mating success ratios of *An. funestus* FUMOZ and FANG strains reared in adult rearing cages manipulated by placing black light contrast visual markers on different sides of the cage as estimated by GLMM.

Strain	Visual Marker	Sample Size	% Insemination [95% CI]	OR [95% CI]	*P*-Values
FUMOZ	No Marker	243	33.1 [26.5, 40.5]	1	
	Side Marker	268	34.8 [28.3, 42.2]	1.08 [0.75, 1.56]	0.682
	Top Marker	256	34.9 [28.3, 42.2]	1.08 [0.74, 1.58]	0.670
	Bottom Marker	**262**	**49.5 [42.2, 56.9]**	**1.98 [1.38, 2.85]**	**<0.001**
FANG	No Marker	265	79.9 [71.3, 86.4]	1	
	Side Marker	247	75.3 [65.8, 82.9]	0.77 [0.51, 1.17]	0.220
	Top Marker	258	76.2 [66.9, 83.6]	0.81 [0.53, 1.23]	0.317
	Bottom Marker	260	78.8 [69.9, 85.6]	0.93 [0.61, 1.43]	0.752

CI – Confidence interval**, OR –** Odd ratios, bold font indicates statistically significant difference from the control cage.

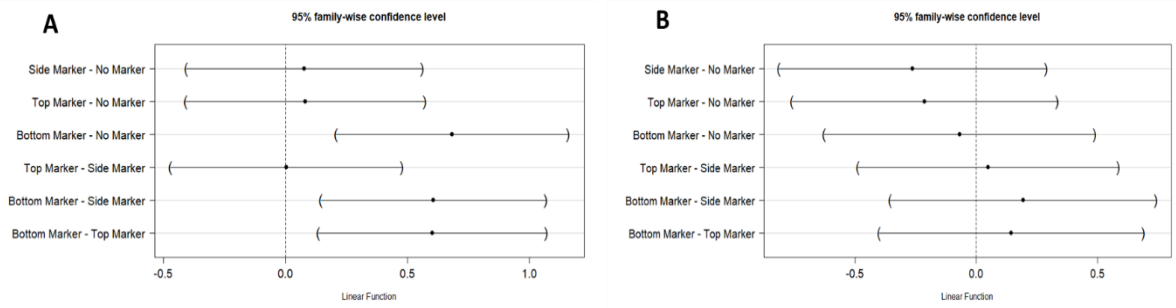

Figure 3.5. Pairwise multiple comparison using Tukey's *post-hoc* test for mating success ratios of *An. funestus* between different artificial light contrast visual markers positioned on the sides of adult mosquito holding cages; **A)** FUMOZ strain, **B)** FANG strain. The dot on the horizontal line represents the mean difference between the two compared groups while the bracketed line represents the measure of precision from the mean (standard error). When the bracketed line does not cross the vertical dotted line, it indicates the two groups are significantly different from each other.

3.4 Discussion

The experiments provided insight into adult *An. funestus* rearing cage modifications that improve their mating in insectariums. These modifications are a vital guide for new attempts to colonise this important malaria vector. The effect of artificial horizons created on adult mosquitoes rearing cages by covering with a black opaque cloth, and placement of visual markers on the sides of the cage was evaluated on the mating success of *An. funestus.* The two strains of *An. funestus* expressed varied preferences to the cage modifications. The top covering of the cage to create a bottom horizon favoured mating of the FUMOZ strain while the side covering favoured the FANG stain mating success ratios (Table 3.1). Likewise, the inclusion of visual markers in adult *An. funestus* cage significantly improved the mating success of the FUMOZ strain but did not have any notable impact on the mating of the FANG strain compared to the standard cages used as control.

The observed improved mating success ratios might suggest increased improved swarming activities induced by the side cover for the FANG strain, and by the top cover and base visual marker for the FUMOZ strain. The observation corelates with field observations where most of the *Anopheles* mosquito swarms are associated with presence of varied ground markers including surfaces such as open ground areas (Harper, 1944; Charlwood *et al.*, 2003; Charlwood, 2011; Kaindoa *et al.*, 2017; Zawada *et al.*, 2018; Kaindoa *et al.*, 2019), burnt ground, and garbage heaps (Kaindoa *et al.*, 2017). It can be assumed that swarming, and hence mating behaviours are influenced by the presence of surfaces that may form contrast to the

fading light at sunset. Furthermore, the presence of environmental features such as vegetation (Charlwood *et al.*, 2003; Zawada *et al.*, 2018), termite mounds (Kaindoa *et al.*, 2017), and man-made structure such as houses (Harper, 1944; Charlwood *et al.*, 2003; Kaindoa *et al.*, 2019) have been associated with *An. funestus* swarming. These features and other standing environmental objects such as hills have the potential to create horizons to the sunset lighting in nature. Therefore, covering the top or vertical half of the mosquito cages in the insectary might have simulated these light horizons inside the cage that induced mating. Considering the natural preference by *Anopheles* to rest in dark places in their natural habitats (Coluzzi, 1964; Clements, 1999; Maramorosch and Mahmood, 2014), the top and side cage covering may have created a dark resting area in relation to the ceiling lights allowing the mosquitoes to reserve additional energy required for increased mating activities. Covering mosquito cages has been shown to improve mating and survival time in *Anopheles* mosquitoes (Mashatola *et al.*, 2017), which might be due to the dark space created by the cover that offer conducive resting hence energy reservations.

The uncovered and bottom-covered cages had low mating success ratios compared to the top covered which might be due to limited favourable resting areas inside the cage. The uncovered cage would have resulted in an even distribution of light inside the cage from the shelf and ceiling lights meaning the mosquitoes inside did not get the naturally required dark resting area. Similarly in the bottom covered cages, even though it might have been possible to create some dark space at the bottom of the cage, the ceiling lights would have still supplied light inside the cage as their light was reaching the cage from the top uncovered side. This might have limited the resting time of the mosquitoes in these cages and hence limiting their energy reserves for mating activities. The lowest mating success in the full covered cage compared to the rest of the cage modifications might have been the effect of interruption to the mosquitoes' circadian clock as the opaque black cloth may have made the inside of the cage constantly dark. Several reported studies have indicated the importance of changing light phases on the mosquito circadian clock dependent behaviour which include mating (Jones *et al.*, 1967; Baik *et al.*, 2020; Manoukis, 2021; Liu *et al.*, 2022). The fully covered cage would have been expected to create dark space that will ensure mosquitoes will have conducive conditions for resting. But the observed low mating success in these cages demonstrate the importance light and changes in the light environment have on the mating activity of *Anopheles*.

The inclusion of artificial visual markers in the form of black coloured discs had a significant effect on the mating success of *An. funestus* FUMOZ strain where a marker put on the base resulted in higher mating success of the strain. A visual marker put on the base might have

induced mating activities by contrasting ceiling lights during the dimming process when the shelf lights were off. The effect of the base marker on the FUMOZ strain correlates with field observation where *Anopheles* swarming has been reported to be induced by the presence of ground markers with the potential of contrasting light from the sunset including the soil colour around the swarm, burned ground, and garbage heaps (Charlwood *et al.*, 2003; Diabate *et al.*, 2003; Charlwood, 2011; Diabaté *et al.*, 2011). Similar to the observed effect of base visual markers on the FUMOZ strain mating success, light contrasting surfaces have been demonstrated to induce swarming activities in *An. gambiae* mosquitoes under semi-field settings (Facchinelli *et al.*, 2015; Niang *et al.*, 2019).

One would expect the two strains to express similar responses to the inclusion of light contrasting surfaces, however, inclusion of artificial visual markers did not have any statistically significant influence on the mating success of the *An. funestus* FANG strain. The observed difference in response to artificial visual markers between the FANG and FUMOZ strains correlates with two previous filed observation (Harper, 1944; Charlwood *et al.*, 2003). Harper (1944), indicated that *An. funestus* swarm were induced artificially by mimicking the features seen on their natural swarming locality in Tanzania, while, Charlwood *et al.* (2003), reported that *An. funestus* swarms in Mozambique usually formed above clear ground and swarms dissolved or did not form on the same location the following day when a light contrasting surface was introduced. The dissociation and avoidance of forming a swarm at the same area observed by Charlwood *et al.* (2003), might have been caused by the disruption of the original cues that the mosquitoes were using as their swarm stimulator and indicates the importance of the light environment on the mating activities of *An. funestus*. The observed differences between the FANG and FUMOZ strains towards artificial horizons and visual markers in the insectary might suggest existence of genetic differences between the two strains.

3.5 Recommendations

From the observed results with the artificial horizon and light contrasting visual markers, *An. funestus* FANG strain had consistently higher mating ratios compared to the FUMOZ strain, and the two strains had varied responses to similar cage conditions. One of the reasons that might have led to the higher mating success ratios in FANG might be the insecticide susceptibility status of the two strains. This study recommends a phenotypic screen by back selection to isolate an insecticide susceptible line of the FUMOZ strain and compare the mating success between the susceptible and resistant lines of the strain. Also, further studies on the manipulation of adult rearing cages by changing the horizon planes and evaluation of the

combined effect of artificial horizon and visual markers. Similarly, further genomic studies should be done on the two strains and strains from other localities to deduce any genotypic differences that might influence the observed behavioural differences.

CHAPTER FOUR

The effect of mosquito rearing cage size and vector density on mating success ratios of reared *An. funestus*

4.1. Introduction

The importance of vectors in disease control has led to semi-field and laboratory rearing of different mosquito species to conduct research and answer operational needs (Coluzzi, 1964; Gillies and De Meillon, 1968; Gerberg, 1970; Charlwood and Jones, 1979; Van Handel, 1986). This has accelerated the study of *Anopheles* bionomics and the establishment of more colonies which has better-informed vector control interventions intending to control the spread of infectious diseases (Coluzzi, 1964; Hunt *et al.*, 2005; Ng'Habi *et al.*, 2010; Maramorosch and Mahmood, 2014; Ng'Habi *et al.*, 2015; Vatandoost, 2021). In the laboratory/insectary rearing of mosquitoes, adult mosquitoes are held and reared in cages/enclosures that are mostly made of fine-meshed netting material and holders of different materials (Coluzzi, 1964; Marchand, 1985; Maramorosch and Mahmood, 2014; Mashatola *et al.*, 2017; Vatandoost, 2021). The common adult mosquito-rearing cages are of different sizes and shapes such as rectangular, square, and cylindrical (Coluzzi, 1964; Marchand, 1985; Maramorosch and Mahmood, 2014; Otienoburu *et al.*, 2016; Mamai *et al.*, 2017; Mashatola *et al.*, 2017; Vatandoost, 2021; Nignan *et al.*, 2022). *Anopheles gambiae* complex has been shown to adapt well in rearing facilities expressing sufficient physiological and reproductive fitness in adult rearing cages (Maramorosch and Mahmood, 2014; Munhenga *et al.*, 2016; Mashatola *et al.*, 2017; Nignan *et al.*, 2022). However, it has proven difficult to attain the same mating success for *An. funestus* reared in the standard cages (Odero *et al.*, 2023).

Adult mosquito rearing cage size has been noted to influence mating success in the laboratory for *An. gambiae* complex where cube cages with at least 30 cm sides are considered the best choice for colony maintenance (Coluzzi, 1964; Marchand, 1985; Maramorosch and Mahmood, 2014; Nignan *et al.*, 2022). Customised cylindrical cages of different sizes have also been used for the rearing of *Anopheles* species (Mashatola *et al.*, 2017). While small cages including small cubic cages with 15 cm sides and small cylindrical cages of two litres are used in laboratory experimentation with *Anopheles* mosquitoes. The density of adult mosquitoes per cage and sex ratios per cage are important determinants of mating fitness reported in *An. gambiae* complex (Coluzzi, 1964; Gerberg, 1970; Maramorosch and Mahmood, 2014; Mamai *et al.*, 2017; Nignan

et al., 2022). To the best of my knowledge, there is no established standard mosquito-rearing cage or vector density per rearing cage for the successful mating of *An. funestus.*

As indicated in Chapter 2, only two strains of *An. funestus* (one from Angola and another from Mozambique) have been fully established and maintained under laboratory conditions (Hunt *et al.*, 2005; Zengenene *et al.*, 2021). The main parameter that has been observed to limit establishment and sustainability of new colonies of *An. funestus* from field collections has been their low mating success ratios in laboratory cages (Ngowo *et al.*, 2021). Therefore, this chapter investigated cages of different shapes and size to evaluate the effect of cage size and vector density per cage on the mating success of *An. funestus*.

4.1. Materials and Methods

All experiments for this chapter were conducted in the BDMI room number 4B (see section 2.2.1 in this dissertation for more details on the room). Two separate sets of experiments were conducted with six biological replicates each to establish the optimal adult mosquito rearing cage size and density of adult mosquitoes per cage to improved mating success in *An. funestus*. *Anopheles funestus*; FANG and FUMOZ strains were used in the experiments (see section 2.2.2 for more detail on the strains). During the experiment, the insectary was operating at 27 (±2) °C, and 70% (±10) RH.

4.2.1 Effect of adult mosquito rearing cage size on the mating success ratios *An. funestus*

A set of adult mosquitoes rearing cages with different volumes (2, 5, 10 and 20 litres) consisting of cylindrical polypropylene buckets with a clear fine-net-sleeved opening and a clear fine-netting top were used to evaluate the effect of cage size on the mating success of the FANG and FUMOZ strains. An equal number of female and male (50 of each, N = 100) pupae of FANG and FUMOZ were randomly selected from rearing bowls and placed into individual cages. Experiment cages maintenance and dissections were done as described in this dissertation, section 2.2.4 and 2.2.5 respectively. A total of six distinct biological replicates of the experiment were conducted using different mosquito generational cohorts and only one cage per biological replicate was set for each treatment.

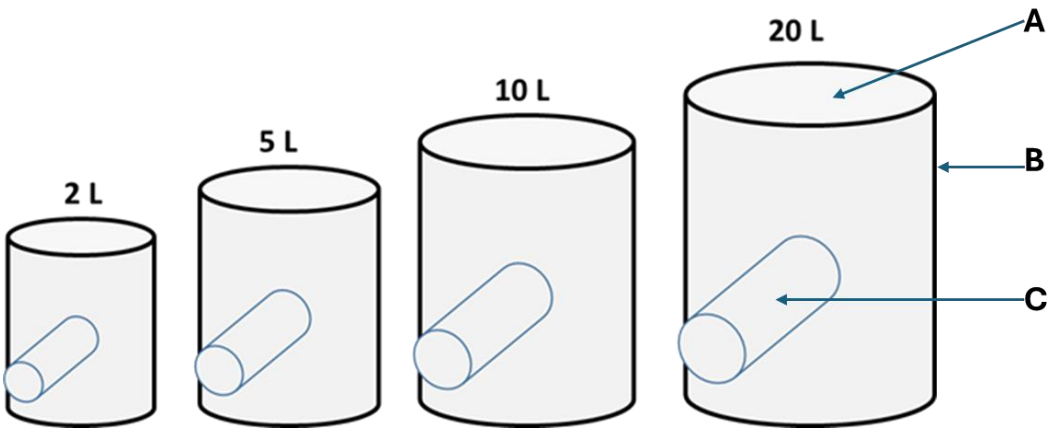

Figure 4.1. A sketch of cylindrical polypropylene cages displaying the different cage size used for the evaluation of the effect of cage size on mating success of *An. funestus*; A) fine-netting cage cover, B) White coloured polypropylene cage wall, and C) Side fine-netting sleeve for transferring material into and out of the cage.

4.2.2 Effect of adult mosquito density inside rearing cages on the mating success ratios of *An. funestus*

Three small cubic cages (15 x 15 x 15 cm; length, width, height (3.4L)) and three small cylindrical polypropylene (2 L) mosquito cages were prepared and an equal number of male and female *An. funestus*; FANG and FUMOZ pupae were randomly selected and added to separate cages at 30:30 (N = 60), 60:60 (N = 120), and 120:120 (N = 240) (female:male) ratios respectively. Experiment cages maintenance and dissections were done as described in this dissertation, section 2.2.4 and 2.2.5 respectively. A total of six distinct biological replicates of the experiment were conducted using different mosquito generational cohorts and only one cage per biological replicate was set for each treatment.

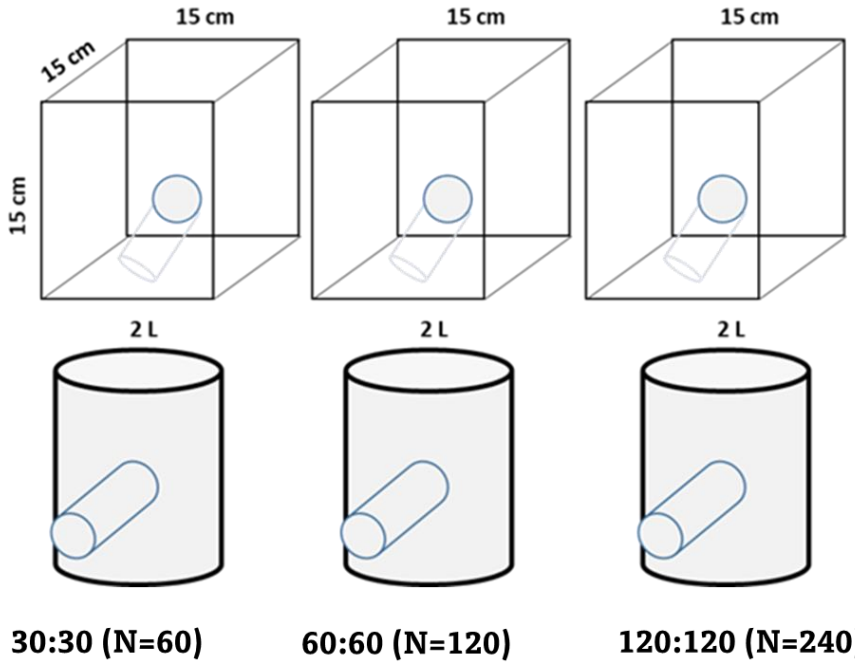

Figure 4.2. Schematic representation of the small cubic and cylindrical cages used to evaluate the effect of mosquito density per cage on the mating success of *An. funestus*; FANG and FUMOZ. The number of mosquitoes used is indicated under each cage set.

4.2.3 Data analysis

All statistical analysis, comparisons and plotting were done in R statistical software as described in section 3.2.4 in this dissertation. Generalized linear mixed model was fitted with mating success was set as a binomial variate with cage size and vector density as fixed effect, while biological replicates were treated as the random effects for the cage size and vector density experiments respectively. For the cage size experiment the small cage (2L) was set as the reference when extracting model estimates while the low vector density (30:30) was set a reference in the vector density analysis. Pairwise multiple comparison was performed using Tukey's *post-hoc* test to determine the individual statistically significant differences between the cage sizes and vector densities. Log likelihood ratio tests (LRT) were used to test the significance of each variable of interest in all models. Data analysis and figure were plotted in R statistical software as indicated previous in section 2.2.6.

4.2. Results

4.3.1 Effect of adult mosquito rearing cage size on the mating success ratios of *An. funestus*

Anopheles funestus mating was differently influenced by the size of rearing cages within and between strains. Cage size significantly influenced the mating success of the *An. funestus* FANG stain ($\chi^2 = 18.39$, $p < 0.001$, Table 4.1) but did not have a significant effect on the FUMOZ strain ($\chi^2 = 5.01$, $p = 0.17$, Table 4.1). The mating success ratios of the FANG strain were higher in the 20-litre cylindrical cage (80.1%, n = 256), and lower in the 2-litre cylindrical (62.5%, n = 251), comparing to the 5L and 10L cages (Table 4.1). However, this was not observed for the FUMOZ strain where mating ratios were similar between the different cage sizes evaluated (Table 4.1). The mating success ratios of the *An. funestus* FUMOZ strain was consistently lower compared to the FANG strain in all categories of cage size (Table 4.1). Following pairwise multiple comparisons using Tukey's *post-hoc* test there was no significant difference in the mating success of FUMOZ strain between cage sizes (Fig 4.3). However, the mating success of the FANG strain was significantly lower in the 2-litre cylindrical cage compared to the 5-litre, 10-litre, and 20-litre cylindrical cages at 95% CI (Fig. 4.3, Table 4.1).

Table 4. 1. The GLMM estimate proportions of mating success of *An. funestus*, FUMOZ and FANG strains reared at equal numbers of male and female (N = 100) in cylindrical cages of different volume.

Colony	Cage Specification	Sample Size	% Insemination [95% CI]	OR [95% CI]	P-Values
FUMOZ	2 L	256	21.0 [14.5, 29.3]	1	
	5 L	251	26.7 [19.1, 36.0]	1.38 [0.92, 2.07]	0.124
	10 L	277	25.1 [17.9, 34.0]	1.26 [0.84, 1.90]	0.255
	20 L	267	19.6 [13.5, 27.6]	0.92 [0.60, 1.40]	0.691
FANG	2 L	251	62.5 [55.0, 69.3]	1	
	5 L	**275**	**76.8 [70.3, 82.2]**	**1.99 [1.31, 3.02]**	**0.001**
	10 L	**282**	**74.5 [60.0, 80.0]**	**1.76 [1.17, 2.64]**	**0.007**
	20 L	**256**	**80.1 [73.7, 85.3]**	**2.42 [1.56, 3.77]**	**<0.001**

CI – Confidence interval**, OR –** Odd ratios, bold font indicates statistically significant difference from the control cage.

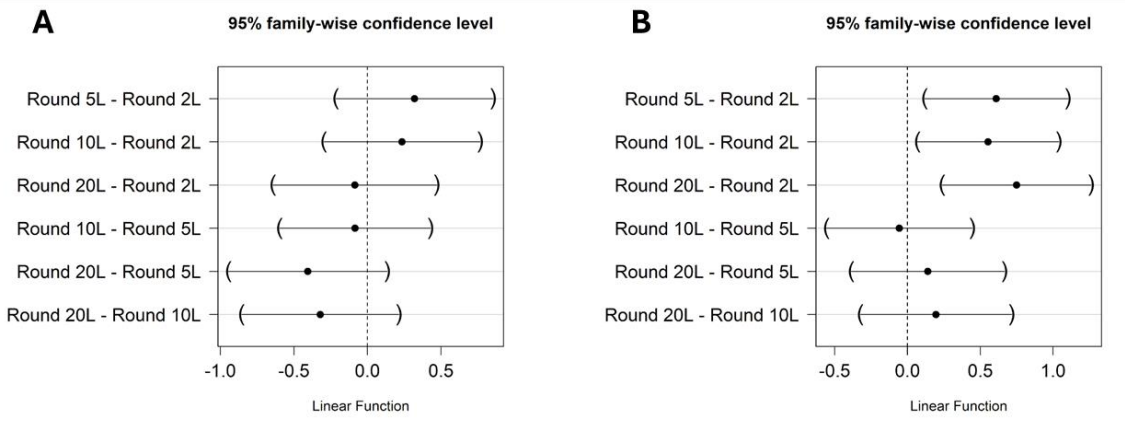

Figure 4.3. Pairwise multiple comparison with Tukey's *post-hoc* test for the mating success of *An. funestus* between different cylindrical cage sizes **A)** FUMOZ strain **B)** FANG. The dot on the horizontal line represents the mean difference between the two compared groups while the bracketed line represents the measure of precision from the mean (standard error). When the bracketed line does not cross the vertical dotted line, it indicates the two groups are significantly different from each other.

4.3.2 Effect of adult mosquito density inside rearing cages on the mating success ratios of *An. funestus*

Vector density per cage significantly influenced the mating status of FUMOZ both in small cubic cages (χ^2= 12.72, p = 0.002) and in small cylindrical cages (χ^2 = 8.43, p = 0.0147). Similarly, vector density per cage significantly influenced the mating status of FANG in the small BugDorm cubic cages (χ^2 = 11.03, p = 0.004) *versus* the small cylindrical cages (χ^2 = 5.77, p = 0.056). The highest mating success ratios for the FUMOZ strain were observed in the 120:120 (N = 240) vector ratio in the small BugDorm cubic and cylindrical cage (Table 4.2). However, the mating success ratios in the small BugDorm cubic cages (49.1%, n = 630) were higher compared to the small cylindrical cages (44.2%, n = 601) at 95% CI for the 120:120 vector density (Table 4.2). In contrast, the higher mating success ratio for the FANG strain was observed in the 30:30 vector density (78.5%, n = 160) for the small BugDorm cubic cages and in the 120:120 vector density (73.5%, n = 558) for the small cylindrical cages at 95% CI (Table 4.2). Generally, the mating success ratios were higher for the FANG strain compared to the FUMOZ strain in all categories of cage type and vector density per cage (Table 4.2).

Following pairwise multiple comparisons using Tukey's *post-hoc* test for the *An. funestus* FUMOZ strain the 120:120 vector density had significant higher mating success than the 30:30 vector density (z = 3.42, p = 0.002, Fig. 4.4(A)) in the small BugDorm cubic cages and the

60:60 vector density (z = 2.74, p = 0.016, Fig. 4.4(B)) in the small cylindrical cages. When pairwise multiple comparisons with Tukey's *post hoc* test were done on the *An. funestus* FANG strain mating success ratios were significantly higher in the 30:30 vector density compared to the 60:60 vector density (z = -2.46, p = 0.036, Fig. 4.4(C)) in the small BugDorm cubic cages. Even though the 120:120 vector density had high mating, the mating success ratio for *An. funestus* FANG strain in the small cylindrical cages was not significantly different to the 30:30 and 60:60 vector densities at 95% CI (Fig 4.7(D)).

Table 4. 2. The GLMM estimate proportions of mating success of *An. funestus*, FUMOZ and FANG strains reared at a 30:30, 60:60 and 120:120 female:male ratios in small BugDorm cages and small cylindrical cages.

Strain	Cage Type	Vector Ratio	Sample Size	% Insemination [95% CI]	Odd Ratio [95% CI]	P-Values
FUMOZ	Small Cubic	30_30	159	33.8 [26.2, 42.3]	1	
		60_60	**316**	**43.4 [36.8, 50.1]**	**1.50 [1.01, 2.24]**	**0.045**
		120_120	**630**	**49.1 [43.6, 54.6]**	**1.89 [1.31, 2.72]**	**0.001**
	Small Cylindrical	30_30	156	36.9 [26.8, 48.4]	1	
		60_60	336	34.8 [26.3, 44.5]	0.91 [0.61, 1.36]	0.652
		120_120	601	44.2 [35.3, 53.5]	1.35 [0.93, 1.96]	0.11
FANG	Small Cubic	30_30	160	78.5 [70.9, 84.5]	1	
		60_60	**390**	**64.1 [57.6, 70.1]**	**0.49 [0.32, 0.76]**	**0.001**
		120_120	**761**	**67.8 [62.7, 72.5]**	**0.58 [0.38, 0.87]**	**0.008**
	Small Cylindrical	30_30	135	65.9 [57.5, 73.4]	1	
		60_60	275	66.5 [60.8, 71.9]	1.03 [0.67, 1.59]	0.9
		120_120	558	73.5 [69.7, 77.0]	1.43 [0.96, 2.14]	0.08

CI – Confidence interval**, OR –** Odd ratios, bold font indicates statistically significant difference from the 30:30 (N=60) vector density.

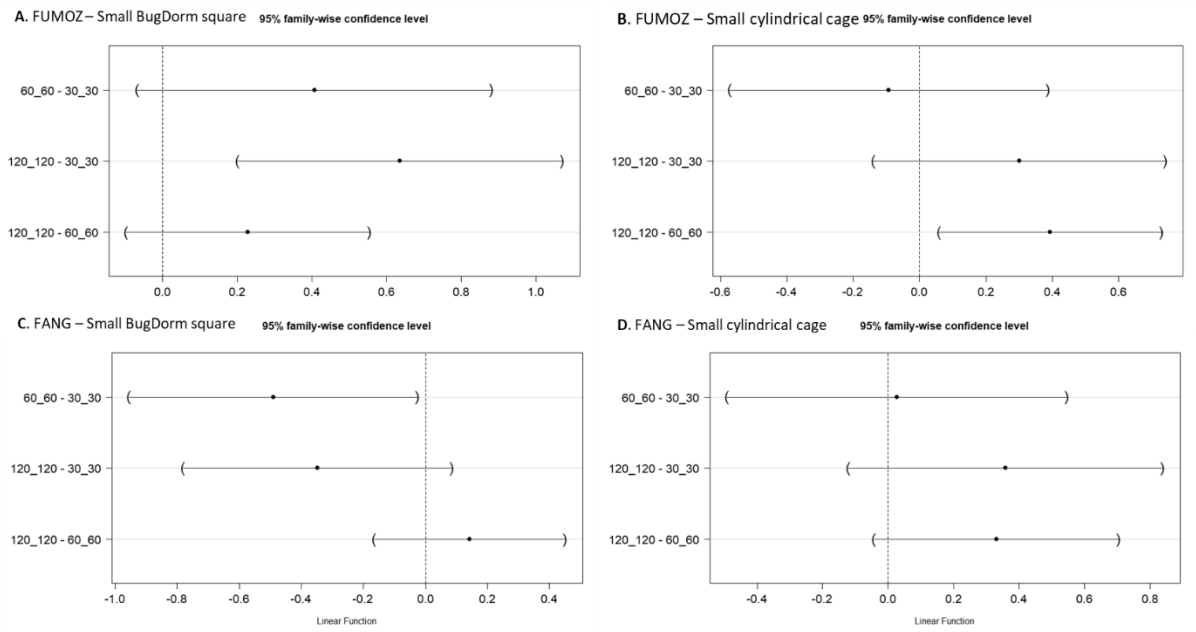

Figure 4.4. Pairwise multiple comparison with Tukey's *post-hoc* test for the mating success of the two *An. funestus* strain at different vector density; **A)** FUMOZ small cubic BugDorm, B) FUMOZ small cylindrical cages, **C)** FANG small cubic BugDorm, **D)** FANG small cylindrical cages. The dot on the horizontal line represents the mean difference between the two compared groups while the bracketed line represents the measure of precision from the mean (standard error). When the bracketed line does not cross the vertical dotted line, it indicates the two groups are significantly different from each other.

4.3. Discussion

In this chapter the effect of cage size and vector density on the mating success ratios of two *An. funestus* strains, FANG and FUMOZ was evaluated. The two strains expressed different responses to the cage size and vector density per cage. Firstly, the *An. funestus* FANG strain mating success ratios was significantly affected by the size of the adult mosquito rearing cage. High mating ratios were observed in the large cylindrical cages (20 litre) while low mating ratios were observed in the small cylindrical cages (2 litre). This observation might have been influenced by surface area availability inside the rearing cages with the large cage providing enough space for mosquitoes to fly freely and initiate mating activities. Large rearing space has been observed to allow *Anopheles* mosquitoes to mate freely in semi-field settings (Coluzzi, 1964; Ng'Habi *et al.*, 2010), and swarms were observed in large experimental chambers (Facchinelli *et al.*, 2015; Achinko *et al.*, 2016; Niang *et al.*, 2019), as well as increased mating in large cages in laboratory experiments (Coluzzi, 1964; Marchand, 1985; Pudar *et al.*, 2021).

Cage size has also been shown to affect the physiological and reproductive fitness of *Aedes* mosquitoes (Coluzzi, 1964; Maramorosch and Mahmood, 2014; Pudar *et al.*, 2021).

Contrastingly, the *An. funestus* FUMOZ strain did not express any significant difference in mating success regardless of the cylindrical cage sizes. In fact, the mating success ratios of the FUMOZ in the cylindrical cages was lower compared to the mating success ratios of the same strain in small cubic cages at the same vector ratios of 50:50 male:female. This observation indicates that the shape or materials used to make the walls of the adult mosquito cage might have had significant effects on the reproductive fitness of the adult FUMOZ strain. The cylindrical cages are made of nonporous white plastic with only the side hole and top cover made of a clear fine net. This might have affected the general air circulation in the experimental cages compared to the cube cages that had four sides made of clear fine net. *Anopheles* mosquito mating selection in swarms has been reported to depend on fast flights and wingbeat sounds (Jaffe, 2002; Maramorosch and Mahmood, 2014; Baeshen, 2022). The nonporous walls of the cylindrical cages might have disrupted the sounds in the cage making it difficult for the strain to harmonise their flight tone which is vital for mating activities (Garcia Castillo *et al.*, 2021; Somers *et al.*, 2022).

Secondly, the effect of vector density per cage was observed to influence mating success ratios for both *An. funestus* FANG and FUMOZ strains in the two small cage shapes. There was a slight variation in the trend of mating success between the two strains across the two cage shapes used. The FANG strain had higher mating success at low density (30:30 male:female ratios, total of 60 mosquitoes per cage) in the small cubic cages while the mating success ratios of the FUMOZ strain consistently increased with increased vector density. High density of mosquitoes per rearing cage has been observed to increase mating success in large mass rearing cages however, high mosquito densities per cage can limit mating which might be due to limited flight activities (Coluzzi, 1964; Maramorosch and Mahmood, 2014; Mamai *et al.*, 2017). In the laboratory, *Anopheles* mating has been observed to be influenced by vector density per cage which indicates the importance of available space for flight activities (Coluzzi, 1964; Mashatola *et al.*, 2017).

4.4. Recommendations

The cage size evaluation experiments in this chapter were conducted in cylindrical cages with varied sizes, while the vector density experiments were done in small cylindrical (2 litre) and small square (15 cm) cages. To better understand how the cage size and shape may affect the

reproductive fitness of the two used strains of *An. funestus* further experiments should be conducted. Experiments should be done to evaluate the effect of different sizes of cubic cages and vector density in different sizes of cylindrical and cubic cages on the mating success, survival, and other life table parameters of *An. funestus*. This was not feasible as it required a large number of adult *An. funestus* which were not available during the study.

CHAPTER FIVE

The impact of dimming duration and globe type on the mating success of *An. funestus*

5.1. Introduction

Anopheles mosquito's circadian rhythms are naturally dependent on specific photoperiods (Coluzzi, 1964; Maramorosch and Mahmood, 2014; Moon *et al.*, 2014; Rund *et al.*, 2016; Sheppard *et al.*, 2017; Baik *et al.*, 2020; Sizemore and Dacks, 2020; Rahman *et al.*, 2021; Au *et al.*, 2022). Adult *Anopheles* feeding, resting, and mating activities are stimulated by specific light environments (Jones *et al.*, 1967; Maramorosch and Mahmood, 2014). Specifically, *Anopheles* mating activities coincide with the sunset where the fading light is known to stimulate swarming which is the main initiator of mating activities (Jones *et al.*, 1967; Charlwood and Jones, 1979; Charlwood *et al.*, 2003; Diabate *et al.*, 2003; Charlwood, 2011; Diabaté *et al.*, 2011; Achinko *et al.*, 2016; Kaindoa *et al.*, 2017; Zawada *et al.*, 2018; Kaindoa *et al.*, 2019; Manoukis, 2021; Baeshen, 2022). Simulations of the important photoperiods have been observed to improve the in-house maintenance of *Anopheles* colonies (Coluzzi, 1964; Hunt *et al.*, 2005; Maramorosch and Mahmood, 2014; Villarreal-Treviño *et al.*, 2015). Even though sufficient mating success ratios have been observed in insectaries where light is set at 12:12 hours of dark – light cycles, some insectaries have been installed with dimming systems (Hunt *et al.*, 2005; Benedict *et al.*, 2009; Maramorosch and Mahmood, 2014; Villarreal-Treviño *et al.*, 2015). However, even though specific details on how colours and space in mosquito insectaries should be standardize (Maramorosch and Mahmood, 2014), there are no standardised light types, dimming duration or dimming mechanisms.

Dimming duration varies between insectaries which can last from a few minutes to more than an hour. Systems both with and without dimming time have been reported to significantly enhance mating in *An. gambiae* complex species while it has been difficult to enhance mating in *An. funestus* (Hunt *et al.*, 2005; Ngowo *et al.*, 2021; Odero *et al.*, 2023). Given the uniqueness of the swarming behaviour of the two vectors in their natural environment (Harper, 1944; Charlwood and Jones, 1979; Charlwood *et al.*, 2003), it is worth considering that they might have a unique response to artificial lights used in the insectaries. At the same time, the promising effectiveness of sterile insect technique and gene drive based technology in malaria vector control as a solution to overcome insecticide resistance (Munhenga *et al.*, 2016), calls for improved systems to manage mass production of the targeted malaria vectors. Hence, there is a need to improve rearing systems to improve the mating success ratios of *An. funestus* in

captivity. This objective focused on evaluating the impact of dimming duration on the mating success ratios of *An. funestus* in captivity by comparing mating success ratios at 30- and 90-minutes dimming durations.

5.2. Material and methods

Experiments for this objective were conducted in two separate rooms in BDMI to compare mating success of *An. funestus* at 30- and 90-minutes dimming durations. One room was operating at 30-minutes dimming duration and the second room had a 90-minute dimming duration. The cage alterations with improved mating success ratios for *An. funestus,* FANG and FUMOZ established in objective two were used (chapter three in this dissertation). Side-covered cages were used to evaluate the mating success of the FANG strain while top-covered cages with a base visual marker were used for the FUMOZ strain. A separate uncovered cage was used as a control for each of the two strains and experiments were conducted simultaneously. Mating success evaluation in this chapter were conducted as described in chapter three but with the selected cages and different diming durations. A total of three distinct biological replicates of the experiment were conducted using different mosquito generational cohorts and only one cage per biological replicate was set for each treatment.

5.2.1 Experiment room settings

Room one was BDMI room 4B and it was operating under the normal light conditions of 12:12 hours dark:light cycles with 30 minutes dimming duration before and after the light phase as described in chapter two section 2.2.1 in this dissertation. The regular lights in the BDMI Room 7 were uninstalled and the room was set to 12:12 hours dark:light cycles with a 90 minute dimming duration using desk lamps. Six desk lamps were used to create the photoperiods by placing them on the shelves one side of the room opposite the shelves with experimental cages. Three desk lamps were put on a lower shelf and set at 12:12 hours dark:light phases and the other three desk lamps were put on the top shelf and tilted to direct their light to the ceiling (Fig. 5.1). The photoperiods were set by means of digital switches where the three bottom bench lamps were connected to a digital switch set at 12-hour light on and 12-hour light off. The 90-minute dimming duration was set by connecting the three dimming lamps to separate digital switches set to off and on in series at 30-minute intervals at the onset of dark phase and light phase respectively.

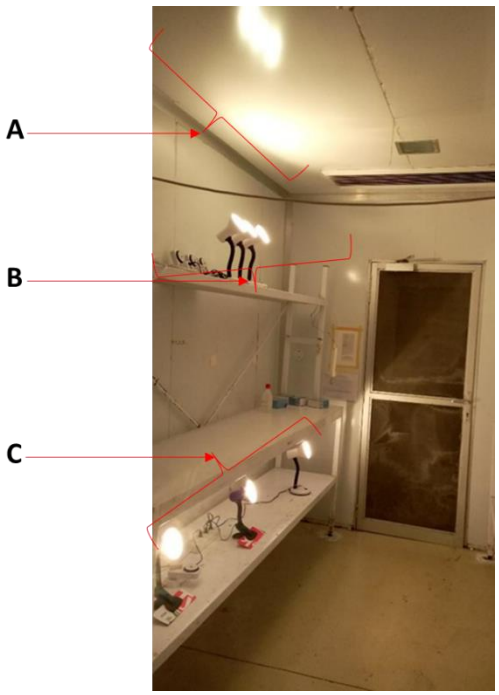

Figure 5.1. A section of the BDMI Room 7 demonstrating the experimental setting with, **A)** light directed to the ceiling from the tilted top shelf lamps, **B)** desk lamps on the top shelf to simulate photoperiods with a 90-minute dimming time, and **C)** desk lamps put on the bottom shelf to mimic bench lights as in room 4B.

5.2.2 Data analysis

Data were analysed in R studio statistical software where logistic regression models using the *lme4* package were used to estimate the mean mating success ratios for *An. funestus* and compare mating success ratios between the 30- and 90-minutes dimming durations for the FANG and FUNOZ strains. Mating successes were modelled as a binomial variate with dimming duration as fixed effects while the experiment replicates were treated as random effects. The final model: Mating success ~ Dimming duration + (1|EXP_Replicate)-1, family = binomial(link = "logit"), data =). The 30-minute diming duration was set as intercept when running the model. Tukey *ad hoc* was used to conduct multiple comparisons between the 30- and 90- minute diming duration for the FANG and FUMOZ strains.

5.3. Results

After five biological replicates the mating success ratios of *An. funestus* were compared between the 30-minute and 90-minute dimming duration per treatment. The mating success ratios of the two *An. funestus* strains were not significantly different between the 30-minute and 90-minute dimming durations both in the control cages, FUMOZ ($\chi^2 = 0.42$, $p = 0.52$), FANG ($\chi^2 = 2.13$, $p = 0.14$), and in the modified cages, FUMOZ ($\chi^2 = 0.01$, $p = 0.92$), FANG ($\chi^2 =$

0.34, p = 0.56). Mating success ratios were then compared between the modified and unmodified cages within the same dimming duration. The FANG strain had significantly higher mating success ratios in the modified cage (54.1%, n = 219, Table 5.1), compared to the control cage when tested under the 30-minute dimming duration (χ^2 = 10.1, p = 0.001). However, the mating success ratios of FANG strain were not significantly different when compared between the modified and control cage under 90-minute dimming duration (χ^2 = 1.61, p = 0.2). The mating success ratios of the FUMOZ strain were not significantly different between the control cage and the modified cage under 30-minute (χ^2 = 1.28, p = 0.26), and 90-minute (χ^2 = 0.56, p = 0.46), dimming durations. Similarly, when multiple comparisons of means were done with Tukey *post hoc* only the mating success ratios of the FANG strain between the control cage and the modified cage were significantly different (z = 3.15, p = 0.002, Fig. 5.2). Generally, the mating success of the FANG strain was higher compared to the FUMOZ strain (Table 5.1). It is worth noting that the FANG mating success was much lower compared to previous chapters in this dissertation.

Table 5. 1. The GLMM estimate proportions of mating success of *An. funestus*, FUMOZ and FANG strains when reared in rooms with different dimming durations (30 versus 90 minutes) using the cage modifications with high mating success ratios from chapter three in this dissertation.

Strain	Cage specification	Dim Time (minute)	Sample Size	% Insemination [95% CI]	Odd Ratio [95% CI]	P-Values
FUMOZ	No Cover	30	228	35.9 [21.2, 53.9]	1	
		90	227	32.9 [19.0, 50.6]	0.87 [0.58, 1.13]	0.52
	Top Cover	30	226	30.6 [19.3, 44.8]	1	
		90	228	30.2 [19.0, 44.3]	0.98 [0.65, 1.47]	0.9
FANG	No Cover	30	194	36.6 [19.2, 58.2]	1	
		90	225	44.0 [24.7, 65.3]	1.36 [0.90, 2.06]	0.15
	Side Cover	30	219	54.1 [37.9, 69.5]	1	
		90	219	51.2 [35.2, 67.0]	0.89[0.60, 1.32]	0.6

CI – Confidence interval**, OR –** Odd ratios

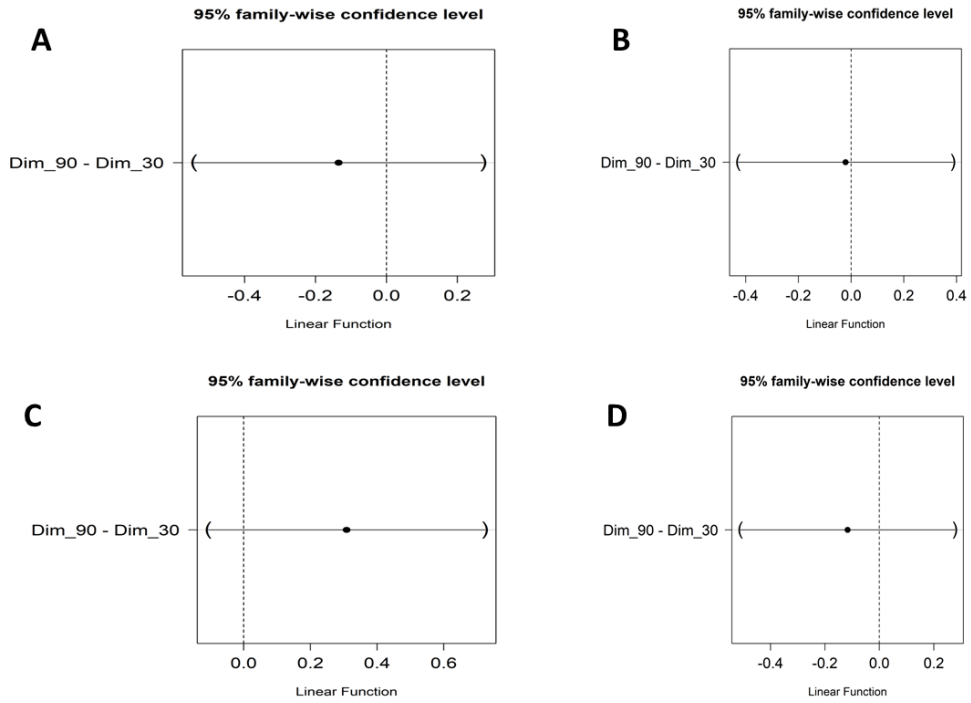

Figure 5.2. Mating success ratios of *An. funestus*, FUMOZ and FANG strains when reared in rooms with different dimming durations: 'Dim_30' for 30 minutes diming duration and 'Dim_90' for 90 minutes diming duration) using the cage modifications with high mating success ratios from chapter three in this dissertation. **A)** Standard (uncovered) cage for FUMOZ strain, **B)** Top covered cage with base visual marker for FUMOZ strain, **C)** Standard (uncovered) cage for FANG strain, **D)** Side covered cage for FANG strain. The dot on the horizontal line represents the mean difference between the two compared groups while the bracketed line represents the measure of precision from the mean (standard error). Since all bracketed line crossed the vertical dotted line, it indicates mating between the two diming durations were not significantly different.

5.4. Discussion

The aim of this objective was to optimise dimming duration to improve mating success of *An. funestus* by using two available laboratory strains. From the current experiments the study, conclusions on the optimal dimming duration required for effective mating of *An. funestus* in mosquito rearing facilities could not be defined. The experiments were done by using modified adult mosquito holding cages that were observed to improve mating success ratios of the two strains in a previous experiment (Chapter three in this dissertation). From the previous experiments, the mating success ratios of the FUMOZ strain were found to increase significantly when the top-half of the cage was covered by a black opaque cloth and when a black visual marker was placed on the base of the cage (Chapter three in this dissertation). The

two modifications were merged and expected to synergise each other and further improve the mating success of the FUMOZ strain. Similarly, in the same chapter (Chapter three in this dissertation), the mating success ratios of FANG strain were significantly improved by covering the side-half of an adult cage. However, results from this objective were lower than those observed in chapter three both in the control and modified cages.

The observed decrease in mating success ratios might have been due to the variation in temperature and relative humidity in the BDMI insectary at the time of these experiments (chapter five) compared to when experiments for chapter two in this dissertation were conducted. High temperature variations were recorded (varying between 20 – 30 °C) and relative humidity reached as low as 36%. The insectary conditions were further destabilised by the failing ventilation which forced the management to temporarily change the air flow systems. The ventilators in the insectary are usually run continuously but due to high temperatures and low relative humidity they were set to run at time intervals to improve humidity in the insectary. Since the two strains used in this study (FANG and FUMOZ) have been reared in the insectary and the previous experiments in chapter three were done when the ventilation was stable the mosquitoes might have adapted to the insectary noise and air flow. Sound frequency plays an important role in initiation of mating activities in *Anopheles* mosquitoes (Garcia Castillo *et al.*, 2021; Somers *et al.*, 2022).The change in the ventilation cycle might have affected the air flow in the insectary as well as noise levels which may have negatively affected the behaviour of mosquitoes including flight and mating initiation. Temperature and relative humidity are important determinants of mosquito's physiological fitness including flight and longevity (Maramorosch and Mahmood, 2014), hence their disruption might have stressed the mosquitoes and negatively affected their mating.

5.5. Challenges and Recommendations

During the time when chapter five experiments were conducted to compare dimming durations the climate conditions (temperature and relative humidity) as well as air circulation in the BDMI insectary were unstable. For instance, when chapter five experiments were conducted, the ventilators were readjusted to circulate air at time intervals from the routine setup where ventilators were set to run continuously. Therefore, it is important that these experiments must be repeated when the BDMI insectary climate conditions are stable again. This will enable evaluation of the cage modifications in a similar environment to the one they were optimised in as well as in different diming durations. Having the findings on the effect of diming durations on the mating success of *An. funestus* will complement the findings on cage modifications so that it is possible to optimise number of days needed for optimal insemination.

CHAPTER SIX

6.1. GENERAL DISCUSSION AND CONCLUSION

Anopheles funestus is now the main malaria vector responsible for active and residual transmission of the disease in many regions of the African continent (Coetzee and Koekemoer, 2013; Msugupakulya *et al.*, 2023; Odero *et al.*, 2023). Studies on this vector group were for a long time by the lack of effective method to characterise its species (Koekemoer *et al.*, 2002; Coetzee and Koekemoer, 2013). Even after the establishment of a molecular techniques to discriminate species in the *An. funestus* group (Koekemoer *et al.*, 2002), and successful colonisation of two strains of *An. funestus* (Hunt *et al.*, 2005; Zengenene *et al.*, 2021), studies on the vector has been limited by lack of additional colonised strains. The main limitation of colonisation of this species remains the low mating success under artificial settings (Coetzee and Koekemoer, 2013; Ngowo *et al.*, 2021). The rise in vector resistance against the mainstay vector control interventions and the development of new control interventions that are based on mass release have increased the need for additional colonies. To match the criteria and increase the effectiveness of the mass release programs such as sterile insect technique and gene drive it is recommended to use mosquitoes originated from the local vector populations to avoid the introduction of alien genotypes to the local population.

This study capitalised on the two only existing fully established strains of *An. funestus* (FANG and FUMOZ) to evaluate the mating success of this important vector in insectaries. This study provided valuable insights into important aspects of adult mosquito housing conditions in terms of cage and light environment that may improve the mating success ratios of *An. funestus*. The conditions identified in this study will be useful in guiding studies on the mating behaviour of *An. funestus* and when attempting colonise species from local/wild population. It is very interesting to see that two strains obtained from different populations of the same species expressed some unexpected response to similar mating cues and rearing conditions. For example, while both artificial horizon and inclusion of visual marker in adult mosquito cages significantly improved mating success ratios of the FUMOZ strain, it is only the inclusion of artificial horizon that influenced mating success ratios for the FANG strain. These findings highlight that adult rearing conditions must be well tailored to fit a specific population of *An. funestus* when attempting to colonise or sustaining the species in captivity. Field studies have reported contrasting swarming characteristics between closely related *Anopheles* species

(Gueye *et al.*, 2023), but this is the first reported observation on the existence of differences in the mating behaviour within the same species.

The FANG strain which originated in Angola had consistently higher mating ratios compared to the FUMOZ strain originated from Mozambique. The highest mating success ratios of the FANG strain in this study were about 80% which is slightly higher compared the previous mean mating ratios of 74.8% reported on the same species also in a cylindrical cage (Zengenene *et al.*, 2021). However, the observation in this current study was with a vector density of 100 mosquitoes in a 20-litre cylindrical cage compared to the 600 mosquitoes in a five-litre cage in the previous study by Zengenene *et al.* (2021). The findings suggest that a 20-litre cylindrical cage might be the best option for rearing the FANG strain, but it can also be an adaptation as the strain is reared in the same cage size for routine maintenance. However, the significant improvement of mating success ratios for the FANG strain in the side covered small cubic cage may better indicate the added effect of cage modification contrary to the 20L cylindrical cage which used for rearing the strain in routine colony maintenance.

The highest observed mating success ratios for the FUMOZ strain in this study (49.5%) were less compared to findings from previous studies that reported more than 70% insemination ratios on the same species (Ngowo *et al.*, 2021; Felamboahangy *et al.*, 2022; Maharaj *et al.*, 2022). In the two studies (Ngowo *et al.*, 2021; Felamboahangy *et al.*, 2022), they used large cubic cages (30 x 30 cm), and they dissected a randomly selected subsample of females from the experimental cage while for this study highest mating ratios were in small cubic cages (15 x 15 cm) and all surviving females per cage were dissected after the mating time. Even though Maharaj *et al.* (2022) used the small BugDorm cages as in this study, they started their experiments with newly emerged mosquitoes allowed them to mate for 10 to 12 days. Experiments in this study were set with mosquitoes at the pupa stage and mating was evaluated after 10 days. Pupa were used to ensure adult mosquitoes will emerge and stay in specific experimental conditions of cage modification and vector density.

The two strains used in this study were colonised around the same time and for this study the mosquitoes were obtained from colonies reared in the same insectary. The observed differences in mating success ratios might not have resulted from their adaptability to the insectary condition. However, one cannot exclude a possibility that the FUMOZ stain may be more sensitive to the unstable insectary conditions experienced during the time of this study. During the whole time of this study South Africa was experiencing load shedding (power cuts) which greatly affected the light environment in the insectary. Load shedding might have as well placed

an additional burden on machinery systems in the insectary including the ventilators resulting in system failure that affected climate conditions during the last part of experiments.

Alternatively, the observed mating success differences between the two strains might be due to their varied geographical originality or other evolutionary genomic differences. Several studies have reported evidence of population segregation influenced by geographical landmarks and ground distance between the two populations (Koekemoer *et al.*, 2006; Amvongo-Adjia *et al.*, 2020; Kaddumukasa *et al.*, 2020; Odero *et al.*, 2023). Similarly, field observations have reported notable differences in swarming behaviour within the same species of *Anopheles* mosquitoes across different geographical regions (Charlwood, 2011; Sawadogo *et al.*, 2013; Baeshen, 2022). For example, *Anopheles gambiae* swarms in the southeastern side of the African continent (Charlwood, 2011; Baeshen, 2022), were observed to differ in swarm timing, height from the ground and swarm markers from those on the western side of the continent (Charlwood, 2011; Gueye *et al.*, 2023).

Moreover, it has been observed that mosquito populations of the same species from different geographical conditions or localities may express fine genomic differences that can influence their adaptation and survival, especially against insecticides (Miles, 2021; Odero *et al.*, 2023). These genomic differences have been reported to affect the physiological and reproductive fitness of *Anopheles* mosquitoes. For example, the presence of target-site mutations for insecticide resistance has been linked to lowering the reproductive fitness of male mosquitoes (Platt *et al.*, 2015). The *Anopheles funestus* FANG strain is known to be susceptible to insecticides while FUMOZ express metabolic resistance able to survive the WHO 5x discriminating dose (Hunt *et al.*, 2005; Coetzee and Koekemoer, 2013; Venter *et al.*, 2017; Tchouakui *et al.*, 2018; Tchouakui *et al.*, 2021; Menze *et al.*, 2022; Nolden *et al.*, 2022). Expression of metabolic resistance has been linked to reducing the reproductive fitness in *An. funestus* FUMOZ strain (Tchouakui *et al.*, 2018; Tchouakui *et al.*, 2021). Therefore, the observed difference in the two strains of the same species might be connected to their difference in geographical origin or other underlying genomic differences including their susceptibility to insecticides. However, it is unlikely that the observed decrease in insemination ratios for the FUMOZ strain was due to susceptibility status since Zengenene *et al.* (2021) and Felamboahangy *et al.* (2022) reported higher mating success ratios using the same colony in the BDMI but prior to insectary climate control instability. Therefore, future studies should consider comparing the mating success ratios of the FANG and FUMOZ strain when the BDMI conditions are stable or by using mosquitoes from a more stable mosquito rearing facilities such as the WRIM insectary.

The findings in this study demonstrate the importance of light and changes in the light environment on the mating success of *An. funestus*. These findings further indicate that designs/modifications aimed at improving the mating of *Anopheles* in captivity should consider the position of the dimming lights as it's a determinant of the light environment inside the mosquito cage. The observed difference in mating success ratios between FUMOZ and FANG might be due to the different geographical origins of the two colonies or underlying genetic differences. Therefore, adult *An. funestus* housing should be customized when attempting to colonise new strains in insectariums as well as in mass production of malaria vectors for release programmes. The current study proposed to optimise the number of days required for maximum mating in *An. funestus* but due to the mentioned instability in the insectary conditions that was not accomplished. The findings from the current study will pave the way for more optimisation on the mating success ratios of *An. funestus*. This is vital as malaria vectors of the same species but from different geographical localities or populations may express fine biological/genetic differences that can impact their physiological and reproductive fitness. Moreover, further studies should be done especially with the FUMOZ strain to evaluate the observed decrease in mating success ratios when compared to previous studies.

APPENDICES

Appendix. B. University of the Witwatersrand Animal Ethics Research Committee ethics waiver

ANIMAL RESEARCH ETHICS COMMITTEE

Registration number: AREC-101210-002

Date: July 1, 2019

Certificate reference: 20190701-7O
Category: O
Re: Waiver from the Animal Research Ethics Committee of the University of the Witwatersran

Reference : Animal Ethics to complete
Re: Waiver from the Animal Research Ethics Committee of the University of the Witwatersrand
HoD applicant : Lizette Koekemoer
Staff number : 09700304
Degree : PhD/Msc Candidates
Study Title : "Studies on malaria vectors".
Department: Wits Research Institute for Malaria;
laboratory
Supervisor: Lizette Koekemoer, Givemore Munhenga, Yael Dahan-Moss, Shune Oliver, Basil Brooke and Maria Kaiser
Email: lizette.koekemoer@wits.ac.za
Address: 7 York Road, Parktown, Johannesburg, 2001
Tel: + 011-717 2486;
Full clearance certificate from the AREC of the University of the Witwatersrand is NOT required.
Reason: This study uses mosquitoes (invertebrates).
Comment/Notes :
1) **This is a General Waiver for the PI and listed Supervisors.**
2) **Individual researchers and MSc & PhD candidates may require Ethics Clearance in the own name according to applicable rules of the Post-Graduate and other Committees.**
3) **Feeding of the mosquitos should be specified.**

Please contact me should you require further information.

GP Candy

Geoffrey Candy PhD
Chair : Animal Research Ethics Committee

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