UNIVERSITY OF THE WITWATERSRAND

OPTIMISING LABORATORY-REARING PARAMETERS FOR *ANOPHELES FUNESTUS* TO ENHANCE SCALABILITY TOWARD THE STERILE INSECT TECHNIQUE

by

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Johannesburg, 03 November 2023

DECLARATION

I, Lalasoa Niain'ny Felamboahangy, declare that this Thesis is my own, unaided work. It is being submitted for the Degree of Doctor of Philosophy 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)

03 of November in 2023

DEDICATION

Grace upon grace (John1:16)
“Ny hazo no vanon-ko lakana, ny tany naniriany no tsara”

A Lord almighty,

Allowed be your name

My parents, Mr Rakotomamonjy Andriamparisoa N. & Mrs Ranivosoaniaina Herilalao

Mr & Mrs Harrington Michael & Jennifer

Find in this work the fruit of your long years of sacrifices and prayers

Your unconditional support is so precious

Much love and profound gratitude

My lovely nephews

Having you working with me is a precious encouragement for me

Find a pathway on this work, go far more than I do

In loving memory of my grandparents

You shared with me your precious Malagasy moral value

Death leaves a heartache no one can heal, love leaves a memory no one can steal (Irish headstone)

All family members, friends, and knowledge who supported me in the completion of this second thesis

Find here my gratitude

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   Role: Initiated the idea of participating in the conference, conducted the experiment, prepared the slides and presented at the conference.

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ABSTRACT

The plateauing of gains in the fight against malaria, partly due to insecticide resistance in malaria vector mosquitoes, is a threat to malaria control. Additional vector control tools like the sterile insect technique (SIT) are being evaluated. *Anopheles funestus*, a main malaria vector in Africa, has not yet been evaluated for control using SIT partly due to difficulties in its colonisation. To proceed with SIT for this species, knowledge of their optimal laboratory-rearing conditions is critical.

To optimise *An. funestus* laboratory-rearing conditions, this study investigated the effect of using an artificial blood-feeding system or anaesthetised guinea pig on fecundity and fertility. Different larval diet doses and larval rearing densities were determined using a life-table approach. Finally, a range of irradiation doses were used to determine the optimal dose which induces sterility in *An. funestus* males without impacting the ability of the sterile males' to compete with fertile males to mate with fertile females. Subsequently, mating competitiveness under laboratory conditions of *An. funestus* males irradiated at 120 Gy across three different ratios of fertile: irradiated males were performed.

Results showed that fecundity was three times higher in females blood-fed on anaesthetised guinea pig compared to those blood-fed on the artificial blood-feeding system using bovine blood. Increasing the blood-meal frequency using the artificial blood-feeding system increased egg production, although it was not statistically significant. There were no significant differences between the egg fertility from females that fed on guinea pig or those fed on the artificial blood-feeding system using bovine blood. *Anopheles funestus* larvae fed with a food dose of 0.04 mg/larva and reared at a density of 0.48 larvae/cm^2 resulted in optimal developmental time and pupal production. Furthermore, irradiation of male pupae at different doses did not affect adult emergence regardless of the dose used, however, it correlated negatively with longevity and fertility. Irradiation at doses greater than 100 Gy resulted in a significant difference in both fecundity and fertility. The average mating competitiveness value of *An. funestus* males irradiated at 120 Gy was 1.24, decreased from 2.63 to 0.27 for different ratios of sterile male: fertile male: fertile female 1:1:1 to 3:1:1.

In conclusion, these findings can be used to improve the rearing of *An. funestus* under laboratory conditions, improving evaluations for SIT. Feeding defibrinated bovine blood through an artificial system will reduce the dependency on live animals for blood-feeding. Optimised larval feeding and rearing density will reduce developmental time and ensure

maximal pupal production. An irradiation dose of 100 Gy can be used to induce sterility of *An. funestus* without significantly affecting mating competitiveness.

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LIST OF ABBREVIATIONS and SYMBOLS

ACTs	Artemisinin-based Combination Therapies
Ae.	*Aedes*
AL	Artemether-Lumefantrine
a.m	ante meridien
An.	*Anopheles*
ANOVA	ANalysis Of Variance
ATP	Adenosine TriPhosphate
AW-IPM	Area-Wide Integrated Pest Management
Bs	*Bacillus spaericus*am
Bti	*Bacillus thuringiensis israelensis*
b/p/n	bites per person per night
CDC	Centers for Disease Control and prevention
cm	centimetres
χ^2	chi-square
C.I	Confidence Interval
COVID-19	COronaVIrus Disease 2019
Cx.	*Culex*
°	degree
°C	degree Celsius
DDT	Dichloro-Diphenyl-Trichloroethane
DEET	N, N-diethyl-meta-toluamide
ect.	et cetera
e.g.	"*exampli gratia* " means for example
EIR	Entomological Inoculation Rate
ELISA	Enzyme-Linked Immuno Sorbent Assay et al.
et al. (et alia)	and others
FUMOZ	*FUnestus* from MOZambique
g/dL	grams per decilitre
Gy	Gray
HAT	Human African Trypanosomiasis
HBI	Human Blood Index
HBR	Human Biting Rates

HLC	Human Landing Catch
hrs	hours
IAEA	International Atomic Energy Agency
ib/p	infected bites per person
i.e	“id est” means that is
IQR	Interquartile Range
IRS	Insecticide Residual Spraying
ITNs	Insecticide Treated Nets
IU	International Unit
L	Litres
L1 to L4	First instar larva to fourth instar larva
$L1/cm^2$	First instar larvae per centimetres square
L1/ tray	First instar larvae per tray
LLIN	Long-Lasting Insecticidal bed Net
LSM	Larval Source Management
m	metres
µg	micrograms
µL	microlitre
mg/dL	milligrams per decilitre
mg/larva	milligrams per larva
mL	millilitre
mm	millimetres
min	minutes
NICD	National Institute for Communicable Diseases
NWS	New World Screwworm
%	Percent
P.	*Plasmodium*
PCR	Polymerase Chain Reaction
pH	potential of Hydrogen
RBC	Red Blood Cells
RIDL	Release of Insect carrying a Dominant Lethal
RO	Reverse Osmosis
RTS,S/AS01	First generation of purified recombinant circumsporozoite protein vaccine, malaria candidate vaccine
sec	second

SD	Standard Deviation
SE	Standard Error
SIT	Sterile Insect Technique
s.l.	*senso lato*
s.s.	*senso stricto*
sp	*species*
SP	Sulfadoxine-Pyrimethamine
US$	United States dollar
VCRL	Vector Control Reference Laboratory
WHO	World Health Organization

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 BACKGROUND ON MALARIA

1.1.1 Malaria disease

In tropical regions of the world, malaria is a devastating and important disease in public health (Bloland and William, 2002). Malaria is an infectious human disease caused by the *Plasmodium (P.)* parasite transmitted by mosquitoes as discovered in 1897 by Ronald Ross (Cox, 2010). The parasite is usually transmitted through the bite of an infected *Anopheles (An.)* female mosquito (Marcus, 2009; Cox, 2010; Wirth, 2017). Malaria disease is provoked by one or more parasite species, including *P. vivax, P. ovale, P. malariae, P. knowlesi,* and *P. falciparum* (Grassi, 1900 as provided by Cox, 2010; Stephens, 1922; Cox-Singh et al., 2008). From all of these species, *P. falciparum* can be highlighted as it is widely distributed across the world, and it is one of the deadliest species. Another transmission route for malaria includes blood transfusions and transmission via the placenta during pregnancy (Bloland and William, 2002) which might increase malaria incidence. Delayed treatment due to late diagnosis results in clinical complications, which can result in death (Fattorusso and Ritter, 2004). Malaria is still considered a public health problem, with an estimated mortality of 627 000 recorded globally in 2020 (WHO, 2021[a]). This corresponds to a growth of 12% compared to 2019. Pregnant women and young children were identified as the most vulnerable group by the World Health Organization (WHO, 2021[a]).

Globally, malaria affects 85 endemic countries, and an estimated 241 million malaria cases were reported, with 10 deaths per 100 000 population at risk in 2020 (WHO, 2021[a]). Africa reports the most malaria cases annually, accounting for 95% of the world's malaria cases and 94% of global malaria deaths. Malaria deaths were estimated at 7.6 million from 2000 – 2019 (WHO, 2020). However, the incidence of malaria reduced from 368 cases/1 000 in the at-risk population in 2000, to 222 cases/1 000 of those at risk in 2019 and increased to 232 cases/ 1 000 population at risk in 2020 because of disturbance of services during the corona virus disease 2019 (COVID-19) pandemic lockdown (WHO, 2020; WHO, 2021[a]). The global pandemic caused by COVID-19 started in 2019 and spread rapidly from China to the rest of the world in 2020 (Hollingsworth and Xiong, 2021; Sills, 2021) affecting malaria disease control and prevention. The travel restrictions or re-allocation of funding (WHO, 2020) employed to reduce the spread of COVID-19 delayed or prevented the implementation of malaria vector

control interventions including the distribution of long-lasting insecticidal bed nets (LLINs) and insecticide residual spraying (IRS), which resulted in increased incidence of malaria cases and malaria deaths (WHO, 2021[a]).

Currently, several countries are working towards malaria elimination, while several others have successfully eliminated malaria, such as China, El Salvador and Malaysia. China has not recorded any indigenous malaria cases since 2017. In 2021, the WHO declared China and El Salvador malaria-free (WHO, 2021[b,c]; Zhou, 2021[a]). Malaysia was declared malaria-free in 2022, due to the absence of malaria since 2018 (WHO, 2021[d]; Muhammad et al., 2022). The European region has been declared malaria-free since 2015 (WHO, 2020).

In addition to health problems, malaria also affects the economic and social aspects of countries. Malaria causes poverty in countries on the African continent due to the loss of income owing to reduced productivity caused by morbidity and disability (Markley and Edmond, 2009; Hailu et al., 2017). In Mozambique, for example, the overall cost of malaria care (household and health care costs), in a high transmission district, was US$ 7.80 per uncomplicated case while it was US$ 107.64 per severe malaria patient (Alonso et al., 2019). In the district of Mopeia containing 1 373 households, nine severe malaria cases and 697 uncomplicated malaria cases; 514 malaria cases treated in the private health facilities or by traditional health care centres were usually recorded per month. This translated to malaria burden cost of on average US$ 27 690.52 per month.

1.2 MALARIA VECTORS

1.2.1 Background on *Anopheles*

The word *Anopheles* was derived from an ancient Greek word and means “harmful” or “useless” (Carnevale and Robert, 2009). It was first used to describe this genus of mosquitoes by German entomologist Johann Wilhelm Meigen in 1818 (Meigen, 1818 as provided in Harbach, 2013). The genus *Anopheles* belongs to the phylum: Arthropoda, Order: Diptera, Family: Culicidae.

There are 3 698 species and subspecies of mosquitoes throughout the world, 484 were formally identified as *Anopheles* species, with 187 subgenera and another 50 unnamed species (Meigen, 1818; Harbach, 2004; Harbach, 2013; Knols, 2021). While a small number of these (80 species) have been implicated in malaria transmission, 41 of them are known as malaria vector species or species that can impact public health (Carnevale and Robert, 2009; Hay, 2010;

Sinka et al., 2012; Williams and Pinto, 2012; Knols, 2021). The global distribution of these species is summarised in Figure 1.1 (Sinka et al., 2010; Sinka et al., 2012). Variations in environmental and geographic factors determine the distribution of anophelines and therefore contribute to malaria transmission heterogeneity. Temperature, humidity, fauna, and flora are all factors that can impact vector behaviour and distribution (Minakawa et al., 1999; Afrane et al., 2005; MacHault et al., 2009; Williams and Pinto, 2012; Rejmánková et al., 2013; Emidi et al., 2017; Ondiba et al., 2019).

1.2.1.1 Life cycle of Anopheles

The *Anopheles* mosquito life cycle (Figure 1.2) consists of an aerial stage for the adult and aquatic stages for eggs, larvae, and pupae (Carnevale and Robert, 2009). The *Anopheles* adult will emerge two to three days after pupation and will rest for a period to sexually mature before flying away to mate and contribute to the next generation of mosquitoes (Carnevale and Robert, 2009).

The adult body is divided into the head, thorax (containing one pair of wings and three pairs of legs), and an abdomen of 10 segments (Williams and Pinto, 2012). Anopheles adults rest at an angle of between 50 – 90° to the resting surface (Williams and Pinto, 2012). The mouthparts of males and females are composed of two maxillary palps and the proboscis (sucking mouthpart), used to pierce substrates (e.g. females pierce the skin to obtain blood) to ingest food (Carnevale and Robert, 2009; Cox, 2010; Williams and Pinto, 2012). The palps and proboscis in anophelines are almost equal in length (Williams and Pinto, 2012). Both male and female adults feed on nectar and plant sugars, however, most females need a blood meal for egg development and maturation (Carpenter and LaCasse, 1955; Charlwood et al., 1995; Tripet et al., 2003). Females mate only once in their lifetime (Baimai and Green, 1987; Charlwood et al., 2003; Magnusson et al., 2011). Males and females can easily be differentiated by observing the antennal morphology. The male antennae are bushy and plumose, while the females have simple pilose antennae (Figure 1.3).

Anophelinae eggs are black, oval-shaped and 0.5 mm in length. They are usually laid individually on the water surface by the females. The egg is characterised by lateral floats (Carnevale and Robert, 2009). Egg laying continues throughout their lifespan, after each blood meal. The developmental time needed for the cycle from egg to emerging adult, as well as the lifespan of the adult, varies from species to species. Environmental conditions, e.g. temperature

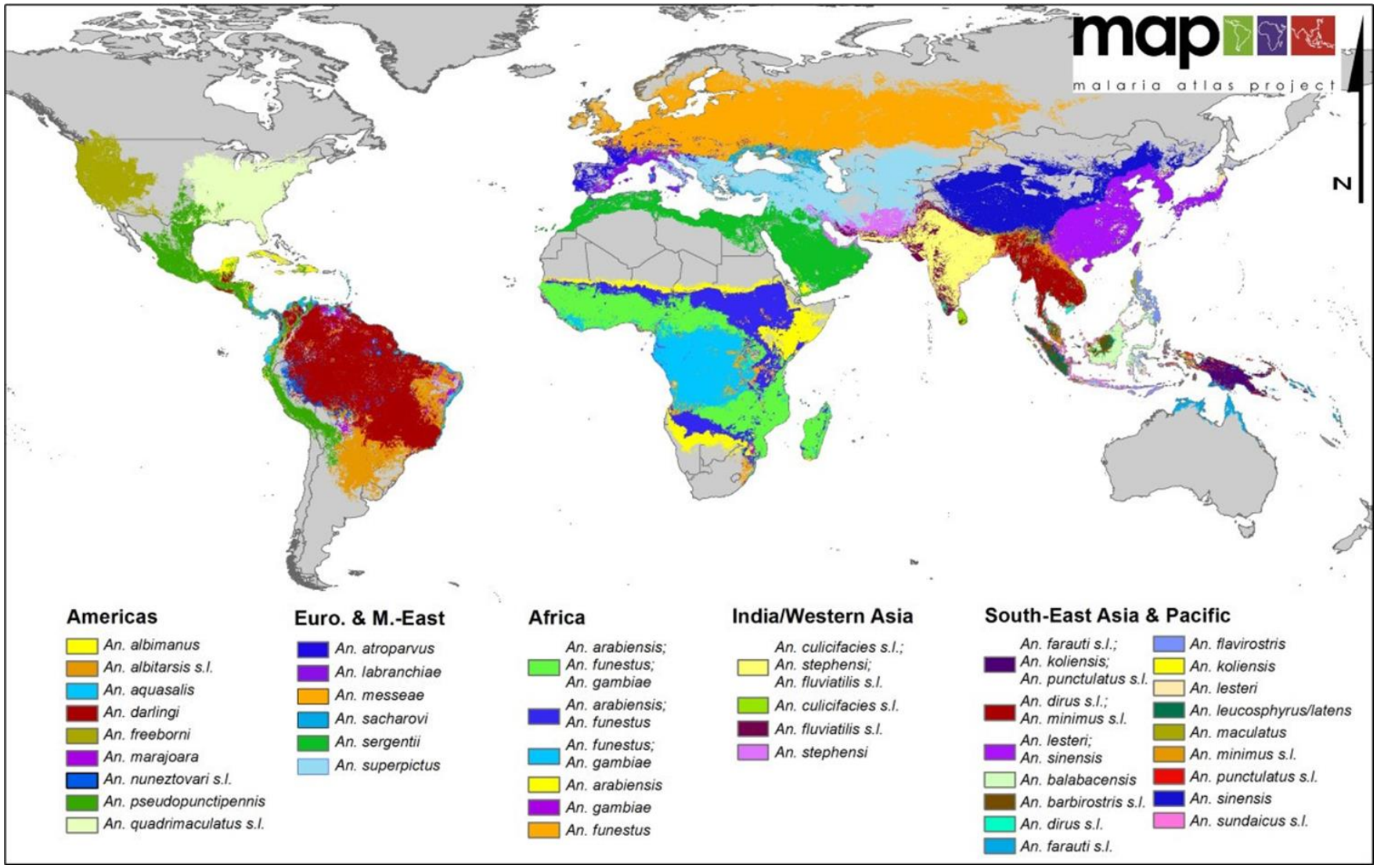

Figure 1.1: Distribution of main malaria vector species in the world (Source Sinka et al., 2012). Note, "*An. gambiae*" reflects the presence of both *An. gambiae s.s.* and *An. coluzzii* which were raised to species level in 2013 (Coetzee et al., 2013 [a])

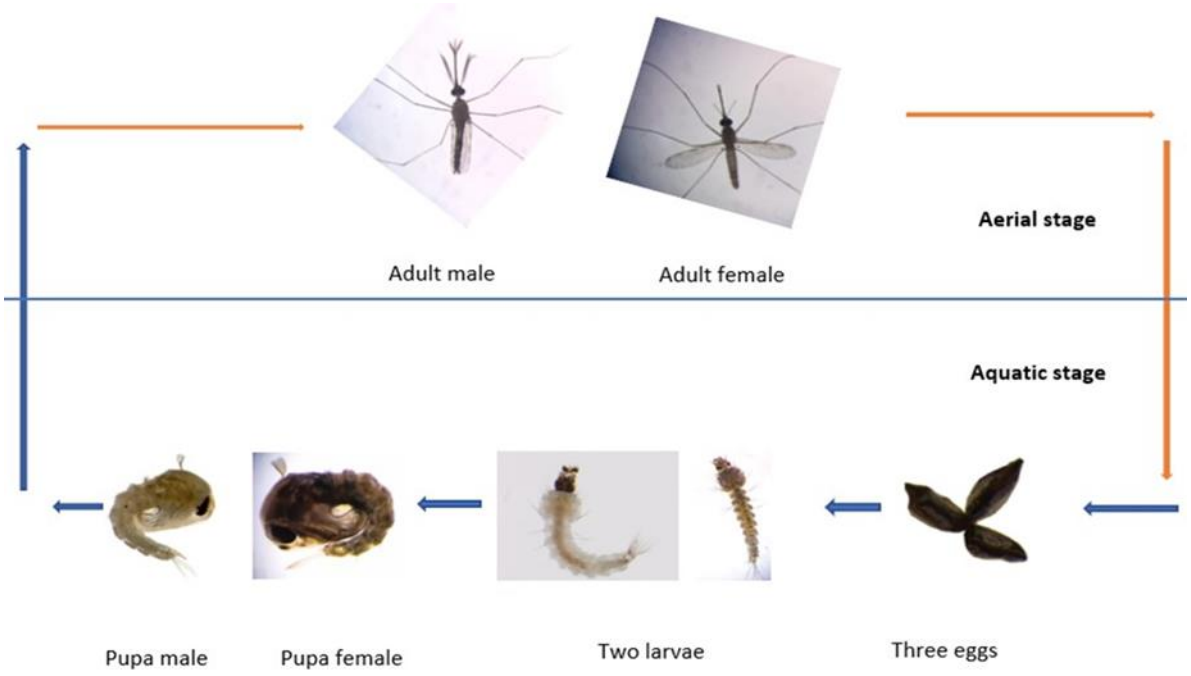

Figure 1.2: Life-cycle of Anopheles mosquito (adapted from Carnevale and Robert, 2009)

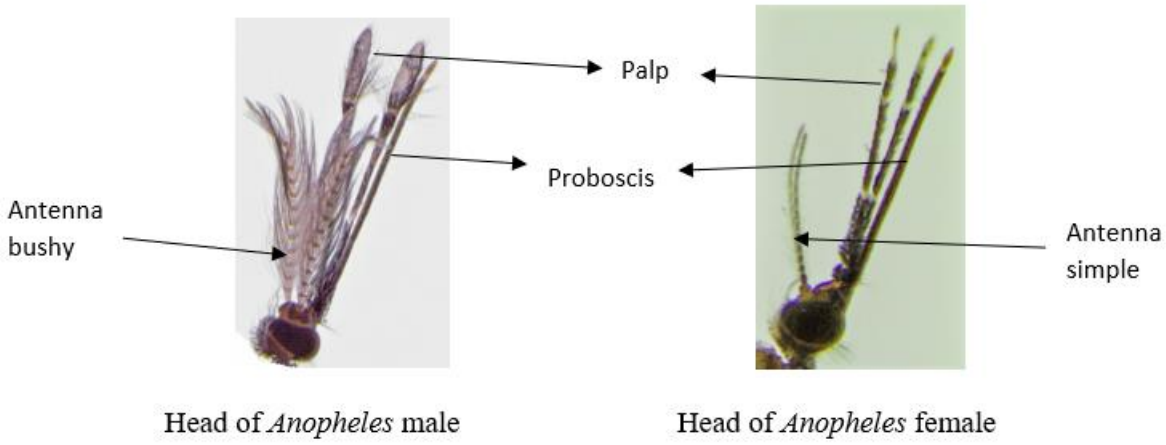

Figure 1.3: Differences between heads of *Anopheles* males and females

also affects the time needed to complete the lifecycle (Ralisoa et al., 1992). The eggs will hatch into larvae after two to three days (Carnevale and Robert, 2009).

The larvae live in water and predominantly lie horizontally on the water surface (Carnevale and Robert, 2009). The larval stage is active and larvae feed on microorganisms such as algae, protozoa, and organic debris filtered from the water. Mosquito larval habitats are species-specific and vary from rain pools, hoof prints, mineral springs, and bodies of saline water (Mastbaum, 1957; Mutero et al., 2000; Muturi et al., 2006). *Anopheles* larvae have a preference for small temporary habitats with or without algae and aquatic vegetation, including

habitats such as rice fields (Githeko et al., 1994; Bayoh et al., 2010). Larvae will moult four times before the adult mosquito emerges. There are four larval instars (first instar (L1) to forth instar (L4)), followed by an inactive pupal stage.

The pupae have a comma-shaped morphology, and do not feed. The pupal stage is the stage during which the mosquito metamorphoses into the adult form (Carnevale and Robert, 2009). Mosquito pupae can move by contraction of their abdominal segments. The sex can easily be determined during the pupal stage using the last abdominal (terminalia) segment below the paddles (Carvalho et al., 2014).

1.2.1.2 Identification of Anopheles species

To identify species within the genus *Anopheles*, morphology, cytotaxonomy and molecular methods are used (Grjebine, 1966; Gillies and De Meillon, 1968; Gillies and Coetzee, 1987; Coetzee, 2020). A reference key for morphological identification has been developed (Grjebine, 1966; Gillies and De Meillon, 1968; Gillies and Coetzee, 1987; Coetzee, 2020). The polymerase chain reaction (PCR) technique was also established to differentiate between morphologically identical or similar species, like observed for the *An. gambiae* complex and *An. funestus* group (Scott et al., 1993; Koekemoer et al., 2002).

Africa is home to 150 *Anopheles* species, including members of the *An. funestus* group and the *An. gambiae* complex, known for their involvement in malaria transmission (Figure 1.1). Malaria vectors can be divided into secondary and primary malaria vectors, depending on their role in malaria transmission.

1.2.2 Secondary malaria vectors

Secondary malaria vectors are species that contribute to malaria transmission, however to a lesser extent, usually contributing less than 5% of the transmission in a given geographical setting (Afrane et al., 2016; WHO, 2019[a]). Secondary malaria vectors are not efficient in transmitting malaria because of their behaviour, most are exophilic (resting outdoors), exophagic (bite outdoors), and zoophilic (prefer animals) (Antonio-Nkondjio et al., 2006; Durnez and Coosemans, 2013; Killeen, 2014; Chaccour and Killen, 2016; United States Agency for International Development (USAID), 2020). They become crucial under residual malaria transmission settings because they are difficult to control using the current vector control methods such as LLINs and IRS (Gillies, 1964; Antonio-Nkondjio et al., 2006; Meza et al., 2019). In sub-Saharan Africa, *An. merus*, *An. melas* and *An. bwambae*, belonging to *An.*

gambiae complex are regarded as minor or secondary malaria vectors (Gillies and Coetzee, 1987; Hunt et al., 1998; Sinka et al., 2010; Brooke et al., 2013; Coetzee et al., 2013[a]). Within the *An. funestus* group, *An. rivulorum* (Gillies and Smith, 1960; Wilkes et al., 1996; Temu et al., 2007; Kawada et al., 2012; Derua et al., 2015), *An. parensis* and *An. vaneedeni* (Temu et al., 2007; Burke et al., 2017; Burke et al., 2019) have been reported to be infected with *P. falciparum* and can circumstantially be classified as minor vectors. *Anopheles leesoni* has also been implicated as a secondary malaria vector by Temu et al., (2007). In addition, *An. pharoensis* (Gillies, 1964), *An. coustani* (Lobo et al., 2015), *An. ziemanni* (Vincke and Jadin, 1946) and *An. squamosus* (De Meillon, 1947) have been implicated as secondary vectors.

1.2.3 Main malaria vectors

The main malaria vectors are responsible for 95% of malaria transmission in Africa (Mouchet et al., 2004). The six main malaria vector species in Africa are *An. gambiae s.s.* (called *An. gambiae* going forward), *An. arabiensis, An. coluzzii, An. funestus, An. nili,* and *An. moucheti* (Ingram et al., 1927; Gillies and De Meillon, 1968; Sinka et al., 2012; Amvongo-Adjia et al., 2018; Braack et al., 2020). *Anopheles gambiae, An. coluzzii* and *An. arabiensis* are the main vectors from the *An. gambiae* complex (Coetzee et al., 2013[a]), while *An. funestus* belongs to the *An. funestus* group.

Anopheles gambiae, An. coluzzii, An. arabiensis and *An. funestus* are co-dominant and co-exist across large parts of Africa (Figure 1.1) (Gillies and Coetzee, 1987; Hunt et al., 1998; Kaindoa et al., 2017[a]; Debrah et al., 2021). The main vector species in the *An. gambiae* complex contribute to most malaria transmission in Africa because of their adaptability to different ecosystems (Meza et al., 2019; Olabimi et al., 2021). Vectors that are adapted to feeding indoors (endophagic) and on humans (anthropophagic) like *An. gambiae*, *An. coluzzii* and *An. funestus* have the greatest potential as vectors because these behaviours improve the chance that they will transmit the malaria parasite. However, *An. arabiensis* will feed indoors as well as outdoors (exophagic) on both humans and animals (mainly cattle) (zoophagic) and will rest both indoors as well as outdoors (exophilic) (Mastbaum, 1957; Gillies and De Meillon, 1968; Onyabe et al., 2001; Muturi et al., 2006; Muriu et al., 2008).

The focus of this study is on *An. funestus*, and hence this species will be discussed further in more detail.

1.2.3.1 *Anopheles funestus*

Anopheles funestus is the main malaria vector predominant in east and southern Africa, but it is also found in the western and central regions of Africa (Gillies and De Meillon, 1968; Cohuet et al., 2003; Coetzee and Fontenille, 2004; McCann et al., 2014; Kaindoa et al., 2017[b]). *Anopheles funestus* has twelve African siblings: *An. rivulorum*, *An. leesoni*, *An. vaneedeni*, *An. parensis*, *An. confusus*, *An. aruni*, *An. fuscivenosus*, *An. brucei*, *An. funestus*-like, *An. rivulorum-like, An. longipalpis* type A, and *An. longipalpis* type C (Gillies and De Meillon, 1968; Gillies and Coetzee, 1987; Cohuet et al., 2003; Harbach, 2004; Spillings et al., 2009; Choi et al., 2012; Vezenegho et al., 2013; Dia et al., 2013; Mouatcho et al., 2018). Most of these species can be differentiated using genetic sequence differences (Koekemoer et al., 2002; Cohuet et al., 2003; Vezenegho et al., 2013).

Anopheles funestus is a highly efficient malaria vector (Coetzee and Koekemoer, 2013[b]). In the humid savannah of Burkina Faso, a study of malaria transmission by *An. funestus* observed a peak of the human biting rate (HBR) of 45 to 50 bites per person per night (b/p/n) (Dabiré et al., 2007). It was lower, but still high, in Nigeria, where HBR corresponded of 14.6 b/p/n (Oyewole et al., 2007). The infectivity rate (presence of malaria parasites in the mosquito salivary glands) of *An. funestus* varies from 1 – 2% (Oyewole et al., 2007). Seasonal differences (wet and dry seasons) did not result in significant differences in infection rates with 1.8% infection rate recorded during the rainy period (April-October) and 1.6% in the dry climate (November – March) in Nigeria (Oyewole et al., 2007). In Kenya infection rates varied between 1 – 2% (Debrah et al., 2021). However, large variation in the entomological inoculation rate (EIR) between seasons was observed in Cameroon. The EIR varied from 2.6 infective bites per person (ib/p) in the dry season to 12.8 ib/p in the rainy season (Cohuet et al., 2004). *Anopheles funestus* was the major malaria vector, responsible for 88% of malaria transmission with a 6.5% *P. falciparum* infection rate, which confirmed its role as a dominant malaria vector in Cameroon (Cohuet et al., 2004).

1.2.3.2 Anopheles funestus habitat and adult distribution

As *An. funestus* is a major malaria vector, the knowledge of its habitat, distribution, and behaviour is essential to formulate appropriate vector control strategies (Rozendaal, 1997).

Anopheles funestus habitat:

The larval and adult habitat can be characterised by factors such as elevation above sea level, temperature, and rainfall, as these can be species-specific for Anopheline species

(Minakawa et al., 1999; Afrane et al., 2005; MacHault et al., 2009; Williams and Pinto, 2012; Rejmánková et al., 2013; Emidi et al., 2017; Ondiba et al., 2019). Successful *An. funestus* sites have been reported to mainly range from 309 m to 1 531 m above sea level in Burkina Faso, Kenya, Tanzania, Madagascar, and Mali (Cohuet et al., 2003; Dabiré et al., 2007; Emidi et al., 2017; Kaindoa et al., 2018; Debrah et al., 2021). Furthermore, *An. funestus* favours tropical climates (Sinka et al., 2010; Cohuet et al., 2004) with mean temperatures ranging from 20°C to 35°C in Cameroon, Tanzania, and Kenya (Cohuet et al., 2004; Kaindoa et al., 2017[b]; Debrah et al., 2021). Generally, *An. funestus* transmits malaria where annual rainfall ranges from 600 mm to 2 000 mm (Cohuet et al., 2004; Niain'ny Felamboahangy, 2010; Seyoum et al., 2012; Emidi et al., 2017; Debrah et al., 2021).

Environmental factors influence the choice of *An. funestus* larval sites. Larval sites may be natural (swamps, edges of lakes, pools in streams, slow-moving rivers) or human-made (ponds, rice fields, brick pits (Walker and Lynch, 2007; Gillies and De Meillon, 1968; Mwangangi et al., 2007; Williams and Pinto, 2012; Debrah et al., 2021 Marrama et al., 1995; Niain'ny Felamboahangy, 2010; Toure, 1982; Emidi et al., 2017; Nambunga et al., 2020). The permanency of *An. funestus* larval sites vary from semi-permanent wetlands to permanent clear freshwater bodies (containing organic matter, mineral salts, algae), and small streams/ rivers in low-lying and irrigated areas. Vegetation that provides light shade is a common feature on most *An. funestus* larval habitats.

Human impact due to agricultural practices, like the establishment of rice fields, generate additional artificial larval sites for this species. These large water bodies unintentionally increase malaria transmission (Gillies and De Meillon, 1968; Walker and Lynch, 2007; Nambunga et al., 2020; Debrah et al., 2021). In Kenya, *An. funestus* larval sites were characterised and of these, 36% were artificial sites (Debrah et al., 2021). Debrah et al. (2021), found that the presence of rice fields intensified malaria transmission. Understanding the physiochemical characteristics (e.g. pH, microbiological composition, etc.) of these artificial larval sites is therefore important so their impact on oviposition choice, larval development, survival, and abundance of *Anopheles* species can be predicted (Garba and Olayemi, 2015).

Adult distribution:

Anopheles funestus is widespread in Africa as shown in Figure 1.4 (Kyalo et al., 2017; Irish et al., 2020). Reviews of the geographical distribution of this species in the African region

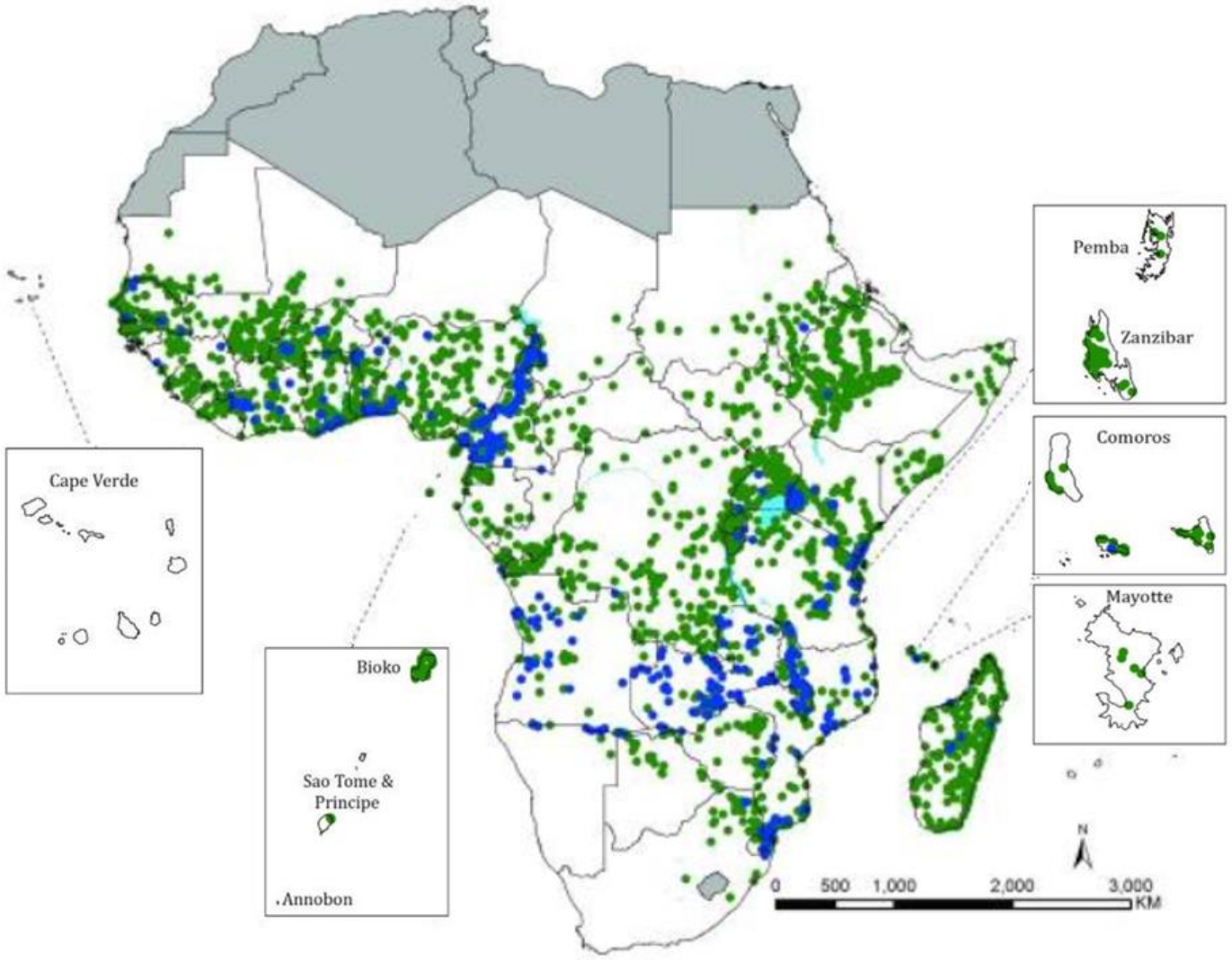

Figure 1.4: Distribution of *An. funestus* in Africa and surrounding islands – *An. funestus* group (green), *An. funestus* s.s. (blue) *(Source Kyalo et al., 2017)*

spanning the years 1900 – 2016 showed its existence in southern, eastern, central, and west Africa and the surrounding islands (Gillies and De Meillon, 1968; Tchen et al., 2006; Kyalo et al., 2017; Irish et al., 2020). *Anopheles funestus* occurs in sympatry with other species of the *An. funestus* group as observed in Burkina Faso (*An. leesoni* and *An. rivulorum-like* in Soumousso) (Dabiré et al., 2007). In Zambia, *An. funestus* was reported to be sympatric with *An. rivulorum*, *An. parensis*, *An. vaneedeni* and *An. leesoni* (Figure 1.4) (Seyoum et al., 2012; Meza et al., 2019). However, *An. funestus* can also occur in sympatry with members of *An. gambiae* complex or *Culex sp*. as was reported in Tanzania (Russell et al., 2011; Kaindoa et al., 2017[b]), Zimbabwe (Sande et al., 2015) and Kenya with *An. rivulorum*, *An. arabiensis*, *An. gambiae*, *Culex sp*, *An. coustani* and *An. funestus* occupying the same niche (Debrah et al., 2021).

1.2.3.3 Anopheles funestus adult behaviour

The widespread distribution of *An. funestus* in Africa highlights its importance in malaria transmission and it is, therefore, important to understand its behaviour to design malaria vector control strategies. One needs to be aware of preferred feeding and resting behaviours, as well as their breeding time and place. This knowledge will assist in choosing appropriate vector control methods (WHO, 1975; Curtis, 2002).

A blood meal ELISA (Beier et al., 1988) or molecular DNA methods (Kent and Norris, 2005) can be used to determine the blood meal source. In *An. funestus*, the human blood index fluctuates between 75% and 100%. However, designs and analyses vary between studies and this needs to be taken into consideration when interpreting these results (Fontenille et al., 1997; Cohuet et al., 2004; Kaindoa et al., 2017[b]; Meza et al., 2019; Debrah et al., 2021).

Adult female biting behaviour varies according to the biotope or environment but in general, *An. funestus* feeds when people are in their deepest sleep. This means their feeding on humans often goes undisturbed, increasing the mosquitoes' chances of transmitting malaria (Gillies and De Meillon, 1968; Seyoum et al., 2012; Amvongo-Adjia et al., 2018). This is supported by a study in Nigeria, where *An. funestus* peak biting rate was observed at 03:00 am indoors and 8:00 pm outdoors (Oyewole et al., 2007). Similarly, *An. funestus* were found feeding indoors in Cameroon between 01:00 am and 6:00 am (Amvongo-Adjia et al., 2018). After feeding, *An. funestus* females tend to rest inside houses (endophilic) (Gillies and De Meillon, 1968; Charlwood et al., 2003; Dabiré et al., 2007; Oyewole et al., 2007; Gatton et al.,

2013). However, a few studies have reported exophagic behaviour in An. funestus. In Tanzania, the proportion of indoor b/p/n was 60.8% in 1997, and this decreased to 29.8% in 2009, equating to a 50.5% decrease in the proportion of indoor contact with *An. funestus* indicating possible behavioural adaptation (Russell et al., 2011).

The resting and feeding behaviours of *An. funestus* females are relatively well documented, however, female mating behaviour and mating in general are understudied. *Anopheles funestus* is eurygamic (mate in flight) (Gillies and De Meillon, 1968). The female will enter a male swarm (males aggregating in flight in a large group) and mate with a male (Tripet et al., 2003; Ng'habi et al., 2008; Howell and Knols, 2009; Magnusson et al., 2011). Only a few studies to date have reported information on swarming in *An. funestus* (Harper, 1944; Charlwood et al., 2003; Zawada et al., 2018; Kaindoa et al., 2019). Mating occurs approximately 11 min after the beginning of swarming (Charlwood et al., 2003). Male *An. funestus* swarms were reported to occur 2 – 4 m above the ground (Charlwood et al., 2003; Zawada et al., 2018). In a subsequent study in Tanzania, swarming heights were confirmed to be 1.7 m above ground and lasted for 12.9 min (7.9 – 17.9 min) (Kaindoa et al., 2019). Swarming re-occurred repeatedly in the same location (Zawada et al., 2018; Kaindoa et al., 2019). Understanding male swarming might provide an opportunity to target adult males to reduce the reproductive fitness in *An. funestus* (Howell and Knols, 2009). *Anopheles funestus* females are monogamous, meaning that mating happens once during a female's lifetime. The spermatozoa from the male is kept in the female spermathecal capsule and eggs are fertilised as they are oviposited (Tripet et al., 2003).

1.3 MALARIA CONTROL

1.3.1 Disease control

Considering the impact of malaria on the economy and health, its prevention, treatment, and control are essential. Vector control, malaria case diagnosis and management (treatment), are the key elements for successfully controlling malaria. Malaria control aims to reduce the disease's incidence, prevalence, morbidity, and/or mortality (WHO, 2019[a, b]). Malaria is not yet eliminated due to various challenges of implementing and maintaining disease control. These challenges include insecticide resistance (discussed in section 1.3.1.2), drug resistance, and the unavailability of vaccines. Several countries have reported therapeutic failures caused by resistance in the parasite (Dondorp et al., 2009; Ashley et al., 2014; Gobbi et al., 2016; Adegbite et al., 2019; WHO, 2021[a]). Partial resistance to artemisinin, where artemisinin-based combination therapies (ACTs) are used, has been observed in Africa (WHO, 2021[a]). For

example, a study on the efficacy, tolerability, and safety of arthemether-lumefantrine (AL) and artesunate-amodiaquine, drugs, which were used to treat uncomplicated malaria caused by *P. falciparum* in Gabon from October 2017 to March 2018, showed an efficacy rate of 97% and 95% in children for those years, respectively (Adegbite et al., 2019). Unfortunately, from 2017 – 2019 in Wuhan, China, a cross-sectional study of migrants from Africa showed evidence of AL resistance. However, chloroquine, amodiaquine and mefloquine were still effective (Cheng et al., 2021). Drug resistance can be detected through parasite surveillance and screening for the mutation associated with treatment failure (Hussien et al., 2020; Cheng et al., 2021).

Research into malaria vaccine development has increased over the past decades, but remains complicated (Hoffman et al., 2015). The first generation of a purified recombinant circumsporozoite protein vaccine, the malaria candidate vaccine (RTS,S/AS01 vaccine) is promising in preventing malaria cases and deaths. This vaccine gives protection for one year (Stoute et al., 1997; Garcon et al., 2007). An evaluation of RTS,S/AS01 vaccine is in phase three with trials having been carried out in eight African countries targeting infants and children aged from six weeks to 17 months (Kaslowa and Biernaux, 2015). Based on the outcome of the trials, the WHO called for the implementation of RTS,S/AS01 in pilot sites in 2016 in the Sub-Saharan Africa region. Results of the trials showed that the efficacy of vaccine RTS,S/AS01 was comparable to the use of four annual chemoprevention treatments with sulfadoxine-pyrimethamine (SP) and amodiaquine (AQ). The malaria candidate vaccine (RTS,S/AS01), is now available and recommended by WHO to prevent malaria disease in children living in countries with moderate to high malaria transmission (WHO, 2021[a]).

Furthermore, visitors travelling to malaria-endemic areas should also use appropriate chemoprophylaxis to prevent malaria infection (Behrens et al., 1998; WHO, 2014; Rodrigues et al., 2019). The use of repellents from early evening and sleeping under LLINs is also recommended as a personal protection method (WHO, 2014). This study is focused on *An. funestus*, one of the most efficient malaria vectors, therefore the next section will explore challenges in malaria vector control.

1.3.2 Vector control

The WHO is actively promoting the use of LLINs as well as IRS for vector control interventions, amongst others (WHO, 2020). Malaria vector control aims to prevent malaria transmission by reducing contact of infected malaria vectors with humans, by killing the

mosquitoes that transmit *Plasmodium* species (WHO, 2019[a,b]). Different vector control methods have been available since the beginning of the 20th century and some are still being used today (Swellengrebel et al., 1931; Walker and Lynch, 2007; Raghavendra et al., 2011; Benelli and Beier, 2017; Derua et al., 2019; WHO, 2020). Personal protection, wearing long sleeved clothes for example, prevents being bitten by mosquitoes. The individual protection in vector control has been enhanced with the use of insecticide (permethrin, pirimiphos-methyl), or repellent (DEET (N, N-diethyl-meta-toluamide)) impregnated clothing (Pennetier et al., 2010; WHO, 2014; CDC, 2019). Personal safety and skin sensitivity should be monitored when using these products. In addition, the emergence of insecticide resistance when using these products should be considered (Pennetier et al., 2010). However, the cornerstone of malaria vector control remains the use of IRS and LLIN (McCarroll and Hemingway, 2002; Brooke et al., 2013; WHO, 2020).

Mass distribution of LLINs in endemic countries alone or in combination with IRS have reduced malaria transmission significantly (Protopopoff et al., 2018; WHO, 2019[a]; Bhatt et al., 2015). However, insecticide resistance continues to pose a problem for malaria control programmes which rely heavily on insecticide-based approaches such as LLINs and IRS. It is therefore important that non-insecticide-based methods like the sterile insect technique (SIT), gene drive initiatives, and the use of biopesticides are to be considered in an integrated vector control strategy to supplement these standard vector control methods.

The use of biopesticides like the entomopathogenic fungi, *Metarhizium anisopliae* and *Beauveria bassiana* spores, to target adult mosquitoes have shown efficacy (Howard et al., 2010). Both of these fungi species have been evaluated under laboratory and field conditions (Scholte et al., 2004; Howard et al., 2010; Howard et al., 2011; Lee et al., 2019). Research into this area is still ongoing and a further five novel entomopathogenic fungi belonging to *Galactomyces sp*, *Isaria sp*, and *Mucor sp*. have been shown to cause mortality in *An. gambiae* (Accoti et al., 2021). In addition, biopesticides targeting mosquito aquatic stages have traditionally been used for larval source management (LSM). Several reviews of effectiveness and the operational feasibility, followed by effective implementation in the field of larval biopesticides, have been done in Sub-Saharan Africa and Asia (Bond et al., 2004; Walker and Lynch, 2007; WHO, 2013; Derua et al., 2019; Dambach et al., 2020). Biopesticides such as bacterial larvicides: *Bacillus thuringiensis subsp. israelensis* (*Bti*), *Bacillus spaericus* (*Bs*), and *Spinosad* played a significant role in controlling vectors (Killeen et al., 2002; Howard et al.,

2010; WHO, 2013; and see reviews: Thavara et al., 2009; Nartey et al., 2013; Derua et al., 2019; Gimnig et al., 2020).

Larval source management also extends to physical environmental management such as cleaning and modifying potential larval sites (Kelly-Hope et al., 2009; WHO, 2013). In addition, community education plays a central role in LSM. In the agricultural sector (rice cultivation, etc.) farmers can be educated on their role in generating artificial larval sites for vector species. The use of irrigation agro-systems with fast water flow rates might be effective in clearing or preventing mosquito larval development in rice fields.

An additional possible vector control strategy is the push and pull strategy. This involves ensuring the availability of cattle near or in human homes to attract mosquitoes and reduce the number that bite humans in areas where both cattle and humans are present (Muriu et al., 2008). On the contrary, Zeru et al. (2020) showed that the presence of cattle (called bovine blood in remaining document) near the home can increase the likelihood of humans becoming infected with malaria. Therefore, these strategies should be evaluated further to indicate their effectiveness in malaria prevention (Muriu et al., 2008; WHO, 2013; Zeru et al., 2020).

1.3.2.1 Challenges with insecticide resistance

According to the WHO, insecticide resistance is defined as "*the development of an ability in some individuals of a given organism to tolerate doses of a toxicant which would prove lethal to the majority of individuals in a normal population of the same organism*" (WHO, 1957; Cochran, 1995; WHO, 2012). To detect insecticide resistance, susceptibility assays have been developed by WHO (WHO, 2012; 2018; 2022[a]). These consist of exposing mosquitoes to a definite diagnostic concentration of insecticide for a predetermined period. Mortality, with a few exceptions, is then recorded 24 hours after insecticide exposure (WHO, 2020). Mortality higher than 98% indicates susceptibility to the insecticide and if below, then resistance is reported (WHO, 2020).

Insecticide resistance is of major concern, as only five classes of insecticides are available and recommended by WHO for malaria vector control. The most common classes used for IRS are organophosphates, pyrethroids and carbamates. Recently clothianidin, a neonicotinoid insecticide, was approved for IRS (Ngufor et al., 2017; WHO, 2018; Agumba et al., 2019; WHO, 2021[a]; Zoh et al., 2021). Pyrethroids are the most cost effective and have a low level of toxicity in mammals and a residual efficacy of approximately three months

(Chrustek et al., 2018). Pyrethroids are mainly used for LLINs, but other classes of insecticides are also now being used (WHO, 2006; 2012; 2021[a]). Pyrethroids occupy 25% of the world's insecticide market for all sectors including the agricultural sector, households, forestry, and horticulture, and this is the insecticide class most used for preventing malaria in Africa (Bradberry et al., 2005; Soderlund, 2012; Costa, 2015; Ngufor et al., 2017).

The organochlorine dichloro-diphenyl-trichloroethane (DDT) is still available for public health but very few countries still use this class for malaria control (WHO, 2012; 2018). In Côte d'Ivoire, larval and adult resistance to DDT and permethrin was observed (Elissa et al., 1994). Insecticide resistance was also observed with the use of carbamate (CX) and organophosphate (OP) insecticides to control malaria vector (Alout et al., 2008). A mutational resistance to DDT and pyrethroids was observed in western, and central Africa (Jones et al., 2012; Winkler et al., 2012; Fossog Tene et al., 2013; Djègbè et al., 2014; Edi et al., 2017; Lynd et al., 2018) and east Africa (Stump et al., 2004; Kabula et al., 2012; Kaindoa et al., 2017).

Judicious control of insecticide resistance can reduce or prevent the spread of insecticide resistance (WHO, 2012; 2021[a]). It consists of a number of strategies including rotation between different insecticide classes, mosaic application of insecticides i.e. simultaneous use of two different classes in the same area, or involves combining two or more insecticides with different modes of action (WHO, 2012; Benelli and Beier, 2017; Zhou et al., 2021[a]). Due to the limited number of insecticides available and the lack of effective insecticide resistance management strategies resulting in selection pressure most countries face insecticide resistance challenges. From 2010 to 2020, 28 out of 88 malaria-endemic countries reported multiple insecticide resistance while 78 countries confirmed insecticide resistance in at least one malaria vector (WHO, 2021[a]). An example of such countries includes Tanzania, where multiple insecticide resistance (to pyrethroids, dieldrin and DDT) was observed in *An. funestus* (Kaindoa et al., 2017[b]; Kaindoa et al., 2019). Due to the focus on *An. funestus* in this thesis, insecticide resistance in this species will be discussed in more detail.

1.3.2.2 Anopheles funestus insecticide resistance

Anopheles funestus has exhibited insecticide resistance since the 20th century (Toure 1982; Brown, 1986; Hargreaves et al., 2000; Coetzee and Koekemoer., 2013[b]; Chanda et al., 2020). The first report of insecticide resistance in *An. funestus* was in the 1970s when resistance to organophosphates was identified in western Africa, central Africa, and eastern Africa (Toure 1982; Brown, 1986). Since then, the reported geographical range of insecticide resistant

populations of *An. funestus* has expanded to include countries in southern Africa. In southern Africa, insecticide resistance to pyrethroids and carbamates in *An. funestus* was first reported in 2000 (Hargreaves et al., 2000; Hunt et al., 2010; Coetzee and Koekemoer., 2013[b]; Chanda et al., 2020). It expanded between 2000 and 2009, with increased resistance to pyrethroids was reported in Mozambique and South Africa (Brooke et al., 2001; Casimiro et al., 2007; Cuamba et al., 2010; Kloke et al., 2011). Similarly, a survey in Zambia between 2009 – 2010 showed pyrethroid and DDT resistance in this species, (Chanda et al., 2011). Further follow up studies since the first report of insecticide resistant populations of *An. funestus* in west Africa showed that this species has developed resistance to DDT (83.5% mortality) and pyrethroids (94.5%) (Anto et al., 2009) and in Benin, it was extremely resistant to DDT (Djouaka et al., 2011).

Evaluation of the resistance mechanisms to pyrethroid, carbamate, and DDT insecticides in *An. funestus* have been conducted and reported (WHO 2006; Chrustek et al., 2018; WHO, 2021[a]). Numerous studies to determine the mechanisms of resistance were subsequently carried out (Coetzee and Koekemoer, 2013[b]; Chanda et al., 2020; Sandeu et al., 2020; Matowo et al., 2021). These mechanisms include different point mutations according to the insecticide class (Feyereisen, 1995) or biochemical mechanisms inducing modification of binding protein sites (Jurat-Fuentes et al., 2021). Mosquito behavioural changes, like the changes in biting hours after using pyrethroids on LLINs were observed (Sougoufara et al., 2014). This highlights the broad extent of insecticide resistance in *An. funestus*, which may affect the future sustainability of insecticide-based malaria vector control (Gnankiné et al., 2013). It is, therefore, imperative to investigate additional vector control tools for this species.

1.3.2.4 Additional vector controls methods

Some additional vector control tools are novel or still under evaluation and include the genetic transformation of vectors. These genetic changes vary and include prevention of mosquitoes from transmitting the malaria parasite to humans by driving mosquitoes refractory to infection into the population as well as sex distortion through driving a male-biased sex-distorter into the natural population (producing unisex populations that eventually collapse). The study on transgenic mosquitoes for a malaria vector control strategy was initiated as early as 2003 with the aim of either replacement or suppression of wild vector populations (Moreira and Jacobs-Lorena, 2003; Knols et al., 2007). These types of interventions are appropriate for use in a gene drive approach to spread the transformed genes through the wild population (Curtis and Townson, 1998; Burt et al., 2018; Adolfi et al., 2020; Hammond et al., 2021; Kranjc et al., 2021). In addition, the use of non-transgenic approaches like SIT, has provided further

options for vector control (Curtis and Townson, 1998; Helinski et al., 2006; Munhenga et al., 2011; Oliva, 2012; Mashatola et al., 2018). The following section will give information about the SIT, as this is important for the scope of this thesis.

1.4 BACKGROUND ON THE STERILE INSECT TECHNIQUE

Insect control is becoming more challenging in both the health and agricultural sectors due to insecticide resistance. Non-chemical vector control methods that are environmentally acceptable, such as the sterile insect technique (SIT) are ideally suited to address this gap (Knipling, 1955; Curtis, 1971; Parker and Mehta, 2007). The SIT, applied as part of the area-wide integrated pest management (AW-IPM) approach offers an environmentally friendly control intervention (Klassen, 2005; Catteruccia et al., 2009; Nolan et al., 2011). The history of SIT started in the 1940's when Serebrovsky (1940) from the Soviet Union and Knipling (1955) from America started to work on SIT for the control of the new world screwworm (NWS), *Cochliomyia (C.) hominivorax*. This pest caused serious livestock damage in America (Bushland and Hopkins, 1953; Knipling, 1955; Lindquist, 1955). Using SIT, this species was successfully eradicated in 1954 on a Caribbean island called Curaçao (Lindquist, 1955; Knipling, 1985). Subsequently the NWS was eradicated from Florida and the southeast United States in 1959 (Lindquist, 1963; Wyss, 2000; Baumhover, 2002).

SIT generally involves the mass-rearing of the target insect species, the removal of female insects (sex separation), and the sterilisation of males by irradiation (Dame et al., 1981; Klassen, 2005; Dunn and Follett, 2017). The sterile males are then released at a specific ratio to the targeted species in nature (wild population). This release ratio is typically high (over-flooding ratio) to ensure that the sterile males inundate the target population and outcompete fertile wild males for successful mates with the wild females (Dame et al., 1981; Klassen, 2005; Dunn and Follett, 2017). The females mated to the sterile males will produce sterile offspring. The systematic release of sufficient numbers of sterile males in a target area, for a specified period, will reduce, suppress or eliminate the target pest (Dame et al., 1981).

As mentioned above, the well-known SIT success story involved a 45-year area-wide SIT campaign for the suppression and finally the eradication of NWS in the United States of America, Mexico, North America, central America, South America, and north Africa (Wyss, 2000; Vargas-Terán et al., 2005). This eradication resulted in a huge economic benefit of up to ≥ US$ 1.3 billion to the livestock producers (Vargas-Teran et al., 2005; Skoda et al., 2018). Another successful example from the crop-producing industry includes the eradication of the

Mediterranean fruit fly *(Ceratitis capitata*) in California, Chile, and some parts of central America (Klassen and Curtis, 2005). These results demonstrate the incontestable success of SIT in the agricultural sector, regardless of the pest species, and provided support for using this technology in the public health sector. The eradication of the tsetse fly, *Glossina austeni*, observed in 1996 in Zanzibar using SIT was one of the first success stories for public health (release of sterile males started in August 1994) and improved the general economy of Zanzibar (Vreysen et al., 2000; Feldmann et al., 2013). The success of the tsetse fly eradication programme is noted as the incidence of trypanosomiasis disease decreased from up to 25 000 cases in the year 2000 to 7 106 in 2012 and less than 1 600 in 2017 (Simarro et al., 2015; Franco et al., 2017; WHO, 2017; Feldmann et al., 2021). In Africa, the number of human African trypanosomiasis (HAT) cases is lower than the target set in WHO roadmap for HAT elimination (Franco et al., 2020).

1.4.1 SIT for mosquito control

Learning from the SIT's success on livestock pests and fruit flies (Knipling, 1985; Wyss, 2000; Klassen and Curtis, 2005; Vargas-Terán et al., 2005; Catteruccia et al., 2009; Nolan et al., 2011; Bassène et al., 2017; De Beer et al., 2020; Vreysen et al., 2021; Mastrangelo et al., 2021), the first successful SIT pilot project with mosquitoes was conducted in the 1970s, against the malaria vector, *An. albimanus* in El Salvador (Breeland et al., 1974; Dame et al., 1974; Lofgren et al., 1974; Weidhaas et al., 1974; Dame et al., 2009). One hundred percent sterility was established in a wild population with a 99% reduction of the *An. albimanus* population. Five months after releasing sterile males, a reduction in parasite transmission in 97% of the targeted population was achieved (Seawright et al., 1978; Dame et al., 1981; Benedict and Robinson, 2003). Unfortunately, the work stopped and four months later, the density of the mosquitoes was similar to the density before the releases (Lofgren et al., 1974).

Detailed baseline studies to fill knowledge gaps is necessary before SIT can be fully implemented against mosquito species (Weidhaas et al., 1962; Dame et al., 1964; Breeland et al., 1974; Klassen, 2005; Dame et al., 2009). This includes, amongst others, the development of mosquito mass-rearing protocols and determination of the irradiation doses required for male sterilisation. The dynamics of the targeted population is species-specific and therefore, SIT needs to be developed specifically for each target species (Yahouédo et al., 2014; IAEA 2017). Furthermore, SIT requires multi-year planning, commitment and coordination among stakeholders and, importantly, public acceptance studies are needed prior to implementation of this technology (Oliva et al., 2014; Klassen et al., 2021). Apart from the research activities

during the development of SIT for a vector control intervention, as detailed above, community engagement should accompany the implementation of SIT (Oliva, 2012; Oliva et al., 2014; Guissou et al., 2020; Manana et al., 2021).

The preliminary studies on SIT against mosquito vector species now includes studies on various species such as *Aedes albopictus* (*Ae. albopictus*) (Bellini et al., 2007; Zhang et al., 2018), *An. arabiensis* (Helinski et al., 2006; Helinski et al., 2008; Munhenga et al., 2011; Oliva, 2012; Munhenga et al., 2016; Mashatola et al., 2018) and *An. gambiae* (Davidson et al., 1970; Yahouédo et al., 2014). These studies are about trials, irradiation and dose-response competition. Currently there are 34 mosquito SIT pilot site trials around the world spanning four species: *Ae. aegypti, Ae. albopictus, An. arabiensis, An. coluzzii* (Bouyer and Vreysen, 2020). In 1999, one of the earlier trials was conducted in Italy, against *Ae. albopictus* (Bellini et al., 2007). This study released approximately 1 000 – 1 500 sterile males pupae per hectare, per week, over five years (2005 – 2009) (Bellini et al., 2007). This resulted in the reduction of female fertility of up to 70% of the target population which had a positive impact on reducing population density (Bellini et al., 2013). In Africa, there are two pilot trials, one in La Reunion Island and the other in Mauritius, both to control *Ae. albopictus* populations. These trials were promoted against a backdrop of an outbreak of chikungunya and dengue in the southwest Indian Ocean region in 2005 – 2006 (WHO, 2006). During the Mauritian trials, irradiated sterile males were released at the pilot site to reduce the population size of the target species (Iyaloo et al., 2014; Iyaloo and Facknath, 2017). The field trial showed the possibility of integration of SIT as a vector control tool based on the reduced egg production in the release site. For this study, data was recorded weekly from the beginning of implementation of the sterile male releases, the only mosquito control in the pilot site within specific eco-climatic conditions and within a low-density pest population (Iyaloo et al., 2019[a]; Iyaloo et al., 2019[b]; Iyaloo et al., 2020). Researchers from La Reunion island also aimed to implement SIT for the control of *Ae. albopictus* and a road map for designing, implementing, and evaluating a pilot site was developed (Gouagna et al., 2020; Oliva et al., 2021). The work is still ongoing.

SIT against malaria vectors has not yet been implemented as a vector control intervention, however, as mentioned above, various research projects have been initiated, in Sudan, Mauritius, La Reunion island, and South Africa (Helinski et al., 2006; Munhenga et al., 2011; Oliva, 2012; Ageep et al., 2014; Mashatola et al., 2017; Mashatola et al., 2018). A feasibility study of SIT for *An. arabiensis* was initiated in Sudan in 2006, however, political instability in the country has affected its progress (Helinski et al., 2006[a,b]; Helinski et al., 2008;

Malcolm et al., 2009, Azrag et al., 2016), resulting in its temporary stoppage in 2017 before sterile males could be released. However, before the stoppage, a mark-release-recapture of *An. arabiensis* was conducted using in insectary reared and irradiated males. The results showed that irradiated males were participating actively in swarms over three consecutive nights. This meant that the sterile males from the laboratory were ready to mate with wild females and might be effective in an SIT programme (Ageep et al., 2014; Hassan et al., 2014). These early initiatives in the northern state of Sudan affirmed positive lessons and acted as knowledge building for technology against malaria vectors (Azrag et al., 2016; Ali et al., 2018; Elaagip and Adedapo, 2022). Similar studies were initiated in South Africa in 2011 (Munhenga et al., 2011). This study started with the identification of field sites, long term longitudinal entomological surveillance on *An. arabiensis* between the target (SIT intervention) and control (no intervention) sites, population size estimation, as well as the development of quality control, protocols for mass-rearing, sex-separation and sterilisation (Munhenga et al., 2016; Dandalo et al., 2017; Dandalo et al., 2018; Mashatola et al., 2017; Mashatola et al., 2018; Kaiser et al., 2021). This provided the basis for a pilot trial in South Africa and this is one of the first countries that has undertaken pilot releases of sterilised *An. arabiensis* in 2021/2022 (Munhenga et al. unpublished).

1.5 RATIONALE FOR THIS STUDY

Several research activities have been initiated to develop additional vector control interventions against the main African malaria vectors, including SIT for *An. arabiensis* (Munhenga et al., 2011; Oliva et al., 2012; Munhenga et al., 2016; Mashatola et al., 2018) and gene drive initiatives for *An. gambiae* (Tabachnick et al., 2003; Assogba et al., 2010; Nolan et al., 2011; McArthur et al., 2014; Hammond et al., 2016; Bernardini et al., 2018; Dong et al., 2020; Kranjc et al., 2021). However, these interventions will have limited impact on the eradication of malaria unless all the main African malaria vectors, including *An. funestus*, are targeted. The lack of research into SIT for *An. funestus* is due to several challenges, including the difficulty in colonising this species, the lack of knowledge of colonisation parameters and optimal rearing conditions (Gillies and De Meillon, 1968). This study was, therefore, initiated to address knowledge gaps in *An. funestus* laboratory rearing that currently exist. In addition, the optimal irradiation dose range needed to sterilise *An. funestus* males without affecting their fitness or mating competitiveness is unknown and will be determined. Filling these gaps will allow further studies into the feasibility of using SIT against *An. funestus*. This will increase the number of control tools already in place and can help circumvent the problem of insecticide

resistance (Hargreaves et al., 2000; Hunt et al., 2005; Dabiré et al., 2007; Coetzee and Koekemoer, 2013[b]; Kaindoa et al., 2017[b]; Kaindoa et al., 2019).

1.6 AIM AND OBJECTIVES

This study aimed to determine the parameters for successful laboratory rearing of *An. funestus* (Diptera: Culicidae) and irradiation dose-response of the species. These serve as initial steps to evaluate the potential of the sterile insect technique for vector control against this major African malaria vector.

To address this aim, the following five specific objectives were investigated:

1) Evaluating the impact of blood-feeding using a membrane feeding system on fecundity and fertility in colonised *An. funestus.*

2) Measuring the impact of different larval food doses on physiological and reproductive parameters (larval development, adult emergence, adult body size, fecundity, fertility).

3) Determining the optimum larval density that does not compromise the number of adults that emerge.

4) Determining the optimal irradiation dose for inducing sterility in *An. funestus* males.

5) Assessing mating competitiveness of irradiated *An. funestus* males against non-irradiated (fertile) *An. funestus* males.

CHAPTER 2

EVALUATING THE USE OF AN ALTERNATIVE BLOOD MEAL SOURCE FOR COLONISED *ANOPHELES FUNESTUS*

This chapter formed part of a publication (Niain'ny Felamboahangy et al., 2023; Appendix A, but more details are provided here).

2.1 INTRODUCTION

2.1.1 Background

The first report of an *An. funestus* laboratory colony was in 1954 and it was maintained for only three years (Service and Oguamah, 1958). During this time, *An. funestus* was regarded as eurygamic (did not like mating in confined spaces) and laboratory colonisation proved difficult (Gillies and De Meillon, 1968). According to the authors, sustainable colonisation failed due to insufficient egg production even though the females were offered blood from both humans and guinea pig (Service and Oguamah, 1958; Gillies and De Meillon, 1968). *Anopheles funestus* adult females, like other anophelines, are monogamous (Baimai and Green, 1987; Charlwood et al., 2003; Magnusson et al., 2011), and mate once in their lifetime (Tripet et al., 2003). They are also anautogenous and require a blood meal (vertebrate blood) to ensure egg production, development, and maturation (Briegel, 1990; Clements, 1992; Tripet et al., 2003; Hansen et al., 2014). Thus, a blood meal is a critical component for mosquito reproduction (Lyimo and Ferguson, 2009; Harrison et al. 2021). It is therefore, important to understand the nutritional composition of blood and how this impacts mosquito fecundity and fertility. In addition, how this blood is provided to mosquitoes is also a critical aspect during laboratory rearing conditions.

Vertebrate blood is composed of blood cells (45%) suspended in blood plasma or blood fluid (55%) (De Smet, 1978; Watson, 2005; Farley et al., 2012). Of the blood cells, the majority are red blood cells or erythrocytes (containing haemoglobin for oxygen transport). There are also white blood cells or leukocytes (known for protection against the invasion of foreign bodies) and platelets or thrombocytes (necessary for blood clotting) (Lewis, 1992; Watson, 2005; Munro, 2009; Farley et al., 2012; Celkan, 2020). The blood fluid (plasma) provides a medium to transport excretory products and, it constitutes mainly water (92% by volume), and proteins. Protein components in plasma include mainly albumin, amino acids (e.g. isoleucine) and haemoglobin, which are important in mosquito egg development and maturation (Briegel,

1985; Harrington et al., 2001). Although blood composition differs between vertebrates, there are similarities between them (Table 2.1).

Table 2.1: Comparison between human, guinea pig, avian, and bovine blood characteristics

Blood characteristics	**Human***	**Guinea pig****	**Avian *** (chicken/duck)**	**Bovine****
Clotting time	6 – 12 min	2.5 min	> 1 min	12.4 – 30.1 sec
Concentrations of coagulation factors in plasma: Factor I (fibrinogen)	303 (250 – 400 mg/dL of plasma	263 mg/dL of plasma	346 mg/dL of plasma	125 – 697 mg/dL of plasma
Erythrocyte ranges: red blood cell (RBC)	4 – 5.5 × 10^6/µL	4.93 × 10^{12}/µL	2.5 – 3.5 × 10^6/µL	4.9 – 7.5 × 10^{12}/µL
Haemoglobin concentrations (varies according to age and sex)	12 – 16 g/dL	13.5 g/dL	7 – 13 g/dL	8.4 – 12.0 g/dL
Leukocyte counts: white blood cell (WBC)	5 – 10 × 10^3/µL	10.2 × 10^3/µL	12 – 30 × 10^3/µL	5.1 – 13.3 × 10^3/µL
Platelet count	150 – 450 × 10^3/µL	434 × 10^3/ µL	7.78 – 10 × 10^3/µL	160 – 650 × 10^3/µL
Total protein	6.3 – 7.7 g/dL	5.78 g/dL	3.0 – 4.9 × 10^{-3} g/dL	7.5 g/dL
Albumin	3.2 – 4.4 g/dL	2.13 g/dL	1.17 – 2.74 g/dL	3.1 g/dL

Source: Bigland, 1961*** – Heuwieser et al., 1989**** – Lewis, 1992** – Meluzzi et al., 1992 *** – Bounous and Stedman, 2000*** – Kawamoto et al., 2003* – Wood and Quiroz-Rocha, 2010**** – Roland et al., 2014**** – Cecchinato et al., 2018 **** – Morrison et al., 2018* – Odunitan-Wayas, 2018****

Guinea pig blood and human blood share several similarities: concentrations of coagulation factors in plasma, erythrocyte ranges, haemoglobin concentrations and platelet count are all within the same ranges (Table 2.1) (Lewis 1992; Washington and Van Hoosier, 2012). The leukocyte counts of guinea pig blood compared to human blood overlap (Table 2.1). The total protein and albumin content of guinea pig blood is slightly lower than in human blood (Lewis, 1992). This explains why guinea pig blood has been used successfully under many

experimental conditions to provide an alternative blood meal source for mosquitoes in the absence of human blood (Kawamoto et al., 2003; Morrison et al., 2018) (Table 2.1). Although avian blood and bovine blood share some qualities with guinea pig blood, some characteristics differ greatly, e.g. the platelet count is much lower in avian blood compared to other blood (Table 2.1). The total protein content, as well as albumin of bovine blood, is similar to that in human blood (Heuwieser et al., 1989; Wood and Quiroz-Rocha, 2010; Roland et al., 2014; Odunitan-Wayas, 2018).

Factors such as the age or sex of the vertebrate host can also play a role in the quantity or quality of the blood components. Female animals have higher concentrations of haemoglobin than males (Anderson et al. 1930). In addition, Anderson et al. (1930) showed that the quantity of haemoglobin in mammal blood depended on the age of the animal (it decreased with age). Age impacts on the total protein content, which in guinea pig, stabilises around 30 days of age (1 month). However, other blood component concentrations (like albumin, haemoglobin, and glucose) decreased with age (Genzer et al., 2019). These studies highlight the complex role of blood-meals in mosquito feeding (Anderson et al., 1930; Genzer et al., 2019). Researchers often compare feeding rates and fecundity in mosquitoes using different hosts, this will be debated below.

The blood-feeding rates (proportion of females that blood-feed) of female *An. dirus*, *An. cracens* and *An. minimus* fed on live guinea pig and direct feeding by humans were compared and no significant differences were reported (Phasomkusolsil et al., 2013). However, the proportion of females that blood-fed alone does not paint the full picture. Nutritional composition of blood can influence mosquito fecundity and consequently mosquito productivity. The impact of blood nutritional composition and source of the blood is generally overlooked during maintaining mosquito colonies under laboratory conditions. It is established that high-packed red cell volume in blood and nucleated red blood cells containing haemoglobin, improve egg production (Bennett, 1970). Therefore, blood-feeding females on avian blood can result in the same fecundity (number of eggs) as when fed on guinea pig or human blood since the range in haemoglobin composition of these three hosts overlaps (Table 2.1) (Bennett, 1970; Phasomkusolsil et al., 2013; Stier et al., 2013; Takken and Verhulst, 2013). The quality of blood is known to deteriorate as blood ages. In a study in which *An. dirus* and *Ae. aegypti* females were fed human blood 15 days post its expiry date and compared to females that were fed on blood that was 10 days post its expiry date, a marked decrease in fecundity was observed when females were fed the older blood (Pothikasikorn et al., 2010).

The reduction in blood quality with age of blood is due to depletion of red blood cells (RBC) and essential nucleic acids (e.g. adenine nucleotides) as well as RBC fractionation as blood ages (Hosoi, 1959; Sawant et al., 2007).

Several studies have shown that the blood meal source does not result in significant differences in fecundity and fertility. Gunathilaka et al. (2017) compared the fecundity of *Ae. aegypti* females fed on different defibrinated blood meal sources (human, bovine, and avian) as well as the fertility of the resultant eggs. In this study, no differences in fecundity or fertility were observed and the authors, recommended the use of bovine blood due to the ease of obtaining it from local slaughterhouses, low cost, and convenience. Similarly, no significant differences in the fecundity and fertility for *Culex quinquefasciatus* were observed when females were fed on different live warm-blooded hosts (mouse, rabbit, and pigeon) or cold-blooded hosts (lizard, toad) (Suleman and Shirin, 1981). However, a difference in fecundity (egg production) between *An. gambiae* and *An. quadriannulatus* was observed between females blood-fed directly on humans versus those which were artificially blood-fed on bovine blood. Subsequently, the females that had blood-fed on a human produced more eggs compared to those blood-fed on bovine (Takken et al., 2002; Takken and Verhulst, 2013).

Without artificial blood, various animal blood sources (defibrinated sheep, horse, and bovine blood as well as expired donated human blood (no longer suitable for human use)) are still being used for artificial membrane feeding systems. It is important to note that during defibrination, the coagulation factors (fibrin) are removed to prevent blood from clotting during blood-feeding and this may reduce the blood nutritional components available for egg production. Richards et al. (2012) reported that feeding processed blood meals (defibrinating bovine blood) decreased feeding rate, fecundity, and fertility over two gonotrophic cycles in *Cx. quinquefasciatus* compared to feeding on a live chicken or chicken blood in Alsever's solution (Alsever, 1941).

These studies, however, are limited in application as it will not be sustainable to use live animals for laboratory mosquito rearing. It is therefore necessary to explore how the blood will be delivered to mosquitoes under insectary rearing conditions. The methods used to provide the blood-meal to the mosquitoes will most likely also have to be evaluated and optimised. One method of providing blood through a feeding system will be discussed in the next paragraph.

Artificial membrane feeding system:

More than a decade ago, studies were initiated to promote artificial membrane feeding systems to reduce the need of using live animals to provide blood meals to laboratory-reared mosquitoes (Benedict, 2009; National Research Council (US), 2011). The method used to provide mosquito females with a blood-meal can impact the blood-feeding rate which will impact further downstream on the fecundity of a colony (Lyski et al., 2011; Iyaloo and Facknath, 2017). Most delivery methods maintained the blood temperature at 37°C with a 30 min feeding duration, (Gunathilaka et al., 2017; Iyaloo and Facknath, 2017). Temperature control can be provided through various methods and one of the most general artificial feeding systems used is the Hemotek® artificial feeding system (Figure 2.1). This system uses an electrical energy source with a small aluminium blood plate with a capacity of holding 5mL of blood. The membrane was attached to the aluminium plate by the rubber O ring (Figure 2.1). The aluminium plate holds the blood using any artificial or natural membrane such parafilm or sausage skin respectively (Iyaloo and Facknath, 2017). The procedures for setting up the Hemotek® artificial feeding system used in this study is shown in Figure 2.2 (a, b, c and d). Other artificial feeding systems include the use of a heated rice bag placed on top of a metal plate containing blood bound by a membrane Figure 2.3 (a, b, c) (Gunathilaka et al., 2017) or providing warm blood contained in a sausage skin membrane (Figure 2.4).

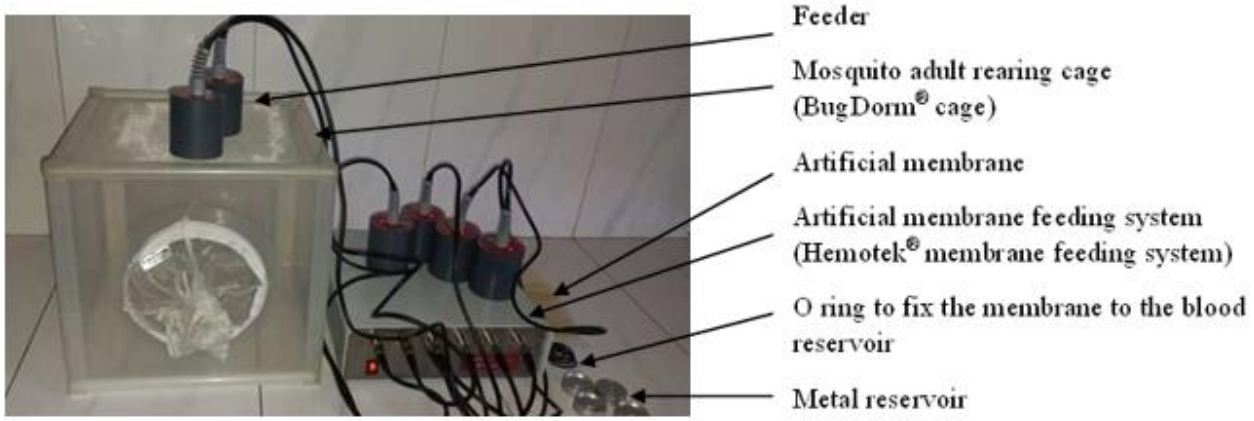

Figure 2.1: Hemotek® membrane feeding system with accessories.

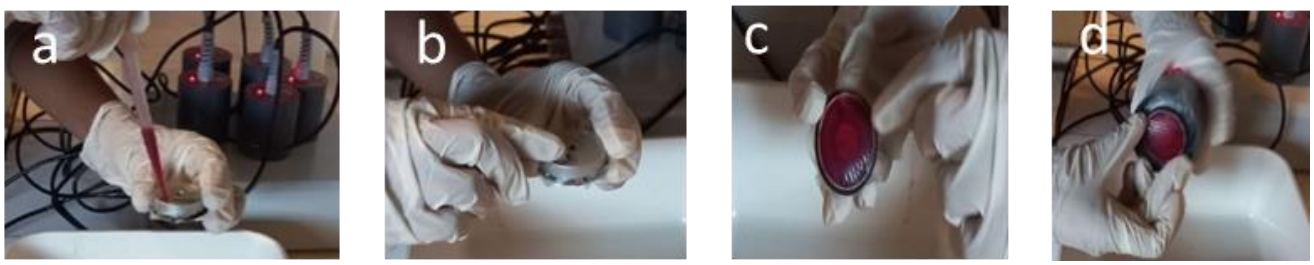

Figure 2.2: Procedures for setting up the Hemotek® feeding system (a, b, c, d) : a: Blood filling in the reservoir covered with artificial membrane; b: Plug the hole used to fill the blood; c: Reservoir filled with 5mL of bovine blood ; d: Reservoir fixed to the heated feeder, which is then placed on top of mosquito cage

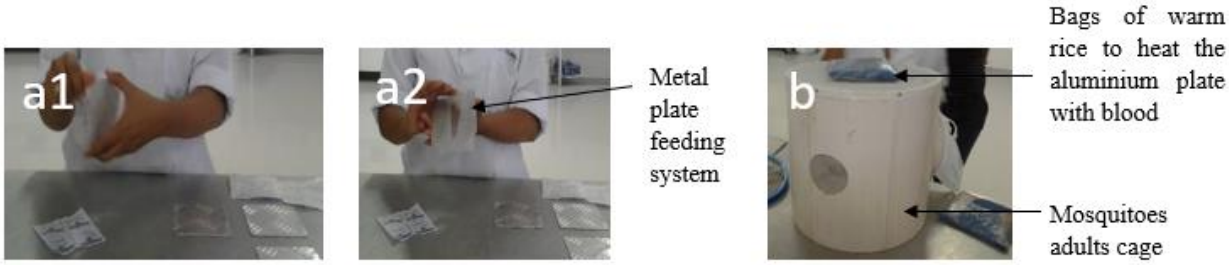

Figure 2.3: Procedure for setting up the metal plate feeding system (a, b, c): a: Materials of metal plate feeding system; b: Creating a bag using parafilm attached to aluminium plate and filled with blood; c: Aluminium plate placed on the mosquito cage, heated using warm rice

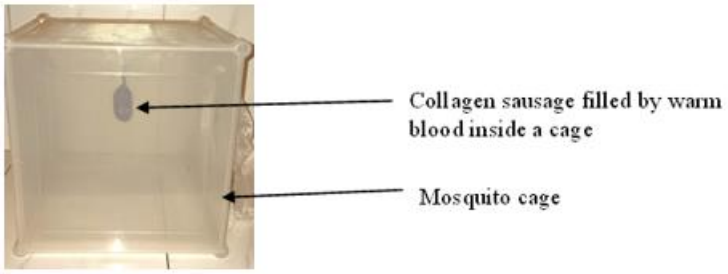

Figure 2.4: Sausage skin feeding system placed inside the mosquito cage

2.1.2 Rationale for this study

Most blood-feeding optimisation studies have been done on *Aedes* (*Ae. aegypti*, *Ae. albopictus*) (Gunathilaka et al., 2017; Iyaloo and Facknath, 2017) and *Culex (Cx. quinquefasciatus)* (Suleman and Shirin, 1981) and some anophelines (*An. arabiensis*, *An. dirus*, *An. cracens*, *An. minimus* (Damiens et al., 2013; Phasomkusolsil et al., 2013).

However, very limited information is available on different blood delivery approaches for *An. funestus*. This is due to the scarcity of *An. funestus* colonies that can be used to conduct blood-feeding studies. Developing methods to blood feed *An. funestus* under laboratory conditions is vital for the development of novel technologies, like SIT which rely on the availability of a stable colony that can be mass-reared, sterilised, and released (males only) at a large scale to reduce or suppress a target population. The current approach of using live hosts to provide blood-meals is not cost effective and has a lot of ethical considerations because it requires keeping live animals. Maintaining the mosquito colony on an artificial membrane feeding system, such as the Hemotek® system is therefore crucial to ensure colony sustainability. This chapter evaluated the impact of feeding defibrinated bovine blood through an artificial membrane system on fecundity and fertility of *An. funestus* compared to guinea pig fed controls. This information will provide valuable insight into the possibility of maintaining and potentially mass-rearing this anthropophagic species on this artificial membrane feeding system.

2.1.3 Aim

This study aimed to evaluate the potential use of an alternative blood meal source and delivery method for an *An. funestus* (Diptera: Culicidae) colony.

2.1.4 Objectives

The objective of this study was to determine the impact of blood-feeding on colonised *An. funestus* using an artificial system by measuring female fecundity and egg fertility. Two sub-objectives were investigated:

1) Determining the percentage of blood-fed females, fecundity, and fertility of *An. funestus* fed using Hemotek® providing defibrinated bovine blood compared to direct skin blood-feeding using anaesthetised guinea pig.
2) Evaluating the effect of multiple blood meals artificially delivered using a Hemotek® feeder on the percentage of blood fed females, fecundity, and fertility of *An. funestus*.

2.2 MATERIALS AND METHODS

2.2.1 Biological materials and routine colony maintenance

Biological material for this study originated from the *An. funestus* colony, FUMOZ *(An. **fu**nestus* from **Moz**ambique) maintained at the Botha De Meillon insectary, at the Vector Control Reference Laboratory (VCRL), National Institute for Communicable Diseases (NICD) in Johannesburg, South Africa. This strain was collected from southern Mozambique in July

2000 and subsequently colonised (Hunt et al., 2005). The routine rearing conditions for the colony are described below.

2.2.1.1 Adult rearing conditions

The FUMOZ colony was maintained at a temperature of 25°C (± 2°C) with relative humidity of 80% (± 10%) and twelve-hour dark: light regime including 45 min dusk and dawn transitions (Hunt et al., 2005). Adult males and females were kept in cages that were constructed by connecting two 25 L plastic buckets (dimensions of each bucket: 27 cm [high] × 34 cm [top diameter] × 30 cm [bottom diameter]) (Figure 2.5). The plastic bottom of the top cage had been cut out (15 cm diameter) and replaced with netting to allow for blood-feeding while the sides of the cages were removed and replaced with netting sleeves to allow for handling of the mosquitoes and egg plates (Figure 2.5).

Figure 2.5: Double bucket used as a cage for rearing *An. funestus* adults

Ten percent sugar solution was available in cotton wool placed inside the cages as energy source for males and females and these were replaced twice a week. For routine blood-feeding the NICD uses anaesthetised guinea pig (NICD animal ethics, Appendix B) and adults are allowed to feed for 10 – 20 min twice a week after dusk.

Cages were egg-plated twice a week, two-to-three days after blood-feeding. Egg plates consisted of a round bowl (6 cm [height] × 12 cm [top diameter] × 9 cm [bottom diameter]), hereafter referred to as egg plates, filled with 100 mL of reverse osmosis (RO) water as well as a drop of algae collected from a fishpond. Algae quantities were not quantified and are included routinely as part of the rearing protocol. However, for experimental purposes the design below (section 2.2.1.2) algae were excluded to insure no additional variation was included in the

experiment. RO water is water purified without large particles much as contaminants and sediments such as chlorine, salt, and dirt. Egg plates were kept in the cage overnight to allow females to oviposit and removed the following day. The egg plates were covered with a lid and left on a shelf until the eggs hatched (one to three days after harvesting). Once the eggs hatched, larvae aged one day were transferred into a standard larval rearing bowl (21 cm [length] × 15 cm [width] × 8 cm [height]) and reared as described in section 2.2.1.2.

2.2.1.2 Larval routine rearing conditions

The larvae were fed with dog biscuits (Beeno®) and brewer's yeast (Vital®) crushed finely at a weight to weight ratio of 3:1 respectively (Hunt et al., 2005, Zengenene et al., 2022). To prepare the larval food the dog biscuits are crushed in a Croydon grinder and subsequently in a Retsch laboratory mill, (Retsch South Africa, Verder Scientific Pty Ltd, Johannesburg) to ensure all food particles (dog biscuits as well as yeast) are homogenous, 0.10 µm size. The dog biscuits have the following nutritional value as stated by the manufacturer i.e. 16% protein, 10% moisture, 2.5% crude fibre, 2.5% crude fiber, 1.5% phosphorus, 3 mg vitamin C, 10 IU vitamin E and 10 µg organic selenium.

Second instar larvae were divided into one or more standard larval bowls (21 cm [length] × 15 [width] × 8 cm [height], containing 600 – 800 mL of RO water, as needed to prevent overcrowding. When larvae reached the L3 – L4 stages they were transferred to large larval rearing trays (32.5 [length] × 25 [width] × 14 cm [height], with 1.5 L – 2 L of RO water. The bowls were covered with a net and emerging adults collected every day using a mouth aspirator. Adults were placed into a cage for routine feeding as described in section 2.2.1.1.

2.2.2 Experimental design

All experimental work was conducted using standard cube shaped BugDorm® cages (32.5 [length] × 32.5 [width] × 32.5 cm [height], NHBS GmbH, Siemens Str. 10, 53121, Bonn Germany), constituted by plastic walls with a netted opening to access the cage. The bottom and top consist of mesh allowing for blood-feeding. Each cage contained 100 *An. funestus* FUMOZ newly emerged virgin females and 100 newly emerged adult males (giving a total number of adults/cage of 200). The mosquitoes were offered 10 % sugar solution *ad libitum* as soon as they were placed into the cage. Adults were allowed to mate for 10 consecutive days to allow for optimal mating (Maharaj et al., 2022). Three biological replicates were performed, with three technical repeats (same biological material within the three

replicates) unless otherwise stated. The insectary room temperature, relative humidity, and dark: light cycle were the same as for the routine colony rearing (section 2.2.1.1).

Impact of blood meal sources on fecundity and fertility

Blood meals were offered after the mating period on days 11 and 14 at dusk to improve the feeding response (Damiens et al., 2013). The mosquitoes were starved 24 hrs before a blood meal to encourage feeding. A control cage (fed on an anaesthetised guinea pig) and three treatment cages (females fed using defibrinated bovine blood) were set up for each biological repeat. To determine the feeding success, all blood-fed females were counted after the last blood-feeding and expressed as % of the total mosquitoes in each cage.

An artificial blood-feeding system (Hemotek® membrane feeding system: Hemotek Ltd, Unit 5 Union Court, Great Harwood Business Zone, Blackburn BB67FD, United Kingdom) using defibrinated bovine blood (Figure 2.1) was used to feed female mosquitoes. Six feeders consisting of aluminium heating plates (3.7 cm diameter and 1.3 cm thick) connected to a power unit covered with hog membrane (used by general public to make sausages) purchased from a local butchery (secured by an O-ring) were filled with 5 mL of defibrinated bovine blood provided from an accredited slaughterhouse. Blood was frozen after harvesting for long term storage (3 months). Once defibrinated blood is stored in a freezer until use, it can be kept for several months. Frozen blood was defrosted in cold water for 2 – 3 hrs before feeding. The aluminium plates were heated to 35 ± 1°C as described by Damiens et al., 2013. Two plates were placed on the top of each cage for 30 min to allow females to feed (Figure 2.6) (Cosgrove et al., 1994, Iyaloo and Facknath, 2017). Females from this cohort constituted the treatment group.

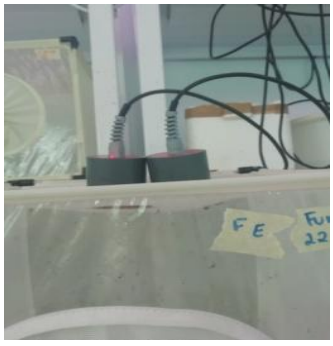

Figure 2.6: Blood-feeding of females in a BugDorm® cage using the Hemotek® membrane feeding system with two plate feeders (35.± 1°C) with bovine blood.

Living host blood meal*:* An anaesthetised shaved guinea pig as per standard NICD protocol (Hunt et al., 2005) was left on top of the rearing cage for 15 min, to provide a blood meal for *An. funestus* females. The anaesthesia agent used was a mixture of xylazine (5 mg) and ketamine (25 mg), administered subcutaneously by a trained and qualified animal handler. This amount of anaesthetic is suitable for about 45 min of anaesthesia (Dang et al., 2008). This is routinely performed by the NICD animal handler to maintain the colony. To limit the use of animals as well as biological material, only one control cage was fed (one technical replicate) per biological repeat.

Egg harvesting: After the second blood meal, all males as well as any unfed females, were taken out of the cage to allow egg collection from gravid females. The number of engorged females was recorded. An egg plate containing 100 mL of RO water was placed into each cage two days after the last blood-feeding on day 16 for oviposition. Egg collection took place 24 hrs post-egg-plating. Collected eggs were counted by using a manual magnifying lens to determine fecundity.

Insemination rate*:* After eggs were harvested, 10 females per cage (10% of the original females) were randomly selected and removed from the cage. Females were placed into a cup and frozen before their spermathecal capsules were dissected to determine the percentage of inseminated females (*i*). To adjust for fecundity the percentage of inseminated females was calculated following the formula describe by Niain'ny Felamboahangy et al. (2023).

Fecundity: the fecundity was calculated following formula from Niain'ny Felamboahangy et al. (2023); fecundity (*F*) = number of eggs (*ne*) / adjusted female number (*afn*). The adjusted female number (*afn*) = *fn* (number of females fed) × *i* (percentage of female inseminated).

Fertility: a random subsample of 30% of the harvested eggs, or a minimum of 100 eggs per cage, were transferred into new egg plates containing 100 mL of RO water. For 14 consecutive days, the larvae that emerged were counted and transferred daily to a standard larval tray to complete their metamorphosis. The total number of eggs hatched over the 14 days was noted (Munhenga et al., 2016). The number of larvae obtained over the 14 days divided by the number of eggs laid gives the fertility expressed as a percentage.

Impact of increased feeding frequency using the Hemotek® system on fecundity and fertility

The second experiment on adult feeding was to determine the impact of increased blood-feeding frequency using Hemotek® machine on eggs per female and hatching rate as described above. The experimental method and the measured parameters were the same as previously described. The treatment group cages were fed four times (Monday (day 11), Tuesday (day 12), Thursday (day 14), and Friday (day 15)) while the control group cages were blood-fed twice (Monday (day 11) and Thursday (day 14)) using the Hemotek® feeder as previously described. An egg plate was made available two days after the last blood meal, on day 16 for the control cage and day 17 for the treatment cage. Due to the routine guinea pigs feeding, it was not possible to synchronise egg plating for this experiment. The rest of the procedures and monitoring parameters were the same as those described previously.

2.3 DATA ANALYSIS

Statistical analyses and graphs were completed using the statistical StataCorp software, version 14, 2015 and the level of significance cut-off was 0.05. Shapiro-Wilk normality tests were conducted for each variable (Shapiro and Wilk, 1965). The data was found to be normally distributed and the percentage of blood-fed females, fecundity, and fertility were summarised as the mean percentage of females that blood fed ± SD (standard deviation), the number of eggs produced ± SD, and the mean proportion of eggs hatched ± SD. The effects of blood source and artificial blood-feeding frequency on fecundity and fertility were analysed using independent two-sample t-tests.

2.4 RESULTS

2.4.1 Evaluation of the effect of blood-feeding source on *An. funestus* fecundity and fertility

Details on Appendix C: Raw data of the impact of blood-feeding source for *An. funestus* are summarised in the supplementary table.

2.4.1.1 Blood fed females

Of the females offered an anaesthetised guinea pig, the percentage that blood fed (mean % ± SD) was 53.00% ± 4.58 (n = 300). It was 48.77% ± 11.04 (n = 900) for females that blood fed on the artificial system. There was no statistically significant difference in the percentage of females that blood-fed between the two different blood-feeding methods (Independent t-test, $t(10) = 0.63$, $p = 0.544$) (Table 2.2).

Table 2.2: Summary of blood-feeding outcome of *An. funestus* females fed on a live animal host (control: anaesthetised guinea pig) and artificial membrane feeding system (treatment)

Blood-feeding approach (N)	Mean % of female blood-fed mean ± SD (n)	Insemination rate (%) (mean ± SD) (n)	Number of eggs/female mean ± SD (n)	% Fertility (mean ± SD) (n)
Anaesthetised guinea pig (300)	53 ± 4.58 (159)	83.33 ± 15.27 (30)	10.94* ± 0.77 (1 460)	79.56 ± 9.34 (826)
Artificial feeding using Hemotek® feeder (900)	48.78 ± 11.04 (439)	62.22 ± 23.33 (90)	2.88* ± 2.09 (664)	77.33 ± 14.44 (664)

N = total number of females used; n = number of females that fed/ were inseminated/ number of eggs laid/ number of eggs hatched. * Significant difference between the two cohorts

2.4.1.2 Insemination rate

The percentage of inseminated females (mean % ± SD) drawn from cages where females were fed on anaesthetised guinea pig (control) was 83.33% ± 15.27 (n = 30), while it was 62.22% ± 23.33 (n = 90) for those drawn from cages that were fed by bovine blood through Hemotek® (Table 2.2). No significant statistical difference in female insemination rate between guinea pig-fed females and females fed bovine blood through the Hemotek® system was observed (Independent t-test, t (10) = 1.44, p = 0.180).

2.4.1.3 Fecundity

Egg production was higher from females fed on anaesthetised guinea pig (mean number of eggs/female ± SD) 10.94 ± 0.77 (n = 1 460) than those fed on the artificial membrane feeding system, 2.88 ± 2.09 (n = 664). A statistically significant difference was observed in independent two-sample t-test (t (10) = 6.37, p = 0.0001) (Table 2.2).

2.4.1.4 Fertility

The fertility (mean % fertility ± SD) of eggs produced from females blood-fed on guinea pig was 79.56% ± 9.34 (n = 826) compared to 77.33% ± 14.44 (n = 664) for females that fed on the artificial membrane blood-feeding system. There was no statistically significant difference in fertility between the guinea pig fed females and those fed using the artificial membrane blood-feeding system (independent two-sample t-test: t (10) = 0.25, p = 0.810) (Table 2.2).

2.4.2 Evaluation of the impact of increased feeding frequency using an artificial blood-feeding system on female fecundity and fertility

The effect of increasing the blood-meal frequency was investigated using the same experimental process as described above. Table 2.3 summarises the results of blood-feeding of *An. funestus* females fed twice using the artificial blood-feeding system (control) and fed four times using the artificial blood-feeding system (treatment). The raw data on the impact of blood meal frequency using the Hemotek® membrane feeding system is presented in Appendix D.

2.4.2.1 Blood fed females

The mean percentage of females (mean % ± SD) that blood-fed when offered two blood meals with the Hemotek® membrane feeding system was 46.33% ± 13.65 (n = 300) compared to 51.44% ± 10.98 (n = 900) for females offered four blood meals. No statistical significant difference was noted in the percentage of females that took a blood meal between the two cohorts (independent t-test, t (10) = -0.66, p = 0.522) (Table 2.3).

Table 2.3: Summary of blood-feeding outcome of *An. funestus* females fed two blood meals vs four blood meals through an artificial blood-feeding system

Blood-feeding method (N)	% Female blood-fed mean ± SD (n)	Insemination rate (%) mean ± SD (n)	Number of eggs/female mean ± SD (n)	% Fertility mean ± SD (n)
Artificial membrane feeding using Hemotek® feeder for two blood meals (300)	46.33 ± 13.65 (139)	90 ± 10.00 (30)	2.30 ± 1.58 (317)	81.28 ± 16.06 (317)
Artificial membrane feeding using Hemotek® feeder for four blood meals (900)	51.44 ± 10.98 (463)	71.11 ± 24.72 (90)	4.61 ± 2.67 (1 554)	83.85 ± 13.13 (1 554)

N = total number of females used; n = number of females that fed/ inseminated females / number of eggs laid/ number of eggs hatched

2.4.2.2 Insemination rate

Since mating took place before feeding, it was expected that the percentage of females inseminated (mean % ± SD) between the control and treatment cages were similar. Indeed, the percentage of inseminated females was 90.00% ± 10.00 (n = 30) for females that were blood fed twice while it was 71.11% ± 24.72 (n = 90) for females blood-fed four times, using the

Hemotek® system. The percentage of females inseminated did not differ significantly between the two female groups (independent t-test, t (10) = 1.26, p = 0.238) (Table 2.3).

2.4.2.3 Fecundity

The mean fecundity (mean number of eggs/female ± SD) of females blood fed twice was 2.3 ± 1.58 eggs/female (n = 317) while it was 4.61 ± 2.67 eggs/female (n = 1 554) for those fed four times using the artificial feeding system. No statistical significant difference was observed in fecundity between the two groups (Independent t-test, t (10) = -1.39, p = 0.098) (Table 2.3).

2.4.2.4 Fertility

Fertility from females blood fed twice showed a mean fertility (mean % ± SD) of 81.28% ± 16.06 (n = 317) and the eggs laid by females blood-fed four times had a mean fertility of 83.85% ± 13.13 (n = 1 554). No statistically significant difference was observed in fertility between the two cohorts (independent t-test, t (10) = -0.28, p = 0.783) (Table 2.3).

2.5 DISCUSSION

This study aimed to measure the impact of an alternative blood-source and delivery system on key reproductive parameters in a colonised *An. funestus* (Diptera: Culicidae) strain. It constitutes the first report of blood-feeding optimisation for *An. funestus*, and provides valuable information to fill the knowledge gap on blood-feeding for laboratory colony maintenance for this species. The first part of the work compared the percentage of females engorged, the number of eggs per female and egg hatching rate from females blood-fed with two different blood meal sources (live anaesthetised guinea pig and defibrinated bovine blood via an artificial Hemotek® membrane feeding system). There were differences observed in the percentage of females blood-fed on the two blood-feeding methods. Females fed on guinea pig produced more eggs. However, no difference in egg fertility was observed between egg batches from the two female cohorts fed in two different ways. These findings demonstrate that *An. funestus* can be maintained using alternative blood delivery methods such as Hemotek® feeding using defibrinated bovine blood, but a period of adaptation may be with required.

Results on the impact of artificial blood-feeding using Hemotek® on fecundity tallies with the findings by Richards et al. (2012) where a decrease in the fecundity of *Cx. quinquefasciatus* was observed when females were fed on defibrinated bovine blood compared to those fed on a live avian. The same trend was observed in another study

(Phasomkusolsil et al., 2013) where feeding on defibrinated sheep blood consistently produced fewer eggs in *An. dirus*, *An. cracens*, *An. minimus* and *An. sawadwongporni* compared to blood-feeding on live guinea pig blood. In the same study Phasomkusolsil et al. (2013) found no difference in fecundity of females fed on a live hamster compared to defibrinated guinea pig blood, except for *An. minimus* where eggs per female was higher for females fed on a live host.

The decrease in fecundity observed during the experiment when *An. funestus* was fed on defibrinated blood that could be explained by a sudden switch from guinea pig blood to artificial blood-feeding. The colony used is routinely blood-fed on guinea pig and will probably need to acclimatise to artificial blood-feeding. Zengenene et al. (2021) showed an *An. funestus* FUMOZ colony adapted to artificial blood-feeding over six successive generations of blood-feeding with defibrinated blood. Selection to blood-feeding on debrinated bovine blood by *An. funestus* was also demonstrated by Maharaj et al. (2022). However, in this study an acclimation period was not provided which may explain the low fecundity. In addition, defibrination removes fibrin and the process of freezing and thawing increases protein denaturation (isoleucine degradation) which lowers the quality of the blood (Duarte et al., 1999; Gonzales and Hansen, 2016), that could also have affected the fecundity. Another possible reason for low fecundity was the temperature of the feeding plates of the Hemotek® membrane feeding system used in this study. It was confirmed as 35.1°C using a reference thermometer. The temperature normally used on the NICD units which is 37°C. Therefore, low temperature used during artificial feeding using Hemotek® feeding system might be considered a limitation of the study, as the average temperature of bovine, guinea pig and human ranges from 37 – 39.5°C. However, the proportions of engorged females did not differ between the two methods, suggesting that this may not have played a role in reduced fecundity.

The second part of the study evaluated the result of increased blood-feeding frequency on the fecundity and fertility of *An. funestus.* Fecundity of females that fed on defibrinated bovine blood via Hemotek® effectively doubled when blood meals were offered four times before oviposition instead of twice. However, no statistical significant difference was observed. In reality, doubling of eggs is operationally significant and translates to an increase in the actual number of larvae and therefore the number of mosquitoes that will emerge which will increase the number of females available to produce eggs in the next generation. According to the results of this work, if *An. funestus* is selected for artificial blood-feeding then increasing the frequency of blood meals before oviposition can have a positive effect on egg production. This phenomenon is stated on Damiens et al. (2013) findings, who showed that *An. arabiensis*

fecundity increased significantly with increased feeding frequency when fed bovine blood via an artificial feeding system. However, the colony used by Damiens et al. (2013) was routinely fed on an artificial membrane feeding system and had already been selected for this method.

Usually, bovine blood is used as a blood source for *Anopheles* females reared in insectaries (Damiens et al., 2013; Mamai et al., 2017; Maharaj et al., 2022) due to its availability and relative affordability. However, the regulations needed to know the origin of the blood being used and whether the health and veterinary-controlled slaughterhouse where the blood is coming from or if trained technical personnel are being used during slaughtering are important points which should be considered (Gunathilaka et al., 2017). Other important areas that should be considered before transition to defibrinated bovine blood is the quality of blood being obtained. It would be advantageous if the erythrocyte range of the bovine blood being used is similar to the range seen in guinea pigs, which will make adaptation of strains easier and ensure consistent egg production. Lastly, it is established that the total protein of bovine blood might be influenced by defibrination (Richards et al., 2012). Therefore, it is critical that defibrination methods used alter blood protein levels as little as possible to avoid the lowering blood quality. All these facts should be borne in mind when choosing bovine blood to be used as a candidate replacement for guinea pig blood during mosquito colony maintenance in the laboratory.

In conclusion, four defibrinated bovine blood meals weekly, offered through an artificial blood-feeding system, could potentially be used as an alternative to a live host for maintaining an *An. funestus* colony. The increased cost of feeding four times per week is likely going to be compensated for by increased mosquito productivity. This is based on the assumption that the colony will adapt to the defibrinated bovine blood. It is recommended that future research should focus on nutritional value of defibrinated blood and its impact on fecundity and fertility. The addition of adenine nucleotides, adenosine monophosphate (AMP) or adenosine triphosphate (ATP) to blood is another potential area for further investigation (Galun, 1967; Pothikasikorn et al., 2010; Luo, 2014). Chang and Judson (1977) showed the benefit of blood additives like isoleucine that can improve fecundity.

CHAPTER 3

ASSESSMENT OF LABORATORY LARVAL REARING PARAMETERS FOR *ANOPHELES FUNESTUS*

This chapter formed part of a publication and the results from experiments on larval food and larval density were included in (Niain'ny Felamboahangy et al., 2023; Appendix A, but more details are provided here).

3.1 INTRODUCTION

3.1.1 Background

Mosquito larval rearing is key in the mosquito rearing cycle. Larvae are essential in the maintenance of a colony because they determine whether sufficient adults are produced to perpetuate subsequent generations (Reisen 1975; Khan et al., 2013; Somda, et al., 2017). Larval food of sufficient quantity and quality is an important parameter for successful larval rearing (Damiens et al., 2012; Khan et al., 2013; Balestrino et al., 2014; Jong et al., 2016; Somda et al., 2017). Another important parameter during larval rearing is rearing density. Larval rearing density should be low enough to prevent overcrowding and competition for food (Lyimo et al., 1992; Balestrino et al., 2014; Zengenene et al., 2022), however, optimising the use of available resources (water) is also a concern in mass production setting. A review on *An. funestus* larval habitats was provided in chapter 1, here only the key larval rearing parameters for insectary rearing, e.g. larval diet dose and larval density will be discussed.

Research on the optimal larval food components began as early as 1947 focusing on *Ae. aegypti* (Golberg and De Meillon, 1947; Golberg and De Meillon, 1948). This research continued and was strengthened with the onset of interest in SIT on mosquito species (Soman and Reuben, 1970; Zahiri et al., 1997; Bond et al., 2017). Researchers developed various formulations of larval food that were inexpensive, locally available and suitable to be used under laboratory rearing conditions for *An. stephensi* (Khan et al., 2013), *Ae. albopictus* (Puggioli et al., 2013; Jong et al., 2016), *An. arabiensis* (Damiens et al., 2012; Somda et al., 2017) and *Ae. aegypti* (Bond et al., 2017). Table 3.1 provides an overview of larval diet components evaluated for *An. arabiensis* as an example. Common components for mosquito larval diets are proteins (which can be derived from animal and/or vegetable sources), brewer's yeast, and vitamins (Table 3.1). Each diet component has a role in mosquito development.

Table 3.1: Larval diets with percentage of each component that was evaluated in *An. arabiensis* according to Damiens et al. (2012) and Somda et al. (2017)

	Main component of larval diet					Additives			
Species	**Bovine liver powder**	**Brewer's yeast**	**Koi fish food**	**Squid liver powder**	**Tuna meal**	**Ascorbic acid**	**Spirulina**	**Vitamin mix (Vanderzant vitamin)**	**Reference**
An. arabiensis	100%								Damiens et al ., 2012
		100%							Damiens et al ., 2012
			100%						Damiens et al ., 2012
				100%					Damiens et al ., 2012
					100%				Damiens et al ., 2012
							100%		Damiens et al ., 2012
	50%				50%	25 µg			Damiens et al ., 2012
	50%				50%		25 µg		Damiens et al ., 2012
	50%				50%			92 µg	Damiens et al ., 2012
	50%				50%				Somda et al., 2017
					100%				Somda et al., 2017
	30%				70%				Somda et al., 2017
	15%	15%			70%			additive	Somda et al., 2017

In general, larval diets are mostly composed of protein. Proteins are required for the larvae to grow with excess protein being converted to fat. These stored fat reserves are used during the non-feeding pupal stage and for transformation into adulthood (Briegel et al., 1990; Yahouédo et al., 2014). In *An. arabiensis* diets ranged from 0.05 – 0.4 mg/larva/day (Damiens et al., 2012; Puggioli et al., 2017). Yeast contains elements that promote growth, which adds value in terms of larval development and can be mixed with beans, rice or wheat to shorten larval development. However, it can decrease the survival of L1 to pupal and adult stages (Khan et al., 2013). Vitamin requirements vary according to the genus being reared and act to produce bigger adults and also promotes faster larval development. In studies done by Damiens et al. (2012) and Somda et al. (2017), *An. arabiensis* larvae reared without vitamins had a longer larval developmental time and the resultant adults were smaller compared to those reared with vitamins in their food. Similarly, different amino acids combinations in addition to other nutritional requirements are also essential for egg maturity, larval growth and pupal production in *Ae. aegypti* (Singh and Brown, 1957).

Larval rearing density is another parameter essential for optimal larval development. Rearing density has an impact on the development of the mosquito and has down-stream effects on body size, egg production and body fat reserves (used for flight and swarming behaviour, needed for mating) (Baldal et al., 2005; Baleba et al., 2019; Gilles et al., 2011; Silva et al., 2021). Larvae reared in overcrowded conditions experience cannibalism, starvation, death, and the resultant adults are often small (Beserra et al., 2009; Briegel, 1990; Price et al., 2015). This is supported by studies done on the effect of high larval density and high temperature during *An. gambiae* laboratory rearing (Lyimo et al., 1992). Results of this study showed an effect of larval rearing density on adult body sizes. In another study on *An. gambiae*, larvae reared at high densities showed increased development time, reduced fourth instar and adult sizes, and lower pupal production (Gimnig et al., 2002; Tchuinkam et al., 2011). Similarly, high larval density reduced all immature stage survival, adult emergence and adult body size (Muriu et al., 2013). Epopa et al. (2018) also demonstrated the effect of larval rearing densities on *An. coluzzii* in which adult emergence and adult body size decreased when the density of larvae increased. In *An. funestus,* Zengenene et al. (2022) evaluated the impact of larval density on the life-history traits of insectary colonised and obtained similar results as obtained for *An. gambiae*. Tchuinkam et al., (2011) defined as larval optimal larval density for *An. gambiae* about as 1 larva/cm^2, which is a double the density of recorded by Niain'ny Felamboahangy et al. (2023) fund with *An. funestus*.

3.1.2 Rationale for this study

Currently, there are no standard rearing parameters/protocols for the general rearing of *An. funestus.* In the previous chapter, the impact of an artificial blood-feeding system on fecundity and fertility of reared *An. funestus* was investigated as a sustainable alternative to guinea pig blood-feeding. The success of genetic based vector control methods under development such as the SIT programme, are dependent on efficient larval rearing that can ultimately produce healthy adults capable of competing with wild males (Khan et al., 2013; Bond et al., 2017). Larval diet dose and larval rearing density are some of the critical parameters that can impact on the quality of the adult mosquitoes produced. A locally available diet which has been used to maintain *Anopheles* colonies for more than five decades, including the 20-year colony of *An. funestus* FUMOZ, has never been optimised to determine the optimal dosage. Dosage optimisation will standardise the rearing procedure to ensure the consistent production of the same quality males regardless of the personnel responsible for the rearing. In this work, a locally available diet is used with benefits including ensured availability and reduced costs (not necessary to import larval diet). In addition to larval diet dose optimisation, larval rearing density was also optimised. Zengenene et al. (2022) recently published work on the effect of larval density in *An. funestus* strain from Angola (FANG). This study was probably among the first initiatives in maintaining laboratory colonies as there are not many published records available. There is a need for follow up studies using a different strain from a different geographical location to support the findings of Zengenene et al. (2022).

3.1.3 Aim

This study aimed to determine the optimum larval rearing parameters for laboratory rearing of an *An. funestus* strain.

3.1.4 Objectives

Different experiments were carried out to determine the optimal diet dose and larval rearing density for *An. funestus*. The specific objectives of this study were:

1) Evaluating the impact of different doses of the standard NICD larval diet on *An. funestus* larval development
2) Assessing the optimal larval rearing density among five different densities for rearing *An. funestus* larvae under laboratory conditions

3.2 MATERIALS AND METHODS

3.2.1 Biological materials: Details are provided in section 2.2.1.

3.2.2 Methods

3.2.2.1 Larval diet dose experimental design

The standard larval diet used at the NICD is a powdered diet consisting of dog biscuits (Beeno® Original peanut butter flavour containing vitamins, minerals, immune boosters, and antioxidants, sold in a pack of 1 kg) and brewer's yeast (containing B-vitamins, protein, trace amounts of chromium and zinc) mixed at a ratio of 3:1. Details are provided in section 2.2.1.2. The food was weighed using an analytical balance (KERN ABT 120-5DM, KERN and SOHN GmbH, Ziegelei 1, 72336 Balingen, Germany), and stored in aluminium foil packs containing enough diet to feed 100 larvae at the respective doses.

One hundred (n = 100) first instar larvae were put into a standard larval tray (21 cm [length] × 15 cm [width] × 8 cm [height]) with 750 mL RO water resulting in a larval density/surface area of 0.315 larvae/cm^2 (~0.32 larvae/cm^2). Larvae were fed with one of the predetermined larval diet doses (Table 3.2). The baseline dose was determined from experience of NICD colony maintenance in which it has been observed that larvae reared with very low quantities of food developed slowly and those reared at a high food quantity died before pupation. This has also been reported in several publications on *Anopheles* species (Damiens et al., 2012; Khan et al., 2013; Somda et al., 2017). Five different diet doses were selected and the doses were increased daily as the larvae developed (Table 3.2). The full range of larval diet doses were evaluated in two independent experiments, referred to as phase 1 (0.02 – 0.03 – 0.04 mg/larva/feeding) and phase 2 (0.04 – 0.06 – 0.08 mg/larva/feeding). Specified doses were given two times a day to reared larvae, as a large amount of food provided at once is detrimental, especially for small larvae. Three biological replicates were performed. Each biological replicate consisted of nine technical repeats. .

For both phases 1 and 2, the same physiological and reproductive parameters were evaluated and included: development time (time in days from first instar larvae to pupae), pupal production, pupal weight, adult emergence and wing length. These experiments were conducted at the VCRL.

- ***Larval development time***

Developmental time of larvae was measured from the first instar larvae time to pupae emergence. The development time was recorded daily and survival analysis was used to calculate the median time taken to develop from larvae into pupae.

Table 3.2: The feeding regimen using NICD larval food to determine the impact of different larval diet doses on an *An. funestus* strain acronym FUMOZ (for each dose mosquito larvae were fed twice daily)

Treatment/ dose	Day of feeding														
	D1	D2	D3	D4	D5	D6	D7	D8	D9	D10	D11	D12	D13	D14	D15
T1	0.02	0.03	0.04	0.06	0.08	0.10	0.12	0.14	0.20	0.26	0.32	0.40	0.50	0.50	0.40
T2	0.03	0.04	0.05	0.07	0.09	0.12	0.15	0.20	0.25	0.30	0.35	0.40	0.50	0.50	0.40
T3	0.04	0.05	0.06	0.08	0.10	0.14	0.18	0.22	0.28	0.32	0.40	0.45	0.50	0.50	0.40
T4	0.06	0.08	0.10	0.14	0.18	0.24	0.30	0.40	0.50	0.60	0.70	0.80	1.00	1.00	0.80
T5	0.08	1.00	0.12	0.16	0.20	0.28	0.36	0.44	0.56	0.64	0.80	0.90	1.00	1.00	0.80

Diet dose / feeding

Diet dose/feeding (mg/larva) T: Treatment D: Day

- ***Pupal production***

Pupation was recorded daily and overall pupal production was calculated as the ratio of the total number of pupae obtained in comparison to the total amount of first instar larvae used, expressed as a percentage.

- ***Pupal weight***

All pupae produced were brought to the laboratory, and a subsample randomly selected to determine their sex by observing the abdominal terminalia morphology as described by Benedict et al., (2007) using a microscope (OLYMPUS, model BX50F4 from Olympus optical co. LTD. Japan) fitted with a camera. Thereafter, 30 males and 30 females were used to determine pupal weight. In detail, a pupa was placed onto a piece of filter paper to absorb the excess water before being individually weighed in analytical balance. A piece of filter paper was used to transfer the pupa from the filter paper to remove excess water and put it on the balance plate (Figure 3.1). The overall mean pupal weight was calculated from the 60 pupae

weighed per treatment. After the pupae were weighed, they were excluded from subsequent experiments.

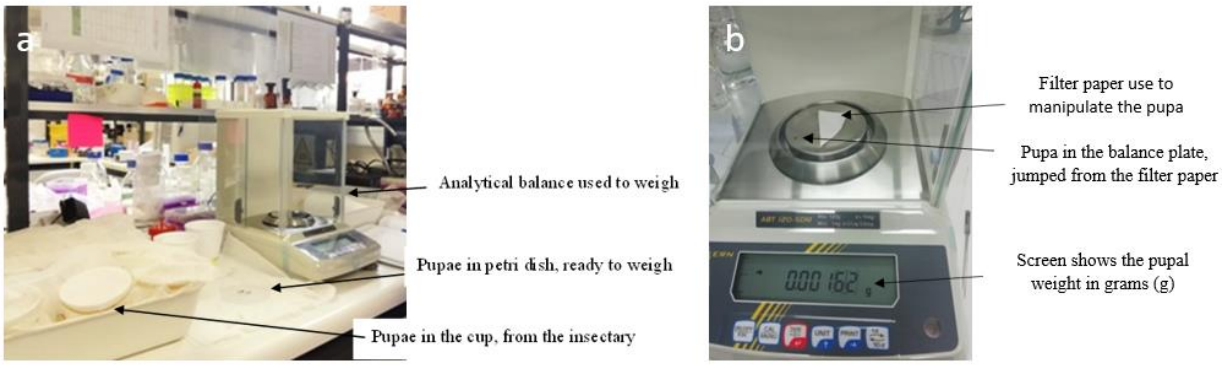

Figure 3.1: Pupal weighing process (a, b): a: Batch of biological material (pupae) in the cup, in the petri dish and on analytical balance; b: Pupal weighing

- ***Adult emergence***

The remaining pupae following pupal weighing were used to monitor the proportion of adults that emerged. Adult emergence rate was considered as the number of emerged adults divided by the number of pupae, expressed as a percentage.

- ***Wing length***

The wing length was measured from a subsample of 30 adult males and 30 adult females, which emerged at different times, and selected at random. Wing length is used as a proxy of adult body size (Fish, 1985; Ng'habi et al., 2005; Muriu et al., 2013; Epopa et al., 2018). To measure the wing length, *An. funestus* adults were anaesthetised in a refrigerator at 4°C for 15 min and then placed onto a glass slide with the dorsal side of the mosquito body facing upwards. Wing length gave an estimation of body size. Wing length was measured for each individual under the microscope by dissecting the right wing gently from the body. The wing was spread out on the glass slide and measured under a microscope (SZ2-ILST, Olympus Corporation Tokyo, Japan) fitted with a camera (S2X7) and stream essential software (Olympus Stream Image Analysis Software Version 510_UMA-Olystream 192-Mekong_en_00_21072014 – Olympus Soft Imaging Solutions GmbH, Johann-Krane-Weg 39, D-48149 Münster) (Figure 3.2). The measurement was taken from the distal edge of the alula (a lobe of the posterior margin of the wing) to the end of the radius vein, excluding the fringe scales as they might be damaged during the process (Marina et al., 1999; Kassim et al., 2012).

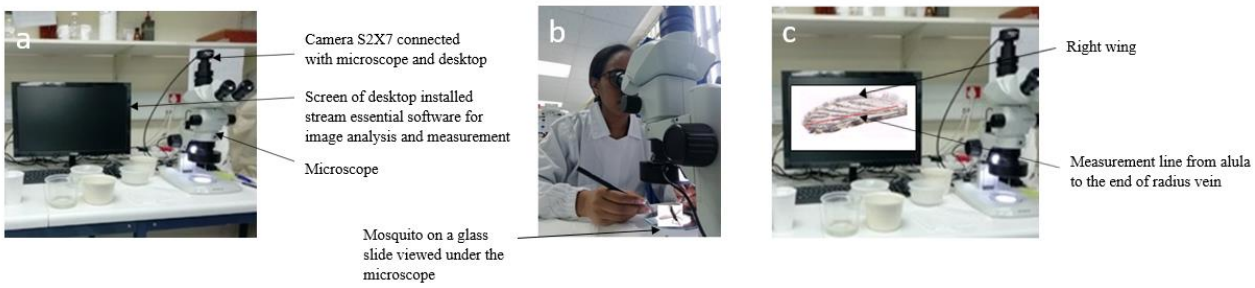

Figure 3.2 : Materials and process of wing length measurement (a, b, c): a: Materials of wing length measurements: microscope with accessories (camera and software in desktop) to measure wing length; b: Dissection of right wing from the body to be measured by the software; c: Measurement of right wing of *An. funestus*

3.2.2.2 Larval density experimental design

The experimental design was similar to the larval diet dose experiments (section 3.2.2.1). Five different larval densities were evaluated, 80 L1/ tray (0.25 larvae/cm^2) – 100 L1/tray (0.32 larvae/cm^2) – 150 L1/tray (0.48 larvae/cm^2) – 200 L1/tray (0.64 larvae/cm^2) – 400 L1/tray (1.27 larvae/cm^2). The choice of these densities was guided by the experimental design used by Tchuinkam et al., 2011. Mosquitoes were reared using standard rearing conditions described in section 3.2.2.1 with a larval diet dose of 0.04 mg/larva/feeding fed two times a day. The dose increased every day as shown in Table 3.2. Three biological replicates with three technical replicates were performed. The same parameters as in the above experiment on larval diet dose were assessed. Due to limitations in space and the availability of biological material, the larval density experiment was conducted in two phases.

In phase 1, three different larval densities 0.25, 0.32, and 0.48 larvae/cm^2 were evaluated. Phase 2 was set up with 0.32 larvae/cm^2, 0.64 larvae/cm^2 and 1.27 larvae/cm^2. The parameters measured were the same as those measured for larval diet dose.

- ***Development time, pupal production and adult emergence***

The measurement of these three parameters, was similar to larval diet dose evaluation experiments and is as described in section 3.2.2.1.

- ***Pupal weight and wing length***

The sample size of pupae used for pupal weight and wing measurement was based on the number of L1 in the larval rearing tray for each density. Therefore, 30 pupae were used for the larval density of 0.25 larvae/cm^2. The number of pupae for the other densities were

calculated as 10% of the total number of larvae (L1) in the tray at the beginning of the experiment: i.e. 30 for 0.32 larvae/cm^2, 45 for 0.48 larvae/cm^2, 60 for 0.64 larvae/cm^2 and 120 individuals for 1.27 larvae/cm^2.

3.3 DATA ANALYSIS

Statistical analysis and graphs were computed using StataCorp, version 14, 2015, and the level of significance cut-off was 0.05. A Shapiro-Wilk test was used to determine the normality of the variable distribution (Shapiro and Wilk, 1965). For normally distributed data ANOVA (Anscombe, 1948) was used to analyse the production of pupae, the emergence of adults, pupal weight and wing measurement between all cohorts. When result showed a statistical significant difference, Tukey's honestly significant difference (HSD) *post hoc* test was carried on to evaluate differences between pairs. If the data were not normally distributed, Kruskal-Wallis tests (Kruskal and Wallis, 1952) were used to determine differences for each variable, subsequent pairwise comparisons using χ^2 tests were performed to detect any significant differences. Kaplan-Meier survival analysis (Kaplan and Meier, 1958) was used to estimate the development time, and a log-rank test was used to determine statistically significant differences.

3.4 RESULTS

3.4.1 Effects of larval diet doses on rearing parameters

- ***Larval development time*** (phase 1)

The development time was the time spent from the first instar larvae to pupation. The median development time in days (IQR: [Q1 – Q3]) for *An. funestus* fed at a diet doses of 0.02 mg/larva was 16 [15 – 17] days and 15 [14 – 17] days for larvae fed at 0.03 mg/larva and 0.04 mg/larva (Table 3.3), graph on Appendix E: Figure 3.3. Further analysis of development time for phase 1 using the log-rank test stated a significant difference in development time between the three larval diet doses ($\chi^2_{(2,\, n\,=\,2\,189)}$ = 79.45, $p < 0.0001$). Consequent pairwise comparisons revealed significant differences in development time between all three cohorts 0.02, 0.03 and 0.04 mg/larva ($p < 0.0001$). Details are shown in Table 3.4.

In phase 2 the development time of *An. funestus* fed on diet doses of 0.04 to 0.08 mg/larva was 17 [16 – 17] days for those fed 0.04 mg/larva, 12 [12 – 15] days for 0.06 mg/larva and 13 [12 – 16] days for those fed 0.08 mg/larva (Table 3.3), graph on Appendix E: Figure 3.4. Further analysis of development time in phase 2 using the log-rank test showed a significant difference in development time between the three larval diet doses

($\chi^2_{(2, n = 766)} = 159.85$, $p < 0.0001$). Consequent pairwise comparisons revealed significant difference in development time between all three cohorts 0.04, 0.06 and 0.08 mg/larva ($p < 0.0001$) (Table 3.4).

Table 3.3: Summary of different parameters evaluated according to the different larval diet doses used to feed *An. funestus* FUMOZ

Diet dose (mg/larva/ feeding)	Development time (day) median ± IQR	Pupal production (%) mean ± SD	Pupal weight (mg) mean ± SD	Adult emergence (%) mean ± SD	Wing length (mm) mean ± SD
Phase 1					
0.02	16 ± [15 – 17][a]	74.11 ± 10.07[b]	1.34 ± 0.10	93.76 ± 3.76	3.28 ± 0.16
0.03	15 ± [14 – 17][a]	80.22 ± 10.18	1.36 ± 0.11	96.82 ± 2.59	3.27 ± 0.12
0.04	15 ± [14 – 17][a]	88.89 ± 5.86[b]	1.35 ± 0.12	96.58 ± 2.96	3.31 ± 0.17
Phase 2					
0.04	17 ± [16 – 17][a]	75.33 ± 18.01	1.42 ± 0.14	89.25 ± 7.14	3.37 ± 0.12
0.06	12 ± [12 – 15][a]	0.67 ± 1.12	1.30 ± 0.16	NA	NA
0.08	13 ± [12 – 16][a]	9.11 ± 17.95	1.40 ± 0.14	NA	NA

[a] superscript notes a statistically significant difference between all cohorts for development time
[b] superscript notes a statistically significant difference from the cohort in pupal production
NA = not applicable

Since the diet dose of 0.04 mg/larva was included in both phase 1 and 2, the developmental times between the two phases were compared. In phase 1, developmental time was faster (15 days ± [14 – 17]) than in phase 2 (17 days ± [16 – 17]). The difference in median development time between the two phases was significant ($\chi^2_{(1, n = 1\,478)} = 188.86$, $p < 0.0001$) The two phases were conducted at different times (Appendix E).

- ***Pupal production*** (phase 1)

The percentage of pupae produced (% pupae produced ± SD) from larvae reared at larval diet doses 0.02 mg/larva to 0.04 mg/larva (phase 1) ranged from 74.11% ± 10.07, to 88.89% ± 5.86 (Table 3.2) respectively. A significant difference in pupal production through the different larval diet doses was observed in phase 1 ($F_{(2, 24)} = 6.22$, $p = 0.007$). Subsequently, Tukey's HSD test for multiple comparisons found that the mean pupal production was significantly different among 0.02 mg/larva and 0.04 mg/larva ($p = 0.005$, 95% C.I = [0.04, 0.54]). The pupal production from larvae reared on 0.04 mg/larva was higher than pupal production from larvae reared on 0.02 mg/larva.

Table 3.4: Pairwise comparisons (χ^2 test) of development time according to the different larval diet doses used to feed *An. funestus* FUMOZ larvae, bold font values indicate a statistically significant difference between treatments

Diet dose (mg/larva/feeding)	χ2(1)	p-value
Phase 1		
0.02 vs 0.03	**31.68**	**0.0000**
0.02 vs 0.04 (3.1)	**83.93**	**0.0000**
0.03 vs 0.04 (3.1)	**10.18**	**0.0014**
Phase 2		
0.04 (3.2) vs 0.06	**150.04**	**0.0000**
0.04 (3.2) vs 0.08	**72.37**	**0.0000**
0.06 vs 0.08	**61.56**	**0.0000**

Pupal production from phase 2 experiments showed that larvae reared at diet doses 0.04 mg/larva to 0.08 mg/larva, was 75.33% ± 18.02 pupae for those fed at 0.04 mg/larva; 0.67% ± 1.12 pupae for larvae reared at 0.06 mg/larva and 9.11% ± 17.95 pupae for larvae reared at 0.08 mg/larva (Table 3.2). Larval cohorts fed at doses of 0.06 and 0.08 mg/larva produced very few pupae (Table 3.3) and were unadvised for colonising *An. funestus*. Therefore, these were not analysed further for pupal weight, time-to-first adult emergence, adult emergence and wing length. No statistical significant difference in pupal production from larvae reared at 0.04 mg between phase 1 and 2 ($H_{(1)} = 2.123$, $p = 0.145$).

- ***Pupal weight*** (phase 1)

The mean pupal weight in (mg ± SD) was obtained from larvae fed three different larval diet doses was 1.34 mg ± 0.10, 1.36 mg ± 0.11, and 1.35 mg ± 0.12 respectively for pupae originating from larvae fed at 0.02, 0.03, and 0.04 mg/larva/feeding respectively. Statistically, similar weights were detected between pupae originating from larvae fed at the different diet doses ($H_{(2)} = 2.518$, $p = 0.284$).

- ***Adult emergence*** (phase 1)

Mean percentage of adults emerged from larvae fed with different larval diet doses of 0.02, 0.03, and 0.04 mg/larva/feeding was 93.76% ± 3.76, 96.82% ± 2.59 and 96.58% ± 2.96, respectively (Table 3.3). The mean percentage adult emergence amongst the different treatments did not differ significantly ($F_{(2,24)} = 2.65$, $p = 0.091$).

- ***Wing length*** (phase 1)

Mean wing length (mm ± SD) obtained from adults emerging from larvae fed the three different larval diet doses was 3.28 mm ± 0.16 (for larvae fed at 0.02 mg/larva), 3.27 mm ± 0.12 (0.03 mg/larva) and 3.31 mm ± 0.17 (0.04 mg/larva), respectively (Table 3.3). No statistical significant differences were found in wing length measurements between the three adult groups, Kruskal-Wallis analysis ($H_{(2)} = 3.529$, $p = 0.171$).

3.4.2 Effects of larval rearing density on rearing parameters

- ***Development time***

The longest median development time (IQR (day): [Q1 – Q3]) of 19 [17 – 21] days, was observed at the lowest density i.e. 80 larvae/tray (or 0.25 larvae/cm^2) (Table 3.5), graph on Appendix F: Figure 3.5. In phase 1, the median developmental times were 17 [16 – 19] and 16 [15 –17] days for 100 larvae/tray (0.32 larvae/cm^2) and 150 L1/tray (0.48 larvae/cm^2), respectively. Log-rank test analysis indicated significant differences in development time through the different rearing densities ($\chi^2_{(2,\ n\ =\ 2\ 001)} = 405.21$, $p < 0.0001$). Further pairwise comparisons displayed a significant differences in development time among 0.25 larvae/cm^2 and 0.32 larvae/cm^2 ($\chi^2_{(1,\ n\ =\ 973)} = 149.23$, $p < 0.0001$), between 0.25 larvae/cm^2 and 0.48 larvae/cm^2 ($\chi^2_{(1,\ n\ =\ 1\ 367)} = 369.83$, $p < 0.0001$) and amongst 0.32 larvae/cm^2 and 0.48 larvae/cm^2 ($\chi^2_{(1,\ n\ =\ 1\ 237)} = 78.78$, $p < 0.0001$).

In phase 2, the longest development time of 15 [14 – 16] days was recorded in larvae colonised at the density of 0.32 larvae/cm^2, while it was 14 [13 – 15] days for larvae reared at 0.64 larvae/cm^2. The shortest development time was 12 [11 – 13] days, which was recorded in larvae reared at 1.27 larvae/cm^2 (Table 3.5), graph on Appendix F: Figure 3.6. The difference in developmental time was statistically significant different between the cohorts during phase 2 (log-rank test; $\chi^2_{(2,\ n\ =\ 2\ 454)} = 26.93$; $p < 0.0001$). Subsequent pairwise analyses revealed significant differences among 0.32 larvae/cm^2 and 0.64 larvae/cm^2 ($\chi^2_{(1,\ n\ =\ 1\ 467)} = 4.73$, $p = 0.030$), 0.32 larvae/cm^2 and 1.27 larvae/cm^2 ($\chi^2_{(1,\ n\ =\ 1\ 639)} = 20.30$, $p < 0.0001$),

0.64 larvae/cm^2 and 1.27 larvae/cm^2 ($\chi^2_{(1, n=1\,802)}$ = 4.32, p = 0.038). The two phases were conducted at different times.

Table 3.5: Summary of the effects of different larval rearing densities on the developmental parameters of *An. funestus* larvae

Larval rearing density Larvae/cm^2 (Larvae/tray)	Development time (day) median ± IQR	Pupal production (%) mean ± SD	Pupal weight (mg) mean ± SD	Adult emergence (%) mean ± SD	Wing length (mm) mean ± SD
Phase 1					
0.25 (80)	19 ± [17 – 21]^a	47.08 ± 24.28^a	1.36 ± 0.18	81.30 ± 20.35	3.36 ± 0.15
0.32 (100)	17 ± [16 – 19]^a	70.44 ± 12.29^a	1.37 ± 0.16	86.07 ± 9.85	3.33 ±0.14
0.48 (150)	16 ± [15 –17]^a	76.07 ± 14.53^a	1.37 ± 0.16	91.85 ± 6.66	3.33 ± 0.15
Phase 2					
0.32 (100)	15 ± [14 – 16]^b	72.44 ± 15.95^b	1.31 ± 0.15^a	89.18 ± 7.74	3.30 ± 0.13^a
0.64 (200)	14 ± [13 – 15]^b	45.28 ± 26.08^{bc}	1.35 ± 0.14^b	76.03 ± 20.54	3.28 ± 0.12^b
1.27 (400)	12 ± [11 – 13] ^{b}	27.42 ± 29.41^c	1.29 ± 0.12^a	86.44 ± 8.92	3.24 ± 0.11^c

^{a,b,c} superscript notes a statistically significant differences between treatments, if there are no letters or symbols there were no significant differences between treatments

- ***Pupal production*** (phase 1)

The mean pupal production (% ± SD) from L1 larvae reared at 0.25 larvae/cm^2 was 47.08% ± 24.28 , 70.44% ± 12.29 for pupae originating from larvae reared at 0.32 larvae/cm^2, and 76.07% ± 14.53 for pupae originating from larvae reared at 0.48 larvae/cm^2 (Table 3.5). Significant difference on pupal production depending on the larval rearing density ($H_{(2)}$ = 7.308, p = 0.026) was noted. Subsequent combination tests showed pupal production between pupae originating from larvae reared at 0.25 larvae/cm^2 and those originating from larvae reared at 0.32 larvae/cm^2 differed significantly p = 0.020, 95% C.I = [3.78, 38.69]. Similarly pupal production from larvae colonised at 0.25 larvae/cm^2 and 0.48 larvae/cm^2 differed significantly p = 0.007, 95% C.I = [4.50, 24.49]. Pupal production from larvae colonised at 0.25 larvae/cm^2 was lower than a pupal production from larvae reared at 0.48 larvae/cm^2; pupal production

increased with the larval rearing density. However, pupal production from larvae reared at 0.32 and 0.48 larvae/cm^2 did not differ significantly.

- ***Pupal production*** (phase 2)

The percentage of pupae produced from phase 2 experiments ranged from 27.42% ± 29.41 (for pupae from larvae reared at 1.27 larvae/cm^2) to 72.44% ± 15.95 for pupae originating from larvae reared at 0.32 larvae/cm^2 (Table 3.3). Pupal production from larvae creared at 0.32 larvae/cm^2 was higher than a pupal production from larvae reared at 0.64 and 1.27 larvae/cm^2; pupal production decreased with the larval rearing density in phase 2. The mean percentage pupal production differed significantly between the three larval rearing densities ($H_{(2)}$ = 10.31, p = 0.005). Subsequent pairwise comparisons for 0.32 larvae/cm^2 and 0.64 larvae/cm^2 p = 0.017, 95% C.I = [27.09, - 3.09] and 0.32 larvae/cm^2 and 1.27 larvae/cm^2 p = 0.001, 95% C.I = [- 4.52, -7.64] differed significantly.

The larval density of 0.32 larvae/cm^2 was used in both phase 1 and phase 2 experiments. For phase 1, the pupal production at a larval density of 0.32 larvae/cm^2 was 70.44% ± 12.20 while it was 72.44% ± 15.95 for phase 2. Statistically, no significant difference in pupal production was observed between the two phases when larvae were reared at 0.32 larvae/cm^2 ($F_{(1,16)}$ = 0.09, p = 0.769). Based on this result, data from phase 1 and 2 were combined (Table 3.5). Overall, a significant difference in pupal production was observed amongst the five different larval rearing densities ($H_{(5)}$ = 21.161, p = 0.0008). A weak significant relationship among larval density and pupal production in phase 2 ($r^2_{(53)}$ = 0.1379, p = 0.006). High larval rearing density produced less pupal production.

- ***Pupal weight***

The mean pupal weights (mg ± SD) of pupae obtained from larvae reared through different densities in phase 1 were 1.36 mg ± 0.18, 1.37 mg ± 0.16, and 1.37 mg ± 0.16 for larvae reared at 0.25 larvae/cm^2, 0.32 larvae/cm^2 and 0.48 larvae/cm^2, respectively (Table 3.5). No significant difference in the weight of pupae among the different larval densities was observed ($H_{(2)}$ = 0.709, p = 0.701).

The mean pupal weight obtained in phase 2 from the different larval densities was 1.31 mg ± 0.15, 1.35 mg ± 0,14, and 1.29 mg ± 0.12 for pupae originating from larvae reared at 0.32 larvae/cm^2, 0.64 larvae/cm^2 and 1.27 larvae/cm^2, respectively (Table 3.5). A statistically significant difference was observed in mean pupal weight between pupae derived from the

different larval rearing densities ($H_{(2)}$ = 30.59, p = 0.0001). Subsequent pairwise comparisons of pupal weight showed a statistically significant difference between pupae derived from larvae reared at 0.32 larvae/cm^2 and 0.64 larvae/cm^2, ($H_{(1)}$ = 7.380, p = 0.007). The weight of pupae from larval densities of 0.64 larvae/cm^2 and 1.27 larvae/cm^2 were statistically significant different ($H_{(1)}$ = 30.94, p = 0.0001). Comparisons of the pupal weight when pupae were derived from larvae reared at a density of 0.32 larvae/cm^2 and 1.27 larvae/cm^2 did not differ significantly ($H_{(1)}$ = 2.79, p = 0.095).

The highest mean pupal weight from the two phases was 1.37 mg ± 0.16 recorded from pupae coming from larvae reared at 0.32 larvae/cm^2 density and the lowest pupal weight was 1.29 mg ± 0.12 from pupae originating from larvae reared at a density of 1.27 larvae/cm^2. No correlation among larval density and pupal weight, for both phases ($r^2_{(902)}$ = 0.0037, p = 0.068) was observed.

- ***Adult emergence***

The mean percentage adult emergence (% ± SD) in phase 1 for adults coming from larvae reared at different larval densities of 0.25 larvae/cm^2 to 0.48 larvae/cm^2 ranged from 81.30% ± 20.35 to 91.85% ± 6.66 (Table 3.7). Statistically, the adult emergence between the three larval rearing densities ($H_{(2)}$ = 1.326, p = 0.537) was similar. In phase 2, the mean percentage adult emergence varied from 76.03% ± 20.54 to 89.18% ± 7.74 (Table 3.5) with no significant difference between the different densities ($F_{(2,16)}$ = 1.82, p = 0.194).

- ***Wing length***

Mean wing measurement (mm ± SD) obtained in phase 1 was 3.36 mm ± 0.15, 3.33 mm ± 0.14, and 3.33 ± 0.15 for wings obtained from adults derived from larvae reared at densities of 0.25 larvae/cm^2, 0.32 larvae/cm^2 and 0.48 larvae/cm^2, respectively (Table 3.3). No significant difference in wing measures amongst the different groups was observed ($H_{(2)}$ = 2.299, p = 0.317).

In phase 2, mean wing lengths were 3.30 mm ± 0.13, 3.28 mm ± 0.12, and 3.24 ± 0.11 for adult wings coming from adults derived from larvae reared at 0.32 larvae/cm^2, 0.64 larvae/cm^2 and 1.27 larvae/cm^2, respectively (Table 3.5). A statistically significant difference was observed in wing length for phase 2 ($H_{(2)}$ = 39.74, p = 0.0001). Subsequent two by two combination tests displayed that wing measurements differed significantly among adults originating from larvae reared at larval densities of 0.32 larvae/cm^2 and 0.64 larvae/cm^2

($\chi^2_{(1, n = 433)} = 5.30$, $p = 0.0213$). There were significant differences in wing lengths between adults derived from larvae reared at larval densities 0.32 larvae/cm^2 and 1.27 larvae/cm^2 ($\chi^2_{(1, n = 475)} = 38.36$, $p = 0.0001$), and those from larval densities 0.64 larvae/cm^2 and 1.27 larvae/cm^2 ($\chi^2_{(1, n = 548)} = 15.34$, $p = 0.0001$). A significant relationship between larval rearing density and wing length for phase 2 ($r^2_{(727)} = 0.0395$, $p < 0.0001$) was noted. Larval rearing density increased as wing measurement decreased.

3.5 DISCUSSION

This chapter focused on *An. funestus* larval rearing parameters, including the larval diet dose and larval density. Based on the literature search, this is the first study that measured the impact of these rearing parameters in the colony (FUMOZ) using a life table approach.

The first experiment investigated the larval developmental time of *An. funestus* FUMOZ larvae fed five different doses of NICD standard larval diet. Development time reduced with increasing larval food availability up to a plateau dose of 0.04 mg/larva. Doses higher than 0.04 mg/larva became detrimental. The percentage of pupae produced from larvae reared at 0.04 mg/larva was more than three quarters of the initial larvae used. The adult emergence was the highest from larvae fed at 0.04 mg/larva, almost 90% emerged. Pupal weight was not affected by the dose larvae were fed, however the highest pupal weights (big pupae and high mean weight) were obtained from larvae fed 0.04 mg/larva. Similarly, changing larval diet dosage did not affect adult sizes as measured using their wing length, nevertheless the biggest adults (longest wing lengths and mean length) were also obtained from a diet dose of 0.04 mg/larva. The larval food dose of 0.04 mg/larva is, therefore, considered an optimal dose because it produced the most pupae in the shortest time. When the diet dose was increased to 0.06 – 0.08 mg/larva, pupal production was considerably reduced. These higher larval diet quantities were not beneficial in terms of development time for *An. funestus*. In addition, too few pupae were obtained for downstream analyses and these doses were, therefore, deemed unsuitable.

Some of the results of this study differ from literature. In this study, larvae developed faster than those from the study by Ngowo et al. (2021), who recorded a developmental time of 22 [21 – 23] days for the same strain (FUMOZ). The difference might be attributed to differences in experimental design. The phenomenon of increasing larval diet dose beyond a certain point resulting in decreased pupation as reported is not limited to this study. The same effect, was also observed in other species of *Anopheles* such as *An. arabiensis* and *An. stephensi*

(Reisen, 1975; Gilles et al., 2011; Damiens et al., 2012). This might be due to mortality in the L1 due to overfeeding which results in excess food on the water surface. Overfeeding can cause larval suffocation by reducing the oxygen available to the larvae. This is because anophelines use siphons to breathe air at the water's surface, blocking of the water surface by excess food interferes with breathing resulting in suffocation. Surprisingly, overfeeding decreased the development time. It can be speculated that the death of most larvae after overfeeding resulted in less competition on the remaining larvae resulting in enough resources to develop faster.

Although no statistical significant differences in the adult sizes (using wing length as a proxy) between different larval diet doses were detected, generally the adult sizes observed in this study were larger than reported in other studies, including Mwangangi et al. (2004), Ngowo et al. (2021) and Zengenene et al. (2022). All of the above mentioned studies measured the wing in the same way i.e., from the apical side to the auxiliary margin without the fringe scales. Therefore, the difference in adult sizes observed in these studies compared to Niain'ny Felamboahangy et al. (2023) might be due to the larval food quantity used. In Niain'ny Felamboahangy et al. (2023) the quantity could be, above the daily nutritional requirements for larvae at all doses and therefore did not restrict adult size. Body size has a role to play in mating, it is the reason of importance of body size measurement. Larger females mate more successfully than smaller ones; successful mating is crucial for *An. funestus* colony stability (Nasci, 1987; Packer and Corbet, 1989; Renshaw et al., 1994; Hurd et al., 1995).

In this study, the optimal dose of the NICD diet for *An. funestus* was investigated and determined to be 0.04 mg/larva/feeding. FUMOZ needs a small but consistent quantity of food (Niain'ny Felamboahangy et al., 2023). Overfeeding has a negative impact on their development (Niain'ny Felamboahangy et al., 2023). These results showed that FUMOZ larvae most likely suffocated and died at the first or second instar larva when they are overfed using NICD standard larval food. The use of other larval diet food with similar or different nutritional composition will need to be evaluated for *An. funestus* in the future.

The second part of this chapter evaluated the effect of different larval rearing densities in *An. funestus* FUMOZ laboratory rearing. The criteria to select the optimal larval rearing density were shorter developmental time and higher pupal production (Niain'ny Felamboahangy et al., 2023). The optimum larval density was observed to be 0.48 larvae/cm^2 (150 larvae/tray) which gave an average development time of 16 days and pupal production of 76.07%. The percentage adult emergence was also highest at this larval density. Rearing larvae

at densities higher than 0.48 larvae/cm^2 resulted in shortened larval development time and reduced pupal and adult production. The first three larval rearing densities (0.25 larvae/cm^2 – 0.32 larvae/cm^2 – 0.48 larvae/cm^2) did not have any impact on pupal weights, however, rearing larvae at higher densities had a negative impact on resultant pupal weights. This observation differed from previous studies (Tchuinkam et al., 2011; Zengenene et al., 2022).

Zengenene et al. (2022) working on *An. funestus* FANG, found a very interesting result on the relationship between developmental time and larval density. They showed that developmental time increased with larval density from 0.42 – 3.33 larvae/cm^2 and that high larval density decreased the pupal production (Tchuinkam et al., 2011; Zengenene et al., 2022). In other species like *An. arabiensis*, *An. gambiae* and *An. coluzzii*, a similar result was observed; higher larval density decreased the proportion of pupae produced (Gilles et al., 2011; Muriu et al., 2013; Epopa et al., 2018). This difference might be strain specific or related to the availability of larval diet and research to evaluate both FANG and FUMOZ strains concurrently under the same conditions may provide more insight. Studies on larval density and developmental time in *An. gambiae* (Lyimo et al., 1992) tallied with results from this study where high larval rearing density shortened the developmental time and produced more pupae (89.5%). Larval rearing density played a major role in pupal production, adult emergence and developmental time in this study.

Another crucial parameter evaluated was the pupal weight. The pupal weight was constantly high in pupae originating from larvae reared at densities ranging from 0.25 – 0.48 larva/cm^2. Pupal weight from larvae reared at a high density i.e. above 0.48 larva/cm^2 decreased with increased density. The pupae produced from a high larval density were smaller. This small size of pupae might be due to a lack of larval appetite induced by the high larval density in the tray. However, this will need to be investigated before any conclusions can be drawn. Pupal weight might be important as it is associated with adult body size (Koenraadt, 2008). The pupal weight can indirectly indicate fitness of the pupa and adult fitness (Nguyen et al., 2019). This might impact on adult fecundity; the bigger size is more productive (Armbruster and Hutchinson, 2002), and on survival during irradiation to induce sterility.

It was assumed that the process of weighing the pupae might negatively affect the percentage adult emergence (due to handling during weighing), hence in this study, the pupae used to determine pupal weight were not involved in the adult emergence analysis. In this study, the highest percentage adult emergence was recorded from adults emerging from larvae reared

at 0.48 larva/cm^2. However, adult emergence seems not to be affected by rearing larvae at different densities.

Literature on *An. funestus* larval rearing density was very limited and this study provided some additional information to fill the knowledge gaps for this species. The results of this work may be used as a guideline for future *An. funestus* FUMOZ larval rearing. The optimal parameters for larval rearing were a diet dose of 0.04 mg/larva fed two times a day and a larval density 0.48 larvae/cm^2. However, when the larval density was consistent, the median time to pupation was 16 days, 76.07% of L1 become pupae and 91.85% of pupae became adults (Niain'ny Felamboahangy et al., 2023) and according to Zengenene et al. (2022) high larval density increased the time to pupation and decreased the adult production. To improve rearing at a large scale, a standard operating procedure (SOP) may need to be further developed as *An. funestus* is a notoriously difficult species to colonise and maintain in the laboratory.

CHAPTER 4

IRRADIATION DOSE OPTIMISATION FOR *ANOPHELES FUNESTUS*

4.1 INTRODUCTION

The success of SIT is dependent on, amongst others, the mass-rearing and sterilisation of male insects. These sterilised males are released to compete with wild fertile males to mate with wild fertile females (Dyck et al., 2005; Bouyer and Vreysen, 2020; Bouyer et al., 2020, Dyck et al., 2021). Male sterilisation for SIT application can be achieved through irradiation (or chemical treatment) (Dame et al., 1981; Klassen, 2005; Dunn and Follett, 2017). Irradiation remains the favoured approach for sterilisation of mosquitoes. However, the greatest challenge of this approach is that irradiation is not specific and damages both gametic and somatic cells. Damage of somatic cells has unintended consequences, affecting physiological fitness of the males. Therefore, an optimal dose, which should effectively sterilise the males without affecting physiological parameters such as shortening their lifespan, flight ability (important during swarming and dispersal of sterilised males), or their ability to locate and mate with wild females (Culbert et al., 2020; Dyck et al., 2021) is needed. Irradiation can damage cells responsible for reproductive behaviour in the males which can affect swarming behaviour of the males, compromising their mating ability (Lance et al., 2000; Barry et al., 2003; Kraaijeveld and Chapman, 2004). Response to irradiation varies from species to species and can even vary within the same strain from different geographical locations (Table 4.1). The sterilising irradiation dose needed for *Ae. albopictus* is lower (15 – 25 Gy), compared to the dose used for *Ae. aegypti* (50 Gy) (Bond et al., 2019; Bond et al., 2021). The optimal irradiation dose is also strain dependent, for example Morlan et al. (1962) recorded 110 – 180 Gy for *Ae. aegypti* and 35 – 50 Gy for *Ae. albopictus*. Contrary, Yamada et al. (2019) showed an optimal dose of 70 Gy for *Ae. Aegypti* strains from Brazil, Indonesia, France and Thailand, and 35 Gy as an optimal dose for *Ae. albopictus*. These optimal doses differ from recent work where *Ae. albopictus* required optimal doses as low as 15 – 25 Gy (Bond et al., 2019). Higher doses induced complete sterility, however, they negatively affected flight ability of the sterilised males (Bond et al., 2019).

Irradiation doses used for *Anopheles* species are higher compared to those used for *Aedes* species and vary from 70 – 120 Gy depending on the species (Table 4.1). Within the anophelines dose variation exists but is more similar to doses used to sterilise culicines. The optimal irradiation doses that can induce sterility without impacting adult emergence or

longevity range between 70 – 100 Gy in *An. arabiensis* (Helinski et al., 2008[a]; Munhenga et al., 2016; Dandalo et al., 2017). The optimal sterilising irradiation dose for *An. gambiae* and *An. quadrimaculatus* was recorded at 120 Gy (Weidhaas et al., 1962; Andreasen and Curtis, 2005; Dame et al., 2009), while for *An. stephensi* optimal doses vary from 80 – 120 Gy (Sharma et al., 1978; Andreasen and Curtis, 2005) (Table 4.1).

Table 4.1: Summary of irradiation doses used to induce sterility according to mosquito species with their references

Mosquito species	**Irradiation dose which induced sterility**	**References**
Ae. aegypti	50 Gy	Bond et al., 2019; Bond et al., 2021; Ranathunge et al., 2022
	70 Gy	Yamada et al., 2019
	110 – 180 Gy	Morlan et al., 1962
Ae. albopictus	35 – 50 Gy	Bond et al., 2019; Yamada et al., 2019; Bond et al., 2021
An. arabiensis	70 – 100 Gy	Helinski et al., 2008 ; Munhenga et al., 2016; Dandalo et al., 2017; Yamada et al., 2019
An. gambiae	120 Gy	Andreasen and Curtis, 2005
An. stephensi	80 – 120 Gy	Sharma et al., 1978; Andreasen and Curtis, 2005
An. quadrimaculatus	120 Gy	Weidhaas et al., 1962; Dame et al., 2009
Cx. quinquefasciatus	60 – 120 Gy	Patterson et al., 1975; Patterson et al., 1977 ; Ramhadani et al., 2020

Irradiation can be performed either at the pupal or adult stages. Although mosquito irradiation at the adult stage is less harmful to the mosquito, it is easier to irradiate mosquitoes at the pupal stage as pupae are more manageable, without the risk of escaping by flight (Andreasen and Curtis, 2005). Irradiation of pupae can cause low mating competitiveness compared to unirradiated mosquitoes (Andreasen and Curtis, 2005). The pupal age used for irradiation varies slightly between different research publications and ranges between 22 – 30 hrs old (Patterson et al., 1975; Patterson et al., 1977; Helinski et al., 2006; Munhenga et al., 2016). In pupae irradiated at ages younger than 22 hrs old, adult emergence, longevity as well

as fecundity were reduced (Davis et al., 1959; Abdel-Malek et al., 1967; Curtis 1976; Balestrino et al; 2010). Most irradiation studies were performed to compare the longevity between irradiation of young pupae aged 16 – 24 hrs, 24 – 40 hrs and 40 – 48 hrs to those irradiated at older than 30 hrs, Balestrino et al. (2010). In a study using *Ae. albopictus* longevity was reduced when using 80 Gy compared to unirradiated control group (Balestrino et al., 2010). Competitiveness studies of adult males from pupae which were irradiated different doses (15 – 90 Gy) compared to wild males of 24 – 36 hrs for *Ae. aegypti* and *Ae. albopictus* were carried out (Bond et al., 2019). Male pupae of *An. arabiensis* aged 24 ± 2 hrs were irradiated at different doses (50 – 160 Gy) to evaluate mating ability after sterilisation (Culbert et al., 2020). These studies showed that mosquito pupal age impacted the quality of sterilised males and their mating competitiveness (Hooper, 1972).

4.1.1 Rationale for this study

Currently in literature, there is no information regarding the optimal irradiation dose for *An. funestus* although this is one of the main African malaria vector species. This information will be important if this species is to be considered for SIT. Therefore, this study investigated different irradiation doses to determine the optimal dose to induce sterility in *An. funestus* males while limiting the impact of irradiation on male physiological fitness.

4.1.2 Aim

This study aimed to determine the irradiation dose-response of an *An. funestus* to establish the optimum irradiation dose needed to sterilise males without affecting their physiological fitness.

4.1.3 Objectives

The objectives of this study were:

1) Using different irradiation doses to induce sterility in male *An. funestus* at the pupal stage
2) Evaluating the effect of irradiating *An. funestus* males at different irradiation doses on physiological fitness (emergence and longevity)
3) Determining the impact of mating irradiated males with fertile females on reproductive parameters of fertile females (fecundity and fertility)

4.2 MATERIALS AND METHODS

4.2.1 Biological material: Details of the *An. funestus* strain used are provided in section 2.2.1

4.2.2 Irradiation process

Newly emerged pupae were collected and sexed according to terminalia morphology (see section 3.3.2). Approximately 200 male pupae (24 – 30 hrs old) were placed into 150 mL of RO water before being irradiated using a Gammacell 220 (GC220) with a Cobalt 60 (Co60) source (MDS Nordion, Ottawa, Canada) (Figure 4.1). The dose rate of the Gammacell 220 at the time of irradiation was 31.09 Gy/min. This was adjusted downwards by 1% each month due to radioactive decay (Bakri et al., 2005). The specific irradiation times required to achieve each dose were calculated according to the dose rate at the time of irradiation.

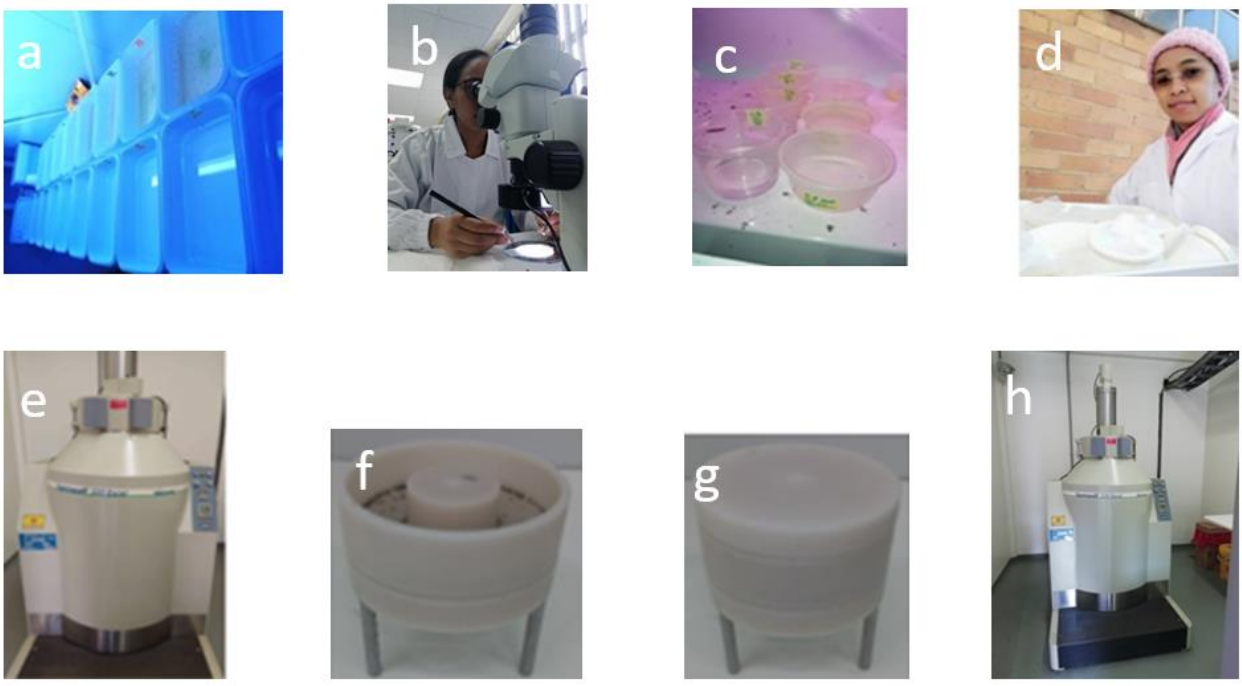

Figure 4.1: Procedures for setting up the irradiation of *An. funestus* FUMOZ using a Gammacell 220 irradiator (a, b, c, d, e, f, g, h): a: Pupae picked from FUMOZ colony reared according previous parameters determined; b: Separation of male and female pupae under microscope; c: Batch of 200 male pupae aged 24 – 30 hrs in 150 mL deionised water; d: Batch of male pupae in the cup covered by net, transported to the irradiation facility; e: Gammacell 220 irradiator Co60 source; f: Open irradiation jig with 200 male pupae to irradiate; g: Irradiation jig with pupae ready for irradiation; h: Set the exposure time of the Gammacell 220 and run the irradiator

4.2.3 Experimental process: Determining the optimal irradiation dose for inducing sterility in *An. funestus* males

Six irradiation doses (30; 60; 90; 100; 110 and 120 Gy) were evaluated. Depending on the availability of pupae, batches of 170 – 209 pupae were irradiated for the six different irradiation doses. The optimal irradiation dose was determined following two independent experiments. The first phase (phase 1) used 30 Gy intervals, while the second phase (phase 2) used 10 Gy intervals. Each experiment included at least one control consisting of 200 – 400 unirradiated male pupae (insectary control, and/or transport control). Controls are detailed in the next paragraph.

Phase 1: Batches of pupae were irradiated at different doses ranging from 30 Gy to 120 Gy (with 30 Gy intervals) as described above. In phase 1, two unirradiated controls were included. The first control consisted of pupae that were sexed and remained in the insectary (insectary control). The second unirradiated control consisted of pupae that were sexed and subsequently transported to the irradiation facility (300 m walk from the insectary) and back to the insectary (transport control). To calculate the induced sterility, an average of the percentage of eggs hatched from the unirradiated control cages was used for this phase 1. Three biological replicates were performed for each irradiation dose.

Phase 2*:* The highest dose from phase 1 (120 Gy) induced the highest sterility without reducing longevity, therefore it was selected and the experiment was repeated using 10 Gy intervals for fine scale analysis (100, 110 and 120 Gy). In phase 1, no statistical differences were detected between the insectary and transport controls (see results section for details), therefore only one control (insectary control) was included during phase 2.

4.2.4 Biological parameters

- ***Adult emergence***

After irradiation, male pupae from each dose were allowed to emerge in separate BugDorm® cages (as described in section 3.2.2.1). Adult male emergence was recorded daily for four consecutive days or until all the pupae metamorphosed to adults. One hundred emerged adult males from each dose were randomly removed from each cage and used in experiments to measure fecundity, fertility and insemination rate when mated to fertile females (methodology already described in section 3.2.2.1), while 50 adult males were used to evaluate longevity

(Helinski et al., 2009; Munhenga et al., 2016). The unirradiated control cages and sterile control cages each contained 100 fertile males and 100 fertile females.

- ***Fecundity, fertility, and insemination rate***

Each cage contained 100 *An. funestus* newly emerged virgin females and 100 newly emerged adult males (total number of adults/cage = 200). The mosquitoes were offered 10% sugar solution *ad libitum* as soon as they were placed into the cage. Adults were allowed to mate for 10 consecutive days to allow for optimal mating (Maharaj et al., 2022). After the mating period, females were blood fed twice a week (Monday and Thursday) using an anaesthetised guinea pig during routine NICD colony feeding. The blood fed females were used to calculate the rate of inseminated females and it was extrapolated to all remaining females in the cage to get the total percentage of inseminated females from three biological repeats. Each biological repeat consisted of three technical replicates unless otherwise stated. Procedures used to determine fertility, fecundity, and insemination is similar to those detailed in section 2.2.2. In all instances, three biological repeats were conducted. Each biological repeat consisted of three technical replicates unless otherwise stated.

- ***Induced sterility (IS)***

The induction of sterility in eggs by sterilised males was calculated by comparing the fertility of eggs laid from virgin females mated with virgin fertile males (unirradiated control males) to virgin females mated with irradiated males (treatments). The induced sterility was calculated using the formula:

*IS = (1 – [Ho/Hn]) * 100* where *Hn* is the percentage of eggs hatched from the unirradiated control cage (insectary control and transport control for phase 1, and insectary control for phase 2); *Ho* is the observed percentage of eggs hatched from females mated with males irradiated at different doses (Fried, 1971; Bellini et al., 2013).

- ***Longevity***

A sub-sample of 50 newly emerged adult males was randomly selected from each dose, transferred into separate BugDorm® cages, and monitored daily until 100% mortality was achieved in each respective cage. Sugar solutions (10%) were available to the males for the duration of the experiments. Longevity was defined as the number of days that adult males survived. Every day, the number of dead males was noted and removed.

4.3 DATA ANALYSIS

To assess the optimal irradiation dose for inducing sterility in *An. funestus* males, the following parameters were used, adult emergence summarised as mean percentage (± SD), fecundity, summarised as the mean number of eggs per female (± SD), and fertility summarised as the mean percentage of egg hatching (± SD). In all instances, normality test using Shapiro-Wilk tests performed by Kruskall-Wallis analysis or ANOVA were used to evaluate the differences in adult emergence, percentage of females inseminated, fecundity, and fertility between the treatments and controls. Pairwise comparisons were subsequently performed using Bonferroni or χ^2 tests. Kaplan-Meier survival analysis was operated to estimate the longevity of irradiated and unirradiated adult males. Log-rank tests were used evaluate whether there were statistical differences in survival time between the different cohorts. Statistical analyses and graphs were computed utilising software StataCorp, version 14, 2015 and the level of significance cut-off was 0.05. Three replicates were performed per experiment.

4.4 RESULTS

4.4.1 Evaluate the optimal irradiation dose for inducing sterility in *An. funestus* males

Results for adult emergence, percentage of females inseminated, fecundity and fertility of females mated with irradiated males as well as longevity of irradiated males were used to determine the optimal sterility inducing irradiation dose for *An. funestus* males. These experiments were carried out in two phases as described in section 4.2 and results for each phase were presented separately (Appendix G – Appendix H).

Phase 1:

Adult emergence: The number of male pupae irradiated per biological replicate ranged from 171 – 204 (Appendix G). The mean percentage adult emergence for the insectary control was 89.71% ± 8.94 (n = 565), while it was 91.20% ± 5.63 (n = 531) for the transport control. No significant difference was observed in the percentage of emerged adults between the two controls ($H_{(1)} = 0.48$, $p = 0.83$). Adult emergence in the treatment cohorts (irradiated pupae) ranged from 84.88% ± 9.45 (in pupae irradiated at 120 Gy) to 87.39% ± 10.99 (in pupae irradiated at 30 Gy) (Table 4.2). No statistically significant differences in adult emergence were observed through any of the irradiated males (treatments) or when they were compared against the two controls (insectary and transport controls) ($F_{(5, 12)} = 0.20$, $p = 0.95$).

Table 4.2: Physiological and reproductive parameters of unirradiated and irradiated *An. funestus* males mated with *An. funestus* females as measured during phase 1

Treatment	Emergence rate			Longevity			Reproductive parameters								
	Mean (%)	**± SD (95% C.I)**	**n**	**Median (days)**	**IQR [Q1 – Q3]**	**n**	**Mean IR (%)**	**± SD (95% C.I)**	**n**	*** Mean number of eggs**	**± SD (95% C.I)**	**n**	**Mean EH (%)**	**± SD (95% C.I)**	**n**
IC	89.70	8.94 (68 – 112)	565	39	20 – 58.5	105	83.33	5.77 (69 – 98)	212	8.01	3.24 (-0.51 – 16)	24	80.51	5.96 (66 – 95)	456
TC	91.20	5.63 (77 – 105)	531	39	20 – 8.5	96	80.00	10 (55 – 105)	218	7.00	1.11 (-4.2 – 9.8)	21	84.10	4.90 (72 – 96)	362
30 Gy	87.39	7.89 (68 – 107)	558	38	19 – 57	85	73.33	5.77 (59 – 88)	202	7.37	6.89 (-9.7 – 24.5)	22	74.67 [b]	4.50 (64 – 86)	431
60 Gy	87.69	10.99 (60 – 115)	552	38	19 – 57	95	83.33	11.55 (55 – 112)	216	3.92	2.89 (-3.3 – 11.1)	12	39.35 [b]	16.64 (-2.0 – 81)	260
90 Gy	86.23	9.06 (64 – 109	551	34 [a]	17 – 51	89	76.67	15.28 (39 – 115)	196	5.24	3.92 (-4.5 – 15)	16	27.69[b]	10.51 (1.6 – 54)	257
120 Gy	84.55	9.45 (61 – 108)	555	33 [a]	17 – 9.5	92	73.33	5.77 (59 – 88)	203	1.00	1.73 (-3.3 – 5.3)	3	0 [b]	NA	100

Abbreviations: IC = insectary control, TC = transport control, SD = standard deviation, IR = insemination rate, *mean number of eggs = mean number of eggs laid per female, EH =egg hatch rate, NA = not available n = sample size

[a] superscript notes a statistically significant difference from the control in longevity

[b] superscript notes a statistically significant difference from the control in egg hatch rate

Insemination rate: From a total of 212 blood-fed female that was permitted to mate with unirradiated insectary control males, 177 females were mated (83.33% ± 5.77). Similarly, from the 218 blood-fed females allowed to mate with transport control males 172, (80.00% ± 10) females were mated (Appendix G), (Table 4.2). No statistically significant difference was observed between the insectary and transport controls ($H_{(1)}$ = 0.19, p = 0.64). The proportion of females inseminated by sterile males at different doses ranged from 73.33% ± 5.77 – 83.33% ± 11.55 (Table 4.2). No statistical significant differences in the percentage of inseminated females were observed between females mated with irradiated males (treatments) and mated with irradiated males (treatments) and mated with unirradiated males (controls) ($H_{(5)}$ = 3.57, p = 0.56).

Fecundity: The number of blood-fed *An. funestus* females that had mated with insectary and transport control males ranged between 212 and 218. These subsequently laid an average of seven to eight eggs per female. Specifically, females that were mated with the insectary control males laid 8.01 ± 3.24 eggs/female, (n = 1 459), which was similar to the transport control that laid 7.00 ± 1.11 eggs/female, (n = 1 206) (Table 4.1). Fecundity ranged from 1.00 ± 1.73 eggs/female (120 Gy) to 7.37 ± 6.89 eggs/female (30 Gy) for irradiated *An. funestus* (Table 4.2). There were no statistical significant differences in fecundity between females mated with either unirradiated insectary or transport controls ($H_{(1)}$ = 0.43, p = 0.51). In addition, there were no statistical significant differences in fecundity between females mated with males irradiated at any of the irradiation doses (30, 60, 90, and 120 Gy) and the unirradiated controls ($H_{(5)}$ = 8.11, p = 0.15).

Fertility: The mean egg hatch rate (fertility) from females mated with the insectary control males was 80.51% ± 5.97, (n = 456), while in the females that mated with the transport control males it was 84.10% ± 4.90, (n = 362) (Table 4.2). Statistically, no significant difference in fertility between the eggs laid by females mated with insectary and transport control males ($H_{(1)}$ = 0.43, p = 0.51) was noted. A range of 100 – 431 eggs that were laid by females that had been mated with males irradiated at 30 – 120 Gy were used to determine egg hatch rate (fertility) (Appendix G). Fertility of the eggs originating from females that had been mated with the irradiated group ranged from 74.67% ± 4.50, n = 431 (30 Gy) to 0%, n = 100 (120 Gy) (Table 4.2). Significant difference was observed in egg hatch rate between treatments (30 – 120 Gy), ($H_{(3)}$ = 9.46, p = 0.02). A statistically significant difference in egg hatch was also observed among the two cohorts (treatments and control) ($F_{(5, 12)}$ = 44.76, p = 0.000). Subsequent pairwise comparisons showed that a higher egg hatch rate, when they were obtained

from females mated with unirradiated males compared to eggs from females mated with irradiated males (60 Gy to 120 Gy) (Table 4.3). In addition, the fertility of eggs from females mated with males irradiated at 60 Gy, 90 Gy and 120 Gy differed significantly ($p < 0.05$) between all cohorts (Table 4.3). A significant and positive strong relationship among fertility and the irradiation dose ($r^2_{(12)} = 0.88$) was observed.

Table 4.3: Pairwise comparisons of a fertility of *An. funestus* FUMOZ females mated with irradiated males and their corresponding control cohorts with significant *p*-values in bold font in brackets

Irradiation doses	**I C**	**T C**	**30 Gy**	**60 Gy**	**90 Gy**
TC	3.59 (1.000)				
30 Gy	-5.84 (1.000)	-9.43 (1.000)			
60 Gy	-41.17 (**0.001**)	-44.76 (**0.001**)	-35.32 (**0.005**)		
90 Gy	-52.82 (**0.000**)	-56.41 (**0.000**)	-46.98 (**0.000**)	-11.66 (1.000)	
120 Gy	-80.51 (**0.000**)	-84.10 (**0.000**)	-74.67 (**0.000**)	-39.35 (**0.002**)	-27.69 (**0.035**)

Abbreviations: IC = insectary control, TC = transport control

Induced sterility: Induced sterility in *An. funestus* females mated with males sterilised at 30 Gy – 120 Gy varied from 9.28% ± 5.46 to 100% ± 0 (Table 4.4). A statistical significant difference in the induced sterility was observed between females mated with males irradiated at different irradiation doses ($F_{(3, 8)} = 28.17$, $p = 0.0001$). Consequent pairwise comparisons (Table 4.5) displayed that induced sterility in females mated with 30 Gy irradiated males was significantly lower ($p < 0.0001$) than in the other treatments (60 Gy, 90 Gy, and 120 Gy). In addition, induced sterility was significantly lower from males irradiated at 60 Gy and 90 Gy than it was from males irradiated at 120 Gy. There was a near perfect positive linear relationship between induced sterility in females mated with irradiated males and irradiation dose received by these males ($r^2_{(4)} = 0.96$). Increasing the irradiation doses resulted in a corresponding increase in the sterility they induced in females.

Table 4.4: Induced sterility of *An. funestus* FUMOZ males irradiated at different irradiation doses

Irradiation doses	Induced sterility (%)	± SD
30 Gy	9.28	± 5.46
60 Gy	52.20	± 20.22
90 Gy	66.36	± 12.76
120 Gy	100.00	± 0.00

Table 4.5: Pairwise comparison by Bonferroni for induced sterility of *An. funestus* FUMOZ males irradiated at different irradiation doses with significant *p*-values in bold font in brackets

Irradiation doses	30 Gy	60 Gy	90 Gy
60 Gy	42.92 (**0.016**)		
90 Gy	57.08 (**0.003**)	14.16 (1.000)	
120 Gy	90.72 (**0.000**)	47.80 (**0.008**)	33.64 (0.060)

Adult longevity: The longevity of emerged adults from irradiated pupae and those that emerged from unirradiated controls are summarised in Figure 4.2 and Table 4.2. The longevity of males emerging from both insectary and transport control pupae were similar 39 days [20 – 58.5], (n = 105). No statistically significant difference in median longevity between the males from the insectary control pupae and those from the transport control pupae ($\chi^2_{(1, n = 201)} = 1.29$, $p = 0.255$) was observed. Longevity varied from 33 days [17 – 49.5], (n = 92) for adults emerged from male pupae irradiated at 120 Gy to 38 days [19 – 57], (n = 85), (n = 95) for adult males emerged from pupae irradiated at 30 Gy and 60 Gy, respectively. A statistically significant difference in median adult longevity between the treatment cohorts, ($\chi^2_{(3, n = 361)} = 40.47$, $p = 0.0001$) was noted. Subsequent pairwise comparisons (log-rank test) of survival times between the various treatments and control cohorts showed that adults from pupae irradiated at 90 Gy and 120 Gy had significantly lower survival times than those from controls ($p < 0.05$). The longevity of males irradiated at 30 Gy and 60 Gy differed significantly from the longevity of males irradiated at 90 Gy and 120 Gy ($p < 0.05$) (Table 4.6). No

significant differences in median longevity were observed among adults originating from pupae irradiated at 90 Gy and 120 Gy or when comparing longevity between 60 and 30 Gy.

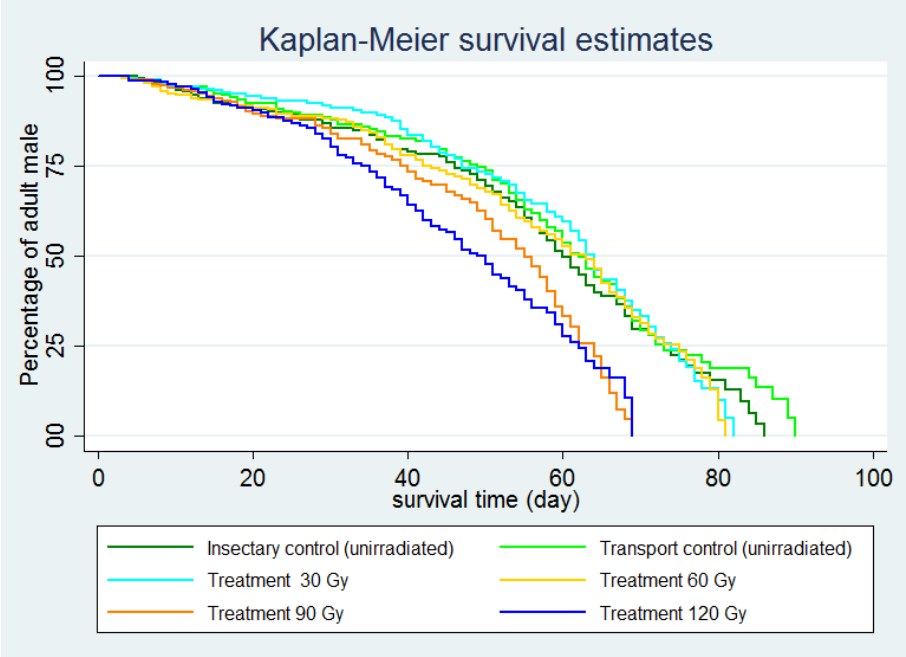

Figure 4.2: Survival curves of *An. funestus* adult males coming from pupae irradiated at different doses and corresponding adults coming from unirradiated pupae (insectary control, transport control)

Table 4.6: Pairwise comparisons of the median longevity of *An. funestus* FUMOZ males irradiated at different irradiation doses using χ^2 tests with significant *p*-values in bold font in brackets

Irradiation doses	**Insectary control**	**Transport control χ^2 (*p*)**	**30 Gy χ^2 (*p*)**	**60 Gy χ^2 (*p*)**	**90 Gy χ^2 (*p*)**
Transport control	1.29 (0.255)				
30 Gy	0.30 (0.586)	0.06 (0.805)			
60 Gy	0.06 (0.812)	1.03 (0.317)	0.88 (0.348)		
90 Gy	11.86 (**0.0006**)	17.45 (**0.0001**)	21.75 (**0.0001**)	12.55 (**0.0004**)	
120 Gy	16.61 (**0.0001**)	22.83 (**0.0001**)	27.58 (**0.0001**)	17.05 (**0.0001**)	1.02 (0.312)

Phase 2:

Results from phase 1 showed that higher levels of induced sterility were obtained when pupae were irradiated at 90 Gy (66.36%) and 120 Gy (100%). To further optimise the irradiation dose, doses > 90 Gy but ≤ 120 Gy were evaluated. Pupae were irradiated at 100 Gy; 110 Gy and 120 Gy. Only the insectary control was included as the two unirradiated controls used in phase 1 did not show any differences.

Adult emergence*:* A range of 608 – 609 male pupae per batch were irradiated at 100 – 120 Gy, and adult emergence ranged from 89.51% ± 3.09 to 85.90% ± 2.71 (Table 4.7; Appendix H), respectively. The adult emergence in the insectary control was 85.80% ± 8.75 (n = 604). Amongst the different irradiated treatments and the control ($H_{(3)}$ = 1.103, p = 0.776), no statistically significant difference was shown in adult emergence.

Insemination rate: Of 155 females that mated with unirradiated insectary control males, 87 females were inseminated (56.67% ± 20.82) (Table 4.7). Of 146 – 166 females allowed to mate with males irradiated at doses ranging from 100 – 120 Gy, 83 – 101 females were inseminated 46.67% ± 45.09 – 70% ± 26.46. No statistical significant differences in the percentage of inseminated females mated by irradiated males through the different irradiation doses (100 Gy – 110 Gy – 120 Gy) or the females mated with unirradiated control males were observed ($H_{(3)}$ = 1.231, p = 0.7328).

Fertility: The fertility of 383 eggs produced by females mated with insectary control males (unirradiated) was 80.77% ± 7.12, whereas egg fertility from females that were mated with males irradiated between 100 Gy and 120 Gy, ranged between 0 – 0.84% (Table 4.7). No statistically significant differences in fertility were observed amongst the treatments (100 Gy, 110 Gy, 120 Gy) ($F_{(2, 6)}$ = 1.00, p = 0.422). A statistically significant difference in the fertility of eggs from females between those females mated with unirradiated (insectary control) males and females mated with irradiated males was observed ($F_{(3, 8)}$ = 367.57, p = 0.0001).

Induced sterility: Induced sterility in An. funestus females mated with irradiated males ranged from 98.85% ± 2.00 to 100% ± 0. Statistically, no significant difference in induced sterility among eggs from females mated with males irradiated at the three different doses was observed ($H_{(2)}$ = 0.600, p = 0.37).

Table 4.7: Physiological parameters of *An. funestus* FUMOZ males irradiated at different doses and reproductive parameters of *An. funestus* FUMOZ females mated with irradiated males and their corresponding control cohort

Treatment	Emergence rate			Longevity			Reproductive parameters								
	Mean (%)	± SD (95% C.I)	n	Median (days)	IQR [Q1 – Q3]	n	Mean IR (%)	± SD (95% C.I)	n	*Mean number of eggs	± SD (95% C.I)	n	Mean HR (%)	± SD (95% C.I)	n
IC	85.80	± 8.75 (64 – 108)	604	43	22 – 57	150	56.67	20.82 (5 – 108)	155	14.31	11.00 (13 – 42)	43	80.77	7.12 (63 – 98)	312
100 Gy	89.52	± 3.09 (82 – 97)	608	38 [a]	20 – 53	145	56.67	20.82 (5 – 108)	161	1.22 [b]	1.42 (-2 – 5)	4	0.84 [c]	1.46 (-3 – 5)	2
110 Gy	87.08	± 6.00 (72 – 102)	609	41	22 – 54	150	70	26.46 (4 – 102)	146	0 [b]			NA		
120 Gy	85.90	± 2.71 (79 – 93)	609	37[a]	19 – 49	150	46.67	45.09 (-65 – 159)	166	0 [b]			NA		

Abbreviations: IC = insectary control, SD = standard deviation, IR = insemination rate, HR = egg hatch rate, NA = not applicable, n = sample size, CI = confidence interval

[a,b,c] superscript notes a statistically significant difference compared to the control

Adult longevity: Longevity of insectary control males was 43 days [22 – 57], (n = 150), while it was 37 days [19 – 49], (n = 150) and 41 days [22 – 54], (n = 150) for males irradiated at 120 Gy and 110 Gy, respectively (Figure 4.3 and Table 4.8). Statistically, a significant difference in the median adult longevity of males between the irradiated cohorts and unirradiated males ($\chi^2_{(3,\ n\ =\ 595)} = 40.47$, $p = 0.0001$) was stated. Pairwise comparisons of the longevity between males emerged from unirradiated pupae (insectary control) and adult males emerging from irradiated pupae (100 Gy, 110 Gy, and 120 Gy) using χ^2 tests are summarised in Table 4.8.

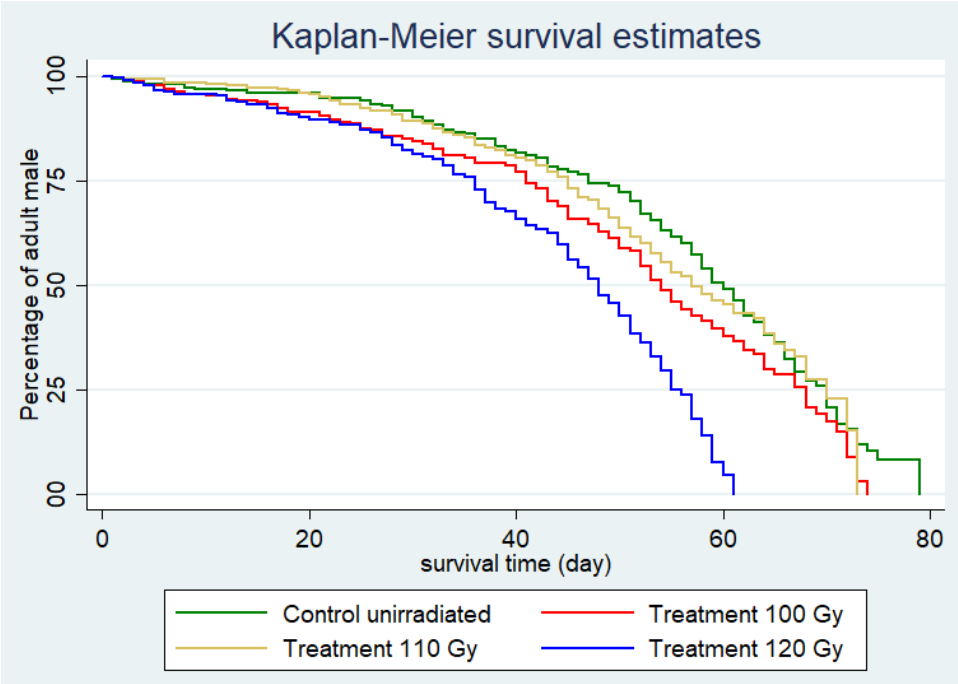

Figure 4.3: Kaplan-Meier survival curve for adult *An. funestus* males emerged from pupae irradiated at 100 Gy, 110 Gy, and 120 Gy and their corresponding control (adults emerged from unirradiated pupae) reared under standard insectary conditions.

Table 4.8: Pairwise comparisons of the median longevity of male *An. funestus* irradiated at different irradiation doses in phase 2 using χ^2 tests with significant *p*-values in bold in brackets.

Irradiation doses	**Insectary control χ^2 (*p*)**	**100 Gy χ^2 (*p*)**	**110 Gy χ^2 (*p*)**
100 Gy	4.23 (**0.0398**)		
110 Gy	3.56 (0.0593)	0.10 (0.7470)	
120 Gy	17.02 (**0.0000**)	2.86 (0.0905)	7.77 (**0.0053**)

4.5 DISCUSSION

Optimisation of irradiation dose constitutes a critical step in the development of SIT as an alternative or complementary vector control approach. Against a background of no prior information on how *An. funestus* responds to irradiation, the objective of this study was to assess the response of male *An. funestus* pupae to range of irradiation doses to determine the minimum sterilising irradiation dose to male sterility without affecting their physiological and reproductive fitness. This study constitutes the first report of using irradiation on male *An. funestus* to induce sterility.

The first parameter that was evaluated was adult emergence after *An. funestus* pupae were irradiated. The range of irradiation dose used in this study had no effect on adult emergence. Adult *An. funestus* emergence rates ranged from 84.55 – 89.52%, indicating that this species resists a variable range of irradiation doses. This is comparable with other main African malaria vector species like *An. arabiensis*. Helinski et al. (2006) and Munhenga et al. (2016) showed a high (> 80%) adult emergence rate for *An. arabiensis* when pupae were irradiated at doses between 25 – 100 Gy. This is an important finding from an SIT application perspective as most of the *An. funestus* pupae irradiated will emerge as adults and thus enhance the production capacity during the implementation of the SIT.

The second parameter evaluated was the effect of irradiation on the male mating ability. Ideally, irradiation should not have a significant impact on male mating behaviour as any effect on mating will reduce SIT success. In this study, unirradiated male pupae and male pupae irradiated at doses from 30 Gy to 120 Gy did not affect the female insemination by the males (mating success). This is in line with other studies that showed that those irradiation doses did not impact on mating success in *An. arabiensis* (Helinski and Knols, 2008[b]; Munhenga et al., 2016).

Thirdly, the impact on fecundity in females mated with irradiated males was evaluated. In this study, a significant reduction in the fecundity of *An. funestus* females mated with irradiated males compared to females that mated with unirradiated males was observed. Irradiation of males affects the sperm production which can consequently reduce egg production by females mated with these irradiated males (Calkins and Parker, 2005; Pérez-Staples et al., 2013). The result of this study tallies with Munhenga et al. (2016), in that there was no statistical significant difference in fecundity between females mated with males irradiated at different doses. The similarity might be caused by the damaged sperm production,

which could have an impact on quantity and quality of egg production (Calkins and Parker, 2005). The failure of female reproduction might not depend on irradiation dose of sterile males, but on female behaviour (Pérez-Staples et al., 2013). An interesting finding in this study is the significant impact higher irradiation doses had on fecundity. Very few eggs/female were produced from females mated with males that were irradiated at high doses. Although this outcome is favourable from an SIT application perspective it presented challenges when evaluating fertility. The number of eggs available to determine fertility between the different treatments was very low. This observation of very low fecundity was in contrast to those reported in other anophelines particularly in. *An. arabiensis,* where irradiation of male pupae at 70 – 100 Gy did not affect the fecundity, egg production from females was above 20 eggs/female (Munhenga et al., 2016). However, the non-production of eggs cannot be attributed to irradiation only but may be associated with the strain used (Yamada et al., 2019). The low fecundity was also observed in *An. funestus* females that were mated with unirradiated males as part of this study (Chapter 4). This unusual result warrants further investigation. Regardless, of the low fecundity of females mated with irradiated males (from 100 Gy) the fertility of the eggs from these eggs was evaluated successfully.

Egg fertility was evaluated to determine the induced sterility in females mated with males irradiated at the pupal stage. The egg hatching rate (fertility) was low for *An. funestus* females mated to males emerged from pupae that were irradiated. This was observed for all doses tested in both phase 1 and phase 2 i.e., irradiation doses ranging from 60 Gy – 120 Gy. This result confirms that irradiation causes lethality in male gametes that results in embryo development failure. The induction in sterility increased with increased irradiation dose. The egg fertility was as low as 0.84% when females were mated with males irradiated at 100 Gy and 27.69% from eggs originated from females mated to males irradiated at 90 Gy. As expected, a correlation between irradiation dose received by males and the amount of sterility they induced, was observed. These results are similar to several dose- response experiments from various mosquito species. However the level of sterility induced and the subsequent response at a given irradiation dose differs from species to species and even at strain level. For example, when *An. arabiensis* females mated with irradiated males (100 Gy) egg fertility was 0.5% (Munhenga et al., 2016), while Poda et al. (2018) reported fertility of 49% at 95 Gy for the same species. *Anopheles gambiae* males sterilised by irradiation at 120 Gy and mated to fertile females produced 0.51% eggs fertile (Andreasen and Curtis, 2005). These findings in *An. arabiensis, An. funestus* and *An. gambiae* showed that they are almost fully sterile when irradiated at doses of 100 Gy and higher.

The final parameter evaluated was the impact of irradiation on adult longevity. Understanding male longevity following irradiation is an important aspect for SIT technology (Calkins and Parker, 2005). If longevity of males used in an SIT programme is significantly shorter than the normal male lifespan, it will negatively impact on the operational implementation of the programme. The probability of potentially compromised males surviving to participate in swarms and mate before they succumb would be reduced and more frequent releases would be required to mitigate for this. Therefore, the better the longevity, the higher the probability will be for males to successfully mate, and possibly mate more than once, which will enhance the population suppression effect. A negative impact of irradiation on male longevity at higher irradiation doses i.e. 90 – 120 Gy was noted during this experiment. Irradiation at these doses shortened male survival compared to unirradiated controls. Although this was a significant reduction from a median age from of 39 – 43 days (phase 1 and 2, without irradiation) to a median age of 33 – 34 days at 120 Gy (phase 1 and phase 2 respectively). It would not have any effect at an operational level as *An. funestus* optimal mating success is 10-days (Zengenene et al., 2021). Sawadogo et al. (2013) showed that the optimal mating age of *An. gambiae s.s*. males was reached at four to eight days, to the best of my knowledge there are no data published for *An. funestus.* This result however, is different from findings of other anopheline species targeted for SIT. For example, unirradiated *An. arabiensis* median longevity is approximately 10 – 16 days and after irradiation at 70 Gy, it varied from 13 – 16 days (Munhenga et al., 2011; Munhenga et al., 2016; Dandalo et al., 2017). This difference in irradiation dose-response is probably genetically controlled and species-specific. However, the reduction in longevity observed in these studies might be due to somatic damage caused by irradiation as alluded to in most studies (Morlan et al., 1962; Proverbs, 1969; Helinski et al., 2006).

To conclude, the optimal dose required to sterilise male *An. funestus* was between 100 – 120 Gy. The use of small interval doses will interesting to determine the optimal dose that induced sterility without impacting sterile male fitness parameters. Although median longevity and fecundity were lower, this was not identified as a major risk. To evaluate the impact of 120 Gy on mating competitiveness of irradiated males, a study was conducted and the results presented in chapter 5.

CHAPTER 5

MATING COMPETITIVENESS OF STERILISED *ANOPHELES FUNESTUS* MALES

5.1 INTRODUCTION

The effectiveness of a mosquito SIT strategy is highly dependent on successful mating of the native female population by sterile males. As mentioned in chapter 4, the successful mating of wild females by laboratory reared and sterilised males depends on a number of factors including mating competitiveness. Sterile male mating competitiveness is defined as the chance that a wild female is mated by a sterile male when the sterile male is competing with wild males at equal ratios (Fried, 1971; Bouyer and Vreysen, 2020). The mating competitiveness of an insect species/strain targeted for SIT releases determines the sterile male to wild male ratio required and ultimately guides on the amount of males that should be released to ensure enough sterility is evolved in the native population to reach the goal of reducing the population (Madakacherry et al., 2014). Therefore, the knowledge of the mating competitiveness value of a species/laboratory strain marked for SIT releases is a cornerstone of any SIT programme.

Mating competitiveness is influenced by a number of factors. One of the factors is that the released males should first be reproductively compatible with native females. As sterilised males normally originate from a colonised strain, the colony would have gone through several genetic bottlenecks, which could result in the loss of certain behaviours such as the ability to locate a mating swarm. Furthermore, strains used for SIT releases are reared in artificial cages (e.g. Bugdorm® cages or specialised adult mass rearing cages). This can change insect courting behaviour and even flight ability that can make them lose their natural behaviours, reducing mating competitiveness (Culbert et al., 2020). Such behavioural changes were observed by Spates and Hightower (1967) where laboratory reared sterile males of the New World screwworm were more aggressive compared to the wild males and harassed wild females during mating. Contrary to this, barriers to mating were not observed in other insect species like the codling moth (Tare et al., 2010) and *Bactrocera oleae* (Olive fruit fly) (Ahmad et al., 2018) which were capable to successfully mate with their wild counterparts (Zervas and Economopoulos, 1982). Irradiation also affects mating competitiveness of sterilised males.

Although irradiation of males results in the intended goal of inducing dominant lethal mutations in germ cells. It also damages somatic cells, which can result in reduced mating competitiveness partly induced by the reduction of lifespan. The process of deterioration of

cells division and growth induced an acceleration of lifespan reduction (Aly et al., 1996). This highlights the need to evaluate mating competitiveness of a strain after determining optimal irradiation dose.

There are a number of approaches that can be used to evaluate mating competitiveness. The first approach is to set up competition experiments and determine the competitiveness index. This index can be used to calculate release ratios of sterile males to wild males to mate with fertile females. The success of SIT also depends on knowledge of the wild population size (Dame et al., 2009; Nolan et al., 2011; Ahmed, 2022; Becker et al., 2022). This information (population size) is important as it will guide the number and the frequency of releases of sterilised males once the competitiveness ratios have been determined experimentally. Therefore, while developing SIT for an insect pest, testing a range of ratios of sterilised to wild males is important (Munhenga et al., 2016; Becker et al., 2022). This information combined with the population size information will then guide the number of sterilised males required for SIT implementation stages. However, generally the higher the sterile male release ratio, the better the success.

The competitiveness index can be established through laboratory and semi-field competition experiments (Munhenga et al., 2016; Becker et al., 2022). It is worth noting that most competitiveness evaluations are conducted in the laboratory using cages or semi-field cages. Competitiveness assays performed this way might be perceived as unreliable in their ability to indicate the performance of sterile males under natural field conditions (Katsoyannos et al., 1999; Itô et al., 2021; Vreysen, 2021), however, laboratory analysis is easier to conduct and provides useful information. The use of semi-field cages provides an alternative mating competitiveness evaluation compared to small-scale evaluations in small laboratory rearing cages and has been used for studies. For example, semi-field cages have been used for *Ae. albopictus* and *An. arabiensis* to evaluate mating competitiveness (White et al., 1977; Zapien et al., 1983; Katsoyannos et al., 1999; Cayol et al., 2002; Oliva et al., 2012; Damiens et al., 2016; Munhenga et al., 2016).

Studies of mating competitiveness in mosquitoes have been previously done in various species. In the malaria vector, *An. coluzzii,* Maïga et al. (2014) showed that males sterilised at 90 Gy needed an overflooding proportion of 2:1 (sterile: fertile males) to effectively compete with wild-type males. The overflooding ratio of released sterile males to compete with wild males is one of the successful keys of the SIT vector control method. This efficiency of

competition among sterile males and wild males needs to be maintained during population suppression. For *An. arabiensis*, the insemination rate and fecundity were not affected by the ratio of sterile males to fertile males (Munhenga et al., 2016). A high sterile male ratio (10:1) (sterile: fertile) induces a higher rate of sterility and has the potential of population suppression for *An. arabiensis* (50.6 % sterility) (Munhenga et al., 2016). Although data on mating competitiveness and induced sterility exists for some key anopheline species, there is no data for *An. funestu*s.

5.1.1 Rationale for this study

In chapter 4, the dose-response of *An. funestus* to irradiation from 30 Gy to 120 Gy was tested and information on the sterilising irradiation dose for this species was obtained. However, information on the optimal irradiation dose alone is insufficient. The sexual competitiveness of an irradiated insect is also central to any SIT programme. To my knowledge, the gap of information about *An. funestus* competitiveness was unknown. Very few studies based on biology have been undertaken for this species. This current chapter aimed to fill this gap by evaluating the competitiveness ratio between sterile males and fertile males to mate with fertile females. The radiation dose of 120 Gy was selected to analyse the competitiveness of colonised sterile males versus colonised fertile males of *An. funestus*. This dose was selected as it induced complete sterility (induced 100% sterility in chapter 4). The sterile males capability to compete with fertile males is crucial for the implementation of SIT as a vector control method.

5.1.2 Aim

This study aimed to determine the relative mating competitiveness of irradiated colonised *An. funestus* males.

5.1.3 Objectives

The objectives of this study were:

1) Evaluating the amount of sterility induced into fertile females when mated with sterilised males in competition with fertile males
2) Assessing the impact of increased sterilised male to fertile male ratios on egg fertility
3) Determining the mating competitiveness index of *An. funestus* males irradiated at 120 Gy.

5.2 MATERIALS AND METHODS

5.2.1 Biological material: The *An. funestus* strain used for these experiments is fully described in section 2.2.1.

5.2.2 Irradiation

The irradiation process is similar to the one described in section 4.2.2 except that only one irradiation dose (120 Gy) was used. In summary, male pupae aged 24 – 30 hrs were irradiated at 120 Gy with Gammacell 220 irradiator following the procedures described in section 4.2.2. After irradiation male pupae were allowed to emerge in a Bugdorm® cage.

5.2.3 Mating competitiveness assays

Two to five days post-adult emergence, the sterilised males were placed into a Bugdorm® cage previously mentioned. Unirradiated fertile males (n = 50) and virgin fertile females (n = 50), of the same age, were introduced into these cages with different ratios of sterilised males as detailed in Table 5.1. Concurrently, two control cages referred to as fertile and sterile controls were also set up as tabulated in Table 5.1.

Table 5.1: Experimental design used to determine mating competitiveness of different ratios of irradiated males through competing with fertile males for fertile females of *An. funestus*

Cage number	**Ratio of sterile male: fertile male: female**	**Number of sterilised males**	**Number of fertile males**	**Number of virgin fertile females**	**Total mosquitoes/cage**
Fertile control	0: 1: 1	00	50	50	100
Sterile control	1: 0: 1	50	00	50	100
1	1: 1: 1	50	50	50	150
2	3: 1: 1	150	50	50	200
3	5: 1: 1	250	50	50	350

For each experimental cage mating was allowed to occur for 10 consecutive days. Following the 11 days of mating, females were fed on defibrinated bovine blood using a Hemotek® feeding system as previously stated in section 2.2.2. Blood-feeding was done on four different days within the same week (Monday, Tuesday, Thursday and Friday). Forty-eight

hours after the last blood meal, an oviposition plate consisting of a petri dish, painted black and containing 50 mL of RO water, was placed inside each cage to allow gravid females to lay eggs *en masse*. The day after inducing oviposition a subset of 10 females were selected at random from each cage to evaluate the insemination rate of females. Concurrently, eggs from each cage were harvested by collecting the oviposition plate, and fecundity (the number of eggs/female) was calculated (adjusted based on the number of live females which were present on the night of oviposition). Subsequently, fertility (eggs hatch rate), and the females insemination rate were determined as previously described in section 2.2.2. Three replicates were used per mating ratio experiment and for each control.

Calculation of induced sterility and competitiveness index

The effect of different ratios of sterile to fertile males in inducing sterility in females was determined by calculating induced sterility in each mating cage as described in section 4.2.4.

The competitiveness index was calculated after computing the expected egg hatch rates and observed hatch rates using procedures described by Fried, 1971 and Bellini *et al.*, 2013. In summary the competitiveness index (C) was obtained by comparing the observed mean egg hatch rate in the competition cages with mean egg hatch rate from control cages using the formula $C = (Hn - Ho) / (Ho - Hs) \times (N/S)$

The egg hatch rate from the fertile control cage is represented by *Hn* ; *Hs* is the egg hatch rate from sterile control cage; Ho is the observed egg hatch rate in each of the mating treatments (1:1, 3:1, and 5:1 sterilised male: fertile male ratios); *N* is the number of fertile males and *S* is the number of sterile males (in the competition cages).

5.3 DATA ANALYSIS

The data were analysed using the software StataCorp, version 14 (2015) at a 0.05 significance level. The outcome measures were: female insemination rate, fecundity, mating competitiveness index, and induced sterility were summarised as mean ± SE. Shapiro-Wilk test was used to determine data normality. Kruskall Wallis was used to evaluate differences between treatments and controls. Pairwise comparisons were performed when the analysis showed a significant difference. The competitive index and expected egg hatch were calculated utilising the formula of Fried, 1971.

5.4 RESULTS

5.4.1 Insemination rate

The female insemination rate in cages where sterile males (irradiated males) exclusively mated with virgin females (sterile control) was 52.78% ± 5.55 while the mean female insemination rate for the fertile controls i.e. fertile females exclusively mated with fertile males (unirradiated males) was 81.11% ± 5.88 (Appendix I). Statistically, a significant difference in the percentage of females inseminated with control sterile males and control fertile males ($p = 0.046$) was noted. The proportion of inseminated females in cages where sterile males were competing with fertile males for fertile females at different ratios ranged from 74.44% ± 9.15 to 85.55% ± 9.88 (Table 5.2). No significant statistical difference in the female insemination rates between the competitiveness cohorts ($H_{(4)} = 6.94$, $p = 0.14$) (Table 5.2), was observed.

Table 5.2: Reproductive parameters of *An. funestus* FUMOZ females mated with sterile and fertile males at different ratios and their corresponding controls

Ratio of sterile male: fertile male: female	***% Insemination rate (n)* ± SE**	***Number of eggs/female* ± SE**	***% fertility (n)* ± SE**
1:0:1 (sterile control)	52.78 ± 5.55	0.16 ± 0.16	0
0:1:1 (fertile control)	81.11 ± 5.88[a]	7.05 ± 1.06[a]	95.64 ± 2.64[a]
1:1:1	84.55 ± 10.84	0.48 ± 0.48[b]	26.33 ± 26.33
3:1:1	85.55 ± 9.88	10.00 ± 1.9[a]	52.67 ± 14.44
5:1:1	74.44 ± 9.15	13.27 ± 4.28[a]	18.85 ± 14.78[b]

[a]superscript letters within each column show significant difference at 95% significance level with sterile control

[b]superscript letters within each column show significant difference at 95% significance level with fertile control

5.4.2 Fecundity

The mean fecundity in females exclusively mated with irradiated males (sterile control) was 0.16 ± 0.16 eggs/female, while it was 7.05 ± 1.06 eggs/female for females that mated with unirradiated males (fertile control) (Appendix I). A statistically significant difference was observed in fecundity between the sterile and fertile controls ($p = 0.046$). The mean fecundity of females in cages in which they were mated with irradiated (sterile) males that were

competing with fertile (unirradiated) males at different ratios was significantly correlated with the number of sterile males in a mating cage, with fecundity increasing as the number of sterile males in the cage increased ($r^2 = 0.47$; $p = 0.046$). The fecundity of these females mated with sterile males ranged from 0.48 ± 0.48 to 13.27 ± 4.28 eggs/female (Table 5.2). Overall, a significant statistical difference in fecundity among treatment and control cages ($H_{(4)} = 11.07$, $p = 0.02$) was observed. Subsequently, pairwise comparisons stated a statistical significant difference between the fecundity of females from sterile control cages compared to the fecundity of females from competitiveness cages at the ratio of 5:1:1 ($p = 0.016$). A significant difference in fecundity among females mated with sterile males and fertile males at ratios of 1:1:1 and 5:1:1 ($p = 0.019$).

5.4.3 Fertility

The total number of eggs laid by females from the sterile control was 19, but did not hatch. Eggs laid by females from the fertile control cages had a mean fertility of 95.64% ± 2.52; while the eggs laid by females in cages in which they were mated with sterile males that were competing with fertile males ranged from 18.85% ± 14.78 to 52.67% ± 14.44 (Table 5.2). Statistically, significant difference among cohorts ($F_{(4,10)} = 6.08$, $p = 0.009$) was observed. Subsequently, pairwise comparisons showed a significant difference between sterile and fertile controls ($p = 0.001$). Statistically significant differences were also stated in the fertility of eggs originated from females in fertile control cages compared to eggs of females from the competitiveness ratio 5:1:1 cage ($p = 0.047$).

5.4.4 Induced sterility

The induced sterility in eggs from females from 1:1:1; 3:1:1 and 5:1:1 mating cages varied from 44.68% to 79.48% (Table 5.3) (Appendix I). Statistically, no significant difference was noted in induced sterility amongst the three mating cages in which sterile males were competing with fertile males ($H_{(2)} = 1.69$, $p = 0.43$).

5.4.5 Mating competitiveness

The mating competitiveness values were 0.27 and 2.63 for 3:1:1 and 1:1:1 cages, respectively. No statistically significant difference was observed ($H_{(2)} = 2.00$, $p = 0.37$) (Table 5.3) (Appendix I).

Table 5.3: Induced sterility and mating competitiveness values for *An. funestus* males irradiated at 120 Gy competing with fertile males and females under laboratory conditions in Bugdorm® cages

Ratio of sterile male: fertile male: female	***Observed hatch (%) ± SE***	***Induced sterility (%)***	***Competitiveness value* (C)***
1:0:1 (sterile control)	0		
0:1:1 (fertile control)	95.64 ± 2.52[a]		
1:1:1	26.33 ± 26.33	72.91	2.63
3:1:1	52.67 ± 14.44	44.68	0.27
5:1:1	18.85 ± 14.78[b]	79.48	0.81

[a]superscript letters within each column show significant difference at 95% significance level with sterile control

[b]superscript letters within each column show significant difference at 95% significance level with fertile control

*$C = (Hn – Ho) / (Ho – Hs) \times (N/S)$

5.5 DISCUSSION

The fulfilment of any SIT programme stands on the release of sterilised males that can competitively mate with native or wild females. This study constituted the first study on the sterile male competitiveness of *An. funestus* and provides insight into the potential of using SIT to control this vector species. This study gathered baseline data on the competitiveness of *An. funestus* males irradiated at a dose that induced sterility without affecting mating vigour based on insemination rate. This study was conducted under laboratory conditions using rearing cages (BugDorm® cage).

Males irradiated at a dose of 120 Gy were allowed to compete with wild-type males (fertile insectary males) for wild-type females (fertile insectary females) under laboratory conditions at different sterile to wild-type (fertile) ratios. Fecundity and induced sterility were determined and the relative competitiveness index was subsequently calculated. This study showed no difference in female insemination rates between these competitiveness cohorts overall. The insemination rates were not affected by ratio showing that both irradiated males and wild-type males were equally capable of mating with the females. This tally with several

studies that show that irradiation does not affect insemination ability (Munhenga et al., 2016; Dandalo et al., 2017). This confirms that the irradiation dose chosen in Chapter 4 i.e. 120 Gy is a good dose as it did not affect insemination ability.

However, fecundity of females and fertility of eggs from the different mating ratios differed from those in the controls as well as between the cages at the different mating ratios. Females that mated with sterile males (sterile control cage) showed a 44 times reduction in egg production compared to the fertile control females (0.16 eggs/female versus 7.05 eggs/females). This has been previously reported in a number of studies (on different mosquito species) which showed that irradiation has an impact on sperm quality which subsequently affects egg production (Calkins and Parker, 2005; Munhenga et al., 2016; Dandalo et al., 2017). Surprisingly, it was interesting that the fecundity from females in the cages increased as the ratio of sterilised males increased. Females from the 3:1:1 and 5:1:1 treatment cages showed fecundity similar to the fertile control cage. However, no significant difference in fecundity was observed between the females from the 1:1:1 cages and the females in the sterile control cages. This can be attributed to a number of factors. It could be possible that the few females mated with fertile males had very high fecundity masking the effect of low fecundity in females mated with sterile males. A possibility that the irradiation did not affect sperm quality in some of the sterile males is not exceptional. Mutations, as a result of irradiation, are random and do not affect all males in the same manner (Pérez-Stapler et al., 2013; Vreysen, 2021).

As expected the fertility of eggs between the sterile and fertile control cages differed significantly. Irradiation at 120 Gy induced enough sterility, resulting in no egg hatching and supporting the findings in chapter 4. The fertility of eggs from the treatment cages was significantly lower than that of the fertile control cage. The proportion of five sterile males to one fertile male and one fertile female (5:1:1 cage) resulted in the lowest fertility. The dose used to sterilise males damaged reproductive cells induced a partial or total inability to reproduce (Lance and McInnis, 2021). The overflooding of sterile males to compete with fertile males to mate with females resulted in the production of unviable embryos (Robson, 2005). Although, the overall induced sterility as well as the competitiveness value varied, no significant differences between the different ratios tested were observed. Even though the induced sterility did not show a statistically significant difference, it showed that it still increased by increasing the ratio between sterile males and fertile males in 1:1:1 and 5:1:1. This was probably due to the low number of eggs obtained, also due to the small sample size. Munhenga et al. (2016) reported similar results with *An. arabiensis*, where induced sterility

increased when the ratio of sterile to fertile males mated with fertile females increased (Munhenga et al., 2016). The mating competitiveness values varied with the ratio of sterile male: fertile male. The sterile *An. funestus* males were two times more competitive than fertile males (C = 2.63) in the same ratio of sterile males and fertile males (1:1:1). The hatch rate of this ratio was 26.63% compared to 18.85% for a ratio 5:1:1. At a ratio of 5:1:1 sterile males were almost as competitive as the fertile males (C = 0.81). This finding shows that *An. funestus* have a high competitiveness value (mean competitiveness value was 1.24, calculated from the value of ratios 1:1:1, 3:1:1 and 5:1:1) which might due to their ability to tolerate a high irradiation dose (120 Gy) for sterilisation. In contrast, the competitiveness index of *An. arabiensis* irradiated at 75 Gy in laboratory conditions was low (0.1) (Munhenga et al., 2016). Comparing these two studies, it shows the variable response of each species in radio-sensitivity, fecundity and fertility. Furthermore, sterile males were sufficiently competitive in mating with fertile females giving potential for success for SIT *An. funestus* trial. However, the competitiveness in a large cage would need to be investigated further.

The challenge with using laboratory conditions (using Bugdorm cages) is that swarming and mating behaviour might be impacted (Charlwood et al., 2003; Zawada et al., 2018; Kaindoa et al., 2019). However, overall the competitiveness value for *An. funestus* are similar to those of other studies (Munhenga et al., 2016; Maïga et al., 2014). Another limitation of the study was to obtain large numbers of pupae (> 1 000 pupae) of a specific age from the colony for conducting larger experiments with technical replicates or inclusion of a 10:1:1 ratio cage. This challenge can be overcome during semi-field cage evaluations using a larger team, including technicians to assist with rearing, sexing and conducting the experiment.

Based on the induced sterility results for *An. funestus*, the ratio 5:1:1 is recommended to use in future SIT projects. These findings also indicate that irradiation may have impacted the mating vigour of sterilised *An. funestus* males in laboratory experiment when in competition with fertile males, and the use of semi-field cages is advisable for future studies to confirm laboratory results for both mating vigour and induced sterility. This study provided a road map for the future research that will be needed for using SIT to control *An. funestus*. The irradiation dose at 100 Gy which is considered as in the interval of optimal irradiation dose of *An. funestus*, might be performed to see if this dose will improve the competitiveness value.

CHAPTER 6

CONCLUSION

Anopheles funestus, a major malaria vector, is notoriously difficult to rear in the laboratory. This is mainly due to lack of mating between adults under laboratory conditions. However, recently Ngowo et al. (2021) and others have initiated research to understand the colonisation bottlenecks in the hopes of improving the colonisation-ability of this species from various geographical areas. This will exponentially increase research outputs for this species and will be vital for understanding insecticide resistance mechanisms as well as enabling the evaluation of additional vector control tools based on the release of laboratory reared mosquitoes (e.g. SIT). This study was initiated to address some of the *An. funestus* colonisation challenges and to fill knowledge gaps on some biological aspects of developing SIT against this species. This was done through evaluating some key larval rearing and adult maintenance parameters of a colonised strain of *An. funestus*. In addition, the optimisation of irradiation dose based on the irradiation dose-response and the mating competitiveness of sterilised males was investigated. The study concluded that colonised *An. funestus* might be maintained on defibrinated bovine blood fed using an artificial membrane feeding system. This feeding method will alleviate the dependency on live animal hosts for blood meals. It was also shown that larval diet dose and larval rearing density plays a key role in optimised *An. funestus* laboratory rearing. Furthermore, both reproductive (fertility) and physiological (adult median survivorship) irradiation responses were irradiation dose-dependent.

Blood-feeding is required to ensure the continuous production of mosquito offspring (Briegel, 1990; Clements, 1992; Tripet et al., 2003; Hansen et al., 2014). The mosquito blood source depends on the available host species and on the feeding system available at the rearing facility (Vittor et al., 2006; Lyimo and Ferguson, 2009). This study showed no significant difference in egg fertility for *An. funestus* fed on live animals or fed using the artificial feeding system. The usual adult feeding condition was by direct feeding on an anaesthetised guinea pig, but a substitution to defibrinated bovine blood offered by an artificial membrane feeding system was proposed. Increasing the blood-meal frequency using the artificial membrane feeding system before induction of oviposition increased fecundity. A transition period allowing the colony to adapt to the new feeding method will most likely be required. In fact, *An. funestus* from Angola (FANG) fed on bovine blood showed an increase in fecundity over six generations

(Zengenene, 2021) and subsequently an *An. funestus* (FUMOZ) colony is being maintained on bovine blood (Maharaj et al., 2022).

Operating procedures for *An. funestus* colony maintenance i.e. larval and adult rearing are not yet standardised. As part of developing of standard operating procedures (SOPs) and standardisation of laboratory rearing for this species, food dose requirements per larva and the number of larvae per rearing tray to optimally rear this species were evaluated. The major outcomes used to assess these factors were the number of pupae that were successfully obtained from first instar larvae (or the highest pupal production rate) and adult sizes using wing length as a proxy. From the results, the optimal larval feeding dose using the standard NICD larval food was 0.04 mg/larva/feeding at two feedings per day. This regime resulted in a shorter development time from first instars to pupae (15 days) and high pupal production (almost 90%). Interestingly, feeding *An. funestus* at doses both higher and lower than the optimum dose had negative impacts on the output measures (larval developmental time, pupal production and adult sizes). This highlights the importance of measuring larval food quantity during mosquito rearing. This is particularly useful in a mass production setting where each rearing tray can then be standardised to obtain mosquitoes of consistently good quality. Larval density is another larval rearing parameter found to be critical in this study. The optimal *An. funestus* larval density was determined to be 0.48 larvae/cm^2. This density resulted in a short larval development time of an average of 16 days and high pupal production of almost 80%. Although this optimal density can be used to rear *An. funestus* larvae there is potential to increase rearing density by mitigating for larval overcrowding through the provision of additional anchoring surfaces, such as shown by Zengenene et al. (2022). Overall, using results from this study, and those of Zengenene et al. (2022) and Niain'ny Felamboahangy et al. (2023), SOPs can begin to be developed and refined to rear *An. funestus* in standard laboratory conditions. This is a vital step towards developing mass-rearing procedures for this species for future SIT applications.

The cornerstone of SIT programmes is to induce enough sterility in the target population to affect population suppression or eradication. In this study, the impacts of different irradiation doses on male longevity and the subsequent impact on their mating competitiveness was evaluated. This study showed that, although *An. funestus* is more radio-tolerant compared to some other anophelines, its response to irradiation was similar to other anophelines such as *An. arabiensis* (Helinski et al., 2008; Munhenga et al., 2016; Dandalo et al., 2017; Poda et al., 2018) and *An. coluzzii* (Maïga et al., 2014). Higher irradiation doses were deleterious to physiological fitness, although they induced the highest sterility. In this work an irradiation

dose of 120 Gy was found to be optimal to perform the first mating competitiveness evaluation for *An. funestus* under laboratory conditions. However, this dose resulted in lower competitive values compared to other studies. Previous studies (reviewed in chapter 1) have shown that reducing irradiation dose improves the mating competitiveness of sterile males (Hooper, 1972; Toledo et al., 2004; Parker and Metha, 2007). It is therefore recommended that additional laboratory mating competitiveness studies be evaluated at a dose of 100 Gy to see if this will improve the competitiveness value. It will then guide the expansion of competitive studies to semi-field cage evaluation using a ratio 3:1:1, 5:1:1 (sterile male: fertile male: female) and a higher 10:1:1 ratio, prior to field trials.

This study evaluated some key parameters for successful laboratory rearing of *An. funestus* and confirmed that male *An. funestus* can be sterilised by irradiation at an optimum dose range of 100 to 120 Gy. It is critical, prior to embarking on the releases, to assess doses between 100 and 120 Gy (105, 110, 115 Gy) and select a dose that induced more than 99% since 100% sterility obtained with 120 Gy may greatly impact the quality of the sterile males. There are still a large number of key aspects that will need to be evaluated before SIT and mass rearing becomes a reality for this species. This includes (but is not limited to) the evaluation of different colonies to determine if there are colony specific differences for both the insectary rearing parameters as well as the optimal irradiation dose needed for sterilisation. The mating ability of laboratory reared and sterilised males will also need to be investigated in the field.

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APPENDICES

APPENDIX A: Published article

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Optimisation of laboratory-rearing parameters for *Anopheles funestus* larvae and adults

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ABSTRACT

Anopheles funestus is one of the major malaria vectors in Africa. As with the other main vectors, insecticide resistance in this species threatens existing vector control strategies. Unfortunately, scientific investigations, which could improve understanding of this vector species or lead to the development of new control strategies, are currently limited by difficulties in laboratory rearing of the species. In an attempt to optimise laboratory-rearing conditions for *An. funestus*, the effect of an artificial blood-feeding system for adults, different larval diet doses, and a range of other rearing conditions on the life history traits of an existing colony were investigated. Firstly, fecundity and fertility in *An. funestus* adult females fed on either live guinea pigs or bovine blood supplied through an artificial membrane feeding system were assessed. Secondly, a life-table approach was used to assess the impact of larval food dose (mg/larvae), larval density (larvae/cm^2), and the depth of water used for larval rearing on life history traits. Fecundity was significantly higher when females were blood-fed on live anaesthetised guinea pigs than when fed on defibrinated bovine blood. However, the fertility of these eggs did not differ significantly between the two feeding methods or blood meal sources. Mosquitoes fed on defibrinated bovine blood using the artificial membrane feeding system showed an increase in egg production when the blood-feeding frequency was increased, but this difference was not statistically significant. The quantity of larval food influenced both time-to-pupation and pupal production. Increasing the larval densities resulted in reduced both time-to-pupation and pupal productivity. An optimal larval density of 0.48 larvae/cm^2 was vital in preventing overcrowding. Increased water depth in the larval trays, was associated with significantly lower pupal production and reduced pupal weight. In conclusion, these results show that *An. funestus* can be reared using defibrinated bovine blood delivered via an artificial membrane feeding system. The quantity of larval food, optimal larval density, and depth of water used for larval rearing are critical factors influencing colony productivity. These findings can be used to improve current guidelines for rearing *An. funestus* under insectary conditions.

1. Introduction

In 2020, malaria deaths increased by 12% compared to 2019, partly affected by disruptions to malaria control efforts during the COVID-19 pandemic (WHO, 2021). *Anopheles funestus* is recognised as a primary malaria vector that is highly anthropophagic (human feeding) and endophilic (resting indoors) (Gillies and De Meillon, 1968; Manga et al., 1997; Coetzee and Koekemoer, 2013). These behavioural traits make this species highly susceptible to indoor-insecticidal interventions, notably long-lasting insecticide-treated nets and indoor residual spraying. However, the rapid escalation of insecticide resistance has become a significant threat to the sustainability of these interventions (Fontenille

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et al., 1990; Coetzee and Koekemoer, 2013; Hancock and Hendriks, 2020; Moyes and Athinya, 2020). Because of this and other challenges, several alternative interventions are being developed. amongst these are genetic bio-control options such as the sterile insect technique (SIT) and genetically modified mosquitoes (Benedict and Robinson, 2003; James, 2005; Bourtzis et al., 2016). Some of these technologies will require a greater understanding of the biology of the target vector species and the colonisation of the vector species to support field release.

Unfortunately, for *An. funestus*, the scarcity of viable laboratory colonies and its refractoriness to colonisation presents a considerable challenge for developing bio-control strategies against this species (Service and Oguamah, 1958; Service, 1960; Gillies and De Meillon, 1968). There is limited knowledge on optimal laboratory rearing conditions for this species compared to other vector species, limiting the viability of bio-control technologies. Other than the early attempts in Nigeria in the 1950s (Service and Oguamah, 1958), only two stable *An. funestus* colonies, one from Mozambique in 2000 and the other from Angola in 2002, have been successfully established starting from wild material (Hunt et al., 2005; Zengenene et al., 2021). While these two colonies (which have been widely shared with multiple laboratories) enable key studies on this vector species, there remains a desire to colonise and study local *An. funestus* populations in different countries. To achieve this and potentially enable mass-rearing, the critical parameters that promote *An. funestus* proliferation under laboratory conditions need to be investigated and optimised.

One critical rearing parameter for successfully colonising mosquito species is having a sustainable method of providing a blood meal to adult female mosquitoes to facilitate egg development. Most *Anopheles* mosquitoes are anautogenous and depend on blood for reproductive success (Hansen et al., 2014). The blood provides key amino acids and proteins required for egg production (Briegel, 1990; Clements 1992; Takken et al., 1998; Olayemi et al., 2011; Gonzales and Hansen, 2016). *Anopheles funestus* feeds primarily on humans (Kahamba et al., 2022), a characteristic that may become a significant bottleneck to colonisation, especially in laboratories that rely on non-human vertebrates for the blood meal sources. Successful colonisation at scale may also require a change from direct human blood-feeding to animal or artificial membrane feeding systems (Gerberg, 1970; Benedict et al., 2007; Olayemi et al., 2011; LaFlamme, 2011). Current and past *An. funestus* colonies have been successfully maintained on either guinea pigs alone or both guinea pigs and human blood (Service and Oguamah, 1958; Hunt et al., 2005; Ngowo et al., 2021). This presents a challenge due to the large number of animals required, ethical concerns and cost implications associated with such blood delivery approaches (Gonzales and Hansen, 2016).

Besides blood-feeding, optimal larval rearing conditions are also necessary to ensure the consistent production of "high-quality" healthy adults (Damiens et al., 2012; Somda et al., 2017). The quality and quantity of larval food affect the developmental time and the number of emerging adults (Damiens et al., 2012; Somda et al., 2017). Harold (1930) initiated the first research on larval food for *Anopheles*, *Aedes* and *Culex* mosquitoes (*Culicidae*) almost a century ago. Since then, various studies to optimise larval rearing conditions have been conducted on multiple anophelines, including *An. darlingi* (Araujo et al., 2012), *An. stephensi* (Reisen et al., 1975) and *An. arabiensis* (Gilles et al., 2011; Damiens et al., 2012; Somda et al., 2017). Increased larval densities can also lead to extended larval development periods (Zengenene et al., 2022). This phenomena of extended larval development time and smaller *An. gambiae* adults, was also observed when water-rearing depths were increased (Phelan and Roitberg, 2013). However, there remains a limited number of larval rearing studies conducted on *An. funestus* (Ngowo et al., 2021; Zengenene et al., 2021, 2022).

To address the gaps, blood-feeding and larval rearing of *An. funestus* were investigated. These studies constituted an attempt to optimise laboratory-rearing conditions for this vector species.

2. Materials and methods

2.1. Biological material

All experiments were conducted using mosquitoes from the FUMOZ (*An. funestus* from Mozambique) colony (Hunt et al., 2005). Aquatic stages were maintained on a standard powdered larval diet consisting of crushed dog biscuits (Beeno®, South Africa, http://www.beeno.co.za) and brewer's yeast (Vital®, Vital Health Foods, South Africa), mixed at a 3:1 ratio respectively. The nutritional composition of the dog biscuits is provided in Zengenene et al. (2022). Adult mosquitoes were sustained on a 10% sucrose solution, and blood meals were provided using anaesthetised guinea pigs. Adult females were fed twice a week, and egg plates were provided after at least two blood meals.

2.2. Assessing the effect of different blood meal sources on fecundity and fertility

A total of 200 adults (100 virgin males and 100 virgin females) were allowed to mate in a BugDorm-4S3030 cage (32.5 cm x 32.5 cm x 32.5 cm; BugDorm®, Megaview Science Co., Ltd, Taichung, Taiwan, hereafter referred to as BugDorm cage) for 10 consecutive days with ad libitum access to 10% sugar solution. After the mating period, the 10-day-old females were sugar-starved for 24 h and provided with either defibrinated bovine blood (for 30 min) via an artificial membrane feeding system (Hemotek®, Hemotek Ltd, United Kingdom) or a live shaven anaesthetised guinea pig (for 15 min). The use of live guinea pigs, being the standard blood-feeding method used to maintain *An. funestus* colonies at the Botha de Meillon insectary, at the National Institute for Communicable Diseases, Johannesburg, was considered as the control cohort.

Artificial membrane blood feeding was done by adding 5 ml of defibrinated bovine blood to a preheated (37 °C) aluminium heating plate (3.7 cm in diameter and 1.3 cm thick) covered with hog casing membrane and connected to the Hemotek® membrane feeding system (Cosgrove et al., 1994; Damiens et al., 2013).

Feeding occurred in a dark room when adults were 11 days old (first blood meal) and again when they were 14 days old (second blood meal). Blood-fed females were not removed after first blood feeding to mimic routine rearing procedures for the colony. However, males and unfed females were removed after the second blood meal. The blood-fed females were counted to determine the feeding success. Subsequently, fecundity of these blood-fed females and fertility of resultant eggs was determined. These experiments were repeated three times using different biological material (biological repeats). Each biological repeat consisted of three replicates (for the artificial membrane feeding system) or one replicate (guinea pig feeding to limit the number of guinea pigs needed) (Appendix Table A1a).

Egg harvesting: Egg plates containing purified water were provided 48 h after the second (last) blood meal to induce females to oviposit. Eggs were harvested 24 h later. Water used was purified using reverse osmosis, which filters ionic contaminants, most organic compounds and all particulates from the water. However, the hardness of the water used for larval rearing was not analysed.

Percentage insemination: After egg harvesting, the spermathecae of 10 randomly-selected females from each cage were dissected to determine the percentage of inseminated females.

Fecundity: Fecundity (F) for the first gonotrophic cycle was calculated as the number of eggs per female after adjusting the total number of females (fn) with percentage insemination rate (i), as follows: Adjusted number of females (afn) = fn x i and Fecundity (F) = number of eggs (ne)/afn. The number of females used to calculate fecundity was the number alive in a cage when the cage was egg plated. It was not possible to determine whether females that died 24 hrs post egg plating contributed to egg production or not, therefore, no adjustment for female mortality was done post egg platting.

Fertility: Harvested eggs were transferred into small round larval bowls (9 cm base radius x 12 cm top radius with a height of 6 cm) filled with 100 ml of purified water. The eggs were monitored for hatchlings daily for 14 days. Fertility was calculated as the number of larvae hatched divided by the total number of eggs used (Munhenga et al., 2016).

2.3. Assessing the effect of blood feeding frequency using the artificial membrane feeding system on fecundity and fertility

Female *An. funestus* were either fed twice (Monday and Thursday) or four times (Monday, Tuesday, Thursday, and Friday) on defibrinated bovine blood using the artificial membrane feeding system as described in Section 2.2. Fecundity and fertility were estimated using the same cages and adult density as described in Section 2.2 above (see Appendix Table A1b).

2.4. Assessing the effects of larval diet dose on time-to-pupation, pupal production, adult emergence, pupal weight and wing length

One hundred first instar larvae were placed into standard larval trays (21 cm (length) × 15 cm (width) × 8 cm (height), containing 750 ml of purified water, resulting in a larval density of 0.32 larvae/cm^2. Five food weights (doses) (0.02; 0.03; 0.04; 0.06; 0.08 mg/larva) of the standard larval diet described in Section 2.1 were evaluated. Larvae were fed twice daily, with food quantity increasing proportionally daily until pupation (Table 1). One biological repeat comprising of three technical replicates and a total of three biological repeats were conducted.

Time-to-pupation: larvae were monitored daily until pupation. The number of days between the first instar stage of larvae to pupae was recorded and survival analysis was used to calculate the median time to pupation. As the ambient temperature was controlled and recorded, the water temperature was not recorded.

Pupal production: The total number of pupae obtained was recorded and represented as a proportion of the total number of first instar larvae used.

Pupal weight: Due to limited pupal numbers, a subset of 30 male and 30 female pupae were randomly selected from each treatment. Each pupa was placed onto filter paper to absorb and remove excess water before being weighed using an analytical balance (KERN ABT 120–5DM, Balingen, Germany). Overall mean weight was calculated.

Adult emergence: Adult emergence was monitored and recorded for all pupae after excluding those used for weight measurements. Percentage emergence was calculated as the number of emerged adults divided by the number of pupae for each diet dose.

Wing length: Upon emergence, a subsample of 60 adults (30 females and 30 males) per treatment were immobilised in a refrigerator (4 °C), and used for wing length measurements (as a proxy for adult size). The right wing was gently dissected from the body using forceps, placed onto a glass slide, and then viewed under a microscope (SZ2-ILST, Olympus Corporation Tokyo, Japan) fitted with a camera (S2 × 7). Wing length was measured using Olympus Stream Essentials 1.9.4 from the distal edge of the allula to the end of the radial vein, excluding the fringe scales (Marina et al., 1999).

2.5. Assessing the effects of larval densities on time-to-pupation, pupal production, adult emergence, pupal weight and wing length

Five different larval densities (0.25, 0.32, 0.48, 0.64, 1.27 larvae/cm^2) were evaluated under standard rearing conditions described in Section 2.1. Larvae were fed twice daily at a dose of 0.04 mg/larva of the standard larval diet (Table 1), based on the diet dose experiment above. The experiments were divided into two stages. Stage one evaluated 0.25, 0.32 and 0.48 larvae/cm^2, while stage two investigated 0.32, 0.64 and 1.27 larvae/cm^2. One biological repeat comprised of three technical replicates, and a total of three biological repeats were conducted. The parameters assessed were the same as in the diet dose experiment (Section 2.4).

2.6. Assessing the effects of water depth on time to pupation, pupal production adult emergence, pupal weight and wing length

Three different water depths (1, 3, and 5 cm) were compared. Larvae were reared as described above (Section 2.5) with a larval density of 0.48 larvae/cm^2 and were fed 0.04 mg/larva of larval food twice a day. The evaluation consisted of three biological repeats, each comprising five technical replicates. The parameters assessed were the same as in the diet dose experiment (Section 2.4).

2.7. Data analysis

Statistical analyses were completed using statistical software StataCorp, version 14 (2015) at a 0.05 significance level. The Shapiro-Wilk normality test was used to assess normality of variables (Shapiro and Wilk, 1965). Two-sample t-tests were used to compare fecundity and fertility. Kaplan-Meier survival analysis (Kaplan and Meier, 1958) was used to estimate the time-to-pupation. Log-rank tests were used to test for equality of survivor functions between the different treatments. A one-way analysis of variance (ANOVA) (Anscombe, 1948) was used to compare proportions of pupae produced, proportions of adults produced, wing length and pupal weight between treatments and controls. Where a significant difference was observed, Tukey's "honestly significant difference" (HSD) *post hoc* test was used to determine differences between pairs. For data that did not fit the assumptions of normality, Kruskal-Wallis tests (Kruskal and Wallis, 1952) were used to determine differences for each variable. Differences between pairs were then analysed using pairwise χ^2 tests.

2.8. Ethics

The blood-feeding protocol for routine colony rearing was approved by the National Health Laboratory Service, Animal Ethics Committee (AESC: 1993–047).

3. Results

3.1. Effects of different blood meal sources on fecundity and fertility

The percentage of females that were successfully fed (% blood-fed ±

Table 1
Anopheles funestus larval feeding regimen using standard larval diet. Larvae were fed the amounts indicated in mg/larva twice a day i.e. once each in the morning and afternoon.

Diet dose / feeding	Treatment	D1	D2	D3	D4	D5	D6	D7	D8	D9	D10	D11	D12	D13 until end
	T1	0.02	0.03	0.04	0.06	0.08	0.10	0.12	0.14	0.20	0.26	0.32	0.40	0.50
	T2	0.03	0.04	0.05	0.07	0.09	0.12	0.15	0.20	0.25	0.30	0.35	0.40	0.50
	T3	0.04	0.05	0.06	0.08	0.10	0.14	0.18	0.22	0.28	0.32	0.40	0.45	0.50
	T4	0.06	0.08	0.10	0.14	0.18	0.24	0.30	0.40	0.50	0.60	0.70	0.80	1.00
	T5	0.08	1.00	0.12	0.16	0.20	0.28	0.36	0.44	0.56	0.64	0.80	0.90	1.00

Diet dose / feeding (mg/larva): larvae were fed twice a day using the quantity of food indicated in the table.
T: Treatment; D1: Day one, etc.

S.D.) did not differ significantly between females provided with anaesthetised guinea pigs (53.00% ± 4.58; $n = 300$) and those provided with defibrinated bovine blood using the artificial membrane feeding system (48.78% ± 11.04; $n = 900$) (Independent t-test, $t_{(10)} = 0.63$, $p = 0.5442$) (Table 2; Appendix Table A1a). Furthermore, the percentage of females inseminated (% insemination ± S.D.) amongst mosquitoes fed on guinea pigs was 83.33% ± 15.27, ($n = 30$), and 62.22% ± 23.33, ($n = 90$) for those fed on bovine blood using the artificial membrane feeding system. These differences were not statistically significant (independent t-test, $t_{(10)} = 1.44$, $p = 0.1799$).

Females fed on guinea pigs produced significantly more eggs per female (10.94 ± 0.77; $n = 1460$) than those fed on bovine blood via the artificial membrane feeding system (2.88 ± 2.09; $n = 664$); ($t_{(10)} = 6.37$, $p = 0.0001$). However, the egg fertility (% of eggs hatching into larvae) did not differ between blood sources (guinea pigs (79.56% ± 9.34; $n = 826$); bovine blood (77.33% ± 14.44; $n = 664$); two sample t-test ($t_{(10)} = 0.25$, $p = 0.8105$)).

3.2. Effects of blood feeding frequency using the artificial membrane feeding system on fecundity and fertility

The percentage of females that took a blood meal (% blood-fed ± S.D.) when fed twice using the artificial membrane feeding system (46.33% ± 13.65, $n = 300$) did not differ significantly from those fed four times (51.44% ± 10.98, $n = 900$), (independent t-test, $t_{(10)} = -0.66$, $p = 0.5223$), (Table 2, Appendix Table A1b). The percentage of females inseminated (% insemination ± S.D.) were also similar between the two groups (two meals: 90.00% ± 10.00 ($n = 30$); four meals: 71.11% ± 24.72 ($n = 90$); independent t-test ($t_{(10)} = 1.26$, $p = 0.2377$)). However, the number of eggs produced per female was doubled in females fed four times (4.61 ± 2.67; $n = 1554$) compared to females fed twice (2.3 ± 1.58; $n = 317$), even though this difference was not statistically significant (independent t-test, $t_{(10)} = -1.39$, $p = 0.0977$). The mean fertility (% of eggs hatching ± S.D.) of eggs laid by females that were blood-fed twice was 81.28% ± 16.06 ($n = 317$) compared to 83.85% ± 13.13 ($n = 1554$) in eggs from females blood-fed four times, this difference was statistically insignificant ($t_{(10)} = -0.28$, $p = 0.7829$).

3.3. Effects of larval diet dose on time-to-pupation, pupal production adult emergence, pupal weight and wing length

Time-to-pupation: Larval development time (time-to-pupation) was calculated as the time taken by the first instar larvae to develop into pupae. The median times-to-pupation in days (IQR: Q1 - Q3) for *An. funestus* reared on 0.02, 0.03, and 0.04 mg/larva of the larval diet were 16 (15 - 17), 15 (14 – 17), and 15 (14 – 17) days respectively (Table 3). Further analysis of larval survival distributions using the log-rank test showed that the time-to-pupation differed significantly between the three larval doses ($\chi^2_{(2,\ n\ =\ 2189)} = 79.45$, $p < 0.0001$). The larvae fed on the lowest diet dose (0.02 mg/larvae) had the longest time-to-pupation, i.e. 16 (15 - 17) days (Table 3). Subsequent pairwise comparisons showed a significant difference in time-to-pupation between 0.02 mg/larva and either 0.03 mg/larva ($\chi^2_{(1,\ n\ =\ 1389)} = 31.68$, $p < 0.0001$) or 0.04 mg/larva ($\chi^2_{(1,\ n\ =\ 1467)} = 83.93$, $p < 0.0001$). This was also true when comparing time-to-pupation between 0.03 mg/larva and 0.04 mg/larva ($\chi^2_{(1,\ n\ =\ 1522)} = 10.18$, $p = 0.0014$). In the second stage experiments, the median times-to-pupation were 17 (16 - 17), 12 (12 – 15) and 13 (12–16) days when larvae were fed 0.04, 0.06 and 0.08 mg/larva of the diet, respectively (Table 3). However, no subsequent statistical analysis was conducted due to low pupal production (see below).

Pupal production: Pupal production between the diet doses differed significantly ($F_{(2,\ 24)} = 6.22$, $p = 0.0067$) in the first stage experiments. The highest pupal production (% pupae produced ± S.D.) was from larvae fed at 0.04 mg/larva (88.89% ± 5.86, $n = 800$), and the lowest from larvae fed at 0.02 mg/larva (74.11% ± 10.07, $n = 667$) (Table 3). In the second stage experiments, the 0.06 and 0.08 mg/larva doses yielded only 0.67% and 9.11% of pupae, respectively (Table 3), and were considered unsuitable for rearing *An. funestus*. Downstream analysis was impossible and therefore excluded in subsequent data analyses.

Pupal weight: The mean pupal weight (± S.D.) was 1.34 ± 0.10; 1.36 ± 0.11 and 1.35 mg ± 0.12 mg for larvae fed at 0.02, 0.03 and 0.04 mg/larva respectively. These were not statistically different (Kruskal-Wallis; $H_{(2)} = 2.518$, $p = 0.2840$; $n = 540$).

Adult emergence: Adult emergence ranged from 93.76% ± 3.76 ($n = 487$) for larvae fed at a dose of 0.02 mg/larva to 96.82% ± 2.59 ($n = 542$) for those fed at 0.03 mg/larva. Feeding larvae at a higher dose of 0.04 mg/larva produced 96.58% ± 2.96 ($n = 599$) emergence of adults from the pupae. There were no significant differences in emergence between the larval diet doses (ANOVA: $F_{(2,\ 24)} = 2.65$, $p = 0.0915$).

Wing length: Mean wing lengths (± S.D.) for larvae reared at 0.02 ($n = 180$), 0.03 ($n = 180$) and 0.04 mg/larva ($n = 180$) of the diet were 3.28 ± 0.16, 3.27 ± 0.12 and 3.31 mm ± 0.17 respectively (Table 3). The Kruskal-Wallis analysis showed no significant differences in wing lengths between these diet doses ($H_{(2)} = 3.529$, $p = 0.1713$).

3.4. Effects of larval densities on time-to-pupation, pupal production adult emergence, pupal weight and wing length

Time-to-pupation: Larvae reared at a very high density of 400 larvae per tray (1.27 larvae/cm^2) had the shortest time-to-pupation. The median (IQR: Q1 – Q3) times-to-pupation were 19 (17 - 21), 17 (16 - 19), and 16 (15 - 17) days for the three rearing densities 0.25, 0.32 and 0.48 larvae/cm^2, respectively (Table 4). Log-rank test analysis indicated significant differences in time-to-pupation between the rearing densities ($\chi^2_{(2,\ n\ =\ 2001)} = 405.21$, $p < 0.0001$). Larvae reared at the lowest density (80 larvae/tray or 0.25 larvae/cm^2) had the longest time-to-pupation from first instar larvae to pupae. Subsequent pairwise comparisons showed there were significant differences in time-to-pupation when 0.25 larvae/cm^2 were compared to 0.32 larvae/cm^2 ($\chi^2_{(1,\ n\ =\ 973)} = 149.23$, $p < 0.0001$) and 0.48 larvae/cm^2 ($\chi^2_{(1,\ n\ =\ 1367)} = 369.83$, $p < 0.0001$). Time-to-pupation of larvae reared at 0.32 larvae/cm^2 was significantly longer than larvae reared at a density of 0.48 larvae/cm^2 ($\chi^2_{(1,\ n\ =\ 1662)} = 78.78$, $p < 0.0001$). Similarly, analysis for stage two showed significant differences in time-to-pupation between the three larval rearing densities (Log-rank test: $\chi^2_{(2,\ n\ =\ 2454)} = 26.93$, $p < 0.0001$). Subsequent pairwise analyses also showed significant differences between 0.32 larvae/cm^2 and 0.64 larvae/cm^2 ($\chi^2_{(1,\ n\ =\ 1467)} = 4.73$, $p = 0.0297$), 0.32 larvae/cm^2 and 1.27 larvae/cm^2 ($\chi^2_{(1,\ n\ =\ 1639)} = 20.30$, $p < 0.0001$), and 0.64 larvae/cm^2 and 1.27 larvae/cm^2 ($\chi^2_{(1,\ n}$

Table 2
Summary of blood feeding outcome of *An. funestus* females fed on A) live animal host (control) and artificial blood feeding system (treatment) and B) two blood meals vs four blood meals through an artificial blood feeding system.

	Blood feeding approach (N)	% females blood fed ± S.D. (n)	% females inseminated ± S.D. (n)	Number of eggs laid/ female ± S.D.	% Egg fertility ± S.D. (n)
A	Anaesthetised guinea pig (300)	53 ± 4.58 (159)	83.33 ± 15.28 (30)	10.94 ± 0.77	79.56 ± 9.34 (826)
	Artificial feeding using Hemotek feeder (900)	48.78 ± 11.04 (439)	62.22 ± 23.33 (90)	2.88 ± 2.09	77.33 ± 14.44 (664)
B	Artificial feeding using Hemotek feeder for two blood meals (300)	46.33 ± 13.65 (139)	90 ± 10.00 (30)	2.30 ± 1.58	81.28 ± 16.06 (317)
	Artificial feeding using Hemotek feeder for four blood meals (900)	51.44 ± 10.98 (463)	71.11 ± 24.72 (90)	4.61 ± 2.67	83.85 ± 13.13 (1554)

N– total number of females used; *n*– number of females that fed, inseminated or number of laid eggs and hatched.

Table 3
Summary of developmental parameters of *An. funestus* larvae reared at different larval diet doses.

	Diet dose (mg/larva)	Total number of L1 used	Developmental parameter monitored: Median time-to-pupation in days (IQR)	Total number of pupae from L1	Mean proportion of L1 developing into pupae (S.D.)%	Mean pupal weight (S.D.) mg	Total number of pupae used for adult emergence *	Total number of adults emerged	Mean proportion of pupae developing into adults (S.D.)%	Mean wing length (S.D.) mm
Stage 1	**0.02**	900	16 [15 - 17]^{a}	667	74.11 (10.07)^{a}	1.34 (0.10)	487	455	93.76 (3.76)	3.28 (0.16)
	0.03	900	15 [14 - 17]^{b}	722	80.22 (10.18)^{ab}	1.36 (0.11)	542	524	96.82 (2.59)	3.27 (0.12)
	0.04	900	15 [14 - 17]^{c}	800	88.89 (5.86)^{b}	1.35 (0.12)	620	599	96.58 (2.96)	3.31 (0.17)
Stage 2	**0.04**	900	17 [16 –17]	678	75.33 (18.01)	NA	NA	NA	NA	NA
	0.06	900	12 [12 - 15]	6	0.67 (1.12)	NA	NA	NA	NA	NA
	0.08	900	13 [12 - 16]	82	9.11 (17.95)	NA	NA	NA	NA	NA

* indicates the total number of pupae available for adult emergence after pupae were removed for pupal weight.

Table 4
Summary of the effects of different larval rearing densities on the developmental parameters of *An. funestus* larvae.

	Larval rearing density (larvae/cm^2), (larvae/tray)	Total number of L1 used	Developmental parameter monitored: Median time-to-pupation in days (IQR)	Total number of pupae from L1	Mean proportion of L1 developing into pupae (S.D.)%	Mean pupal weight (S.D.) mg	Total number of pupae used for adult emergence□	Total number of adults that emerged	Mean proportion of pupae developing into adults (S.D.)%	Mean wing length (S.D.) mm
Stage 1	**0.25 (80)**	720	19 [17–21]^{a}	339	47.08 (24.28)^{a}	1.36 (0.18)	161	128	81.30 (20.35)	3.36 (0.15)
	0.32 (100)	900	17 [16–19]^{b}	634	70.44 (12.29)^{b}	1.37 (0.16)	454	392	86.07 (9.85)	3.33 (0.14)
	0.48 (150)	1350	16 [15–17]^{c}	1027	76.07 (14.53)^{b}	1.37 (0.16)	757	696	91.85 (6.66)	3.33 (0.15)
Stage 2	**0.32 (100)**	900	15 [14–16]$^{\#}$	652	72.44 (15.95) $^{\#}$	1.31 (0.15)^{a}	472	421	89.18 (7.74)	3.30 (0.13)^{a}
	0.64 (200)	1800	14 [13–15]*	815	45.28 (26.08) $^{\#*}$	1.35 (0.14)^{b}	509	406	76.03 (20.54)	3.28 (0.12)^{b}
	1.27 (400)	3600	12 [11–13]$^{\&}$	987	27.42 (29.41)*	1.29 (0.12)^{a}	570	501	86.44 (8.92)	3.24 (0.11)^{c}

□ before pupae were taken for weighing

Superscript letters and symbols indicate significant differences between treatments, if there are no letters or symbols there were no significant differences between treatments.

$_{= 1802)} = 4.32$, $p = 0.0377$).

Pupal production: The percentage of pupae (mean% pupae produced ± S.D.) produced per treatment from stage one experiments ranged from 47.08 ± 24.28 ($n = 339$) to 76.07% ± 14.53 ($n = 1027$) (Table 4). The mean percentage pupal production differed significantly depending on the larval rearing density for stage one (Kruskal Wallis; $H_{(2)} = 7.308$, $p = 0.0259$). Pairwise comparisons within stage one showed that the pupal production from larvae reared at 0.25 larvae/cm^2 was significantly different from those reared at 0.32 larvae/cm^2 ($p = 0.0202$, 95% C.I. = [3.78 −38.69]) and 0.48 larvae/cm^2, ($p = 0.0073$, 95% C.I. = [4.50 - 24.49]). Pupal production did not differ between larval densities of 0.32 and 0.48 larvae/cm^2 ($p = 0.3865$).

The pupal production (% ± S.D.) from the second phase evaluations was lowest (27.42% ± 29.41) when larvae were reared at the highest density (1.27 larvae/cm^2) (Table 4). There were statistically significant differences in mean percentage pupal production between the different larval rearing densities (Kruskal Wallis; $H_{(2)} = 10.31$, $p = 0.0058$). Pairwise comparisons within stage two, revealed significant differences in mean percentage pupal production between larvae reared at a density of 0.32 larvae/cm^2 (72.44% ± 15.95) and 0.64 larvae/cm^2 (45.28% ± 26.08) ($p = 0.0169$, 95% C.I. = [−27.09, −3.09]). This was also true when comparing 0.32 larvae/cm^2 (72.44% ± 15.95) and 1.27 larvae/cm^2 (27.42% ± 29.41) ($p = 0.001$, 95% C.I. = [−24.52, −7.64]). Generally, there was a weak but significant relationship between larval rearing density and pupal production (r^2 (53) = 0.1379, $p = 0.0057$); pupal production decreased as the larval rearing density increased.

Pupal weight: The weight (mean weight ± S.D.) of pupae reared at different larval densities during stage one experiments were generally similar (1.37 mg ± 0.16 observed in the 0.48 and 0.32 larvae/cm^2 trays and 1.36 mg ± 0.18 in the 0.25 larvae/cm^2 tray). Kruskal-Wallis test showed no significant differences ($H_{(2)} = 0.71$, $p = 0.7014$).

However, in the second stage experiment, the lowest weight was obtained from the 1.27 larvae/cm^2 trays (1.29 mg ± 0.12, $n = 417$). The mean pupal weights for larvae reared at 0.32 and 0.64 larvae/cm^2 were 1.31 mg ± 0.15 ($n = 180$) and 1.35 mg ± 0.14 ($n = 306$) respectively, these differences being statistically significant (Kruskal-Wallis, $H_{(2)} = 30.59$, $p = 0.0001$). Pairwise comparisons showed a statistical difference between pupae reared at 0.64 and 0.32 larvae/cm^2 ($\chi^2_{(1,\ n\ =\ 486)} = 7.38$, $p = 0.0066$). Pupal weight from larvae reared at 0.64 larvae/cm^2 and 1.27 larvae/cm^2 also differed significantly ($\chi^2_{(1,\ n\ =\ 723)} = 30.94$, $p = 0.0001$). Comparisons of the pupal weight when larvae were reared at a density of 0.32 larvae/cm^2 and 1.27 larvae/cm^2 did not differ significantly ($\chi^2_{(1,\ n\ =\ 597)} = 2.79$, $p = 0.0952$). There was no correlation between larval density and pupal weight from stage two experiments ($r^2_{(902)} = 0.0037$, $p = 0.0680$).

Adult emergence: In stage one, the percentage of adult emergence ranged from 81.30 to 91.85% (Table 4), and there were no significant differences between the three larval rearing densities (Kruskal-Wallis; $H_{(2)} = 1.3256$, $p = 0.5372$). In stage two, the mean percentage of adult emergence ranged between 76.03 and 89.18% (Table 4), with no significant difference between these cohorts ($F_{(2,16)} = 1.82$, $p = 0.1944$).

Wing length: The mean wing length of adults emerging from larvae

reared at 0.25 larvae/cm^2 was 3.36 mm ± 0.15 (n = 92), 3.33 mm ± 0.14 for those reared at 0.32 larvae/cm^2 were (n = 180) and 3.33 mm ± 0.15 for those reared at 0.48 larvae/cm^2 (n = 2700). There were no statistically significant differences in wing lengths between the different cohorts (Kruskal-Wallis, $H_{(2)} = 2.30$, $p = 0.3169$). However, in the stage two experiments, there were significant differences in wing lengths between the cohorts (Kruskal-Wallis, $H_{(2)} = 39.74$, $p = 0.0001$) (Table 4). Pairwise comparisons showed that wing lengths in adults emerging from larvae reared at 0.32 larvae/cm^2 (3.30 mm ± 0.13, n = 180) differed significantly from those reared at 0.64 larvae/cm^2 (3.28 mm ± 0.12, n = 253), ($\chi^2_{(1,\ n\ =\ 433)} = 5.30$, $p = 0.0213$) and those reared at 1.27 larvae/cm^2 (3.24 mm ± 0.11, n = 295), ($\chi^2_{(1,\ n\ =\ 475)} = 38.36$, p = 0.0001). This was also true for wing lengths of adults emerging from larvae reared at 0.64 larvae/cm^2 and compared to wing lengths of adults from larvae reared at 1.27 larvae/cm^2 ($\chi^2_{(1,\ n\ =\ 548)} = 15.34$, p = 0.0001). There was a significant relationship between larval rearing density and wing length for stage two ($r^2_{(727)} = 0.0395$, $p < 0.0001$), with wing length decreasing as larval rearing density increased.

3.5. Effects of water depth on time-to-pupation, pupal production, adult emergence, pupal weight and wing length

Time-to-pupation: The median time-to-pupation (IQR: Q1 – Q3) for larvae reared at a depth of 1 cm was the longest at 18 days (14 - 19), followed by 16 days (15 - 19) and 16 days (15 - 17) for larvae reared at 3 cm and 5 cm depths respectively (Table 5). A log-rank test showed that time-from first instar larvae to pupation differed significantly between the different rearing water depths ($\chi^2_{(2,\ n\ =\ 1915)} = 58.10$, $p < 0.0001$). Subsequent pairwise comparisons showed time-to-pupation differed significantly when larvae were reared at a water depth of 1 cm compared to those reared at a water depth of 3 cm ($\chi^2_{(1,\ n\ =\ 1110)} = 22.29$, $p <$ 0.0001), and between larvae reared at water depths of 3 cm and 5 cm ($\chi^2_{(1,\ n\ =\ 1334)} = 70.08$, $p < 0.0001$). There was no significant difference in time-to-pupation for larvae reared at a water depth of 1 cm compared to those reared at a depth of 5 cm ($\chi^2_{(1,\ n\ =\ 1386)} = 0.64$, $p = 0.4187$).

Pupal production: Percentage pupal production (% pupae produced ± S.D.) was highest when larvae were reared at a depth of 3 cm (63.51% ± 25.68, n = 1429) followed by a depth of 1 cm (56.39% ± 30.23, n = 1269), and the lowest pupal production was obtained at a depth of 5 cm (28.62% ± 23.69, n = 669) (Table 5). Statistical analysis showed a significant difference in pupal production between the three treatments (Kruskal-Wallis, $H_{(2)} = 11.06$, $p = 0.0040$). Pairwise comparisons revealed that the differences in pupal production were between larvae reared at water depths of 1 cm and 5 cm ($\chi^2_{(1,\ n\ =\ 392)} = 5.688$, p = 0.0171), as well as between water depths of 3 cm and 5 cm ($\chi^2_{(1,\ n\ =\ 434)}$ = 10.602, $p = 0.0011$). There was no significant difference between the pupal production when larvae were reared in water 1 cm or 3 cm deep ($\chi^2_{(1,\ n\ =\ 209)} = 0.190$, $p = 0.6632$).

Pupal weight: Pupal weights (Mean weight in mg ± S.D.) were higher for larvae reared in water 1 cm deep (1.25 mg ± 0.22, n = 270) compared to those reared at depths of 3 (1.16 mg ± 0.18, n = 270) and 5 cm (1.09 mg ± 0.16, n = 270). Pupal weight differed significantly between treatments (Kruskal-Wallis, $H_{(2)} = 16.15$, $p = 0.0003$).

Subsequent pairwise comparisons showed that pupal weight from larvae reared in water 1 cm deep differed statistically from those reared at water depths of 3 cm ($\chi^2_{(1,\ n\ =\ 120)} = 4.419$, $p = 0.0347$) and 5 cm ($\chi^2_{(1,\ n\ =\ 120)} = 15.126$, $p = 0.0001$). The pupal weight of larvae reared at a water depth of 3 cm also differed statistically from those reared at a water depth of 5 cm ($\chi^2_{(1,\ n\ =\ 120)} = 4.665$, $p = 0.03$). There was a significant relationship between water depth and pupal weight ($r^2_{(180)}$ = 0.1029, $p < 0.0001$), with pupal weight decreasing as water depth increased.

Adult emergence: The proportion of adults emerging from pupae from the three water depths did not differ statistically (ANOVA: $F_{(2,\ 6)} = 1$, p = 0.4227) and ranged from 90.18 to 94.41% across the different treatments (Table 5).

Wing length: The wing sizes of adults emerging from larvae that were reared at a depth of 1 cm (2.49 mm ± 0.13, n = 270) were similar to those of larvae reared at depths of 3 (2.51 mm ± 0.13, n = 270) and 5 cm (2.49 mm ± 0.11, n = 270). Statistically, there were no significant differences in the wing lengths regardless of the depth of the water in which the larvae were reared ($H_{(2)} = 0.48$, $p = 0.7876$).

4. Discussion

This study reports on crucial laboratory rearing parameters for *An. funestus* and was an attempt to contribute towards the optimal rearing conditions of the species. It is the first report evaluating the impact of blood meal sources on fecundity and fertility and the effect of larval diet and water depth on *An. funestus* growth and development.

The study compared the fecundity and egg fertility of *An. funestus* females that were offered blood meals from two different delivery systems. Blood was provided to *An. funestus* females via a live host (anaesthetised guinea pig) or an artificial membrane feeding system using defibrinated bovine blood. The females' blood-feeding rates when using artificial system and live animals were comparable. However, the number of eggs per female (fecundity) obtained from the two blood-fed cohorts differed significantly. Fecundity in females blood-fed using the artificial membrane feeding system was considerably lower than in females fed on live animals (guinea pigs). The higher fecundity in females that fed on guinea pigs could be an adaptation of the strain to guinea pig blood feeding. However, it cannot be excluded that the difference in fecundity might be due to the use of defibrinated blood in the artificial membrane feeding system. It is established that defibrination results in a loss of a protein component, mainly fibrinogen, and might explain the reduction in fecundity reported here (Johnstone and Thorpe, 1987; Wotkuh-Wocadkuu, 1970). Fecundity was improved by increasing blood-feeding frequency using the artificial membrane feeding system,

Table 5
Summary of different developmental parameters of *An. funestus* larvae reared at three different water depths.

Rearing water depth (cm)	Total number of L1 used	Developmental parameter measured: Median time-to-pupation in days [IQR]	Total number of pupae from L1	Mean proportion (%) of L1 developing into pupae (S.D.)	Mean pupal weight (S.D.) mg	Total number of pupae for adult emergence*	Total number of adults emerged	Mean proportion of pupae developing into adults (S.D.)%	Mean wing length (S.D.) mm
1 cm	2250	18 [14–19]^a	1269	56.39 (30.23)^a	1.25 (0.22)^a	1209	1125	94.05 (3.06)	2.49 (0.13)
3 cm	2250	16 [15–19]^b	1429	63.51 (25.68)^a	1.16 (0.18)^b	1369	1235	90.18 (2.37)	2.51 (0.51)
5 cm	2250	16 [15–17]^a	669	28.62 (23.69)^b	1.09 (0.16)^c	609	589	94.41 (5.87)	2.49 (0.11)

L1 = first instar larvae.
* indicates the total number of pupae available for adult emergence after pupae were removed for pupal weight (as per Table 2 in manuscript)
Superscript letters that differ indicate significant differences between treatments, if there are no letters there were no significant differences between treatments.

but this was not statistically significant.

Even where fecundity is low, it is possible to improve this through continuous selection. Mosquitoes are known to adapt to different blood-feeding sources depending on circumstances and the availability of the host blood meal (Bruce-Chwatt et al., 1966; Phasomkusolsil et al., 2013; Gunathilaka et al., 2017; Khan et al., 2021). This was recently confirmed by Zengenene (2021), who successfully increased fecundity in an *An. funestus* colony from Angola after six generations of selection on an artificial blood-feeding system using defibrinated bovine blood. After this study, a FUMOZ colony was sustained on bovine blood (Maharaj et al., 2022). Although the experimental procedures were different, from this study, Maharaj et al. (2022) reported a fecundity of 48.11 ± 4.12 eggs per female on the same FUMOZ colony blood-fed on defibrinated cattle blood. Supporting the notion that selection has improved the egg production in this species. However, it is unknown if defibrinated bovine blood will be suitable for establishing a new colony from wild females.

Another critical parameter for successful maintenance of *An. funestus* colonies under insectary conditions is optimising larval rearing conditions. This study investigated the impact of larval diet dose, density and water rearing depth on five key physiological parameters (time-to-pupation, pupal production, pupal weight, adult emergence success and adult wing length [as a proxy for body size]). Larval diet dose significantly affected the median larval time-to-pupation for *An. funestus*. Increasing larval food quantities initially had a significant positive impact on larval survivorship until a critical point where further increases became detrimental. Higher doses (0.06 and 0.08 mg/larva) resulted in significantly lower pupal production. The optimal larval food dose with the highest pupal productivity (almost 90% of larvae pupated) was 0.04 mg/larva. The decreased pupation at higher food doses is not peculiar to this study and was previously reported in *An. arabiensis* and *An. stephensi* (Reisen, 1975; Gilles et al., 2011; Damiens et al., 2012). The high larval mortality at high food doses might be attributed to larval suffocation as it will result in excess food on the water surface, making it impossible for anopheline larvae to breathe. It could be possible that using a liquid diet might mitigate this challenge; further investigation to explore this is recommended (Damiens et al., 2012)

Feeding larvae different food doses did not affect the mean pupal weight, adult emergence and wing length. However, the limited sample size used for measuring pupal weight and wing length might preclude this result. Never the less, this observation contrasts with results obtained for *An. arabiensis* where higher quantities of larval diet produced larger *An. arabiensis* adults (Damiens et al. (2012). Interestingly the wing sizes reported in this study were bigger than previously published studies on *An. funestus* (Mwangangi et al., 2004; Ngowo et al., 2021; Zengenene et al., 2022). Therefore, it is possible that the quantity of food provided was above the daily nutritional requirements for the larvae.

The ideal larval rearing density was investigated with faster time-to-pupation and high pupal productivity as the key indicators for successful rearing. These criteria were met at a larval density of 0.48 larvae/cm^2, and lower or higher densities decreased pupal production. The lower pupal production at higher larval densities might be explained by predation or, more likely larval suffocation due to overfeeding. This might be mitigated by maintaining the total larval food dose per day but feeding a lower dose (mg/larva at a time) at more frequent intervals (remaining with the same amount of food in a 24 h feeding cycle). It is difficult to explain the decrease in pupal production at low larval density. One hypothesis is that increased larval mortality resulted from first instar larvae perhaps being less mobile and unable to feed properly in large containers, possibly due to uneven or sporadic distribution of food on the water surface.

This study also concluded that a water depth of 3 cm fulfils the key criteria for successful larval rearing (fastest time-to-pupation and highest pupal production). Interestingly, the pupal weight decreased with increased water depth, although no differences in adult wing sizes were recorded. Tchuinkam et al. (2011) showed that water depths of 3 cm or less produced more *An. gambiae* pupae (>70%) compared to water depths of 6, 10, 15 and 30 cm. In deeper water, larvae and pupae most likely use more energy reserves when swimming to the surface, resulting in decreased resources available for development (Tchuinkam et al., 2011).

In conclusion, *An. funestus* females can produce viable eggs if fed on defibrinated bovine blood. Therefore, an artificial membrane feeding system is a possible alternative adult blood-feeding system to maintain colonies. Optimal larval feeding, density and water depth can ensure maximum pupal productivity in relatively short periods. These optimised parameters could be used in standard operating procedures for laboratory-reared *An. funestus* and provides vital information for the future design of mass rearing equipment for this species. However, these should be evaluated against local populations and other laboratory strains where and when available.

Declaration of Competing Interest

The authors declare no conflict of interest.

Data availability

Data will be made available on request.

Author statement

The authors contributed to the drafting the corrections on the attached revised manuscript.

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Disclosures

LLK conceptualised the project, LNF; GM; MLK and LLK designed the project; LNF and MPZ conducted the experiments; LNF, PMZ, MLK, GM and LLK analysed data; LNF, MLK, FO, GM and LLK wrote first and subsequent versions of the manuscript. All authors read and approved the final manuscript.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.actatropica.2022.106785.

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APPENDIX B: Ethic clearance

ANIMAL RESEARCH ETHICS COMMITTEE
Registration number: AREC-101210-002

December 6, 2018

To whom it may concern,
Reference : Waiver Felamboahangy Koekemoer 201812060
Re: Waiver from the Animal Research Ethics Committee of the University of the Witwatersrand

Student : *Lalasoa Niain'ny Felamboahangy*
Student number : 1941769
Degree : PhD Candidate
Study Title : "Evaluating the potential use of sterile insect technique for the future control of the major African malaria vector, *Anopheles funestus* (Diptera: *Culicidae*)".
Email: 1941769@students.wits.ac.za

Department: Student house, 1 Modderfontein Road, NICD/NHLS, Sandringham 2131

Supervisor: Dr Maria Kaiser, Dr Givemore Munhenga, Prof Lizette Koekemoer ; Email: Lizette.Koekemoerwits.ac.za
Wits Research Institute for Malaria, National Laboratory Health Services, Sandringham 2131
Tel: + 011 5550303; 060-4518491;

Full clearance certificate from the AREC of the University of the Witwatersrand is NOT required.

Reason: This study uses mosquitoes (invertebrates).

Comment/Notes : Full ethics application was made – waiver issued.

Please contact me should you require further information.

Yours sincerely

GP Candy

Geoffrey Candy
Chair : Animal Ethics Research Committee, University of the Witwatersrand

Geoffrey P Candy PhD
Professor, Department of Surgery, Faculty of Health Sciences, University of the Witwatersrand, *7 York Road Parktown 2193, Johannesburg; Tel: 27-11-717-2574; Email: geoffrey.candy@wits.ac.za*

APPENDIX C: Raw data the impact of blood-feeding source on the percentage of females blood fed, percentage of females inseminated, fecundity and fertility

Control = guinea pig , biological replicate: 1,2,3

ABF=Artificial blood-feeding; ABF 1.1 = Biological replicate 1, technical replicate 1 – ABF 1.2 = Biological replicate 1, technical replicate 2 – ABF 1.3 = Biological replicate 1, technical replicate 3 – ABF 2.1 = Biological replicate 2, technical replicate 1 and so on by ABF 3.3

Source of blood feeding	Number of females	Number of females fed	Blood feeding rate (%)	number of females dissected for insemination rate	Insemination Rate (%)	Number of eggs	Number of females to calculate Number of eggs	Number of eggs/female = fecundity	Number of eggs used	Number of 1st instar larvae	Fertility in (%)
Control 1	100	52	52.00%	10	80%	486	41.6	11.68	100	90	90.00%
Control 2	100	49	49.00%	10	70%	348	34.3	10.15	100	72	72.00%
Control 3	100	58	58.00%	10	100%	626	58	11	626	480	76.68%
Overall	**300**	**159**	**53.00%**	**30**	**83%**	**1460**	**133.9**	**10.94**	**826**	**642**	**79.56%**
ABF 1.1	100	42	42.00%	10	50%	12	21	0.57	12	12	100.00%
ABF 1.2	100	67	67.00%	10	60%	121	40.2	3.01	121	92	76.03%
ABF 1.3	100	46	46.00%	10	30%	102	13.8	7.39	102	84	82.35%
ABF 2.1	100	57	57.00%	10	80%	50	45.6	1.10	50	46	92.00%
ABF 2.2	100	43	43.00%	10	40%	71	17.2	4.13	71	61	85.92%
ABF 2.3	100	28	28.00%	10	40%	43	11.2	3.84	43	27	62.79%
ABF 3.1	100	56	56.00%	10	80%	53	44.8	1.18	53	39	73.58%
ABF 3.2	100	51	51.00%	10	90%	117	45.9	2.55	117	63	53.85%
ABF 3.3	100	49	49.00%	10	90%	95	44.1	2.15	95	66	69.47%
Overall	**900**	**439**	**48.78%**	**90**	**62%**	**664**	**283.8**	**2.88**	**664**	**490**	**77.33%**

APPENDIX D: Raw data the impact of blood-feeding frequency on the percentage of females blood fed, percentage of females inseminated, fecundity and fertility

2ABF1 = 2 blood meals on artificial feeding system; biological replicate 1 and so on for 2ABF2 and 2ABF3

4ABF 1.1 = 4 blood meals on artificial feeding system; biological replicate 1, technical replicate 1 –… – 4ABF3.3 = 4 blood meals on artificial feeding system; biological replicate 3, technical replicate 3

Source of blood	Number of females	Number of females fed	Percentage of females blood fed	number of females dissected for insemination	Females inseminated (%)	Number of eggs	Adjusted female number (afn)	Number of eggs/ female= fecundity	Number of eggs used	number of 1st instar larvae	Fertility (%)
2ABF1	100	37	37.00%	10	80%	16	29.6	0.54	16	15	93.75%
2ABF2	100	40	40.00%	10	90%	130	36	3.61	130	113	86.92%
2ABF3	100	62	62.00%	10	100%	171	62	2.76	171	108	63.16%
Overall	**300**	**139**	**46.33%**	**30**	**90.00%**	**317**	**127.6**	**2.30**	**317**	**236**	**81.28%**
4ABF1.1	100	48	48.00%	10	50%	83	24	3.46	83	61	73.49%
4ABF1.2	100	49	49.00%	10	60%	158	29.4	5.37	158	92	58.23%
4ABF1.3	100	39	39.00%	10	30%	33	11.7	2.82	33	31	93.94%
4ABF2.1	100	67	67.00%	10	80%	468	53.6	8.73	468	435	92.95%
4ABF2.2	100	35	35.00%	10	50%	133	17.5	7.60	133	125	93.98%
4ABF2.3	100	52	52.00%	10	80%	48	41.6	1.15	48	36	75.00%
4ABF3.1	100	49	49.00%	10	90%	298	44.1	6.76	298	272	91.28%
4ABF3.2	100	67	67.00%	10	100%	109	67	1.63	109	86	78.90%
4ABF3.3	100	57	57.00%	10	100%	224	57	3.93	224	217	96.88%
Overall	**900**	**463**	**51.44%**	**90**	**71%**	**1554**	**345.9**	**4.61**	**1554**	**1355**	**83.85%**

APPENDIX E: Kaplan-Meier survival curves illustrates the different times to pupation according to the different larval diet doses (Figure 3.3 – Figure 3.4)

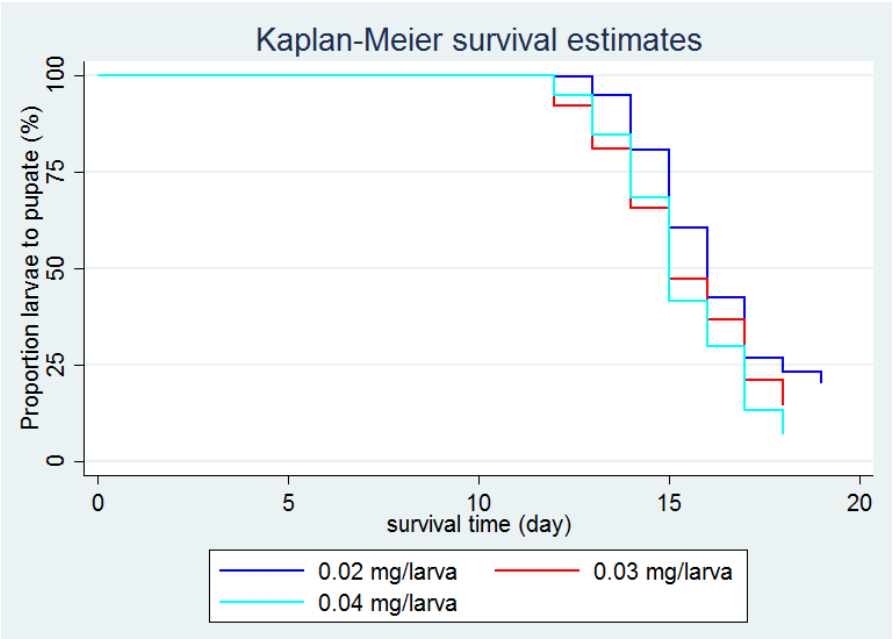

Figure 3.3: Kaplan-Meier survival curve of development time from first instar larvae to pupae of *An. funestus* FUMOZ feed on 0.02, 0.03, and 0.04 mg/larva/feeding (phase 1)

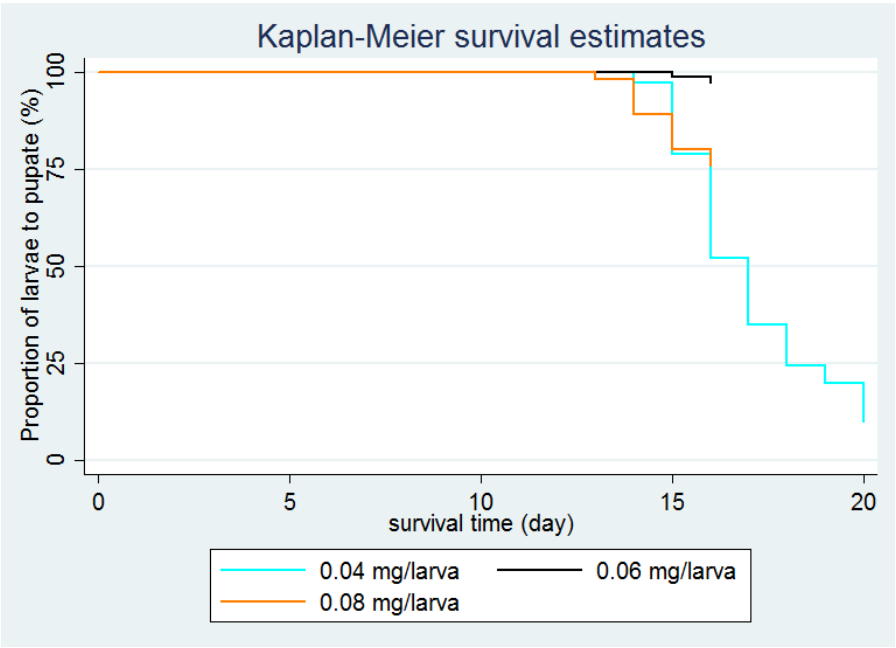

Figure 3.4: Kaplan-Meier survival curve of development time from first instar larvae to pupae of *An. funestus* FUMOZ fed on 0.04, 0.06, and 0.08 mg/larva/feeding (phase 2)

APPENDIX F: Kaplan-Meier survival curves illustrates the different times to pupation according to the different larval densities (Figure 3.5 – Figure 3.6).

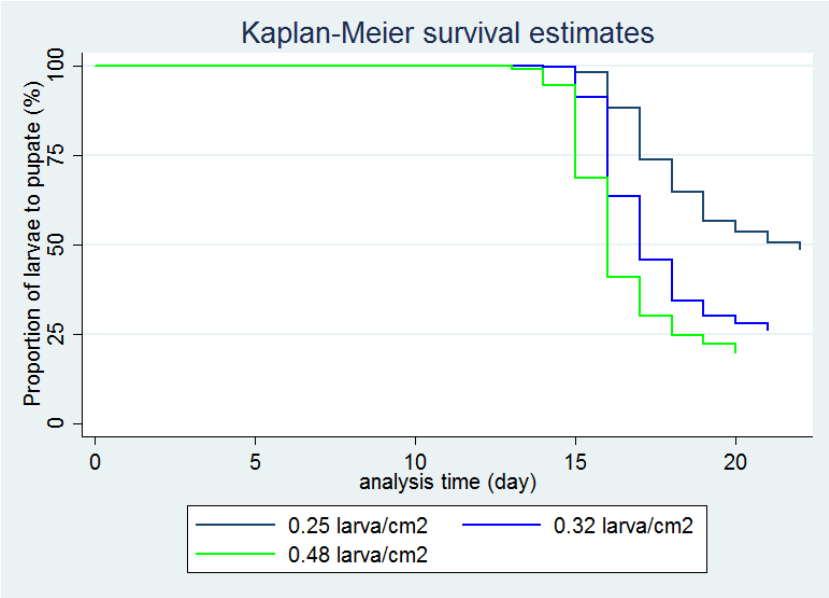

Figure 3.5: Kaplan-Meier curve of development time from first instar larvae to pupae of *An. funestus* FUMOZ reared in phase 1 (0.25, 0.32, and 0.48 larvae/cm^2)

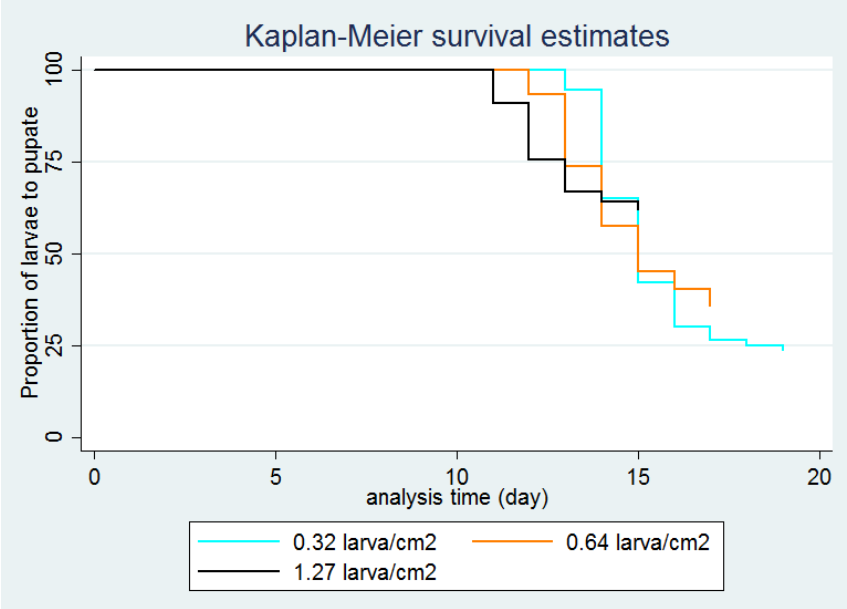

Figure 3.6: Kaplan-Meier curve of development time from first instar larvae to pupae of *An. funestus FUMOZ* reared in phase 2 (0.32, 0.64, and 1.27 larvae/cm^2)

APPENDIX G: Raw data of dose-response on emergence, fecundity, fertility, and insemination rate of *An. funestus* pupae irradiation on 30, 60, 90 and 120 Gy with two controls unirradiated

Rep	Treatment	Pupae irradiated	Pupae Emerged	% emergence	Total number of Eggs	Eggs used (30% or 100)	Insemination Rate (%)	number of female laid eggs	Eggs per Female	Total hatched day 14	Hatching rate day 14
1	1	180	173	96.11	393	118	80	48	8	101	85.59
1	2	174	170	97.70	340	102	90	59	6	86	84.31
1	3	179	164	91.62	97	97	70	37	3	76	78.35
1	4	173	162	93.64	25	25	90	43	1	6	24.00
1	5	172	151	87.79	280	100	80	36	8	17	17.00
1	6	171	156	91.23	0	0	80	45	0	0	0.00
2	1	200	159	79.50	793	238	90	71	11	176	73.95
2	2	175	154	88.00	419	126	80	56	7	112	88.89
2	3	198	155	78.28	780	234	70	51	15	163	69.66
2	4	200	150	75.00	308	100	70	55	6	37	37.00
2	5	200	153	76.50	277	100	60	38	7	38	38.00
2	6	204	151	74.02	0	0	70	48	0	0	0.00
3	1	185	173	93.51	273	100	80	58	5	82	82.00
3	2	182	160	87.91	447	134	70	57	8	106	79.10
3	3	181	167	92.27	257	100	80	61	4	76	76.00
3	4	179	169	94.41	449	135	90	81	6	77	57.04
3	5	179	169	94.41	57	57	90	78	1	16	28.07
3	6	180	160	89.39	166	100	70	55	3	0	0.00

APPENDIX H: Raw data of dose-response on emergence, fecundity, fertility, and insemination rate of *An. funestus* pupae irradiation on 100, 110 and 120 Gy with a control unirradiated

Rep	Treatment	Pupae irradiated	Emerged	% emergence	Total number of Eggs	Eggs used for fertility	Insemination Rate (%)	Number of female laid eggs	Eggs per Female	Total hatched_D14	Hatching_rateD14
1	1	208	181	87.02	395	119	80	38	10	87	73.11
1	2	208	179	86.06	0	0	80	40	0	0	0.00
1	3	209	175	83.73	0	0	60	27	0	0	0.00
1	4	209	177	84.69	0	0	50	26	0	0	0.00
2	1	200	153	76.50	546	164	40	20	27	143	87.20
2	2	200	181	90.50	19	19	40	22	1	0	0.00
2	3	200	167	83.50	0	0	50	27	0	0	0.00
2	4	200	168	84.00	0	0	0	0	0	0	0.00
3	1	196	184	93.88	165	100	50	28	6	82	82.00
3	2	200	184	92.00	79	79	50	29	3	2	2.53
3	3	200	188	94.00	0	0	100	47	0	0	0.00
3	4	200	178	89.00	0	0	90	57	0	0	0.00

APPENDIX I: Raw data of fecundity, fertility, insemination rate, induced sterility, and mating values for *An. funestus* pupae irradiated on 120 Gy with a control unirradiated

Treatment	Fecundity	Insemination rate %	Fertility	Induced sterility	Mean induced sterility	Mean competitiveness value
Sterile control	0.475	58.33	0.00			
Sterile control	0	58.33	0.00			
Sterile control	0	41.67	0			
Fertile control	6.1	70.00	90.71			
Fertile control	5.88	90.00	99.0			
Fertile control	9.18	83.33	97.20			
1:1:1	0	90.00	0	100	72.908	
1:1:1	0	100.00	0	100		
1:1:1	1.45	63.64	79.0	18.724		2.63
3:1:1	13.76	90.00	64.0	29.445	44.682	
3:1:1	8.75	100.00	70.0	29.292		
3:1:1	7.51	66.67	24.0	75.308		0.27
5:1:1	21.6	90.00	48.0	47.084	79.482	
5:1:1	10.8	75.00	8.55	91.363		
5:1:1	7.41	58.33	0	100		0.81