



The biology of *Anopheles funestus* (Diptera:Culicidae):
effects of the sugar diet – advances towards SIT

Student:

Mbuyi Nelly Kalonji

1043541

Masters by research

School of Pathology

Supervisors:

Prof Lizette L. Koekemoer

PhD (WITS)

Wits Research Institute for Malaria (WRIM) &

Vector Control Reference Unit, National Institute of Communicable Diseases

Dr Yael L. Dahan-Moss

PhD (WITS)

Wits Research Institute for Malaria (WRIM) &

Vector Control Reference Unit, National Institute of Communicable Diseases

A thesis submitted to the Faculty of Health Science, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Master of Science in Medicine on 18 November 2021

Declaration

I Mbuyi Nelly Kalonji declare that this thesis is my own, unaided work. It is being submitted for the Degree of Master of Science 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)

_____18th_____day of_____November_____2021_____in_____Johannesburg_____

Dedication

To my late dad, Oscar Ngoyi Kalonji, for instilling in me the love for education and science at a young age and ensuring his girl child gets the best foundation in education available

To my mother, Josephine Bibomba Wa Leja, for sacrificing her finances to pay for my undergraduate degree and always being an entire support system

To my lovely husband, Khombomoni Keith Chuma, for being understanding and helping me deal with stress throughout the degree and always being a present help

Abstract

Malaria is a severe disease caused by parasites of the *Plasmodium* genus and is transmitted by specific *Anopheles* vector mosquitoes. Different vector control methods are implemented in an effort to reduce malaria transmission. Due to the threat of insecticide resistance in some *Anopheles* vector populations, the sterile insect technique (SIT), amongst other methods, has been seen as an attractive control method to include in integrated malaria vector control programs. The success of SIT relies heavily on the mass rearing of the vector species and its competitiveness in the wild. *Anopheles funestus* is a major malaria vector in Africa and targeting this vector for SIT is essential to reduce the spread of malaria. The aim of this study was to examine the impact of different sugar water concentrations (0 to 40%) on longevity, flight ability and mating of colonised *An. funestus*. The impact of male to female ratio and male age on mating success were evaluated. Furthermore, the potential of one male to inseminate multiple females when adults were fed with different sugar water concentrations was also assessed.

Increased sugar water concentrations had an overall positive impact on male and female *An. funestus* longevity, flight performance and mating success. Results showed that a higher mating success was observed in cages with a higher male to female ratio when compared to the control cage. Increasing the number of males (3:1 male to female ratio) fed with 10% sugar water increased the mating success to the same level as the males (3:1 male to female ratio) fed with the 40% sugar water. The age of the male adults affected mating success for 10% sugar water fed males, however no differences were recorded in mating success for males of different ages fed with 40% sugar water.

In conclusion, results from this study indicate that keeping *An. funestus* adults on a 40% sugar water solution will be advantageous in a mass rearing SIT program.

Keywords: flight performance, longevity, male age, male to female ratio, mating success, SIT, sugar water diet, vector control

Acknowledgements

I would firstly like to thank my supervisors (Prof LL Koekemoer and Dr. YL Dahan-Moss) for conceiving the project and providing me with all the material and required resources to bring this project to completion and tireless editing of my previous drafts. Additionally, Prof LL Koekemoer for training on colony maintenance.

For the flight test device used in this study, I would like to thank Culbert *et al.* (2020) (IAEA) for the design of the device, Dr. H Yamada (IAEA) for facilitating the shipment process and Dr G Munhenga for bringing the flight tubes to South Africa from Austria.

I would like to thank Mr N Venter for his support and training on colony maintenance and information on the FUMOS strain at the Maureen Coetzee Insectary and training on spermathecal dissections.

Mr N Venter is further thanked along with Miss AS Aswat for assistance with longevity data when I was away.

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List of symbols and abbreviations

°C	Degrees Celsius
<i>Ae.</i>	<i>Aedes</i>
<i>An.</i>	<i>Anopheles</i>
Bs	<i>Bacillus sphaericus</i>
Bti	<i>Bacillus thuringiensis israeliensis</i>
cm	Centimetre
DDT	Dichloro-diphenyl-trichloroethane
h	Hours
IRS	Indoor residual spraying (of insecticides)
ITN	Insecticide treated bed net
LSM	Larval source management
m	Metre
MDA	Mass drug administration
ng/mg	Nanogram per milligram
<i>P.</i>	<i>Plasmodium</i>
PABA	Para-aminobenzoic acid
<i>Pers comm</i>	Personal communication
RO	Reverse osmosis
SE	Standard error
w/v	Weight per volume
WHO	World Health Organisation
µg/kg	Microgram per kilogram
µm	Micrometres

CHAPTER ONE

1. BACKGROUND

Malaria is one of the most important human vector borne diseases with symptoms such as chills, fever and headaches (WHO, 2020). Severe malaria in children can lead to respiratory distress, cerebral malaria and severe anaemia. In adults, severe malaria can lead to multi-organ failure (WHO, 2020). Partial immunity can occur in malaria endemic regions, allowing for asymptomatic infections (WHO, 2020).

The number of estimated malaria cases in 2019 was 229 million (WHO, 2020). Approximately 409,000 people died from malaria in the same year. The WHO Africa Region accounted for about 94% of these deaths. Five countries from the WHO African Region collectively reported about 50% of all the global malaria deaths. Nigeria and the Democratic Republic of the Congo accounted for 23% and 11% of the global malaria deaths, respectively. The United Republic of Tanzania accounted for 5% of the global deaths, while three countries, Burkina Faso, Mozambique and Niger, each accounted for 4% of the malaria deaths. Among the recorded deaths in the WHO African Region, 69% were children under 5 years (WHO, 2020).

Malaria is caused by the *Plasmodium* parasites (WHO, 2020). The *Plasmodium* parasite, *P. falciparum*, accounted for the highest estimated malaria cases in the year 2018 (WHO, 2020). The estimated *P. falciparum* cases for various regions were 99.7% from the WHO African Region, 71% from the WHO Eastern Mediterranean Region, 65% from the WHO Western Pacific Region and 50% from the WHO South-East Asia Region (WHO, 2020). Another major malaria parasite, *P. vivax*, accounted for 75% of the estimated malaria cases in the WHO Region of the Americas (WHO, 2020). Other parasites of lower prevalence include *P. malariae*, *P. knowlesi* and *P. ovale* (WHO, 2020). There are various *Plasmodium* species but the five mentioned above are known to cause malaria in humans (WHO, 2020).

High malaria burden countries spent approximately 40% of their public health budget on malaria prevention and treatment (Narasimhan and Attaran, 2003). Between 2010 and 2018, 70% of funds for malaria control and elimination in endemic countries were provided by international sources

(WHO, 2020). The impact of malaria on the economies and governments include missed workdays by employees due to the disease, and expenditures by governments on malaria prevention and treatments and the disease negatively impacts on workforce mobility (Sachs and Malaney, 2002). Globally, the distribution of gross domestic product per capita in 1995 (adjusted for purchasing power) showed a distinct correlation between malaria and poverty as the geographical distribution of poverty matched that of malaria transmission (Sachs and Malaney, 2002). It has been recognised that malaria is a cause of both social inequality and poverty, and one of the outcomes of poverty itself (Ren, 2019). Given the strong correlation between malaria and poverty, controlling the spread of the disease is necessary to reduce poverty.

Interventions that target both the *Plasmodium* parasites and mosquito vectors are important for effective malaria control. Some antimalarial drugs used in Africa to treat *P. falciparum* malaria include dihydroartemisinin-piperaquine, artemetherlumefantrine and artesunate-amodiaquine (WHO, 2020). The average efficacy of these drugs ranged from 98.0% to 99.4% for *P. falciparum*, malaria with a low treatment failure rate (<10%) (WHO, 2020). Although mutations have been found in *P. falciparum* in some African countries, the drugs used remain effective and further investigations relating to the effects of the mutations on drug treatments are still required (WHO, 2020). The use of insecticide-treated mosquito nets (ITNs) and indoor residual spraying (IRS) are some of the methods that prevent the spread of malaria by targeting the vectors (WHO, 2020). According to data supplied to WHO by manufactures, 253 million ITNs were delivered to malaria endemic countries and 84% of these were delivered to sub-Saharan African countries (WHO, 2020). Between 2000 and 2019, the global population sleeping under an ITN increased from 2% to 46% (WHO, 2020). The use of IRS declined between 2000 and 2019, with 180 million people being protected from malaria in 2000 compared to about 98 million in 2019.

The extensive use of insecticides resulted in selection pressure on malaria vectors and insecticide resistance developed rapidly over the past few decades (Coetzee *et al.*, 2013; Dai *et al.*, 2015; Sun *et al.*, 2017). Globally, insecticide resistance has been seen in various disease vectors such as *Aedes aegypti* (Lima *et al.*, 2011; Badolo, *et al.*, 2019), *Ae. Albopictus* (Marcombe *et al.*, 2014; Yougang *et al.*, 2020), *Culex tritaeniorhynchus* (Karunaratne and Hemingway, 2000), *An. gambiae* (Edi *et al.*, 2012; Casimiro *et al.*, 2014) and *An. funestus* to mention but a few. This increase in insecticide resistance in vector populations has led to a reduction in the effectiveness of IRS and ITNs and

their role as malaria vector control strategies (N'Guessan *et al.*, 2007; Ranson *et al.*, 2011; Coetzee and Koekemoer, 2013; Hancock *et al.*, 2018).

1.1 Malaria Vectors

Specific mosquitoes of the *Anopheles* genus transmit malaria. African malaria vector species are mainly species from the *Anopheles gambiae* complex and *Anopheles funestus* group (Gillies and De Meillon, 1968; Gillies and Coetzee, 1987; Sinka *et al.*, 2012). The distribution of these vectors differs between regions. In the WHO African regions, *An. arabiensis*, *An. coluzzii* and *An. gambiae* from the *An. gambiae* complex and *An. funestus* from the *An. funestus* group (Gillies and De Meillon, 1968; Battle, 2012; Sinka *et al.*, 2012; Coetzee *et al.*, 2013; Akogbéto *et al.*, 2018) are the main malaria vectors.

Anopheles gambiae complex

The *An. gambiae* complex consists of nine species that are almost identical in morphology but differ in vectoral capacity, genetics and behaviour (Gillies and Coetzee, 1987; Hunt *et al.*, 1998; Coetzee *et al.*, 2013). In Africa, these species are mainly found in the afro-tropical region (Coetzee *et al.*, 2000). The major African malaria vectors in this species complex are *An. gambiae* s.s., *An. coluzzii* and *An. arabiensis* (Coetzee *et al.*, 2013). The minor vectors are *An. merus*, *An. melas* and *An. bwambae* while *An. quadriannulatus*, *An. fontenillei* and *An. amharicus* do not transmit malaria and are thus non-vectors (White, 1974; Coetzee *et al.*, 2000; Coetzee *et al.*, 2013; Barrón *et al.*, 2019; Coetzee, 2020). *Anopheles gambiae* and *An. coluzzii* are strongly anthropophilic (prefer human hosts) in all their distributions and *An. arabiensis* is both anthropophilic and zoophilic (prefer non-human animals as hosts), in most of its distribution given the abundance of the availability of hosts (Gillies and Coetzee, 1987). Although *An. merus* and *An. melas* display intermediate anthropophily, they are normally zoophilic and *An. quadriannulatus* and *An. amharicus* are zoophilic (Gillies and Coetzee, 1987; Coetzee *et al.*, 2013).

***Anopheles funestus* group**

The *An. funestus* group consists of at least 11 species similar in morphology at the adult stage and are largely distributed in the afro-tropical region (Gillies and De Meillon, 1968; Coetzee and Fontenille, 2004). The major malaria vector in this group is *An. funestus* (*An. funestus sensu stricto*) (Gillies and De Meillon, 1987).

Anopheles rivulorum is mainly distributed in the west and east Africa with their larvae found in slow-flowing water or in aquatic vegetation. The species is zoophilic, rests outdoors and is seldom found inside houses (Gillies and De Meillon, 1968). However, in Kenya and Tanzania, *An. rivulorum* has been implicated in malaria transmission (Wilkes *et al.*, 1996; Kawada *et al.*, 2012).

Anopheles parensis has recently been implicated as a potential malaria vector in South Africa (Burke *et al.*, 2019). Although mainly zoophilic, *An. parensis* has been found resting indoors in both South Africa and Kenya (Kamau *et al.*, 2003; Mouatcho *et al.*, 2018). *Anopheles lesoni* is distributed in the eastern and western African savannah and southern Africa and is believed to be zoophilic (Gillies and De Meillon, 1968, Tawe *et al.*, 2017) although it has also been collected in human dwellings (Evans, 1931). Specimens of *An. lesoni* (Temu *et al.*, 2007) have been found infected with *P. falciparum* in the wild. However, Dahan-Moss *et al.* (2020) showed that if *An. gambiae* complex samples are mistakenly identified using the *An. funestus* group PCR, these samples will be misidentified as *An. lesoni*. Since this was not investigated by Temu *et al.* (2007), further studies are needed to confirm the importance of *An. lesoni* in malaria transmission. The human blood index (HBI) of *An. lesoni* in Equatorial Guinea was recorded at 0.81 (Pappa *et al.*, 2011). In the presence of other animal hosts, *An. lesoni* can feed on humans (Pappa *et al.*, 2011).

Anopheles vaneedeni has mainly been recorded in South Africa (Gillies and Coetzee, 1987; De Meillon *et al.*, 1977; Burke *et al.*, 2017), Kenya (Kweka *et al.*, 2013, Ogola *et al.*, 2018) and some parts of southern Africa (Kyalo *et al.* 2017). *Anopheles vaneedeni* is often caught in the early hours of the night, biting humans while outdoors (De Meillon *et al.*, 1977). The larval habitat of *An. vaneedeni* is similar to that of *An. funestus* (Gillies and Coetzee, 1987). The species has also been found infected with *P. falciparum* in the wild (Burke *et al.*, 2017).

Little is known about *An. confusus*. The species is assumed to be zoophilic and prefers resting outdoors. The distribution of *An. confusus* south of the Sahara stretches from Ethiopia and Kenya to South Africa and its larvae are found in slow-moving water (Gillies and De Meillon, 1968). *Anopheles fuscivenosus* has been collected in Zimbabwe and little is known about the species (Gillies and De Meillon, 1968). *Anopheles brucei* and *An. aruni* are also members of the *An. funestus* group. *Anopheles funestus*-like have only been recorded from Malawi (Spillings *et al.*, 2009; Vezenegho *et al.*, 2009), while *An. rivulorum*-like have been recorded from Cameroon (Cohuet *et al.*, 2003) and South Africa (Mouatcho *et al.*, 2018).

Anopheles funestus sensu stricto (An. funestus from this point)

Anopheles funestus was historically a major malaria vector in South Africa. This species is thought to be the most important species in the *An. funestus* group that is capable of transmitting malaria in South Africa (Coetzee and Koekemoer, 2013). *Anopheles funestus* is generally linked to epidemics and outbreaks in areas affected by malaria (Coetzee and Hunt, 1998, Hargreaves *et al.*, 2000). This species is the most widely distributed and anthropophilic (preferring to bite humans even in the presence of alternative hosts like domestic animals) compared to other members of the *An. funestus* group (Gillies and De Meillon, 1968). *Anopheles funestus* mainly rests indoors and prefer to bite late during the night (Gillies and De Meillon, 1968). Historically, the human blood index in *An. funestus* has been recorded ranging from 94% to 99% (Pull and Grab, 1974; Boreham, 1975; Chandler *et al.*, 1975). However, more recently Degefa *et al.* (2017) recorded indoor HBI of *An. funestus* ranging from 58.3% to 72.7%. Additionally, Machani *et al.* (2020) recorded HBI of indoor resting *An. funestus* ranging from 75% to 83%. The difference in the HBI found in the historical and recent data could be due to the improved methods and developments in molecular identification to differentiate between species in the *An. funestus* group that were not available in the 1970s.

The preferred breeding sites for *An. funestus* are semi-permanent and permanent bodies of water (Gillies and De Meillon, 1968). *Anopheles funestus* larvae can remain submerged under water for extended amounts of time, making them difficult to find in their natural habitats (Gillies and De Meillon, 1968). Adult *An. funestus* populations fluctuate through the year and usually peak at the

beginning of the dry season (Gillies and De Meillon, 1968). Post blood feeding, the first gonotrophic cycle for *An. funestus* lasts for 4-5 days and the cycles that follow last for 2-3 days and females require a second blood meal to mature the egg batch (Brenques *et al.*, 1968; Brenques and Coz, 1973).

Anopheles funestus showed a positivity rate for *P. falciparum* in Kenya at 9.5% (38 out of 402) (Shililu *et al.*, 1998). Malaria parasite rates of 2.5% (Mendis *et al.*, 2000) and 5.3% (Mulamba *et al.*, 2014) have been reported in Mozambique and Uganda, respectively. *Anopheles funestus* parasite positivity rates of 22% (De Meillon, 1933) were recorded prior to the implementation of vector control in South Africa (SA). During the malaria outbreak in KwaZulu-Natal, SA, in 2000, a 5.4% *P. falciparum* infection rate was reported (Hargreaves *et al.*, 2000). Despite all this evidence, research on *An. funestus* has not been adequate as more efforts have been focused on *An. gambiae*.

1.2 *Anopheles funestus* Vector Control in South Africa (SA)

Historically, SA used dichloro-diphenyl-trichloroethane (DDT) until the 1994's for malaria vector control. Pyrethroids replaced DDT in the control program resulting in a major malaria outbreak in 1999/2000 with almost 65,000 cases being reported (Coetzee *et al.*, 2013). The rapid return of DDT, in combination with pyrethroids, into the control program reduced the number of malaria cases to averages between 5,000 – 10,000 cases in the subsequent years (Coetzee *et al.*, 2013). In SA, insecticide resistance in *An. funestus* has historically been accompanied by an increase in the transmission of malaria (Hargreaves *et al.*, 2000; Coetzee *et al.*, 2013). This poses a major challenge for sustainable vector control. South Africa continued to use a mosaic approach (pyrethroids in combination with DDT) to reduce vector populations and insecticide resistance pressure. This species is resistant to both pyrethroids and carbamates (Brooke *et al.*, 2001). The only alternative insecticides to control this vector are organophosphates, neonicotinoids and DDT. Due to cost implications, organophosphates and neonicotinoids are not currently feasible alternatives. *Anopheles funestus* has mainly been eliminated from South Africa (Brooke *et al.*, 2011). Currently, *An. arabiensis* is the main malaria vector in SA, however, *An. funestus* remains a threat to SA as it is a major vector in neighbouring countries such as Mozambique and Zimbabwe

(Mendis *et al.*, 2007, President's Malaria Initiative, 2018). The threat of insecticide resistance highlights the need for research into alternative control interventions; some of these are discussed further below.

1.3 Alternative Vector Control Methods

Over the past few decades, malaria vector control methods have mainly been insecticide based (Bhatt *et al.*, 2015). The addition of complementary non-insecticide based interventions is required to counteract the negative impact of insecticide resistance in mosquito populations on malaria control programs. Some of these alternative control methods include the use of endectocides (Yakob, 2016; Khaligh *et al.*, 2021), gene drive technologies (Macias *et al.*, 2017), larval source management (WHO, 2013) and the sterile insect technique (SIT). Some of these complementary methods are discussed below.

Endectocides

Endectocides are drugs administered to an animal to kill endo- and ectoparasites, transmitted by blood-feeding arthropod vectors (Yakob, 2016). Endectocides are mainly used to control nematodes in vertebrates (including humans), but they can be toxic to malaria vectors, e.g. *Anopheles* species, that feed on hosts with endectocides in their bloodstream (Foy *et al.*, 2011). Ivermectin (IVM) is an endectocide that inhibits the glutamate-gated chloride neuromuscular transmission channels in insects (Kane *et al.*, 2000). The use of IVM began as early as the 1980s in a mass drug administration (MDA) against onchocerciasis in West Africa, however at that time, the impact of IVM on the vector's (*Similium* species) survival was not monitored. Ivermectin was revisited as a vector endectocide intervention when Chaccour *et al.* (2010) administered an oral dose of 200 µg/kg of IVM to humans and allowed mosquitoes to feed on them. This resulted in reduced *An. gambiae* survivorship even four days post IVM administration as a cumulative mortality of 89% was recorded over this period (Chaccour *et al.*, 2010).

During an MDA campaign in parts of Senegal between 2008 and 2009, 150 µg/kg of IVM was administered to humans in different villages (Sylla *et al.*, 2010). *Anopheles gambiae* were then

collected from these villages for survivorship experiments and the conclusion was that significant reduction in *An. gambiae* survivorship was recorded up to six days after MDA in each village (Sylla *et al.*, 2010). Ivermectin is readily administered to agricultural livestock and Fritz *et al.* (2013) conducted a study on IVM toxicity on *An. gambiae* that fed on IVM-treated cattle and found that 90% of the mosquitoes died within ten days post blood feeding. The search for other drugs that can be used as endectocides is ongoing (Burrows *et al.*, 2017) and additional studies on the use of IVM as part of MDA for malaria control are ongoing in West Africa (Dabira *et al.*, 2020).

Gene drive technologies

Gene drive is a technology used to increase the prevalence of desired genes and their corresponding phenotypes faster than Mendelian inheritance (Benedict *et al.*, 2008). Gene drive can either be used in a suppression or in a replacement strategy (Burt, 2003; Marshall and Akbari, 2016). Population suppression drives limit the population of the *Anopheles* mosquitoes either by sex ratio bias or spreading sterility and lethal genes (Burt, 2003), but is not a permanent genetic change. Modified mosquitoes will need to be released at regular intervals. Population replacement drives on the other hand can restrict the *Anopheles* mosquito's capability to transmit *Plasmodium* parasites (Marshall and Akbari, 2016) and the population will eventually have more individuals incapable of transmitting the parasite. Gene drive technologies are actively being studied for field applications in Africa (African Union, 2018). Public engagement is a critical factor in the success of gene drive technologies (Hartley *et al.*, 2019). Much of development is still required for this method and ongoing public consultations are underway and release of these modified organisms will depend on social norms, culture and traditions of local communities (Collins, 2018) and not simply the scientific evidence.

Larval source management for malaria control

Larval source management (LSM) is a method of vector control that targets the aquatic juvenile stages of mosquitoes (the larvae and pupae) by preventing their maturation into adults, thus reducing the adult vector prevalence (WHO, 2013). The treatment or elimination of most larval habitats could reduce the number of infective bites per person (since there would be fewer adult

female mosquitoes) and thus malaria transmission (WHO, 2013; Tusting *et al.*, 2013). There are four types of LSM: (1) habitat modification, (2) habitat manipulation, (3) larviciding and (4) biological control.

Habitat modification involves changing the land permanently (WHO, 2013), for example, filling an *Anopheles* breeding site with sand and repurposing the area for planting or another activity. The construction of dams can reduce the number of breeding sites for mosquitoes because their smaller breeding sites are combined into a vast water body (WHO, 2013). Larvae cannot survive in dams unless there is some floating aquatic vegetation or if the banks of the dam are shallow enough (WHO, 2013). Such permanent changes to larval habitats are helpful for malaria control.

Habitat manipulation is a repeated activity to temporarily alter the environment (WHO, 2013) such as canal water management and flushing streams. In ancient history, the Greeks and Romans used habitat manipulation strategies as they realised the link between stagnant water and swamps with fevers (reviewed by Konradsen *et al.* 2002). The drainage plans and system lead to an improvement in the health of the local communities (reviewed by Konradsen *et al.* 2002). Kibret *et al.* (2014) conducted a study in Ethiopia and discovered that poor canal water management was linked to increased malaria transmission in the irrigated villages that were studied.

Larviciding involves systematically applying chemicals or biological agents to larval habitats (Tusting *et al.*, 2013). Field research has been conducted in Africa and proven that larviciding reduces adult vector abundance and malaria prevalence (Fillinger and Lindsay, 2006; Fillinger *et al.*, 2009; Tusting *et al.*, 2013). There are various larvicides such as oils, chemicals and microbial larvicides. Oils may be used to create a film on the water body to suffocate the larvae (WHO, 2013), whereas chemicals such as chlorpyrifos and pirimiphos-methyl (which are organophosphates interfering with the nervous system of larvae) are used to negatively affect the mosquito's central nervous system by inhibiting cholinesterase (WHO, 2013). Due to the use of insecticides over time, adult mosquitoes have been observed to alter their behaviour (Kamdem *et al.*, 2017; Sougoufara *et al.*, 2017) – this makes larviciding advantageous because larvae cannot alter their behaviour when a control intervention targets their habitat (Killeen *et al.*, 2002). This method targets both indoor and outdoor biting *Anopheles* mosquitoes, unlike IRS which is only useful for controlling *Anopheles* indoors (Derua *et al.*, 2019). The most common microbial

larvicides used are *Bacillus thuringiensis israeliensis* (Bti) and *Bacillus sphaericus* (Bs) and their mode of action is the release of toxins into the gut of larvae, preventing them from eating which increases mortality (WHO, 2013). Becker (1997) monitored the use of Bti in various larval habitats in Germany over time and recorded annual adult mosquito reductions of over 90% and no evidence of negative impacts on the ecosystems. Barbazan *et al.* (1998) treated various water bodies in Maroua (Cameroon) with Bs and noticed that malaria incidence at a healthcare facility in the town decreased and the malaria transmission season was delayed by two months.

Familiarity with breeding sites and their dynamics are crucial for the success of larviciding using Bs (Barnazan *et al.*, 1998). However, treatment of all or most breeding sites is unlikely to be achieved in sub-Saharan Africa (WHO, 2013), hence LSM should be employed as a supplementary method of vector control instead of being the only control method used.

Biological control for malaria control is the process of introducing the natural predators of mosquito larvae and pupae such as predatory invertebrates and fish and organisms that cause disease to the target pest (WHO, 2013). The success of biological control depends on the breeding site being clear and defined and the water suitable for biological control agents (WHO, 2013).

The sterile insect technique (SIT)

The sterile insect technique (SIT) was developed during the 1930's and 1940's and can essentially be described as the introduction of sterility in a wild population of pest insects to control them (Klassen *et al.*, 2005). Various people individually conceived this idea in three main fields and the principal investigators were: E. F. Knipling of the United States Department of Agriculture, A. S. Serebrovskii at Moscow State University, and F. L. Vanderplank at a rural tsetse fly field research station in modern-day Tanzania (Klassen *et al.*, 2005). Serebrovskii investigated pest control by way of chromosomal translocations to control pest population but this did not succeed, in part, due to the calamity brought about in the Union of Soviet Socialist Republics during the Second World War and he died after the war. Vanderplank (1948) conducted research on cross-breeding in tsetse-flies in a large field setting and used the sterility of the hybrids to successfully control a tsetse-fly population but lacked the funds and resources to continue with this work. Discoveries by Muller (1941) regarding the use of ionising radiation to induce mutations and sterility (in *Drosophila*)

prompted E.F. Knipling to further this work leading to one of the best successes of the area-wide control of the New World screwworm fly (*Cochliomyia hominivorax* (Diptera: Calliphoridae)) from the United States of America, to Mexico and the Caribbean (Knipling, 1959; Knipling *et al.*, 1968). The sterile insect technique has also been successfully applied as a control method in field conditions for various pests such as the codling moth in 21 orchards in British Columbia (Proverbs *et al.*, 1977) and the fruit fly from the island of Kume, part of the Okinawa archipelago (Iwahashi, 1977).

The sterile insect technique relies on the correct identification of the species (which will then be mass-reared), successful sterilisation of the males and the mass releases of these sterile males (Knipling, 1959; Knipling *et al.*, 1968). The sterile insect technique is species-specific and thus has no negative impacts on the environment reported to date (Benedict and Robinson, 2003) as other species are not affected by the release of the sterile males.

The SIT method cannot be used as a standalone method and its success depends on making this part of an area-wide integrated pest management approach (Hendrichs *et al.*, 2007). Insects (usually male) can be sterilised by irradiation (Dyck *et al.*, 2005), chemo-sterilisation (Sharma *et al.*, 1978; Dame *et al.*, 1981) and methods that involve genetic manipulation (Alphey *et al.*, 2002). Since male mosquitoes are not the vectors of disease, sterilizing and releasing males does not add to the disease burden. Furthermore, wild *Anopheles* females will mate once in their lifetime and mating with a sterile male would prevent the development of the eggs and lead to a reduction in the vector population.

Male sterilisation can, unfortunately, have a negative impact on its biology and survival. El-Gazzar and Dame (1983) found that *Culex quinquefasciatus* males were less competitive under laboratory conditions after sterilisation. Diet optimisation could thus reduce some of the negative impacts of sterilisation and increase the competitiveness of sterile males (Oliva *et al.*, 2012). Ensuring high competitiveness in the sterile males is an important requirement of SIT to ensure the male can identify and mate with the wild-type female, without being outcompeted by their wild-type fertile counterparts (Dame *et al.*, 2009). The effects of sterilisation on longevity vary between species (El-Gazzar and Dame, 1983; Gato *et al.*, 2014). Oliva *et al.*, (2012) investigated the mating success of irradiated and fertile wild-type male *Ae. albopictus* provided with 10% sugar water and reported

no significant difference in mating success for males aged between 1-5 days old. Information regarding sterilization on *An. funestus* and its effect on the male fitness is lacking. However, understanding the effects of adult diet on longevity and mating success will improve mass rearing conditions for *An. funestus*. This will be one of the first steps to evaluate the potential of using SIT for *An. funestus* control.

1.4 Biological Importance of Sugar for Adult Mosquitoes

Mosquitoes and other insects feed primarily on plant nectar in the wild (Foster, 1995; Scott and Takken 2012). A large composition (sometimes more than 90%) of nectar is sugar (Brewer *et al.*, 1974; Torres and Galetto, 2002; Herrera *et al.*, 2006). Sugar concentration in nectar ranges between 5% and 75% with the majority ranging between 25% and 40% (Huang, 2010). Some of the sugars found in various plants include sucrose, galactose, maltose, fructose and glucose with the mass totalling between 1,783 ng/mg and 7,581 ng/mg for certain flowers (Nyasembe *et al.*, 2012). Under laboratory conditions, mosquitoes are usually fed on a sucrose solution (Hunt *et al.*, 2005; Zengenene *et al.*, 2021).

Biosynthesis (conversion of substrates into complex molecules in living organisms) and metabolism (chemical reactions that convert ingested food into energy) occur in the insect fat body (Law and Wells, 1989). Other functions of this organ include innate immunity (Buchon *et al.*, 2014) and homeostatic control (Zheng *et al.*, 2016). The insect fat body encircles the insect gut and reproductive organs and is distributed along the insect's body (Dean *et al.*, 1985). This organ is usually composed of thin lobes and it is located in the haemolymph (a fluid in invertebrates that is equivalent to blood in vertebrates) – allowing the insect to promptly adjust its activities to the concentration of energy precursors in its body (Dean *et al.*, 1985). The insect fat body has no vertebrate analogue (comparable organ) and is thus unique in insects (Law and Wells, 1989).

Adipocytes are the primary metabolite storage cells that the insect fat body is composed of (Dean *et al.*, 1985). These are distinguished by having many lipid droplets. These droplets, and glycogen, increase in the insect's intracellular space after feeding (Dean *et al.*, 1985). Mobilisation of metabolites is vital in insects for the energy required for various activities, including flying and is

controlled by the endocrine system (Gäde, 2004). The adipokinetic hormone (AKH) is a family of neuropeptides (with over 40 peptide isoforms) and has been characterised multiple times in various orders of insects, including Diptera (Gäde *et al.*, 1997; Van der Horst, 2003; Gäde, 2004). The principal function of AKH is metabolite mobilisation to the haemolymph from storage in the adipocytes (Dean *et al.*, 1985). Glucose is stored as glycogen in insects and other animals and is, in this polymeric form, readily broken down as a glycolytic fuel (Steele, 1982).

The process of metamorphosis (in mosquitoes, this is the transformation from the aquatic larval stage to the terrestrial adult stage) can practically deplete the glycogen reserves because no feeding occurs during the insect pupal stage (the intermediate stage between the larval and adult stage) (Boggs and Freeman, 2005; Boggs, 2009). Newly emerged adult mosquitoes survive for a few days only when deprived of sugar and fed with just water under laboratory conditions (Nayar and Pierce, 1980; Andersson, 1992). Glycogen, often stored as trehalose (a sugar composed of two glucose molecules), is mobilised for use by other tissues (Thompson, 2003). The action of glycogen phosphorylase, which provides glucosyl residues for trehalose synthesis, is required for glycogen utilisation (Steele, 1982, Thompson, 2003). When insects are not feeding, trehalose is utilised to sustain energy metabolism (Thompson, 2003; Shi *et al.*, 2017). Gary and Foster (2006) conducted a study to assess sugar feeding in *An. gambiae* and found that males feed more frequently than females. Male *An. gambiae* fed twice daily, and this was significantly more than virgin females whose mean sugar feeding per day was less than 1 (Gary and Foster, 2006). Sugar digestion in mosquitoes is marked by an increase in the activity of the enzyme α -glucosidase enzymes as these enzymes break down carbohydrates and simple sugars (Souza-Neto *et al.*, 2007).

Mosquitoes restore their energy reserves by sugar feeding (Foster, 1995). Adult mosquitoes require frequent sugar meals, especially males, as they cannot supplement their sugar feeding with blood feeding (Foster, 1995; Gouagna *et al.*, 2014). Male mosquitoes and autogenous female mosquitoes (females that can undergo normal ovarian development without an external protein source) require sugar reserves for mating and in addition, non-autogenous female mosquitoes (females that require an external protein source to undergo normal ovarian development) require sugar for host-seeking leading to blood-feeding, egg development and egg-laying (Yuval, 1992; Foster, 1995). England and Evans (1997) assessed the egg production of the parasitic wasps *Bathyplectes curculionis* by supplementing their alfalfa (*Medicago sativa*) diet with pea aphid honeydew (sugar source) and

found a significant increase in *B. curculionis* egg production (11-15% increase) compared to those that only had access to water and alfalfa.

Mosquitoes mate by forming swarms in the wild (Yuval *et al.*, 1994; Zawada *et al.*, 2018). Flight is necessary for swarming because it is a mating system whereby males aggregate aerially to form sites for females searching for mates (Cooter, 1989). Swarming requires sugar to provide the mosquitoes with energy for flight and mating (Nayar and Van Handel, 1971; Foster, 1995; Gary and Foster 2001). Maïga *et al.* (2014) found that *An. gambiae* males [*An. coluzzii* and *An. gambiae* s.s. (previously referred to as M and S form, respectively)] that were collected during the early stage of swarming had significantly higher total sugar contents than those collected in the late stages of the swarm. This demonstrated that swarming has an energetic cost and males need to restore these sugar reserves to increase their longevity and reproduction. Similar results have previously been observed in *An. freeborni* (Yuval *et al.*, 1994). Metabolic rates in flying insects increase more than 50 fold (Beenakkers *et al.*, 1984). The insect fat body replenishes energy precursors in the insect flight muscles as very few are available in the muscle (Beenakkers *et al.*, 1984). Long-distance flyers, such as locusts (Van der Horst *et al.*, 1980) and mosquitoes begin their flight with trehalose as an energy source and then turn to lipids after flying for several hours (Kaufmann and Brown, 2008). It is thus vital for mosquitoes to have higher energy reserves for flight activity to be sustained for as long as required.

First described by Rowley *et al.* (1968), flight-mill systems have often been used to assess mosquito flight performance. Briefly, a mosquito is mounted on one of the two arms of the flight-mill and another mosquito, with glued wings (thus unable to fly) is mounted on the other arm as a counterbalance. Every 30 seconds, a computer records the number of flight-mill revolutions by the mosquito over a given period and this is used to calculate the flight distance, duration and frequency of the mosquito. Over the years, it was suggested that a better system that is less time consuming and easier to use should be developed. Culbert *et al.* (2018) developed a flight test device that can assess mosquito flight ability in two hours and is relatively easy to use and inexpensive to produce.

Sugar feeding is vital for both flight energy to swarm and mating. Stone *et al* (2009) found a reduced percentage insemination in the sugar-deprived males in a semi-field experiment in a study

comparing percentage insemination in *An. gambiae* between 10% sugar water fed and sugar-deprived adults. When comparing the effects of sugar feeding on mating competitiveness between sterile and fertile males *Ae. albopictus*, Bellini *et al.* (2014) found that 10% sugar feeding compared to sugar deprivation increased mating competitiveness in young males, whether sterile or fertile.

1.5 Effects of Male Age on Mating Success and the Possibility of Multiple Mating in *An. funestus*

The age of the male is one of the factors that influences mating success in some mosquito species such as *Ae. aegypti* and *An. gambiae*. Ponlawat and Harrington (2009) conducted a study on laboratory-reared *Ae. aegypti* and found that older males (5 days old) inseminated significantly more females than younger males (1 day old). Similar results were also observed in a 2007 study by the same authors when they compared male age to the mean number of sperm stored in the reproductive organs of male *Ae. aegypti* (Ponlawat and Harrington, 2007). Sawadogo *et al.* (2013) studied the effects of male age on percentage insemination in *An. gambiae* and found that females that mated with older males (4-8 days old) had significantly higher percentage of insemination than those that mated with younger males (2 days old). These studies showed that male age is positively correlated with high rates of insemination.

Although there were no records published of multiple mating in male *Anopheles* (to my knowledge), studies have been conducted to explore the possibility of one female mating with more than one male. Female *Anopheles* mate once and it is not known whether males can mate with more than one female. Charlwood *et al.* (2003) studied the pre-gravid behaviour of female *An. gambiae* in São Tomé and Príncipe and found only two females (in copula) with already developed ovaries. However, the authors could not determine whether the advanced development of the ovaries was due to: (1) the females having mated before or (2) the females failing to mate the night prior to the mosquito collections. Even if the females had mated before, Charlwood *et al.* (2003) reported that it would have been 1% of the females in São Tomé that probably mated more than once. This is not significant enough to claim that that female *Anopheles* can mate multiple times.

1.6 Rationale of the Study

In this study, the effects of the sugar diet was evaluated in adult males to determine whether it could improve the fitness of adult male *An. funestus*. There is a lack of data on the effects of the sugar diet on *An. funestus* under insectary conditions because the species is difficult to colonise (Gillies and De Meillon, 1968). It is beneficial to understand the specific ways in which diet can be optimised for the *An. funestus* biological events such as mating capacity and longevity during laboratory rearing. Optimising the mating success is also important to laboratory rear the species in a relatively shorter time (less than the current 6-8weeks), which could also reduce costs significantly – this is especially important in resource-scarce regions (Koekemoer, *pers comm*). Relevant to this study, the impact of sugar on the longevity, ability of the males to swarm and mating success, will be assessed.

1.7 Study Aims and Objectives

1.7.1 Aim

The aim of this study was to examine the impacts of different sugar concentrations on colonised *An. funestus* longevity, flight ability and mating.

1.7.2 Objectives

Objective 1: Determine the impact of different sugar water concentrations on colonised *An. funestus* longevity and flight performance.

Objective 2: Investigate the effect of different sugar water concentrations on mating in colonised *An. funestus*.

Objective 3: Identification of critical factors that can influence mating success. These include the male to female ratio, age of males and potential of one male to inseminate multiple females when fed with different sugar water concentrations.

CHAPTER TWO

2. METHODS

2.1. Laboratory Reared *An. funestus* Species.

The species of interest for this study was *An. funestus* (Diptera: Culicidae). The *An. funestus* colony, FUMAZ, was reared at the Maureen Coetzee Insectary at the University of the Witwatersrand Health Science Campus, Parktown. Wild-caught adults from southern Mozambique were used to establish the original colony (Hunt *et al.*, 2005). FUMAZ is kept under the standard insectary conditions of $26 \pm 1^{\circ}\text{C}$, 84% relative humidity and 12h:12h Light: Dark cycles with 45-minute gradual crepuscular transitions to scotophase from photophase. The colony was routinely maintained as described in more detail below.

2.1.1 Colony maintenance

Oviposition

Egg plates (Figure 2.1 A) were prepared by painting Petri dishes (diameter of 6 cm, height of 1 cm) with black spray paint and leaving the dishes to air dry for several days before use. Absorbent cotton wool (HGRA2805, Lassec, South Africa) was placed into the Petri dish. A 70 mm diameter grade 3hw filter paper (lot: 20-104, Munktell, Sweden) was placed on top of the cotton wool and saturated with reverse osmosis (RO) water for each egg plate. The prepared egg plates were placed into the *An. funestus* adult cages overnight for ovipositioning after the second blood meal. The eggs were harvested the following day and placed into 250 ml egg bowls (Figure 2.1 B) (diameter of 12 cm and height of 7 cm) containing about 100 ml RO water. The eggs were swirled around daily using a wash bottle containing RO water. This washing step stimulated hatching and removed eggs that were stranded on the sides of the bowl which might have died due to desiccation.

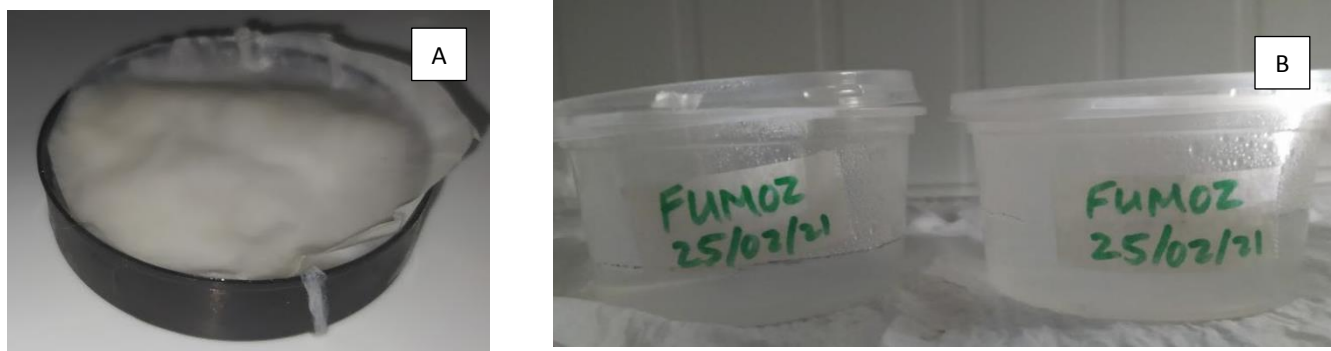


Figure 2.1 Images of the egg plate used to induce oviposition in female *An. funestus* (A); the egg bowls that contained the harvested eggs. Colony name and date of egg harvest are indicated on the bowls (B).

Larval rearing

Two to three days post egg harvesting, most of the eggs had hatched and the 1st instar larvae were transferred to 2 L plastic larval containers (12 cm (L) x 7 cm (W) x 6 cm (H)) (Figure 2.2) with approximately 500 mL RO water. When most of the larvae were 2nd – 3rd instar larvae, they were all transferred to 8 L larval bowls (8 cm (H) x 30 cm (W) x 40 cm (H)) (K05715, Plastpro Scientific, South Africa) containing 1.5 L RO water and covered with a Perspex lid, with the density of larvae not exceeding 1,000 per bowl. When the larvae started to pupate, the Perspex lid was replaced with netting to facilitate adult harvesting from the larval bowls and prevent adults from escaping.

Larval food was prepared using ground dry dog biscuits (Beeno®, original flavour, South Africa) and ground yeast tablets (Vital Brewer's Yeast, South Africa) in a 3:1 ratio by volume, respectively. A household food blender was used to grind the ingredients into a fine powder. The ground larval food was kept dry in a closed plastic container. In order to feed the larvae, the larval food was transferred into small vials with a modified lid made of a net and plastic ring. The vial was tapped several times over the larval bowl to add food to the water surface for the larvae to feed. The food vials were stored in the insectary and the food was changed weekly. The larvae were fed three times a day.



Figure 2.2 A 2 L container used to rear 1st – 2nd instar larvae. The label was important to keep track of the colony name (FUM0Z) and the date the eggs (which have developed into larvae at this stage) were originally harvested.

Adults

Depending on how many adults emerged per day, male and female adult *An. funestus* were removed every morning using a mouth aspirator and transferred to either 5L buckets or large Bug-Dorm cages (dimensions 30 cm (L) x 30 cm (W) x 30 cm (H); Bug-Dorm1, MegaView Science Co., Ltd., Taiwan). Adults were provided with 10% sugar water containing 0.5 g/L para-Aminobenzoic acid (PABA) (para-Aminobenzoic acid, A9878, Sigma-Aldrich, South Africa) to feed *ad libitum*. The PABA sugar water was placed into a modified 50 ml centrifuge tube (CLS430829, Sigma, South Africa). The tubes were cut into two equal pieces to produce two sugar feeding containers. One sugar feeding container was placed into the cage for the adults. The PABA sugar water was replaced every three days.

Adult female *An. funestus* that were 7 – 10 days old were blood-fed using a Hemotek membrane feeder (1917, Hemotek, United Kingdom) twice a week. Blood feeding took place during scotophase (in the dark). Bovine blood, stored in 50 mL falcon tubes in a -20°C freezer, was thawed and defrosted in cool water at room temperature before use. Salt preserved bovine intestines were washed with running cold tap water to remove the salt and were then used as a membrane to cover the feeding reservoirs (Figure 2.3A). Using a handheld pipette, 3 mL of blood was added into each reservoir prior to attaching them to the FU1 feeders connected to the PS6 power unit (Figure 2.3B). The device was temperature controlled (temperature range: 36.5-37.1 °C). The females were

allowed to feed for 40 minutes. Egg plates were placed in the cages or buckets on the third day after the second blood meal.

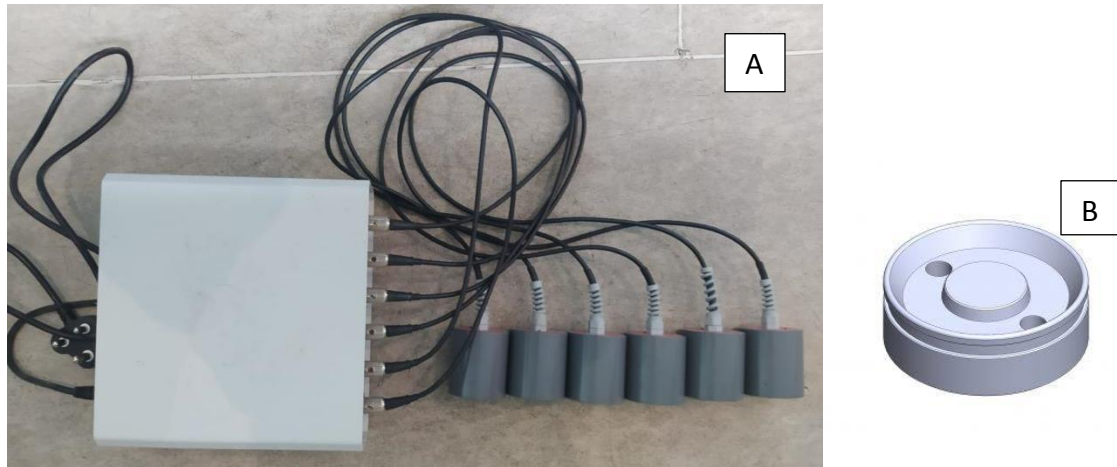


Figure 2.3 Hemotek membrane feeder system PS6 power unit connected to 6 FU1 feeders (A) and membrane feeder reservoir (3mL) (B).

2.1.2. Sugar water preparation and sugar feeding for experimental work

The sugar water was prepared using white sugar (Selati, South Africa) and reverse osmosis (RO) water. Ten, 20, 30 and 40% sugar water (w/v) was made. The different solutions were stirred until all the sugar dissolved completely. The solutions were then transferred to sterile glass bottles, labelled and kept in a 4°C refrigerator, and used within a week.

In this study, *An. funestus* fed with the 10% sugar water served as the control group because it is the current standard sugar water concentration used for FUMOS colony maintenance (Section 2.2) (Hunt *et al.*, 2005). Cotton wool (HGRA2805, Lassec, South Africa) was soaked in the designated sugar water concentrations and placed in 2 cm diameter Petri dishes. The dishes were then placed on top of each cage, faced down, for mosquitoes to feed (Figure 2.4). The different sugar water concentrations were replaced every third day.



Figure 2.4 Illustrating the placement of sugar water on the top of 17.5cm (L) x 17.5cm (W) x 17.5cm (H) Bug-Dorm cage for *An. funestus* feeding.

2.2 Experimental Design and Data Analyses

2.2.1 Longevity in colonised *An. funestus*

2.2.1.1 The effects of various sugar water concentration on the longevity of colonised male *An. funestus*

Male *An. funestus* adults were cleared upon emergence from the larval bowls, and transferred to experimental cages of 17.5 cm (L) x 17.5 cm (W) x 17.5 cm (H) (BugDorm-4M1515, MegaView Science Co., Ltd., Taiwan) and fed with the designated sugar water concentration (0 (0% sugar water), 10, 20, 30 and 40%) *ad libitum*. The lack of sugar in the 0% co-horts acted as a negative control for longevity experiments. Each cage contained an initial number of 50 newly emerged male *An. funestus*. Each day, the dead mosquitoes were removed from the cages and the number of these mosquitoes was recorded on a datasheet attached to the cage. This procedure was repeated ten times (ten biological replicates) for each sugar water concentration. On the occasion that there were small numbers of mosquitoes emerging on a particular day (due to the difficulty maintaining the *An. funestus* colony under insectary conditions), it was not possible to set up all five cages for one biological repeat on the same day. However, since the adults emerged from the same cohort of eggs, a biological repeat was set up within five days or less. Data were analysed using the survival and survminer packages and the Kaplan-Meier survival analysis (Kaplan and Meier, 1958) was performed. Descriptive statistics were obtained and p-values using the pairwise_survfit

function (log-rank test) with R and R Studio. Data analyses were performed in R (v. 3.4.3) with R Studio (v. 1.1.423) and significance was set at $\alpha=0.05$.

2.2.1.2 The effects of various sugar water concentration on the longevity of colonised *An. funestus* male and female (concurrent males versus females experiment)

Results obtained from the male longevity experiment necessitated the longevity experiment also to include *An. funestus* females that were fed with different sugar concentrations and compare their longevity with their male counterparts. This comparison was only possible by conducting longevity experiments between *An. funestus* males and females concurrently. The *An. funestus* male and female longevity experiments were conducted the same day for each sugar concentration. This longevity assay was done as described in section 2.2.1.1 above but the exception was that each cage contained 60 males and 60 females.

All biological replicates of the longevity of *An. funestus* that were fed a specific sugar concentration were compared. Data were analysed using the survival and survminer packages and the Kaplan-Meier survival analysis (Kaplan and Meier, 1958) was performed. Descriptive statistics were obtained and p-values using the pairwise_survfit function (log-rank test) with R and RStudio. Data analyses were performed in R (v. 3.4.3) with R Studio (v. 1.1.423) and significance was set at $\alpha=0.05$.

2.2.2 Effects of sugar water concentration on flight performance in colonised *An. funestus*

A flight testing device similar to the one used by Culbert *et al.* (2020) was shipped from the International Atomic Energy Agency (IAEA) in Austria and used for this objective.

Brief device specifications:

The flight test device (FTD) had three major components (Figure 2.5 A, B, C). The largest cylinder (A) had an 18cm diameter (covered with a 1 mm² mesh net at the top and a sleeve at the bottom) and was 40cm in length. A 12cm diameter hole 5cm from the bottom of the cylinder covered with a 30cm sleeve made with 1 mm² mesh net)

A smaller inner cylinder (B) with a dimension of 8 cm diameter and 29 cm length was filled with 24 transparent acrylic plastic flight tubes with a length of 25 cm and a diameter of 1 cm and held together by silicone gel. A 1 cm diameter hole, 3 cm from the bottom of (B) allowed for aspiration of mosquitoes into the FTD. The inner cylinder (B) was placed on a plastic base. The function of the 7.8 cm diameter plastic base (C) was to close off the 1cm hole at the bottom of the tube where the mosquitoes were aspirated. This also reduced the amount of space the mosquitoes had in the chamber, forcing them to fly out.

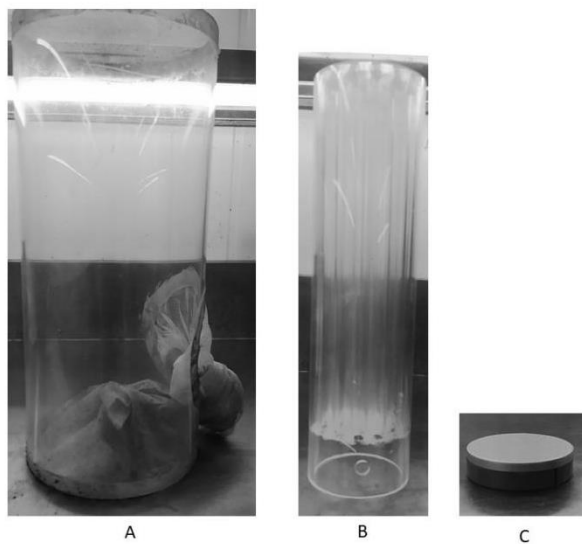


Figure 2.5 Flight test device (FTD). The figure shows (A) the larger tube that escaped mosquitoes were in, (B) the casing containing the 24 acrylic plastic tubes that mosquitoes had to fly through and (C) the sleeve allowing access to the acrylic tubes.

Flight test methodology

Newly emerged *An. funestus* male and female mosquitoes were kept in separate experimental cages with dimensions of 17.5 cm (L) x 17.5 cm (W) x 17.5 cm (H) (BugDorm-4M1515, MegaView Science Co., Ltd., Taiwan) and fed with ten, 20, 30 or 40% sugar water and were assessed for flight performance using the FTD when adults were 2 – 3 days old. Sugar water was available for the mosquitoes to feed for the duration of the experiment. Each experimental cage

contained 45 mosquitoes and of those, 30 mosquitoes were used to perform the flight experiment. All the mosquitoes from each treatment (male only or female only fed with the same sugar water concentration) were transferred into the device using a mouth aspirator and allowed two hours to exit the tubes. This procedure was repeated five times for each group (male or female) for each sugar treatment. Flight ability (P) was calculated by dividing the number of mosquitoes that escaped from those that were placed into the device at the beginning of the experiment using the formula $P=n/N$, where n is the number of mosquitoes that escaped the flight tubes and N is the total number of mosquitoes aspirated into the FTD. This method was used because Culbert *et al.* (2018) and Culbert *et al.* (2020) proved a correlation between flight ability and male fitness using this flight device.

Pearson's correlation was computed to test whether there was a correlation between flight performance of male and female *An. funestus* compared to sugar water concentration. The Shapiro-Wilk test (Shapiro and Wilk, 1965) was used to test for normality of the data and a one-way ANOVA was used for analysis because the data were normally distributed. The comparison was made between males only or females only across the four sugar water concentrations and then males vs females for each sugar water concentration. A post hoc Tukey HSD test was used to determine the significant differences between the various treatments. Data analyses were performed in R (v. 3.4.3) with R Studio (v. 1.1.423) and significance was set at $\alpha=0.05$.

2.2.3 Effects of sugar water concentration on mating success in colonised *An. funestus*

Newly emerged male and female *An. funestus* were transferred into experimental cages in a 1:1 ratio (25 males (m) and 25 females (f)) and were fed with either 0, 10, 20, 30 or 40% sugar water. The sugar water was changed every three days and was available to the mosquitoes for the duration of the experiment. The mosquitoes were allowed ten days to mate, after which the live females were collected from each cage, and spermathecal dissections were performed on these females. Spermathecal dissection was performed under a dissecting microscope (SZ2-ILST, Olympus, Tokyo, Japan). In this procedure, a drop of phosphate-buffered saline (abbreviated PBS) solution was added to the slide and the female was placed into the drop. Two dissecting needles were used for the spermathecal dissection. One needle was placed horizontally on the fifth segment and the second needle was used to pull the cercus away to remove the spermatheca between the sixth and

seventh segments. The dissected spermatheca was covered with a cover slip and then viewed using the 40x objective of a compound microscope (Model BX53F, Olympus, Tokyo, Japan). Insemination was deemed successful if the sperm was present in the spermatheca (Figure 2.6 A). If the sperm was absent from the spermatheca, insemination did not occur (Figure 2.6 B). The experiment was repeated five times for each sugar water concentration. No data is available for the 0% sugar water concentration because over 90% of the mosquitoes died within four days.

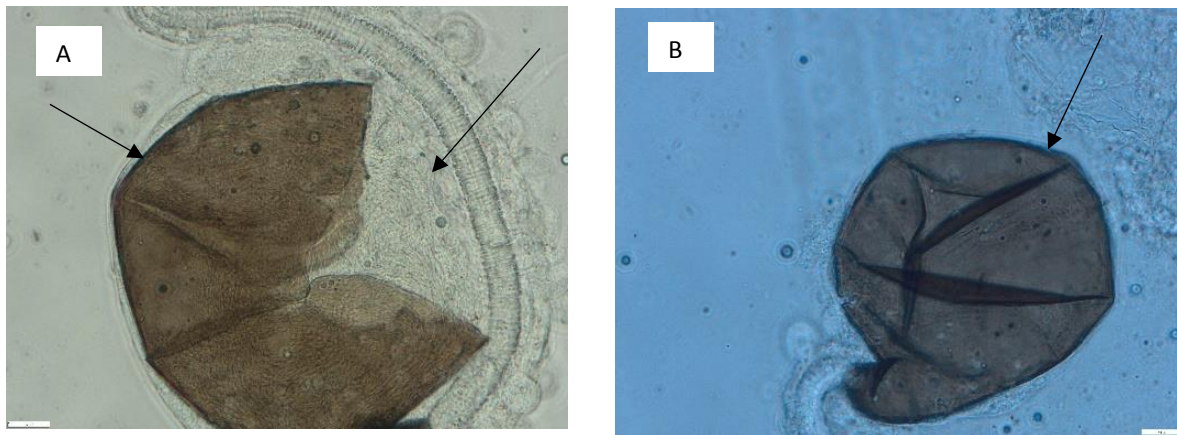


Figure 2.6 Spermathecal capsule visualised under 40x objective of a compound microscope of (A) a mated and (B) unmated female *An. funestus*. The arrows show the spermathecal capsules and sperm in (A). The length of the scale bar represents 10 μm .

Pearson's correlation was computed to test whether there was a correlation between mating success in *An. funestus* compared to sugar water concentration. The Shapiro-Wilk normality test (Shapiro and Wilk, 1965) was performed to test for normality. A one-way ANOVA test was performed to compare differences in percentage insemination (mating success) of *An. funestus* females fed with the different sugar water concentrations. Furthermore, a post hoc Tukey HSD test was performed to determine if there is a significant difference between the treatments. Data analyses were performed in R (v. 3.4.3) with R Studio (v. 1.1.423), and significance was set at $\alpha \leq 0.05$.

2.2.4 Effects of sugar water concentration and male to female ratio on mating success in colonised *An. funestus*

Similar methods were used as in section 2.2.3 but the ratio of males (m) to females (f) were 1m:3f (15m:45f), 1m:2f (20m:40f), 1m:1f (30m:30f), 2m:1f (40m:20f) and 3m:1f (45m:15f) keeping 60 mosquitoes in total per cage constant. Five biological repeats were performed.

Pearson's correlation was computed to test whether there was a correlation between male to female ratio and percentage insemination. A one way ANOVA test was used to 1) compare percentage insemination in females that were in different male to female ratios and fed with the same sugar water concentration and 2) compare female percentage insemination for each male to female ratio that were fed with the two different sugar water concentration, i.e., 10% sugar water fed 1m:1f vs 40% sugar water fed 1m:1f. Data analyses were performed in R (v. 3.4.3) with R Studio (v. 1.1.423) and significance was set at $\alpha=0.05$.

2.2.5 Effects of male age and sugar water concentration on mating success in colonised *An. funestus*

One hundred newly emerged males were collected and transferred to holding cages (30 cm (L) x 30 cm (W) x 30 cm (H); Bug-Dorm1, MegaView Science Co., Ltd., Taiwan). The mosquitoes were fed with either the 10 or 40% sugar water concentration throughout this experiment. For each biological repeat, six males were collected from the holding cages when they were either newly emerged (0 days)/ 5 days old/ 10 days old or 15 days old. Males were transferred into different experimental cages with six newly emerged virgin females (1:1 ratio). The mosquitoes were allowed to mate for 10 days and then the females were removed from the cages on the 10th day. Spermathecal dissections were performed and the spermathecae were viewed using the 40x objective of a compound microscope (Model BX53F, Olympus, Tokyo, Japan) as described above in section 2.2.3. Methods adopted from Culbert *et al.* (2018) and Culbert *et al.* (2020) to include age and diet as factors influencing mating. The experiment was repeated nine times.

Pearson's correlation was computed to test whether there was a correlation between male age and mating percentage insemination. A one-way ANOVA test was used to compare percentage

insemination of females that mated with males of different ages provided with either ten or 40% sugar water. Comparisons were also made between the percentage insemination of females that mated with males of a specific age and were fed different sugar water concentrations. Data analyses were performed in R (v. 3.4.3) with R Studio (v. 1.1.423) and significance was set at $\alpha=0.05$.

2.2.6 The effects of sugar water concentration on multiple mating in *An. funestus* males

This experimental design necessitated the use of smaller containers (cups) to accommodate one male with either five or ten females to answer this objective. This was a three way experiment: Three small disposable cups (6 cm diameter and 9 cm height)) were simultaneously prepared: (1) one male and five females, (2) one male and ten females and (3) six males and six females which were used as the control (Figure 2.7). Only newly emerged mosquitoes were used for this experiment. The mosquitoes were fed with ten and 40% sugar water. The females were removed from the cups after ten days to be dissected and their spermathecae were viewed using the 40X objective of a compound microscope (Model BX53F, Olympus, Tokyo, Japan). Females that died before the ten day period ended were removed. Mating success was calculated by counting the number of females inseminated by the same male over the ten day period (for the experiments with a single male per cup) or counting the number of inseminated females per cup (for the 1:1 male:female ratio). Three biological repeats were performed. Comparisons were also made between the percentage insemination of females that mated with males in cages with a specific ratio and were fed different sugar water concentrations. A one-way ANOVA analysis was performed. Data analyses were performed in R (v. 3.4.3) with R Studio (v. 1.1.423) and significance was set at $\alpha=0.05$.



Figure 2.7 Illustration of one biological repeat of the multiple mating experimental design for each sugar water concentration.

CHAPTER THREE

3. RESULTS

The impact of different sugar water concentrations on key lifetable parameters on adult *An. funestus* were evaluated. These parameters included the impact on longevity, flight performance and mating success.

3.1 Longevity in Colonised *An. funestus*

3.1.1 The effects of various sugar water concentration on the longevity of colonised male *An. funestus*

Longevity of male *An. funestus* fed with various sugar water concentrations was recorded and the data were presented in the form of a Kaplan-Meier plot (Figure 3.1), and in Table 3.1 and Table 3.2. The longevity range of colonised *An. funestus* is provided in Appendix A.

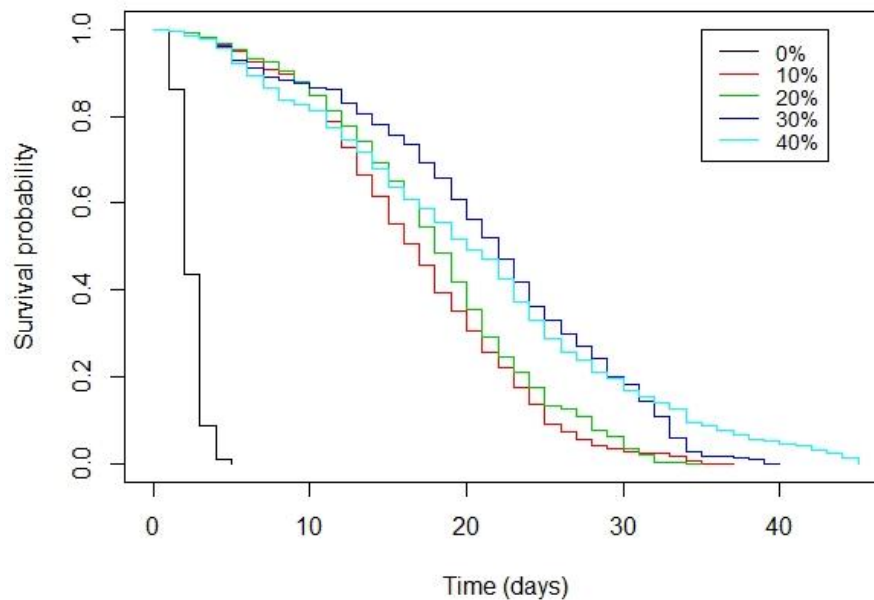


Figure 3.1 Kaplan-Meier survival analysis of male *An. funestus* fed with various sugar water concentrations. All treatments were significantly different from the control (presented in Table 3.1).

The median survival (Table 3.2) for control (10% sugar water fed) males was 17 days (95% CI: 16-18). The males fed on 0% sugar water had a median survival of 2 days (95% CI: 2-2). This showed an 82.24% significant decrease in median days of survival for males fed on 0% sugar water compared to the control ($p<0.001$) (Table 3.1). The median longevity of 20% sugar water fed males was 18 days (95% CI: 18-19) and increased by 5.56% and this was significantly different from the control ($p<0.05$). The 30% sugar water fed males lived for a median was 22 (95% CI: 21-23) days, resulting in a 22.73% significant increase in survival compared to the control ($p<0.001$). The median survival of males fed on 40% sugar water was 20 days (95% CI: 19-22). This resulted in a 15.00% increase in survival compared to the control. This increase in survival was statistically significant ($p<0.001$). Median longevity was higher in 30% and 40% sugar water fed males than those fed with 20% sugar water ($p<0.001$). There was no statistically significant difference ($p>0.05$) between the mean survival time of males fed on 30% sugar water and those fed on 40% sugar water (Table 3.1).

Table 3.1 Pairwise comparison of the longevity of male *An. funestus* fed with various sugar water concentrations.

Sugar water concentration (%)	0	10	20	30
10	<0.001	-		
20	<0.001	<0.05	-	
30	<0.001	<0.001	<0.001	-
40	<0.001	<0.001	<0.001	>0.05

Table 3.2 Median number of days (and 95% CI) that male *An. funestus* lived based on the sugar water concentrations.

Sugar water concentration (%)	0	10	20	30	40
N	500	500	500	500	500
Median survival in days (95% CI)	2 ^a (2-2)	17 (16-18)	18 ^b (18-19)	22 ^c (21-23)	20 ^c (19-22)

N= total sample size

The superscripts denote significant differences in longevity between males fed with the different sugar concentrations, versus males fed with control sugar concentration (10% sugar water).

3.1.2 The effects of various sugar water concentrations on the longevity of colonised male and female *An. funestus* (concurrent males vs. females experiment)

Due to the statistical difference in male survival between the different sugar water concentration treatments, especially the notable increase in longevity in the males as their sugar water concentration diet increased, the impact of the sugar diet on females was also evaluated. The longevity experiment was repeated with both males and females, however 60 males and 60 females *An. funestus* were concurrently placed into each cage. This was done to provide the same adult density/cage for mating experiments described in section 3.4.

The Kaplan-Meier plot and tables with statistical analyses are provided in Figure 3.2 A, Table 3.3 and Table 3.4 When looking at the males, survival analysis first revealed that males (n=60) fed on 10% sugar water had a median survival of 17 days (95% CI: 16-18). These results were corresponding to the previous experiment where 50 males were used (Table 3.2) indicating that the increase in mosquito numbers per cage did not impact survival analysis in the control group. The median survival of males fed on 0% sugar water was two days (95% CI: 2-2). Survival decreased by 88.24% in 0% sugar water fed males compared to the control and this was a significant decrease ($p < 0.001$). Compared to the control, the median survival increase in 20% sugar water males was 19 days (95% CI: 17-21). This was a significant increase in median survival by 11.76% ($p < 0.001$). Median survival for 30% sugar water fed males was 20 days (95% CI 18-21). This resulted in a 17.64 % significant increase in median longevity compared to the control

($p < 0.01$). There was no statistically significant difference between the median survival of males fed on 20% sugar water and those fed on 30% sugar water ($p > 0.05$). The highest median survival was recorded for the 40% fed males was 22 days (95% CI 21-23). Compared to the control, median survival in these 40% sugar water fed males increased by 29.41%, and this increase was significantly higher than the median survival of control males ($p < 0.001$). Median survival in 40% sugar water fed males was also significantly higher compared to 20% ($p < 0.01$), however, median survival between 30% sugar water fed ($0 < 0.001$) *An. funestus* males.

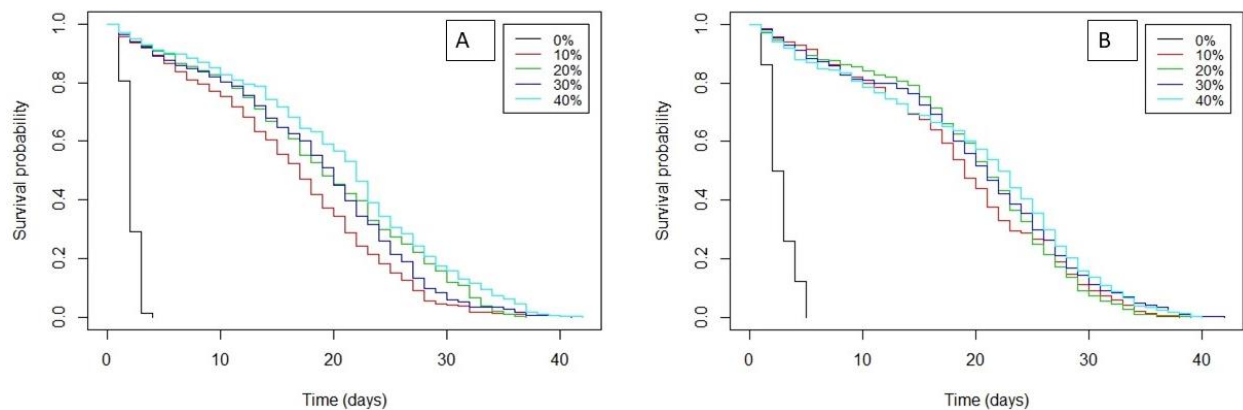


Figure 3.2 Longevity of male *An. funestus* fed with various sugar water concentrations (60 mosquitoes per cage) in the males (A) vs females (B) longevity experiment.

Table 3.3 Pairwise longevity comparison of male and female *An. funestus* (60) fed with various sugar water concentrations in the males vs females longevity experiment.

Sugar water concentration (%)	Males				Females			
	0	10	20	30	0	10	20	30
10	$p < 0.001$	-			$p < 0.001$	-		
20	$p < 0.001$	$p < 0.001$	-		$p < 0.001$	$p > 0.05$	-	
30	$p < 0.001$	$p < 0.01$	$p > 0.05$	-	$p < 0.001$	$p < 0.05$	$p > 0.05$	-
40	$p < 0.001$	$p < 0.001$	$p < 0.01$	$p < 0.001$	$p < 0.001$	$p < 0.01$	$p < 0.05$	$p > 0.05$

Table 3.4 Median number of days (and 95% CI) that male and female *An. funestus* lived based on the sugar water concentrations.

Sugar concentration (%)	N	Median survival in days (95% CI) of males	Median survival in days (95% CI) of females	P-value (males vs females)
0	300	2 ^a (2-2)	2.5 ^v (2-3)	p<0.001
10	300	17 (16-18)	19 (18-20)	p<0.01
20	300	19 ^b (17-21)	21 ^{wx} (20-22)	p>0.05
30	300	20 ^b (18-21)	21 ^{xy} (20-22)	p>0.05
40	300	22 ^c (21-23)	22.5 ^{yz} (21-24)	p>0.05

N= total sample size

The superscripts denote significant differences of longevity between males fed with the different sugar concentrations, versus males fed with control sugar concentration (10% sugar water). Values with the same superscript were not significantly different from each other.

The Kaplan-Meier plot and tables with statistical analyses for females fed on different sugar water concentrations are provided in Figure 3.2 B, Table 3.3 and Table 3.4 above. Control female *An. funestus* had a median survival of 19 days (95% CI: 18-20). The 0% sugar water fed females had a median survival of 2.5 days (95% CI: 2-3). This decrease was statistically significant (p<0.01). The median survival of 20% sugar water fed females was 21 days (95% CI: 20-21). This resulted in a 10.53% increase in survival that was not significantly different from the control (p>0.05). The 30% sugar water fed females also had a median survival of 21 days (95% CI: 20-22). This 10.53% increase in longevity was, however, statistically significant compared to the control (p<0.05) given the high variation within the treatments. The highest median survival compared to the control was recorded for 40% sugar water fed females, and this was 22.5 days (95% CI 21-24). This resulted in an increase in median days of survival in female *An. funestus* of 18.42% for 40% sugar water fed females compared to the control, and the difference was statistically significant (p<0.01). There was no significant difference in median longevity between 20% and 40% sugar water fed *An.*

funestus females and between 30% and 40% sugar water fed females ($p>0.05$). However, 40% sugar water fed females lived longer than 20% sugar water fed females.

A comparison between male and female *An. funestus* fed on the same sugar water concentration showed a statistically significant difference in median survival of males and females fed with 0% and 10% sugar water. Median survival between in female *An. funestus* fed with 0% sugar water increased compared to males by 25% ($p<0.001$) and by 11.76% for the control (10% sugar water fed) group ($p<0.01$). There was no statistically significant difference between male vs female *An. funestus* fed with 20%, 30% and 40% sugar water ($p>0.05$) (Table 3.4).

3.2 Effects of Sugar Water Concentration on Flight Performance in Colonised *An. funestus*

The study examined whether the increased sugar concentration influenced *An. funestus* activities such as flight, which is also vital for swarming and thus mating, and host and food searching. The 0% sugar water group was not included because more than 90% of adults die within three days, and the numbers would not have been sufficient for this experiment.

A positive correlation was found between sugar water for concentration and escape proportion from the FTD for males (Pearson's correlation: $r=0.0.70$, $p<0.001$) and females (Pearson's correlation: $r=0.0.66$, $p<0.01$). Not all concentrations were significantly different from the control when comparing flight performance (Figure 3.3, Table 3.5, and Table 3.6).

The mean escape proportion between control and 20% sugar water fed males was equal at 0.56 ± 0.004 and 0.56 ± 0.006 . Although not statistically significant, escape proportions in 30% sugar water fed males compared to the control increased by 17.86% to 0.66 ± 0.004 ($p>0.05$). Escape proportions increased significantly by 25.21% in 40% sugar water fed males with a mean escape proportion of 0.69 ± 0.004 compared to the control ($p<0.05$). There was, however, a statistically significant difference between males fed with 20% sugar water and those fed with 40% sugar water ($p<0.05$) (Table 3.5). There was no significant difference between male escape proportions for 20% and 30% sugar water fed males ($p>0.05$) and 30% and 40% sugar water fed males ($p>0.05$).

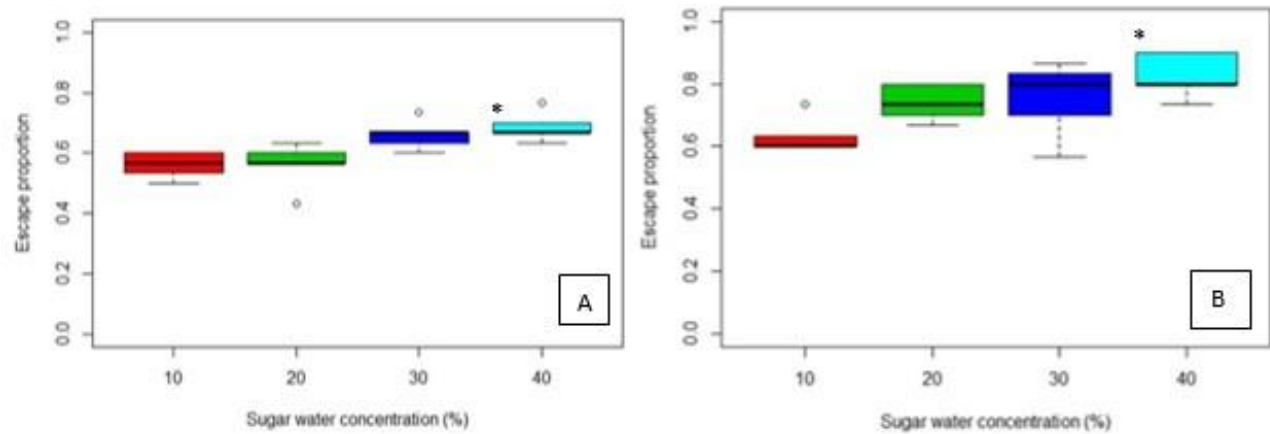


Figure 3.3 Mean escape proportions for the flight performance experiment of male (A) and female (B) *An. funestus* fed with various sugar water concentrations. Compared to the control, only the 40% sugar water treatment resulted in a significantly higher male *An. funestus* escape proportion from the flight test device. The * denotes a significant difference from the control.

Table 3.5 Pairwise comparison of proportions of male and female *An. funestus* that flew out of the flight tubes of the FTD in a period of two hours.

Sugar water concentration (%)	Males			Females		
	10	20	30	10	20	30
20	p>0.05	-	-	p>0.05	-	-
30	p>0.05	p>0.05	-	p>0.05	p>0.05	-
40	p<0.05	p<0.05	p>0.05	p<0.01	p>0.05	p>0.05

Table 3.6 Mean $\pm$ standard error (SE) escape proportions of male and female *An. funestus* that escaped out of the FTD in a period of two hours.

Sugar water concentration (%)	N	Mean $\pm$ SE escape proportion of male <i>An. funestus</i>	Mean $\pm$ SE escape proportions of female <i>An. funestus</i>	p-value (males vs females escape proportions)
10	150	0.56 $\pm$ 0.004	0.63 $\pm$ 0.005	p>0.05
20	150	0.56 $\pm$ 0.006	0.74 $\pm$ 0.005	p<0.05
30	150	0.66 $\pm$ 0.004	0.75 $\pm$ 0.010	p>0.05
40	150	0.69 ^a $\pm$ 0.004	0.83 ^w $\pm$ 0.006	p>0.05

N= total sample size

The superscripts denote significant differences of proportions between the control (10% sugar water fed *An. funestus*) and treatments for each gender. Where there are no superscripts, statistically significant differences were not found after analysis.

The mean escape proportion for control females (Table 3.6) was 0.63 $\pm$ 0.005 and 0.74 $\pm$ 0.005 for 20% sugar water fed females, which was indicative of a non-significant increase of 17.46% (p>0.05) (Table 3.5). A mean escape proportion mean of 0.75 $\pm$ 0.010 for the 30% sugar water fed females, a 19.05% increase compared to the control. However, this was also not significantly different (p>0.05). Similar to the males, female *An. funestus* fed with 40% sugar water escaped the flight tubes faster than the control group with a mean escape proportion of 0.83 $\pm$ 0.006 and a significant increase in the escape proportion of 31.75% (p<0.05). Escape proportions from the flight tubes did not differ between 40% sugar water fed females compared to 20 and 30% sugar water treatments (p>0.05) (Table 3.5). The 30% and 20% sugar water fed groups did not differ in escape proportions (p>0.05).

A comparison between male and female *An. funestus* revealed that females were generally able to escape the flight tubes faster than males. Escape proportion increased, although not significantly,

by 12.50% for the female control group compared to the male control group ($p>0.05$). Escape proportions in female *An. funestus* fed with 20% sugar water were 32.14% significantly higher than males fed with the same sugar water concentration ($p<0.05$). For the 30% and 40% sugar water fed males and females, escape proportions of females compared to males increased by 25.00 % and 20.29%, respectively. These two increases were not significantly different.

3.3 Effects of Sugar Water Concentration on Mating Success in Colonised *An. funestus*

Both males and females were combined into an experimental cage and allowed to mate to determine if sugar concentration also impacts on mating success. Different sugar water concentrations were used as described in section 2.2.3 and the impact on mating success was evaluated. The 0% sugar water fed mosquitoes were excluded as most of them die within three days and data for this objective depended on mosquitoes to live for at least ten days.

There was a steady increase in percentage insemination of the females who mated with males when both were fed on increasing sugar water concentration (Figure 3.4). Pearson's correlation showed a strong positive correlation between sugar water concentration and percentage insemination (Pearson's correlation: $r=0.82$, $p<0.001$). The p-values and mean $\pm$ SE are recorded in Table 3.7 and Table 3.8 respectively. The mean percentage insemination $\pm$ SE for the control was 46.62% $\pm$ 1.70 (n=95) while insemination for the 20% sugar fed *An. funestus* was 56.81% $\pm$ 0.89 (n=99). The difference between percentage insemination in 20% sugar water fed *An. funestus* compared the control was an increase of 28.29% but it was not statistically significant ($p>0.05$).

Percentage insemination in 30% sugar water fed *An. funestus* was 71.16 $\pm$ 0.54% (n=108). Compared to the control group, this resulted in an increase of percentage insemination of 52.64% and was significantly higher ($p<0.01$). The percentage insemination for the 40% sugar water fed *An. funestus* was 79.15 $\pm$ 0.36% (n=91) and resulted in a 69.78% increase in percentage insemination compared to the control. This increase was statistically significant from the control ($p<0.001$). There was no statistical difference between the mating success of adults fed on either 30% or 40% sugar water solution ($p>0.05$) or between adults fed with 20% or 30% sugar water solution. However, a statistically significant difference in mating success was observed when comparing adults that were fed with 20% and 40% sugar solution ($p<0.05$).

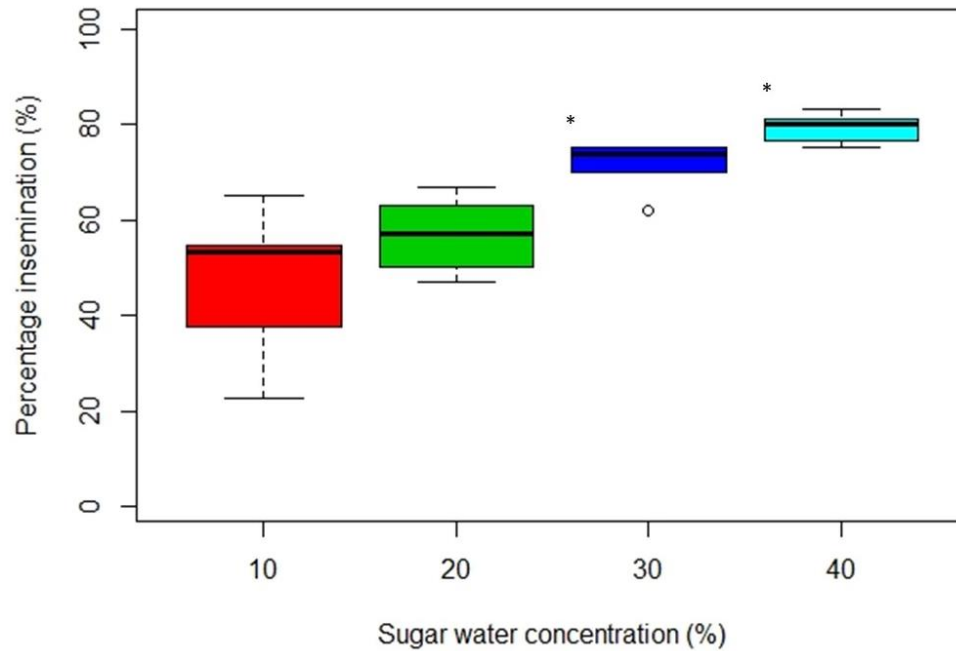


Figure 3.4 Mating success of *An. funestus* fed with different sugar water concentration. Compared to the control, percentage insemination increased significantly with the 30 and 40% sugar water concentration. * denotes significant difference from control

Table 3.7 Tukey HSD pairwise comparison of the mating success of *An. funestus* fed with different sugar water concentrations.

Sugar water concentration (%)	10	20	30
20	p>0.05		
30	p<0.01	p>0.05	
40	p<0.001	p<0.05	p>0.05

Table 3.8 Mean and Standard Error $\pm$ SE of percentage insemination of *An. funestus* fed with various sugar water concentrations. Superscripts denote significant difference.

Sugar water concentration (%)	10	20	30	40
N (n dissected)	150 (95)	150 (99)	150 (108)	150 (91)
Mean percentage insemination $\pm$ SE	46.62 $\pm$ 1.70	56.81 ^a $\pm$ 0.89	71.16 ^{bc} $\pm$ 0.54	79.15 ^c $\pm$ 0.36

N= total sample size at the beginning of the experiment.

n= total number of live mosquitoes on day 10, hence total number of mosquitoes dissected on day 10.

Superscripts denote significant difference from control. Values with the same superscript were not significantly different from each other.

3.4 Effects of Sugar Water Concentration and Male to Female Ratio on Mating Success in Colonised *An. funestus*

The biggest difference in longevity, flight performance and mating were between 10% and 40% sugar water fed *An. funestus*. Thus from this point, only 10% and 40% sugar water were used for the experiments. Overall, the ratio between males and females had an impact on percentage inseminations in *An. funestus* fed with both 10 and 40% sugar water concentrations (Figure 3.5). Pearson's correlation showed a strong negative correlation between sugar water concentration and male female ratio as the number of males in each cage decreased (from high male to female ratio to low male to female ratio) (Pearson's correlation: $r = -0.99$, $p < 0.001$ for 10% sugar water and $r = -0.87$, $p < 0.001$ for 40% sugar water fed *An. funestus*). Higher percentage insemination was observed for the groups where the ratio of males was equal to or higher than the females (Table 3.9, Table 3.10).

The control cages had an equal ratio of males to females (1:1; 30m:30f). A Mean percentage insemination of $45.37 \pm 0.15\%$ ($n=77$) was recorded for *An. funestus* adults fed with a 10% sugar water solution. The mean percentage insemination in 10% sugar water fed *An. funestus* of $31.26 \pm 0.46\%$ ($n=110$) was recorded for cages with a 1:2 male to female ratio (20m:40f), compared to

the control cage. This reflects a 31.10% decrease significantly different compared to the control cages ($p<0.05$). A male to female ratio of 1:3 (15m:45f) showed an even lower percentage insemination of only $12.91\pm0.75\%$ ($n=111$) in 10% sugar water fed *An. funestus*. This was a statistically significant decrease ($p<0.001$) in percentage insemination of 71.55% compared to the control cage. The mean percentage insemination in 10% sugar water fed *An. funestus* compared to the control in cages with the 2:1 male to female ratio was $58.36\pm0.26\%$ ($n=53$). This was an increase in percentage insemination by 28.63% compared to the control. This increase was significantly different from the control ($p<0.05$). The mean percentage insemination in 10% sugar water fed *An. funestus* in cages with the 3:1 male to female ratio (45m:15f) was recorded at $75.48\pm0.67\%$ ($n=41$). This was a significant increase in percentage insemination by 66.37% compared to the control (30m:30f) ($p<0.001$). Percentage insemination also differed significantly between the rest of the male to female ratios in this experiment ($p<0.01$)

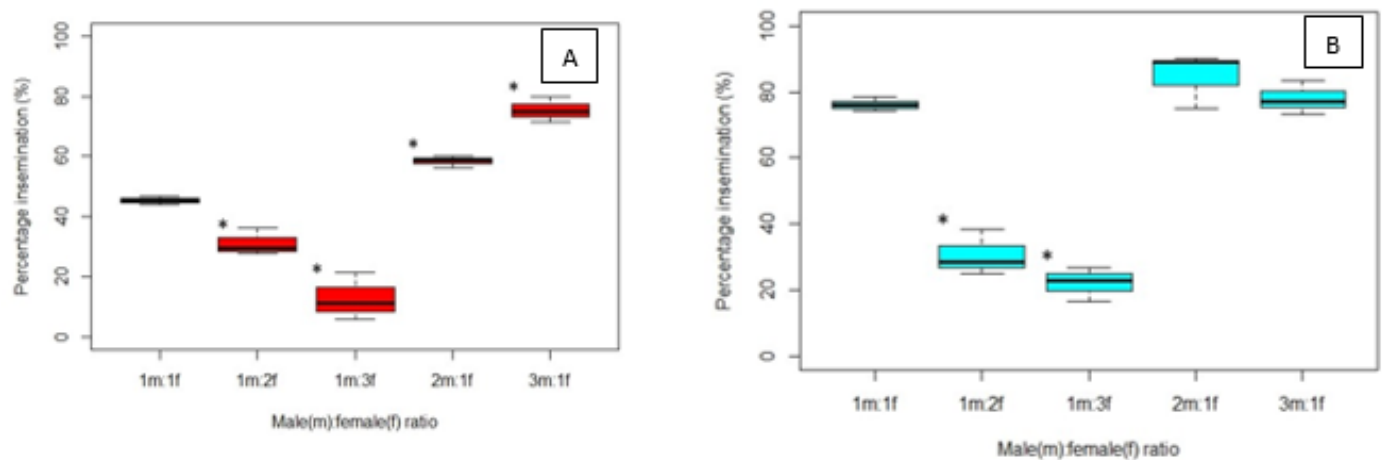


Figure 3.5 Percentage insemination of (A) 10% and (B) 40% sugar water fed *An. funestus* reared in various male to female ratios. * denotes significant difference from control.

Table 3.9 Pairwise comparison of percentage insemination of *An. funestus* between different ratios fed with 10% and 40% sugar water.

Male: female ratio	10% sugar water fed <i>An. funestus</i>				40% sugar water fed <i>An. funestus</i>			
	1:3 (15m:45f)	1:2 (20m:40f)	1:1 (30m:30f)	2:1 (40m:20f)	1:3 (15m:45f)	1:2 (20m:40f)	1:1 (30m:30f)	2:1 (40m:20f)
3:1 (45m:15f)	p<0.001	p<0.001	p<0.001	p<0.01	p<0.001	p<0.001	p>0.05	p>0.05
2:1 (40m:20f)	p<0.001	p<0.001	p<0.05		p<0.001	p<0.001	p>0.05	
1:1 (30m:30f)	p<0.001	p<0.05			p<0.001	p<0.001		
1:2 (20m:40f)	p<0.01				p>0.05			

Table 3.10 Mean and Standard Error (SE) of percentage insemination of *An. funestus* between different ratios fed with 10% sugar water.

Male (m):female(f) ratio	N(n) (10% sugar water fed females dissected on day 10)	N(n) (40% sugar water fed females dissected on day 10)	Mean percentage insemination ± SE of 10% sugar water fed females	Mean percentage insemination ± SE of 40% sugar water fed females	p-value (males vs females percentage insemination)
1:1 (30m:30f)	90(77)	90(75)	45.37±0.15	76.11±0.24	p<0.001
1: 2 (20m:40f)	129(96)	129(110)	31.26 ^a ±0.46	30.68 ^w ±0.66	p>0.05
1:3 (15m:45f)	135(111)	135(101)	12.91 ^b ±0.75	22.22 ^x ±0.52	p>0.05
2:1 (40m:20f)	60(53)	60(54)	58.36 ^c ±0.26	84.63±1.14	p<0.001
3: 1 (45m:15f)	45(41)	45(40)	75.48 ^d ±0.67	77.86±0.80	p>0.05

N= total sample size of females at the beginning of the experiment.

n= total number of live mosquitoes on day 10, hence total number of mosquitoes dissected on day 10.

Superscripts denote significant difference from control and in addition to being significantly different from the control. Where there are no superscripts, statistically significant were not found after analysis.

When analysing the effects of 40% and male to female ratio in 40% sugar water fed *An. funestus*, a mean percentage insemination of $76.11 \pm 0.24\%$ ($n=75$) was recorded for the control cage (30m:30f ratio). The mean insemination percentage compared to the control was $30.68 \pm 0.66\%$ ($n=110$) in cages with a 1:2 male to female ratio (20m:40f). This resulted in a significant decrease in percentage insemination of 59.69%, ($p < 0.001$). The mean insemination was $22.22 \pm 0.52\%$ ($n=101$) in cages of *An. funestus* with a 1:2 male to female ratio (20m:40f) and significantly decreased by 70.81% compared to the control ($p < 0.001$). A mean percentage insemination in 40% sugar water fed *An. funestus* of $84.63 \pm 1.14\%$ ($n=54$) was recorded for cages with a male to female ratio of 2:1 (40m:20f). This reflects an 11.19% increase compared to percentage insemination of the control cages, however, this did not significantly differ ($p > 0.05$). The mean mating success of $77.86 \pm 0.80\%$ ($n=40$) for the 3:1 male to female ratio (45:15) compared to the control and was only 2.30% higher than that of the control cages. No statistically significant difference ($p > 0.05$). Percentage insemination in the 1:2 compared to the 1:3 treatments did not differ ($p > 0.05$). In the 3:1 and 2:1 treatments, percentage insemination also did not differ ($p > 0.05$). However, there were significant differences between percentage insemination in male biased compared to female-biased treatments and male-biased treatments had the higher percentage insemination ($p < 0.001$).

A comparison of the mean mating success between the *An. funestus* adults fed on 10% sugar water and 40% sugar water at different male to female ratios revealed some interesting results. The control cage with a ratio of 1:1 (30m:30f), showed that feeding on 40% sugar solution increased the mean percentage insemination by 67.75% from $45.37\% \pm 0.15$ to $76.11\% \pm 0.24$ compared to feeding on 10% sugar water. This increase in mating success was statistically significant ($p < 0.001$). The percentage insemination of the 1:2 male to female ratio (20m:40f) increased by 1.86% in 40% sugar water fed compared to 10% sugar water fed *An. funestus* and this difference was not statistically significant ($p > 0.05$). The 1:3 male to female ratio (15m:45f) cages showed a 3.15% increase in percentage insemination in 40% sugar water fed *An. funestus* compared to 10% sugar water fed *An. funestus*. This difference was not statistically significant ($p > 0.05$). The percentage insemination of the 2:1 male female ratio (30m:20f) cages showed an increase in percentage insemination of 45.01% in 40% sugar water fed mosquitoes compared to those fed on 10% sugar water. This was a significant difference between the two sugar water concentrations ($p < 0.001$).

The percentage insemination of the 3:1 male to female ratio (45m:15f) cages increased by 3.15% in 40% sugar water fed *An. funestus* than those fed with 10% sugar water. This difference in mating success was not statistically significant ($p>0.05$). Regardless of sugar water concentration, mating success was significantly lower when females outnumbered the males. It was unknown if older males will increase mating or if one male can inseminate multiple females. These questions were investigated in section 3.5 and 3.6.

3.5 Effects of Male Age and Sugar Water Concentration on Mating Success in Colonised *An. funestus*

An observed overall decrease in percentage insemination was recorded in aged males versus newly emerged males that fed on 10% sugar water solution and were allowed to mate with the females for ten days (Pearson's correlation: $r = -0.41$, $p < 0.05$) (Figure 3.6 A). The effects of male age on percentage insemination were less pronounced when *An. funestus* adults that were fed on 40% sugar water solution although a slight decrease in percentage insemination was observed, the correlation between age and percentage insemination was not significantly different from 0 (Pearson's correlation: $r = -0.24$, $p > 0.05$) (Figure 3.6 B). Detailed statistical analyses can be found below for each sugar water treatment in Table 3.11 and Table 3.12.

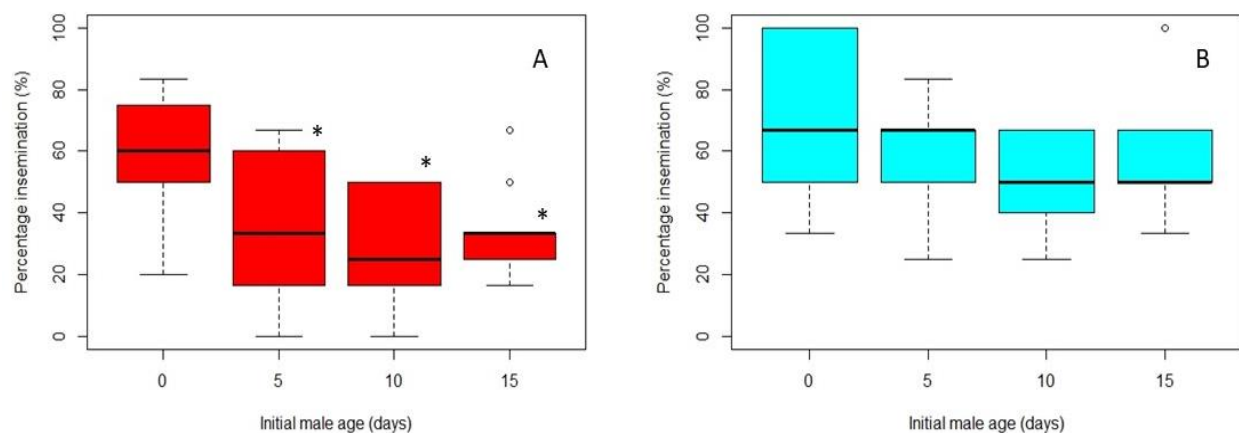


Figure 3.6 Percentage insemination of (A) 10% and (B) 40% sugar water fed female *An. funestus* mated with male *An. funestus* of various age groups. * denotes significant difference from control.

Table 3.11 Pairwise comparison of percentage insemination in females that mated with males of various age groups in *An. funestus* fed with 10% and 40% sugar water.

Initial male age (days)	10% sugar fed <i>An. funestus</i>			40% sugar fed <i>An. funestus</i>		
	5	10	15	5	10	15
0	p<0.05	p<0.05	p<0.05	p>0.05	p>0.05	p>0.05
5		p>0.05	p>0.05		p>0.05	p>0.05
10			p>0.05			p>0.05

Table 3.12 Mean and Standard Error (SE) of percentage insemination in females that mated with males of various age groups in *An. funestus* fed with 10% and 40% sugar water.

Initial male age (days)	N(n) (10% sugar water fed females dissected on day 10)	n (40% sugar water fed females dissected on day 10)	Mean percentage insemination $\pm$ SE of 10% sugar water fed females	Mean percentage insemination $\pm$ SE of 40% sugar water fed females	p-value (males vs females percentage insemination)
0	54(45)	54(36)	60.37 $\pm$ 3.11	70.00 $\pm$ 4.21	p>0.05
5	54(46)	54(45)	32.96 ^a $\pm$ 3.90	59.26 $\pm$ 2.70	p>0.05
10	54(50)	54(51)	29.82 ^a $\pm$ 2.59	49.82 $\pm$ 2.12	p >0.05
15	54(44)	54(40)	33.33 ^a $\pm$ 2.43	58.52 $\pm$ 2.95	p>0.05

N= total sample size of females at the beginning of the experiment.

n= total number live mosquitoes on day 10, hence total number of mosquitoes dissected on day 10.

Superscripts denote significant difference from control and in addition to being significantly different from the control, values with a similar superscript were not significantly different from each other. Where there are no superscripts, there were no significant differences.

The control group was in cages containing newly emerged males (newly emerged males when the experiment commenced). The mean percentage insemination in females that mated with newly emerged males for the 10% sugar water treatment was $60.37\% \pm 3.11$ (n=45). Percentage insemination in females that mated with 5-day old males decreased significantly by 45.40% to $32.96\% \pm 3.90$ (n=46) compared to the control ($p < 0.05$). The mean percentage insemination in females mated with 10-day old males was $29.82 \pm 2.59\%$ (n=50) and significantly decreased by 50.60% ($p < 0.05$). The mean percentage insemination in females that mated with 15-day old males was $33.33 \pm 2.43\%$ (n=44), resulting in a significant decrease in percentage insemination of and 44.79% compared to the control ($p < 0.05$). There were no statistically significant differences in five, ten and 15-day old males ($p > 0.05$) (Table 3.11).

Results obtained for 40% sugar water fed *An. funestus* were different from those of 10% sugar water fed *An. funestus*. There was no difference between percentage insemination for the control and any of the treatments (male age groups) in the 40% sugar water fed *An. funestus* ($p > 0.05$). The mean percentage insemination in females that mated with newly emerged males was $70.00 \pm 4.21\%$ (n=36) and decreased by just 15.34% to $59.26 \pm 2.70\%$ (n=45) in females that mated with 5-day old males. The mean insemination percentage in females mated with 10-day old males compared to the control decreased by 28.83% to $49.82\% \pm 2.12$ (n=51). The mean percentage of insemination in females that mated with 15-day old males decreased by just 16.40% to $58.52\% \pm 2.95$ (n=50). A pairwise comparison between 10% sugar water fed and 40% sugar water fed *An. funestus* of the same age group revealed that there was no significant difference ($p > 0.05$) between treatments with males of the same age (for example, newly emerged males fed with either 10% or 40% sugar water solution did not differ when compared to each other).

3.6 The Effects of Sugar Water Concentration on Multiple Mating in *An. funestus* Males

Thus far, higher sugar water concentrations increased longevity, mating success and flight performance in the laboratory-reared *An. funestus*. To my knowledge, no data or publications was indicating whether male *An. funestus* can mate multiple times. This study examined the possibility of one male mating with multiple *An. funestus* females (5 and 10 females) and if the concentration of sugar diet can impact these mating events. Adults were fed with either 10% or

40% sugar solution for all experiments. The control in this experiment were cages with a 1:1 male to female ratio (6m:6f). Raw data is provided in Appendix B. The mean percentage insemination for the 10% sugar water fed *An. funestus* (control) was $64.26 \pm 1.05\%$ (n=45) (Figure 3.7 A, Table 3.14). Compared to the control, mean percentage insemination decreased significantly by 83.57% to $10.56 \pm 0.61\%$ (n=40) in cages with a 1:5 male to female ratio (1m:5f). ($p < 0.001$). The mean percentage insemination for cages with a 1:10 male to female ratio (1m:10f) decreased by 96.34% to $2.35 \pm 0.24\%$ (n=75), which was significantly different from the control ($p < 0.001$). There was no statistically significant difference in mean percentage insemination of 10% sugar water fed females of the 1m:5f and 1m:10f treatments ($p > 0.05$).

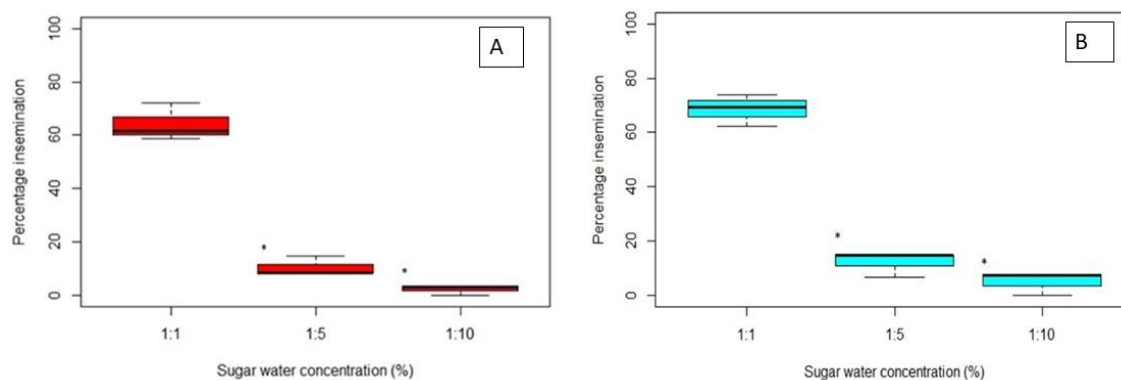


Figure 3.7 Test for multiple mating in *An. funestus* fed with (A) 10% and (B) 40% sugar water in a 6m:6f, 1m:5f and 1m:10f male female ratio. With the 1:5 (1m:5f) and 1:10 (1m:10f) ratio groups cages containing one male each and five or ten females respectively.

Table 3.13 Tukey HSD pairwise comparison of the mating success *An. funestus* fed with 10% and 40% sugar water in a 6m:6f, 1m:5f and 1m:10f male female ratio. With the 1m:10f and 1m:5f ratio groups cages containing 1 male each and 5 or 10 females respectively.

Male:female ratio	10% sugar water fed		40% sugar water fed	
	1m:5f	1m:10f	1m:5f	1m:10f
6m:6f	p<0.001	p<0.001	p<0.001	p<0.001
1m:10f	p>0.05		p>0.05	

Table 3.14 Mean and Standard Error (SE) of percentage insemination of *An. funestus* fed with 10% and 40% sugar water in a 6m:6f, 1m:5f and 1m:10f male female ratio. With the 1m:5f and 1m:10f ratio groups cages containing 1 male each and 5 or 10 females respectively.

Number of males and females respectively per cage	N(n) (10% sugar water fed females dissected on day 10)	n (40% sugar water fed females dissected on day 10)	Mean percentage insemination $\pm$ SE of 10% sugar water fed females	Mean percentage insemination $\pm$ SE of 40% sugar water fed females	p-value (males vs females percentage insemination)
1:1 (6m:6f)	54(45)	54(44)	64.26 $\pm$ 1.05	68.52 $\pm$ 0.89	p>0.05
1:5 (1m:5f)	45(40)	45(37)	10.56 ^a $\pm$ 0.61	12.22 ^w $\pm$ 0.79	p>0.05
1:10 (1m:10f)	90(75)	90(79)	2.35 ^a $\pm$ 0.24	5.12 ^w $\pm$ 0.50	p>0.05

N= total sample size of females at the beginning of the experiment.

n= total number live mosquitoes on day 10, hence total number of mosquitoes dissected on day 10.

Superscripts denote significant difference from control and in addition to being significantly different from the control, values with a similar superscript were not significantly different from each other.

Similar results were observed in 40% sugar water fed *An. funestus* compared to 10% sugar water fed *An. funestus*. The mean percentage insemination for the 40% sugar water fed *An. funestus* control cages was 68.52 $\pm$ 0.89% (n=44) (Figure 3.7 B and Table 3.14 above). Compared to the control, mean percentage insemination decreased significantly by 82.17% to 12.22 $\pm$ 0.79% (n=37) (p<0.001) (Table 3.13). The mean percentage insemination for cages with a 1:10 male to female ratio in 40% sugar water fed *An. funestus* (1m:10f) decreased by 92.53% to 5.12 $\pm$ 0.50% (n=79), which was significantly different from the control (p<0.001).

A comparison between 10% sugar water fed and 40% sugar water fed *An. funestus* revealed no significant difference between treatments with the same male to female ratio (for example, in *An. funestus* in a 1:1 (6m:6f) male to female ratio fed with either 10% and 40% sugar water concentration, percentage insemination did not differ significantly).

CHAPTER FOUR

4. DISCUSSION

Sugar water concentrations (above the 10% control) had an overall positive impact on the key biological parameters evaluated in this study: longevity, flight performance and mating success. The greatest differences in these biological parameters were seen between the control 10% sugar water and the highest sugar water concentration used in this study (40% sugar water). Increased sugar water concentration leads to increased longevity, flight performance and mating success. To further improve mating success and to understand factors that are critical for successful mating, different ratios of males and females and the age of males were further evaluated. Increasing the number of males to double or three times as much as the females enhanced the mating success. Furthermore, the effect of increasing the number of males (3:1 male to female ratio with 45 males and 15 females per cage) fed with 10% sugar water increased the mating success to the same level as the males (3:1 male to female ratio with 45 males and 15 females per cage) fed with the 40% sugar water. *Anopheles funestus* male age did not affect on percentage inseminations of the females when both males and females were fed with 40% sugar water. However, in 10% sugar water fed *An. funestus*, significantly higher percentage inseminations were recorded when newly emerged males versus aged males (5 to 15 days post eclosion) were allowed to mate for ten days with newly emerged females.

4.1 The Effects of Various Sugar Water Concentration on the Longevity of Colonised *An. funestus*

It is well known that when mosquitoes are sugar-fed, their survivorship increases compared to those fed with just water (Briegel and Kaiser, 1973, Gary and Foster, 2001; Gary *et al.*, 2009; Kessler *et al.*, 2015). This is also true for other insects such as the endoparasitoids *Meteorus pulchricornis* (Sheng *et al.*, 2019) and *Pteromalus puparum* (Xiong *et al.*, 2020) and honey bees (*Apis mellifera*) (Abou-Shaara, 2017).

The 0% sugar water treatment resulted in the lowest observed longevity of male and female *An. funestus* when compared to all sugar water fed *An. funestus* (were *An. funestus* were provided with water only), confirming previous studies which showed that sugar is necessary for mosquitoes to survive (Foster 1995; Bellinni *et al.*, 2014). As sugar concentration increased, so did the longevity of both male and female *An. funestus*. Adult *An. funestus* that were provided with water alone survived for no more than five days and had a median survival of only two days but this ranged between 17 days to 22 days in adult males and 19 days to 22.5 days in females provided with 10% and 40% sugar water respectively. The median survival of female *An. funestus* were significantly higher than male *An. funestus* when provided with water only (an increase of 25% in median survival was recorded) and in the control group (fed with 10% sugar water) where a significant increase of 11.76% was recorded. . Furthermore, there were no difference in median survival between male and female *An. funestus* when fed with 20% and 30% sugar water.

The adult sugar water diet did not only have a significant increase in male longevity, but it also increased female longevity. This could be because, males primarily have to find a sugar source and form swarms to mate whereas females have need to live longer to find a blood meal after the sugar source, develop the eggs and find a suitable habitat to lay eggs. The RO water (0% sugar water) treatment results showed that female *An. funestus* are naturally longer-lived than their male counterparts as they had a significantly longer median survival time than males. There was interreplicate variations (standard error) in the longevity of *An. funestus* between mosquitoes fed with distilled water as compared to those that were sugar-fed. This could be because with higher sugar water concentrations, the males lived longer and data was collected over a longer period of time whereas for the 0% sugar water treatment, 100% mortality was recorded by day 5. The eggs and larvae were reared in the same insectary using the same protocol, it is possible that the higher variation in survival with increasing sugar water concentration is because these male mosquitoes had approximately the same amount of teneral reserves but due to the differential sugar water intake frequencies between individuals or to a lesser extent, possible competition between individuals, effects of sugar concentrations differ. Assuming that some males fed more than others and thus exploited the full effects of the diet they were fed.

England and Evans (1997) supplemented the diet of the parasitoid wasp *Bathyplectes curculionis* with a sugar source (pea aphid honeydew) and found that the longevity of the *B. curculionis* fed

with this additional sugar source was approximately 50% higher than those that did not receive the supplementary sugar source. Sheng *et al.* (2019) reared the parasitoid wasp *Meteorus pulchricornis* with a diet of various sugar types and distilled water. Sugar significantly increased longevity of the wasps, regardless of the sugar type, compared to the distilled water treatment.

Similar results were observed by Nayar and Pierce (1980) for *Culex nigripalpus*. Male and female *C. nigripalpus* were fed with varying sugar concentrations between 5—25% and lived for 19.8—23.7 and 37.5—53.4 days respectively. Gary and Foster (2001) assessed the survival of *An. gambiae* females and found that those supplied with water only survived for a median of 7 days but this increased significantly to 20 days when fed with 10% sugar water. Apart from studies in mosquitoes, other dipteran studies confirm the results obtained for *An. funestus*. Ajayi and Fadamiro (2016) found that longevity in three different species of male and female *Pseudacteon* (Ps.) flies (Diptera: Phoridae), also called ant-decapitating flies, namely, *Ps. cultellatus*, *Ps. curvatus* and *Ps. obtusus* increased when they were fed with 20% and 40% sugar compared to flies that were fed with just water. Zengenene *et al.* (2021) maintained *An. funestus* (FANG strain) on 10% sugar water and recorded a median longevity of males and females of 44 days and 24 days, respectively. Contrary to the findings by Zengenene *et al.* (2021), female *An. funestus* (FUMOZ strain) in my current study lived 2 days longer than male *An. funestus* when comparing the median survival for the control.

In the current study, there was no significant difference in females from the 2040% sugar water groups and the male 20% and 30% sugar water groups. Similar results were obtained by Xiong *et al.* (2020) who conducted a study on the endoparasitoid *Pteromalus puparum* and reared newly emerged females with various sugar water concentrations ranging from 1.25% to 40% sugar water and recorded an increase in longevity of female *Pt. puparum* as sugar water concentration increased. However, this species showed no increased survival when sugar water concentration was above 20%. In my current study, increasing sugar water concentration, as afore-mentioned, increases longevity in insects, including *An. funestus*, but the effect is lessened as possible upper threshold sugar water concentration is approached. A possible upper threshold was approached in female *An. funestus*, because there was no significant difference in median survival of females fed with 20% sugar water compared to those provided with 30% sugar. As sugar water concentration increased further there was no significant difference between females provided with 30% sugar

water and 40% sugar water. Further studies, however are required with inclusion of sugar water concentrations above 40% to confirm the upper threshold.

4.2 Effects of Sugar Water Concentration Flight Performance in Colonised *An. funestus*

As 10% sugar water is the control and standard sugar water concentration for FUMOS rearing, differences were not expected in escape rate between males and females because flight is important in both sexes. At the 20% sugar water concentration, however, a higher proportion of females could fly out of the flight tubes of the FTD compared to the males. With the increased sugar water concentration, female *An. funestus* were probably able to metabolize the sugar better than male *An. funestus* to benefit from it in terms of energy for flight. Better flight performance might have been observed in females compared to males because in the wild, males need to fly to begin and maintain swarms for about 17 minutes. (Zawada *et al.*, (2018) observed *An. funestus* swarms between 18:05 and 18:27 in Zambia) – although the use of infra-red cameras might detect swarm formation earlier and correct duration as opposed to observations with the naked eye. Females fly into the swarms to select a mate and search for a blood meal after mating to ensure egg development. Females will then have to fly and search for suitable oviposition sites prior to searching for the next bloodmeal. These are energy-consuming endeavours and having a better flight performance will therefore be very beneficial.

Sugar water concentrations of 30 and 40% had an equal effect on improved escape proportions in males and females. At these much higher concentrations, the gender differences in flight performance were not evident. In swarming male *An. gambiae*, it has been recorded that they expend 5.9 times more sugar-derived energy while swarming compared to being in a state of rest (Maïga *et al.*, 2012). Should the sugar concentration increase beyond a certain threshold, sugar-derived energy being lost due to flight might not result in a substantial decrease in the minimum threshold of sugar reserves. Historical data on male and female *An. funestus* in the wild showed that males swarm at heights of about 2 m above the ground (Gillies and Wilkes, 1976) and however, in a separate study, when host-seeking, females were able to fly above a 6 m high netting fence (Gillies and Wilkes, 1978). Historical data has to be treated with caution as many species were

probably misidentified as morphology was the only available method to distinguish between species.

Kaufmann and Briegel (2004) assessed flight performance in female *An. gambiae* s.s and *An. atroparvus* using flight mills. The females were either blood fed, sugar fed or starved. The flight distance of sugar-fed *An. gambiae* was 9km and starved *An. gambiae* could only fly less than 3km. In *An. atroparvus*, Kaufmann and Briegel (2004) recorded a flight distance of up to 12 km sugar-fed mosquitoes and less than 3.5 km for starved mosquitoes. On average, *An. gambiae* females used 38% of their sugar reserves for flight whereas *An. atroparvus* used 41% of sugar reserves for flight.

As noted above, female *An. funestus* escaped the flight tubes at significantly higher proportions than males on the 20% sugar water concentration diet, it would be important to find out if they also escape the flight tubes at a faster rate than the males to add to the body of knowledge regarding the difference in gender, but this was outside of the scope of the current study. Future studies should also investigate if males and females use similar percentage of sugar reserves during flight or if gender specific differences are present.

4.3 Effects of Sugar Water Concentration on Mating Success in Colonised *An. funestus*

It is imperative to understand the parameters that influence mating success in vectors in the wild prior to implementing novel control methods (Maïga *et al.*, 2014) and using the laboratory-reared *An. funestus* as a representative of wild species is a start.

In pairwise comparisons between percentage insemination in females that mated with males in a 1:1 ratio (25m:25f) fed with various sugar water concentrations, significant differences were only observed between the control (10% sugar water fed *An. funestus*) and the higher sugar water concentrations in this study (30% and 40% sugar water concentrations). Statistically significant differences in percentage insemination in females mated with males in a 1:1 male to female ratio (25m:25f) were between the 20% and 40% sugar water-fed *An. funestus*. However, there was no significant difference in percentage insemination between 30% and 40% sugar water fed *An. funestus*. It is possible that a threshold sugar water concentration is approached in this experiment.

Percentage insemination increased significantly in 30% and 40% sugar water fed *An. funestus* by 52.64% and 69.78% respectively compared to the 10% sugar water-fed control.

In this study, male *An. funestus* fed with higher sugar water concentrations (30% and 40% sugar water) inseminated more females and had longer survivorship compared to the control (10% sugar water). Phasomkusolsil *et al.* (2017) found that sugar intake (10% sucrose or glucose) and multivitamins in *An. campestris*, *An. dirus*, and *An. sawadwongporni* increased mating success because it affects either sperm or seminal secretions, but could not determine the exact reason for increased mating success. Mating success in *An. funestus* in the context of this study is likely due to diet affecting the male's reproductive biology rather than those in the female, however, this will need to be investigated further. Magia *et al.* (2014) showed that about half the sugar reserves in male *An. gambiae* ss and *An. coluzzii* were used up for 25 min of swarming activity. Mating has an energetic cost besides flight in males such as the production of the copulatory plugs post mating, common in *An. funestus* and *An. gambiae*, also lead to the consumption of energy in the male.

Research shows that supplementary sugar feeding in some female parasitic wasps increases their reproduction ability (England and Evans, 1997). Although egg production was not assessed in this study, percentage insemination was higher for *An. funestus* fed with higher sugar water concentrations (30% and 40% sugar water).

Zengenene *et al.* (2021) maintained *An. funestus* (FANG strain) on 10% sugar water and recorded a mean insemination percentage of $74.8\% \pm 8.2$ in 2L cylindrical cages. In this study, using *An. funestus* (FUMOZ strain), insemination percentage for the 10% sugar-fed mosquitoes was recorded between 45% and 47% in experiments with males and females in a 1:1 ratio (with 25 males and 25 females or 30 males and 30 females in experimental cages with dimensions of 17.5 cm (L) x 17.5 cm (W) x 17.5 cm (H)). The percentage insemination in females mated with males was >60% experiments using six males and six females regardless of rearing container (experimental cage mentioned above or small disposable cup with dimensions of 6cm diameter x 9 cm (H)). Percentage insemination in this study above 70% was only recorded for *An. funestus* fed with 30% and 40% sugar water. The difference might be due to the FANG strain being an insecticide susceptible colony whereas the FUMOZ colony is insecticide-resistant.

A plausible reason is that Zengenene *et al.* (2021) reared the mosquitoes in 2L cylindrical cages and the experimental cages in my current study had external dimensions of 17.5 cm (L) x 17.5 cm (W) x 17.5 cm (H) (5L capacity). In addition to this, different people reared the colonies and different blood meals are provided to the colonies and the insectary conditions also differ, with the Botha De Meillon Insectary where FANG is reared having a more stable temperature and humidity setting. In this study 25 males and 25 females (Section 2.4) or 30 males and 30 females (Section 2.3) were transferred to experimental cages, while Zengenene *et al.* (2021) used 300 males and 300 females. The number of mosquitoes per cage might impact percentage insemination and should be investigated further in more detail. However, in a study assessing mating success in *An. arabiensis*, Mashatola *et al.* (2017) found no significant difference in percentage insemination when different rearing container shapes and sizes using equal numbers of males and females. Based on this study, using more males could offset the use of higher sugar water concentration, but to maximise this mating success, higher sugar water concentrations (30% and 40% sugar water) are necessary.

4.4 Effects of Male to Female Ratio on Mating Success

In the wild, the expected male to female ratio is about 50% of each gender. This is also true for laboratory-reared *An. funestus*. An artificially female-biased sex ratio (1:2 (20m:40f) and 1:3f (15m:45f) male to female ratios in this study) does not necessarily mean that percentage insemination will increase. In the wild, males form a swarm and females enter the swarm to select a mate (Yuval and Bouskila, 1993) and therefore, swarms are naturally male-biased. Not every male that emerges in the wild (or under laboratory conditions) will get an opportunity to mate due to individual variation and intraspecific competition (Yuval *et al.*, 1994; Diabaté *et al.*, 2011; Sawadogo *et al.*, 2013; Maïga *et al.* 2014) hence even the 3:1 (40m:20f) male to female ratio did not result in 100% insemination success.

Anopheles funestus provided with 10% sugar water while varying on the male to female ratios yielded percentage insemination significantly different from the control 1:1 male to female ratio (30m:30f). The highest mean percentage insemination was recorded in cages with more males than females (3:1 male to female ratio with 45 males and 15 females) – it was an increase of 66.37% in

percentage insemination compared to the control. In cages with a lower male to female ratio (more females than males), percentage insemination decreases significantly by as low as 71.55% compared to the control. However, in 40% sugar water fed *An. funestus*, the decrease in percentage insemination was only observed in cages with a low male to female ratio and no significant differences were observed in cages with a high male to female ratio compared to the control. Increasing male numbers did not impact on percentage insemination in 40% sugar water fed *An. funestus*.

Similar results were found by Terzian and Stahler (1954), who conducted a study to assess the effects of male to female ratio on mating success in *An. quadrimaculatus*. Terzian and Stahler (1954) found that the number of inseminated females was highest in experiments where male numbers were higher than their female counterparts. Gou *et al.* (2019) conducted a study on greenhouse pests, black fungus gnat flies (*Bradysia difformis*) and found that manipulating the male to female ratio also influenced insemination, leading to increased insemination in treatments with a 1:1, 2:1 and 3:1 male to female ratios.

Mating success was already increased by diet and was further increased by manipulating the sex ratio with a male-biased ratio in this study. It was unknown whether older males will perform better and increase mating success or if one male can mate with more than one female. This was investigated to provide some insight into the low mating success recorded in cages with a low male to female ratio (1:2 male to female ratio (20m:40f) and 1:3 male to female ratio (15m:45f)).

4.5 Effects of Male Age and Sugar Water Concentration on Mating Success in Colonised *An. funestus*

The males that were used for the experiment on the day they emerged (newly emerged) mated with the highest number of females and this was significantly different from all other treatments for 10% sugar water fed *An. funestus*. There was no significant difference in percentage insemination in 40% sugar water fed females mated with males of different ages when the experiment commenced (i.e. males that were newly emerged and males that were aged for 5, 10 or 15 days before the commencement of this mating experiment).

Sawadogo *et al.* (2013) investigated the effects of age on *An. gambiae* mating success and the number of spermatocysts and the proportion of sperm in the reservoir of wild-caught male testis were used to characterise male age. They found that the optimum age for increased insemination success in the laboratory was 4–8 days old and older males (>4 days old) were more abundant in swarms (Sawadogo *et al.*, 2013). Mahmood and Reisin (1994) evaluated the age for maximum mating success in *Anopheles culicifucies* and found it to be 5–7 days old in males. As the male aged, the number of mature spermatocysts decreased. In this study, male age did not have a significant increase or reduction in percentage insemination in laboratory-reared *An. funestus* fed with 40% sugar water.

High sugar water concentration (40% sugar water) has been seen to increase percentage insemination even in experiments with a female-biased ratio (discussion section 4.4 above) and has also been found to diminish the reduced mating success of aged males in this laboratory study. As sugar is mobilised for mating and other activities in mosquitoes, this mobilisation might increase with male age and given enough sugar; males might still have high mating success even when aged.

4.6 The Effects of Sugar Water Concentration on Multiple Mating in *An. funestus* Males

According to the results in this experiment where one *An. funestus* male was transferred to a cage with either five or ten newly emerged *An. funestus* female for each biological repeat, multiple mating did not occur. For each cage with one male, either one female was inseminated or insemination did not occur at all. In both 10% and 40% sugar water fed *An. funestus*, percentage insemination in cups with a single male and either 5 or 10 females decreased by more than 82% for both treatments (Appendix B). The result that would have supported multiple mating would be the presence of more than one mated female in a cup that had only one male mosquito. According to my knowledge, there is no research exclusively proving or disproving the occurrence of multiple mating in male *An. funestus*.

It is known that *An. funestus* mate in the wild by forming swarms. Multiple males are required to initiate the swarm and create a “site” for females to select a mate. A lone male thus might be the

reason mating did not occur in some cups, but further research will be required. The small sample size was a limitation in this study as one male had to be used per cup to test for multiple mating. In order to rule out lack of swarming by a single male as the reason for male *An. funestus* failing to mate multiple times under laboratory conditions, radio-isotope marked males (Helinski *et al.*, 2007) would have to be used and mating success would then be measured by the number of isotope males that were able to mate with females compared to unmarked males.

CHAPTER FIVE

5. CONCLUSION

This study provides knowledge on lifetable parameters in colonised *An. funestus*. These gave insights into the survival of *An. funestus* and their reproduction capacity based on diet, male to female ratio and male age. It is imperative to understand these factors to have a successful rearing system. Rearing conditions (such as temperature and humidity) and diet need to equate to or exceed the minimum environmental and nutritional requirements of the mass reared species to optimise survival and reproduction (Portilla *et al.*, 2014).

The sterile insect technique depends on successful colonisation of the target species (Knippling, 1959; Knippling *et al.*, 1968) and being able to rear the target species in large numbers. This will require high mating success and high reproductive success. The next phase in SIT will be to induce sterility in the pest species without negatively impacting its fitness. Sterilisation by high radiation levels can have negative impacts on male insects such as reduced survival (Proverbs, 1969; Sharma, 1978) and mating competitiveness (El-Gazzar and Dame; 1983). Any significant improvement to the survival and ability of the mosquitoes to mate is therefore crucial for mass rearing and thus SIT success. This study explored the effects of diet on adult male and female longevity and found that a high sugar water concentration of 40% significantly increased longevity as well as mating success. Longer lived females will allow for increase number of gonotrophic cycles i.e. increase egg production age. If a female can produce multiple egg batches before she dies, the reproductive potential of the strain will be high and it will be easier to upsize the colony to numbers needed for mass releases. However, this was not evaluated in the current study and should be investigated in future. The same will be true for understanding factors that increase mating success.

Furthermore, the increase age of males will theoretically allow increase probability for mating, however this was not supported in the current study and future research will be needed. However, the increased flight performance of males fed on 40% sugar water, indicated that these males might perform better during swarming. This is vital since SIT will rely on the release of sterilized males that will need to compete with wild fertile males for mating with females. If the 40% sugar water

solution can provide a fitness advantage for released males, it might increase their mating success with wild females. This still needs to be investigated and confirmed.

A high sugar water concentration increased mating success in *An. funestus*. Increased mating success will ensure that more females will be able to produce eggs during mass rearing and this will directly increase the number of insects available for releases. Although the mating success of male-biased (more males than females) cages was higher even for 10% sugar water fed *An. funestus*, this will not be a suitable solution during mass rearing. This will increase the labour required and is not recommended. However, this might be an appropriate solution to increase the mating success when new colonies are to be established.

An increase in the sugar diet of *An. funestus* adults is recommended for future SIT studies and future studies should investigate if this diet also increase the mating competitiveness of sterilised males to mate with wild females.

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APPENDICES

Appendix A

Table 1 A Minimum, median and maximum longevity of *An. funestus* males fed on various sugar water concentrations in the experiment with 50 males per cage.

Sugar water concentration (%)	Minimum longevity (days)	Median longevity (days)	Maximum longevity (days)
0	1	2	5
10	1	17	37
20	1	18	35
30	1	22	40
40	1	20	45

Table 2 A Minimum, median and maximum longevity of *An. funestus* males fed on various sugar water concentrations in the experiment with 60 males per cage in the concurrent males vs females experiment.

Sugar water concentration (%)	Minimum longevity (days)	Median longevity (days)	Maximum longevity (days)
0	1	2	4
10	1	17	37
20	1	19	37
30	1	20	41
40	1	22	42

Table 3 A Minimum, median and maximum longevity of *An. funestus* females fed on various sugar water concentrations in the experiment with 60 females per cage in the concurrent males vs females experiment.

Sugar water concentration (%)	Minimum longevity (days)	Median longevity (days)	Maximum longevity (days)
0	1	2.5	5
10	1	19	38
20	1	21	39
30	1	21	39
40	1	22.5	40

Appendix B

Raw data of the experiment assessing the possibility of multiple mating in provided with 10% and 40% sugar water

Table 1 B Raw data of the experiment assessing the possibility of multiple mating in provided with 10%. Three biological repeats with three technical repeats each.

REP 1

Ratio	1:1 (6m:6f)			1:5 (1m:5f)			1:10 (1m:10f)		
Mated	5	2	4	0	0	1	0	0	1
Unmated	1	1	2	5	4	3	6	8	9

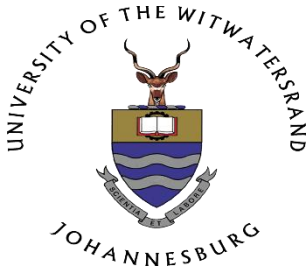
REP 2

Ratio	1:1 (6m:6f)			1:5 (1m:5f)			1:10 (1m:10f)		
Mated	3	3	2	1	1	0	1	0	0
Unmated	2	1	2	4	3	5	8	9	8

REP 3

Ratio	1:1 (6m:6f)			1:5 (1m:5f)			1:10 (1m:10f)		
Mated	3	4	3	1	0	0	0	0	0
Unmated	2	2	3	3	5	4	10	7	8

ANNEXURE A



ANIMAL RESEARCH ETHICS COMMITTEE

Registration number: AREC-101210-002

Date: 07/05/2019

Certificate reference: Waiver 02-05-2019-O

Category: O

Applicant: Mbuyi Nelly Kalonji

Department: Wits Research Institute for Malaria (University of the Witwatersrand)

Tel: 011 717 2668; **Email:** francesca.parrini@wits.ac.za

Re: Waiver from the Animal Ethics Research Committee of the University of the Witwatersrand

This letter is to confirm that Mbuyi Nelly Kalonji, student (#1043541) at the WITS Research Institute for Malaria under the supervision of Prof Lizette L. Koekemoer (Wits Research Institute for Malaria), Dr Yael L. Dahan-Moss (Vector Control Reference Unit, National Institute for Communicable Diseases), does not require full Animal Ethics Research Committee clearance to undertake the work titled **'The biology of *Anopheles funestus* (Diptera:Culicidae): Effects of the sugar diet'**.

Reason for waiver

This study involves the examination of the biology of invertebrates, the mosquitoes in the study. There will be no other animal involvement.

Details of the study

Male and female *Anopheles funestus* (FUMOS strain) will be used for this study. Mosquitoes will be fed with various sugar water concentrations and their longevity (males), mating success and flight performance (males) will be assessed.

Arrangements will be made with VCRU to acquire uninfected mosquitoes of other species from the *An. funestus* group for longevity assessments: *Anopheles parensis*, *Anopheles vaneedeni* and *Anopheles rivulorum* (this will depend on which species are available)

The individuals covered by the waiver are Mbuyi Nelly Kalonji, Lizette L. Koekemoer and Yael L. Dahan-Moss.

Conditions

An ethics waiver from WITS is given on condition that:

- i. The mosquitoes used in this project will be sourced from the Wits Medical School. Arrangements have been made with the head of the VCRU at the Wits medical school to acquire uninfected mosquitoes
- ii. NSPCA will be notified of the dates that the mosquitoes will be required within a period of a little over a month before the dates.
- iii. Ensure that all requisite biosafety and biosecurity measures are adhered to.
- iv. Ensure that Human ethics waiver stipulations are adhered to.

Please contact me should you require further information.

Yours sincerely



Dr Frederic Michel
Deputy Chair: Animal Research Ethics Committee
University of the Witwatersrand