CHARACTERISATION OF *ANOPHELES FUNESTUS* (DIPTERA: CULICIDAE) COLONISATION PARAMETERS

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

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DECLARATION

I Munyaradzi Prince Zengenene declare that this dissertation is my own, independent work. It is being submitted for the Degree of Master of Science in Medicine at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other University.

_____

(Signature of candidate)

On this 14th___day of__October__in_2021_____

DEDICATION

To my wife Idea Albertina, my mother Chemedzai and my son Tafara Kaylum

In memory of my late father

Erinasi Michael

(1955 – 2018)

PUBLICATIONS AND PRESENTATIONS ARISING FROM THE STUDY

Publications

1. **M.P Zengenene**, G. Munhenga, G. Chidumwa and L.L Koekemoer. 2020. Characterisation of life history parameters of an *Anopheles funestus* (Diptera: Culicidae) laboratory strain, *Journal of Vector Ecology* **46**(1): 24-29.

Contribution to publication:

M.P Zengenene carried out all the laboratory experiments and wrote the first and subsequent drafts of the manuscript.

2. **M.P Zengenene**, G. Munhenga, and L.L Koekemoer (2020) Effect of larval density on the life history traits of a laboratory colonised *Anopheles funestus* (Diptera: Culicidae). *Medical and Veterinary Entomology* (In Prep)

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M.P Zengenene carried out all the laboratory experiments and wrote the first and subsequent drafts of the manuscript.

Publications indirectly originating from the study:

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Contribution to publication:

Due to my MSc training and subsequent involvement in *An. funestus* rearing, I had the opportunity to participate in the WHO AFRO Vector Control Needs Assessment and a capacity-

building workshop carried out in Zimbabwe. Data collected during the workshop led to the publication. M.P Zengenene carried out field and laboratory experiments, and wrote the first and subsequent drafts of the manuscript.

Oral conference presentations

1. **M.P Zengenene**, G. Munhenga and L.L. Koekemoer. Characterisation of the life history parameters of a laboratory reared *An. funestus* colony. 2019. 21st Congress of the Entomological Society of Southern Africa. Durban, South Africa.

Contribution to presentation:

M.P Zengenene prepared the PowerPoint presentation and orally presented during the conference.

2. **M.P Zengenene**, G. Munhenga, and L.L. Koekemoer. Characterisation of the life history parameters of a laboratory reared *An. funestus* colony. 2019. 5th South African Malaria Research Conference. Pretoria, South Africa

Contribution to presentation:

M.P Zengenene prepared the PowerPoint presentation and orally presented during the conference.

ABSTRACT

The knowledge on several biological and ecological aspects of malaria vectors continues to grow as numerous stable laboratory colonies in different geographical regions are available. However, knowledge of these aspects on *Anopheles funestus*, a major African malaria vector, is limited due to difficulties in colonisation of this species. The aim of this dissertation is to characterise and understand critical baseline parameters that promotes successful sustenance of *An. funestus* under laboratory conditions using an already colonised strain. Understanding such parameters can be used as a reference point for colonisation of other strains from different geographical areas. The life history parameters of a colonised *An. funestus* strain were characterised in relation to colonisation. The adaptability of the strain to an artificial blood feeding system was evaluated and two larval diets were assessed on its development. The effect of larval density on its life history traits was also investigated. The age specific life table attributes of *An. funestus* under laboratory conditions and their relation to colonisation showed high re-colonisation potential of this species. Adaptability of this species to an artificial cow blood feeding system using Parafilm-M® membrane was asserted. A standard Vector Reference Laboratory larval diet was shown to be appropriate both from a biological and economical perspective. However, it is worth noting that the strain used has been reared on this diet for over a decade and this might have an impact on the results. Overcrowding larvae in rearing containers negatively influenced the development and physiology in ways that have consequences for successful laboratory colonisation. The addition of an extra anchoring surface seemingly altered the adverse effects of overcrowding. The baseline data produced by this study suggests high re-colonisation potential of *An. funestus* under laboratory conditions. The data can be extrapolated and used as a reference point for colonisation of populations from other geographical regions. Further studies on characterisation and optimisation of parameters that promote colonisation of *An. funestus* are recommended.

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NOMENCLATURE

%: Percent

±: Plus or minus

®: Registerdtrademark

χ^2: Chi square

µm: micrometre

ANOVA: Analysis of Variance

ATP: Adenosine Triphosphate

CA: California

CDC: Centre for Disease Control

cm: Centimetre

cm^2: Square centimetre

CA: California

COI: Cytochrome oxidase I

Corp: Corporation

Cum: Cumulative

DNA: Deoxyribonucleic acid

***et al.*:** *et alia*

FANG: *Anopheles funestus* Angola strain

FAO: Food and Agricultural Organisation

FUMOZ: *Anopheles funestus* Mozambique strain

HSD: Honest Significant Difference

IAEA: International Atomic Energy Agency

IBM: International Business Machines

Inc: Incorporated

IPCL: Insect Pest Control Laboratory

kg: Kilogram

L1: First instar larva

M: Molar

mm: millimetre

ml: millilitre

MR4: Malaria Research and Reference Reagent Resource Centre

N: Total sample size

n: Sample size

NADH: Nicotinamide Adenine Dehydrogenase

ND5: Nicotinamide Adenine Dehydrogenase Subunit 5

NICD: National Institute for Communicable Diseases

°C: Degrees Celsius

P: Probability level

PBS: Phosphate Buffered Saline

PCR: Polymerase Chain Reaction

SD: Standard Deviation

SIT: Sterile Insect Technique

SPSS: Statistical Package for Social Sciences

s.s.: Sensu stricto

USA: United States of America

VRL: Vector Reference Laboratory

WHO: World Health Organization

w/v: Weight per Volume

X: Times

CHAPTER 1

LITERAURE REVIEW

1.1 The global malaria burden

Malaria is one of the leading and life threatening vector-borne diseases in the world with nearly half of the world's population at risk. The World Health Organization (WHO) data estimated 228 million cases of malaria and 405,000 deaths in 2018 (WHO, 2019). Although malaria cases have gone down from 71 to 57 cases per 1,000 populations at risk between 2010 and 2018, data from 2014 to 2018 reveal that no significant progress in reducing global malaria cases was achieved in this period. According to the World Malaria Report, most cases (93%) were recorded in Africa (WHO, 2019). Although Africa has a disproportionately high share of the global malaria burden, the disease is still found in Asia and the Americas (Figure 1.1). Of the population at risk, children under the age of five years old are the most vulnerable. In 2018, they accounted for 67% of malaria mortalities globally (WHO, 2019).

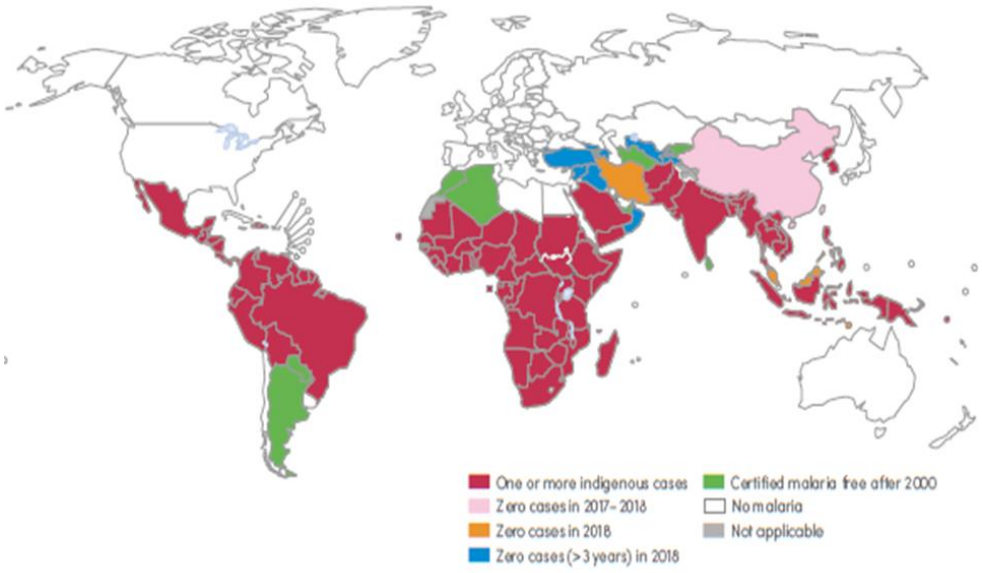

Figure 1.1: World map showing the World Health Organization's malaria zones where transmission still occurred in 2018 (adapted from 2019 World Malaria Report (WHO 2019)).

Malaria has a major health impact in Africa where it diminishes social and economic growth by making affected persons' sick thus removing them from the labour force and causing a strain on state resources as a consequence of medical treatment (WHO, 2005). Malaria in pregnancy jeopardises the mother's health and puts her at greater risk of death. It affects the health of the fetus, leading to prematurity and low birth weight, major contributors to neonatal and infant mortality (WHO, 2019). In 2018, 11 million pregnant women in sub-Saharan Africa were infected with malaria and, as a result, nearly 900,000 children were born with a low birth weight (WHO, 2019). Furthermore, infection in children has a negative impact on the learning capacity, cognitive ability and in some instances it results in these children becoming dependant on state welfare (WHO, 2005). Another negative impact the disease has on economic growth is the cost incurred in combating the disease. In 2018, the estimated cost invested in malaria control and elimination efforts globally was US$ 2.7 billion, an amount that falls short of the US$ 5.0 billion estimated to be required globally to stay on track towards the Global Technical Strategy for malaria 2016-2030 milestones (WHO, 2019).

1.2 Malaria vectors in Africa

1n 1897, Ronald Ross proved that female mosquitoes act as intermediate hosts for bird and human malaria parasites (Harrison, 1978). Further research revealed that malaria parasites are transmitted from human to human by various mosquito species belonging to the genus *Anopheles* (Gillies and De Meillon, 1968; WHO, 2005). In Africa, the main malaria vectors are *Anopheles coluzzii* (Coetzee *et al.*, 2013), *Anopheles gambiae*, *Anopheles arabiensis*, and *Anopheles funestus s.s.* (Sinka *et al.*, 2012; Coetzee *et al.*, 2013). These mainly belong to two taxonomic groups: the *Anopheles gambiae* complex (*An. gambiae*, *An. arabiensis*, *An. coluzzii*) and the *Anopheles funestus* group (*An. funestus s.s.*) (Gillies and De Meillon, 1968; Gillies and Coetzee, 1987; Coetzee *et al.*, 2013). This dissertation is mainly focused on *An. funestus s.s.* (hereafter referred to as *An. funestus*), therefore, the description of the biology and distribution is limited to this species and details of the *An. gambiae* complex are not discussed further.

1.3 *Anopheles* laboratory biology and culturing

Vector control is an essential component to mitigate mosquito borne diseases as interruption of pathogens is the most effective disease control strategy. Most vector control interventions require the study of biological, ethological and ecological variables that drives the dynamics of disease transmission and vector control. To achieve this, it is essential to conduct research on vector biology, parasite interactions and insecticide susceptibility among others. For this

reason, it is important to maintain mosquito colonies in the laboratory since the above-mentioned studies hinge on having sufficient amounts of laboratory-reared mosquitoes.

1.3.1 Behaviour and physiology of anophelines in the laboratory

Information on mosquito behavior and physiology is vital in making decisions during rearing mosquitoes under standard insectary conditions. Mosquito species-specific behavior determines choice of food, blood, egg laying, insectary supplies, insectary space demanded and cage sizes amongst many other things to be used during colonisation (Benedict *et al.*, 2009; MR4, 2015). Moreover, knowing mosquito behavioral intricacies can also be useful in understanding why mosquitoes are not proliferating or behaving as anticipated.

1.3.1.1 Eggs

Anopheles eggs usually have flattened upper surfaces and convex lower surfaces (Figure 1.2). The egg-shell is made of an inner wall known as the endochorion which tans overtime. The outer wall (exochorion) has a transparent material that might have frills, bosses and floats with 14-18 air chambers, extending over 1/2 to 2/3 length of egg on either side (Gillies and De Meillon, 1968). Most eggs often hatch after two to three days, but during adverse conditions, this might be delayed (Gillies and De Meillon, 1968). The eggs are laid singly or in clusters directly on water, as they are not resistant to desiccation and need contact with water in order to survive and hatch (Dia *et al.*, 2013; MR4, 2015). This egg-laying pattern differs from that of other species such as *Culex* and *Aedes* thus rendering it instrumental in predicting cross genus contamination. When anopheline mosquito eggs are laid, they are white and typically darken and harden within a few hours. The rate at which they change colour and harden hinges on the strain and temperature. Eggs that fail to melanise and or fail to float (sink) do not hatch (Clements, 1992; MR4, 2015). This makes detection of egg viability a critical factor in mosquito colonisation easy.

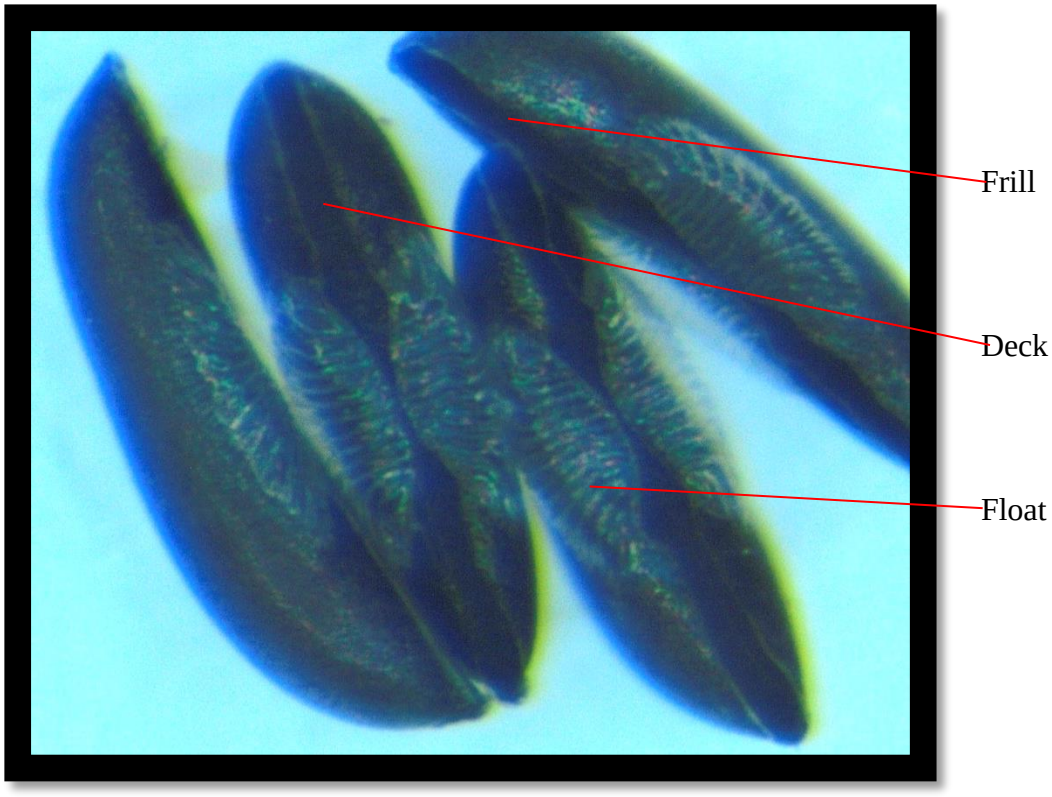

Figure 1.2: *Anopheles* eggs showing flattened upper and convex-shaped lower surfaces as seen under an Olympus light microscope at 150X magnification.

1.3.1.2 Larvae

Larvae hatch from eggs and develop through four instars before metamorphosing into pupae. Between each instar, they shed their rigid outer skin to increase size (Gillies and De Meillon, 1968). The discarded skin is termed an exuvium/exuvia. Most mosquito species larvae have a prominent dark coloured head, large thorax and segmented abdomen with hairs on their body (Figure 1.3). The larval instars are determined by the size of the head capsule, rather than their body length. In contrast to *Culex* and *Aedes* mosquitoes, *Anopheles* larvae lack a respiratory siphon and breathe through spiracles at the posterior end of the eighth abdominal segment (Ponlawat and Harrington, 2009; MR4, 2015). Modified setae on the thorax and abdomen allow them to cling to the meniscus of the water, lying parallel to the surface where they feed on food particles (suspended in the water column or at the water surface) to develop and store enough energy for the transformation and development that occur at the pupal stage (Clements, 1992; MR4, 2015). They habitually feed at the upper water surface by rotating their head 180 degrees

and feeding from below the water micro layer. The size of the particle that larvae can feed on increase with the size and age of the larvae with a minimum and maximum particle size of 0.45 µm and 3.9 µm respectively (Merritt *et al.*, 1992; Timmermann and Briegel, 1993; MR4, 2015). Therefore, factors such as larval size and instar should be considered when determining which larval food to use since different larval foods have varying particle sizes. Larvae swim either by jerky movements of the entire body or through propulsion with the mouth brushes and they dive below the surface typically when disturbed. Development from first instar larvae into pupae is between 10 to 15 days depending on ambient temperature and strain (Gillies and De Meillon, 1968; Gillies and Coetzee, 1987; MR4, 2015).

Figure 1.3: *Anopheles* fourth instar larva showing the dark coloured head, large thorax, segmented abdomen and hairs on the body as seen under an Olympus light microscope, at 150X magnification.

1.3.1.3 Pupae

After the fourth larval instar completed the larvae moults into pupae. Within the body casing of the pupae, the immature tissues break down and adult tissues form. Its lateral view is comma-shaped (Figure 1.4). The cephalothorax, which is made up of the fusion of head and thorax, is exaggerated in size as compared to the rest of the body. Although the pupae are active, they do not feed but use food stored at the larval stage (Gillies and De Meillon, 1968; Clements, 1992). They breathe through a pair of respiratory trumpets on the dorsal side of the cephalothorax. The duration of the pupal stage is dependent on temperature but is generally between 2-3 days. Once the adult tissues have developed and it is time for emergence, the pupae swims to the water surface and stretches itself out to full length, the pupal skin splits along the back and the teneral adult mosquito emerges above the water surface (Gillies and De Meillon, 1968).

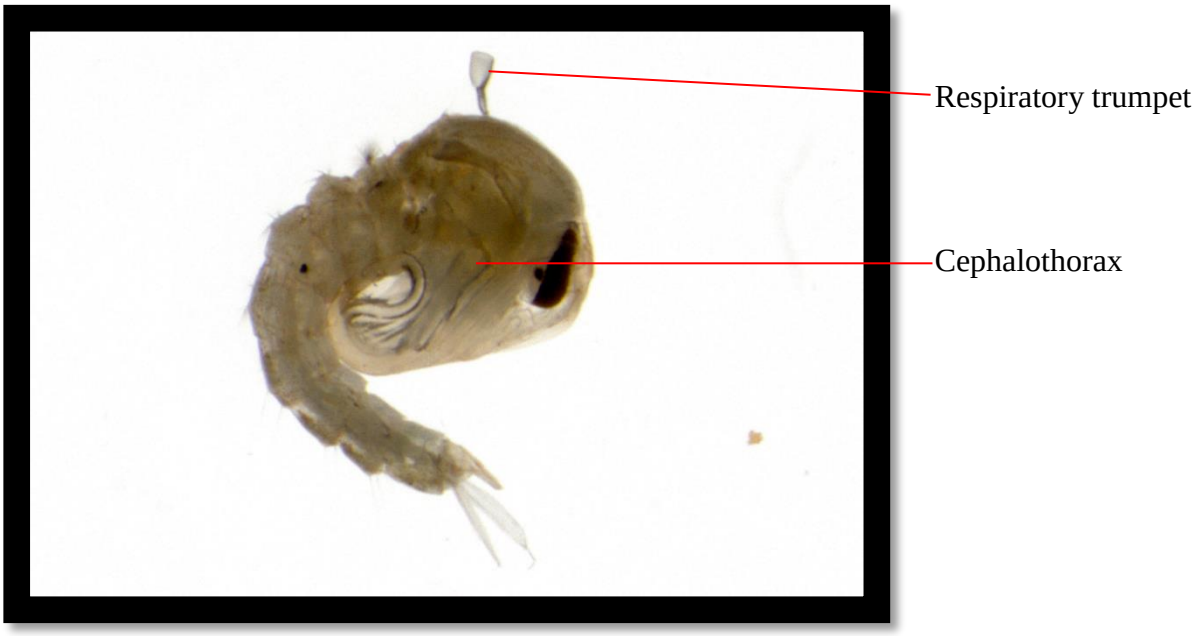

Figure 1.4: *Anopheles* pupae with a comma-shaped lateral view showing exaggerated cephalothorax with respiratory trumpet as seen under an Olympus light microscope at 150X magnification.

1.3.1.4 Adults

After emerging from the pupal casing, the adult mosquito rests on the water surface for a short time allowing its wings and body to dry, before flying off in search of a mate and nourishment. Like all mosquitoes, *Anopheles* adults have three sections: head, thorax and abdomen (Figure 1.5). The head is specialized for acquiring sensory information and for feeding while the thorax is specialized for locomotion with three pairs of legs and a single pair of wings attached to it. The abdomen is specialized for food digestion and egg development (Clements, 1992). Adult mosquitoes usually mate within a few days after emerging from the pupal stage. The males form swarms, usually around dusk, females enter the swarms where they form mating pairs for copulation (Gillies and De Meillon, 1968). Females only mate once in their lifetime. Once successfully mated, the female obtains a blood meal to acquire nutrients for egg production. A single female can lay between 80 to 140 eggs per batch (Gillies and De Meillon, 1968). Males feed on nectar and other sources of sugar, while females feed on sugar sources for energy and blood as a source of proteins and amino acids for egg development. Once the eggs are fully developed, the female oviposits and seeks another blood meal to sustain another batch of eggs. The cycle repeats itself until the female dies. Duration of adult survival depends on the energy source, temperature and humidity (Gillies and De Meillon 1968; Gillies and Coetzee, 1987; Clements, 1992).

Figure 1.5: *Anopheles* adult mosquito with three sections (head, thorax and abdomen), three pairs of legs and a pair of wings attached to the thorax as seen under an Olympus light microscope at150X magnification.

1.4 The *An. funestus* group

The *An. funestus* group is comprised of at least 13 morphologically similar species present in the Afrotropical region. Molecular and morphological descriptions placed these species within three subgroups: *An. funestus, An. minimus,* and *An. rivulorum* (Harbach, 2013; Dia *et al.*, 2013). The *An. funestus* subgroup consists of seven species (*An. funestus, An. funestus*-like, *An. aruni, An. confusus, An. parensis, An. vaneedeni* and *An. longipalpis* type C). The *An. minimus* subgroup comprises *An. leesoni* and *An. longipalpis* type A while the *An. rivulorum* subgroup is composed of *An. rivulorum*, *An. rivulorum*-like, *An. brucei* and *An. fuscivenosus* (Dia *et al.*, 2013). Some members of the *An. funestus* group only have visible differences at specific developmental stages (Gillies and De Meillon, 1968; Gillies and Coetzee, 1987; Coetzee, 2020). A detailed taxonomic and molecular characterisation of each new species will be imperative for understanding their biology and may have implications in designing vector control interventions. The most efficient malaria vector in the group is *An. funestus* (Gillies and De Meillon, 1968; Gillies and Coetzee, 1987; Dia *et al.*, 2013).

1.4.1 Distribution and biology of *An. funestus*

Anopheles funestus is widely distributed across most African malaria-endemic areas with suitable breeding habitats. It is traditionally present/found throughout sub-Saharan Africa, and its range spreads from southern Mozambique to South Africa and Madagascar up to south Sudan and west to Senegal (Gillies and De Meillon, 1968; Gillies and Coetzee, 1987; Coetzee and Hunt, 1998, Sinka *et al.*, 2012). Being predominantly a savannah mosquito (Ayala *et al.*, 2009), this malaria vector is extant in high altitude zones and forested areas (Ayala *et al.*, 2009; Tanga *et al.*, 2010; Adja *et al.*, 2011). Additionally, it can inhabit extremely dry environments in the Sahel, when suitable breeding places are available, such as man-made irrigation zones (Labbo *et al.*, 2004). *Anopheles funestus* was eliminated entirely in several parts of Africa after adverse climatic conditions and or effective vector control interventions (Mouchet *et al.*, 1996; Coetzee and Koekemoer, 2013). However, this mosquito progressively re-emerged once control measures were halted; insecticide resistance emerged or suitable ecological conditions re-appeared (Fontenille and Rakotoarivony, 1988; Hargreaves *et al.*, 2000; Dia *et al.*, 2013). This corroborates its extraordinary environmental plasticity and dispersion capacity.

Like all mosquitoes, *An. funestus* goes through four stages in its life cycle: egg, larva, pupa and adult. The first three stages are aquatic and together last 14–16 days, depending on the ambient temperature (Gillies and De Meillon 1968; Gillies and Coetzee, 1987). Only the adult stage

which is an intermittent ectoparasite is not aquatic. The adult female lays about 83 to 139 eggs per batch (depending on age and size of the mosquito) which hatch into larvae when fertile. In contrast to *An. gambiae* larvae, which tends to inhabit temporary pools of water, *An. funestus* tends to favour inhabiting more permanent waters such as lakes and swamps (Gillies and de Meillon 1968; Gillies and Coetzee, 1987), and may develop in locations along sluggish streams and rivers where there is vegetation. *Anopheles funestus* larvae can also be found in artificial habitats such as rice fields, wells and domestic water containers (Evans, 1938; Gillies and De Meillon 1968; Dia *et al.*, 2013). Furthermore, *An. funestus* larvae can promptly dive and remain submerged for prolonged periods, often longer than many other species (Evans, 1938; personal observation). The key limiting factors to their development include salinity, extreme temperatures and sometimes, heavy rains (Gillies and De Meillon 1968; Dia *et al.*, 2013; Koekemoer *et al.*, 2014).

Anopheles funestus is one of the most anthropophilic and endophilic mosquitoes known, in many areas biting humans even in the presence of abundant alternative animal hosts such as cattle and sheep (Gillies and De Meillon 1968; Dia *et al.*, 2013). In common with a number of other important anophelines, the main biting activity of *An. funestus* takes place at night, usually between midnight and the early hours of the morning (Gillies and De Meillon, 1968; Oyewole and Awolola, 2006; Oyewole *et al.*, 2007). The great bulk of feeding takes place inside houses partly because, as described above, it feeds mainly during the night when most people are indoors, and partly because it shows a lesser tendency to feed outdoors (Gillies and De Meillon 1968; Awolola *et al.*, 2005; Dia *et al.*, 2013). In parallel with its anthropophagic tendencies, *An. funestus* shows a closer adaptation to human dwellings than other members in the *An. funestus* group. It spends the greater part of its adult life in houses, which has made it one of the most susceptible species to control by indoor residual spraying using insecticides (Gillies and De Meillon, 1968).

Anopheles funestus, like other anophelines, mates through creating swarms, which usually occur at sunset in relatively open areas. Swarm formation begins when one or more males begin flying concurrently, then the swarm numbers promptly increase, reaching a maximum size and density within approximately five minutes (Charlwood *et al.*, 2003). Subsequently, a female enters the swarm and mates with one of the swarming males. Studies have shown that females choose a specific mate (Ng'habi *et al.*, 2008; Howell and Knols, 2009). The harmonisation of wing beat frequencies between males and females seemingly plays a role in successful mating amongst others (Pennetier *et al.*, 2010). Laboratory colonisation largely depends on swarm

formation and mating success in the confines of cages and the amount of adults in a cage may affect mating behaviour (Benedict *et al.*, 2009; MR4, 2015). The adult energy source, as well as blood source and blood-feeding system, also affect colonisation. A lot of research has been done on how these factors impact colonisation (Benedict *et al.*, 2009; MR4, 2015) of most *Anopheles* species but the same cannot be said for *An. funestus*.

1.4.2 Molecular genotypes of *An. funestus*

Molecular studies using mitochondrial clade analysis have revealed genetic structuring in *An. funestus* populations (Michel *et al.*, 2005; Choi *et al.*, 2013). Phylogenic studies based on mitochondrial DNA led to the description of two cryptic subdivisions in *An. funestus*, clade I (widespread across Africa) and clade II, known only from Mozambique, Tanzania, Madagascar and Zambia (Choi *et al.*, 2013; Kweka *et al.*, 2018; Zawada *et al.*, 2018). Mitochondrial clade analysis is based on the DNA sequence analysis of the *Nicotinamide Adenine Dehydrogenase (NADH) subunit 5 (ND5)* and *Cytochrome Oxidase I (COI)*. The two clades can be separated through a hydrolysis probe analysis (Taqman assay) that combines PCR amplification reaction and visualisation in a single step using primers and probes designed from *COI* (Choi *et al.*, 2013). Currently, it is not known whether mitochondrial sub-populations are resulting in population isolation. In southern African countries where clade types have been reported to coexist in single populations, there is no evidence that they have behavioural differences with regard to insecticide resistance or vector competence (Choi *et al.*, 2013; Zawada *et al.*, 2018).

1.4.3 Colonisation of *An. funestus*

Anopheles funestus is extremely difficult to rear in the laboratory. Attempts to colonize this species commenced in 1954 and continued for three years. During 1955-56 the first successful mosquito colony was established by Service and Oguama (1958) in Nigeria from eggs laid by wild-caught females of *An. funestus*, given alternative feeds on human and guinea pig blood. The egg production of this colony was of short duration, and the colony ultimately died out after a few months. A year later, the same authors established another colony in Nigeria from wild-caught, gravid females maintained on guinea pig blood meals only, which was more successful. Eggs were laid on filter paper immersed inside water, and larvae were reared in tap water with floating waterweed and fed with pure dried yeast, while adults were provided with a sugar solution. Some eggs were transported to London to establish another colony, but successful colonisation did not materialise, and the colony was never maintained (Service, 2010). In 2005, Hunt and his fellow researchers successfully established the first two long term

An. funestus strains from eggs laid by wild caught females from Mozambique and Angola, given alternative feeds on human and guinea pig blood. These colonies have since adapted to feeding on guinea pig blood and have been under colonisation since 2000 and 2002. Eggs of these colonies have since been shared and various sub-colonies have subsequently been established by the Biodefense and Emerging Infections Research Resources Repository, Liverpool School of Tropical Medicine, Ifakara Health Institute and Cameroon (L.L Koekemoer, personal communication)

1.5 Mosquito colonisation

There are two means of starting a colony in the insectary. These either are by colonisation using mainly eggs from an established colony or from field-collected mosquitoes. It is easier to establish a colony from an existing laboratory colony than from field collected material, as the mosquitoes are adapted to the insectary conditions. The donor laboratory receives *Anopheles* eggs that can be dispensed into the water and rears them to adults (Benedict *et al.*, 2009; MR4, 2015).

Rearing mosquitoes from wild-caught material is difficult as the conditions in the insectary differ vastly from those in nature. While the aquatic rearing may be less problematic, the adults may find it difficult to acclimatise to the cage setting (Benedict *et al.*, 2009). The space limitations, high density and many other confining factors will inevitably influence the natural behaviour of the mosquitoes. Females may lay few or no eggs at first, which may be due to reduced mating activity as well as reluctance to blood-feeding from an artificial blood source. However, field-collected material has closer semblance of behavioural and genetic traits to the wild population than those from existing laboratory colonies (Benedict *et al.*, 2009; MR4, 2015). Endophilic *Anopheles* females are collected from human dwellings or other resting sites by mouth aspiration. The live females are transported to the insectary, where, after morphological identification (Coetzee, 2020), they are placed into individual oviposition vials to induce oviposition (Choi *et al.*, 2014). After oviposition, the female is identified morphologically and if needed also molecularly (Gillies and Coetzee, 1987; Scott *et al.*, 1993; Koekemoer *et al.*, 2002). Once the identity of the mother is confirmed, eggs and respective larvae of the same species are combined and reared through to adults. Alternatively, larvae and pupae can be collected directly from the field for rearing to adulthood and breeding after species identification. However, this approach is more laborious as each mosquito requires to be genotyped individually to avoid cross-contamination. Furthermore, larvae and pupae are not

easily visible in the dirty water of many larval habitats, and pupae hide in dark niches. The larvae of some mosquito species can also stay submerged for a long time, thus complicating the sampling procedure (Evans, 1938; Gillies and De Meillon, 1968).

1.5.1 Evolution and genetics of colonised mosquitoes

Under insectary conditions, genetic alleles preserved in the wild may be selected against or lost under insectary conditions due to the bottleneck effect. In addition to this, the laboratory rearing conditions are stable, and no seasonal variations are experienced, unlike in the wild (Norris *et al.,* 2001; Benedict *et al.,* 2009). Furthermore, colonised mosquitoes are not subject to stochastic biotic and abiotic threats as those found in natural field settings. Laboratory colonies established from field-collected mosquitoes generally undergo genetic drift, selection, and/or bottleneck and subsequently may not be archetypal of the source population (Norris *et al.*, 2001; Benedict *et al.*, 2009; MR4, 2015). As time since colonisation increases, genetic variances can compound. From this standpoint, colonies become increasingly homogeneous entities that vary genetically from wild populations and whose competitiveness is assumed to decline (Norris *et al.*, 2001; Benedict *et al.*, 2009; Olivia *et al.*, 2011). As a result, assessing the similarity between colony and field populations and whether founder effects or bottlenecks have occurred are imperative before interpreting results from experiments with colonized mosquitoes. Researchers have proposed approaches for conserving genetic and phenotypic diversity within laboratory colonies by exposing mosquitoes to semi-field conditions or by introducing wild adults back into the colony (Benedict *et al.*, 2009).

1.5.2 Key environmental factors for mosquito colonisation

Anophelines are among the successful groups of animals, particularly in conferring most of the ecological conditions. However, in laboratory settings, there is a need to adjust the microclimates such as temperature, humidity, and lighting to mimic the natural environments (Benedict *et al.*, 2009; MR4, 2015). Temperature is a critical parameter in an insectary as it affects larval development. For each specific species, there is a temperature range in which its development is optimal. Outside this range, growth and development alter significantly. Therefore, it is critical to control the temperature to achieve predictable culturing (Clements, 1992; Timmermann and Briegel, 1993; MR4, 2015). Insectaries used for mosquitoes are usually maintained within a temperature range of 25–27 °C.

Adult mosquitoes' high surface area to volume ratio makes them susceptible to desiccation at low humidity levels. Studies on mosquito desiccation revealed that extremely low levels of relative humidity are fatal to mosquitoes thus humidity regulation is mandatory (Clements, 1992; Benedict *et al.*, 2009; MR4, 2015). A range of 80 ± 10% relative humidity is an often mentioned optimal value to maintain adults (Benedict *et al.*, 2009; MR4, 2015).

Light intensity and photoperiod have an impact on the development of various mosquito life stages. Previous studies have reported significant interactions of insects with photoperiod revealing that it affects traits such as developmental time (Yee *et al.*, 2012), immature survivorship (Leimar, 1996), metabolic rates (Lanciani and Anderson, 1993) and larval growth indices (Leimar, 1996; Ukubuiwe *et al*., 2017). Other traits affected by light intensity and photoperiod include adult survivorship (Leisnham *et al.*, 2011), adult size (Costanzo *et al*., 2015), fecundity (Carmine and Ronald, 1993), longevity (Lanciani and Anderson, 1993), mating and gonotrophic cycles (Oda and Nuorteva, 1987) as well as diapause induction (Lounibos *et al.*, 2003). Some species may require a crepuscular period to mate, which may necessitate controls for automatic gentle dimming of lights to provide a dusk effect and gradual increase in brightness to simulate dawn (Oda and Nuorteva, 1987; MR4, 2015). A photoperiod of 12 hours of light and 12 hours of darkness is often appropriate (Jordan and Bradshaw, 1978; MR4, 2015).

1.6 Rationale

Research on biological and ecological aspects of most malaria vectors continues to grow owing to the successful colonisation of numerous laboratory colonies in different geographical regions. The biology of these colonies under insectary conditions is now well established. This has led to immense advancement in malaria research. Although knowledge on the biology and ecology of most African malaria vectors continues to grow rapidly, the same cannot be said for *An. funestus.* Lack of advancement in basic research on *An. funestus* is attributed to difficulties in the colonisation of this species. Laboratory colonisation of *An. funestus* remains challenging due to its tendency to avoid mating in confined places, making its proliferation in laboratory cages challenging (Gillies and De Meillon, 1968). Additionally, there is a relative paucity in information on *An. funestus* larval biology under insectary settings. Factors such as larval food and larval density affect larval development and laboratory colonisation and their impact on most anophelines rearing is clearly elucidated. However, there is no literature on how these factors affect *An. funestus* rearing. Therefore, there is a critical need to understand the impact

of different larval diets and larval density on *An. funestus* rearing under laboratory conditions. Such information can be used as baseline information for standardised *An. funestus* rearing making colonisation of this species achievable.

Currently there are only two stable colonies, *An. funestus* colonies: FUMOZ (*Anopheles funestus* strain from Mozambique) and FANG (*Anopheles funestus* strain from Angola) (Hunt *et al.*, 2005). FUMOZ was established in 2000 using the progeny of wild caught females from southern Mozambique while FANG originated from field collections conducted in southern Angola and has been kept in colony since 2002 (Hunt *et al.*, 2005). The successful colonisation of these two *An. funestus* strains paved new avenues for research on several biological aspects of this important malaria vector. However, subsequent efforts to colonise *An. funestus* from various localities have been fruitless (R.H Hunt; N Venter, Unpublished data). The factors that impede these attempts remain elusive. Additionally parameters that permitted successful colonisation of the two *An. funestus* strains (FUMOZ and FANG) have never been described. Rearing of these strains predominantly relies on experience and information passed from individual to individuals through word of mouth. This study seeks to investigate and identify critical parameters that promotes successful sustenance of an established *An. funestus* laboratory strain. The hypothesis is that understanding parameters that promotes sustenance of a well-established strain can be used as a reference point for colonisation of other *An. funestus* strains from different geographical areas. This will be addressed through characterising baseline age specific life table attributes and baseline colonisation parameters of an already colonised *An. funestus* strain. The information generated from this study will elucidate information on the colonisation of *An. funestus* and pave the way for the investigations of novel vector control interventions against this species, such as the Sterile Insect Technique (SIT) and self-limiting gene drive initiatives that rely on colonising mosquitoes and would be limited by failure to colonise this species.

1.6.1 Aim and objectives

The aim of the study is to characterise and understand baseline parameters permissive to the successful colonisation of *An. funestus*.

Specific objectives were:

1. To characterise the life history parameters in a colonised *An. funestus* strain.
2. To assess the impact of different larval diets on the development of *An. funestus*.
3. To optimise and adapt *An. funestus* females to an artificial membrane blood-feeding system.
4. To assess the effect of larval density on the development of *An. funestus*.

CHAPTER 2

CHARACTERISATION OF THE LIFE HISTORY PARAMETERS OF A COLONISED *AN. FUNESTUS* STRAIN

**This work was published in the Journal of Vector Ecology (Appendix D).*

2.1 Introduction

Challenges to successfully colonise *An. funestus* have been hampered by inadequate knowledge on the biological factors that promote its colonisation and therefore, resulting in limited research on this species (Coetzee and Koekemoer, 2013). When intending to colonise any insect, it is often necessary to have prior knowledge of its biology and predict occurrences such as death, pupation, and adult emergence (Benedict *et al.*, 2009; MR4, 2015). Furthermore, knowledge of attributes such as fecundity, developmental rates, survival rates, age-specific or time-specific mortality and longevity is fundamental in understanding baseline colonisation parameters.

During the colonisation of insect species, including mosquitoes, the first bottleneck is early instar mortality owing to intra-specific competition (Southwood *et al.*, 1972; Alto and Juliano, 2001; Tun-Lin *et al.*, 2000). Going over this hurdle is therefore critical for successful colonisation (Mamai *et al.*, 2018). After this, low mortality rates at each developmental stage increases the likelihood that a mosquito strain undergoing colonisation will emerge in enough numbers to perpetuate subsequent generations. Another important factor that determines the stability of a mosquito colony is the adult survival rate. Adult survival affects the total egg production of a female during her lifetime and consequently the propensity of mosquitoes to proliferate under laboratory rearing conditions. All these parameters can be extrapolated from life tables.

Information from life table characterisation gives a baseline reference for successful colonisation as it guides the development, reproduction and survival of a regiment of individuals or an individual of a given species (Mahmood, 1997; Jha *et al.*, 2014; Greco *et al.*, 2017). Surprisingly the life history parameters of *An. funestus* under controlled laboratory settings has never been described despite evidence that such information can facilitate its colonisation. This chapter aimed to fill this research gap by determining the age-specific life table attributes of an already established *An. funestus* strain under laboratory conditions and their relation to colonisation.

2.2 Materials and methods

2.2.1 Biological material/ mosquito strain

An *An. funestus* laboratory strain named FANG (F = *funestus*, ANG = Angola) was used. FANG originates from field collections conducted in southern Angola and has been kept in colony since 2002. It is currently maintained in the Botha De Meillon Insectary under standard insectary conditions of 25-27°C; 80% relative humidity and a 12 hour day/night cycle with a 45-minute dusk/dawn transition period at the Vector Reference Laboratory (VRL) of the National Institute for Communicable Diseases (NICD) using methods described by Hunt *et al* (2005).This strain was used in all the subsequent objectives of this entire study thus the biological material/mosquito strain section will not be mentioned in the following chapters.

2.2.2 Inducing oviposition

A total of 1,800 (300 males and 300 females/replicate over 3 replicates), 24-30 hours old pupae were separated by gender-based on genitalia morphology (Clements, 1992). To allow emergence into adults, the pupae were placed into 55mm (radius) X 120mm (height) cylindrical vials containing 200ml of deionised water (water that has been treated to remove all ions – typically, that means all of the dissolved mineral salts) inside five litre plastic cages. After eclosure, the newly emerged adults were pooled and allowed to mate for 10 consecutive days with a provision of 10% sugar solution. After the mating period, two blood meals (10-minute feeding period per blood meal) were offered over a five-day period with a two-day interval between each blood meal (Hunt *et al.*, 2005). After the first blood meal, females that had successfully blood fed were recorded and transferred into a new cage for the second blood meal. The transition period between the first and second blood meal was two days. After the second blood meal, the mosquitoes were allowed 48 hours for blood digestion and egg development. Using a handheld aspirator, a total of 210 blood-fed females were randomly selected (70 females /replicate/over three replicates) and gently introduced into individual oviposition glass vials (11mm in radius and 50mm in height) (Figure 2.1) containing a damp filter paper disc to induce oviposition (Choi *et al.*, 2014). The vials were monitored daily to ensure the filter paper remained damp. Eggs oviposited by each female from the first gonotrophic cycle were counted using a handheld magnifying glass. Females that did not lay eggs during the first 24 hours were kept for an additional 10 days before insemination and egg load in the ovaries were determined.

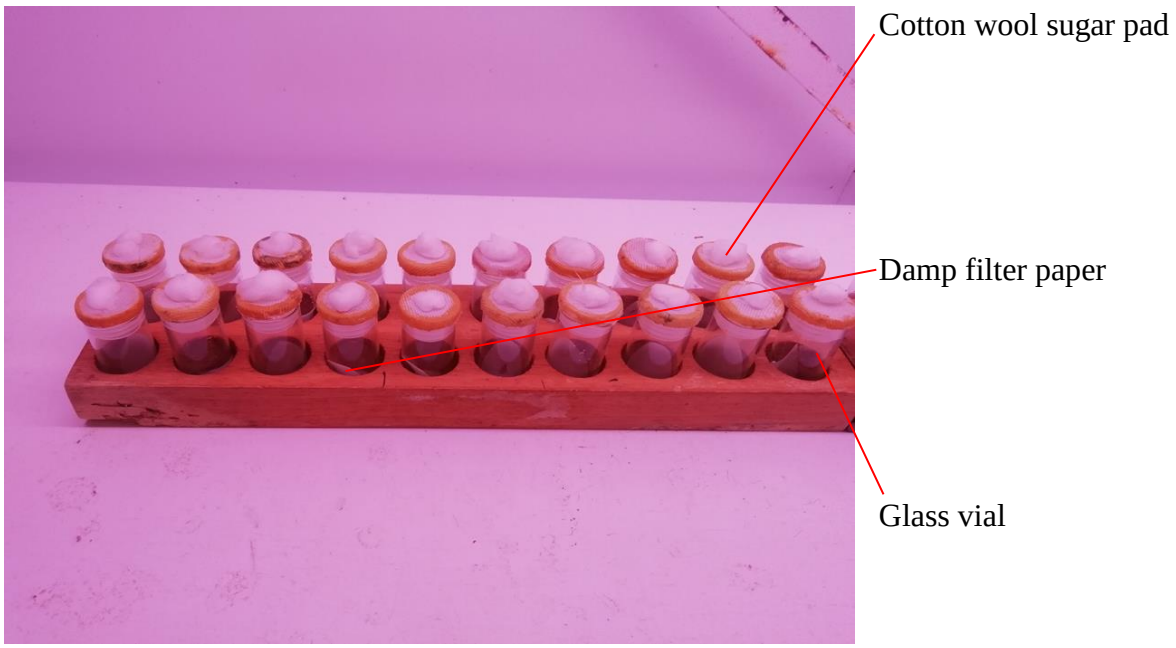

Figure 2.1: Oviposition vials containing damp filter paper with one female placed in each and cotton wool soaked in 10% sugar solution provided to each female for the duration of the experiment (Captured by M.P Zengenene at NICD).

2.2.3 Spermathecal capsule and ovary dissections

Immediately after each female had oviposited, spermathecae dissections were carried out on all females (n = 210) to determine insemination (mating success) according to the protocols of MR4 (2015). Each female was immobilised at -20°C for 30 minutes and placed in a drop of phosphate-buffered saline (PBS) on a clean microscope slide using sterile forceps. The abdomen of each of these females was opened using a pair of fine tweezers (BioQuip Products Inc., Rancho Dominguez, Califonia (CA), United States of America (USA)) under a dissecting microscope (OLYMPUS SZX7, Olympus America Inc., Center Valley, CA, USA). During dissection, the spermathecal capsule was removed together with the terminalia. Subsequently, the spermathecal capsule was separated from the rest of the tissues, and a coverslip was gently placed on the capsule. Slides with the isolated capsule were photographed with an Olympus SC50 digital camera under the microscope using the cellSens software package (Olympus). Insemination success was recorded as presence of long thread-like spermatozoa within the capsule appearing as fine concentric threads (Figure 2.2).

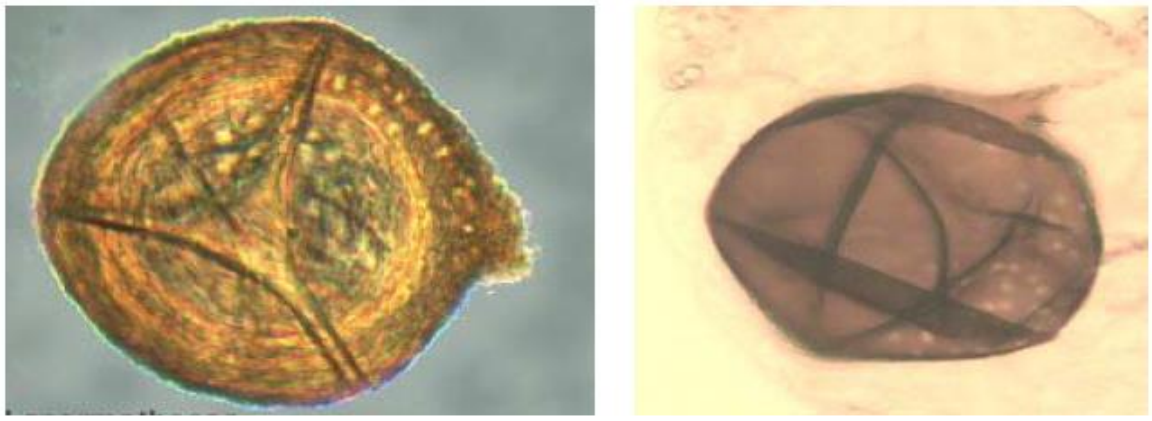

Figure 2.2: Spermathecal capsule of mated (left) and unmated (right) females. The bright ring on the mated female capsule is the bundle of sperm (Adapted and modified from MR4, 2015).

2.2.4 Fecundity (egg load and egg retention)

Occasionally, after a female mosquito completes oviposition, one or more eggs remain in the ovaries. Several physical and chemical factors influence this behaviour, including tactile, olfactory and visual cues (Maharaj, 2003). Furthermore, gravid females can retain mature eggs when oviposition behaviour is interrupted by oviposition repellents or when there is an immediate lack of a suitable oviposition medium (Seenivasagan *et al.*, 2014). Therefore, a more appropriate way to determine egg production would involve counting the number of eggs oviposited as well as those retained. Immediately after determining insemination rates, egg retention in females was investigated. To determine egg retention in females that had oviposited eggs as well as those that did not, abdomens were dissected using a pair of fine tweezers (BioQuip Products Inc., Rancho Dominguez, CA, USA) under a dissecting microscope (OLYMPUS SZX7, Olympus America Inc., Center Valley, Pennyslvania, USA) to access the ovaries. The number of eggs retained in the ovaries were counted and recorded and fecundity was calculated as total egg load per female i.e. total between eggs laid plus any eggs retained in the ovaries after oviposition.

2.2.5 Fertility (egg hatch rates)

To determine fertility, eggs from individual females that had been recorded as inseminated were combined and 300 eggs per replicate over three replicates (n = 900) were randomly selected and used to determine egg hatch rates. In detail, eggs were transferred to into

cylindrical larval rearing bowls (60mm radius X 50mm height) containing 150ml of deionised water. Upon hatching, first instar (L1) larvae were counted daily by transferring them to new larval rearing bowls (60mm in radius and 50mm in height) using a three ml plastic pipette. This was done to avoid double counting of newly hatched L1s. Eggs were allowed to hatch for 14 consecutive days under standard insectary conditions. Hatch rates were calculated as the proportion of eggs hatching in each replicate.

2.2.6 De-chorination of unhatched eggs for embryo development observations

Anopheline eggs fundamentally hatch after the completion of embryonic development, two to three days post oviposition. However, staggered hatching has been observed whereby some eggs showed marked variation in time-to-hatch (18 days post oviposition or later). The mechanism facilitating delayed hatch has not been clearly expounded but is likely arbitrated by environmental and genetic influences that either induce diapause or slow embryo development (Kaiser *et al.*, 2014). To investigate staggered hatching in FANG, all unhatched eggs from the three replicates (n = 120) were decalcified 14 days post oviposition and observed under the microscope to determine presence and status of embryos using methods described by Kaiser *et al.* (2014). In detail, eggs were collected on a strip of filter paper and covered in a few drops of fixative solution (3.6 M formaldehyde; 0.87 M glacial acetic acid; and 8.5 M absolute ethanol), for 30 minutes. Once eggs had been fixed the filter paper strip with eggs was placed into a 1.5 ml reaction tube and covered with one ml of decalcifying solution (0.59 M sodium hypochlorite and 0.35 M glacial acetic acid in deionised water). This successfully fixed the eggs to inhibit hatching and bleached the chorion so that embryos could be viewed under the microscope. After decalcification, eggs were qualitatively checked by observation for the presence of an embryo and level of development of the embryo under a stereomicroscope (OLYMPUS SZ2-ILST, Olympus Corporation, Tokyo, Japan) at 150X magnificatio

2.2.7 Larval development time

Newly hatched FANG larvae (L1) of the same age (three replicates, n=300/replicate) were placed in rectangular larval rearing bowls (120mm width X 200mm length X 70mm height) containing 750ml deionised water. Larvae were fed twice daily on a mixture of finely crushed dog biscuits and brewer's yeast (at a daily rate as shown in Table 2.1) until pupation. Larval food regimen was amended according to early instar mortality. The number of larvae pupating and day of pupation were recorded daily until all larvae pupated. The proportion of larvae surviving to pupation was

calculated as the number of pupae pupated compared to the total of L1 larvae used. Time to pupation was calculated as the time taken to develop from L1 until pupation.

Table 2.1: Daily larval feeding regiment of *An. funestus* larvae reared to monitor larval development in FANG (Optimised by Felambihangy, unpublished data).

Day of feeding	**Morning dose (mg/ larva)**	**Evening dose (mg/larva)**
1	0.04	0.04
2	0.05	0.05
3	0.06	0.06
4	0.08	0.08
5	0.10	0.10
6	0.14	0.14
7	0.18	0.18
8	0.22	0.22
9	0.28	0.28
10	0.32	0.32
11	0.40	0.45
12	0.45	0.50
13	0.45	0.50
14	0.45	0.50
15	0.40	0.30
16	0.40	0.30
17	0.40	0.30
18	0.40	0.30

2.2.8 Adult emergence

Upon pupation of larvae from larval development experiments above (section 2.2.7), pupae were grouped according to day of emergence and adult eclosion was monitored daily. The number, day of emergence and gender of adults emerging were monitored daily per replicate until the emergence of the last pupae. Only adults that successfully emerged and had the capacity to fly were recorded as emerged. The number of adults emerging was recorded. Pupal productivity was calculated as the mean proportion of pupae surviving to the adult stage, and the time to emerge was recorded.

2.2.9 Adult longevity

Newly emerged males and females from adult emergence experiments above (section 2.2.8) were combined by day of emergence and gender (n=75 per gender/replicate; three replicates) and placed into different two-litre cylindrical adult cages (62.5mm in radius and 135mm in height). All adults were maintained under standard insectary conditions with a 10% sugar solution that was replaced every three days. A daily count and removal of dead adult mosquitoes was conducted until 100% mortality was reached. The longevity of the resultant adults was calculated using Kaplan Meier survival analysis (Kaplan and Meier, 1958).

2.3 Data analysis

Data were managed in Microsoft Excel analysed using IBM SPSS Statistic software (IBM Corp., Armonk, New York), version 21. All data was first checked for normality using the Sharpiro-Wilk test. A p-value <0.05 was considered statistically significant. Data on egg hatch rates, the proportion of larvae surviving to pupation, insemination status and adult emergence were summarised as the mean proportion of eggs hatching, the mean proportion of larvae surviving to pupae, mean proportion of females that contained sperm in their spermathecae and mean proportion of pupae surviving to adult stages respectively. Kaplan Meier analysis was used to determine the probability that each developmental stage will survive beyond any given specified time. A one-sample t-test was used to assess if the emergence of adult males and females deviated from the expected one is to one ratio.

2.4 Results

2.4.1 Insemination rate, fecundity and fertility

The life-history attributes of FANG are summarised in Table 2.2. Mating success, measured as mean insemination rate ranged from 67.1 to 83.4% with a mean of 74.8 ± 8.2% (n = 157) (Table 2.2). Females that were not inseminated (n = 53) did not lay eggs. The fecundity of mated females ranged from 23 to 79 eggs/female with an average egg load of 67.1 ± 17.1 eggs per female. Out of these, 77.1% (n = 121) laid all their eggs while 22.9% (n = 36) retained some eggs in their ovaries. Of the females that retained eggs, 36.1% (n = 13) completely retained all their eggs while 63.9% (n = 23) retained a proportion of the eggs. Fertility determined as the proportion of eggs that successfully hatch into first instar larvae ranged from 81 to 93%, with a mean of 86.7 ± 6.1% (n = 780).

2.4.2 De-chorination of unhatched eggs for embryo development observations

After 14 days, the chorion of most of the eggs was degraded. Consequently, no results could be inferred from this objective.

Table 2.2: Life table characteristics of a laboratory colonised *An. funestus* strain (FANG) reared from eggs to adults under standard insectary conditions

Life history characteristic	Total sample size (N)	Mean/Median ±SE/range
Mean insemination rate (%)	210	74.8 ± 8.2
Mean egg load	157	67.1 ± 17.1
Mean hatch rate/fertility (%)	900	86.7 ± 6.1
Mean larval developmental time (days)	900	16.4 ± 2.5
Mean survivorship, L1 to pupa (%)	900	72.9 ± 13.6
Mean survivorship, pupa to adult (%)	656	78.8 ± 3.0
Sex ratio of resultant adults (females: males)	517	261: 256
Median adult eclosion time, in days (Inter-quartile range)	656	2 (1,3)
Median longevity of males in days (Inter-quartile range)	225	44.0 (21, 55)
Median longevity of females in days (Inter-quartile range)	225	28.0 (22, 33)

2.4.3 Larval developmental time and adult emergence

Mean developmental time from the first-instar larvae to the pupal stage was 16.4 ± 2.5 days (Figure 2.3). The proportion of first instar larvae that survived to pupation ranged from 56 to 94%, with a mean of 72.9 ± 13.6% (n = 656). The mean proportion of pupae that emerged into adults was 78.8 ± 3.0% (n = 517) and ranged from 84 to 89%. The time taken for pupae to emerge into adults ranged from one to three days with a median of two days (Figure 2.4). The sex ratio of the emerged adults

was not significantly different from the 1:1 ratio (Table 2.2) (one-sample t-test; t (661) = -0.31, p = 0.76).

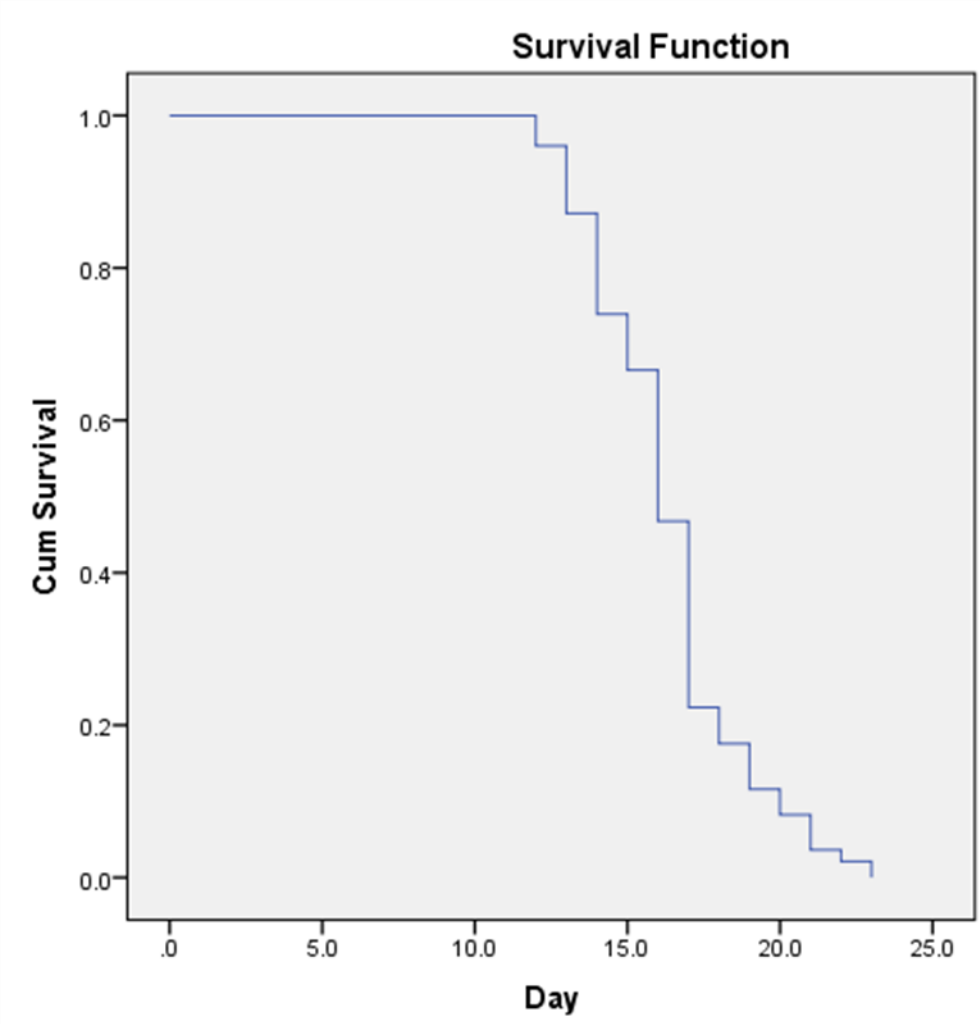

Figure 2.3: Kaplan Meier survivorship curve showing the developmental time from the first instar larvae stage to pupation of FANG larvae reared under standard insectary conditions on a standard larval diet.

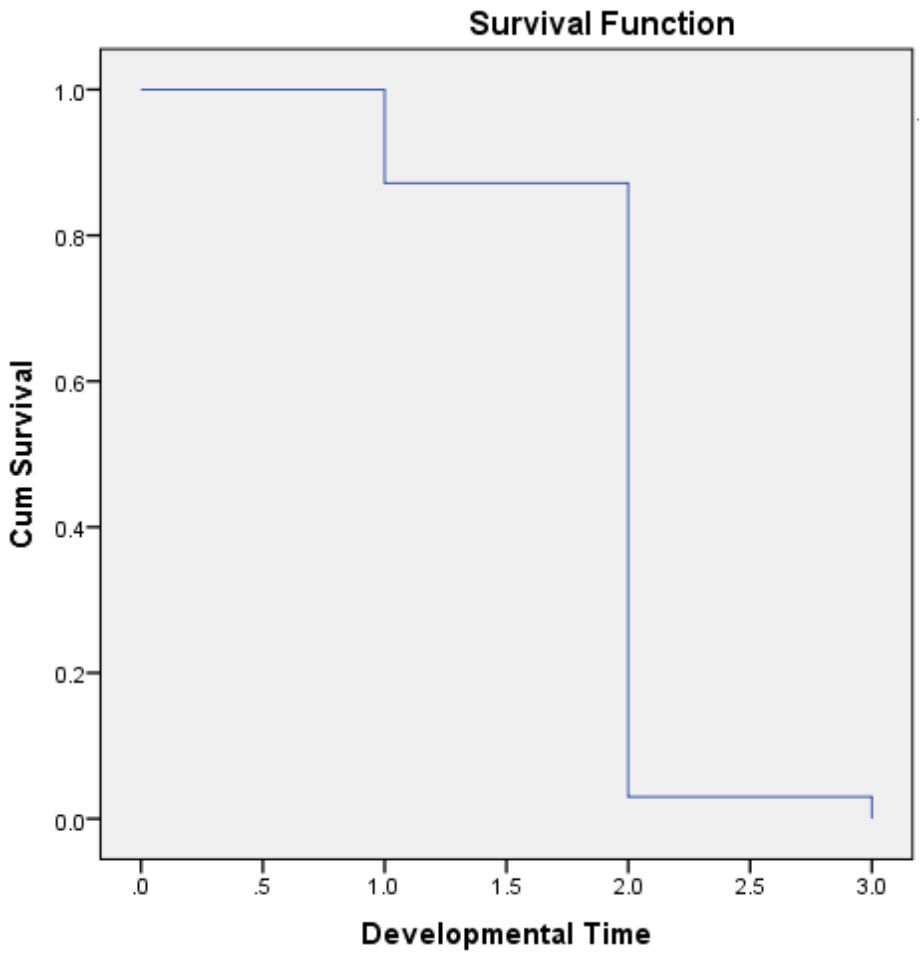

Figure 2.4: Kaplan Meier survivorship curve showing the developmental time of FANG pupae to the adult stage under standard insectary conditions.

2.4.4 Adult longevity

Overall, adult survivorship as a measure of longevity from emergence to death is shown in Figure 2.5. Mortality started occurring on day two for males and day seven for females. There was 100% mortality in females and males on day, 42 and 69, respectively. Overall, the median adult lifespan of males was 44 days (n = 225), while that of females was 28 days (n = 225) (Table 2.2). The difference between the median adult longevity of males and females was statistically significant (log-rank test, $\chi 2 = 174.16$, DF = 1, $p = 0.000001$).

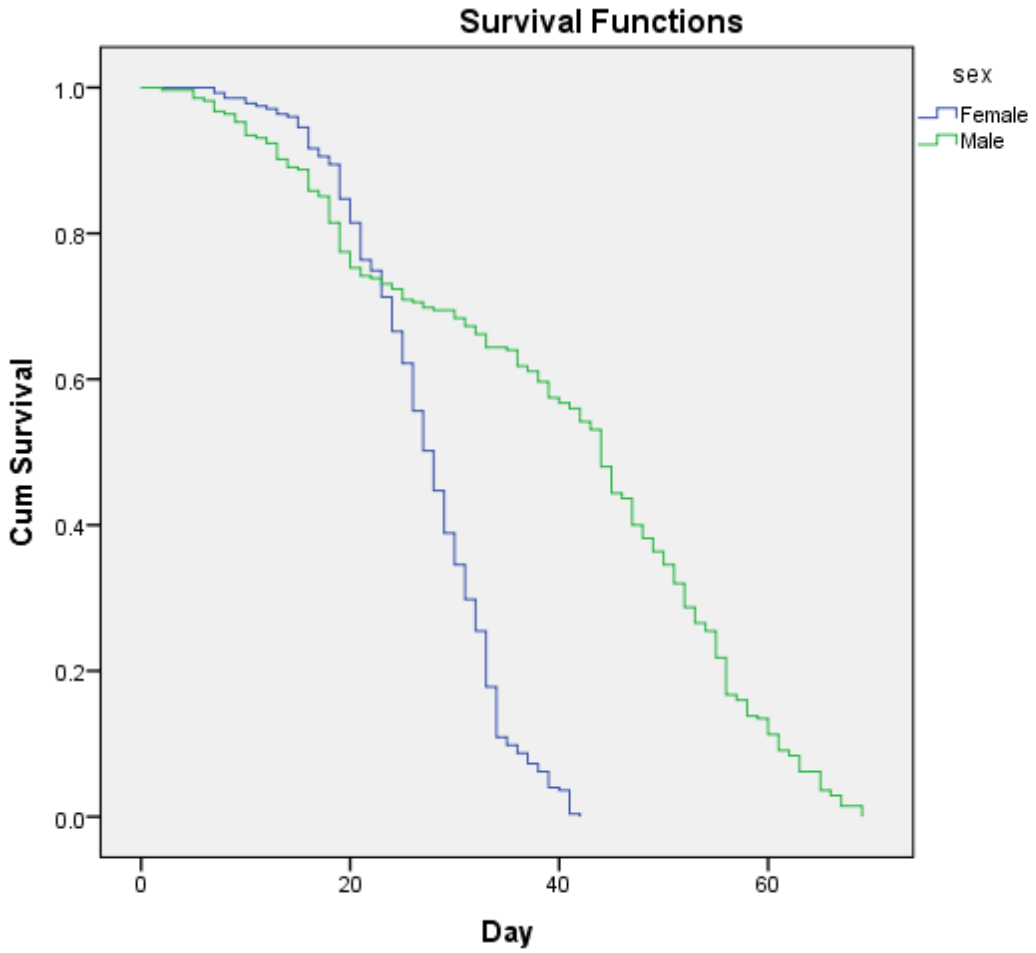

Figure 2.5: Kaplan Meier adult survivorship curve showing survival time in days for male and female FANG adults.

2.5 Discussion

These studies provide insights on the biological factors that can promote re-colonisation (establishing a sub-colony using eggs from the main colony) of the *An. funestus* FANG strain. To understand the potential baseline parameters necessary for successful rearing of *An. funestus*, a life table approach was applied to study the physiological and reproductive characteristics of the FANG strain.

Data from this study showed high female insemination rates (Table 2.2). This result is similar to those observed in colonised *An. gambiae* strains where insemination rates between 72 - 76% have been recorded (Verhoek and Takken, 1994; Stone *et al.*, 2009) but lower than those reported for colonised *An. arabiensis* (89.7 - 93.3%) (Munhenga *et al.*, 2016; Dandalo *et al.*, 2017). This result was unexpected for *An. funestus*, a species considered to be refractory to

colonisation (Gillies and De Meillon, 1968), for which low mating rates success would be expected. The relatively high mating success obtained in this study can be explained by the fact that FANG has been under colonisation for 18 years. Although baseline life-history parameters of this strain are not known because these were not determined at the time of colonisation in 2002, it is possible that individuals with a capacity to mate in confined spaces have been selected over time resulting in efficient mating. It is well established that bottlenecks and selective pressure placed on individuals cause them to adapt to the artificial environment (Howell and Knols, 2009; Rull *et al.*, 2012). Furthermore, successful mating could have been exacerbated by the 10 day mating period, as mating within the first three days is highly unlikely (L.L Koekemoer, personal communication). The high mating success observed here increases the chances of females ovipositing fertile eggs. This is critical for re-colonisation of this strain in other laboratories.

Generally, fecundity is the reproductive capacity of an organism or population and is measured by the number of eggs or asexual propagates (Sowilem *et al.*, 2013). Fecundity is affected by a number of factors, including female age and size, adult diet type and feeding frequency, larval feeding as well as environmental conditions (Briegel, 1990; DeJong *et al.*, 2007). Optimisation of these factors could result in increased fecundity, consequently leading to high reproductive rates. On the other hand, subtle changes to these factors may result in decreased fecundity, leading to colony collapse. The mean egg load recorded during this (Table 2.2) study was approximately half of what was recorded in wild *An. funestus* populations of unknown ages (Davidson, 1954; Gillies and De Meillon, 1968). According to Gillies and De Meillon (1968) an *An. funestus* female lays an average of 123 eggs per gonotrophic cycle. However, these females were of unknown age, oviposition cycle and blood feeding frequency, critical factors that affect fecundity. Although fecundity levels in this study were low compared to natural populations, they fell within the range for other laboratory colonised *Anopheles* strains (Munhenga *et al.*, 2016; Mamai *et al.*, 2017). This could be an adaptive phenomenon. In the wild, females lay more eggs because of adverse environmental conditions and predation that negatively affect their survival and the hatch rates of the eggs (Gillies and De Meillon, 1968). Therefore, more eggs will be required to ensure survival. In contrast, under laboratory conditions, these factors are not limiting (Kaiser *et al.*, 2014). Eggs laid under standard insectary conditions have greater chances of survival compared to those laid in the wild, and therefore there is no adaptive advantage for having large egg batches. Low fecundity observed

in this study will likely not have detrimental effects on the re-colonisation prospective of this species.

Fertility in mosquitoes, as determined using egg hatch rates, affects population growth and consequently the colonisation ability of a species. Low hatch rates indicate slow population growth and difficult colonisation ability while high hatch rates indicate high colonisation potential and rapid population growth (Grieco *et al.*, 2003; Nur-Aida *et al.*, 2008). In this study, fertility was determined by monitoring first instar larvae hatching from eggs and high rates were observed (Table 2.2). This is probably the first report on the fertility of an *An. funestus* laboratory strain. However, research on other anophelines has shown that egg hatch rates greater than 80% are typical for a laboratory colony (MR4, 2015; Mamai *et al.*, 2018). Therefore, results from this study suggest that FANG has a high re-colonisation potential.

Developmental rates of immature mosquito stages and their survivorship can affect population growth and impact adult life traits (Li et al., 2014) and consequently impact colonisation potential. The mean development time from L1 to pupa recorded in this study (15 days) (Figure 2.3), is consistent with data from wild *An. funestus* populations (Gillies and De Meillon, 1968). On the contrary, the larval developmental time recorded for FANG is longer than reported for other anopheline strains such as *An. arabiensis*, *An. gambiae* and *An. albimanus* (7 to 10 days) under laboratory conditions (Mahmood, 1997; MR4 2015; Dandalo *et al.*, 2017; Mamai *et al.*, 2018). Under natural conditions, an extended larval stage is generally associated with an increased mortality risk because of habitat instability and predation (Gillies and De Meillon, 1968; Clements, 1992). The larvae of the *An. gambiae* complex, for example, inhabits temporary pools that can dry up quickly, while *An. funestus* develop in more permanent water bodies. These genetic traits are not affected by laboratory conditions. Therefore, the extended developmental time of FANG recorded during this work has no impact on the capacity to colonise this strain. The mean proportion of L1 that survived to pupation and pupae that emerged to adults was consistent with those obtained from studies involving other laboratory colonised anopheline strains (Dandalo *et al.*, 2017; Somda *et al.*, 2017). Low larval mortalities observed are due to stable climatic conditions and no predation when mosquitoes are bred under laboratory conditions. Low mortality during immature stages increases the probability of the emergence of physiologically fit and adults with high reproductive potential, increasing the chances of successful rearing and re-colonisation.

Adult longevity is a highly influential component of mosquito population models and colonisation potential, given its relationship to average generation time and fecundity. Adults from this study as shown in Figure 2.5 lived longer than observed in nature (Davidson, 1954). This increases colonisation potential as adults survive long enough to reach sexual maturity and reproduce (Benedict *et al.*, 2009). The high longevity is most likely due to laboratory conditions that are near perfect for survival because of the lack of predators, disease, perfect climatic conditions and absence of other hazards that cause mortality in the wild. Furthermore, energy sources required for adult survival are readily available under standard insectary conditions. During this study, there was always a constant supply of a sugar source for the mosquitoes, whereas wild mosquitoes search for energy sources, and availability is uncertain. The mean longevity by gender showed that males lived longer than females. This is similar to observation from other studies, which addressed different objectives using laboratory-reared *An. arabiensis* (Maïga *et al.*, 2016; Mamai *et al.*, 2016; Somda *et al.*, 2017). Having males surviving longer is an interesting observation as females usually survive longer than males under natural conditions (Clements, 1992; Lehman *et al.*, 2006). It could be that the longevity of these males confers them an advantage leading to maximised mating opportunities in cages, thus increasing colonisation potential. From the mean longevity of females, it can be inferred that maximum egg production could be achieved under laboratory conditions as the female would be able to undergo several gonotrophic cycles during the observed survival period.

The information generated in this study gives insight into the developmental pattern of FANG in the absence of environmental stressors, such as inter-species competition, predators and insecticides that affect natural populations. This information helps in understanding the biology of *An. funestus* under laboratory rearing conditions. The data can be extrapolated and used as a reference point for colonisation of *An. funestus* populations from other geographical regions.

CHAPTER 3

OPTIMISING AND SELECTING AN *AN. FUNESTUS* LABORATORY COLONY TO ARTIFICIAL MEMBRANE BLOOD FEEDING

3.1 Introduction

Female mosquitoes have a gonotrophic cycle that begins with taking a blood meal ending with oviposition of eggs. Therefore, ingestion of a blood meal is critical to the mosquito's reproductive cycle. The blood meal is used as a source of protein for the development and maturation of eggs (Clements, 1992; Gunathilaka *et al.*, 2017). When a blood meal is ingested, mosquito midgut trypsin-proteases digest the blood into their constituent amino acids (Graf *et al.*, 1986; Price *et al.*, 2015). These amino acids are transported out of the midgut and taken up by the fat body and other tissues via specialized amino acid transporters (Boudko *et al.*, 2015). A large percentage of the amino acids are metabolised and used for energy production, while the remainder is used for egg production (Sappington *et al.*, 1995; Hansen *et al.*, 2014).

Various studies have revealed that fecundity in mosquitoes is correlated with size of blood meal taken and source of the blood (Briegel, 1990; Taylor and Hurd, 2001; Deng *et al.*, 2012). The nutritional quality of a blood meal and quantity of blood taken impact the number of eggs from a female that develops to maturity and subsequently the number of eggs produced per female (Taylor and Hurd, 2001). This is ascribed to differences in protein composition between blood meal sources (Prasad, 1987). Haematological properties such as amino acid composition and erythrocyte density vary between vertebrate species (Harrington *et al.*, 2001); therefore, the nutritional value of a blood meal and its subsequent impact on mosquito reproduction also varies. The amount of blood ingested during blood feeding is correlated to the quantity of proteins/amino acids available for egg development (Foster and Eischen 1987; Briegel, 1990). Consequently, during laboratory colonisation of mosquitoes, it is important to consider the above factors and select a blood meal source high in nutritional composition and a feeding system that promotes high feeding rates.

Laboratory colonies can be maintained using direct skin feeding from a vertebrate or indirectly using artificial membrane systems (Harrington *et al.*, 2001; Montes *et al.*, 2002). Direct skin feeding on humanss has been the best approach for mosquito colonisation, particularly for the major African malaria vectors such as *An. gambiae* and *An. funestus* (Benedict *et al.*, 2009). However, the use of humans as a blood meal source during mosquito rearing is not sustainable

because of ethical and feasibility concerns, particularly on a large scale. Similarly, the use of live animals is also discouraged because of the same concerns (Kasap *et al.*, 2003; Deng *et al.*, 2012). Mosquito rearing under colony will require the use of alternative blood sources and/or delivery systems other than the human and live animal hosts. Currently, the blood-feeding system being utilised at VRL mainly involves the use of anaesthetised guinea pigs. This poses a challenge due to costs involved in anaesthetics and operating an animal husbandry facility to sustain a consistent blood meal source, especially where rearing involves the large-scale production of mosquitoes. Another drawback is that as the guinea pigs grow, they become resistant to the administered anaesthetic. Furthermore, due to the duration needed to feed numerous cages, they sometimes recover from the anaesthetic effect before the mosquitoes get a sufficient blood meal needed for optimal egg production (Personal observation; Birck *et al.*, 2014). Due to these limitations, it is essential to consider alternatives such as artificial blood feeding systems, particularly in mass rearing.

Several colonies of different mosquito species have been successfully weaned to cow blood using artificial feeding systems (Bousema *et al.*, 2012; Damiens *et al.*, 2013; Sampaio *et al.*, 2016; Mamai *et al.*, 2017) and the use of different types of membranes for artificial blood feeding is well described (Wirtz *et al.*, 1980; Benzon and Apperson 1987; Hagen and Gruneweld 1990). However, these systems are not readily transferable. They require optimisation as several laboratories have reported varying success due to mosquito strains, device/feeding system, blood type/source and membrane. The purpose of this study was to optimise and select for cow blood-feeding in an *An. funestus* laboratory strain (which is currently being maintained on anaesthetised guinea pigs) using an artificial membrane feeding system. Two different membranes were assessed and the most effective membrane was optimised and used for selecting the *An. funestus* strain to cow blood feeding.

3.2 Materials and methods

3.2.1 Comparison of direct feeding using anaesthetised guinea pigs against artificial blood feeding using different membranes

3.2.1.1 Blood delivery system

Direct skin feeding using anaesthetised guinea pigs was compared to indirect membrane feeding using a Hemotek® membrane feeding system (Hemotek PS6A/220) (Figure 3.1). Since all mosquito colonies at NICD are maintained and fed routinely on guinea pigs (ethics number: 1993047 (Appendix C)), the feeding of adults was conducted through routine departmental procedures under the guidance of authorised technicians. The Hemotek® membrane feeding system was selected based on its historical success in routine laboratory feeding of female mosquitoes, operational ease and commercial availability (Wood and Cosgrove, 1996, Damiens *et al.*, 2013). It functions electronically through a power unit that can simultaneously connect multiple temperatures controlled heating plates to aluminium blood reservoirs. This device can maintain a constant temperature of 37°C to mimic that of most live vertebrates. Its capacity to connect to numerous heating plates makes it possible to compare different membranes concurrently.

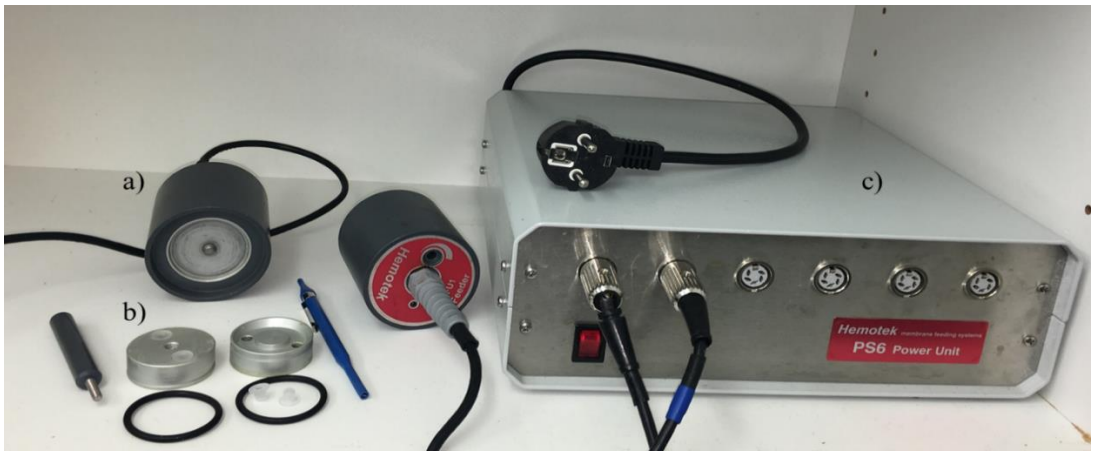

Figure 3.1: Structural components of the Hemotek® feeding system used during blood feeding optimisation of a laboratory *An. funestus* colony: a = heating plate: b = reservoir, O-ring, plug and blue temperature setting device: c = power unit (Captured by M.P Zengenene at NICD).

3.2.1.2 Membranes tested

Two different types of membranes: Parafilm-M® membrane Gunathilaka *et al.*, 2017)) and pig intestineKasap *et al.*, 2003) were compared in this study (Figure 3.2).

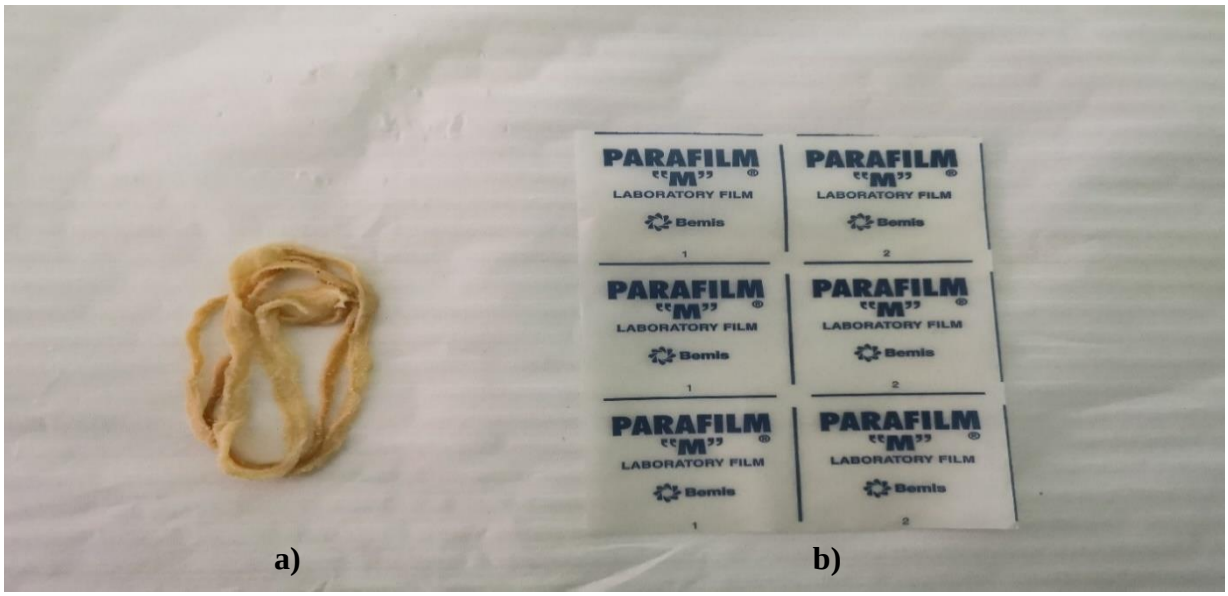

Figure 3.2: Membrane types tested to determine the most effective membrane feeding system that can be optimised for laboratory colonisation at a large scale: a = pig intestine membrane: b = Parafilm-M® membrane (Captured by M.P Zengenene at NICD).

3.2.1.3 Artificial Blood feeding process

The feeding process used is summarised in Figure 3.3. It comprised of enclosing the top surface of the 5ml aluminium reservoir (3.7cm diameter X 1.3cm thickness) with either one of the two types of membranes above and fastening using the O-ring. The pig intestine was first washed as it contained salt, then soaked in water, cut open and stretched across the reservoir. Parafilm-M® membrane was only stretched across the reservoir. Subsequent to this, 5ml of thawed defibrinated cow blood was loaded into each reservoir covered with the respective membrane. Defibrination prevents blood from clotting/coagulating. Soon after the reservoir was filled, the blood was allowed to warm up to 37°C then placed on top of the netted lid of a five litre cage containing mosquitoes. Blood used in this study was recurrently availed by a local abattoir (Karen Beef abattoir, Belfour, Free State Province, South Africa) and frozen in a refrigerator at 2-8°C for use within three months.

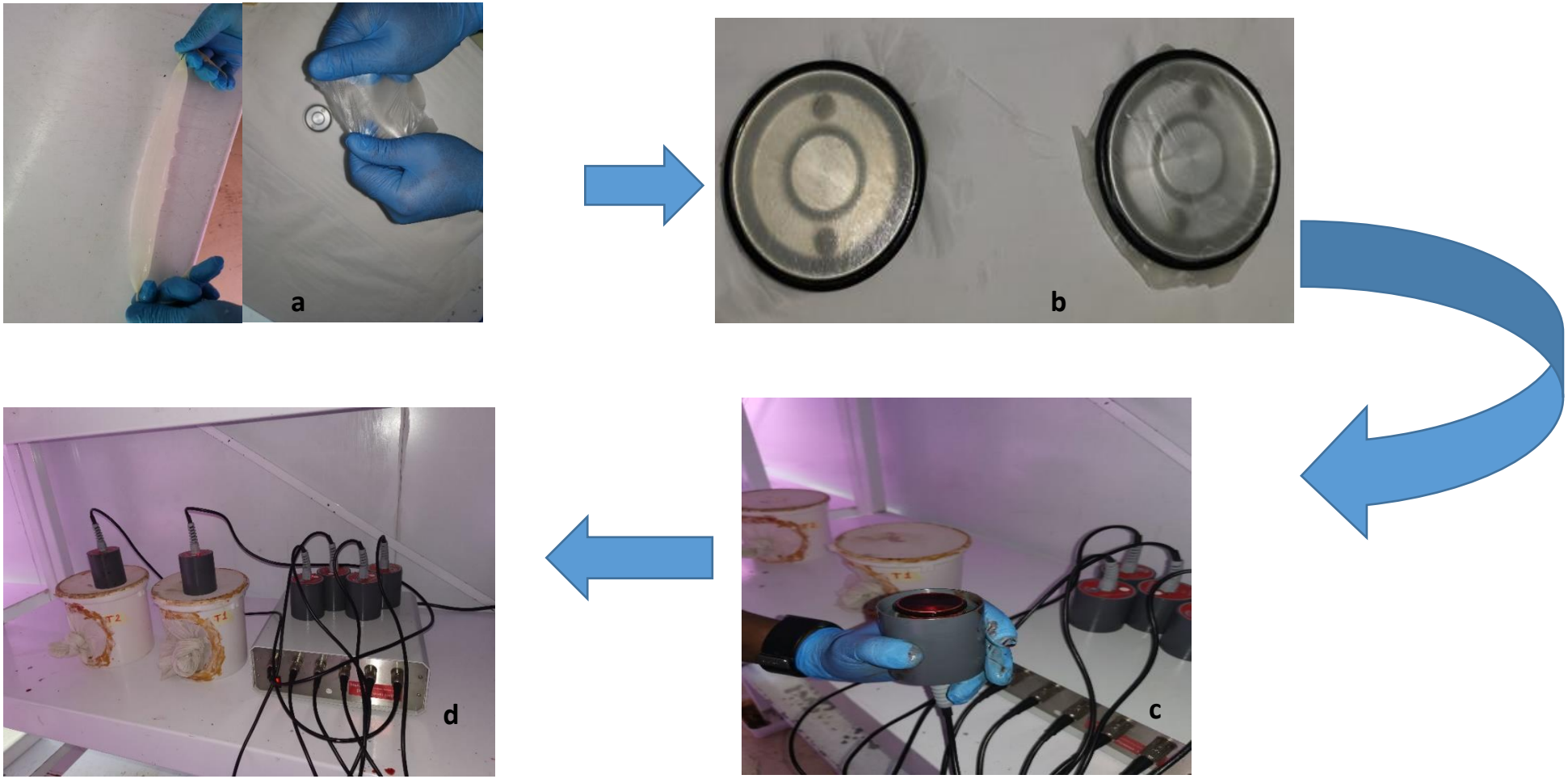

Figure 3.3: Blood feeding procedure used during the study: a = stretching of membranes before use (left is using parafilm and right is pig intestine); b = covering reservoirs with membranes [left is pig intestine vs right parafilm]; c = filling the reservoir with blood and attaching it to heating plate; d = placing heating plate and blood reservoir construct on top of a cage (Captured by M.P Zengenene at NICD).

3.2.1.4 Experimental design

The experimental design for this study involved introduction of an equal ratio of newly emerged adults (300 males: 300 females) into a 20 litre cage and allowing them to mate for 10 consecutive days. Insemination success was determined by removing a subsample of 10 females from each cage (post-mating period) and each female dissected under a microscope to observe the presence of spermatozoa in their spermathecal capsules (Clements, 1992; MR4, 2015). This was done to correct for fecundity as mating success affects feeding success subsequently impacting egg production. Inseminated females were recorded and calculated as a percentage and compared between the treatments and control. After the mating period, 50 females each were randomly selected and introduced into each of the three different treatments (five-litre cages) consisting of guinea pig (control – direct skin feeding) and two membrane types (treatments): treatment 1 (Parafilm-M® membrane) and treatment 2 (pig intestine). Direct skin feeding occurred for 10 minutes (Hunt *et al.*, 2005), while indirect feeding was allowed for two hours (Damiens *et al.*, 2013). After the first blood meal, females that had successfully blood-fed were recorded and put in a separate cage for the second blood meal. Females that did not feed after the first blood meal were counted and excluded for the second blood meal. A second blood meal was provided 48 hours after the first blood-feeding, using similar feeding procedures. Immediately after the second blood meal, the number of females with blood in their abdomen was counted in each cage and calculated as percentage feeding success. Two days after the second blood meal, all live female mosquitoes in each cage were counted, and oviposition sites (egg plates) were provided to induce oviposition. After oviposition, fecundity was determined and calculated as the total number of eggs laid divided by the number of females alive. A sub-sample of eggs from each cage (n = 750/ replicate/treatment) were observed for hatching (fertility) for 14 consecutive days by daily counting and transferring first instar larvae into fresh bowls and a percentage calculated. Each experiment was repeated three times.

3.2.2 Selecting adult female *An. funestus* to artificial membrane feeding

Once the above optimisation studies (section 3.2.1) were completed, the most effective membrane type (in this instance Parafilm-M® membrane) was selected for subsequent colony blood feeding. This was achieved by feeding six successive filial generations (F1 to F6) and monitoring the same parameters as described in section 3.2.1 above. These parameters were monitored over six consecutive generations and compared between generations. The parameters were monitored under standard insectary conditions at a constant temperature and humidity. The same blood batch was used, it was kept frozen in a refrigerator at 2-8°C within three months and thawed in warm water before use.

3.2.2.1 Experimental design

The experimental design for the selection of female *An. funestus* to an indirect artificial membrane feeding method involved the introduction of newly emerged adults (100 males:100 females) from each respective generation into a separate but corresponding cage and allowing them to mate for 10 days. After the mating period, 50 females were randomly aspirated and introduced into a new correlating cage (Figure 3.4). Each cage was blood-fed twice over 5 days with a 48-hour transition period between the first and second blood meal. Both meals were provided for 2 hours using the same procedures described in section 3.2.1.4 above. All experiments were done over three biological repeats, consisting of five technical replicates (n = 750 adults/replicate/ generation).

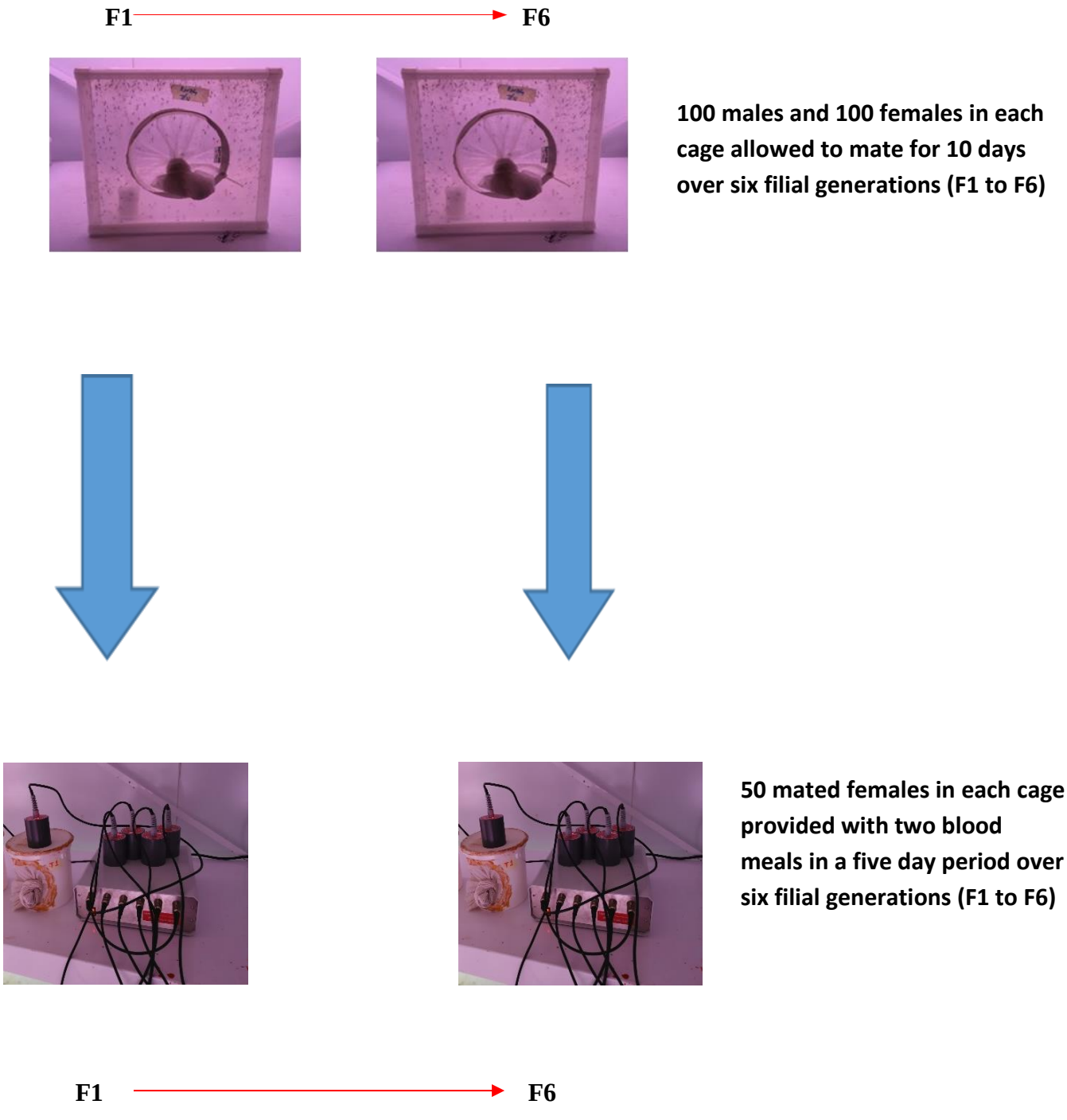

Figure 3.4: Flow diagram showing experimental design used to adapt adult female *An. funestus* to artificial membrane feeding (Captured by M.P Zengenene at NICD).

3.2.3 Data analysis

Data on adult female feeding success after the second blood meal, insemination rate (mating success), fecundity and fertility were summarized respectively as the mean proportion of abdominally engorged females, the mean proportion of females that contained sperm in their spermathecal, mean number of eggs laid per female and mean the number of eggs that hatched. One-Way analysis of variance (ANOVA) was used to analyse the mean difference between fecundity of direct skin vs indirect membrane feeding followed by Tukey's Honest Significant Difference (HSD) post hoc analysis while differences between feeding success, insemination rates and fertility were analysed using the Chi-square test. Effect of colony generation on feeding success was analysed using logistic regression, while that of insemination rates and fertility were analysed using the Chi-square test. Effect of colony generation on fecundity was analysed using One-Way ANOVA, followed by means separation by Tukey's HSD test at 5% level of significance.

3.3 Results

3.3.1 Comparison of direct feeding using guinea pigs against artificial blood feeding using different membranes

The mean number of inseminated females ranged from 63.3% to 65.3% between all the feeding methods (Table 3.1) and there was no significant difference in mating success between feeding methods (χ^2 (2) = 0.18, P = 0.92).

The results for comparing indirect feeding methods to direct skin feeding are summarised in Table 3.1. After the second blood meal, the highest feeding success was obtained from direct skin feeding using an anaesthetised guinea pig (59.1 ± 18.4%, n = 443). Between the two membranes used during indirect membrane blood-feeding success, the Parafilm-M® membrane recorded a higher feeding success (40.8 ± 1.5%, n = 306) compared distantly to pig intestine membrane (16.9 ± 5.4%, n = 127). Statistical analysis showed that the differences in feeding success between the different methods were significant (χ^2 (2) = 296.24, P = 0.00000128).

Fecundity (mean number of eggs laid per female) was highest in the direct skin feeding method using anaesthetised guinea pigs (25 ± 8.2, n =443) followed by Parafilm-M® membrane (11.6 ± 8.7, n = 306). The lowest fecundity was recorded from the use of pig intestine (4.5 ± 2.6, n = 127) (Table 3.1). Statistically, fecundity was significantly different between the three blood-feeding regiments (One Way ANOVA, DF = 2; F = 6.53; P = 0.03). Post hoc Tukey HSD

multiple comparisons revealed that the mean fecundity obtained from the direct skin feeding method using anaesthetised guinea pigs was significantly different from that obtained using pig intestine (P = 0.01).

The mean egg hatch rates (fertility) for direct skin feeding using anaesthetised guinea pig was 82.8 ± 15.4 %(n = 2,250). For indirect membrane blood-feeding methods, the mean fertility was 89.3 ± 14.7% (n = 2,250) and 80.2 ± 7.3% (n = 2,250) for pig intestine and Parafilm-M® membrane respectively (Table 3.1). There was no statistically significant difference in fertility between the feeding regimes (χ^2 (2) = 0.49, P = 0.70).

Table 3.1: Mean values of feeding success, fecundity, insemination rate and fertility of adult females of an *An. funestus* colony blood fed directly on guinea pig abdomen and indirectly using different membranes

Feeding method	**Feeding surface**	**% Feeding success ± SD**	**Fecundity ± SD**	**% Insemination rate ± SD**	**% Fertility ± SD**
Direct	Anaesthetised Guinea pig shaved abdomen	59.1 ± 18.4[a]	25.0 ± 8.2[a]	65.3 ± 10.1[a]	82.8 ± 15.4[a]
	.				
Indirect	Parafilm-M® Membrane	40.8 ± 1.5 [b]	11.6 ± 8.7 [b]	65.3 ± 10.1 [a]	80.2 ± 7.3[a]
	Pig intestine	16.9 ± 5.4[c]	4.5 ± 2.6 [c]	63.3 ± 7.8[a]	89.3 ± 14.7[a]

Different superscript letters within a column indicate statistically significant difference ($p < 0.05$)

3.3.2 Selecting adult female *An. funestus* to artificial membrane blood feeding

Following optimisation of blood-feeding methods (section 3.3.1), the Parafilm-M® membrane was chosen as the optimal membrane because feeding success and fecundity was significantly higher compared to pig intestine. Parafilm-M® membrane was therefore used as a membrane to adapt the colony to indirect blood-feeding over six subsequent generations (F1 to F6), and these results are summarised in Table 3.2. The lowest mean feeding success was recorded in F1 (46.7%, n = 750) and highest in F6 (82.7%, n = 750). Feeding success increased with each generation, and statistical analysis using simple linear regression found a significant difference in the feeding success with each successive generation (R^2 = 0.084, DF = 4,499, p = 0.00000178). Fecundity followed the same trend as feeding success by recording the lowest mean egg production per female in F1 and the highest in F6 and showing a significant statistical difference between the subsequent generations (R^2 = 0.808, DF = 17, p = 0.0000213). The mean insemination rate was above 56% for all generations and showed no statistical significant difference (χ^2 (20) = 18.40, P = 0.56). Mean fertility varied between 80.8% and 86.3% and also showed no significant difference (χ^2 (85) = 92.28, P = 1.00).

Table 3.2: Mean percentages of feeding success, fecundity, insemination rates and fertility of adult laboratory reared females of an *An. funestus* colony selected for indirect blood feeding administered through Parafilm-M® membrane over six subsequent generations (F1 to F6)

Colony generation	Feeding success ± SD	Fecundity ± SD	Insemination rate ± SD	Fertility ± SD
F1	46.7 ± 8.33[a]	9.79 ± 3.68[a]	60.0 ± 10.0[a]	86.0 ± 4.43[a]
F2	50.00 ± 2.00[a]	8.83 ± 2.18[a]	60.0 ± 20.0[a]	84.1 ± 1.68 [a]
F3	53.3 ± 8.33[a]	10.7 ± 1.19[a]	63.3 ± 5.77[a]	82.8 ± 3.8 [a]
F4	61.3 ± 3.06[b]	15.4 ± 0.96[a]	66.7 ± 5.77[a]	84.7 ± 1.38 [a]
F5	71.3 ± 3.06[c]	19.0 ± 1.47[b]	56.7 ± 20.8[a]	80.8 ± 1.14 [a]
F6	82.7 ± 3.06[d]	21.9 ± 1.94[b]	63.3 ± 11.5[a]	86.3 ± 2.07 [a]

Different superscript letters within a column indicate statistically significant difference ($p < 0.05$)

3.4 Discussion

Several artificial feeding systems have been developed, and their efficacy differs with respect to blood meal source and membrane type (Damiens *et al.*, 2013; Sampiano *et al.*, 2016; Mamai *et al.*, 2017). This study investigated the possibility of adapting membrane feeding using cow blood that is easily available from local abattoirs and thus can be readily obtained if needed in large quantities. Various parameters including blood-feeding efficiency, the fecundity of females fed on the artificial membranes and fertility of resultant eggs were monitored after different feeding regiments.

Anaesthetised guinea pigs had significantly higher feeding success compared to both Parafilm-M® and pig intestine membranes (Table 3.1). This can be attributed to the fact that the mosquito strain used in the study is highly adapted to feeding on anaesthetised guinea pigs and may not readily feed on an artificial feeding system. For the artificial blood feeding membranes evaluated in this study, the Parafilm-M® membrane had significantly higher feeding success compared to pig intestine. This is congruent with other artificial blood feeding studies that showed relatively high feeding rates on *Anopheles* and *Aedes* species when the Parafilm-M® membrane was used (Nasirian *et al*., 2006; Gunathilaka *et al.*, 2017). Conversely, some studies on the efficacy of different artificial blood feeding membranes suggested that animal-derived membranes such as pig and calf intestine provide better feeding success than artificially derived membranes (Kasap *et al.*, 2003; Ooi *et al.*, 2005; Pothikasikorn *et al.*, 2010; Lyski *et al.*, 2011). The assumption is that animal-derived membranes' natural sensation and rough gripping surface result in high feeding success as it possibly mimics direct skin feeding (Ooi *et al.*, 2005; Lyski *et al.*, 2011).

However, feeding success alone is not sufficient in evaluating an artificial feeding system. The blood source needs to result in substantial fecundity. Therefore, another parameter investigated was the fecundity of females that blood-fed from the different feeding systems. There was significantly higher fecundity among females fed on anaesthetised guinea pigs than those fed on cow blood using either membrane (Table 3.1). This was expected as research has shown that direct skin feeding is more efficacious than indirect membrane feeding (Kasap *et al.*, 2003; Deng *et al.*, 2012).

Moreover, this colony has been selected for guinea pig feeding for almost two decades. Comparison of the fecundity of females that blood-fed on cow blood using the Parafilm-M® and pig intestine membranes showed that the Parafilm-M® membrane was a better artificial feeding membrane than pig intestine, resulting in significantly higher fecundity. There is no consensus in the literature regarding the best artificial feeding membrane between the membranes used in this study. While our result is similar to those obtained from several artificial blood feeding studies with different objectives on *An. stephensi, An. cracens* and *An. dirus* as well as *Ae. aegypti* and *Ae. albopictus* species (Tseng, 2003; Nasirian *et al*., 2006; Gunathilaka *et al.,* 2017; Phasomkusolsil *et al.,* 2019) contradicts other researchers who found low fecundity using Parafilm-M® membrane on *Aedes aegypti* and *Aedes albopictus* (Lyski *et al.*, 2011; Luo, 2014). High fecundity levels from the Parafilm-M® membrane in this study may be due to the notion that mosquitoes ingested more blood than pig intestine, considering that the same blood source was used.

Any blood meal need not only result in high egg production, the eggs need to be fertile to perpetuate the next generation. As a result, it was necessary to investigate the fertility of resultant eggs from females fed on the various feeding regiments. Relatively high fertility was recorded throughout all the treatments affirming egg viability (Table 3.1). Although there were significant differences among mosquitoes fed with cow blood and guinea pig blood in terms of feeding success and fecundity, there was no significant difference in fertility. These findings are similar to several studies where different blood sources were assessed and there was also no significant difference in fertility between the blood sources (Kasap *et al.*, 2003; Ooi *et al.*, 2005; Pothikasikorn *et al.*, 2010 Lyski *et al.*, 2011; Luo, 2014). The high fertility attained indicates the substantial viability of the eggs and the reliability of the blood sources.

An efficient way of evaluating optimisation success is feeding the colony at subsequent generations to determine if there are improvements in feeding success and egg production over successive generations. *Anopheles funestus*, was successfully reared over six consecutive generations without feeding on a live host. Furthermore, the significant increase in fecundity and feeding success over successive generations observed after the fourth generation asserted the adaptability of *An. funestus* to the feeding system (Table 3.2). Results suggest that *An. funestus* can be selected, over subsequent generations, to feed on an artificial blood feeding system successfully.

Currently, cow blood is easily accessed from local abattoirs at no cost. Logistically, the Parafilm-M® membrane is locally accessible (www.parafilm.com), stored at room temperature thus can be utilised for prolonged periods. It is also tough and can be stretched easily, making it extremely versatile in blood volume capacity. Although it is locally available, it is imported from the USA, and this affects its financial cost. Parafilm has a mean thickness of 127 ± 2µm before stretching and can be stretched three to four times its original length before breaking translating to approximately 31 to 42µm thickness. This makes it easier for mosquitoes to pierce and access blood compared to pig intestine which has a mean thickness of 73 ± 4µm. A drawback of Parafilm-M® membrane is that it is difficult to stretch to uniform thickness and easily raptures when stretched below 25µm (Cosgrove *et al.*, 1994; Luo *et al.*, 2014). Another concern is that some of the stretched parafilm became dry and brittle at the end of the feeding period. Despite these downfalls, its availability, operational ease, and high feeding success and fecundity results obtained from this study make it a valuable and suitable membrane for artificial blood feeding.

Since FANG displayed a marked preference for a non-animal based membrane, the efficacy of other non-animal based artificial blood feeding membranes such as collagen film (Hagen and Gruneweld 1990) and nylon net (Hagen and Grunewald, 1990) need to be investigated. Further work needs to be done to evaluate the effect of mosquito age on feeding success as it impacts feeding success (Nasirian *et al*., 2006). The feeding time and feeding frequency may also have an impact on feeding success; thus, further work on these aspects is recommended. The addition of phago-stimulants (compounds detected by insect chemoreceptors that stimulate insects to imbibe their meals to full engorgement) such as natural odour ligands from human skin (Ghaninia *et al.*, 2008), L-lactic acid (Sanz *et al.*, 2008) as well as adenosine triphosphate (ATP) (Galun, 1967) may improve feeding success and need to be explored. The outcome of this study and other future works could be useful in the mass production of *An. funestus* in the insectary and consequently facilitate the speedy implementation of novel vector control interventions for area-wide control of the vector in the near future.

CHAPTER 4

ASSESSMENT OF TWO LARVAL DIETS ON THE DEVELOPMENT OF A LABORATORY-REARED *AN. FUNESTUS* COLONY

4.1 Introduction

When maintaining mosquitoes under laboratory conditions, optimal larval rearing conditions relating to climate and diet are needed to ensure sustained productivity, synchronised development and adequate adult size (Benedict *et al.*, 2009; Khan, 2010; Hood-Nowotny *et al.*, 2012). Mosquito larvae are omnivorous, opportunistic aquatic feeders that feed on aquatic microbes (detritus, algae and microorganisms) to acquire nourishment for growth and accumulation of excess nutrients in the body for utilisation in later developmental stages (Gillies and De Meillon, 1968; Clements, 1992; Bond *et al.*, 2005). Larval nutrition has an impact on their development and also affects adult physiological and reproductive fitness. It affects larval developmental time, larval growth, pupae weight, metabolic storage reserve, and adult fitness (Gilles *et al.*, 2011; Bond *et al.*, 2017; Benedict *et al.*, 2020).

Research has shown that mosquito larval development requires a diet containing proteins, sugar, polyunsaturated fatty acids, sterols, vitamins and nucleotides (Khan, 2010; Gilles *et al*., 2011; Damiens *et al.*, 2014; Benedict *et al.*, 2020). It is therefore imperative that a larval diet contains all these nutritional components. Furthermore, cost, availability, and quality should be considered when selecting a larval diet (Calkins and Parker, 2005; Puggioli *et al.*, 2013; Somda *et al.*, 2017).

Diets used for laboratory mosquito culturing are not designed to imitate natural diets but typically comprise of readily accessible material that experience has proven to allow consistent rearing. These commonly fall into two categories: simple mixtures of ingredients such as yeast and liver powder that are formulated by the users and commercial formulations of multifarious composition comprising of foods such as fish-food flakes (Carvalho *et al.*, 2014), pellets, hog supplement, cereals, maize pollen and algae (Kivuyo *et al.*, 2014).

Currently, mosquito colonies at the VRL inclusive of FANG are maintained on a diet that consists of a mixture of powdered dog biscuits (West's Beeno Traditional Crunchy Biscuit Treats, Martin and Martin, South Africa) and brewer's yeast (Vital Health Foods, South Africa) prepared at a ratio of 3:1. The food was milled for approximately 30 minutes to a fine powder and stored at room

temperature before use. When using this diet, the larva are fed twice, once each in the morning and evening (Table 2.1). This diet has been used successfully for more than 30 years. In contrast, the Insect Pest Control Laboratory (IPCL) of the Food and Agricultural Organisation (FAO)/International Atomic Energy Authority (IAEA) Agriculture and Biotechnology Laboratories, Seibersdorf, Austria, previously maintained their mosquito colonies on a liquid larval diet consisting of tuna meal (T.C. Union Agrotech, Thailand), bovine liver powder (MP Biomedicals, U.S.A) and vitamin mix (Bio-Serv, U.S.A.). This was later modified by replacing the bovine liver powder with soldier fly larvae powder (Mamai *et al.,* 2019). This diet (hereafter referred to as the IAEA diet) is designed to adequately supply all necessary components for larval growth, including fatty acids, proteins, sugars and vitamins. Larvae are fed only once a day when using this diet, making it operationally easier to use than the VRL diet. The IAEA diet has been used to rear several *Anopheles* and *Aedes* colonies (Damiens *et al.*, 2014; Mamai *et al.*, 2017; Mamai *et al.*, 2018), affirming the adequacy of its nutritional value and quality.

Although the IAEA diet has been used to rear several anophelines, it has never been used to rear *An. funestus*. This chapter compared the IAEA larval diet against the VRL diet on the development of a laboratory-reared *An. funestus* colony acronymed FANG.

4.2 Materials and methods

4.2.1 Optimisation of the IAEA larval diet on a laboratory-reared *An. funestus* colony

Before comparing the two diets, it was essential to optimise the IAEA diet on *An. funestus*. This is a critical aspect in developing standardised rearing procedures. Dose optimisation for the IAEA diet was conducted by assessing the larval development time, adult emergence and adult sizes after feeding *An. funestus* to six different doses (Table 4.1). The diet was prepared by mixing soldier fly larvae powder, tuna meal and vitamin mix at 0.50, 0.50 and 0.46% w/v respectively in deionised water. It was stored in 13 ml aliquots and frozen at -20˚C until use. Before feeding, the food was thawed in warm water and shaken to obtain a homogenous mixture. Larvae were fed on different doses of the diet depending on treatment and day of feeding as detailed in Table 4.1. For example, when 1.0 ml of the diet was fed, it resulted in a feeding rate of 0.01 ml/larva/day. Hereafter the diet dose treatments will be presented as the volume of diet provided, i.e. 1.0 ml, 1.5 ml, 2.0 ml,

2.5 ml, 3.0 ml and 3.5 ml. The feeding rate was adjusted accordingly, considering early instar mortality and pupation.

4.2.1.1 Larval development time and adult emergence

Larval developmental time and adult emergence were monitored as outlined in section 2.2.7. Newly hatched/same aged L1 larvae (n = 100/treatment/replicate over three biological repeats) were placed in larval rearing bowls (120mm width, 200mm length and 70mm height) containing 750ml deionised water translating to a larval density of 0.42/cm^2 and fed on six different doses of IAEA larval food until pupation. Each biological repeat had five technical replicates. The first biological repeat comprised four larval doses (1.0 ml, 1.5ml, 2.0ml, and 2.5 ml). The 3.0 ml and 3.5 ml doses were added on the second and third biological replicates to determine the extreme diet limiting larval development.

Table 4.1: Different IAEA larval dose treatments used to rear 100 *An. funestus* larvae in 750ml of deionised water.

	Day								
Treatment/volume of diet fed	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9 onwards**
1.0 ml	1.0	1.0	1.0	1.0	1.0	2.0	2.0	2.0	2.5
1.5 ml	1.5	1.5	1.5	1.5	1.5	2.5	2.5	2.5	3.0
2.0 ml	2.0	2.0	2.0	2.0	2.0	3.0	3.0	3.0	3.5
2.5 ml	2.5	2.5	2.5	2.5	2.5	3.5	3.5	3.5	4.0
3.5 ml	3.0	3.0	3.0	3.0	3.0	4.0	4.0	4.0	4.5
3.5 ml	3.5	3.5	3.5	3.5	3.5	4.5	4.5	4.5	5.0

4.2.1.2 Wing length measurements

Adult mosquitoes {50 adults (25 males and 25 females/treatment/replicate; over three biological repeats} were randomly selected post-emergence. These were immobilised in a refrigerator and their left-wing dissected, and its length measured. Wing length has been shown to give a useful approximation of adult mosquito body size and is routinely used as a proxy to body size (Paaijmas

et al., 2009). The wing length (Figure 4.1) was measured from the distal edge of the alula to the end of the radius vein excluding fringe scales (Damiens *et al.*, 2014) at 200X magnification using an eyepiece micrometre mounted on a dissecting microscope (OLYMPUS SZX7, Olympus America Inc., Center Valley, CA, USA). The mean wing length was compared by gender within and between the IAEA and VRL diet cohorts.

Figure 4.1: Wing length measurement of the left wing from the distal edge of the alula to the end of the radius vein excluding fringe scales (Captured by M.P Zengenene at NICD).

4.2.2 Comparison of the optimised IAEA and VRL larval diets on the development of a laboratory-reared *An. funestus* colony

Once the above optimisation studies (section 4.2.1) were completed, the most effective IAEA diet concentration (in this instance 0.025ml/larva/day i.e. 2.5 ml dose) was compared against an optimised dose of the VRL diet (Felambohangy, unpublished data). Growth and development of *An. funestus* larvae from a laboratory colony (FANG) was compared by monitoring the same parameters as described in section 4.2.1 above.

4.2.2.1 Larval development time and adult emergence

Larval developmental time and adult emergence was monitored as outlined in section 2.2.7. This was done over three biological repeats with 100 larvae /treatment/replicate, five technical replicates per biological repeat. The mean proportion of larvae surviving to pupation, developmental time of L1 larvae to pupation, proportion of pupae surviving to adulthood and time to emergence was compared between the VRL and IAEA Diets.

4.2.1.2 Wing length measurements

Wing length was monitored as outlined in section 4.2.1.2 {50 adults (25 males and 25 females)/treatment/replicate}. The mean wing length was compared by gender within and between treatments.

4.2.3 Data analysis

Data were entered and managed in Microsoft Excel then analysed using IBM SPSS Statistical software (IBM Corp., Armonk, New York), version 21. Data on larval developmental time, larval survivorship, pupal survival, and adult size (wing length) was summarised as mean larval developmental time (L1 to pupa), mean proportion of larvae surviving to pupation, mean proportion of pupae surviving to the adult stage and mean wing length respectively. Survival from larvae to pupation and pupae to adulthood was analysed using Kaplan-Meier analysis. The difference in proportion of L1 surviving to pupation and pupae surviving to adulthood between treatments was analysed using the Chi-square test. General linear modelling (GLM) was used to test the association of larval diet dose with survivorship from L1 to pupa, followed by means separation by Tukey's HSD (honest significant difference) at a 5% level of significance. Adult size (wing length) variances between the different VRL and IAEA diet cohorts were analysed using the independent samples t-test. Results were interpreted at 95% confidence. A one-sample t-test was used to to assess if emergence of adult males and females conformed to the expected one is to one ratio.

4.3 Results

4.3.1 Optimisation of the IAEA larval diet on a laboratory reared *An. funestus* colony

4.3.1.1 Larval development time and adult emergence

There was a difference in larval developmental time and proportion of first instar larvae developing into pupae between the six different doses. Larval developmental time for all treatments ranged from 17 to 23 days (Figure 4.2). Statistical analysis showed that there was a significant difference in developmental time between the 6 doses and a pairwise comparison revealed that differences between all the doses were significant (logrank test, χ^2 = 2,235.08, DF = 5, p = 0.00000748). Larvae reared with the 2.5 ml dose had the highest proportion of L1 developing through to pupa (69.87 ± 1.86%, n = 1,048) and highest pupal survivorship to adulthood (93.89 ± 0.83%, n = 984) (Table 4.2). Statistically there was a significant difference in proportion of L1 developing through to pupae (χ^2 = 1,335.49, DF = 5, p = 0.000000461). A pairwise comparison revealed two groups of doses with the same pupal productivity, i.e. the 1.5 ml dose belonged to its own group. In contrast, the second group consisted of 1.0 ml, 2.0 ml, 2.5 ml, 3.0 ml and 3.5 ml doses. Differences in pupal survivorship to adulthood were significant (χ^2 = 106.19, DF = 5, p = 0.00000803). Pairwise comparison showed two groups of doses with the same adult productivity i.e. 1.0 ml, 1.5 ml, 2.0 ml and 2.5 ml doses, belong to the same group. In contrast, the second group consisted of 3.0 ml and 3.5 ml doses. The median time taken for pupae to emerge into adults in all the diet dose treatments was two days (2 (2, 2), n = 3,747) (Table 4.2) and the difference between the doses was significant (log-rank test, χ^2 = 103.7, DF = 5, p = 0.0000297). According to the General Linear Model (Univariate Analysis of Variance), at a 5% degree of significance, diet dose accurately predicted survivorship of first instar larvae to pupation (R^2 = 0.167, DF = 5, p = 0.0000567).

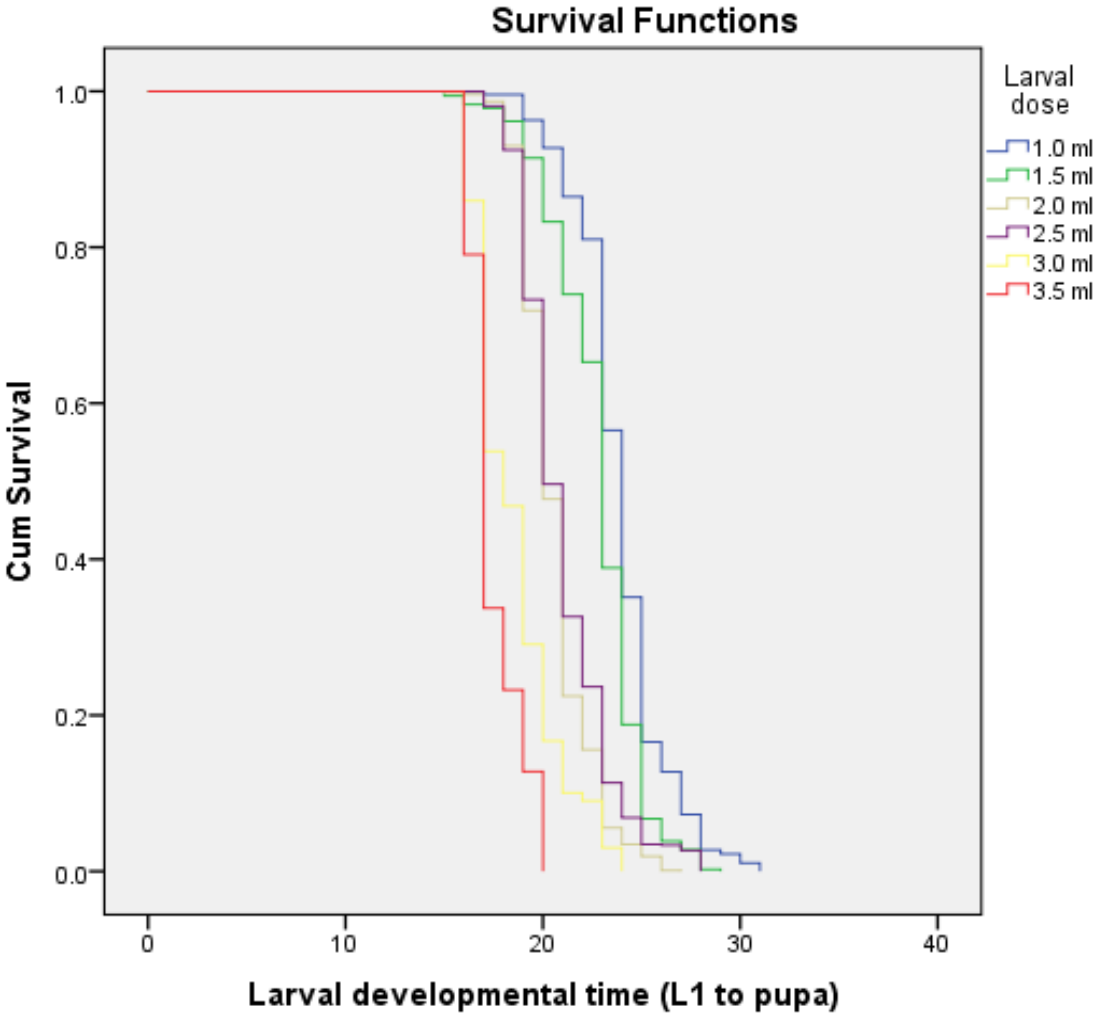

Figure 4.2: Variation of the larval developmental time (from L1 to pupae) of laboratory reared *An. funestus* larvae fed with different diet doses.

Table 4.2: Life history characteristics of colonised *An. funestus* larvae reared to adulthood using different IAEA larval diet doses

Parameter	Feeding rate					
	1.0 ml	1.5 ml	2.0 ml	2.5 ml	3.0 ml	3.5 ml
Mean larval developmental time in days ± SD	23.90 ± 0.07 [a]	22.77 ± 0.08 [b]	20.60 ± 0.06 [c]	20.97 ± 0.07 [d]	18.55 ± 0.12 [e]	17.49 ± 0.14 [f]
Mean proportion (%) of L1 developing into pupa ± SD	63.20 ± 1.51 [a]	53.33 ± 2.07 [b]	65.93 ± 1.79 [a]	69.87 ± 1.86 [a]	29.90 ± 3.06 [a]	8.60 ± 4.97 [a]
Mean proportion (%) of pupae developing to adults ± SD	88.08 ± 0.51 [a]	87.38 ± 0.52 [a]	88.27 ± 0.51 [a]	93.89 ± 0.83 [a]	92.98 ± 0.78 [b]	90.68 ± 0.69 [b]
Median time to emergence in days (interquartile range)	2.0 (2,2) [a]	2.0 (2,2) [a]	2.0 (2,2) [a]	2.0 (2,2) [a]	2.0 (2,2) [a]	2.0 (2,2) [a]
Mean wing length of males in µm ± SD	2,693.26 [a] ± 0.03	2,707.19 [a] ± 0.03	2,753.67 [a] ± 0.04	2,761.87 [a] ± 0.04	2,785.99 [a] ± 0.03	2,768.37 [a] ± 0.04

Different superscript letters within a row indicate statistically significant difference ($p < 0.05$)

4.3.1.2 Wing length measurements

The highest overall wing length was observed from the 3.0 ml dose larval diet treatment (2,789.66 ± 0.04µm, n = 150), while the lowest was reported from the 1.0 ml dose (2,565.24 ± 0.02 µm, n = 150) (Table 4.1). The wing length measurements regardless of sex were not statistically different between the different diet doses, and females were consistently larger than females for all treatments; however, the difference was not significant (One Way ANOVA, DF = 5; F = 0.81; P = 0.55).

4.3.2 Assessment of the IAEA and VRL larval diets on the development of a laboratory-reared *An. funestus* colony

Following larval diet dose optimisation (section 4.3.1), the 0.025ml/larva/day dose (2.5 ml) was selected as the optimal IAEA larval diet dose because the proportion of first instar larvae surviving to pupation and pupae surviving to adulthood were significantly higher than other doses. Furthermore, there was no adverse effect on body size recorded, further affirming its sufficiency.

4.3.2.1 Larval development time and adult emergence

The mean larval developmental time from L1 to pupation was 14.97 ± 1.29 (n = 930) and 20.08 ± 1.94 (n = 1,161) days for larvae fed on VRL diet and IAEA diets respectively (Figure 4.3). This developmental time was statistically significantly between to the two larval diets (log rank test, DF = 1, χ^2 = 1,754.34, p = 0.000031). Of the 1,500 first instar larvae reared per diet, 62.00 ± 0.47% (n = 930) larvae reared on VRL diet survived to pupation while those reared on IAEA diet had a 75.00 ± 0.28% (n = 1,125) survival rate (Table 4.3). The difference in survivorship between larval cohorts fed on the two diets was statistically significant (χ^2 = 185.06, DF = 1, p = 0.00000000451). Proportion of pupae emerging into adulthood was 98.92 ± 0.10% (n = 920) and 96.90 ± 0.18% (n =1,125) for pupae originating from larvae fed on VRL and IAEA diet respectively, the difference was statistically significant (χ^2 = 9.85, DF = 1, p = 0.001). The median time for pupae to emerge into adults was two days for both pupal cohorts (Table 4.2). The sex ratio of the resultant adults did not deviate from the 1: 1 ratio for both the VRL (one-sample t-test, t (919) = 0.07, p = 0.947) and IAEA (one-sample t-test, t (1,125) = 0.06, p = 0.953) diets.

Table 4.3: Life history characteristics of a colonised *An. funestus* strain reared under VRL and IAEA larval diets

Parameter	**VRL Diet**	**IAEA Diet**
Mean larval developmental time in days ± SD	14.97 ± 1.29[a]	20.08 ± 1.94 [b]
Mean survivorship (%) from L1 to pupa ± SD	62.00 ± 0.47 [a]	75.00 ± 0.28 [b]
Mean survivorship (%) from pupa to adult ± SD	98.92 ± 0.10 [a]	96.90 ± 0.18 [b]
Median time to emergence in days (interquartile range)	2 (2,2) [a]	2 (2,2) [a]
Sex ratio of resultant adults (females: males)	463: 456	569: 557
Mean wing length of males in µm ± SD	2,694.81 ± .0.02 [a]	2,701.31 ± 0.03 [a]
Mean wing length of females in µm ± SD	2,719.37 ± 0.02 [a]	2,703.48 ± 0.02 [a]
Mean overall wing length of males and females in µm ± SD	2,701.79 ± 0.02 [a]	2,702.39 ± 0.02 [a]

Different superscript letters within a row indicate a statistically significant difference ($p < 0.05$)

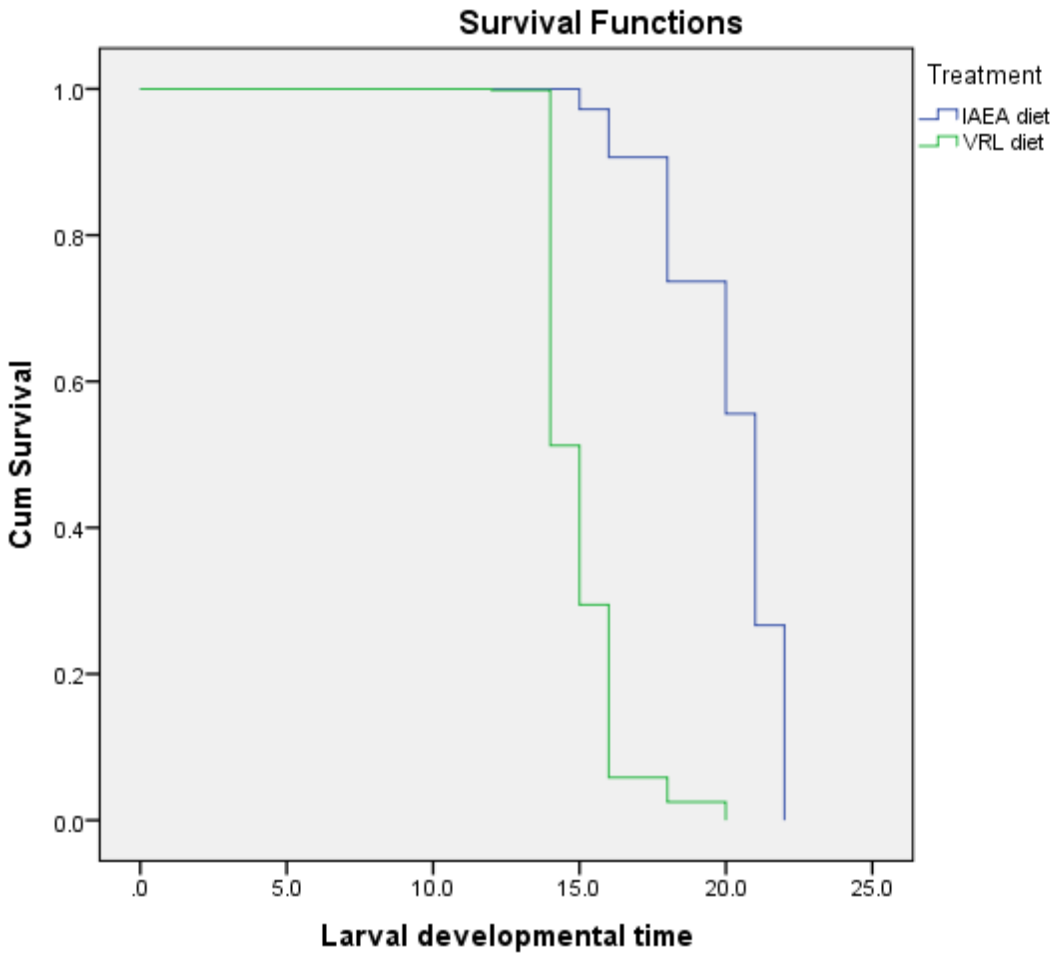

Figure 4.3: Difference in the larval developmental time from first instar larva to pupation of laboratory reared *An. funestus* larvae fed with VRL and IAEA diets.

4.3.1.2 Wing length measurements

The overall mean wing length (n = 150) for adults emerging from larvae fed on VRL and IAEA diets cohorts was 2,701.79 ± 0.02µm and 2,702.39 ± 0.02µm respectively (Table 4.2). The difference in wing sizes was not statistically significant (independent samples t-test, t = -1.58, DF = 10, p = 0.15). Splitting these results by gender showed that females emerging from larvae fed on the VRL diet had bigger wing sizes 2,719.37 ± 0.02 µm (n = 75) compared to those fed on IAEA diets (2,703.48 ± 0.02 µm, n = 75). However, this difference was not significant (independent samples t-test, t = -0.235, DF = 4, p = 0.83). Adult male mosquitoes' mean wing length sizes

followed the same trend, 2,694.81 ± 0.02 µm and 2,701.31 ± 0.03 µm for adults coming from larvae fed on VRL and IAEA diets respectively and this difference was also not statistically significant (independent samples t-test, t = -0.001, DF = 4, p = 1.00).

4.4 Discussion

This study was conducted to determine the effect of two different larval diets (IAEA and VRL diets) on several life-history traits of a laboratory *An. funestus* strain. The objective was to obtain the most effective diet for rearing *An. funestus* to aid the colonisation of this species in other geographical areas. The IAEA diet dose was first optimised by comparing various feeding regimens ranging from very low to high doses. The effect of the optimal dose on larval development was then compared to a standard dose of the VRL diet. The VRL diet has been in use for several decades for the rearing of *Anopheles* mosquito larvae at the NICD as food but has never been compared against other artificial larval diets. This diet (VRL) was recently optimised for *An. funestus* rearing (Felambohangy; Unpublished data).

The larval developmental time of *An. funestus* in the wild is approximately 15 days at about 23.4°C (Gillies and De Meillon, 1968) but this is expected to vary with temperature fluctuations, competition and food availability. On the contrary, environmental parameters under laboratory conditions are controlled and therefore constant. The developmental time is more likely going to differ due to different larval diet nutritional composition and larval rearing density, among other factors. Under the experimental conditions experienced in this study (25-27°C, ±80% relative humidity), it took between 15 to 20 days for L1 larvae to pupate in both diet treatments (Figure 4.3). This range tallies with what was reported by other researchers addressing different objectives on laboratory-reared *An. funestus* using the VRL diet (Okoye *et al.*, 2007; Lyons *et al.*, 2013; Koekemoer *et al.*, 2014) and Tetramin® fish food (Tchigossou *et al.*, 2018; Nambunga *et al.*, 2020). There is no information in the literature on how colonised *An. funestus* strains develop when sustained on an IAEA diet. Larvae fed on the VRL diet had a shorter developmental time compared to larvae fed on the IAEA diet. As reported by several researchers, nutritional reserves, particularly carbohydrates, play a regulatory role in insect development, influencing developmental time (Chambers and Klowden, 1990; Clements, 1992; Bond *et al.*, 2017). The IAEA diet contains a more substantial amount of carbohydrates, proteins, fats, and vitamins than the VRL diet (Appendix B). Therefore, the shorter larval developmental time observed from the

VRL diet could be attributed to the fact the strain used during the experiment is highly adapted to this diet. Assuming an equal diet requirement, a shorter larval developmental time would yield a shorter rearing schedule for obtaining adults and, accordingly, reduced operational cost.

Significant diet-based differences were also observed in the proportion of larvae developing from L1 to pupae and pupal survival to adulthood. Larvae fed on the IAEA diet produced significantly more larvae and pupae than the latter VRL diet cohort (Table 4.3). This may be indicative of a better nutritional value of the IAEA diet as nutrient reserves influence the ability of larvae to pupate and pupae to emerge into adults (Chambers and Klowden, 1990; Bond *et al.*, 2017). Furthermore, the IAEA diet is liquid, and the solid particulates sink to the bottom of the container leaving the water surface clearer. In this regard, it may be speculated that this diet is easily accessible to larvae as it is in a liquid state, leading to higher survival rates. High larval and pupal survival rates are desirable when rearing mosquitoes in a laboratory, as these increase the overall rate of insect production and colonisation potential.

The two larval diets that were assessed did not impact the sex ratio of the resultant adults, with each diet cohort producing a sex ratio of approximately 1:1 (Table 4.3). Generally, male mosquitoes tend to emerge before females in the wild and under laboratory settings (Clements, 1992). Any scarcity or excess nutrient value in a larval diet could preferentially influence the emergence and survival of females over males (Clements 1992; Agnew *et al.*, 2000). The sex ratio of resultant adults in the present study presumably supports the notion that both larval diets contain the sufficient nutritional components required for gender-balanced adult emergence. A balanced sex ratio is desirable when rearing mosquitoes as it promotes mating success, a critical factor for successful colonisation. Indeed, according to literature, a highly male-biased operational sex ratio is associated with sturdy competition for mates (Lambert and Slooten, 1983). The time to adult emergence was not affected by larval diet type (Table 4.3) and remained within the range (two days) observed in both wild and laboratory-reared *An. funestus* mosquitoes (Gillies and De Meillon, 1968; Okoye *et al.*, 2007; Nambunga *et al.*, 2020). According to Tun- Lin *et al.* (2000), emergence from pupae to adults occurs faster when all nutrients are abundant. Time to emergence results recorded in this study indicates adequate nutrients in both larval diets. A shorter time to emergence also shortens the rearing schedule, a desirable attribute in colony maintenance.

Anopheles wing length measurement is often used to provide a reliable indicator of body size. The composition of the diet received in the immature life stages influences the adult size and physiological fitness and has long-term effects which can be passed on to succeeding generations (Lance and McInnis, 2005; Hood-Nowotny *et al.*, 2012). In nature, because of the variable availability of resources, the adult size of mosquitoes varies depending on food source availability. The wing length, and therefore body size, of FANG adult mosquitoes in this experiment, regardless of the larval diet used, was within the normal range (Table 4.3) reported in the natural populations from different geographical areas (Gillies and De Meillon, 1968; Mwangangi *et al.,* 2004; Ayala *et al.,* 2011). Since the adult size values obtained in this study were both within the range reported in wild strains, it may be concluded that both diets provide the required nutrients that provide adequate reserves that result in the emergence of physiologically fit adults.

While mosquito development is of paramount importance when selecting a larval diet, other factors such as financial cost, accessibility, and convenience should also be taken into consideration. The cost of the IAEA diet (approximately R1,347 per kg) exceeds that of the VRL diet (R199 per kg). Also, the vitamin mix has to be imported from Europe, whereas all the ingredients of the VRL diet are locally available. However, Somda *et al* (2019) substituted the vitamin mix with brewer's yeast without compromising the quality of the diet. As a result, the vitamin mix can be substituted with brewer's yeast to reduce the cost of the IAEA diet since tuna meal and soldier fly larvae powder can be locally accessed. The IAEA diet could not be stored at room temperature or in the insectary because it gradually loses its quality under these conditions.

For this reason, the diet was stored in a refrigerator, and daily aliquots which needed to thaw in warm water before use were prepared. This made the preparation and storage of the IAEA diet inopportune in a small scale set-up, particularly for the weekend feeding of larvae. Comparatively, the VRL diet is a dry diet that can be easily prepared and stored dry at room temperature (long term storage) or under insectary conditions (for a short duration-1 week). Conversely, during large scale rearing using specially designed equipment, the IAEA diet will be more operationally easy to use. This liquid diet can be efficiently supplied to larvae using a mechanical system (Benedict *et al.,* 2009; Gilles *et al.,* 2011).

Considering that the IAEA diet is more expensive than the VRL diet and that no significant differences were observed between diets in time to adult emergence, sex ratio and adult size, as

well as the fact that the larval developmental time was significantly shorter in the VRL diet treatment, it is suggested that this standardised diet is appropriate for rearing *An. funestus*. Nevertheless, FANG has been reared using the VRL diet for nearly two decades, and it is therefore adapted to this diet. Since the IAEA diet displayed significantly higher larval and pupal survivorship and showed no significant difference in time to adult emergence, sex ratio and adult size, it is recommended to test the efficacy of this diet over successive generations to see if there will be an improvement in the larval developmental time. Assessing the impact of the modified IAEA diet (Somda *et al.,* 2019) on the development of *An. funestus* under standard insectary conditions is also recommended. The effect of these two diets on fecundity, fertility and adult longevity was not investigated in this study and need to be explored in future.

CHAPTER 5

EFFECT OF LARVAL DENSITY ON THE LIFE HISTORY TRAITS OF A COLONISED *AN. FUNESTUS* STRAIN

5.1 Introduction

The larval stage is the most likely stage at which density-dependent ramifications occur. This is because of inherent larval competition for resources, especially in larvae being bred under confined space and limited resources (Giles *et al.*, 2011). Therefore, larval density may play an important role in population size regulation (Juliano, 2009), particularly under laboratory conditions. The effects of overcrowding larvae during mosquito rearing can be experienced either physically or chemically (Roberts and Kokkinn, 2010). In some mosquito species, the effects of crowding cause the build-up of chemicals (metabolites and allelochemicals) in the rearing water resulting in retardant growth (Moore and Fisher, 1969; Roberts, 1998). Findings from other researchers could not link overcrowding with chemical factors and proposed that density effects result from physical interactions where moving larvae continually disturb each other as they collide (Roberts and Kokkinn, 2010). This, in turn, causes waves of turbulence in the feeding larvae and affects their ability to feed properly.

In laboratory experiments where food is not limiting, high mosquito larval densities have decreased developmental time in *An. gambiae* (Lyimo *et al.*, 1992). On the contrary, other researchers showed an extended developmental time in *An. stephensi*, *An. coluzzii*, *An. gambiae* and *An. arabiensis* (Muriu *et al.*, 2013; Yadav *et al.*, 2017; Epopa *et al.*, 2018; Mamai *et al.*, 2018). Therefore, the effect of crowding on larval developmental time is not well understood. Several studies elucidated that crowding larvae increases early instar mortality (Roberts and Kokkinn, 2010; Epopa *et al.*, 2018). Direct effect of larval density on adult physiological fitness is complex (Lyimo *et al.*, 1992; Schneider *et al.*, 2000). Various studies revealed that adults body size, survival, fecundity, mating success and flight capacity are lowered by high larval densities (Fisher *et al.*, 1990; Ng'habi *et al.*, 2005; Muriu *et al.*, 2013; Epopa *et al.*, 2018). For this reason, it is imperative to rear larvae at a density that is not detrimental to the larvae and the resultant adults.

Although there are numerous studies on the influence of larval density on the development of several mosquito species (Lyimo *et al.*, 1992; Yadav *et al.*, 2017; Epopa *et al.*, 2018), there is a

relative lack of information on the impact of larval density on *An. funestus*. This motivates the need for detailed studies on the effect of larval crowding on this species. Such a study will help to understand optimal larval density for *An. funestus* laboratory colonisation. Another important phenomenon is that in nature, *An. funestus* anchors on swamps and vegetation to avoid periodic flushing by heavy rainfall (Gillies and De Meillon, 1968). Under laboratory conditions, this phenomenon has been adopted through anchoring to surfaces of the rearing container (personal observation). It is speculated that this behaviour may aid the survival of *An. funestus* under crowded conditions provided there is enough anchoring surface. In this work, it was hypothesised that providing an additional anchoring surface in the container may help reduce effects of overcrowding. This chapter aimed to assess the effect of larval density on the development of a laboratory-reared *An. funestus* strain and the impact of providing an additional anchoring surface on reducing the impact of overcrowding.

5.2 Materials and methods

5.2.1 Effect of larval density on the life history traits of a laboratory reared *An. funestus* colony

Larvae were reared at four different densities (100, 200, 400 and 800) in 750ml of deionised water, at a surface area of approximately 0.42, 0.83, 1.67 and 3.33 larvae per $cm^{2,}$ respectively. Each density (treatment) had five technical replicates and repeated over three biological repeats. In detail, first instar larvae were added to 750ml of deionised water in larval rearing containers (120mm width, 200mm length and 70mm height) with an anchoring perimeter of 640mm. The larvae were fed on the VRL diet daily/larvae as described in section 2.2.7 (Table 2.1). The daily feeding rate was adjusted accordingly in the event of mortality and or pupation, such that the food quantity per larvae remained the same. Life-history traits were used to determine the effect of each larval density on the development of *An. funestus* larvae up to adulthood. The following parameters were measured: larval developmental time, larval survival (proportion of L1 developing into pupa), adult emergence (proportion of pupae developing into adults) and wing length of resultant adults using methods described under section 2.2.7. These parameters were calculated and compared between the different treatments (larval densities).

5.2.2 Impact of providing an extra anchoring surface on reducing the effects of overcrowding

Once the above larval density studies (5.2.1) were completed, a similar experiment was set up to evaluate the effect of providing an extra anchoring substance on reducing overcrowding. The anchoring surface was added to the larval density that had the most inimical overcrowding effects, in this instance 3.33 larvae/cm^2. In detail, a rectangular wax paper (www.pnp.co.za) anchoring surface (155mm length, 123mm width) bound together by a non-toxic tape was added to each rearing container comprising 3.33 larvae/cm^2 (Figure 5.1) over three biological repeats, with each biological repeat constituting five technical replicates. The anchoring surface had an outer perimeter of 556mm and an inner perimeter of 476mm, providing an additional 1,032mm of anchoring perimeter. Wax paper was chosen based on its ability to float on water, moisture resistance and ability to stay in water for a prolonged period before losing form. A control experiment was set up where 3.33 larvae/ cm^2 were reared with a total anchoring perimeter of 640mm. The impact of adding an extra anchoring surface on reducing the effects of overcrowding was assessed using parameters described in section 5.2.1 and compared with the control.

5.2.2 Data analysis

Data was summarised and analysed as described in section 4.2.3. Wing length differences between different larval density treatments were analysed using one-way ANOVA. Wing length differences between overcrowded larvae without an additional anchoring surface (control) and overcrowded larvae with a provision of an additional anchoring surface (treatment) were analysed using the independent samples t-test. Results were interpreted at 95% confidence. A one-sample t-test was used to assess if the emergence of adult males and females conformed to the expected one is to one ratio.

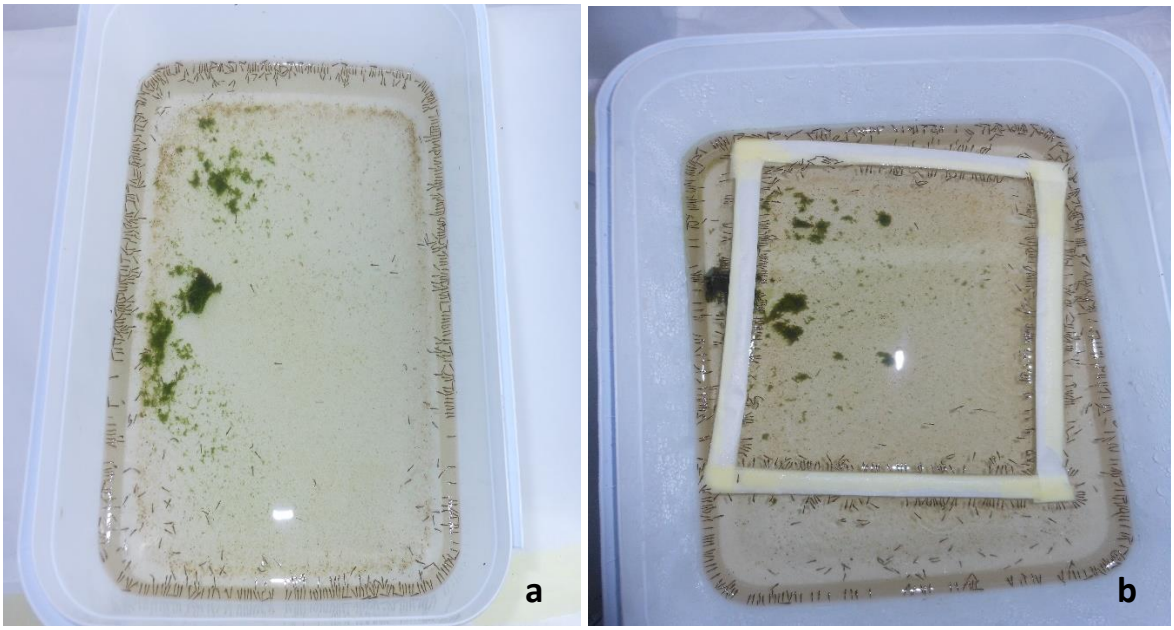

Figure 5.1: Larvae used during the assessment of the impact of adding an anchoring substance on reducing overcrowding: a = 3.33 larvae/cm^2 with a total anchoring perimeter of 640mm without an anchoring surface (Control); b = 3.33 larvae/cm^2 with a wax paper anchoring surface constituting a total of 1,672mm anchoring perimeter (Captured by M.P Zengenene at NICD).

5.3 Results

5.3.1 Effect of larval density on the life history traits of a laboratory reared *An. funestus* colony

5.3.1.1 Larval development time and adult emergence

Larval developmental time and proportion of first instar larvae developing into pupae differed between the four larval densities compared. Larval developmental time ranged from 16 to 20 days for all treatments (Figure 5.2). The 0.42 larvae/mc^2 (100 larvae per 750ml of water) larval density had the least developmental time while the 3.33 larvae/mc^2 (800 larvae per 750ml of water) density had the longest. The differences were statistically significant (log-rank test, χ^2 = 8,572.02, DF = 3, p = 0.000037). There was a positive but weak correlation between larval density and developmental time into pupation (Pearson's correlation analysis, R^2 = 0.38, DF = 22,499, p = 0.0000913).

Results on larval productivity are presented in Table 5.2. Larvae reared at a density of 0.83 larvae/cm^2 had the highest proportion of L1 developing through to pupa (94.67% ± 0.37, n = 11,360) whereas the 3.33 larvae/cm^2 density treatment recorded the lowest (60.46% ± 0.49, n = 7,256). Statistical analysis showed that the differences in the proportion of L1 developing through to pupae between the different treatments was significant (χ^2 = 864.70, DF = 6, p = 0.0000764). Further analysis using Pearson's correlation analysis showed no linear relationship between larval density and pupal productivity (R^2 = 0.03, DF = 15,787, p = 0.00000106). Pupal productivity decreased with increased larval density then tailed off. Differences in proportion of pupae emerging into adults were significant (χ^2 = 167.81, DF = 6, p = 0.00000212). A pairwise comparison revealed two groups of larval densities with the same adult productivity i.e. 0.42, 0.83 and 1.67 larvae/cm^2 belong to the same group, while the second group is comprised of the 3.33 larvae/cm^2 density. According to Pearson's correlation analysis, there was no linear relationship between proportion of pupae emerging into adults and larval density (R^2 = 0.000025, DF = 15,450, p = 0.000000019). The median time taken for pupae to emerge into adults in all the treatments was two days. After statistical analysis, results showed no significant difference in time to adult emergence between the treatments (log-rank test, χ^2 = 87.18, DF = 3, p = 0.37). The sex ratio of the resultant adults did not deviate from the expected ratio of 1:1 (Table 5.1).

Table 5.1: Statistical outcomes of the independent samples t-test to determine if the sex ratio of the resultant adults from different larval density treatments deviated from the expected 1:1 ratio

Larval density treatment	Test statistic (t) value	Degrees of freedom	p-value
0.42 larvae/cm^2	0.92	1,070	0.93
0.83 larvae/cm^2	1.77	2,173	0.17
1.67 larvae/cm^2	1.56	3,850	0.12
3.33 larvae/cm^2	0.18	6,025	0.86

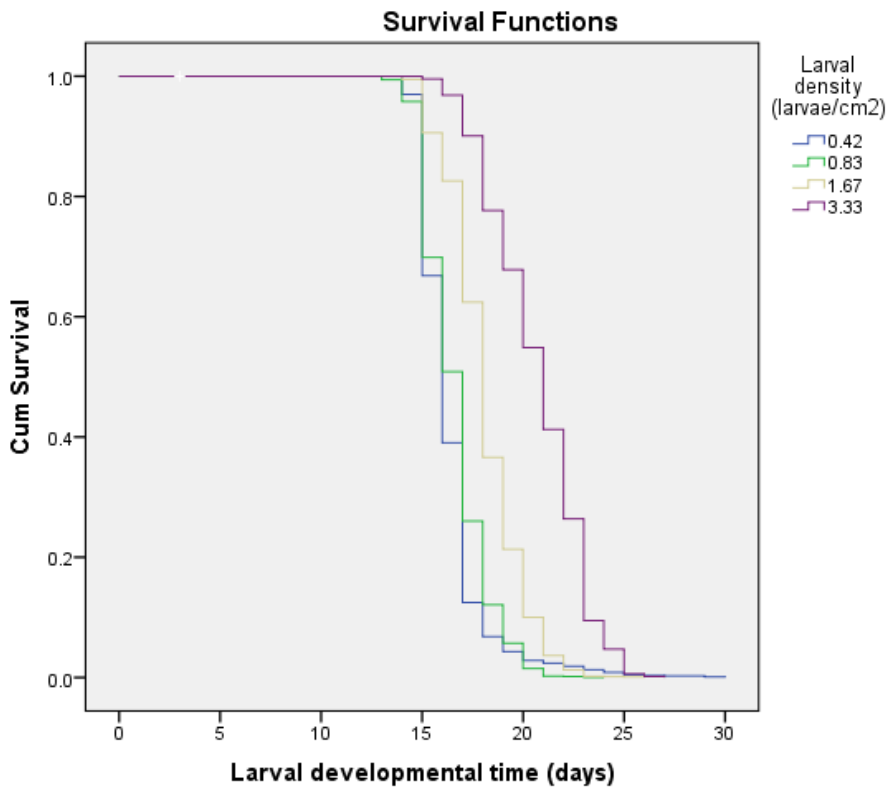

Figure 5.2: Larval developmental pattern from first instar larvae to pupation of colonised *An. funestus* larvae reared under different densities ranging from low to high.

Table 5.2: Life history characteristics of colonised *An. funestus* larvae reared under different densities to assess the effect of larval density on the development

Life history trait	Larval density			
	0.42 larvae/cm^2	**0.83 larvae/cm^2**	**1.67 larvae/cm^2**	**3.33 larvae/cm^2**
Mean larval developmental time in days ± SD	16.37 ± 1.8 [a]	16.62 ± 1.6 [b]	18.08 ± 1.8 [c]	20.69 ± 2.4 [d]
Mean proportion (%) of L1 developing into pupa ± SD	77.67 ± 0.42 [a]	94.67 ± 0.37 [b]	75.46 ± 0.43 [a]	60.46 ± 0.49 [c]
Mean proportion (%) of pupae developing to adults ± SD	91.93± 0.27 [a]	86.51 ± 0.42 [a]	85.05 ± 0.36 [a]	83.03 ± 0.38 [b]
Median time to adult emergence in days (min, max)	2.0 (1,3) [a]	2.0 (1,3) [a]	2.0 (1,2) [a]	2.0 (1,3) [a]
Mean wing length of males in µm ± SD	2,592.42[a] ± 1.10	2,598.93 [a] ± 0.72	2,306.66 [b] ± 0.15	2,303.02 [b] ± 0.34
Mean wing length of females in µm ± SD	2,641.12 [a] ± 0.34	2,647.35 [a] ± 0.36	2,328.76 [b] ± 0.64	2,321.01 [b] ± 0.19

Different superscript letters within a row indicate a statistically significant difference ($p < 0.05$)

5.3.1.2 Wing length measurements

The highest overall wing length was reported from the 0.83 larvae/cm^2 density treatment whereas the lowest was observed from the 3.33 larvae/cm^2 density. The wing length measurements irrespective of gender were statistically different between the different treatments (one-way ANOVA, F= 61.67, DF = 3, p = 0.000000344). A pairwise comparison revealed two treatment groups of adult sizes. The first group were adults coming from larvae reared at 0.42 and 0.83 larvae/cm^{2,} while the second group were adults reared at 1.67 and 3.33 larvae/cm^2. Results of the Pearson correlation indicated a linear relationship between overall adult size and larval density (R^2 = 0.71, DF = 2399, p = 0.00000747). Adult sizes showed that females were generally larger than males in all treatments (Table 5.2). However, this difference was not statistically significant (one-way ANOVA, F= 0.254, DF = 1, p = 0.62). The largest male wing length was recorded in adults emerging from larvae reared at a density of 0.83 larvae/cm^2 (2,598.93 ± 0.72 µm, n = 150), while the smallest was reported from adults originating from larvae reared at 3.33 larvae/cm^2 (2,303.02 ± 0.34 µm, n = 600). Statistically, there was a significant difference between all treatments (one-way ANOVA, F= 17.87, DF = 3, p = 0.001), and a pairwise comparison showed that there were two groups of male adult sizes depending on the larval densities. The first group consisted of adults emerging from larvae reared at a density of 0.42 and 0.83 larvae/cm^2. In contrast, larvae reared at a density of 1.67 and 3.33 larvae/cm^2 constituted the second group of adults. Wing length measurements from females followed the same trend, the larges and smallest wing sizes were recorded from 0.83 and 3.33 larvae/cm^{2,} respectively (Table 5.2).

5.3.2 Impact of providing an extra anchoring surface on reducing overcrowding

Following assessment of the effect of larval density on the life-history traits of a colonised *An. funestus* strain (section 5.3.1), larvae reared at 3.33 larvae/cm^2 were considered most affected by overcrowding. This density resulted in longer developmental time, reduced larval survival and smaller adults. Consequently, an experiment was set up where an extra anchoring surface was added to determine if this could reduce the effects of overcrowding.

5.3.2.1 Larval development time and adult emergence

The mean larval developmental time from L1 to pupation was 15.66 ± 1.67 (n = 6,719) for larvae reared without an extra anchoring surface (control) and 17.87 ± 1.83 (n = 10,530) for those reared at the same density with an extra anchoring surface (treatment) (Figure 5.3). This developmental time was statistically significantly (log rank test, χ^2 = 5,941.36, DF = 1, p = 0.000001). The proportion of larvae developing through to pupae for larvae reared under crowded conditions without an extra anchoring surface as well as those reared with an extra anchoring surface was 55.99 ± 0.50% (n = 6,719) and 87.75 ± 0.33% (n = 10,530) respectively (Table 5.3). This difference in pupal productivity was statistically significant (χ^2 = 2,993.35, DF = 1, p = 0.000000242). Proportion of pupae emerging into adults was 92.95 ± 0.23% (n = 6245) and 92.13 ± 0.28% (n = 9701) for pupae emanating from larvae reared without an extra anchoring surface and from those reared with an extra anchoring surface respectively. Statistically, this difference was not significant (χ^2 = 55.74, DF = 1, p = 0.16). The median time for pupa to emerge into adults was two days for both pupal cohorts (Table 5.3). Sex ratio of the resultant adults did not deviate from the 1: 1 ratio for both the control (one-sample t-test, t = 0.13, DF = 6,244, p = 0.99) and treatment (one-sample t-test, t = 0.11, DF = 9,700, p = 0.91).

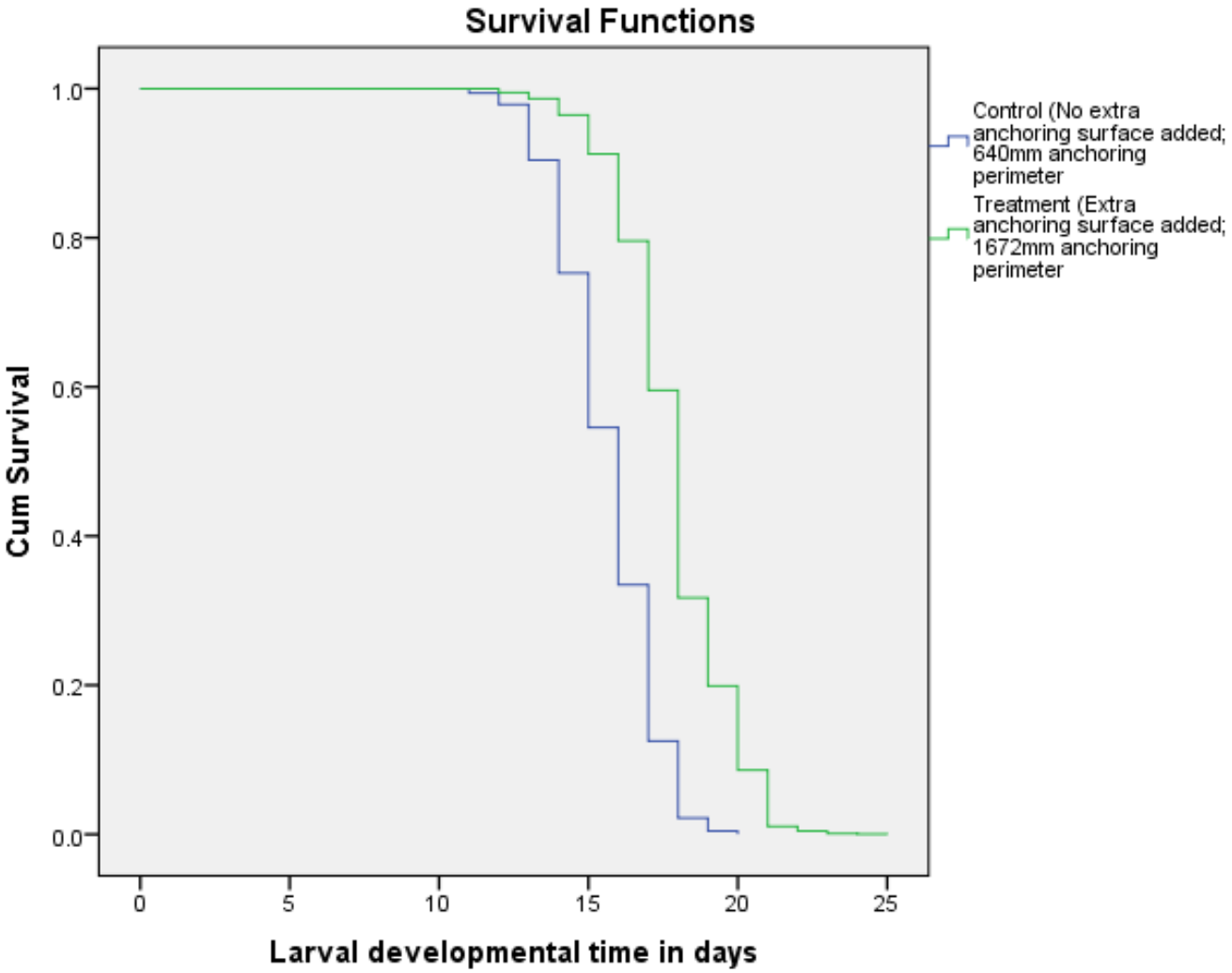

Figure 5.3: Difference in the larval developmental time from first instar larva to pupation of laboratory reared *An. funestus* larvae reared at a density of 3.33/cm^2 with and without an extra anchoring substance added to assess its effect on reducing overcrowding effects.

Table 5.3: Life history characteristics of a colonised *An. funestus* strain reared at a larval density of 3.33 larvae/cm^2 with an extra anchoring surface added to the treatment group to assess its impact on reducing overcrowding

Life history trait	**No extra anchoring surface (Control)** **640mm anchoring surface**	**Extra anchoring surface (Treatment)** **1672mm anchoring surface**
Mean larval developmental time in days ± SD	15.66 ± 1.67[a]	17.87 ± 1.83 [b]
Mean survivorship (%) from L1 to pupa ± SD	55.99 ± 0.50 [a]	87.75 ± 0.33 [b]
Mean survivorship (%) from pupa to adult ± SD	92.95 ± 0.23 [a]	92.13 ± 0.28 [a]
Median time to emergence in days (min, max)	2 (1,3) [a]	2 (1,3) [a]
Sex ratio of resultant adults (females: males)	3,127: 3,118	4,856: 4,845
Mean wing length of males in µm ± SD	2,411.85 ± 11.25 [a]	2,463.62 ± 4.46 [b]
Mean wing length of females in µm ± SD	2,505.58 ± 6.82 [a]	2,523.13 ± 4.08 [b]
Mean overall wing length of males and females in µm ± SD	2,458.72 ± 6.98 [a]	2,493.38 ± 3.37 [b]

Different superscript letters within a row indicate statistically significant difference ($p < 0.05$)

5.3.1.2 Wing length measurements

The overall mean wing length regardless of gender for adults emerging from larvae reared with an extra anchoring surface and larvae reared without an extra anchoring surface was 2,493.38 ± 3.37 µm and 2,458.72 ± 6.98 µm, respectively (Table 5.2). The difference in overall wing sizes was statistically significant (independent samples t-test, t = -4.47, DF = 2,398, p = 0.0000013). Overall, females were consistently bigger than males in both treatments. When adult sizes were split by gender, females emerging from larvae reared with an extra anchoring surface had larger wing sizes 2,523.13 ± 4.08 µm (n = 600) compared to those from larvae reared without an extra anchoring surface (2,505.58 ± 6.82 µm, n = 600), this difference was statistically significant (independent samples t-test, t = -0.207, DF = 1,198, p = 0.03). The same tendency was observed in males, 2,463.62 ± 4.46 µm (n = 600) and 2,411.85 ± 11.25 µm (n = 600) for adults from the treatment and control respectively and this difference was also statistically significant (independent samples t-test, t = -4.28, DF = 1,198, p = 0.000000634).

5.4 Discussion

This study was done to determine the effect of larval density on various life-history traits of a colonised *An. funestus* strain. The objective was to obtain the most appropriate as well the unfavourable density for *An. funestus* rearing. The information is intended to provide guidelines for colonisation of this species in other geographical areas. This was done through comparing several larval densities ranging from low to high. This was followed by evaluating the effectiveness of adding an extra anchoring surface to reduce overcrowding effects. FANG has been in laboratory colony for numerous years, but the suitable larval rearing density in routine colony maintenance has been based on supposition and experience. Also, the effect of larval density on the development of this species has never been studied.

Substantial differences in larval developmental time were observed between larvae reared at different densities. An increase in density significantly prolonged the mosquito species' developmental time (Figure 5.2). The notion that crowding may affect the development time of laboratory-reared mosquito larvae is not new. Larval developmental time may be increased in some instances as observed in *An. arabiensis* (Mamai *et al.*, 2018), *An. gambiae* (Muriu *et al.*, 2013) and *An. stephensi* (Yadav *et al.*, 2017) while being shortened in other cases as seen in *An.*

gambiae (Lyimo *et al.*, 1992). The longer larval developmental time at higher density observed in this study may be attributed to several factors such as suppressed larval daily weight gain (due to insufficient feed intake as a result of competition among the larvae), production of growth retardant chemicals (Ikeshoji and Mulla, 1970), physical disturbance caused by larval collision and increase in production of metabolic wastes (Roberts and Kokkinn, 2010) which disrupt growth. Extended time to pupation results in delayed adult eclosion (Warner and Chesson, 1985), consequently increasing the operational cost of rearing. Moreover, the complexity of the effects of prolonged developmental time is not restricted to the crowded population. Still, it may affect subsequent generations, thus negatively affecting laboratory colonisation success, particularly in mass rearing.

This study also showed a reduced proportion of first instar larvae surviving to pupation as the larval density increased (Table 5.2). The same scenario was true for pupae emerging to adults. This observation is similar to other studies done on *An. arabiensis*, *An. gambiae*, *An. coluzzii* and *An. stephensi* (Giles *et al.*, 2011; Muriu *et al.*, 2013; Yadav *et al.*, 2017; Epopa *et al.*, 2018). Additionally, *An. funestus* prefers permanent large water bodies as breeding habitats (Gillies and De Meillon, 1968) hence, it is likely to be less adaptable against overcrowding stress, presumably due to intraspecific competition due to larval crowding resulting in exhaustion of nutrients. The perception that the production of several toxic wastes by the overcrowded larvae might enhance larval mortality and hamper adult emergence cannot be ruled out (Bedhomme *et al.*, 2005). Furthermore, turbidity in the water surface due to larval waste and microbial growth may result in reduced oxygen diffusion on the water surface and mechanical hindrance of siphonal respiration, adversely affecting the survival of larvae and pupae (Asahina, 1964). High larval and pupal mortality rates are undesirable when rearing mosquitoes in a laboratory as these decrease the overall rate of insect production, negatively impacting laboratory colonisation potential and success.

Adult wing length varied depending on larval density. In summary, larvae reared at low densities produced the largest adults regardless of gender, while those reared at high densities had smaller adults emerging (Table 5.2). This is congruent with several studies that revealed a negative correlation between overcrowded larvae and adult wing size (Ng'habi *et al.*, 2005; Muriu *et al.*, 2013; Epopa *et al.*, 2018). This result indicates that rearing larvae at high densities has a negative impact on physiological fitness of the resultant adults. This in turn adversely affects the potential

of sustainable laboratory colonisation. In nature, smaller females are known to be less fecund (Clements, 1992). Low fecundity levels are unfavourable during laboratory rearing as they may lead to colony collapse. While it is not clear whether large males have better success in mating (a critical parameter in laboratory colonisation) than smaller counterparts, the conception that larger males have a more competitive advantage because of their physiological fitness could constitute reasonable speculation. In this regard, males who are larger at emergence will have an advantage. This will need to be investigated in future research.

Adults emerging from larvae reared at low and high larval densities did not show deviation from the expected male to the female sex ratio of 1:1 (Table 5.1). These observations are congruent with the findings of Mamai *et al.* (2018) were no differences in the sex ratio of *An. arabiensis* adults was observed at alternating densities. Conversely, previous reports on *An. stephensi* showed deviation from the 1:1 sex ratio at higher densities favouring males (Yadav *et al.*, 2017). According to literature, at higher densities, males pupate into low weight pupae while most females die after prolonged larval periods and try to accumulate more reserves to emerge as larger adults (Clements, 1992; Arnaldo, 2017). In this study, crowding could have increased larval mortality, which could have reduced pressure on food supply for the survivors, leading to a balanced sex ratio (a desirable trait when colonising mosquitoes).

Since laboratory colonised *An. funestus* larvae anchor on the edges of the rearing container as adoption from natural behaviour. It was hypothesised that adding an extra anchoring surface to larvae reared at high densities could increase the density of mosquitoes that can be reared per surface area. Adding an extra anchoring surface resulted in extended larval developmental time (Figure 5.3), and an increased proportion of larvae surviving to pupation as well as larger adults (Table 5.3). Shorter larval developmental time in larvae reared without extra anchoring substance could be ascribed to high early instar larval mortalities due to competition for anchoring surface, which later resulted in the availability of more food and less crowding on the remaining larvae subsequently reducing time to pupation. This was previously observed by Yadav *et al.* (2017). A significant decrease in larval mortality after adding an extra anchoring surface probably resulted from reduced competition for anchoring surface. This resulted in reduced early instar mortality which is a bottleneck during rearing larvae at high densities. The significant relationship between adult size and available anchoring surface strengthens the theory that anchoring surface is more

important than water surface area during *An. funestus* larval development. This is particularly important in mass rearing and designing equipment, where large quantities of larvae can be reared with low space requirements. Larval crowding negatively affected larval development and consequently had an impact on adult sizes. In the anchoring experiments, it can be speculated that the additional anchoring surface could have prevented the overcrowding effects leading to emergence of larger adults.

In summary, this study helped to understand the relationship between larval density and several mosquito life-history traits. Under standard laboratory conditions, density-dependent competition and alterations negatively influenced the development and physiology of *An. funestus* in ways that have consequences for successful laboratory colonisation. The addition of an extra anchoring surface presumably increased the density of mosquitoes that can be reared per surface area. This could have subsequently altered the harmful effects of overcrowding by increasing larval survival and adult sizes. It could be possible that the anchoring surface is more important than the surface area at high larval densities. This information will help to standardise the rearing of *An. funestus* under laboratory conditions in different geographical regions. Further studies on the impact of larval density on fecundity, fertility and adult longevity, exploring other materials, and the size of the extra anchoring surface feasible for laboratory rearing are needed.

CHAPTER 6

GENERAL DISCUSSION AND CONCLUSIONS

The biology of most African malaria vectors is known, and several species have successfully been colonised in different geographical areas. However, the same cannot be said for *An. funestus* (one of the most efficient vectors of malaria in humans) because it is challenging to colonise this species. The successful colonisation of local *An. funestus* populations from other geographical areas is essential to understand gene flow between different populations, particularly insecticide resistance alleles flow. Furthermore, novel vector control interventions currently under investigation, such as the Sterile Insect Technique (SIT) and gene drive, exclusively rely on the modification of colonised laboratory strains. In this work, factors that influence the successful colonisation of *An. funestus* were characterised using an already established colonised strain. The study results will be used as a reference to understand biological knowledge of this species' under standard rearing conditions that can be extrapolated to rearing and colonisation of *An. funestus* strains from other geographical regions.

The age-specific life table attributes of *An. funestus* species under laboratory conditions and their relation to colonisation showed exceptionally high female insemination for a species considered to be refractory to colonisation. Such high mating rates increase the chances of females ovipositing fertile eggs, thereby increasing the potential for colonisation. Fecundity levels were within the range reported in other laboratory colonised *Anopheles* strains. This indicates that these fecundity levels might not have deterrent effects to *An. funestus* re-colonisation prospective. High fertility rates were observed, suggesting that the species has a high re-colonisation potential. Larval survival was increased. However, development from L1 to pupation was exceptionally long. Low immature stage mortality increases the chances of successful rearing and re-colonisation. The extended developmental time under laboratory conditions had no impact on early instar mortality thus, it does not reduce the capacity to proliferate under insectary settings. The adults survived for long periods, increasing re-colonisation potential as the adults would survive long enough to reach sexual maturity and reproduce. This information on the life table history attributes of *An. funestus* under laboratory conditions helps in understanding *An. funestus* rearing biology under laboratory conditions.

The next phase of the study investigated the possibility of colonised *An. funestus* females to feed and reproduce on an artificial feeding system. Of the two membranes optimised for artificial blood feeding using a membrane feeding system, the Parafilm-M® membrane was the most effective. It was subsequently used to select for cow blood-feeding on the *An. funestus* (FANG) strain. The selection process showed that feeding rates, female fecundity and fertility of resultant eggs improved with each subsequent generation asserting the adaptability of *An. funestus* to the artificial feeding system. Considering the anthropophagic tendencies of this species, adaptability to an artificial feeding system using cow blood suggests high re-colonisation potential. Additionally, artificial blood feeding systems are less expensive and sustainable compared to the use of live animals. Further studies on the efficacy of other non-animal based membranes should be carried out since FANG exhibited a preference for Parafilm-M®, a non-animal based membrane.

The final part of this study was to investigate larval rearing conditions for colonised *An. funestus*. The study evaluated the impact of larval food and larval density on the development of larvae to adulthood. Comparison of IAEA and VRL diets clearly showed that the VRL diet is more superior from a biological and economic perspective. However, these result might not be conclusive as the strain used have been reared using the VRL diet over a decade, and it may have adapted to this diet. It is recommended to test the efficacy of the IAEA diet over successive generations to see if there will be an improvement of this diet to sustain *An. funestus* rearing. Rearing larvae at high density negatively influenced the development and physiology of *An. funestus* in ways that have consequences for successful laboratory colonisation. This indicated that low larval densities should be implored in future re-colonisation of this species. The addition of an extra anchoring surface seemingly altered the adverse effects of overcrowding. Therefore, adding an extra anchoring surface may be instrumental for reducing crowding effects in instances where overcrowding larvae cannot be avoided during laboratory colonisation. Future studies on the exploration of different materials and size of the extra anchoring surface sustainable for laboratory rearing should be considered.

In conclusion, the baseline data produced by this study suggests the high re-colonisation potential of *An. funestus* in different geographical areas under similar laboratory conditions. The data provided insight into the developmental pattern, blood-feeding, larval diet and larval density of a laboratory-reared *An. funestus* strain in the absence of environmental stressors such as interspecies

competition, predators, and insecticides exerted on natural populations. This helps in understanding *An. funestus* rearing biology under laboratory conditions.

Furthermore, *An. funestus* remains a primary malaria vector in Africa and control of malaria vectors using novel control interventions has regained interest in recent years. These approaches will require mass rearing of mosquitoes and a good knowledge of the factors that influence successful colonisation using an already colonised strain to ensure reliable production of suitable mosquitoes. Further studies on characterisation and optimisation of parameters that promote colonisation of *Anopheles funestus* are highly recommended.

APPENDICES

APPENDIX A

University of the Witwatersrand Animal Ethics Research Committee ethics waiver

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

Date: 07/05/2019

Certificate reference: Waiver 02-05-2019-O
Category: O
Applicant: Munyaradzi Prince Zengenene
Department: School of Pathology (University of the Witwatersrand)
Tel: 011 717 2424; **Email**: 1932617@students.wits.ac.za

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

This letter is to confirm that Munyaradzi Prince Zengenene, 1932617 (WITS), does not require full Animal Ethics Research Committee clearance to undertake the work titled **'Characterisation of Anopheles funestus (Diptera; Culidae) colonization parameters'.**

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

The goals of this study will be achieved by describing current rearing conditions and key biological attributes such as development times, fecundity and egg hatching rates of an An. funestus strain already under colonisation. It is anticipated that information generated will be used to optimize and standardize future colonisation for other An. funestus population. Successful development and documentation of colonisation parameters of An. funestus populations will provide gateway for new avenues into the research of several biological aspects of this important vector such as insecticide resistance. Furthermore, elucidation of An. funestus colonisation will pave way on the investigations of novel vector control interventions such as the Sterile Insect Technique (SIT) and gene drive initiatives which are premised on mass rearing of mosquitoes under laboratory conditions.

Statement of specific objectives/hypotheses
The aim of the study is to characterise and understand baseline parameters permissive to the successful colonisation of An. funestus.
Specific Objectives:
1. To determine the molecular genotypes of a laboratory An. funestus strain.
2. To characterise the life history parameters in a colonised An. funestus strain.

3. To assess the impact of different larval diets and larval density on the development of An. funestus.
4. To optimise and adapt An. funestus females to an indirect membrane blood feeding system.

The individual covered by the waiver is Munyaradzi **Prince Zengenene**.

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

APPENDIX B

Nutritional information of the IAEA and VRL larval diets

Nutrients contained in the VRL diet	Nutrients contained in the IAEA diet
Vitamin B1	Protein
Vitamin B2	Fat
Nicotinamide	Salt
Protein	Ash
Crude fat	Crude protein
Crude fibre	Amino acids
Calcium	Calcium
Phosphorus	Phosphorus
Vitamin C	Potassium
Vitamin E	Magnesium
Organic Selenium	Sodium
	Sulphur
	Copper
	Iron
	Manganese
	Zinc
	Vitamin A
	Vitamin E
	Vitamin B_{12}
	Riboflavin
	Niacinamide
	d-Pantothenic acid
	Choline
	Folic Acid
	Pyridoxine
	Thiamine
	d-Biotin
	Inositol
	Dextrose

APPENDIX C

STRICTLY CONFIDENTIAL **Re-ISSUE**

NATIONAL HEALTH LABORATORY SERVICE

ANIMAL ETHICS CLEARANCE CERTIFICATE.

CLEARANCE CERTIFICATE NUMBER: 1993 | 047

APPLICANT: DR M. COETZEE/ DR R. HUNT

DEPARTMENT/COMPANY: SAIMR/ENTOMOLOGY

PROJECT TITLE: FEEDING OF WILD TANZANIAN MOSQUITOES.

SPECIES	NUMBER	TYPE OF APPLICATION
GUINEA PIG	N/A	ROUTINE

i) Approval is hereby given for the experiment/routine procedure described in the above application.

The use of these animals is subject to the National Code 1990 Guidelines as used by the NHLS AEC. If an application for a routine procedure then the recommended guide lines or SOP must be followed. It is limited to the procedure specified in the application form and to:

SIGNED---------- DATE: 24.05.2007
(Chairperson: Animal Ethics Committee)

ii) I am satisfied that the persons listed in this application are competent to perform the procedures therein, in terms of Section 23(1) (C) of the Veterinary and Para-Veterinary Professions Act (19 of 1982)

SIGNED---------- DATE: 24.05.2007
(Registered Veterinarian)

APPENDIX D

Characterization of life-history parameters of an *Anopheles funestus* (Diptera: Culicidae) laboratory strain

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ABSTRACT: The colonization of the African malaria vector *Anopheles funestus* has been hampered by inadequate knowledge of its mating and development under laboratory conditions. Life-tables are routinely used to provide baseline biological characteristics needed for colonization. This study characterized age-specific life-table attributes of an existing *An. funestus* laboratory strain to gain insight into factors that are critical for its colonization. To achieve this, the *An. funestus* laboratory strain was reared from eggs to adulthood under standard insectary conditions, monitoring and characterizing each developmental stage. The mean insemination rate of females was 74.8% with an average egg load of 67.1 eggs/female and a mean fertility of 86.7%. The mean developmental time from 1st instar larvae to pupation was 16.4 days. The mean proportion of 1st instar larvae (LI) that survived to pupation was 72.9%. On average, 78.8% of the pupae successfully eclosed as adults. The median longevity for adult males and females was 44 and 28 days, respectively. This work constitutes the first report on life-table characterization of an *An. funestus* strain. The larval developmental time was within the range reported for wild *An. funestus* while adult longevity was higher compared to survivorship observed in wild populations. These data demonstrate that the colonized *An. funestus* strain has potential to be re-colonized under standard insectary conditions. The study provides base-line information for further studies on identifying critical parameters for the maintenance of *An. funestus* under artificial conditions. ***Journal of Vector Ecology*** **46 (1): xxx-xxx. 2021.**

Keyword Index: Anopheles funestus, colonization, life table, developmental pattern, rearing conditions, malaria vector.

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