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INVESTIGATING LIFE HISTORY CHARACTERISTICS OF *ANOPHELES ARABIENSIS* INFECTED WITH *MICROSPORIDIA MB,* A *PLASMODIUM FALCIPARUM* BLOCKING SYMBIONT

Godfred Yaw Boanyah

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

DOCTOR OF PHILOSOPHY

May 2025

PLAGIARISM DECLARATION

Thesis title

Investigating life history characteristics of *Anopheles arabiensis* infected with *Microsporidia MB*, a *Plasmodium falciparum* blocking symbiont

Declaration

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Godfred Yaw Boanyah

(Student ID: 2624674)

DEDICATION

I dedicate this work to my late Mum (Deaconess Mary Smith) and Dad (Elder Andrew Kwesi Boanyah) whose immense contribution and unflinching support have brought me this far.

RESEARCH OUTPUTS FROM THIS THESIS

I. PUBLICATIONS IN INTERNATIONAL PEER-REVIEWED JOURNALS

Role: For the following manuscripts, I co-designed the study, performed the laboratory experiments, analysed the data, wrote the original draft and contributed to revived versions.

1. **Boanyah GY**, Koekemoer LL, Herren JK, Bukhari T. Effect of *Microsporidia MB* infection on the development and fitness of *Anopheles arabiensis* under different diet regimes. *Parasites & Vectors*. 2024;17(1):294.

   Impact Factor: 3.3
2. **Boanyah GY**, Koekemoer LL, Bukhari T, Herren JK. Monitoring the capacity of *Microsporidia MB* to spread through *Anopheles arabiensis* populations under laboratory conditions. *Malaria Journal* (In preparation).

II. INTERNATIONAL CONFERENCES

Role: For the following presentations, I performed the experiments, analysed the data, and prepared the slides or poster, incorporated editorial input from my supervisors and presented.

1. **Boanyah GY**, Koekemoer LL, Bukhari T, Herren JK**.** "Investigating the effect of diet on life history characteristics of *Anopheles arabiensis* infected with *Microsporidia MB*, a *Plasmodium falciparum* blocking symbiont". Animal-Microbe Symbioses, Gordon Research Seminar and Conference at Renaissance Tuscany Il Ciocco in Lucca, Italy, 17- 23 June 2023. (Oral and Poster presentation).

2. **Boanyah GY**, Koekemoer LL, Bukhari T, Herren JK. "Investigating the effect of diet on life history characteristics of *Anopheles arabiensis* infected with *Microsporidia MB*, a *Plasmodium falciparum* blocking symbiont". 2023 Malaria Gordon Research Seminar

and Conference at Rey Don Jaime Grand Hotel in Barcelona, Spain, 27 May-2 June 2023. (Poster presentation).

3. **Boanyah GY**, Koekemoer LL, Bukhari T, Herren JK. "Investigating the effect of diet on life history characteristics of *Anopheles arabiensis* infected with *Microsporidia MB*, a *Plasmodium falciparum* blocking symbiont". Anti-Vec Network meeting at Kilifi, Kenya, 26-28 February 2023. (Poster presentation).

4. **Boanyah GY**, Koekemoer LL, Bukhari T, Herren JK. "Investigating the effect of diet on life history characteristics of *Anopheles arabiensis* infected with *Microsporidia MB*, a *Plasmodium falciparum* blocking symbiont". Academic Exchange Session, 2023 SSPH+ Lugano Summer School, Swiss School of Public Health (SSPH+), Lugano, Switzerland, 21-26 August 2023. (Oral presentation).

III. LOCAL CONFERENCE

Role: For this presentation, I performed the experiments, analysed the data, and prepared the slides or poster, incorporated editorial input from my supervisors and presented.

1. **Boanyah GY,** Koekemoer LL, Bukhari T, Herren JK. "Investigating the effect of diet on life history characteristics of *Anopheles arabiensis* infected with *Microsporidia MB*, a Plasmodium falciparum blocking symbiont". South African Society of Biochemistry and Molecular Biology 2024 Congress at the Protea Hotel Ranch Resort, Polokwane, South Africa, 7- 10 July 2024 (Poster presentation).

ABSTRACT

Introduction: *Microsporidia MB* is a naturally occurring symbiont found in *Anopheles arabiensis* that blocks the transmission of the *Plasmodium* parasite without any fitness effect on the mosquito. *Microsporidia MB* proliferates in mosquito gonads leading to its high intensity, which is linked to horizontal (sexual) and vertical (transovarial) transmission from one mosquito to another. Developing this mosquito-symbiont into a control tool is highly needed to complement the core malaria control tools. How diet affects *Microsporidia MB* intensity and the *Microsporidia MB*-infected mosquitoes, leading to the identification of diet regimes for mass production of high *Microsporidia MB* intensity mosquitoes is unknown. Furthermore, how *Microsporidia MB* spread in the infected mosquito population over generations is also unknown. The first aim of this study was to investigate how diet type and quantity affect the *Microsporidia MB-An. arabiensis* life table parameters, namely, larval development and mortality, adult size and survival, as well as *Microsporidia MB* intensity in both larvae and adults. The second aim was to monitor the spread of *Microsporidia MB* in infected *An. arabiensis* populations.

Methods: The research was conducted using *Microsporidia MB*-infected and uninfected first filial generation (F_1) larvae or adults from engorged females collected from the field (G_0). The F_1 larvae were reared together (group lines, GLs) or as individual families (eggs from one female also called isofemale lines, IMLs). GLs were fed on 0.3mg/larva/day of Tetramin, GoCat, and Cerelac respectively as well as 0.07 mg/larva/day of Tetramin. Adult GLs were fed on 1% or 6% glucose and their survival was determined. IMLs, on the other hand, were fed on Tetramin 0.07 and 0.3 mg/larva/d for the larvae and 1% or 6% glucose for the adult experiments respectively. Diet choices were selected based on commonly used diets and previous literature. Finally, three colonies (biological replicates) of *Microsporidia MB*-infected mosquitoes were established from F_1 larvae and maintained until the sixth filial generation (F_6) to observe the

symbiont spread throughout the *Microsporidia MB* colony. Prevalence and intensity of *Microsporidia MB,* wing length of mosquitoes, humidity and temperature of the laboratory were recorded.

Results: *Microsporidia MB* infected *An. arabiensis* fed on Tetramin at a dose of 0.3 mg/larva/day had the quickest larval growth, greatest adult emergence, largest mosquito body size, highest prevalence, and highest density of *Microsporidia MB*. In contrast to 6% glucose, 1% glucose did not prolong the lifespan of adult *Microsporidia MB*-infected mosquitoes. *Microsporidia MB*-infected and uninfected *An. arabiensis* fed on Tetramin at a dose of 0.07 mg/larva/day and 1% glucose had similar development time and adult survival respectively, showing that the fitness benefit conferred to the mosquito host was diet-dependent. Furthermore, the results on the *Microsporidia MB* colony showed two replicates progressed to F_6 successfully while the one collapsed (died out) at F_2. Prevalence of *Microsporidia MB* increased from F_1 to F_3 generation and declined subsequently. Furthermore, the intensity of *Microsporidia MB* was not significantly different between the F_1 and F_6 in both replicates. There was a slight positive correlation between *Microsporidia MB* prevalence and temperature. The size of the male mosquitoes was not reduced across the generations while that of females fluctuated.

Conclusion: Even on restricted diets, *Microsporidia MB* did not adversely affect *An. arabiensis* developmental growth or fitness. Tetramin 0.3 mg/larva/day and 6% glucose are the best diet regimes for rearing large number of *Microsporidia MB*-infected mosquitoes. These findings are crucial for mass rearing of high-intensity *Microsporidia MB*-infected *An. arabiensis* mosquitoes in the laboratory for the purposes of experiments and field releases. Furthermore, this first successful *Microsporidia MB* colony establishment attempt shows the pattern of *Microsporidia MB* prevalence across subsequent generations, and this understanding offers a pathway for enhancing rearing protocols to sustain high *Microsporidia MB* prevalence

mosquito colonies. The maintenance of *Microsporidia MB* intensity in the mosquitoes between the starting and the last generation gives credence to the potential of the *Microsporidia MB* malaria control strategy. Lastly, the fitness of the male *Microsporidia MB*-infected *An. arabiensis* across generations observed in this study demonstrates that a male release strategy is feasible.

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LIST OF ABBREVIATIONS

An.	*Anopheles*
Ae.	*Aedes*
s.l.	*sensu lato*
s.s.	*sensu stricto*
CI	Confidence interval
COVID-19	Coronavirus disease 2019
d	Day
℃	Degree Celsius
Colonisation	Self-replicating generations of *Microsporidia MB* infected *Anopheles arabiensis*
Et al	*Et alia*
F (1,2,3,4,5 & 6)	1^{st}, 2^{nd}, 3^{rd}, 4^{th}, 5^{th} or 6^{th} Filial generation
g	gram
G_0	Wild engorged female mosquitoes from the field
GLs	Group lines
hr	Hour
IMLs	Isofemale lines
IRS	Indoor residual spraying
JAK- STAT	The Janus kinase-signal transducer and activator of transcription
L1	First instar larva
LLINs	Long-lasting insecticide-treated be nets

MB+	*Microsporidia MB* infected mosquitoes
MB	*Microsporidia MB*
MB-	*Microsporidia MB* uninfected mosquitoes
mg	Milligram
ml	Millilitres
P.	*Plasmodium*
PCR	Polymerase Chain Reaction
qPCR	Quantitative Polymerase Chain Reaction
SIT	Sterile insect technique
WHO	World Health Organization

CHAPTER 1

INTRODUCTION

1.1 MALARIA

The spread of malaria is caused by the infectious bite of female *Anopheles* mosquitoes whiles the males on the other hand, pose no threat for disease transmission (Filler *et al.*, 2006; Sinka *et al.*, 2010). The infection is caused by a parasite called *Plasmodium* (Larson, 2019). Although there are five species of *Plasmodium* parasite that can infect humans only two of these species, *Plasmodium falciparum* and *Plasmodium vivax*, are considered to be dangerous due to major medical complications associated with them (Rougeron *et al.*, 2022; Sato, 2021). *Plasmodium falciparum* causes the most lethal form of malaria, the commonest on the African continent, and is responsible for the majority of the global mortality (Bousema & Drakeley, 2011; Neveu & Lavazec, 2021; Snow, 2015). On the other hand, *P. vivax* is the most widely distributed malaria causing parasite (Bousema & Drakeley, 2011; Kevin Baird, 2013).

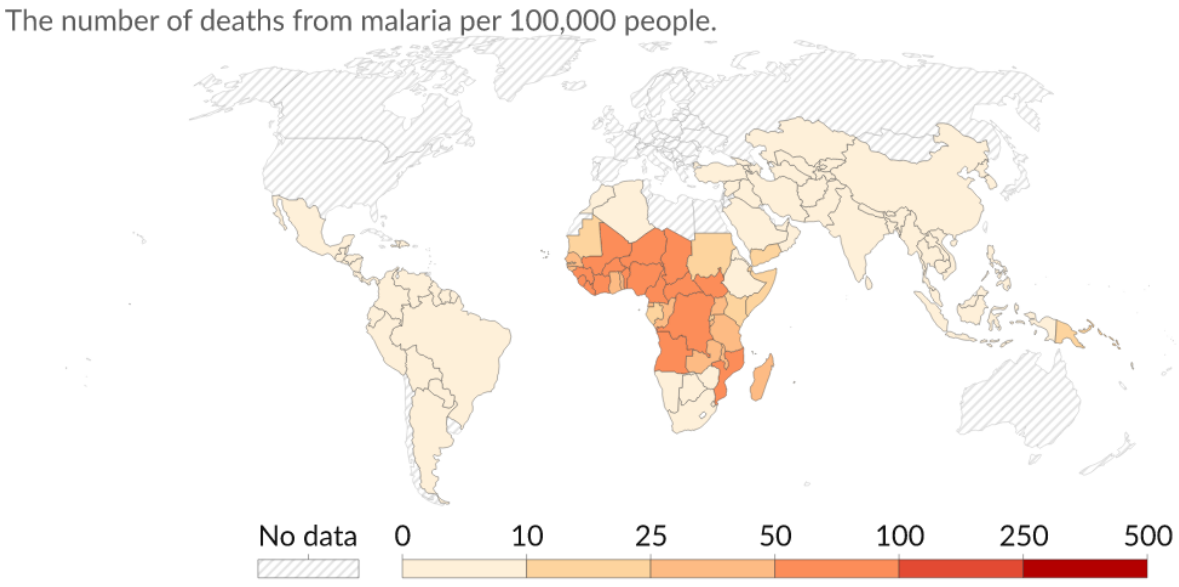


Figure 1: Death rate from malaria, 2021 [Adapted from WHO,(2021)]

Despite significant efforts and investments in malaria control, including enhancements to health systems, vector control measures, and pharmacological treatments, malaria continues to pose a significant public health challenge globally, with a particular emphasis on Africa (Wilson *et al.*, 2020; WHO, 2020). According to the 2023 world malaria report published by the World Health Organization (WHO), there were 608,000 fatalities attributed to malaria worldwide, despite the implementation of interventions by international and national malaria control programmes (WHO, 2023). The use of long-lasting insecticide-treated bed nets (LLIN) and indoor residual spraying (IRS) has led to a significant reduction in malaria transmission in recent years (Abeyasinghe *et al.*, 2012; Rek *et al.*, 2020). Notably, children were identified as the demographic group most severely impacted by this disease (WHO, 2023). The COVID-19 pandemic led to a 12% rise in malaria-related fatalities in 2020, primarily as a result of the limitations imposed on malaria control initiatives (WHO, 2021). The consequences of climate change (Giesen *et al.*., 2020; Leal Filho *et al.*., 2023) and the development of insecticide resistance in regions where the disease is prevalent (Glunt *et al.*., 2018; Hemingway *et al.*., 2016; Lindsay *et al.*., 2021) have impacted the proliferation of mosquito vectors. Furthermore, the influence of chemical insecticides (specifically LLIN/IRS) or repellents have resulted in alterations in mosquito behaviour and physiology, including shift in the time of females biting and outdoor biting, shifts in vector composition, delayed mortality, and other factors that pose a significant challenge to these WHO endorsed primary control strategies (Sougoufara *et al.*, 2020; The malERA Consultative Group on Vector Control, 2011; Wilson *et al.*, 2020). Hence, it is imperative to develop other methods for vector control.

1.2 MAIN AFRICAN MALARIA VECTORS

To determine the likelihood of malaria occurring in a certain location or region, it is necessary to take into account the presence of *Anopheles* vector populations. Out of the 465 species of *Anopheles* mosquitoes that have been formally identified, around 70 of them are capable of transmitting malaria parasites to humans (Warrell & Watkins, 2017). These are often categorised as either primary or secondary vectors via the use of a categorisation system that is not strictly defined, and it is based on the contributions that they have been measured or inferred to the transmission of malaria (Brooke, 2022). The primary vectors of malaria in Africa are classified into four taxonomic groups: the *Anopheles gambiae* complex, the *Anopheles funestus* group, the *Anopheles nili* group, and the *Anopheles moucheti* group (Sinka *et al.*, 2010, 2012). The ranges, habits, and ecological systems of these species complexes or groups are distinct from one another (Sinka *et al.*, 2010, 2012). The acquisition of malaria by blood transfusion is the one and only conceivable exception to infection through an infectious mosquito bite,, an event that is exceedingly uncommon (Mangano *et al.*, 2019).

The *Anopheles gambiae* complex also known as *Anopheles gambiae sensu lato* (*s. l.)* is responsible for the majority of the transmission of malaria in sub-Saharan Africa (Warrell & Watkins, 2017). *Anopheles gambiae senso stricto (An. gambiae sensu s.s.), An. coluzzii*, *An. arabiensis*, *An. melas*, *An. merus*, *An. bwambae*, *An. quadriannulatus*, *An. amharicus*, and *An. fontaneilli* are the nine subspecies that make up this complex. These subspecies are linked to one another and are not identifiable from one another based on their physical characteristics (Barrón *et al.*, 2019). Out of this complex, *An. gambiae s.s.*, *An. coluzzii* and *An. arabiensis* are identified as the major malaria vectors in Africa including *An. funestus* from the *An. funestus* group (Brooke, 2022).

The environmental conditions of a region have a significant impact on the distribution and abundance of these species (Oyewole *et al.,* 2006). In the driest regions, *An. arabiensis* is the most common mosquito species. It is distributed across dry areas south of the Sahara, Horn of Africa, and Namibian savannah. The larvae live in small, transitory, sunny clear and shallow fresh water ponds, stagnant and murky water bodies with or without vegetation, rice fields, and other natural and manmade environments (Ashine *et al.,* 2024; Sinka, 2013). *Anopheles arabiensis*' host preference and biting behaviour vary widely according to location, host availability, and genotypes. It is partially endophagic and exophilic because it bites indoors at night (typically between 19:00 and 03:00 h) and has early-exit behaviour regardless of its meal (Ashine *et al.*, 2024). Despite it being anthropophilic, it has a robust tendency to be zoophilic. *An. arabiensis* is becoming more prevalent in major cities as a result of the fact that the human environment is always evolving and that African cities are expanding (Dabiré *et al.,* 2012; Jones *et al.*, 2012). Furthermore, *An. arabiensis* has been reported as dominant vector for malaria transmission in Western, Eastern, Central and Southern Africa respectively (Dabiré *et al.*, 2012; Jones *et al.,* 2012; Kaiser *et al.,* 2021; Sinka *et al.,* 2010; Zanga *et al.*, 2024).

A recent investigation on the microbiota of two *Anopheles* species revealed that the predominant bacteria in both *An. funestus* and *An. arabiensis* were *Serratia oryzae* and *Elizabethkingia anopheles* (Silva *et al.,* 2021). *Anopheles arabiensis Patton* was found to harbour a symbiotic acetic acid bacterium known as *Asaia spp.* (Epis *et al.*, 2012). In addition to *An. arabiensis, Asaia spp.* has been detected in the microbiota of four other mosquito species, *An. gambiae, An. stephensi, Aedes albopictus, and Aedes aegypti* (Chouaia *et al.*, 2010). *Asaia spp.* is present in various organs of mosquitoes and is transferred either vertically or horizontally between mosquitoes (Rami *et al.,* 2018). Furthermore, this symbiont in *An.*

arabiensis modulates the immune response of the mosquito against malaria parasite and hence has been proposed as a potential vector control tool (Capone *et al.*, 2013).

1.3 MICROSPORIDIA

Cryptomycota, Microsporidia and Aphelida phyla make up the Opisthosporidia superphylum, which are basic Fungi. Despite sharing the evolutionary roots of genuine Fungus (Eumycota), sister phyla are distinguished from genuine Fungi by their common morphological characteristics, such as dual-layered chitinous spore walls and electron-dense anchoring discs (Bass *et al.*, 2018). Microsporidia differ from other Opisthosporidia phyla by having distinct organelles like the polar tube and major metabolic pathway reductions (Bass *et al.*, 2018). The phylum Microsporidia has over 1,300 identified species across 220 genera (Park & Poulin, 2021). This likely reflects just a portion of the actual variety of microsporidia. The placement of the Microsporidia lineage within the tree of life has seen significant alterations (Corradi & Keeling, 2009). The first discovered Microsporidium, *Nosema bombycis* was characterised as a yeast-like fungus and classified within the Schizomycetes, which closely aligns with the contemporary classification of Microsporidia (Corradi & Keeling, 2009). As a result of a taxonomy study that was based on physical characteristics, Microsporidia were subsequently moved to a primitive amitochondrian group termed Archezoa. This was done because of the absence of cell organelles, particularly mitochondria, in the microsporidia. The idea that Microsporidia are, in reality, close relative of Fungi has gained widespread acceptance as a result of the accumulation of molecular data (Bass *et al.*, 2018).

Microsporidia are eukaryotic microorganisms that must always remain inside their host cells (obligate intracellular microbe), the only way for them to live outside of cells is through spore

formation. Morphological identification is often based on spores, which represent the matured infectious stage of the life cycle. It is possible for some species of *Microsporidia* to generate many kinds of spores during their life cycle (Becnel *et al.*, 2005). As an example, the *Aedes aegypti* parasite *Edhazardia aedis* produces four distinct spore types (Becnel *et al.*, 2005). *Microsporidia* have a meront proliferation phase and chitinous cell wall spores that are transmitted from host to host if ingested. Horizontal transmission (mating) species have higher virulence and lower host specificity, whereas horizontal and vertical transovarial transmission species have lower virulence and higher host specificity (Zilio *et al.*, 2018).

Exploitation of the symbiotic association of inherited non-pathogenic *Microsporidia* by a major African malaria vector, *Anopheles arabiensis,* evaluated in Kenya has shown blocking potency against the transmission of the human *Plasmodium* parasite (Herren *et al.*, 2020). This microsporidium was named *Microsporidia MB* by Herren *et al.*, (2020). Moreover, a follow-up investigation showed a high intensity of *Microsporidia MB* in the gonads of *An. arabiensis*. *Microsporidia MB* are transmitted from one mosquito to another through two routes: Horizontal transmission occurs through mating and vertical transmission from mother to offspring (Herren *et al.*, 2020; Nattoh *et al.*, 2021). Vertical transmission of *Microsporidia MB* was shown to be intensity-dependent (Herren *et al.*, 2020). *Microsporidia MB* has also been found in *Anopheles coluzzii* and *An. gambiae s.s* mosquitoes (Ahouandjinou *et al.*, 2024; Akorli *et al.*, 2021; Nattoh *et al.*, 2023)**.**

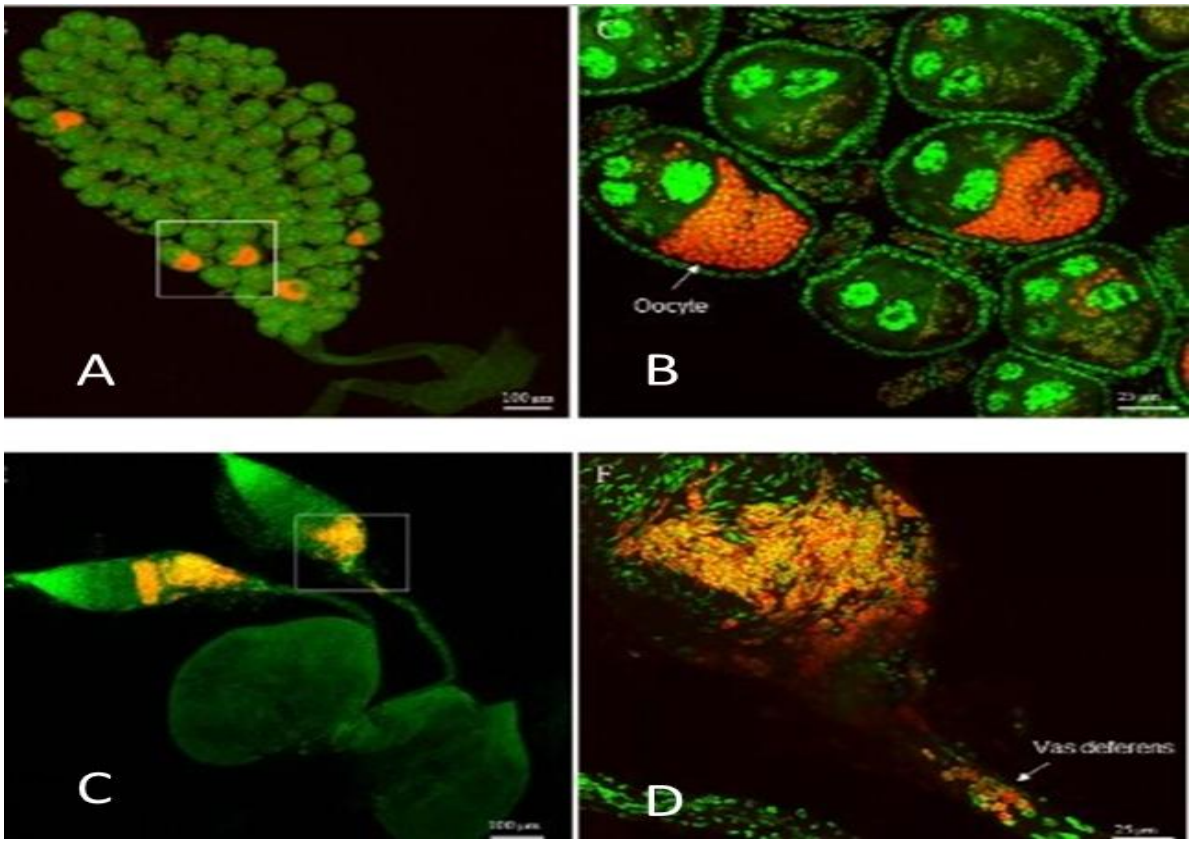


Figure 2: *Microsporidia MB* in the gonads of *An. arabiensis* under confocal microscope using Fluorescence In Situ Hybridization (FISH) (A) MB in female gonad (B) High *Microsporidia MB* intensity in the oocytes (C) *Microsporidia MB* in the male gonad (D) High *Microsporidia MB* intensity in male testes and ejaculatory duct. The images show the *Microsporidia MB* probe-CY5 FISH probe in red and the Sytox-Green general DNA stain in green (Adapted from Makhulu *et al.*, (2024).

1.4 PROBLEM STATEMENT

Several promising non-chemical-based mosquito vector control research discoveries have been made, ranging from paratransgenesis, symbiotic microbes, genetic modification approaches, sterile insect technique (SIT), and many more in the laboratory (Derua *et al.*, 2019; Kaura *et al.*, 2023; W. L. Liu *et al.*, 2022; Marshall & Vásquez, 2022; Tyagi, 2022). However, a few of these have been studied thoroughly for a complete understanding needed for operational implementation (Wilson *et al.*, 2020). SIT uses either irradiation (Ernawan *et al.*, 2022) or a genetic approach (Dilani *et al.*, 2021; Leftwich *et al.*, 2014) to render males sterile. Sterilised males are then released into the environment, where they will mate with wild females, produce infertile eggs and subsequently result in the reduction of the targeted mosquito population

(Lance & McInnis, 2020; Schetelig & Wimmer, 2011). Another example is the existence of symbiosis in mosquitoes that reduces or blocks the transmission of *Plasmodium* to humans (Herren *et al.*, 2020).

Symbiosis refers to a close physical or ecological interaction between two distinct species (Bao & Roossinck, 2013). This association can result in various outcomes for the organisms involved, including commensalism (beneficial to one species), mutualism (beneficial to both species), parasitism (detrimental to one species), or neutrality (no discernible effects) (Smith, 2017). Endosymbionts are diminutive symbiotic organisms that reside within a host organism and reside inside the host's cells (Gray, 2017; Martin *et al.*, 2022). In insects, endosymbionts play a vital role by enhancing nutritional provision and defence mechanisms (Hansen *et al.*, 2020; Waterworth *et al.*, 2020). Additionally, these endosymbionts can have positive or negative effects on the fitness of the insects (Anbutsu & Fukatsu, 2011). However, the stability and density of these symbionts within the insect host, as well as their ability to provide protective or harmful effects, are influenced by environmental factors such as temperature availability of nutrients (Doremus *et al.*, 2019; X. D. Liu *et al.*, 2019; Mouton *et al.*, 2006; B. Zhang *et al.*, 2019).

Vector-pathogen interaction can be manipulated through the symbiotic microbial association of certain microbes, leading to the prevention of transmission of disease pathogens to humans. This phenomenon of spreading symbionts in vector populations can be developed into a novel vector control strategy to mitigate their human disease transmission capacity (Bian *et al.*, 2010). *Wolbachia* is an intracellular bacterium harboured by many insects (Landmann, 2019) and is vertically or maternally transmitted. Its' adoption in *Ae. aegypti* mosquitoes successfully controlled dengue transmission (Dorigatti *et al.*, 2018). According to research, *Wolbachia*

symbionts inhibit viral replication in mosquitoes by modulating the immune system, thereby substantially reducing the prevalence of the human viral disease (dengue) (Dorigatti *et al.*, 2018; Johnson, 2015). In addition to the above, the symbiont uses a reproductive manipulative mechanism called cytoplasmic incompatibility, where the sperms of infected male mosquitoes render eggs from non-carriers females unviable after mating (Werren *et al.*, 2008). This ultimately leads to a dramatic decrease in the size of the mosquito population (Crawford *et al.*, 2020; Dobson *et al.*, 2002). The control of mosquito populations using *Wolbachia* symbionts employs two primary mechanisms: population replacement and population suppression (Alphey, 2009; Yen & Failloux, 2020). The population replacement strategy involves releasing *Wolbachia*-infected female mosquitoes that, upon mating with either infected or non-infected mosquitoes, yield viable offspring (Yen & Failloux, 2020). This facilitates the proliferation of *Wolbachia* within the field population, which contains less competent individuals for disease transmission, while the overall mosquito population size remains constant (Yen & Failloux, 2020). The goal of population suppression is to reduce the overall number of mosquitoes by releasing male mosquitoes that are infected with *Wolbachia* and, after mating with wild females, do not produce viable offspring (Beebe *et al.*, 2021; Werren *et al.*, 2008). Several mathematical models have been established that can be employed in the effective implementation of these two *Wolbachia*-based mosquito population control methods (Lim *et al.*, 2024; Yan *et al.*, 2025; Zhang & Zheng, 2022).

Globally, the method of *Wolbachia* suppression and replacement of mosquito populations has been implemented in countries such as Singapore, Thailand, Mexico, and Australia to significantly reduce arboviral diseases, including dengue and Zika (CDC, 2024). For instance, the release of *Aedes aegypti* mosquitoes infected with the *w*Mel strain of *Wolbachia pipientis* in Indonesia has demonstrated a significant protective efficacy, leading to a significant

reduction in dengue symptomatic patients and hospitalizations (Utarini *et al.*, 2021). The implementation of a *Wolbachia-Aedes aegypti* control strategy in 480,000 households in Singapore resulted in an approximately 80-90% reduction in the mosquito population (National Environmental Agency, 2022). Therefore, in 2022, Singapore increased the household sites for the field release of the symbiotic mosquitoes to an additional 100,000 due to the success of previous releases (National Enviromental Agency, 2022). Furthermore, cytoplasmic incompatibility and SIT have been used in combination with evidence of great success in mosquito population suppression in Mexico, Australia and Indonesia (Beebe *et al.*, 2021; Martín-Park *et al.*, 2022; Tantowijoyo *et al.*, 2020).

Apart from targeting the adults, it is also possible to use non-chemical-based interventions to target the larvae. The implementation of bacterial larvicides for larval source management can reduce the population density of mosquito vectors (Dambach *et al.*, 2020). This methodology employs microbial larvicides, specifically *Bacillus thuringiensis israelensis (Bti)* and *Bacillus sphaericus (Bs),* to effectively target and eliminate mosquito larvae while minimizing any adverse impact on non-target organisms (Dambach *et al.*, 2019). Bacterial larvicides were easily accepted by communities, such as in Burkina Faso (Dambach *et al.*, 2020; Derua *et al.*, 2019).

Malaria control programmes, including the Roll Back Malaria initiative, achieved notable progress with the distribution of insecticide treated bed nets in Africa, but these initiatives have encountered stagnation in recent years due to insecticide resistance and climate change among other factors (WHO, 2018; Yamey, 2004). Hence, it is important to assess the viability of potential strategies such as biological control through natural symbionts in mosquitoes for integration into currently employed strategies (Kamareddine, 2012).

1.5 RATIONALE OF STUDY

Anopheles arabiensis serves as a prominent malaria vector in Africa, including East Africa (Eligo *et al.*, 2024; Hemming-Schroeder *et al.*, 2020; Mustafa *et al.*, 2021). The occurrence of *Microsporidia MB* in the indigenous *Anopheles* mosquito population in Kenya varies from 0 to 9% (Herren *et al.*, 2020). The primary objective of the *Microsporidia MB* malaria control approach is to optimise techniques for the bulk production of *Microsporidia MB*-infected mosquitoes for future field trial releases.

However, how nutrition and environmental conditions affect the intensity and transmission of *Microsporidia MB* in infected *An. arabiensis*, are unknown. Furthermore, how this will impact the life history characteristics of *Microsporidia MB* infected mosquitoes is also unknown. Under standard laboratory mosquito rearing methods, vertical and/or horizontal transmission do not lead to 100% transmission of *Microsporidia MB* from one *An. arabiensis* to another (Nattoh *et al.*, 2021). By understanding how different mosquito diets and rearing regimes affect *Microsporidia MB* prevalence (percentage of individuals carrying *Microsporidia MB*) and intensity, and therefore transmission, optimal rearing protocols can be developed to sustain or increase the prevalence of *Microsporidi*a *MB* in infected *An. arabiensis* colonies. Mosquitoes from these infected colonies could be released into the environment to increase the natural prevalence of *Microsporidia MB* in nature and thereby reducing the spread of malaria.

1.6 AIM AND OBJECTIVES

The aim of this study was to investigate the effect of diet on the life history characteristics of *An. arabiensis* infected with *Microsporidia MB,* a *P. falciparum* blocking symbiont. ‘It was necessary to assess the effect of different diets on *Microsporidia MB* intensity in mosquito

population without compromising the mosquitoes' life table parameters and other aspects of their biology, and as well establish a *Microsporidia MB* infected mosquito colony. The outcome of this study provides a basis for the formulation of protocols for the mass rearing of *Microsporidia MB* mosquitoes. To accomplish the overarching goal, the study examined the three primary objectives in the following manner:

1) To determine the effect of diet regimes on *An. arabiensis* infected with *Microsporidia MB* as well as the effects of diet regimes on *Microsporidia MB* intensity in *An. arabiensis*.
2) To determine the effects of mosquito diet quantity after vertical transmission of *Microsporidia MB*-infected *An. arabiensis.*
3) To determine the prevalence of *Microsporidia MB* in infected *An. arabiensis* under different rearing conditions.

CHAPTER 2

EFFECT OF *MICROSPORIDIA MB* INFECTION ON THE DEVELOPMENT AND FITNESS OF *ANOPHELES ARABIENSIS* UNDER DIFFERENT DIET REGIMES

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2.1 INTRODUCTION

The nutritional adaptations and requirements of the aquatic larvae stage of *Anopheles* mosquitoes differ significantly from those of terrestrial adult-stage mosquitoes (Clements, 1992). The mouth brushes of the omnivorous scavenger larvae are used to collect suspended food particles from submerged surfaces (Clements, 1992). Mosquito larvae in the wild consume organic waste from their surroundings, namely microorganisms such as bacteria, protozoa, and algae, along with crustaceans, plant debris, and insect exuviae (Souza *et al.*, 2019). Adult mosquitoes, on the other hand, gain energy mainly from plant sources such as the nectar of flowers (Müller & Schlein, 2006). Studies have shown that the reproductive capacity of mosquitoes is affected by the type and quantity of diet that either the larval or adult mosquitoes feed on (Nayar & Sauermann, 1975; Zeller & Koella, 2016). Several studies have investigated the effect of diet on *Anopheles* mosquitoes (Carvajal-Lago *et al.*, 2021; Müller & Schlein, 2006; Zeller & Koella, 2016).

One notable finding is that a limited larval diet regime (0.2 mg/larva/day) led to prolonged development time, reduced pupation and adult emergence rates, and diminished adult female

body size in comparison to higher diet regimes (0.3 and 0.6 mg/larva/day), illustrating the influence of larval diet quantity on mosquito life history traits (Dodson *et al.*, 2011). Furthermore, the larval diet's content significantly affects both larval development and adult body size (Damiens *et al.*, 2012). A study on *An. arabiensis* found that fatty acid profiles in mosquitoes were modified by the larval diet they fed on, which controlled mosquito size, phosphorus nutrition, and population size (Hood-Nowotny *et al.*, 2012). Larval diets in nature and the laboratory contain amino acids, carbohydrates and fats essential for larval development and emergence into an adult mosquito (Timmermann & Briegel, 1999; van Schoor *et al.*, 2020). A transcription study on five-day-old female *Aedes albopictus* adult fed with two larval feeding regimes: a low quantity diet (1 mg/larva/day of Tetramin (fish food)) and a high quantity diet (2 mg/larva/day of Tetramin) indicated that the expression of specific immune genes linked to the Toll, JAK-STAT, and Imd pathways was elevated in those subjected to a high larval quantity diet (Mackay *et al.*, 2023). These pathways played very critical roles in the mosquito immune system through the modulation of cytokine receptors and T helper cells (Seif *et al.*, 2017). The inference could be that starvation suppressed gene expression of the immune system to preserve mosquito survival, and when they have optimal or high quantity of diet there is no need to decrease transcription of genes.

Adult *An. stephensi* mosquitoes, emerging from larvae that were fed on a reduced larval diet regime (0.2 mg/larva/day of Tetramin), exhibited a delay in *P. falciparum* parasite development compared to those fed on 0.6 mg/larva/day of same diet (Shapiro *et al.*, 2016). Additionally, the rate at which parasites entered the salivary glands of mosquitoes slowed down, which resulted in an extension of the amount of time it needed for mosquitoes to become infectious (Shapiro *et al.*, 2016). A separate study involving three distinct larval diets—Dr. Clarke's Pool Pellets, Tetramin Fish-Flakes, and Nishikoi Fish Pellets, with protein contents of 47%, 34%,

and 26% respectively, revealed a significantly elevated prevalence and intensity of *P. berghei* in *An. coluzzii* adults that were fed with the larval diet characterised by the lowest protein content (Nishikoi Fish Pellets) during their larval phase, thereby concluding the influence of a low-protein larval diet on the vectorial competence of adult mosquitoes (Linenberg *et al.*, 2016). However, the role of other elements might also play a role and these were not investigated by the authors. In another study, in *Anopheles darlingi*, an increase in larval feed amount was positively correlated with a higher frequency of biting, extended blood meal duration, and increased wing length, hence enhancing vectorial capacity. Anautogenous mosquitoes need blood to lay eggs. Starvation during larval growth resulted small adult mosquitoes that required numerous blood meals for egg production and consequently increased host–vector interactions and disease transmission (Mitchell-Foster *et al.*, 2012). Measuring the wing length of insects is crucial since it serves as a proxy for the determination of body size, which in turn can be used to predict fecundity, the amount of larval food consumed longevity, number of eggs laid and development time (Armbruster & Hutchinson, 2002; Gutiérrez *et al.*, 2020; Mackay *et al.*, 2023; Norry & Loeschcke, 2002)

In contrast to the studies mentioned in the aforementioned paragraph, the adult mosquitoes in nature feed on natural sugar sources from nectar that contain mainly simple carbohydrates for metabolic energy (Kessler *et al.*, 2015; Reyes *et al.*, 2021). The level of metabolic energy obtained by the mosquito through feeding affects the host-seeking and biting behaviour, which consequently impacts the vectorial capacity or disease transmission potency of the adult mosquito (Carvajal-Lago *et al.*, 2021; Zirbel & Alto, 2018). For instance, *An. gambiae* mosquitoes that previously consumed glucose exhibited a heightened biting frequency compared to those fed on a sucrose diet (Kessler *et al.*, 2015). Longevity, body size, and biting

frequency are determined by the quality and quantity of food consumed by the mosquito in its lifetime (Carvajal-Lago *et al.*, 2021).

In addition, adults fed on 10% sucrose or glucose or fructose after 48 hours of starving showed a significant expression of *p400*, piwi4 and *ppo8* genes in *Ae. aegypti* mosquitoes when compared with unfed ones (Almire *et al.*, 2021). The expression of these immune genes after the sugar feeding of the primary arbovirus vector, *Ae. aegypti*, demonstrated an enhancement in gastrointestinal tract integrity and antiviral immunity, consequently inhibiting the development of arbovirus infection and its spread to other tissues, leading ultimately to a decrease in disease prevalence (Almire *et al.*, 2021). This is evidence that sugar diet has an influence on mosquito immunity.

The influence of adult diet affects male and female mosquitoes differently (Vrzal *et al.*, 2010). For example, research showed that mosquitoes consuming nectar rich in both carbohydrates and amino acids did not increase the survival of males when compared to those fed on carbohydrate only, nonetheless, the survival rate of female mosquitoes increased by 5% (Vrzal *et al.*, 2010). Apart from the differences observed in adult survival between males and females, diet affects the microbiota in the mosquito.

Furthermore, diet was found to have a significant influence not just on the mosquito's gut microbiota, but also on the gut microbiota of other insects (Luo *et al.*, 2021; Mugo-Kamiri *et al.*, 2024). When mirid bugs, *Adelphocoris suturalis* were fed with balanced (mungbean sprout only and combined mungbean sprout and aphids) and more uniform (exclusive feeding on aphids) diet, a significant variation of gut microbiota was shown with single diet resulting in

high mortalities of bugs (Luo *et al.*, 2021). Beetles from the family Chrysomelidae that fed on generalist plants had diverse microbiota compared to specialist ones, which could simply be attributed to they picking up diversity through contact with a more diverse set of hosts and thereby demonstrating the effects of nutrition on insect symbionts (Brunetti *et al.*, 2022).

With regards to mosquito rearing in the laboratory, a sugar concentration of 5% or lower was classified as a low-stress adult diet, but that of 20% sugar is categorised as a high-stress diet with 10% sugar diet used mostly for standard laboratory adult rearing regime (Caragata *et al.*, 2016). Some other laboratories also use 6% glucose for normal mosquito maintenance (Herren *et al.*, 2020; Lambrechts *et al.*, 2006; Nattoh *et al.*, 2021).

Nutrition is well recognised as a key factor in influencing the interactions between a host and its symbiotic organisms (Herren *et al.*, 2014; Ponton *et al.*, 2014). Diet composition, for example, protein to carbohydrate ratio has been shown to significantly influence the interaction between *Wolbachia* (which are a maternally inherited bacterial endosymbionts) and it's host insect (Camacho *et al.*, 2017; Caragata *et al.*, 2016; Ponton *et al.*, 2014). This, in turn, influences *Wolbachia* intensity and its impact on the mosquito host's longevity and fertility (Whittle *et al.*, 2021). Three main processes govern the host regulation of endosymbionts. The first suggests that the symbiont intensity is frequently regulated by the dietary requirements of the host: when nutrient availability declines, symbiont intensity may increase to synthesise necessary nutrients for the host (Snyder *et al.*, 2012).

A higher intensity of *Microsporidia MB* infection in *Anopheles* mosquitoes is associated with increased vertical transmission (Herren *et al.*, 2020). Therefore, understanding the factors that

maintain high prevalence and intensity of *Microsporidia MB* in mosquito populations is essential for developing it as a malaria control tool. Additionally, the spread of *Microsporidia MB* may be influenced by its impact on host fitness in various natural environments. My research explored how different diet regimes affect *Microsporidia MB* infection parameters in *An. arabiensis* and investigated how these regimes influence life history traits of *An. arabiensis* in the presence of *Microsporidia MB*.

2.2 AIM AND OBJECTIVES

The main aim in this chapter was to determine the effect of *Microsporidia MB* infection on the development and fitness of *An. arabiensis* under different diet regimes. The three main objectives below encompass experiments to answer this aim:

1. To evaluate the effects of diet regimes on *Microsporidia MB* prevalence and intensity in *An. arabiensis* Group line (GLs).
2. To determine the effect of diet regimes on larval development, mortality, adult emergence and adult survival of *An. arabiensis* Group lines (GLs) infected with *Microsporidia MB*.
3. To determine the effects of mosquito diet quantity after vertical transmission of *Microsporidia MB* in *An*. *arabiensis* isofemale lines (IMLs).

2.3 MATERIALS AND METHODS

2.3.1 Sampling site and mosquito collection

The first filial generation (F_1) originates from engorged wild-caught, also called field-collected females (Generation zero [G_0]) within the period of 2022 and 2023 was utilized for this experiment. Wild blood-fed female *An. gambiae* complex females were collected resting

indoors using aspirators from residential dwellings in the vicinity of the Ahero (–34.9190W, –0.1661N) irrigation scheme, located in Kisumu County, Kenya. In this location, more than 97% of the *An. gambiae* complex was previously identified as *An. arabiensis* mosquitoes (Herren *et al.*, 2020). Females were morphologically identified (Coetzee, 2020).

2.3.2 Mosquito maintenance at the insectary

This experiment was undertaken at the International Centre of Insect Physiology and Ecology (icipe), Thomas Odhiambo Campus (ITOC), Kenya, from August 2023 to July 2024. The mosquitoes were transported to the laboratory located at icipe. For transportation ease, the G_0 females were placed in custom-built cages measuring 30 × 30 × 30 cm, which were covered with a damp towel. The mosquitoes had access to a 6% (w/v) glucose solution. In the laboratory setting, oviposition was induced by placing individual female mosquitoes (G_0) into 1.5 ml microcentrifuge tube. The tube was lined with filter paper and 100 µl of water was added for moisture (Nepomichene *et al.*, 2017). After oviposition, the eggs of each female were placed into water within larval trays measuring 21 × 15 × 8.5 cm (Figure 3). These trays were maintained under semi-field settings (Figure 4) until pupation. The female G_0 individuals that oviposited were identified with PCR assay, as described in section 2.3.4.(Santolamazza *et al.*, 2008). Female G_0 *samples* that were not identified as *An. arabiensis* were discarded and not utilised in the experiments. *Anopheles arabiensis* F_1 eggs were reared through to adults. Emerging F_1 adults were maintained on 6% glucose solution that was replaced every two days in 30 × 30 × 30 cm cage.



Figure 3: Larval trays with dimensions 21 × 15 × 8.5 cm



Figure 4: Screen house for maintaining mosquito larvae

2.3.3 DNA extraction and molecular species identification

DNA extraction was done using the ammonium acetate protein precipitation technique used by Herren *et al.*, (2020). One whole mosquito was homogenised in a 300 µl cell lysis solution on ice. The sample was incubated at 65°C for 2 hrs. At room temperature, 100 µl of Protein Precipitation Buffer (PPS) prepared from 8 M Ammonium acetate from 1 M stock (Sigma-

Aldrich Co., St. Louis, MO [Catalog number 09689]) and 1 mM Ethylenediaminetetraacetic acid (EDTA) from 0.5 M stock (Sigma-Aldrich Co., St. Louis, MO [Catalog number 03677]) was added and vortexed for 30 s. The sample was placed on ice for 15 mins and then centrifuged at 15,000 RPM for 15 mins. The supernatant was removed and placed in a fresh microcentrifuge tube with 300 µl absolute isopropanol, and the pellets discarded. The supernatant was thoroughly mixed by inverting gently 100 times and centrifuging at maximum speed for 1 hr. The supernatant was pipetted off, 300 µl of ice cold 70% ethanol was added and mixed by inverting several times. This was centrifuged for 30 mins and the ethanol pipetted off. The microcentrifuge tube was inverted on tissue paper overnight. The DNA was eluted with 50 µl double distilled water and left in the fridge for 3 hrs and then used or stored in -20°C. DNA extraction was performed for G_0 and F_1 samples. For G_0 samples, 2 µl was utilised for molecular species identification and 2 µl for *Microsporidia MB* screening and intensity, whereas for F_1 samples, 2 µl of DNA was used for *Microsporidia MB* screening and intensity (Section 2.3.4).

A modified method described by Santolamazza *et al.*, (2008) was used to confirm species identity. Molecular species identification was conducted only on G_0 female samples. A forward primer called LikeZianniF, 5'-TCGCCTTAGACCTTGCGTTA-3' and reverse primer called LikeZianniR, 5'-CGCTTCAAGAATTCGAGATAC-3' (Macrogen Europe, Amsterdam, the Netherlands) was used. PCR reactions were carried out in a 10 µl reaction, which contained 0.5 µl of each primer, 2 µl of Blend master mix Ready-to-load, 5 µl of Nuclease-free PCR water (Life Technologies Corporation, 2130 Woodward St. Austin, TX 78744 USA [LOT number 2107464]), and 2 µl of template DNA extracted from a single G_0 mosquito. Thermocycler conditions were 95°C for 15 mins followed by 40 cycles of 95°C for 30 s, 60°C for 45 s and 72°C for 45 s, with a final elongation at 72°C for 5 mins, and a 4°C hold. The resulting products

were analysed on 1.5% agarose gel (1.5 g of agarose in 100 ml of Tris-acetate-EDTA buffer) stained with ethidium bromide (5 µl of stock solution per 100 ml gel), with low and high molecular weight bands corresponding to fragments containing or lacking Gel score: *An. arabiensis* amplicon size =164 bp (Figure of Gel image in Appendix 1). No template control (NTC), two positive known controls, one for *An. arabiensis* and the other for *An*. *Gambiae,* were included.

2.3.4 *Microsporidia MB* screening and intensity determination

The term *Microsporidia MB* intensity in this thesis, refers to the relative abundance or density of the symbiont in the mosquito. Quantitative PCR (qPCR) was performed using DNA extracted from mosquitoes as described in section 2.3.3. The DNA samples of G_0 and F_1 were analysed to identify if they were infected with *Microsporidia MB* using specific primers (MB18SF: 5'-CGCCGG CCGTGAAAAATTTA-3' and MB18SR: 5'-CCTTGGACGTG GGAGCTATC-3') that target the *Microsporidia MB* 18S rRNA region (Herren *et al.*, 2020). The PCR detection process volume was 10 µl. This solution included 2 µl of HOT FIREPol Blend Master mix Ready-To-Load (Solis Biodyne, Estonia with Catalogue number: 04-27-00115), 0.5 µl of forward and reverse primers at a concentration of 5 pmol/µl, 2 µl of the DNA template, and 5 µl of nuclease-free water. The thermocycling protocol consisted of an initial denaturation step at 95°C for 15 minutes, followed by 35 cycles of denaturation at 95°C for 1 minute, annealing at 62°C for 90 seconds, and extension at 72°C for 60 seconds. The final autoextension was performed at a temperature of 72°C for 5 minutes. *Microsporidia MB*-positive samples underwent relative qPCR analysis to measure *Microsporidia MB* relative intensity (Herren *et al.*, 2020). The presence of *Microsporidia MB* in each sample was confirmed with a distinctive melt curve related to the *Microsporidia MB,* MB18SF/ MB18SR primers. The qPCR utilised the MB18SF/ MB18SR primers, with normalisation performed using the *Anopheles* ribosomal protein S7 gene (primers, S7F: 5'-

TCCTGGAGCTGGAGATGAAC-3' and S7R: 5'-GACGGGTCTGTACCTTCTGG-3') as the reference gene. The qPCR was performed using a MIC qPCR cycler (BioMolecular Systems, Australia). The qPCR technique was employed to determine both the prevalence and intensity of *Microsporidia MB* in the experimental mosquitoes. The following controls were used, no template control (NTC), one known *Microsporidia MB* infected (positive control), double distilled water (negative control).

2.3.5 Grouping of mosquitoes for rearing

The F_1 larvae and adults originating from confirmed *An. arabiensis* G_0 females and infected with *Microsporidia MB* were either combined (Group lines (GLs)) called MB+ GLs or reared separately (Isofemale lines (IMLs) called MB+ IMLs depending on the experiment designs (described below). Similarly, uninfected larvae and adults without *Microsporidia MB* were combined (called MB- GLs) or reared separately to serve as controls (called MB- IMLs) for the experiments. It must be noted that MB- IMLs were uninfected lines from an infected G_0 used as controls. All experiments were carried out on either F_1 larvae or adults.

2.3.6.1 Effect of *Microsporidia MB* on developmental fitness under different larval diet regimes

The selection of diet regimes was obtained based on a preliminary dose-response experiment (Dr Tullu Bukhari, *personal communication*). Four treatments or diet regimes (details given below) was used on both MB+ and MB- GLs- (Figure 5). Hence, as indicated in grouping naming in section 2.3.5, the larvae were either *Microsporidia MB*-infected (called MB+ GLs) or uninfected Group lines (MB- GLs). Every regime had three biological replicates. Each biological replicate consisted of 60 unfed 24 h-old *An. arabiensis* larvae that were placed in a larval tray (21 × 15 × 8.5 cm) filled with 1L of distilled water. There were three types of diets (Takken *et al.*, 2013): Tetramin® baby fish diet (Tetra GmbH, Germany), Cerelac® baby diet is

a powdered milk (Nestle Co. Ltd.), and GoCat® diet (Purina®, United Kingdom) that was formulated into four diet regimes. The nutritional content of each diet is available in Table 1. Two doses of Tetramin were tested i.e., 0.3 mg per larva per day (mg/larva/d) and 0.07 mg/larva/d, and one dose of Cerelac and GoCat was tested i.e., 0.3mg/larva/d. Tetramin flakes were ground to reduce the size of the flakes into granules, a routine larval-rearing practice in the laboratory (Linenberg *et al.*, 2016). Tetramin 0.3 mg/larva/d diet is the reference and served as a positive control diet regime and 0.07 mg/larva/d Tetramin diet was tested to determine how low quantity diet availability during larval development (from first instar to pupae) influences the effect of *Microsporidia MB* on mosquito life table parameters (Yan *et al.*, 2021). This is it because larval diet in nature is most likely to be a reduced quantity diet. The diet regime added to the larval trays was adjusted to the number of larvae that remained in the larval trays (There were 60 larvae per tray and hence the initial measurement was made for 60. However, as the larvae numbers reduce upon pupation or mortality, the amount of food was adjusted so that the diet regime remains fixed: Either 0.3 or 0.07 mg/larva/d). The following lifetable parameters of *Microsporidia MB* or the mosquito host were recorded for the GLs:

i) *Microsporidia MB* prevalence and intensity: Prevalence was calculated by the number of mosquitoes with *Microsporidia MB* presence divided by the total number of larvae in each regime. All the mosquitoes in each diet regime were screened for *Microsporidia MB* presence and intensity (described in section 2.3.4). Emerging adults from each treatment group were fed on 6% glucose solution *ad libitum*) and harvested on day 3 for *Microsporidia MB* screening. The three days were allocated to assess the influence of the larval diet regimes of the GLs on *Microsporidia MB* intensity, as the intensity during the larval stages (first, second, third, and fourth instar stages [L1, L2, L3, and L4]) to pupae is highly variable (Nattoh *et al.*, 2021).

ii) Larval mortality: The number of dead larvae was recorded and removed daily and data used to record larval mortality

iii) Larval developmental time: Time taken for larvae to develop from first instar to pupae in days was recorded daily.

iv) Adult emergence: The number of pupae that emerged as adults was recorded daily. Adult emergence rate was calculated using the formula below $\frac{\text{number of emerged adults}}{\text{Total number of larvae per treatment}} x\ 100$).

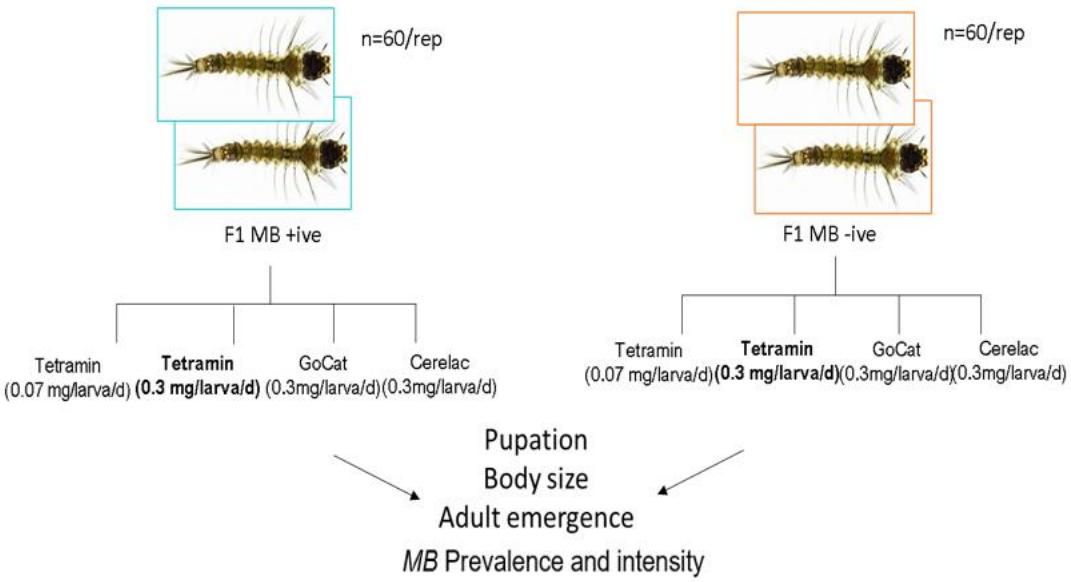


Figure 5: Experimental design to determine the effect of different larval diet regimes on larval development, mortality and adult emergence of *Microsporidia MB* infected *An. arabiensis* as well as the prevalence and intensity of *Microsporidia MB*. MB+ and MB- stands for *Microsporidia MB* infected and uninfected larvae respectively.

Table 1: Nutritional components of Tetramin, GoCat and Cerelac diets

Main Nutritional Composition of diet		
Cerelac	**GoCat**	**Tetramin**
Protein 14%	Protein 30%	Protein 46%
Fat content 9.6%	Fat content 10%	Fat content 11%
Fiber total 1.5%	Crude fibre 3%	Crude fiber 2%
Sodium 0.12		
	Linoleic acid 1.8%	
		Moisture 6%
Vitamin A, B, D & K		
	Arachidonic acid 0.1%	
		Phosphorus 1%
Zinc (trace amount)		
		Vitamin C (446 mg/kg)
		Omega-3-fatty acid 500 mg/kg

2.3.6.2 Effect of *Microsporidia MB* on adult mosquito survival under different adult diet regimes

Larvae from the MB+ GLs were reared on Tetramin 0.3 mg/larva/d for this experiment. This diet regime was selected based on results from previous experiments (Figure 5). At least 80 adults from either *Microsporidia MB* infected or uninfected GLs were used in the experiment. The adult mosquitoes (<1 day old) were divided into two treatment groups (n=40 per cage) and placed in separate cages (15 × 15 × 15 cm). In one cage, mosquitoes had *ad libitum* access to 1% glucose solution and in the second cage, mosquitoes had *ad libitum* access to 6 % glucose solution (Figure 6). The glucose solution was provided in a vial and mosquitoes were fed through a paper towel wick. The glucose vial with a paper wick was replaced every two days. The above procedure was conducted concurrently for both MB+ and MB- GLs. Daily adult mortality was recorded. The removed dead mosquitoes were preserved in 70% isopropanol and then frozen at -20°C. All the dead mosquitoes were screened for *Microsporidia MB* (section 2.3.). The prevalence and intensities of *Microsporidia MB* infection in adults (combined for

males and females) from each diet were recorded. This experiment was conducted in six biological replicates for each diet regimes

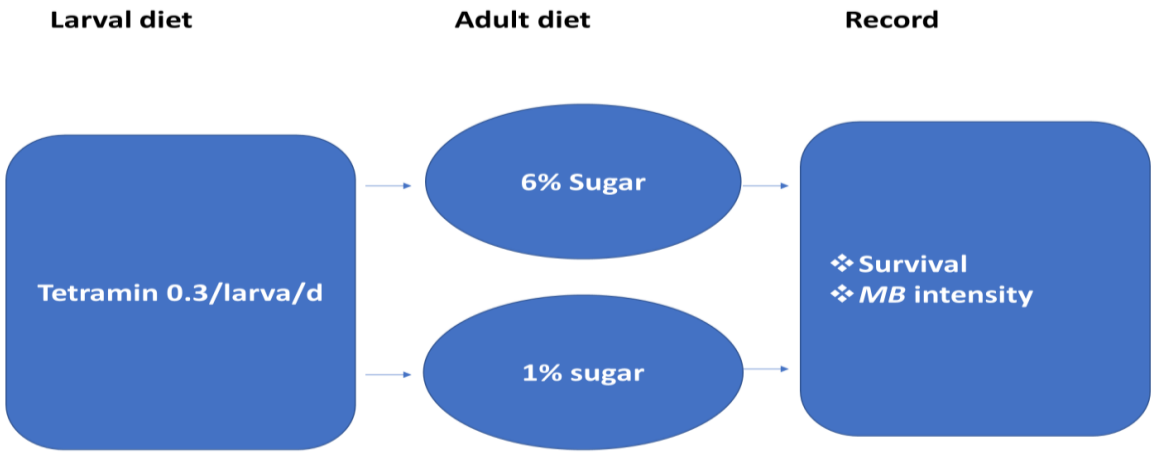


Figure 6: Experimental design to determine the effect of *Microsporidia MB* on adult mosquito survival under different adult diet regimes

2.3.7 Effect of larval and adult diet quantity on *Microsporidia MB* intensity in Isofemale lines (IMLs) after vertical transmission

To determine if the effect of larval diet (different quantities of the same diet) and adult diet on *Microsporidia MB* intensity was not influenced by the genetic background of GLs, the two experiments were conducted with isofemale lines (offspring from the same G_0) referred to as IMLs (Figure 7).

2.3.7.1 Effect of larval diet quantity on *Microsporidia MB* intensity in IMLs

For this experiment (Figure 7A), the two larval diet regimes from Tetramin diet: 0.07 mg and 0.3 mg per larva/d were used (Yan *et al.*, 2021). Larvae from IMLs (Those infected with *Microsporidia MB* called MB+ IMLs while uninfected larvae were called MB- IMLs [being uninfected lines from an infected G_0] were divided into two trays and reared on either a low quantity diet (Tetramin 0.07 gm/larva/d) or high quantity diet (Tetramin 0.3 mg/larva/d).

This experiment was carried out in nine biological replicates, with each replicate consisting of at least twenty unfed 24 h-old IMLs larvae (first instar larvae [L1s]) in a larval tray (21 × 15 × 8.5 cm) with 1 L of distilled water. The diet regime added to the larval trays was adjusted to the number of larvae that remained in the larval trays. Dead larvae were removed daily and recorded. Pupae were collected daily and placed in a 15 × 15 × 15 cm cage. The adults that emerged were fed on 6% glucose solution for three days before being harvested for the following records:

a) *Microsporidia MB* intensity: The intensity of *Microsporidia MB* was determined as described in section 2.3.4.

b) Wing lengths: Twenty adults (10 males and 10 females) were harvested on day 3 from each group and used to determine wing length as a proxy for body size (Huestis *et al.*, 2011; Maïga *et al.*, 2012). The mosquito was placed on a microscope slide under a dissecting microscope. The left wing was cut using a surgical blade and fine-tipped dissection forceps. If the left wing was damaged in the process of cutting, the right wing of the same mosquito was then used. The wing length was measured in mm from the aluta to the most apical point using a Dino-Lite® Premier handheld microscope at a magnification of 32.1 (Huatang Optical Industry Co., Ltd, Taiwan). This was done to determine the difference between MB+ and MB- IMLs males and females respectively.

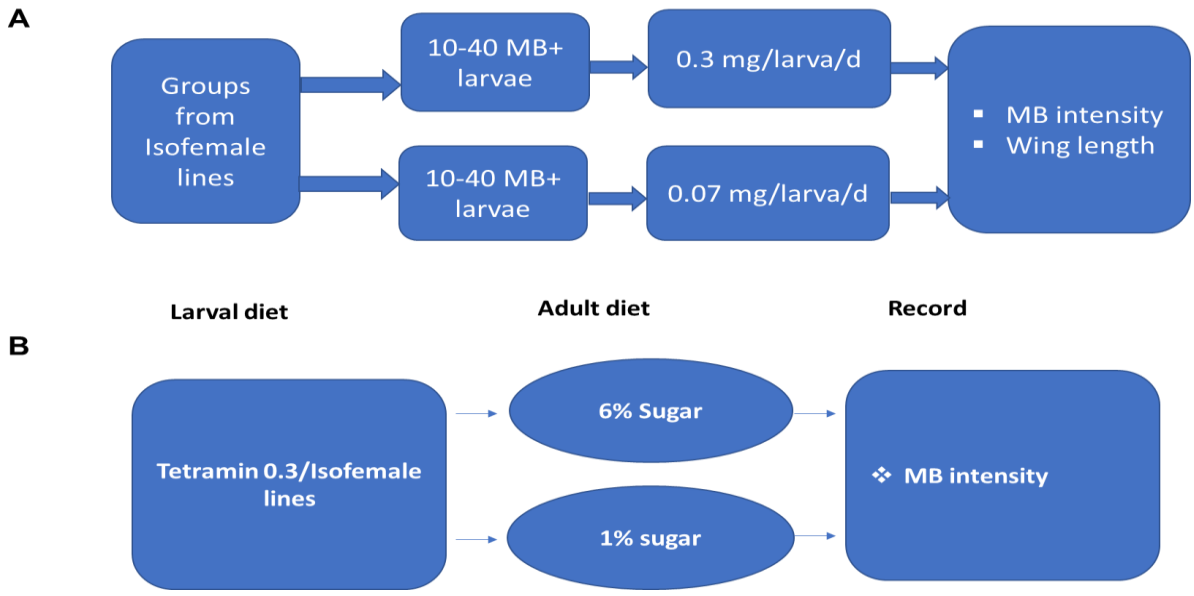


Figure 7: (A) Experimental design to determine the effect of different larval diet quantity on *Microsporidia MB* intensity in the isofemale lines of *An. arabiensis* after vertical transmission (MB+). (B) Experimental design to determine the effect of adult diet quantity on *Microsporidia MB* intensity in isofemale line of *An. arabiensis* after vertical transmission. MB+ stands for An. arabiensis infected with *Microsporidia MB*.

2.3.7.2 Effect of adult diet quantity on *Microsporidia MB* intensity in adult MB+ IMLs

Similar to larval diet quantity, this experiment aimed to determine if the effect of adult diet on *Microsporidia MB* intensity was influenced by the group lines. The adults originated from IMLs larvae that were reared on 0.3 mg Tetramin. The IMLs that produced at least 40 adults were used for this experiment (Figure 7B). The adult MB+ were split in two groups of 20 mosquitoes per cage (15 × 15 × 15 cm). Mosquitoes in one cage were fed on 1% glucose solution and in the second cage on 6 % glucose solution *ad libitum* respectively. Same was done for the adult MB- IMLs which served as controls. The glucose solution was replaced with a fresh one every two days. On day 14, the adult mosquitoes were harvested to quantify *Microsporidia MB* with qPCR (as per methods described in section 2.3.4). The 14 days is optimal for measurement of the impact of the adult diet on *MB* intensity in mosquito (Makhulu *et al.*, 2024).

2.3.8 Ethics

A research permit was sought from the National Commission for Science, Technology and Innovation, Kenya before conducting the research (Ref No. 643670). Ethical approval for the collection of mosquitoes from households was obtained from The Scientific and Ethics Review Unit-Kenya Medical Research Institute (Non-KEMRI protocol number 4520). Ethics certificate number M220622 from the University of the Witwatersrand HREC, South Africa was also obtained (Appendix 2).

2.3.9 Data analysis

Kaplan Meier survival analysis and Cox regression were used to determine the effect of different diet regimes on larval development and adult survival (Kaplan & Meier, 1992). Prevalence and adult emergence data was arcsine transformed and analysed using the Tukey's honestly significant difference (HSD) test of Analysis of Variance (ANOVA) to determine the best-fit diet regime (KendallL, 1938). Non-parametric Mann-Whitney U and Kruskal–Wallis tests (Kruskal & Wallis, 1952) were used to compare the *Microsporidia MB* intensities of the adult and larval diet treatment groups after the data did not pass the normality test (Shapiro & Wilk, 1965). Phenotypic characteristics such as length of wing was tested for significant variation within and across treatment groups using the Generalised Linear Model following gamma distribution (Nelder & Wedderburn, 1972). R software version 4.1.2 (Giorgi *et al.*, 2022) was used for analysis with a p-value < 0.05 considered significant.

2.4 RESULTS

2.4.1 Species identification of G_0 mosquitoes

The table below shows the summary of the three weeks G_0 mosquitoes collected from the field whose F_1offspring were used for all four experiments (2 for GLs and 2 for IMLs).

Table 2: G_0 mosquitoes collected from the field and the number of females confirmed to be *An. arabiensis* and *Microsporidia MB* infected.

Experiment designated for	**Number of** G_0 **mosquitoes that laid eggs (Out of a total of 900** G_0 **collected for each week)**	**Number of individuals confirmed as *An. arabiensis* (%)**	**Number of** G_0 ***An. arabiensis Microsporidia MB* infected mosquitoes (%)**
GLs for larvae	500	450 (90)	52 (11.5)
GLs for adult survival	303	300 (99)	38 (12.6)
IMLs (Both larvae and adult)	410	410 (100)	43 (10.5)

2.4.2 Effect of *Microsporidia MB* on developmental fitness under different larval diet regimes

There were two main experiments under this section as described in section 2.3.6.1 for different larval diet regimes (for MB+ GLs and MB- GLs) and 2.3.6.2 for adult diet impact on survival of MB+ GLs and MB- GLs.

2.4.2.1 Prevalence and intensity of *Microsporidia MB* under different larval diet regimes

Larval diet regimes did not affect the prevalence of *Microsporidia MB* in emerging MB+ GLs adults. There was no statistically significant difference in *Microsporidia MB* prevalence across the four diet regimes (ANOVA after arcsine transformation of the data, $F = 2.655$, df = 3, $P = 0.185$) (Figure 8A). In contrast, the intensity of *Microsporidia MB* was affected by different larval diet regimes (Kruskal Wallis test, $X^2 = 22.85$, df = 3, $P < 0.0001$). Multiple pairwise comparisons showed that three-day-old adults emerging from the larvae feeding on Tetramin 0.3 mg/larva/d had the highest intensity of *Microsporidia MB* when compared to those feeding on Tetramin 0.07 mg/larva/d ($P = 0.007$) and Cerelac 0.3 mg/larva/d ($P < 0.001$) (Figure 8B). There was no significant difference between *Microsporidia MB* intensity under Tetramin 0.3 and GoCat 0.3 mg/larva/d regimes. Furthermore, there was a significant difference between GoCat 0.3 mg/larva/d and Cerelac 0.3 mg/larva/d ($P<0.006$), or between Tetramin 0.07 mg/larva/d and Cerelac 0.3 mg/larva/d ($P > 0.254$).

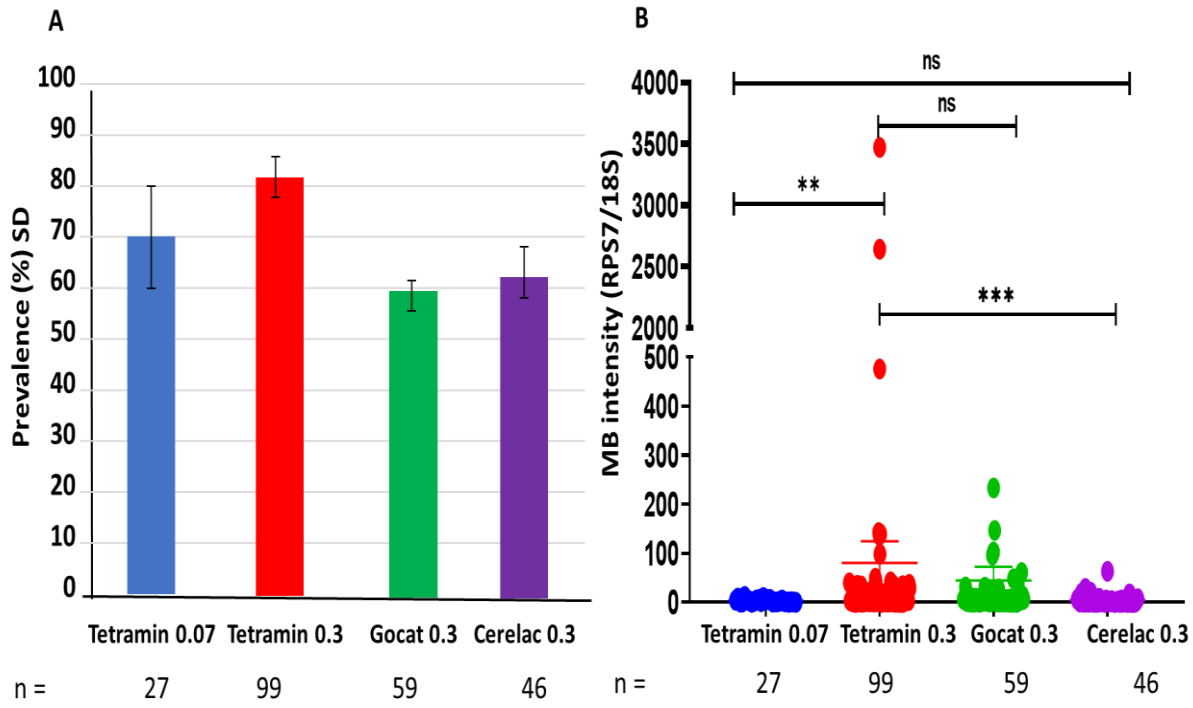


Figure 8: *Microsporidia MB* prevalence and intensity under different larval diet regimes of MB+ GLs. (A) Prevalence (%) *of Microsporidia MB* in 3-day-old F_1 adults reared on different diet regimes. Error bars represent standard deviation. (B) Relative *Microsporidia MB* intensity in 3-day-old F_1 adults reared on different diet regimes using qPCR. Bar represents significant difference between the diet regimes. The number of independent biological replicates was three with each replicate made up of 60 larvae. The corresponding average number of infected individuals are below the x-axis out of the total mosquitoes that emerged in the biological replicates of 60 each. Asterisks show the level of significance *(*P < 0.05, **P <0.001, and ***P < 0.0001)*, while ns indicates no significance.

2.4.2.2 Effect of *Microsporidia MB* on larval mortality, pupation and adult emergence under different larval diet regimes

The highest larval mortality was observed when the larvae were fed on Tetramin 0.07 mg/larva/d. The MB+ GLs cohort showed a 26% larval mortality compared to the MB- GLs (28.33% mortality) (Figure 9A). MB+ GLs larvae fed on GoCat 0.3 mg/larva/d showed a lower larval mortality of 4.45% mortality, while MB- GLs showed a 6.67% mortality. Furthermore, MB+ GLs fed on Cerelac (0.3 mg/larva/d) showed much lower larval mortality of 1.70% and MB- GLs with 2.78% mortality (Figure 9A). Tetramin 0.3 mg/larva/d resulted in a statistically significantly higher median survival with the lowest larval mortality of 1.67 % in MB+ GLs

larvae compared to 15 % mortality in MB- GLs (Log Rank [Mantel-Cox], $X^2 = 15$, $df = 1$, $P < 0.001$). *Microsporidia MB* presence in the larva significantly reduced MB+ GLs larval mortality when the larvae were fed 0.3 mg/larva/d diet regime for both Tetramin and GoCat (Log Rank [Mantel-Cox] $X^2 = 15$, $df = 1$, $P < 0.001$ and $X^2 = 5.43$, $df = 1$, $P = 0.012$ respectively).

The larval development results showed that MB+ GLs larvae developed faster than their MB- GLs counterparts under certain diet regimes (Figure 9B). The *Microsporidia MB* growth rate enhancement advantage to the mosquito host was diet dependent. MB+ GLs larvae only developed significantly faster than their MB- GLs counterparts when fed on the Tetramin 0.3 and GoCat 0.3 mg/larva/d diet regimes (Hazard ratio (HR) = 1.7, 95% CI = 1.4–2.2, $P < 0.001$ and HR = 1.4, 95% CI = 1.1–1.7, $P < 0.01$, respectively). MB+ GLs larvae fed on Tetramin (0.3 mg/larva/d) diet regime had a shorter median development time (days) ± (standard deviation) of 9 ± 0.06 d while the MB- GLs controls was 10 ± 0.15 d (Figure 9B). The hazard ratio of 1.7 showed that MB+ GLs larvae developed 1.7 d faster than MB- GLs. The median development time for MB+ GLs larvae was shorter than MB- GLs when fed on GoCat 0.3 mg/larva/d as well (10 ± 0.12 d and 11 ±0.14 d respectively). The median time for Cerelac 0.3 mg/larva/d was 12 ± 0.12 d and 11± 0.21 d for MB+ GLs and MB- GLs larvae, respectively. However, with Tetramin 0.07 mg/larva/d, the median development time between the MB+ GLs and MB- GLs larvae were 15 ± 0.52 d and 15 ± 0.72 d, respectively.

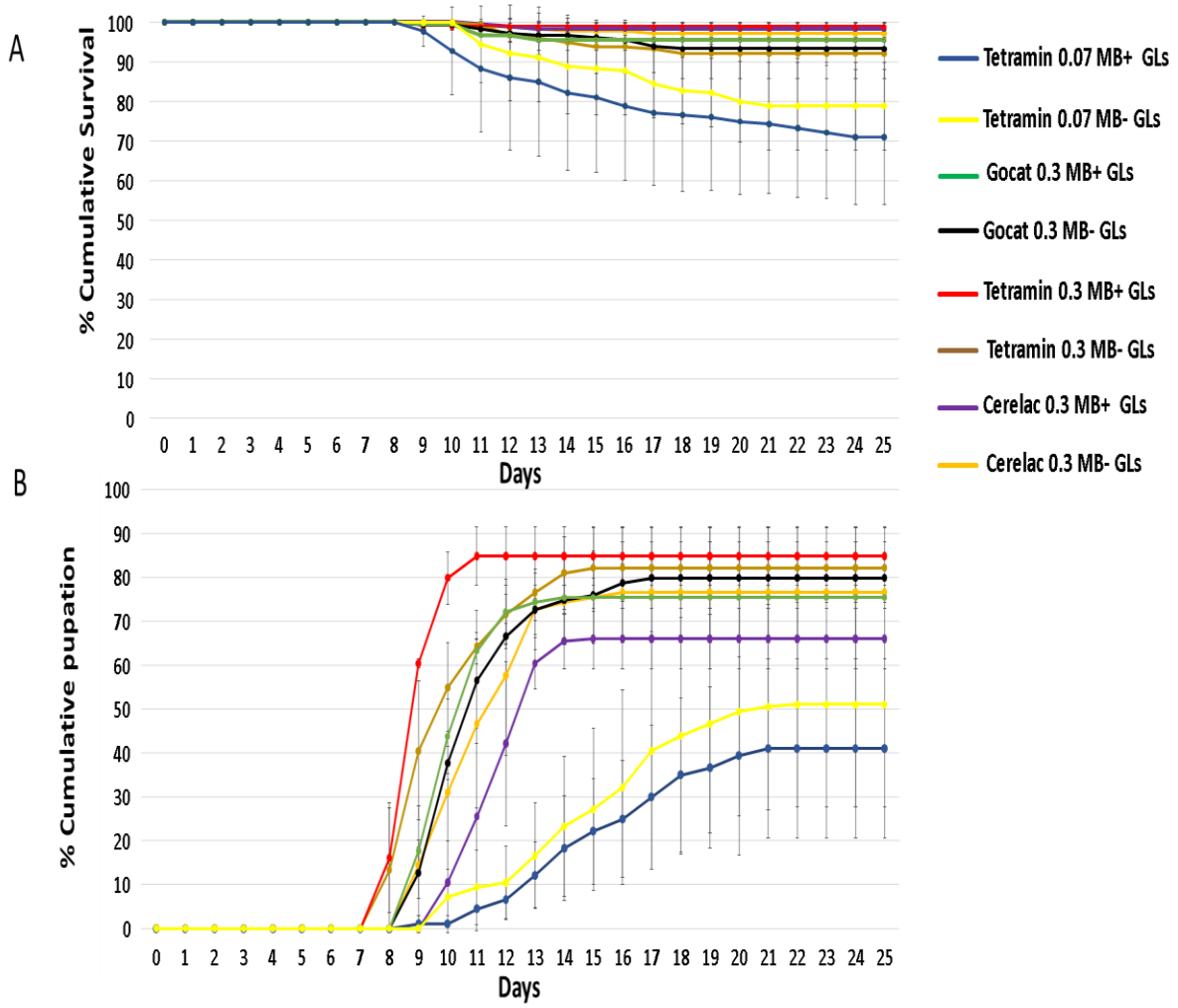


Figure 9: Effect of *Microsporidia MB* on mortality and larval development under different larval diet regimes of GLs. (A): Effect of *Microsporidia MB* on larval mortality under different diet regimes and (B) *Microsporidia MB*'s effect on median larval development time under different diet regimes. Total number of larvae were 180 in three biological replicates (n=60) for each diet regime. Error bars represent standard deviations.

Finally, under this section, the different diets significantly affected the number of GLs larvae that ultimately emerged as adults ($F = 4.66$, $df = 3$, $P = 0.005$, Figure 10). However, regardless of the treatment, there was no significant difference between MB+ verses MB- GLs mosquitoes (ANOVA after arcsine transformation of data $F = 4.66$, $df = 3$, $P = 0.99$).

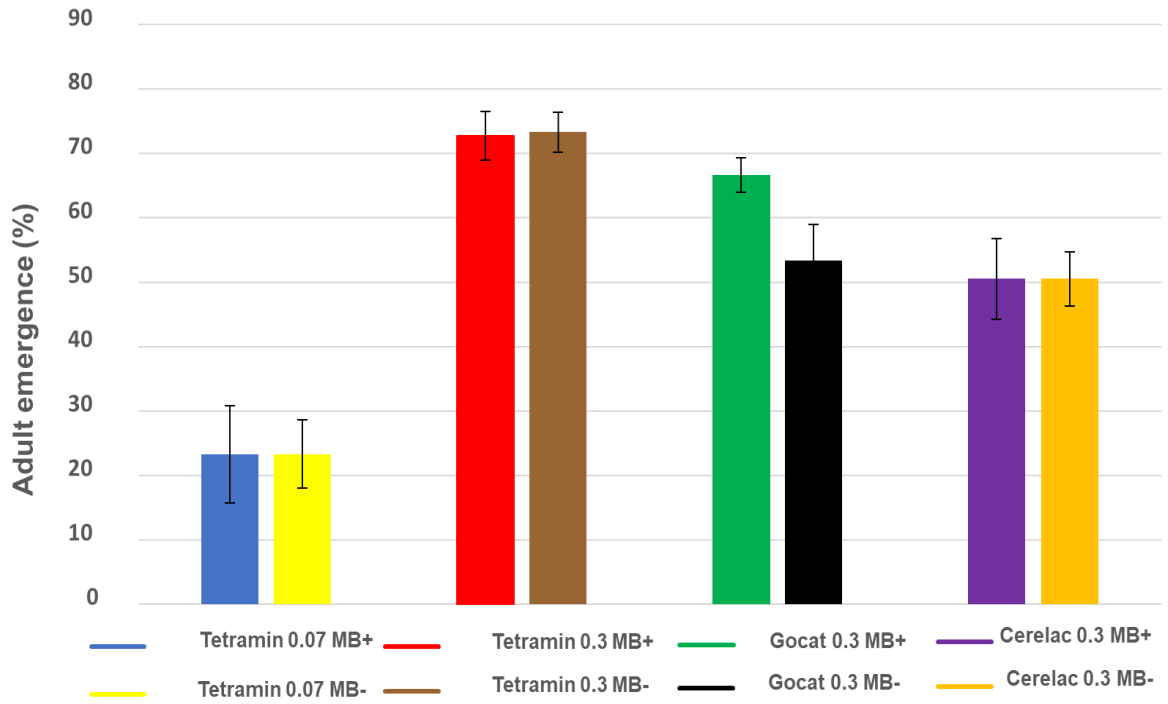


Figure 10: *Microsporidia MB*'s effect on adult emergence under different larval diet regimes of GLs. The data shown is the percentage of larvae that developed into adult mosquitoes from the various diet regimes. This is the total number of adults that emerged from the larvae from each diet. Error bars represent standard deviation.

2.4.2.3 Effect of *Microsporidia MB* on adult mosquito (GLs) survival under different adult diet regimes

Adults used for this experiment originate from F_1 larvae fed on the same larval diet regime of Tetramin (0.3 mg/larva/d). The median survival time (days) ± (standard deviation) for MB+ and MB- GLs adults fed on 1% glucose diet (Figure 11A), was 4 ±0.17 d and 4 ± 0.18 d, respectively. There was no statistically significant difference in the median survival between MB+ and MB- GLs adults (Log-rank, [Mantel-Cox] test, $X^2 = 2.95$, $df = 1$, $P = 0.073$).

On the other hand, those fed on 6% glucose (Figure 11B) showed that MB+ GLs mosquitoes survived significantly longer than their MB-GLs counterparts (Log-rank, [Mantel-Cox] test, $X^2 = 5.84$, $df = 1$, $P = 0.007$). The median survival time for the MB+ GLs adults was 12 ± 0.49 d while that for uninfected was 10 ±0.47 d.

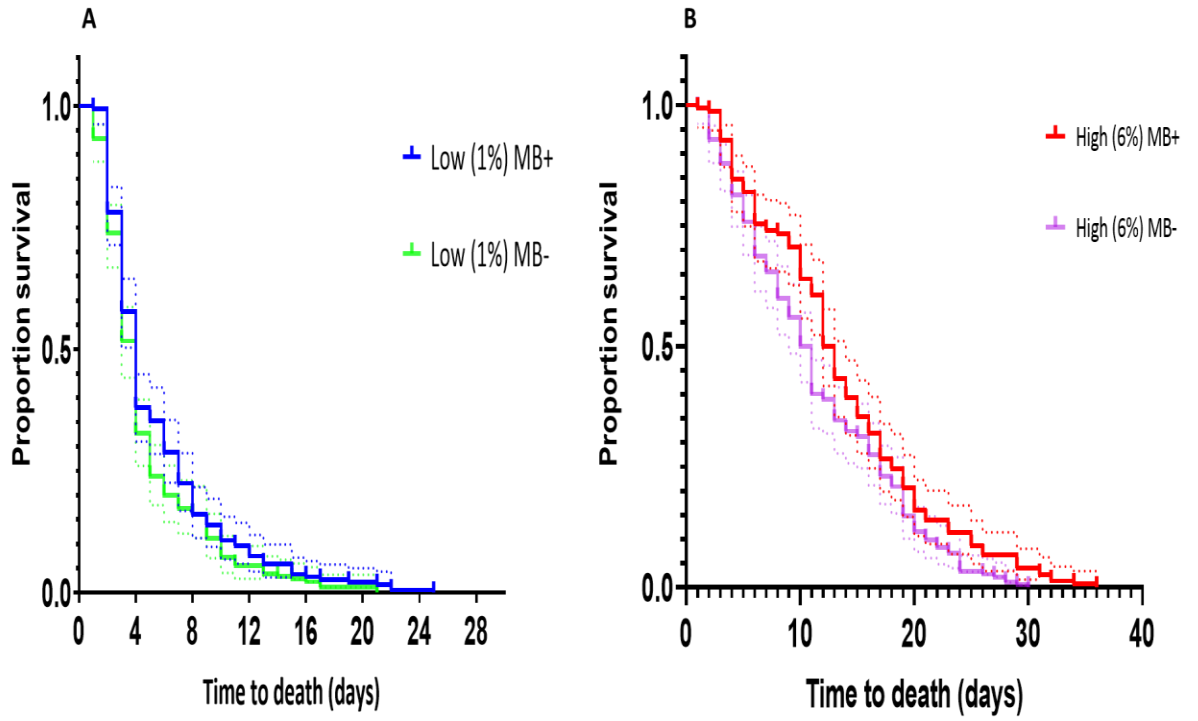


Figure 11: Effect of *Microsporidia MB* on GLs adult mosquito survival under different adult diet quantity regimes (A) 1% glucose diet & (B) 6% glucose diet. The dashed lines are the 95% CL interval while the solid lines are the survival curve for each regime.

2.4.3 Effect of larval and adult diet quantity on *Microsporidia MB* intensity and wing size in Isofemale lines (IMLs) after vertical transmission

2.4.3.1a Effect of larval diet quantity on the *Microsporidia MB* intensity of MB+ IMLs

MB+ IMLs larvae, which were fed on Tetramin 0.07 mg/larva/d and Tetramin 0.3 mg/larva/d concurrently (Figure 12B), showed that the intensity of females was significantly affected by the two larval diets with the later having higher intensity compared to the lower diet quantity (Kruskal-Wallis, $X^2 = 6.38$, $df = 1$, $P = 0.011$). Meanwhile, the males (Figure 12A) showed no significant difference in intensity between both diets (Kruskal-Wallis, $X^2 = 0.30$, $df = 1$, $P = 0.584$). The mean *Microsporidia MB* intensity ± (standard deviation) of male mosquitoes for 0.07 mg/larva/d was 191.4 7S/18S and 0.3 mg/larva/d was 197.6 7S/18S

whiles that for females were 230.7 7S/18S and 469.6 7S/18S for 0.07 mg/larva/d and 0.3 mg/larva/d regimes respectively (Figure 12B).

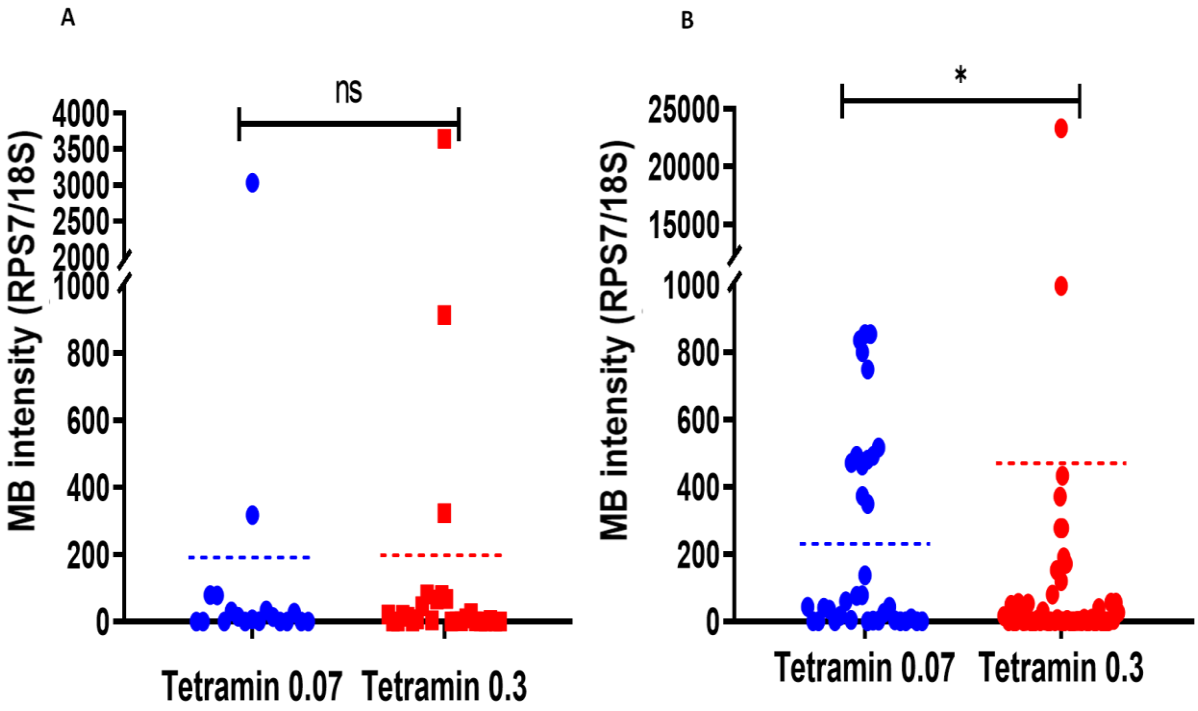


Figure 12: *Microsporidia MB* intensity under different larval diet quantity of MB+ IML. (A) *Microsporidia MB* intensity of adult males and (B) *Microsporidia MB* intensity of adult females that emerged from larvae reared on Tetramin 0.07 mg/larva/d and 0.3 mg/larva/d. The dotted lines represent the mean intensity of each diet regime.

2.4.3.1b Effect of Larval diet quantity on the wing length of MB+ and MB- IMLs

MB+ IMLs larvae fed on Tetramin 0.07 mg/larva/d resulted in significantly smaller wing size (given the size and variation parameter) of adult mosquitoes when compared to the 0.3 mg/larva/d regime (mm) (GLM following family gamma, $t = 4.23$, $df = 1$, $P < 0.0001$, Figure 13). Males fed on 0.07 mg/larva/d and 0.3 mg/larva/d regimes had relatively smaller wing sizes compared to the females on the same treatments (GLM following family gamma, $t = 5.93$, df = 1, $P < 0.0001$). MB+ IMLs female mosquitoes had significantly longer wing lengths under both Tetramin 0.07 mg/larva/d (Kruskal-Wallis, $X^2 = 4.18$, $df = 1$, $P = 0.040$) and 0.3 mg/larva/d (Kruskal-Wallis, $X^2 = 23.21$, $df = 1$, $P < 0.0001$) compared to the respective male counterparts.

Furthermore, the wing length of the female MB+ IMLs mosquitoes was significantly larger compared to the control MB- IMLs (uninfected IMLs from the same infected mother) when fed with Tetramin 0.3 mg/larva/d diet (Kruskal-Wallis, $X^2 = 23$, $df = 1$, $P < 0.0001$, Figure 13). The males under the same diet did not show any difference in size ($U_{(64)} = 3.58$, $P = 0.115$).

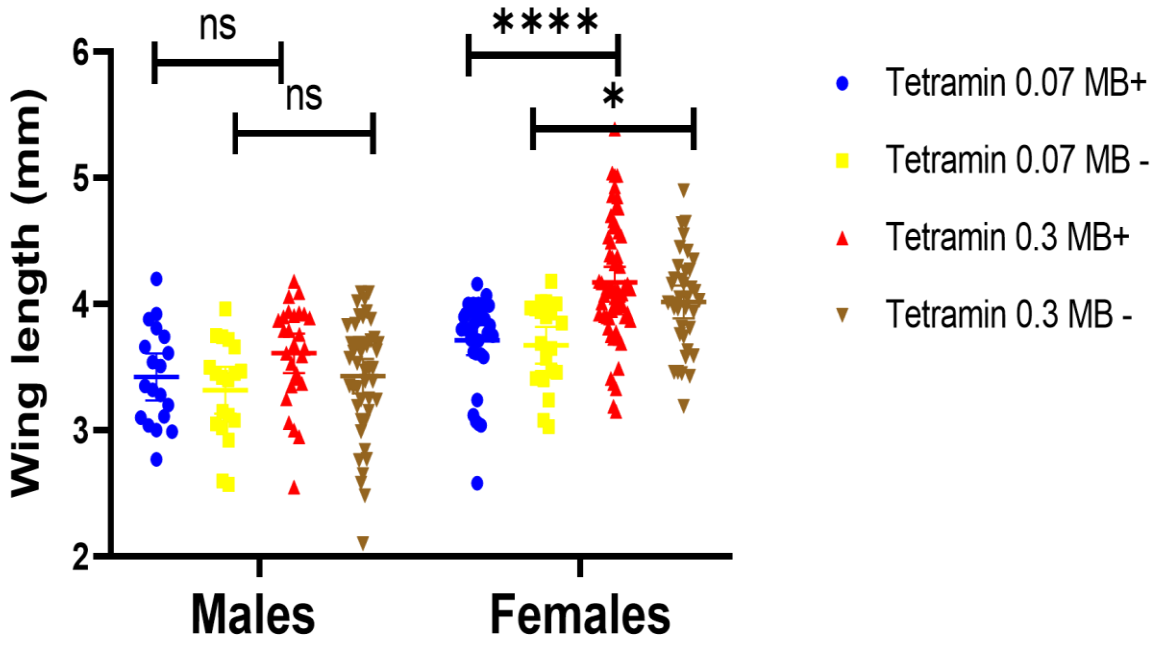


Figure 13: Effects of *Microsporidia MB* on wing length of MB+ & MB- IMLs under larval diet quantity regimes. *Microsporidia MB* significantly increased the length of female wings both under Tetramin 0.07 and 0.3 mg/larva/d respectively. The line and the bars in the middle of each treatment indicate the mean with 95% CI respectively.

2.4.3.2 Adult diet quantity on the *Microsporidia MB* intensity of MB+ IMLs

The analysis conducted here adhered to the methodology outlined in section 2.4.3.1. However, in the absence of significant results among the groups (Figure 14A & B), I proceeded to incorporate analysis of each diet quantity by sex. There was no significant difference in *Microsporidia MB* intensity between adult males fed on 1% and 6% glucose diet (Figure 14A) or females only (Figure 14B) MB+ IMLs mosquitoes ($U_{(62)}$=434, $P = 0.524$ and $U_{(85)}$= 734, $P = 0.153$ respectively). Under a low adult quantity (1% concentration) diet, there was no

significant difference between male and female *Microsporidia MB* intensity ($U_{(39)}$= 7.4, P = 0.430) (Figure 14C). However, when adults were fed with the 6% diet, females had significantly higher *Microsporidia MB* intensity compared to their male IMLs counterparts (Figure 14D, $U_{(46)}$= 12.57, P < 0.009).

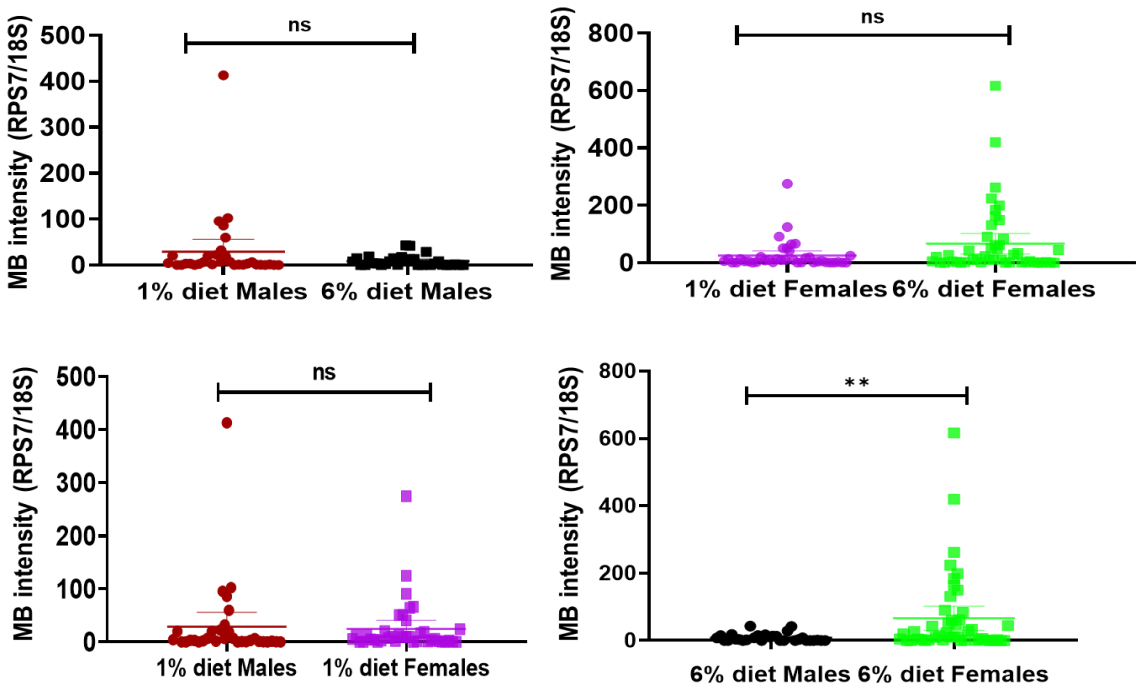


Figure 14: *Microsporidia MB* intensity under different adult diet quantities of MB+ IMLs (A) Effect of low adult diet (1% glucose) on *Microsporidia MB* intensity in male and female IMLs mosquitoes. (B) Effect of high adult diet (6% glucose) on *Microsporidia* MB intensity in male and female IMLs mosquitoes. (C) Effect of low (1% glucose) and high (6% glucose) diet on the intensity of *Microsporidia MB* of male mosquitoes. (D) Effect of low (1% glucose) and high (6% glucose) diet on the intensity of *Microsporidia MB* of female mosquitoes. Bars represent the mean with a 95% confidence interval of the Microsporidia MB intensities of each diet regime.

2.5 DISCUSSION

This is the first study to investigate how host diet regimes and quantities affect the interaction between *An. arabiensis* and *Microsporidia MB*. Understanding the dynamics of this interaction is important for predicting the ability of *Microsporidia MB* to spread naturally in *An. arabiensis* populations. This knowledge is also imperative for establishing optimal methods to rear *Microsporidia MB*-infected *An. arabiensis* for future vector control implementation strategies (Bourtzis *et al.*, 2014; Carvajal-Lago *et al.*, 2021).

Some life history traits were not affected by either *Microsporidia MB* or diet. The prevalence of *Microsporidia MB* was not significantly affected by diet. However, diet conditions or quality could play an essential role in ensuring successful transmission with a significant increase in the intensity of the symbiont. The inference that can be made from the intensity result is that in the absence of Tetramin 0.3 mg/larva/d diet regime, GoCat 0.3 mg/larva/d can be used to achieve a comparable high *Microsporidia MB* intensity in the mosquito host. The low-intensity results seen under Tetramin 0.07 and Cerelac diets could be attributed to the low protein content by quantity (0.07 mg) and composition (14%) ingested by the larvae, respectively.

Microsporidia MB reduced larval mortality in *An. arabiensis* across four different diet regimes, with the greatest impact observed under the Tetramin 0.3 mg/larva/day and GoCat 0.3 mg/larva/day diets. Regarding larval development time, Tetramin 0.3 mg/larva/day resulted in the fastest development, followed by GoCat 0.3 mg/larva/day, Cerelac, and then Tetramin 0.07 mg/larva/day. MB+ GLs larvae pupated 1.7 days faster than MB- GLs (without the symbiont) under the Tetramin 0.3 mg/larva/day diet, though no significant difference was observed under Tetramin 0.07 mg/larva/day, highlighting the role of diet quality and quantity in development

(Araújo *et al.*, 2012; Couret *et al.*, 2014). Larval diet conditions impacted the total number of adult mosquitoes that emerged from first instar larvae. However, *Microsporidia MB* did not affect the emergence of adults from larvae under any of the four diet regimes, aligning with the findings from Herren *et. al.,* (2020).

To conclude, amongst the larval diet regimes tested, Tetramin 0.3 mg/larva/d led to the lowest larval mortality, fastest larval development, and highest adult emergence, supporting previous research indicating that the diet of a mosquito influences the life history characteristics of the mosquito(Carvajal-Lago *et al.*, 2021; Linenberg *et al.*, 2016; Vrzal *et al.*, 2010). I hypothesise that Tetramin's higher protein content, compared to Gocat and Cerelac, was the main dietary factor contributing to the observed differences in development, as prior studies have shown that a protein content above 50% is essential for achieving larger size and weight in mosquitoes (Carvajal-Lago *et al.*, 2021). These results suggest that both the mosquito host and the symbiont are influenced by diet, with the fitness benefits conferred by *Microsporidia MB* being dependent on dietary conditions. Therefore, Tetramin 0.3 mg/larva/day is the most suitable diet for mass-rearing *Microsporidia MB* mosquitoes.

The survival of MB+ GLs adult mosquitoes was also influenced by adult diet concentration. MB+ GLs adult mosquitoes survived longer compared to MB- GLs mosquitoes, reaffirming the diet-dependent nature of the symbiont's fitness benefits, a pattern similar to that seen in *Rickettsia*-infected whiteflies (Himler *et al.*, 2011). It is also worth noting that sugar concentration in the adult diet did not affect the intensity of *Microsporidia MB* in the mosquitoes but rather the presence of *Microsporidia MB* and diet condition determined fitness benefit to the host.

In IMLs, the intensity of *Microsporidia MB* in female mosquitoes was significantly impacted by larval diet, while male mosquitoes showed no such effect. One explanation for this difference could be the distinct protein profiles between male and female mosquitoes, as females possess 682 unique proteins compared to 422 in males (Shettima *et al.*, 2021). However, this is an avenue for further research in the future. Vertically transmitted symbionts strategically manipulate the female host to ensure their effective transmission to the next generation, which might explain the higher intensity of *Microsporidia MB* in females fed on the Tetramin 0.3 mg/larva/day diet, ensuring successful transmission to offspring. Additionally, female mosquito size, which was affected by both diet quantity and sex, also influenced *Microsporidia MB* intensity (Hosokawa *et al.*, 2007; Koga *et al.*, 2021). Research has proven that female mosquitoes with larger body sizes are more likely to become gravid than those with smaller bodies (Lyimo & Takken, 1993). Furthermore, larger females lay more eggs and undergo shorter gonotrophic cycles than small ones (Briegel, 1990; Yan *et al.*, 2021). The quantity of larval diet affects the size of the mosquito, which was also recorded in this study (Yan *et al.*, 2021). The correspondingly higher *Microsporidia MB* intensity with larger body size of MB+ IMLs females confirms the reproductive advantage conferred to the host by the symbiont. Similar reproductive biases have been observed in *Rickettsia*-infected whiteflies (Himler *et al.*, 2011).

Interestingly, the intensity of *Microsporidia MB* in mosquitoes was not significantly affected by adult diet (1% or 6% glucose), but female mosquitoes consistently showed higher symbiont intensity than males, likely due to the combined effects of both larval and adult diets.

Host insects regulate the intensity of obligate intracellular nutritional symbionts depending on their nutritional status (Bryant & Newton, 2020). For example, *Wigglesworthia*, an obligate

mutualist in *Glossina morsitans*, increases in intensity under low-thiamine conditions but decreases with enriched diets (Snyder *et al.*, 2012). As a nutritional symbiont, *Wigglesworthia* synthesises thiamine hence under an enriched diet, the intensity of the symbiont is reduced by the host since there is no need for nutrient provision (Snyder *et al.*, 2010). Similarly, the population intensity of *Buchnera aphidicola* in the pea aphid increases with a nitrogen-rich diet, compensating for host growth (Wilkinson *et al.*, 2007). The proportional increase in *Buchnera* was to increase nutrient provision by the symbiont to compensate for the growth of the insect. *Wolbachia pipientis*, an intracellular proteobacterium that infects about 60% of insects, is both maternally and horizontally transmitted (Bryant & Newton, 2020). It has been demonstrated that the ability of *Wolbachia* symbiont to induce recombination of phenotype from the X, 2nd and 3rd chromosomes in *Drosophila melanogaster* was titer-dependent*Wolbachia* (Bryant & Newton, 2020). *Drosophila* fed on yeast-enriched diets have been shown to reduce *Wolbachia* levels in the germline, while sucrose-rich diets increase them (Serbus *et al.*, 2015). Since *Wolbachia* elevates recombination in a dose-dependent manner, lower densities resulting from yeast-rich diets correspond to reduced recombination rates, whereas higher densities from sucrose intake enhance recombination (Serbus *et al.*, 2015). Elevated recombination is beneficial as it promotes genetic diversity.

In contrast, this study indicated that *Microsporidia MB* is not a nutritional symbiont, resulting in an absence of inversely proportionate changes in intensity throughout the various dietary regimes in the IMLs (Whittle *et al.*, 2021). Insect host regulation of symbionts could be responsible for the restriction of *Microsporidia MB* fitness benefit to *An. arabiensis* under very limited diet conditions, as all resources are channelled to host-symbiont survival (Whittle *et al.*, 2021). However, under normal diet conditions, the metabolism of the symbiont is not

restricted leading to a full expression of fitness advantage seen in both larval and adult diets. The results also show clearly that there was no negative impact on larval development time, adult emergence and adult survival of the host even under very limited diet conditions.

One limitation of this study was not comparing the adult survival of MB+ GLs to MB- GLs adult mosquitoes of the different larval diet regimes. I also acknowledged that while proteins and amino acids play a significant role in mosquito physiology, from digestion to reproduction, other dietary components, including vitamins, phosphorus, zinc, and various micronutrients, may also be crucial in physiological functions, contributing to the observed differences. These are activities for my future research.

In conclusion of this chapter, *Microsporidia MB* has no negative effect on the larval development of *An. arabiensis* even under low diet conditions, often experienced in nature, hence can be used as an effective malaria control tool. Tetramin 0.3 mg/larva/d gives high *Microsporidia MB* intensity in the mosquito. Additionally, the extended survival of the adult MB+ GLs mosquitoes on 6% glucose is an added advantage for the proliferation of *Microsporidia MB* in the MB- GLS mosquito population as males are able to live longer and mate. The diet regimes can, therefore, be utilised for mass rearing of *Microsporidia MB* infected *An. arabiensis*.

CHAPTER 3

MONITORING THE CAPACITY OF *MICROSPORIDIA MB* TO SPREAD THROUGH *ANOPHELES ARABIENSIS* POPULATIONS ACROSS GENERATIONS

3.1 INTRODUCTION

The spread potential of symbionts in mosquitoes is shaped by various factors, including transmission mechanisms, host compatibility, environmental conditions, reproductive dynamics, and host behavioural traits (Beckmann *et al.*, 2021; Bright & Bulgheresi, 2010; Doremus *et al.*, 2019; Niepoth *et al.*, 2018). Symbionts are transmitted either vertically (from parent to offspring) or horizontally (between individuals), with the mode of transmission playing a critical role in their dissemination within populations (Bright & Bulgheresi, 2010; Caspi-Fluger *et al.*, 2012; Chrostek *et al.*, 2017; Koga *et al.*, 2012). Horizontal transmission can occur through oral transfer while feeding or during copulation (male-to-female or female-to-male infection) (Li *et al.*, 2020; Li *et al.*, 2017; Nattoh *et al.*, 2021). Two examples of orally transmitted insect symbionts include *Asaia* infection in white-backed planthoppers (Li *et al.*, 2020) and *Wolbachia* infection in whiteflies following consumption of sap from infected plants (Li *et al.*, 2017). An instance of symbiont transmission between insects during copulation is *Regiella insecticola* in aphids (Moran & Dunbar, 2006). Vertical transmission typically facilitates the establishment of symbionts within populations more efficiently compared to horizontal transmission, some examples are *Microsporidia MB* in *Anopheles* and *Wolbachia* in *Aedes* mosquitoes (Jiggins, 2017; Nattoh *et al.*, 2021). It must be noted that certain symbionts like *Microsporidia MB* and *Asaia* are capable of undergoing both vertical and horizontal transmission in mosquitoes and whiteflies respectively (Li *et al.*, 2020; Rami *et al.*, 2018; Nattoh *et al.*, 2021).

Host compatibility, defined as the symbiont's capacity to flourish in various mosquito species, influences its dissemination (Niepoth *et al.*, 2018; Sanaei *et al.*, 2021; Wang *et al.*, 2012). A symbiont that can associate with several host species has a higher probability of dissemination, which accounts for the successful proliferation of the *Wolbachia* symbiont in numerous host organisms (Sanaei *et al.*, 2021). Environmental factors also substantially affect both symbionts and the host in various ways (Bayoh & Lindsay, 2003; Doremus *et al.*, 2019; Moghadam *et al.*, 2018; Mouton *et al.*, 2006). The diet constitutes a significant aspect of the host-symbiont relationship, as previously addressed in chapter two (Boanyah *et al.*, 2024; Carvajal-Lago *et al.*, 2021). Moreover, environmental factors such as temperature and humidity have been identified as crucial determinants of stability and intensity in symbionts (Doremus *et al.*, 2019; Moghadam *et al.*, 2018; Mouton *et al.*, 2006). A study conducted by Doremus *et al.*, (2019) on *Cardinium* in the parasitic wasp host, *Encarsia suzannae*, showed that elevated temperature of 32 °C diminished *Cardinium* abundance, prolonged pupal maturation time, decreased vertical transmission rate, and weakened both cytoplasmic incompatibility modification and rescue efficacy compared to the control at 27°C. A cool temperature of 20°C, on the other hand, also diminished symbiont density and extended the host pupal stage, illustrating the substantial influence of temperature on symbionts (Doremus *et al.*, 2019). Moreover, *Wolbachia* has been shown to be in high abundance in *Drosophila melanogaster* at 13°C compared to higher temperatures of 23°C and 31°C, respectively (Mouton *et al.*, 2006). The investigation of the impact of temperature on *Wolbachia* abundance at various developmental stages of the *Ae. albopictus* mosquito demonstrated a notable reduction in bacterial intensity after exposure to increased temperature of 37°C and larval density in both sexes (Wiwatanaratanabutr & Kittayapong, 2009).

The reproductive dynamics of the host is also an important factor for the transmission of the symbionts. Reproductive dynamics refers to how the reproductive rates, lifespan, and population density of host insects are manipulated by the symbiont and this determines the symbiont dissemination potential (Beckmann *et al.*, 2021; Cockburn *et al.*, 2013; Hancock *et al.*, 2011; Oliver *et al.*, 2008). Increased reproduction rates may enhance rapid symbiont transmission. Endosymbionts like *Wolbachia* are vertically transmitted via host eggs and modify host biology in various ways, including inducing reproductive manipulations such as sperm-egg incompatibility, male death, parthenogenesis and feminisation (Crawford *et al.*, 2020; Jiggins, 2017; Landmann, 2019).

It is necessary to optimise the rearing of *Microsporidia MB*-infected mosquitoes over generations (colony) through semi-field and laboratory conditions, as temperature and nutrition are critical factors (Boanyah *et al.*, 2024). Considering mosquitoes' tolerance to laboratory conditions, rapid generation time, capacity to thrive on diverse diets, and quantifiable performance characteristics, it is anticipated that qualities pertinent to *Microsporidia MB* can be intentionally enhanced or, at a minimum, preserved, akin to the approach employed in SIT (Benedict *et al.*, 2009). Establishing a laboratory colony of *Microsporidia MB*-infected mosquitoes from field-collected mosquitoes is essential for the mass production of symbiont mosquitoes for field deployment, nevertheless, their adaptation to the laboratory environment presents a significant bottleneck (Rose *et al.*, 2024).

To effectively evaluate the introduction of *Microsporidia MB*-infected mosquitoes in field trials to control malaria, it is imperative to have a comprehensive understanding of the dynamics underlying the sustained transmission of this symbiont across multiple generations, or in

simpler terms, the successful establishment of a colony of *Microsporidia MB* mosquitoes. The outcome of the results from this chapter will inform the feasibility of this symbiont control strategy to curb the transmission of the *Plasmodium* parasite that causes malaria. This study, therefore, seeks to monitor the spread of *Microsporidia MB* in infected *An. arabiensis* under laboratory conditions

3.2 AIM AND OBJECTIVES

The main aim of this chapter was to determine the prevalence of *Microsporidia MB* across generations in *An. arabiensis* colonies under semi-field and laboratory conditions. This was done by optimising the colonisation of *Microsporidia MB*-infected mosquitoes using different rearing conditions.

The specific objectives were:

i. To determine *Microsporidia MB* prevalence and intensity across generations

ii. To evaluate how temperature and humidity affect *Microsporidia MB* prevalence

iii. To determine how phenotypic characteristics such as wing length of mosquitoes change across generations

3.3 MATERIALS AND METHODS

3.3.1 Sampling site and Mosquito collection

G_0 mosquito collection from the field was the same as detailed in section 2.3.1. However, due to some F_1 colonies dying out prematurely, field collection was undertaken four times with the assistance of the icipe field team.

3.3.2 Mosquito maintenance at the insectary

Mosquitoes were maintained following same as details as in section 2.3.2.

3.3.3 Molecular species identification

See section 2.3.3 for the procedure for mosquito DNA extraction and species identification, which was conducted by the icipe molecular team because at that time the research institution mandated that the icipe molecular team be responsible for species identification only, while I completed the remaining tasks.

3.3.4 *Microsporidia MB* screening and intensity determination

Microsporidia MB screening and intensity determination follows the same procedure in section 2.3.4 of this thesis. The qPCR technique employed was consistent in determining both the prevalence and density of *Microsporidia MB* in the experimental mosquitoes from F_1 to F_6

3.3.5 Optimizing colonisation of *Microsporidia MB* mosquitoes under different rearing conditions

This section employed two distinct types of cages under different settings. Large cages measuring 100 cm × 70 cm × 60 cm were utilised in the screen house (semi-field system) to replicate environmental conditions close to those seen in nature. A semi-field system is characterised as an enclosed environment, optimally located within the natural ecosystem of a target insect vector, containing all essential elements for the completion of its lifetime (Ferguson *et al.*, 2008). This study employed a semi-field system consisting of two screen houses, each measuring 6.75 m by 10.85 m in length and 2.5 m in height at the walls. The walls are screened with black fiberglass netting gauze (1.7×1.5 mm) and the roof is covered with fiberglass. One screen house floor was covered with sand to a depth of up to 30 cm, while the other was covered with vegetation to improve humidity and temperature. A small cage (30 × 30 x 30 cm) was utilised in the insectary (laboratory) with uncontrolled temperature and

humidity to maintain conditions similar to those outside the insectary (natural). There were two semi-field settings with the large cages and the insectary setting with the small cages.

For each generation in the small cage (30 × 30 × 30 cm), the left wing of 10 males and 10 females (20 individual *Microsporidia MB* infected mosquitoes) were removed and the length measured as a proxy to examine the difference in mosquito body sizes using a handheld microscope (See section 2.3.7.1b). All the dead mosquitoes were individually placed in 1.5 ml microcentrifuge tubes and screened for *Microsporidia MB* prevalence and intensity according to the procedure used by Heren *et al.*, (2020) described in section 2.3.4. Temperature and humidity were recorded using Temperature and Humidity Data Logger (Elitech®, LogEt 8THE, USA) in the insectary to monitor the climatic conditions in the laboratory as these were not controlled but fluctuated due to the outside environmental conditions and ensured that this was as similar to the semi-field system as possible. The impact of temperature on *Microsporidi*a *MB* prevalence was assessed after the 6th generation was completed. However, these measurements were not taken during the large cage (100 cm by 70 cm by 60 cm) evaluations, as none of the newly initiated colonies progressed to the next generation due to

i) Large cage in screen house without vegetation

Rearing of *Microsporidia MB*-infected mosquitoes was conducted using three different experimental designs mentioned above. Large cage of dimensions 100 cm by 70 cm by 60 cm, referred to as a population cage (Figure 15) was used in the first experimental design (Figure 16). This allowed consecutive generations to be maintained in the same cage. The population cage was stocked with approximately 200-300 F_1 mosquitoes from *Microsporidia MB* positive *An. arabiensis* G_0 within a period of two weeks. The cages were placed in the screen house

(Figure 4) to create semi-field conditions for the mosquitoes. Hence, in this setup, both the larvae and the adult were reared in the screen house. Wet towels were placed on the top of the cage for improved humidity and changed three times a day. Adult mosquitoes were fed with 6% glucose solution in vials with tissue paper. Blood feeding was done by placing a human arm in the cage for 20 mins for five consecutive days (Monday to Friday) per week. Furthermore, clay pots with water were placed in the cage to provide oviposition sites for engorged female mosquitoes. The rack on which the cages were placed had their stands in water basins as a precautionary measure to prevent ants' infestation.

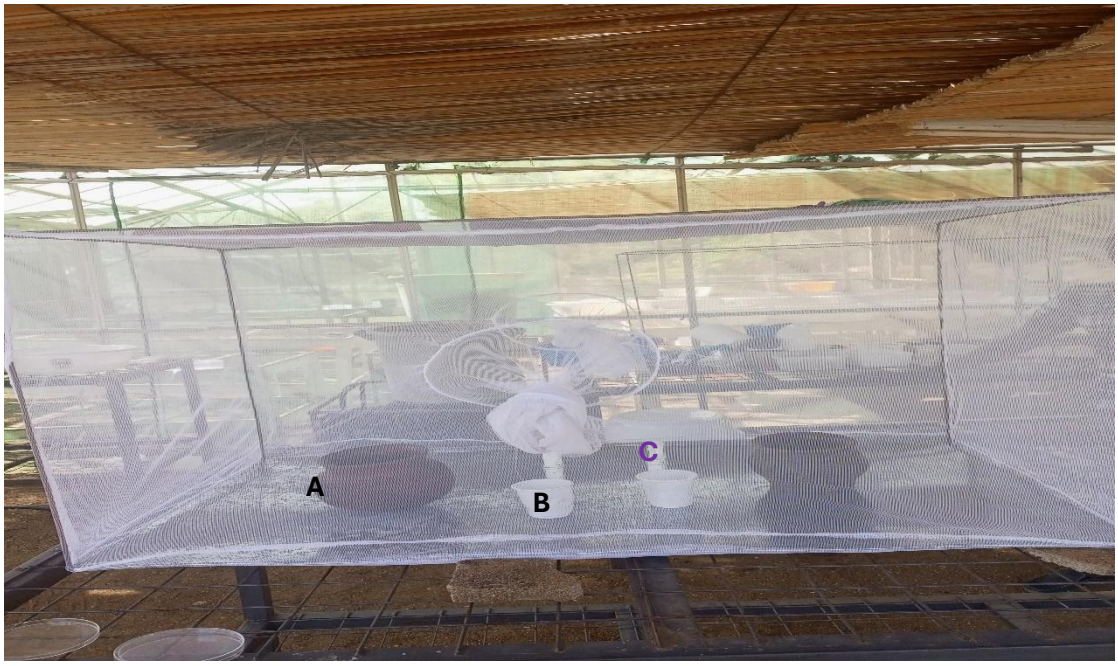


Figure 15: Picture of population cage (100 cm by 70cm by 60 cm): A: clay pot; B: cup containing pupae, C: sugar vial containing 6% glucose solution and moist tissue paper for humidity.

ii) Large cage in screen house with vegetation

The second experimental design followed the first (Figure 16), however, it was conducted in a modified screen house (Figure 17) with plants and a thatched mud-house. A population cage (Figure 15) was stocked with about 200 F_1 mosquitoes from *Microsporidia MB*-infected *An. arabiensis* G_0 s within a period of two weeks. The stocked population of adult mosquitoes were placed in the mud house with enough light from both the sun and an electric bulb that was switched on during the day and switched off during the night. This was to create the day and night cycle experienced by the mosquitoes under natural conditions. The dissections of both *Microsporidia MB*-infected female mosquitoes were not initially included in the experimental design; they were conducted solely to elucidate the absence of reproduction due to lack of insemination. Furthermore, the setup and dissection of uninfected cohorts aimed to demonstrate that the absence of reproduction was not caused by *Microsporidia MB*.

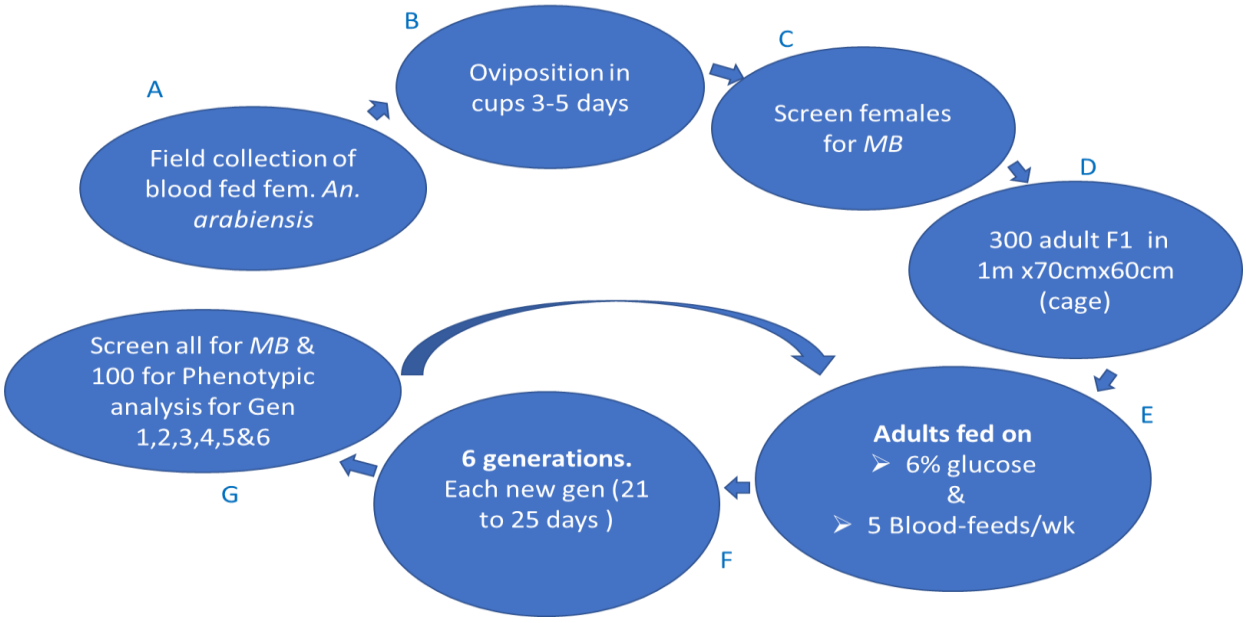


Figure 16: The schematic diagram of experimental design for the spread of *Microsporidia MB* in infected *An. arabiensis* population in large cages (Population cage) under semi-field condition. Alphabetical letters indicate the chronological steps conducted for establishing a large cage.



Figure 17: Picture of the Screen house with plants and thatched mud-house in which population cages were kept to rear mosquitoes.

iii) Standard 30x30x30cm cages under laboratory conditions.

The third and final design was conducted in an insectary without controlled conditions to maintain colonies (laboratory system in which temperature and humidity were not controlled, simulating a semi-field system, Figure 18 and 19).

The experimental design, as shown in Figure 20, was used in this section. In the insectary, a 30 x 30 x 30cm cage (small cages, Figure 19) was stocked with 200 F_1 mosquitoes from *Microsporidia MB*-infected *An. arabiensis* G_0 s within a period of two weeks. Adult mosquitoes were fed with 6% glucose solution in vials with tissue paper similar to the population cages. The cage floor contained small cups with pupae and glass bowls for the oviposition of engorged females. Five blood meals (Monday to Friday) were provided for female mosquitoes per week Three days after the initial blood feeding, the cage was equipped with small water-containing glass bowls that contained cone filter paper. These bowls served as the oviposition sites for female mosquitoes. Eggs laid on the filter paper were removed and placed in larva trays with

1,000 ml of doubly-distilled water. Hatched larvae were fed with Tetramin 0.3 mg/larva/day in the screen house and transferred to the insectary upon pupation.

Unlike the population cage where the experimental design was that one generation overlap in the same cage, the small cages housed one generation at any one time. The subsequent generations were placed into a new cage. This setup followed standard insectary practice. Hence, the generation number (F_2, F_3, etc.) was used to label the corresponding trays and cages. This was to enable easy identification and follow-up analysis of each mosquito generation. Each of the attempts was referred to as a "replicate" or colony. Three biological replicates (three independent attempts) were conducted in this section.

Given the high failure rate of establishing a colony, data recording was only kept and analysed for the colony that progressed beyond F_2 for the following:

i. *Microsporidia MB* prevalence and intensity across generations. All the dead mosquitoes were tubed individually in 1.5 ml microcentrifuge tubes and screened for *Microsporidia MB* according to the procedure in section 2.3.4.
ii. Temperature and humidity measurement in the insectary: Temperature and humidity were digitally recorded every hour in the insectary to monitor the weather conditions in the laboratory as they were not controlled but allowed fluctuations from the environment. This modified insectary settings were to allow it to be close to the semi-field system as possible. The impact of temperature and humidity on *Microsporidia MB* prevalence was assessed afterwards. It must be noted that though the data loggers have the capacity to record data every 30 sec, I chose to use 1 hour for easy data management.

iii. Wing length of mosquitoes across generations: The left wing of 10 males and 10 females (20 individual mosquitoes) was removed and the length measured as a proxy to examine the difference in mosquito body sizes using a handheld microscope.



Figure 18: Picture of the insectary with uncontrolled temperature and humidity



Figure 19: Picture of a rack carrying the 30cmby30cm by 30cm cages in the insectary

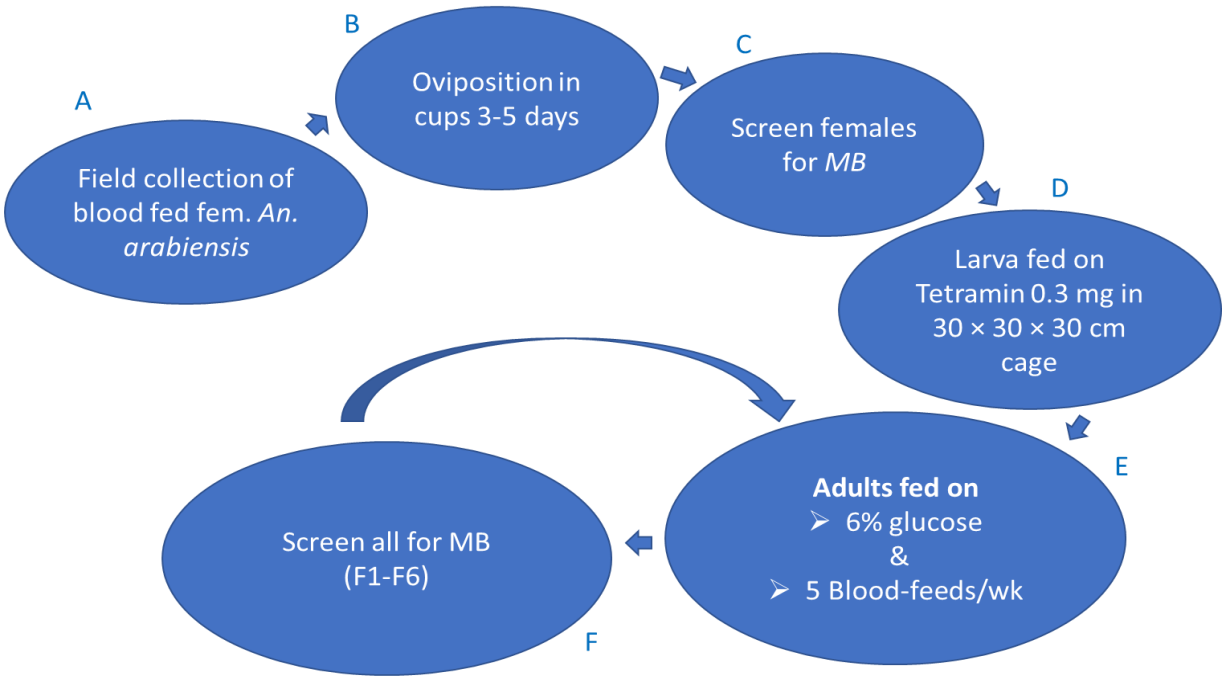


Figure 20: The schematic diagram of the experimental design of the spread of *Microsporidia MB* in infected *An. arabiensis* in small cages (30x30x30cm) in the laboratory. Alphabetical letters indicate the chronological steps

3.3.6 Data analysis

The data was first tested for normality using Shapiro–Wilk test (S. S. Shapiro & Wilk, 1965). Kruskal–Wallis tests and multiple comparisons were used to compare the *Microsporidia MB* intensities between the generation groups (Kruskal & Wallis, 1952). Phenotypic characteristics such as wing size of the mosquitoes tested for significant variation across generations using one way Analysis of Variance (ANOVA). Furthermore, a correlational analysis (Kendall, 1938) was conducted between *Microsporidia MB* prevalence and temperature. Finally, the intensity of *Microsporidia MB* categorised by sex was analysed using a Generalised Linear Model (Nelder & Wedderburn, 1972) with a gamma distribution. R software (Giorgi *et al.*, 2022) version 4.1.2 was used for analysis with a p-value < 0.05 considered significant.

3.4 RESULTS

3.4.1 Optimizing colonisation of *Microsporidia MB* mosquitoes under different rearing conditions

i) *Large cage in screen house without vegetation*

Unfortunately, the adult mosquitoes (n = ± 200) placed in the population cage died within 2 weeks in the screen house (Figure 4). Though they were blood-fed, females did not produce any F_2 offspring. This was most likely due to the harsh environmental conditions such as temperature and humidity. There was no recorded data here as it has been indicated in the methods that due to the high failure rate, until it crossed F_2. This was because all the F_1 offspring died in all the repeated attempts in the large cages. A new screen house with vegetation was then evaluated for use in the next experiment (Figure 17).

ii) *Large cage in screen house with vegetation*

By observation, about 70% of the 200 mosquitoes survived past 30 days (data not recorded). However, they did not reproduce or progress to F_2 generation. Dissection of the spermatheca was performed on the females to ascertain the reasons for their lack of reproduction. The spermatheca of the last 10 surviving female mosquitoes was dissected to determine insemination status. The findings indicated that none (0%) of the females were inseminated, explaining the lack of eggs produced. A negative control cohort (MB-) was established, to determine if the absence of mating was attributable to *Microsporidia MB*-infected females. However, the uninfected cohort also showed a 0% insemination rate from the last 10 surviving females.

iii) *Rearing in small cages under insectary conditions*

Two of the three attempts (replicates) survived beyond F_2 when the small cages were used under insectary conditions. Therefore, analysis was only conducted on the results where more than two generations (F_2 and higher) survived. Hence, the early terminative (referred to as "collapsed") generations in the semi-field systems and replicate 2 in the laboratory system were excluded from the final statistical analysis. *Microsporidia MB* spread in the mosquitoes progressed successfully in two replicates (1 & 3) from the original three laboratory biological replicates.

Figure 21 displays the subsequent generations obtained from the original G_0 females. The sudden decrease of adults from replicate 3, between F_3 (n=281) and F_4 (n=74) was due to the unknown death of 246 adults prior to egg laying. Only 35 adults were alive to contribute to the F_4 generation (Figure 21). Replicate 2 terminated in generation 2 (F_2), as all the adults died without reproducing.

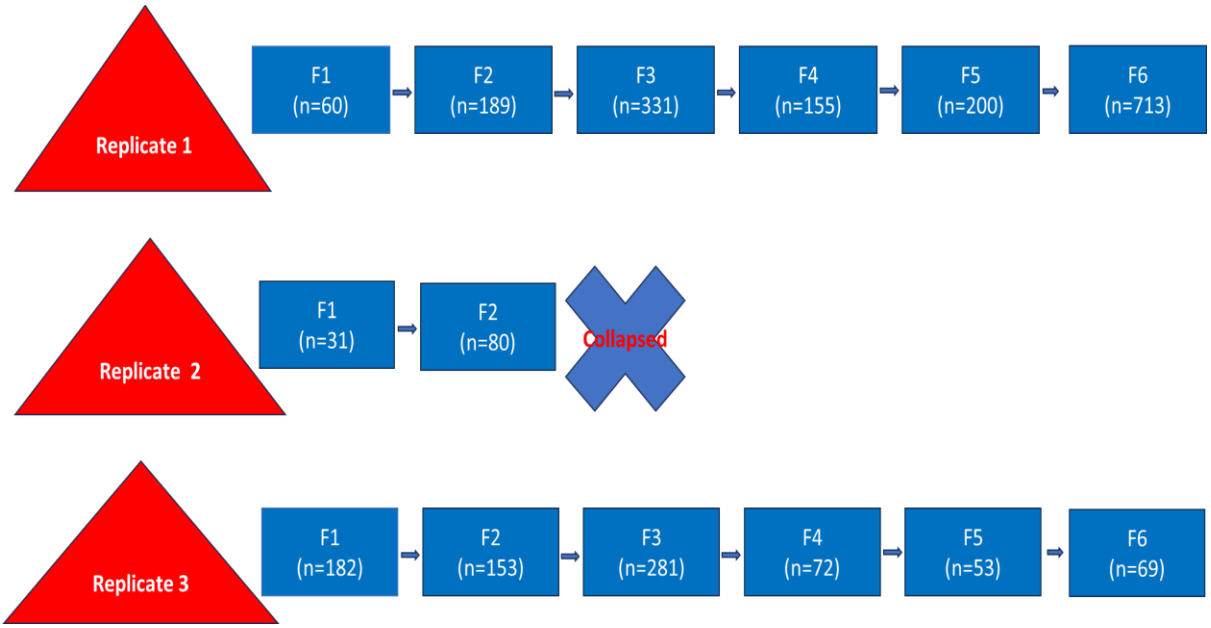


Figure 21: Overview of *Microsporidia MB* spread across generations in *An. arabiensis* in the insectary setting. Total number of adult mosquitoes in each replicate is represented by n. Replicate indicate an independent attempt to colonisation.

3.4.2 *Microsporidia MB* prevalence across generations

Microsporidia MB prevalence was determined to monitor the spread of the symbiont in mosquito populations across generations. The prevalence of *Microsporidia MB* was measured in adults of every generation and within every replicate established (Figure 22). For replicate 1, the *Microsporidia MB* prevalence in the mosquito population increased from F_1 (63.3%) to F_3 (88.2%), while the prevalence for replicate 3 also increased from F_1 (73.6 %) to F_2 (91.5%) (Figure 22). Regardless of the replicate, a decline in the *Microsporidia MB* prevalence was observed over time. Replicate 1 showed a decrease in prevalence from the F_4 generation (87.7 %), while this decline was observed earlier in the F_3 (47 %) generation for replicate 3. The decline in prevalence was rapid in replicate 3 compared to the decline observed for replicate 1 (Figure 22).

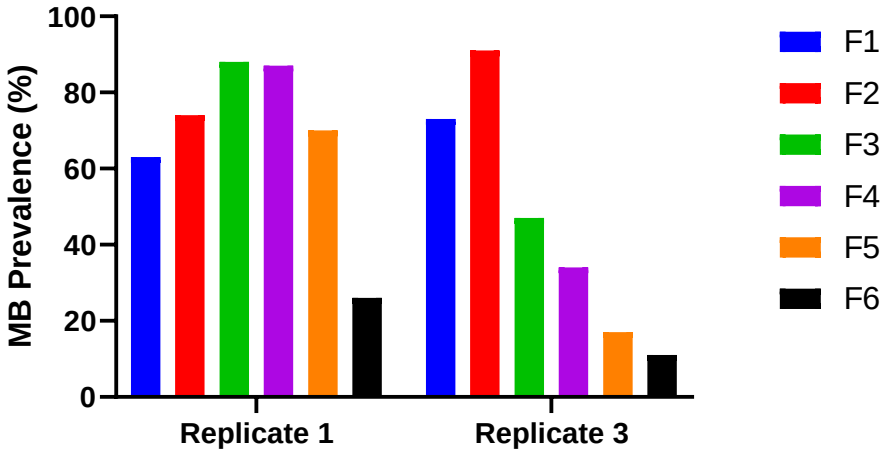


Figure 22: Prevalence of *Microsporidia MB* across generations in *An. arabiensis*

3.4.3 Correlation of *Microsporidia MB* prevalence and temperature

Prevalence increased to a point and declined in both replicates. The decrease in prevalence is seen with a concurrent decrease in temperature as well (Figure 23). A correlational analysis of the data shows a slight positive correlation (*tau* =0.2, *Z*=8.277, *df* =1, *P* < 0.0001) of *Microsporidia MB* prevalence with temperature (Figure 23). This demonstrates the impact of temperature on *Microsporidia MB* prevalence. Humidity data was also collected but did not have significant impact on the prevalence of *Microsporidia MB* (*tau* = 0.07, *Z*= 3.818, *df* =1, *P* < 0.0001). Humidity and temperature weekly average records is attached in appendix 5.

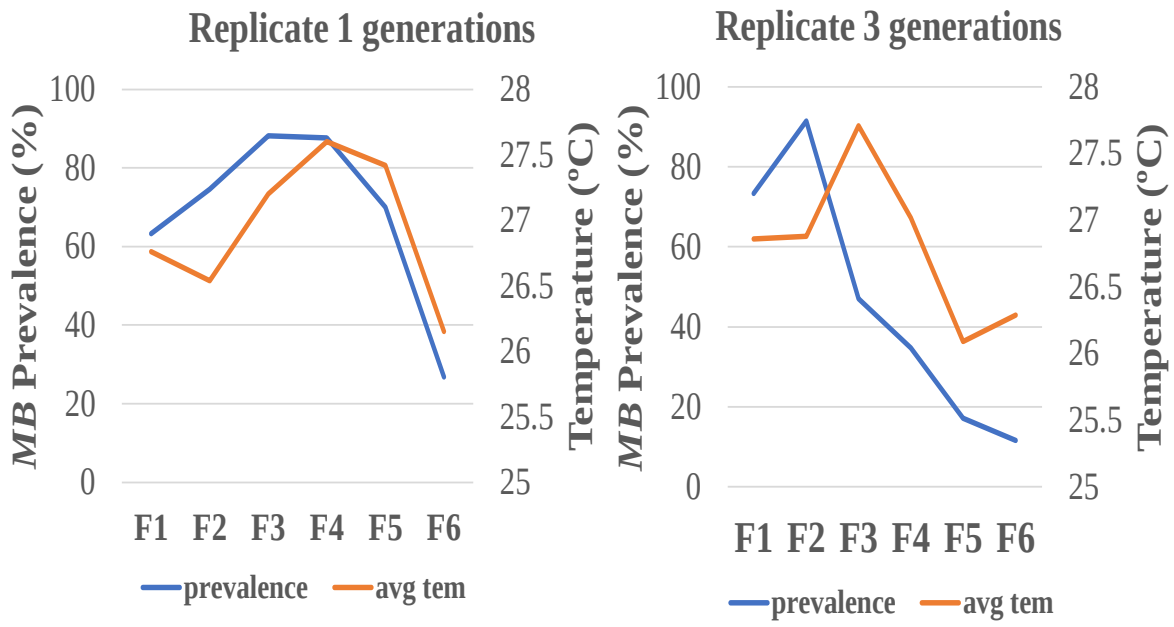


Figure 23: Prevalence of *Microsporidia MB* and temperature across generations in the two replicates (1 & 3).

3.4.4 *Microsporidia MB* intensity

i. Microsporidia MB intensity across generations

For both replicate 1 and 3, *Microsporidia MB* intensities between F_1 and F_6 generations did not change significantly (Figure 24, Appendix 3B & 4B). However, it is worth noting that there was a significant difference between F_6 and the other generations (F_2-F_5) for replicate 1 (Appendix 3B). Furthermore, for replicate 3, there was a significant change in *Microsporidia MB* intensity between F_1 and F_2 and F_3 respectively (Appendix 4B). These results clearly illustrate the consistent preservation of *Microsporidia MB* intensity in the long term as seen between F_1 and F_6 in all the replicates.

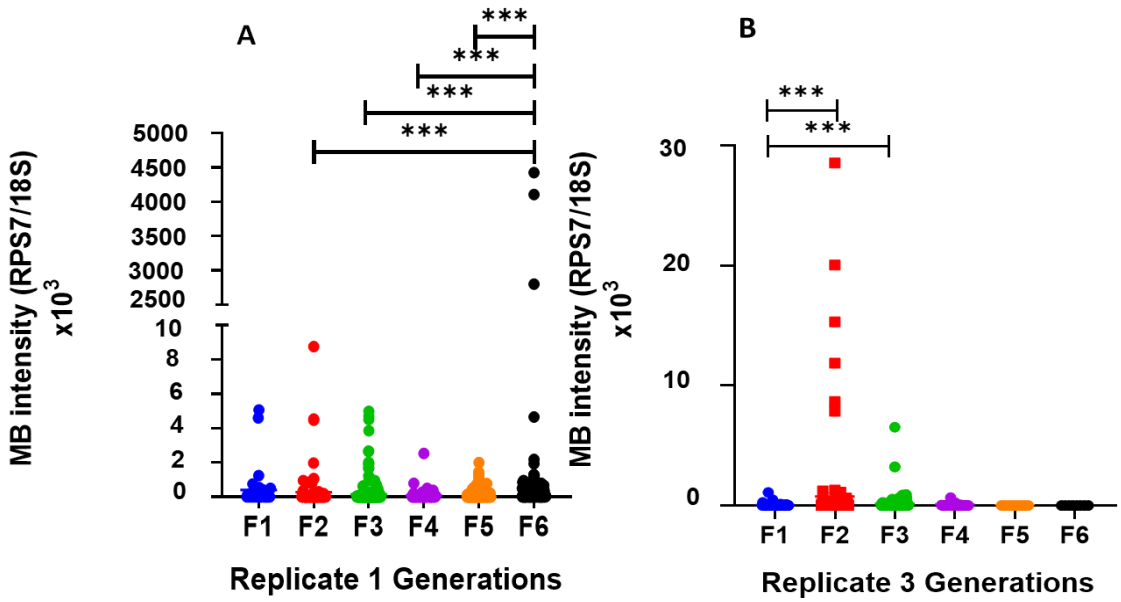


Figure 24: *Microsporidia MB* intensities across generations (A) Replicate 1 intensities (B) Replicate 3 intensities. Asterisks indicate the level of significance *(*P < 0.05, **P < 0 .001, ***P < 0.0001)*; ns indicates no significance.

ii. Microsporidia MB intensity by sex for the combined generations

A GLM analysis showed that mmosquitoes' sex affected the *Microsporidia MB* intensity (Figure 25). In both replicates 1 and 3, female mosquitoes significantly had higher intensity of *Microsporidia MB* compared to the males across generations ($t = 3.223$, $df = 1$, $P = 0.001$ and $t = 3.407$, $df = 1$, $P = 0.0007$ respectively). This was similar to what was observed in Figure 14B.

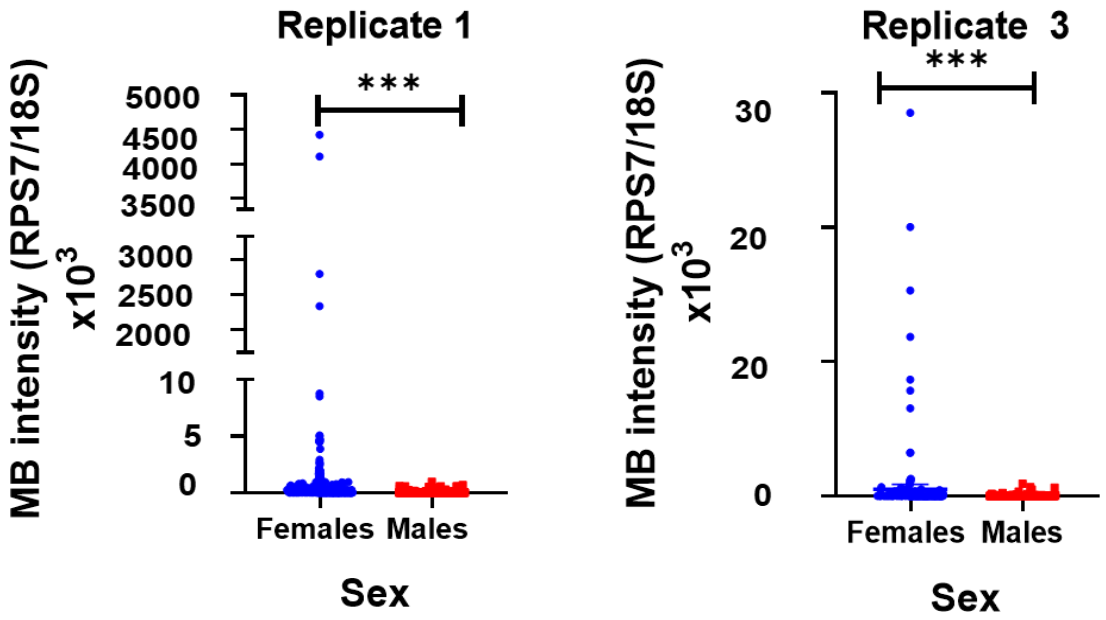


Figure 25: There is a difference in *Microsporidia MB* intensity between the mosquito sexes across generations. The line and the bars on the x-axis indicate the mean with 95% CI, respectively.

3.4.5 Wing size of mosquitoes across generations

One phenotypic characteristic that was measured across generations was the wing length, used as the proxy for the mosquito size in each generation. This physical factor (size of mosquito) is used to determine the fitness of the mosquitoes. Only Replicate 1 was used in this analysis due to replicate 3 not meeting the wing length sampling criteria of 10 male and 10 female mosquitoes per generation in F_6 (hence, it's exclusion). Using the mean size of F_1 mosquitoes as the baseline to compare all the other five generations (Figure 26) showed no significant difference between the males across the different generations ($F = 2.497$, $df = 5$, $P > 0.079$). The mean size and standard deviation of all the males were 3.16±0.123 and the that of all females were 3.69±0.189 respectively. With the exception of F_5, which was comparable to F_1 in size ($F = 5.69$, $df = 5$, $P < 0.0003$), the mean size of the female mosquitoes was much smaller in F_2, F_3, F_4 and F_6 respectively.

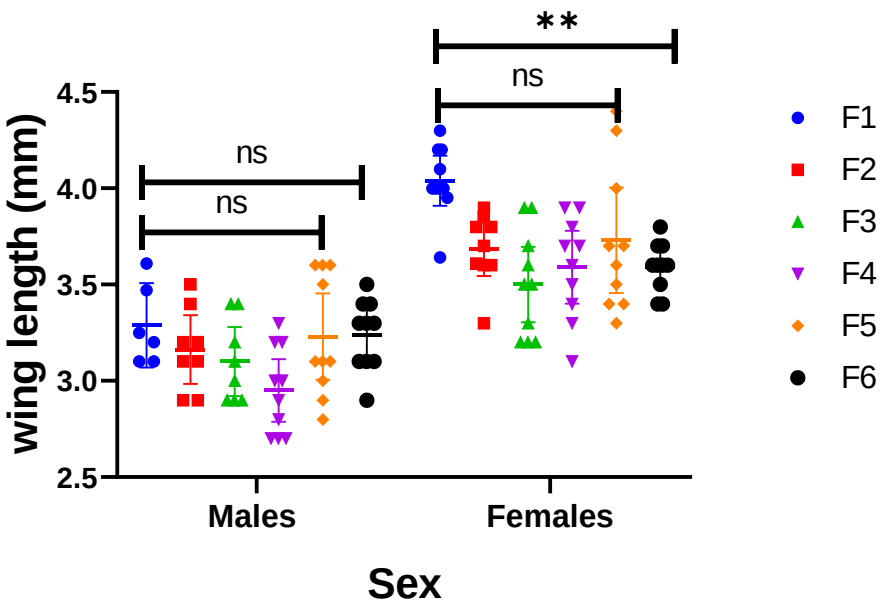


Figure 26: Male and female wing size of mosquitoes across generations for replicate 1. The line and the bars in the middle of each group indicate the mean with 95% CI, respectively. Asterisks indicate the level of significance (*$P < 0.05$, **$P < 0.001$, ***$P < 0.0001$); ns indicates no significance.

3.5 DISCUSSION

This research is the first study to assess if *Microsporidia MB* can be maintained across generations and tests some factors that might be related to its prevalence across generations and semi-controlled conditions. It provides an overview of the spread of the symbiont in mosquito populations under laboratory conditions over several generations. This study also provided essential information to understand how *Microsporidia MB* prevalence and intensity is maintained over generations and the impact of environmental conditions (humidity and temperature) affect *Microsporidia MB* prevalence. It is also worth noting that this was the first successful attempt to establish a *Microsporidia MB* mosquito colony. Previous attempts by others failed (Maina *et al,* unpublished data).

The success of the mosquito SIT projects depended on extensive studies on how to efficiently rear large numbers of healthy mosquitoes and consequently release the sterile males (Lees *et al.*, 2014, 2015; Mashatola *et al.*, 2018; Munhenga *et al.*, 2011). Similarly, the success story of *Wolbachia* mosquitoes-based dengue control strategy was preceded by a comprehensive laboratory study before the field trial (Hoffmann *et al.*, 2011; Jiggins, 2017). To advance the *Microsporidia MB* symbiont-based malaria control strategy, it is crucial to establish a self-sustaining colony of mosquitoes with high prevalence and intensity of the symbiont. While *Microsporidia MB* may be transmitted both vertically and horizontally, this transmission rate is not 100% efficient (Nattoh *et al.*, 2021). Hence, the establishment of a *Microsporidia MB*-infected colony provides a foundation that can then be used to address key questions around prevalence, intensity, and spread through artificial populations.

The lack of mating and death of the adult F_1 generation in both screen houses (with and without

vegetation), derived from G_0 female gravid mosquitoes collected from the field, may be attributable to change in environment. A previous study confirmed that field mosquitoes are often difficult to work with (Rose *et al.*, 2024). In my opinion, field mosquitoes could seek refuge in cooler locations, such as trees and other sheltered areas, during harsh environmental conditions such as elevated temperatures. However, in the screen house, the mosquitoes, despite being shaded, remained restricted to the population cage regardless of the environmental conditions.

The colony overview of the three laboratory replicates demonstrates the promise of using *Microsporidia MB* as a technique for controlling malaria (Herren *et al.*, 2020). To accurately simulate the spread of *Microsporidia MB* and determine the threshold of symbiotic infection, it is crucial to determine prevalence across generations. This was similar to the approach used in the *Wolbachia* mosquito release to assure effective implementation (Hancock *et al.*, 2011). Having knowledge of the point at which prevalence begins to decrease provides valuable information for strategies that could be adapted to optimise high *Microsporidia MB* prevalence in the mosquito colony. This study showed that the *Microsporidia MB* transmission rate to subsequent generations is not 100% under laboratory conditions.

It has been discovered that subsequent generations of *Wolbachia* are influenced by temperatures that are higher than 28 degrees Celsius (Tokash-Peters *et al.*, 2022), in addition to a significant number of other symbiotic microorganisms (Corbin *et al.*, 2017). It is necessary to assess the relationship between the prevalence of *Microsporidia MB* and temperature within the natural mosquito population. This is because temperature and humidity vary in nature.

The relative comparable intensity of *Microsporidia MB* between F_1 and F_6 in both replicates provides evidence that a decline in prevalence does not significantly decrease the intensity of the symbiont in the long term. This is important as the persistent intensity of *Microsporidia MB* ensures the blocking of the *Plasmodium* parasite in the mosquito over generations (Herren *et al.*, 2020). I hypothesise that the significantly higher intensity of *Microsporidia MB* in the female mosquitoes plays two significant roles. Firstly, a bias mechanism to ensure the efficient transfer of the symbiont vertically to offspring. This bias mechanism is observed in a bacteria symbiont in the whitefly (Himler *et al.*, 2011) and confirms my recent findings on diet impact on female *Microsporidia MB*-infected mosquitoes (Boanyah *et al.*, 2024). Secondly, this may represent a strategic advantage offered by *Microsporidia MB* to female mosquitoes, so enhancing human health by preventing the transmission of the *Plasmodium* parasite.

I anticipate future field trial releases of *Microsporidia MB* male mosquitoes as a strategy to propagate the symbiont through mating with the wild population, given that it has been shown that *Microsporidia MB* male mosquitoes can horizontally transmit the symbiont to uninfected female mosquitoes. (Nattoh *et al.*, 2021). The findings of this research indicate that male body size remains consistent throughout generations, suggesting that suggesting a fitness advantage (consistent mating competitiveness) under laboratory conditions. This associated fitness benefit may suggest that the mating competitiveness of male symbiont mosquitoes will remain unaffected when the symbiont disseminates across the mosquito population in consecutive generations. This is because a study has shown that bigger male mosquitoes possess a competitive advantage in mating compared to their smaller counterparts (Maïga *et al.*, 2012). My recent laboratory investigation in Chapter 2 found no substantial disparity in size between *Microsporidia MB*-infected and uninfected male mosquitoes, even when subjected to different

nutritional conditions (Boanyah *et al.*, 2024). Moreover, restricted dietary conditions did not substantially diminish the size of *Microsporidia MB*-infected male mosquitoes; hence, in wild environments with limited diets, the size of *Microsporidia MB* male mosquitoes is likely to be preserved through consecutive generations. However, *Microsporidia MB*-infected males exhibit more competitiveness in mating compared to uninfected males (Maina *et al*., 2025).

The above justifies that *Microsporidia MB-Anopheles* symbiosis could be used to prevent the transmission of the *Plasmodium* parasite from the symbiont-infected mosquitoes (Herren *et al.*, 2020) to humans, hence reducing the malaria prevalence in the communities where these mosquitoes would be released, similar to the approached used with *Wolbachia* symbiosis in *Aedes* mosquitoes to control dengue.(CDC, 2024).

CHAPTER 4

CONCLUDING SUMMARY

4.1 RE-STATEMENT OF THE PROBLEM

Malaria continues to pose a significant threat to lives, particularly among children and pregnant women in endemic countries (WHO, 2023). Since 2015, the reduction of malaria deaths and cases due to chemical-based mosquito control methods, that are the core malaria control interventions, has stalled (WHO, 2023). Furthermore, with the increased global connectivity, the importation of malaria cases into both endemic and non-endemic areas has become a growing public health concern (Mischlinger *et al.*, 2020; S. Sen Zhang *et al.*, 2019). Climate change also exacerbates this issue by influencing the mosquito's proliferation and life cycles, thereby affecting the transmission of malaria (Agyekum *et al.*, 2022; Anoopkumar & Aneesh, 2022; Giesen *et al.*, 2020). In response, continued research efforts are required to discover alternative ways to control the spread of malaria holistically. The discovery of *Microsporidia MB*, a *Plasmodium*-blocking symbiont in the *Anopheles* mosquito is one of such (Herren *et al.*, 2020).

To harness the potential of *Microsporidia MB* as a malaria control tool, it is crucial to address key knowledge gaps to ensure its feasible application. This research aimed to answer several important questions: How does *Microsporidia MB* affect mosquito fitness under various dietary conditions? What diet regimes can be used to mass-rear mosquitoes with high symbiont intensity for future field trials? What is *Microsporidia MB*'s capacity to spread in an *An. arabiensis* population?

4.2 KEY FINDINGS

4.2.1 *Microsporidia MB* does not adversely impact the development and fitness of *An. arabiensis*, even under limited (low quantity) dietary conditions

This study contributes to the expanding knowledge of factors that impact the symbiont-conferred fitness advantages are influenced by environmental factors, which is a pre-requisite for the successful spread of the symbionts in the host population. This addresses the knowledge gap regarding the fitness cost of *Microsporidia MB* on infected mosquitoes under various nutrient conditions (Boanyah *et al.*, 2024). Even under very limited dietary conditions, *Microsporidia MB* did not hinder the growth or fitness of *Anopheles* mosquitoes, so we can assume diet quality or nutrient variation in the field is unlikely to affect *Microsporidia MB* prevalence and intensity.

4.2.2 Optimal diet regime for mass rearing high intensity of *Microsporidia MB* mosquitoes under laboratory conditions

This study highlights the importance of identifying optimal larval and adult diets for producing high-intensity *Microsporidia MB*-infected mosquitoes in laboratory settings for use in laboratory experiments, semi-field and future trial releases. Previously, little was known about how nutrition affects both the mosquito host and the symbiont. This research demonstrated that *Microsporidia MB* confers a fitness advantage to the host under specific dietary conditions. After the evaluation of different larval diets, it was concluded that mass-rearing *Microsporidia MB*-infected *An. arabiensi*s mosquitoes using Tetramin 0.3 mg/larva/day for larvae and 6% glucose for adults provided the best results (Boanyah *et al.*, 2024).

4.2.3 Spread of *Microsporidia MB* in mosquito populations under laboratory conditions

The generational increased prevalence of *Microsporidia MB* from the F_1 to F_3 mosquito population and the gradual decline afterwards gives a clear overview of *Microsporidia MB*

spread under laboratory conditions. This was the first successful establishment of *Microsporidia MB*-infected *An. arabiensis* colonies and the understanding of *Microsporidia MB* prevalence patterns throughout successive generations provide a framework for improving rearing techniques to maintain high *MB* prevalence in mosquito colonies. In addition, maintaining *Microsporidia MB* intensity in the mosquito between the starting and the last generation gives credence to the potential of *Microsporidia MB* malaria control strategy. The consistent fitness levels (in terms of wing size) of male *Microsporidia MB*-infected *An. arabiensis* across generations further suggests the feasibility of using male release strategies in malaria control programmes.

4.3 RECOMMENDATION FOR FUTURE *MICROSPORIDIA MB* MOSQUITO COLONY ESTABLISHMENT

Knowledge on the prevalence pattern of *Microsporidia MB* informs the need to add the progeny of highly infected field-collected mosquito to F_2 *Microsporidia MB* colony and subsequently selecting only the offspring with a high prevalence by pre-screening 10 larvae for *Microsporidia MB* from every batch of eggs laid. This approach allows for the preservation of colonies with a high prevalence of *Microsporidia MB*.

Moreover, I recommend that future attempts to produce *Microsporidia MB* colonies in the screen house (semi-field system) should utilise pre-existing colonies from the laboratory. This may prevent the termination of F_1 generation mosquitoes derived from G_0 collected from the field. This is because laboratory colonies easily adapt to field conditions and not the reverse.

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APPENDICES

Appendix 1: Gel electrophoresis scoring for visualising *An. arabiensis* band

Gel scores: *A. arabiensis* band size =164 bp; *A. gambiae s.s* band size = 194 bp

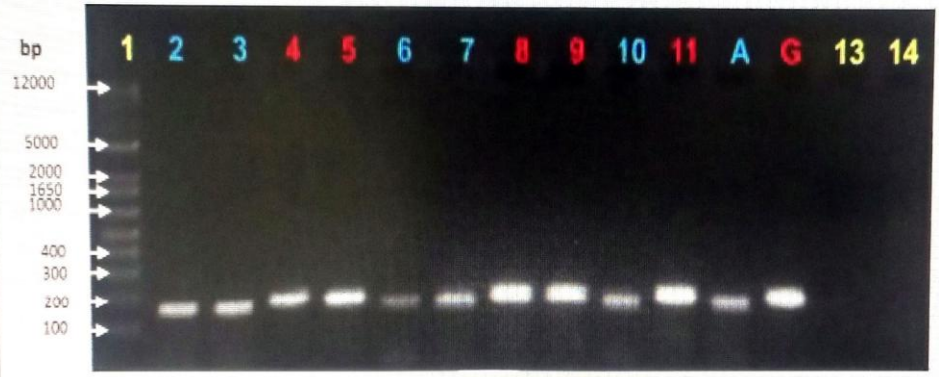


Figure 4.1: A gel representation of amplified products using *Anopheles gambiae* species-specific primers. Lane 1 represents TrackIt™ 1 Kb Plus DNA Ladder (Invitrogen™); lanes 2-11 represent the DNA samples; lane A is an *arabiensis* positive control (164 basepairs) while lane G is an *A. gambiae s.s.* control (194 basepairs). Samples labelled in blue were scored as *arabiensis* and those in red represent *gambiae* samples. Lanes 13 and 14 represent *A. funestus* control and no-template control (NTC), respectively.

Appendix 2: Ethics approval from the Human Research Ethics Committee (Medical), University of the Witwatersrand, South Africa.

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG	HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

Office of the Deputy Vice-Chancellor (Research and Innovation)

TO:	Mr GY Boanyah School of Pathology Wits Research Institute for Malaria Medical School University E-mail: 2624674@students.wits.ac.za
CC:	Supervisor: Professor L Koekemoer; Drs J Herren and ST Bukhari <Lizette.Koekemoer@wits.ac.za> and <HREC-Medical Research Office@wits.ac.za>
FROM:	Mr Iain Burns Human Research Ethics Committee (Medical) Tel: 011 717 1252 E-mail: Iain.Burns@wits.ac.za
DATE:	2022/11/02
REF:	R14/49
PROTOCOL NO:	**M220622** (This is your ethics application reference number. Please quote it in all enquiries, oral or written, relating to this study.)
PROJECT TITLE:	*Investigating the life history characteristics of Anopheles arabiensis infected with Microsporidia MB, a plasmodium falciparum blocking symbiont*

Please find attached the Clearance Certificate for the above project. I hope it goes well and that an article in a recognized publication comes out of it. This will reflect well on your professional standing and contribute to Government funding of the University.

MSWorks2000/Iain0007/Clearscan.wps

UNIVERSITY OF THE
WITWATERSRAND,
JOHANNESBURG

R49 Mr GY Boanyah

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
CLEARANCE CERTIFICATE NO. M220622

NAME: (Principal Investigator)	Mr GY Boanyah
DEPARTMENT:	School of Pathology Wits Research Institute for Malaria Medical School University
PROJECT TITLE:	*Investigating the life history characteristics of Anopheles arabiensis infected with Microsporidia MB, a plasmodium falciparum blocking symbiont*
DATE CONSIDERED:	2022/06/24
DECISION:	Approved unconditionally
CONDITIONS:	
NOTE:	If contact information regarding student study participants is required, please contact the Registrar's office - <Nicoleen.Potgieter@wits.ac.za>
SUPERVISOR:	Professor L Koekemoer; Drs J Herren and ST Bukhari
APPROVED BY:	Dr N Naran, Co-Chairperson, HREC (Medical)
DATE OF APPROVAL:	2022/11/02

This Clearance Certificate is valid for 5 years from the date of approval. An extension may be applied for.

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Research Office secretariat on the 3rd floor, Phillip Tobias Building, Parktown, University of the Witwatersrand, Johannesburg.

I/we fully understand the conditions under which I am/we are authorized to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated from the research protocol as approved, I/we undertake to submit details to the Committee. **I agree to submit a yearly progress report**. When a funder requires annual re-certification, the application date will be one year after the date when the study was initially reviewed. In this case, the study was initially reviewed in **June** and therefore reports and re-certification will be due in the month of **June** each year. Unreported changes to the study may invalidate the clearance given by the HREC (Medical).

Signature of Principal Investigator

4th November, 2022
Date

Appendix 3A: Descriptive statistics for *MB* intensity in Replicate 1 mosquitoes (F_1-6, Chapter 3)

	F_1	F_2	F_3	F_4	F_5	F_6
Number of values	36	142	292	110	187	175
Minimum	0.003927	0.002056	0.001405	0.002729	0.002034	0.03210
25% Percentile	0.5144	0.1851	0.07666	0.1871	0.9999	5.632
Median	6.306	1.769	1.545	6.807	2.721	41.53
75% Percentile	168.6	27.69	24.63	48.57	54.52	197.6
Maximum	5064	11323	4985	2534	890649	4424899
Mean	402.1	273.8	147.1	67.24	12766	78858
Std. Deviation	1123	1314	620.4	259.7	100663	528754
Std. Error of Mean	187.1	110.3	36.30	24.76	7361	39970
Lower 95% CI	22.24	55.76	75.60	18.17	-1756	-30.37
Upper 95% CI	781.9	491.8	218.5	116.3	27288	157747
Mean ranks	528.7	414.3	394.1	447.9	482.9	637.9

Appendix 3B: Kruskal Wallis Multiple comparisons of *MB* intensities in Replicate 1 (F_1-6, Chapter 3)

Number of families	1				
Number of comparisons per family	15				
Alpha	0.05				
Dunn's multiple comparisons test	**Mean rank diff.**	**Significant?**	**Summary**	**Adjusted P Value**	
F_1 vs. F_2	114.4	No	ns	0.3641	A-B
F_1 vs. F_3	134.6	No	ns	0.0766	A-C
F_1 vs. F_4	80.76	No	ns	>0.9999	A-D
F_1 vs. F_5	45.84	No	ns	>0.9999	A-E
F_1 vs. F_6	-109.2	No	ns	0.4237	A-F
F_2 vs. F_3	20.21	No	ns	>0.9999	B-C
F_2 vs. F_4	-33.61	No	ns	>0.9999	B-D
F_2 vs. F_5	-68.53	No	ns	0.3548	B-E
F_2 vs. F_6	-223.6	Yes	****	<0.0001	B-F
F_3 vs. F_4	-53.82	No	ns	>0.9999	C-D
F_3 vs. F_5	-88.73	Yes	**	0.0075	C-E
F_3 vs. F_6	-243.8	Yes	****	<0.0001	C-F
F_4 vs. F_5	-34.91	No	ns	>0.9999	D-E
F_4 vs. F_6	-190.0	Yes	****	<0.0001	D-F
F_5 vs. F_6	-155.1	Yes	****	<0.0001	E-F

Appendix 4A: Descriptive statistics for *MB* intensity in Replicate 3 mosquitoes ($F_{1\text{-}6}$, Chapter 3)

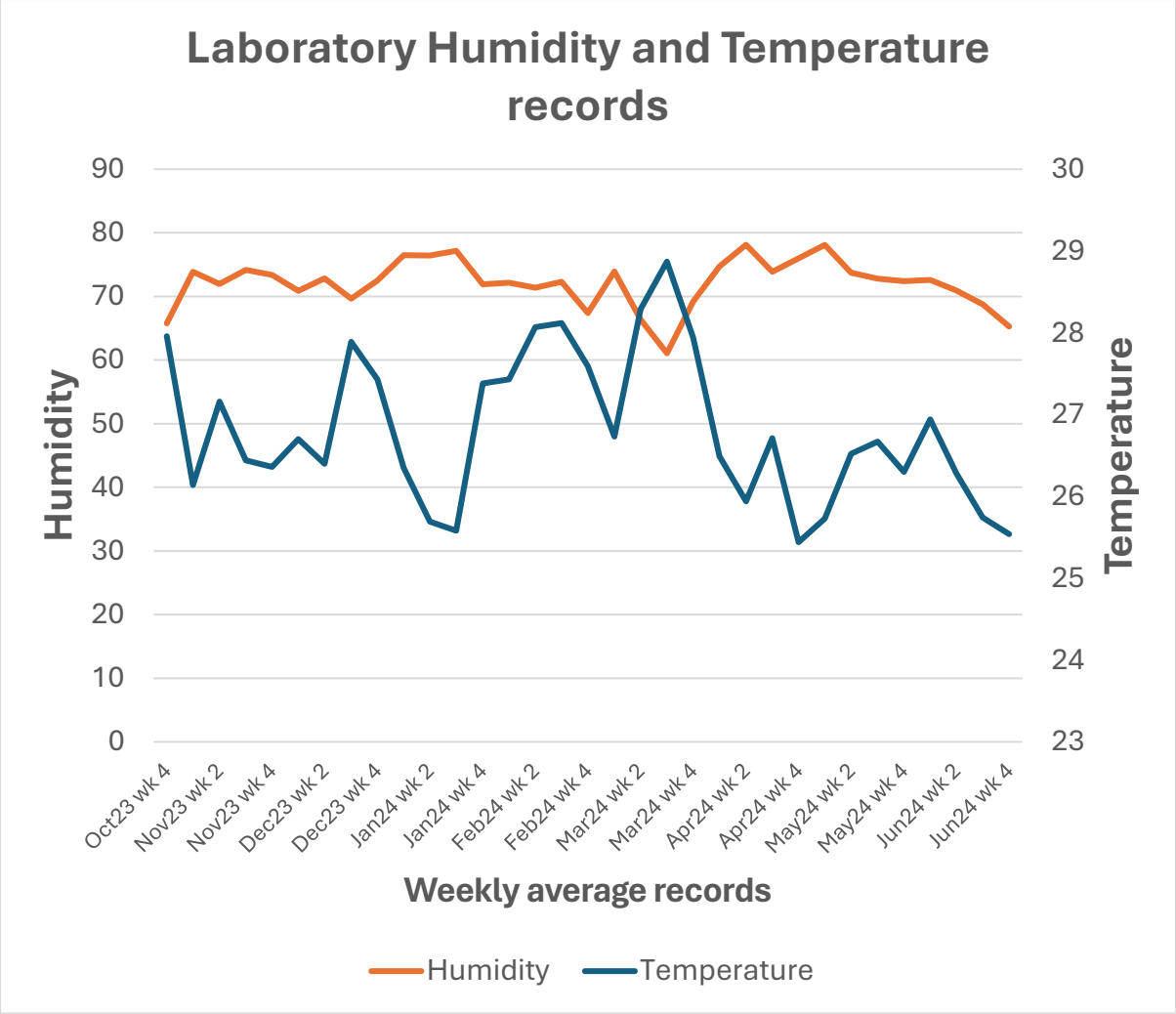
	F_1	F_2	F_3	F_4	F_5	F_6
Number of values	134	139	132	24	9	8
Minimum	0.0005250	0.007755	0.006557	7.400e-005	0.004408	1.770e-008
25% Percentile	0.03115	0.4909	0.3622	0.02464	0.03926	0.1407
Median	0.5015	8.121	6.966	2.034	3.432	0.7058
75% Percentile	10.81	65.15	89.73	56.48	9.957	1.282
Maximum	1094	28545	6534	663.8	11.60	6.559
Mean	23.74	745.3	171.2	70.46	4.776	1.322
Std. Deviation	106.8	3466	697.3	149.9	5.008	2.167
Std. Error of Mean	9.229	294.0	60.70	30.61	1.669	0.7661
Lower 95% CI	5.489	163.9	51.13	7.151	0.9262	-0.4892
Upper 95% CI	42.00	1327	291.3	133.8	8.625	3.134
Mean ranks	169.5	259.4	250.6	208.0	177.9	155.3

Appendix 4B: Kruskal Wallis Multiple comparisons of *MB* intensities in Replicate 3 (F_1**-6, Chapter 3)**

Number of families	1				

Number of comparisons per family	15				
Alpha	0.05				
Dunn's multiple comparisons test	**Mean rank diff.**	**Significant?**	**Summary**	**Adjusted P Value**	
F_1 vs. F_2	-89.91	Yes	****	<0.0001	A-B
F_1 vs. F_3	-81.14	Yes	****	<0.0001	A-C
F_1 vs. F_4	-38.52	No	ns	>0.9999	A-D
F_1 vs. F_5	-8.411	No	ns	>0.9999	A-E
F_1 vs. F_6	14.23	No	ns	>0.9999	A-F
F_2 vs. F_3	8.775	No	ns	>0.9999	B-C
F_2 vs. F_4	51.39	No	ns	>0.9999	B-D
F_2 vs. F_5	81.50	No	ns	0.9902	B-E
F_2 vs. F_6	104.1	No	ns	0.3941	B-F
F_3 vs. F_4	42.61	No	ns	>0.9999	C-D
F_3 vs. F_5	72.72	No	ns	>0.9999	C-E
F_3 vs. F_6	95.36	No	ns	0.6323	C-F
F_4 vs. F_5	30.11	No	ns	>0.9999	D-E
F_4 vs. F_6	52.75	No	ns	>0.9999	D-F
F_5 vs. F_6	22.64	No	ns	>0.9999	E-F

Appendix 5: Humidity and temperature records in the laboratory



Appendix 6: First page of Chapter 2 published paper

Boanyah *et al. Parasites & Vectors* (2024) 17:294
https://doi.org/10.1186/s13071-024-06365-8

Parasites & Vectors

RESEARCH Open Access

Effect of *Microsporidia MB* infection on the development and fitness of *Anopheles arabiensis* under different diet regimes

Godfred Yaw Boanyah[1,2], Lizette L. Koekemoer[2,3], Jeremy K. Herren[1,2*] and Tullu Bukhari[1*]

Abstract

Background *Microsporidia MB* (*MB*) is a naturally occurring symbiont of *Anopheles* and has recently been identified as having a potential to inhibit the transmission of *Plasmodium* in mosquitoes. *MB* intensity is high in mosquito gonads, with no fitness consequences for the mosquito, and is linked to horizontal (sexual) and vertical (transovarial) transmission from one mosquito to another. Maximising *MB* intensity and transmission is important for maintaining heavily infected mosquito colonies for experiments and ultimately for mosquito releases. We have investigated how diet affects the *MB-Anopheles arabiensis* symbiosis phenotypes, such as larval development and mortality, adult size and survival, as well as *MB* intensity in both larvae and adults.

Methods F_1 larvae of G_0 females confirmed to be *An. arabiensis* and infected with *MB* were either combined (group lines [GLs]) or reared separately (isofemale lines [IMLs]) depending on the specific experiment. Four diet regimes (all mg/larva/day) were tested on F_1 GLs: Tetramin 0.07, Tetramin 0.3, Gocat 0.3 and Cerelac 0.3. GLs reared on Tetramin 0.3 mg/larva/day were then fed either a 1% or 6% glucose diet to determine adult survival. Larvae of IMLs were fed Tetramin 0.07 mg and Tetramin 0.3 mg for larval experiments. The mosquitoes in the adult experiments with IMLs were reared on 1% or 6% glucose.

Results Amongst the four larval diet regimes tested on *An. arabiensis* development in the presence of *MB*, the fastest larval development highest adult emergence, largest body size of mosquitoes, highest prevalence and highest density of *MB* occurred in those fed Tetramin 0.3 mg/larva/day. Although adult *MB*-positive mosquitoes fed on 6% glucose survived longer than *MB*-negative mosquitoes, there was no such effect for those fed on the 1% glucose diet. Development time, wing length and adult survival were not significantly different between *MB*-infected and uninfected *An. arabiensis* fed on the Tetramin 0.07 mg/larva/day diet, demonstrating that the *MB*-conferred fitness advantage was diet-dependent.

Conclusions *Microsporidia MB* does not adversely impact the development and fitness of *An. arabiensis*, even under limited dietary conditions. The diet regime of Tetramin 0.3 mg/larva/day + 6% glucose for adults is the superior diet for the mass rearing of *MB*-infected *An. arabiensis* mosquitoes. These results are important for rearing *MB*-infected *An. arabiensis* in the laboratory for experiments and the mass rearing required for field releases.

Keywords *Microsporidia MB*, Larval diet, Adult diet, Life history traits

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