ADDITIVE EFFECTS OF DRAGONFLY (*Pantala flavescens)* NYMPH AND FUNGUS *(Beauveria bassiana)* ON DEVELOPMENT AND SURVIVAL OF MALARIA MOSQUITO (*Anopheles gambiae)*

BY

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DEPARTMENT OF ZOOLOGY

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DECLARATION

I declare that this thesis is my own original work and it has not been presented in any institution of higher learning for the award of a degree or certificate. This thesis or any part of it may not be reproduced without written permission from the author and supervisors.

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DEDICATION

I dedicate this work to my parents, Mr. and Mrs. Ong'wen, together with my siblings who have been supporting me morally, spiritually and even financially.

ABSTRACT

Malaria continues to be a world-wide human health problem. Insecticide resistance challenge efficacy and sustainability of malaria control programs and therefore call for sustainable malaria control strategies. Environmental factors affect mosquito development and survival and should be considered when designing these strategies. However, there is limited knowledge on mosquito ecology, especially on interactions with predator such as dragonfly *Pantala flavescens* nymph and parasite such as *Beauveria bassiana* fungus across stages. This study focused on investigating additive effects of *P. flavescens* and *B. bassiana* on *Anopheles gambiae*. Specific objectives were to determine: predation efficacy of *P. flavescens* nymph against *A. gambiae* larvae; development rate of *A. gambiae* larvae reared in presence of varying densities of *P. flavescens* nymphs; efficacy of *B. bassiana* against *A. gambiae* larvae; and survival of adult mosquitoes exposed to fungus after predator and/or parasite pre-exposure at larval stage. All experiments consisted of survival bioassays quantified either as pupation day or dead larvae and adults. Mosquito eggs were obtained from The Center for Global Health Research, KEMRI; dragonfly nymphs from Ahero Irrigation Scheme, Kenya; and *B. bassiana* spores (IMI- 391510) provided by IN2CARE®, The Netherlands. Predation efficacy investigation involved four replicates of 30 larvae exposed to 1 dragonfly nymph. Development rate investigation involved exposing four replicates of 30 larvae to varying numbers (0-4) of constrained dragonfly nymphs. Four replicates of 30 larvae were exposed to varying spore concentration (0-12 mg) to determine fungal efficacy. Three replicates of 30 adults pre-exposed to predator and/or fungus were exposed to same fungus for adult survival. Predation efficacy test showed significant difference in mean number of dead larvae ($Z=-12.667$, $P<0.001$). Development rate test showed significant difference ($P<0.001$) for groups exposed to 1 or 2 nymphs but group exposed to 4 nymphs ($P=0.227$) was not significantly different. Fungal efficacy test showed that larvae exposed to 3, 6 and 12 mg of fungus had HR, 2.0, 2.5 and 3.5, respectively. In adult survival test, adults not pre-exposed to any factor, those pre-exposed to predator, parasite or both predator and parasite had HR of 45.8, 67.4, 50.9 and 112.0, respectively. It is clear that single and additive effects of the predator and/or parasite affect mosquito development and survival, because it affects mosquito physiology and immunity. However, field studies should be done to prove consistency in the field. The knowledge can then be employed by Ministry of Health for malaria control in areas with *P. flavescens*.

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LIST OF ABBREVIATIONS AND ACRONYMS

CDC	-Centers for Disease Control and Prevention
HR	-Hazard ratio
IVM	-Integrated Vector Management
KEMRI	-Kenya Medical Research Institute
PO	-Phenoloxidase enzyme
LT50	-Median lethal time
ITN	-Insecticide Treated Net/bed net

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CHAPTER ONE

INTRODUCTION

1.1. Background information

Malaria continues to be one of the most important health problems worldwide. The disease is caused by *Plasmodium falciparum, Plasmodium vivax, Plasmodium malariae, Plasmodium ovale* and *Plasmodium knowlesi.* These parasites are transmitted by *Anopheles* mosquitoes, which are their vectors, with the most important being *Anopheles gambiae* (Cohuet, Harris, Robert, & Fontenille, 2010). However, the four species are not typically as life threatening as *P. falciparum.* Despite several control measures aimed at eliminating the disease, the number of malaria cases and deaths is still high. In 2015, for example, there were an estimated 214 million malaria cases and 438,000 deaths. Most of these cases and deaths occurred in the African Region, followed by South-East Asia Region and Eastern Mediterranean Region (WHO, 2015).

Current malaria control strategies are all insecticide-based and mainly target the adult stage of *Anopheles* mosquito. The strategies are challenged by the motility of the adult mosquitoes as well as increasing insecticide resistance (Townson et al., 2005). There is, therefore, an increased interest in control strategies that target the vector's larval stage, because larvae are confined and concentrated in the breeding sites. However, the ideal approach is the Integrated Vector Management (IVM) approach that combines control strategies that target both the larval and adult mosquitoes (Townson et al., 2005) and gives preference to non-chemical methods. However, there is need for a better understanding of larval and adult mosquito ecological interactions in their natural environment. Studies show that there is a high larval mortality in the

natural breeding sites due to climatic conditions, parasitism and predation (Aniedu et al., 1993; Paaijmans et al., 2007; Service, 1993). The selection pressure exerted by the high mortality, due to environmental factors may imply that surviving individuals are better equipped for future climate, parasite and predator challenges, which may be due to pre-exposure. It is actually known that a high proportion of wild *A. gambiae* females are resistant to infection by *Plasmodium* parasite and therefore do not contribute to malaria transmission (Niaré et al., 2002; Riehle, 2006). This resistance is linked to immune response or immune priming of mosquitoes (Niaré et al., 2002; Riehle, 2006), due to pre-exposure and exposure to parasites at either larval or adult stages.

Environmental factors have an effect on mosquito larvae in the breeding sites. These factors may be biotic, involving intraspecific and interspecific interactions such as predation, parasitism and competition, or may be abiotic factors such as climatic conditions. Both biotic and abiotic factors affect development and survival of malaria mosquito (Aniedu et al., 1993; Paaijmans et al., 2007; Service, 1993). For instance, aquatic habitats have different abiotic factors, including water temperature, turbidity, currents and pH (Kenea et al., 2011), which affect development and survival of mosquito larvae. In addition to the direct effects, biotic factors also have indirect effects and therefore, may influence mosquito development and survival differently. However, our understanding of the effects of these factors on the mosquito is limited. Furthermore, as mentioned above, larvae that survive in the breeding sites with different selection pressures may develop into adults that are different in their susceptibility to future challenges such as parasitic infections (Kenea et al., 2011), due to immune enhancement resulting from pre-exposure. This study focused on interaction of two biotic factors; predation and parasitism, with larval stage of

malaria mosquito, and the effect of these factors on the susceptibility of the adult mosquito to the same parasite in a subsequent exposure.

Predators of *Anopheles* mosquito larvae include larvivorous fish, amphibians and insects such as dragonfly nymph (Kamareddine, 2012; Scholte et al., 2004; Walker, 2002), which kill the larvae by directly feeding on them (lethal effect). Dragonfly spends its nymphal stage in water pools. It is a voracious predator of mosquito and other insect larvae, tadpoles and even small fish. For instance, though the widespread *Pantala flavescens* dragonfly nymph has been associated with low densities or absence of *Anopheles arabiensis* larvae in otherwise suitable breeding sites (Gouagna et al., 2012), the results are not attributed to the nymph's predation efficacy or exposure effect on larval development and survival. However, the efficacy of *P. flavescens* nymph against Kisumu strain *A. gambiae* larvae is not known.

Predators also have non-lethal effects (Preisser et al., 2005) against malaria mosquito larvae. For instance, presence of predator can reduce the vector population by affecting the vector immunity through reducing phenoloxidase (PO) enzyme production (Boltaña et al., 2013; Stoks et al., 2006), hence the vector will die if exposed to deadly parasites. Phenoloxidase cascade is involved in clearance of many different pathogens (Braun et al., 1998) by melanization as well as in wound repair (Sugumaran, 2002), and its reduction increases prey susceptibility to pathogens (Boltaña et al., 2013; Stoks et al., 2006). Predators also influence the development of *Anopheline* mosquitoes, as studied in a number of different species including *A. gambiae*, (Kweka et al., 2011), and found out that different predators affect the vector development differently. However,

the effect of the presence of varying densities of *P. flavescens* nymph on the development rate of this strain of *A. gambiae* mosquito larvae is not known.

Several parasites including fungi, microsporidia, oomycetes, flagellates and protists are present in sites that mosquitoes use for breeding. Fungi, microsporidia and oomycetes have been tested in the laboratory and field for their ability to control *A. gambiae* larvae, and have been shown to infect and kill larvae (Boltaña et al., 2013; Stoks et al., 2006), as well as adult mosquitoes by structural damage and endotoxin production (Bukhari et al., 2010). This is because fungal and oomycete infections can be carried from the larval to the adult stage during metamorphosis (Bukhari et al., 2010; Lord & Roberts, 1987; Sandhu et al., 1993; Wilson et al., 1990). Laboratory studies, for instance, have shown that isolates of fungal pathogens such as *B. bassiana* can infect and kill adult mosquitoes including those resistant to chemical insecticides (Heinig et al., 2015), since they are still susceptible to the fungus. This makes the fungus a good candidate for biological control of insecticide resistant mosquitoes. It is an entomopathogenic fungus that grows naturally in soils throughout the world and parasitizes various arthropod species including termites, thrips, whiteflies, aphids, various beetles, bedbugs, and mosquitoes (Barbarin et al., 2012). Despite *B. bassiana* being an effective parasite against insects, including *A. gambiae* (Bukhari et al., 2010), different strains of the same fungal species vary in their virulence and efficacy against different laboratory and field populations of insects, and even mosquitoes. Also, climatic factors such as temperature and humidity of a region influence the efficacy of fungal species. The fungal dosage is another important factor that affects mortality of insects exposed. This is why it was necessary to test the efficacy of varying dosage of *B. bassiana*, strain IMI- 391510 against the Kisumu strain *A. gambiae* larvae reared in Maseno.

Immune response is the last line of defense against parasite infection and determines whether a mosquito is susceptible or refractory to the infection. The refractoriness in mosquitoes can be due to a subsequent infection with the same or another parasite even at different developmental stages, which stimulates vector immune action enhancement against the parasite. For example, infection with the microsporidian *Vavraia culicis* at the larval stage resulted in increased refractoriness to *Plasmodium* infection in the adults that developed from surviving larvae (Bargielowski & Koella, 2009).

Mosquito interaction with other organisms has been reviewed by various researchers (Blaustein & Chase, 2007; Juliano & Lounibos, 2005; Merritt et al., 1992), but there has been need for comprehensive investigations on the effects of interspecific interactions of *A. gambiae* mosquito with *P. flavescens* and *B. bassiana*. In nature, larval habitats can harbor both predators and parasites and mosquito larvae can be exposed to both simultaneously. It is unknown how the contemporaneous presence of both predator and parasite affect mosquito immune system during and after the larval stage. The effects of these interactions can be extremely relevant if the first and/or subsequent exposure/infection is with a parasite such as *Plasmodium falciparum* that causes deadly human disease. Therefore, there is need to know how the single and combined additive predator-parasite pre-exposure effects would influence adult vector susceptibility to the same parasite in future, and even adult vector survival.

1.2. Statement of the problem

Malaria remains an important health problem worldwide. Currently, malaria control programs are mainly insecticide-based, targeting the host seeking and indoor resting female *Anopheles*

mosquito. The efficacy of these programs is increasingly declining as insecticide-resistant mosquitoes and drug-resistant *Plasmodium* parasites emerge. To date, there is no proven long-lasting malaria vector control strategy. The non-target effects of insecticides and malaria mosquito resistance to insecticides have raised the need for an integrated approach, which requires understanding of the vector ecology and its interaction with other organisms. There is the need of knowing how the interaction affects the mosquito from the larval to the adult stage. Also, there is limited knowledge, especially about how larval environment impacts adult susceptibility to parasites. It is also important to understand combined interactions between the vector and environmental factors that are either biotic or abiotic, which affect the life of the vector. These factors, including predator and parasite can be employed in malaria vector control. However, there is the need of finding out effective predator and parasite that affect both larvae and adult mosquito significantly, and which may be used in vector control.

Although the dragonfly *Pantala flavescens* nymph is both ubiquitous in its distribution and is a known predator of mosquito larvae (Gouagna et al., 2012), its predation efficacy against Kisumu strain *A. gambiae* mosquito larvae, and thus potential to reduce mosquito populations remain unknown. Predators can also affect prey negatively in a non-lethal way, and in the case of the dragonfly *P. flavescens* nymph, there is the need of knowing its non-lethal exposure effects on *A. gambiae* larvae development. Amongst fungi, the parasitic, entomopathogenic fungus *Beauveria bassiana* has been proven to infect and kill the malaria vector (Bukhari et al., 2010). It also influences its survival by affecting its immunity, either by compromising it or triggering vector immune priming. However, the efficacy of *B. bassiana,* strain IMI- 391510 against the Kisumu strain malaria mosquito larvae, and the fungal efficacy against adult mosquito survival, even after predator *P. flavescens* nymph and/or fungal parasite pre-exposure, is unknown.

Although integrating larval and adult control has been shown to decrease the population of malaria mosquito by >90% (Fillinger et al., 2008), and despite a history of success, the use of larval control is limited in the current malaria control programs in Africa. This limited use of larval control has mainly been due to the impracticality of finding and treating every breeding site with larvicides. However, the empirical data on the effects of breeding site environment on adult mosquito development and survivorship, even after a successive infection, is lacking. Also, there are major gaps in knowledge on the additive effects of predator and parasite on the malaria vector in its ecology. This study (Figure 1.1) focuses on the additive effects of the predator *P. flavescens* nymph, and the parasite *B. bassiana*, on the development and survival of Kisumu strain *A. gambiae* mosquito.

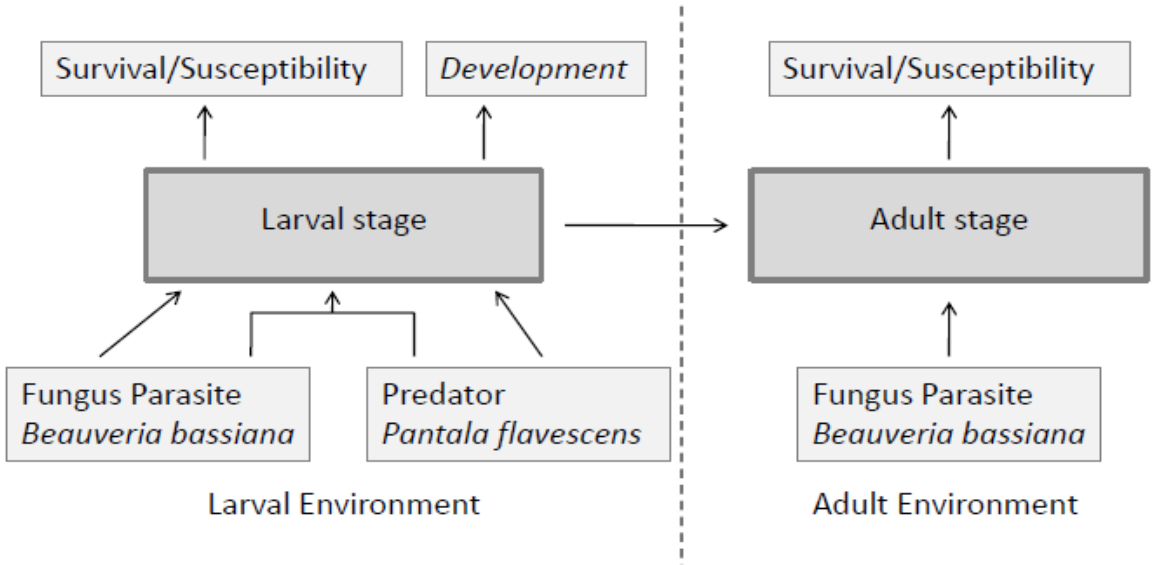

Figure 1.1. A schematic diagram of the study

The study aimed at determining the additive effects of the dragonfly *P. flavescens* nymph, and parasitic fungus *B. bassiana,* on the development and survival of *Anopheles gambiae* mosquito.

1.3. Justification of the study

Integrated vector management approach provides a sustainable and effective approach to malaria control but requires a comprehensive understanding of vector ecology. The combined synergistic or additive effects of various environmental factors in the mosquito larval ecology are very important when it comes to understanding larval development and survival in their natural environment. This is because research has shown that breeding sites vary in terms of their productivity of mosquitoes (Fillinger et al., 2009). This variation may be due to the different biotic and abiotic factors in various larval habitats. For instance, some breeding sites are small and temporary and may only harbor a small number of mosquito larvae and a few microorganisms that will be involved in the interaction. Others are permanent or semi-permanent and can harbor a variety of organisms, including predators and parasites, due to their size and permanency (Fillinger et al., 2009; Fillinger et al., 2004), hence the interaction involves many organisms. As a result, mosquito larvae experience different environment depending on the type of the breeding site they develop in. The interaction of the human malaria vector with environmental factors, such as predator and parasite during the larval stage influence its development and survival, and also the adult susceptibility to parasitic infection and transmission. Predator-prey and parasite-host interactions that are the focus of this study highlight the efficacy and utility of the predator and parasite in directly affecting the vector larvae development and survival, and also influencing adult susceptibility to subsequent parasite infection. This provides an insight into the effect of larval ecology beyond the larval stages of mosquitoes.

1.4. Significance of the study

The findings of this study enhance the existing knowledge of basic predator-prey and parasite-host interactions in mosquito ecology. Furthermore, the information on the single and combined additive effects of *P. flavescens* nymph and *B. bassiana* against *A. gambiae* will guide future studies that will involve adult mosquito infection with human malaria parasite, *P. falciparum*. The results can be highly relevant to malaria epidemiology if the presence of predator and/or parasite during larval development has the same effect on adult susceptibility to *P. falciparum* as it had on adult susceptibility to *B. bassiana*.

Also, this study has underlined the influence of larval control beyond the larval stage of mosquito vector and can be utilized by the Ministry of Health in making informed decisions on effective mosquito control strategies.

1.5. Objectives of the study

1.5.1. General objective

To investigate the additive effects of dragonfly (*Pantala flavescens)* nymph and parasitic fungus (*Beauveria bassiana)* on the development and survival of malaria mosquito (*Anopheles gambiae)*.

1.5.2. Specific objectives

1. To determine the predation efficacy of *P. flavescens* nymph against *A. gambiae* larvae survival.

2. To determine the development rate of *A. gambiae* larvae reared in the presence of varying densities of *P. flavescens* nymph.
3. To determine the efficacy of varying dosage of *B. bassiana* against *A. gambiae* larvae survival.
4. To determine the survival of adult mosquitoes exposed to the fungus *B. bassiana* after they emerged from water with the predator *P. flavescens* nymph, with the parasite *B. bassiana*, and with both the predator and the parasite.

1.6. Null Hypotheses

1. There is no difference in the survival of *A. gambiae* larvae reared either in the presence or absence of *P. flavescens* nymph.
2. There is no difference in the development rate of *A. gambiae* larvae reared in the presence of varying densities of *P. flavescens* nymph.
3. There is no difference in the survival of *A. gambiae* larvae exposed to varying dosage of parasitic fungus *B. bassiana*.
4. There is no difference in the survival of adult mosquitoes exposed to the fungus *B. bassiana* after they emerged from water with the predator *P. flavescens* nymph, with the parasite *B. bassiana*, and with both the predator and the parasite.

CHAPTER TWO

LITERATURE REVIEW

2.1. Malaria

Human malaria continues to be one of the most important health problems worldwide. The disease is caused by *P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale* and *P. knowlesi* that are transmitted by *Anopheles* mosquitoes, which are vectors of the malaria parasite (Cohuet et al., 2010). By 2015, it was estimated that the number of malaria cases was 214 million (range: 149-303 million) and the number of deaths was 438,000 (range: 236,000- 635,000), (WHO, 2015). These statistics are still high hence negatively impact the health, social and economic status of people. Most cases in 2015 were estimated to occur in the WHO African Region (88%), followed by the WHO South-East Asia Region (10%) and WHO Eastern Mediterranean Region (2%) (WHO, 2015). Though other *Anopheline* species transmit malaria, *Anopheles gambiae* Giles is the most efficient vector of human malaria in the African tropical Region (CDC, 2010), and thus it is commonly called the African Malaria Mosquito, (CDC, 2014). It is an important vector of the major human malaria parasite, *Plasmodium falciparum* in Africa where about 90% of all malaria deaths occur (WHO, 2012).

Considering the losses caused by the disease, it is necessary to keep on working to achieve its control. Many control strategies targeting either *P. falciparum* parasite or *A. gambiae* vector were proved to be effective until the emergence of drug resistant malaria parasites and insecticide resistant mosquito strains (Kamareddine, 2012). Also, the chemical agents used as insecticides caused numerous health, environmental and ecological side effects (Kamareddine, 2012) to non-target organisms including humans. Therefore, there has not been a single, effective, long lasting measure to eradicate malaria. This is why it is necessary to work towards

achieving effective integrated strategy that involves the use of a combination of control methods with priority given to the non-chemical methods. A better understanding of mosquito ecology is imperative to developing non-chemical mosquito control methods.

2.2. Larval control of mosquito vector with biological control agents

The main insecticide-based adult mosquito control methods used for malaria control are not sustainable due to development of resistance by mosquitoes against the insecticides (Farenhorst et al., 2009). Compared to adult control, there is a larger arsenal of biological agents that can be used for the larval control of mosquitoes (Bukhari et al., 2013), hence the need to identify the most effective ones for both mosquito stages. It has even been shown that integrating larval control using bacteria *Bacillus thuringiensis,* and *Bacillus sphaericus,* with adult targeting insecticide treated bed nets (ITNs) resulted in a two-fold reduction of new malaria cases in highlands of Kenya compared to only ITN use (Fillinger et al., 2008). In addition, larval control targets the aquatic stages of *Anopheles* mosquitoes, which results in reduced adult emergence from breeding sites, and therefore, reduced numbers of adult mosquitoes in surrounding areas (Bukhari et al., 2013). Use of biological agents to control human malaria mosquito is considered a fundamental part of the malaria eradication program and has so far shown promising results (Kamareddine, 2012). The most promising biological control agents for malaria eradication are mainly predators and parasites. These include larvivorous fish, amphibians, insects, viruses, fungi, bacteria, and nematodes (Jean-Francois & Nielsen-LeRoux, 2000; Kamareddine, 2012; Scholte et al., 2004; Walker, 2002). Among the various ecological factors controlling immature *A. gambiae* vector populations, parasitism and predation appear to be the major factors controlling their population size (Ohba et al., 2010).

Among predators such as larvivorous fish, amphibians and insects (Jean-Francois & Nielsen-LeRoux, 2000; Kamareddine, 2012; Scholte et al., 2004; Walker, 2002), the dragonfly *Pantala flavescens* nymph is thought to be an effective and widely distributed predator of malaria mosquito larvae (Tutt, 1997). Furthermore, the dragonfly nymph is a suitable biological control agent because the nymphal stage takes 1- 2 years in the water site before developing to adults (Tutt, 1997). Consequently, its efficacy as a predator of *A. gambiae* larvae can be expected to be high. However, the efficacy of the predator *P. flavescens* dragonfly nymph against Kisumu strain *A. gambiae* larvae survival is unknown.

Among parasites such as viruses, fungi, bacteria, and nematodes (Jean-Francois & Nielsen-LeRoux, 2000; Kamareddine, 2012; Scholte et al., 2004; Walker, 2002), the parasitic fungus *B. bassiana* is thought to be another effective biological control agent of malaria vector. It is an entomopathogenic fungus that is also widely distributed (Augustyniuk-Kram & Kram, 2012). However, the efficacy of the parasitic fungus *B. bassiana* (IMI-391510) against *A. gambiae* survival is unknown.

2.2.1. Effects of *P. flavescens* nymph against *A. gambiae* larvae

Pantala flavescens, also known as the globe skimmer or wandering glider, is a wide-ranging dragonfly (Tutt, 1997) of the family Lestidae. Most years of its life is spent as a nymph (Plate 2.1) living in fresh water, while the adult stage lives for just a few days or weeks (Tutt, 1997). Dragonflies are predatory both in the aquatic nymphal, and adult stages. The nymphs have lethal effects on prey, whereby they feed on them. Young nymphs feed on a range of fresh water invertebrates while older nymphs can prey on tadpoles and small fish (Dijkstra, 2006).

Plate 2.1. *Pantala flavescens* nymph-. Source: https://www.buglifecycle.com © 2011 (accessed on 05/07/2016).

Like other predators, this predator is also thought to have non-lethal effects (effects against prey after sensing predator presence but where the prey is not killed) against its prey. Chemical recognition of predators by prey may be important for many aquatic insects, especially for those that live in limited visibility habitats or where predators are cryptic (Kats & Dill, 1998). Prey may be able to detect the presence of a predator by their chemical cues (or "kairomones"), which are usually released directly by a potential predator or by injured conspecific prey (Beketov & Liess, 2007). On sensing cues released by potential predators, prey may display diverse changes in behavior, morphology, or even life history (Kats & Dill, 1998), to avoid or escape from the predator.

However, despite there being many predators and parasites of *A. gambiae* larvae, there is the need of determining predators and parasites that are effective in affecting and reducing the

population of this vector, leading to reduced malaria mosquito populations and malaria parasite transmission, low malaria disease cases and deaths. Since *P. flavescens* nymph was thought to be more effective amongst predators, there was need to determine its lethal and non-lethal effects against *A. gambiae* larvae in the laboratory, in order to understand the combined effects of the predator with parasitic fungus on development and survival of malaria mosquito.

2.2.2. Effects of *B. bassiana* against *A. gambiae* larvae

Beauveria bassiana is an entomopathogenic fungus that grows naturally in soils throughout the world. It parasitizes various arthropod species including termites, thrips, whiteflies, aphids, various beetles, bedbugs, and mosquitoes (Barbarin et al., 2012). Entomopathogenic fungi are an important and widespread component of most terrestrial ecosystems (Augustyniuk-Kram & Kram, 2012). For example, *B. bassiana* is reported from tropical rainforest ecosystems (Aung et al., 2008) and survives almost everywhere (Augustyniuk-Kram & Kram, 2012), which increase their chances of contact with mosquitoes. There are different *B. bassiana* strains selected for different insects, which are exposed to the fungus collected from the field and the insect species incubated once it dies (Farenhorst & Knols, 2010), then the growing fungus on the cadaver is collected as an isolate against that insect. The fungal strains selected for mosquitoes, for example, *B. bassiana* (IMI- 391510) will only infect and kill mosquitoes, and not other insects.

When the microscopic spores of the fungus (shown in Plate 2.2) come into contact with the insect host, which forms a substrate, providing nutrients required, they germinate, penetrate the cuticle, and grow inside, killing the insect within 4-8 days (Khan et al., 2013). Afterwards, a white mold emerges from the cadaver and produces new spores. There is also transfer of fungal

infection from larval, to the pupal and to the adult mosquito stages, which may be due to the absence or moderate anti-fungal activity during metamorphosis (Bukhari et al., 2010). This ensures that all the infected insects die at all developmental stages.

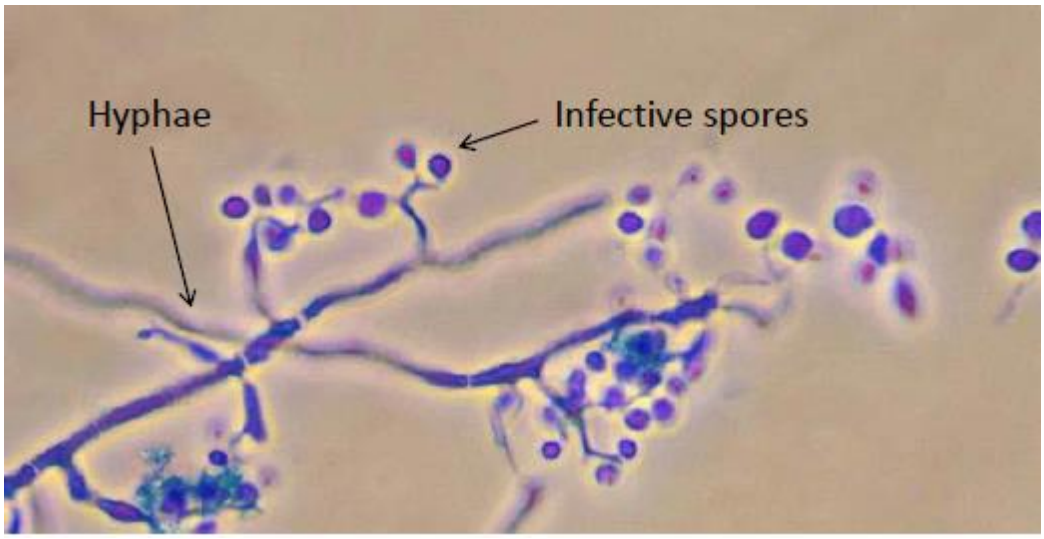

Plate 2.2. *Beauveria bassiana* hyphae with the infective stage spores. Courtesy of Geraldine Kaminsky Medical Mycology Library© 2003, Doctorfungus Corporation (accessed on 05/07/2016).

The fungus has been shown to effectively infect *Anopheles* mosquitoes and significantly reduce their lifespan (Blanford et al., 2005; Scholte et al., 2005; Scholte et al., 2003). However, different fungal strains may have different virulence against different strains of *Anopheles*, depending on other prevailing factors in the vector ecology. Such factors include pre-exposure or exposure to predators and parasites that compromise or prime vector immunity, or affect its physiology. Prior to death, fungal infection can decrease mosquito's malaria transmission potential by reducing its blood feeding propensity, fecundity, *Plasmodium* parasite development and *Plasmodium* sporozoite counts (Blanford et al., 2005), due to altered vector physiology. Fungal

entomopathogens are also effective against insecticide-resistant mosquitoes and increase their susceptibility to insecticides, because they pre-lethally interfere with the expression of insecticide resistance in genetically resistant mosquitoes (Farenhorst et al., 2009). The potential of fungi to kill *A. gambiae* and reduce malaria transmission has resulted in a growing interest to develop practical and sustainable mosquito control methods based on biological control agents that can be integrated into the existing arsenal of malaria control tools.

However, despite *B. bassiana* having been widely studied, there is need to know the efficacy of the strain of *B. bassiana* (IMI-391510) against the Kisumu strain *A. gambiae* larvae survival in Maseno. In addition, the influence of *B. bassiana* (IMI-391510) on this mosquito immunity in terms of susceptibility or refractoriness during a subsequent parasite infection is generally unknown.

2.3. *Anopheles gambiae*

Anopheles gambiae mosquito has four life stages: egg, larva, pupa and adult. Both male and female adult mosquitoes feed on nectar from plants. In addition to the nectar, the female adult mosquito feeds on vertebrate blood to obtain nutrition for oviposition (Foster & Walker, 2002). Although adults can survive for up to one month in captivity, they usually survive for around one to two weeks in the wild (CDC, 2010), an indication that their life span in the wild is shortened by natural environmental factors, such as temperature, predators and parasites. *Anopheles gambiae* adults are active at night, with peak hours of activity at dusk, and from after midnight to just before dawn (Gillies & De Meillon, 1968). Females prefer to blood-feed indoor, but outdoor feeding also occurs (Tuno et al., 2010). These females are anthropophilic (White, 1974), which makes them very efficient vectors of the human malaria parasite and contribute to their status as

one of the most important malaria vectors in the World (CDC, 2010). Adult females lay their eggs on the surface of water in a variety of aquatic habitats, but prefer shallow sunlit pools of standing water (Gillies & De Meillon, 1968). Larvae, (shown in Plate 2.3) hatch from eggs and develop within the aquatic habitat, which can easily be targeted by use of control agents. Studies have also shown that *A. gambiae* larvae can develop in permanent man-made structures such as concrete tanks and drainage canals (Mala et al., 2011), and natural pools such as swamps, hoof prints (Kweka et al., 2012), and marshes (Mala et al., 2011).

Plate 2.3. *Anopheles gambiae* larva. Photographed by Ray Wilson, (Bird and Wildlife Photography). © 2014https://entnemdept.ufl.edu/creatures/AQUATIC/anopheles_gambiae.htm: (accessed on 20/07/2016).

The larvae then develop into pupae which are comma-shaped when viewed from the side. The head and thorax are merged into a cephalothorax with the abdomen curving around underneath. As with the larvae, pupae must come to the surface frequently to breathe, which they do through

a pair of respiratory trumpets on the cephalothorax. After a few days as a pupa, the dorsal surface of the cephalothorax splits and the adult mosquito emerges.

The duration from egg to adult varies considerably among species and is strongly influenced by ambient temperature. Mosquitoes can develop from egg to adult in as little as 5 days but usually take 10-14 days in tropical conditions (CDC, 2015). However, the effect of predator exposure to mosquito larvae on larval development through the pupal stage to adulthood, and also the survival of mosquito larvae, and even adults exposed to parasite after pre-exposure scenarios are unknown.

2.4. The effect of predator-parasite interaction on mosquito larvae

The influence of predators, parasites and temperature on mosquito stages has been studied in a number of different species of mosquitoes, including *Anopheles quadrimaculatus* Say, *Aedes aegypti* Linnaeus, *Culex* and *Anopheles* species, *Toxorhynchites brevipalpis* Theobald and *Wyeomyia smithii* Coquillett (Bayoh, 2001). However, the single and combined interaction effects of predators and/or parasites on the development and survival of *A. gambiae* larvae and developed adults, after predator and/or parasite pre-exposure at the larval stage are unknown.

Larval exposure to predators or parasites may result to immune action or suppression that may determine adult susceptibility or refractoriness to *P. falciparum.* For instance, the susceptibility or refractoriness to a parasite in mosquitoes can be due to a subsequent infection with the same or another parasite of pre-exposure, even at different developmental stages.

Studies show that high mortality of mosquitoes during larval development is due to climatic conditions, parasitism and predation (Aniedu et al., 1993; Paaijmans et al., 2007; Service, 1993). Climatic conditions are majorly influenced by variation in temperature, which has a great influence on mosquito development. Just as temperature is a factor in influencing development of the aquatic stages of *A. gambiae* (Bayoh & Lindsay, 2003), predation and parasitism also are factors influencing their development and hence survival. However, it is not well understood how *P. flavescens* nymph predator presence affect the development of *A. gambiae*, Kisumu strain larvae. Since predator presence reduces phenoloxidase activity which influences vector immunity (Boltaña et al., 2013; Stoks et al., 2006), it may therefore influence mosquito development and survival from larval to adult stage.

Parasites are also thought to influence survival of mosquito larvae. Throughout their life, mosquitoes are exposed to pathogens during feeding, through cracks in their cuticle or pathogen-driven cuticular degradation (Hillyer, 2010). To resist infection, mosquitoes mount innate immune response that is elicited within minutes of exposure, and can lead to pathogen death via three broadly defined mechanisms: lysis, melanization and hemocyte-mediated phagocytosis (Hillyer, 2010). However, it is necessary to understand how pre-exposure to parasites can either compromise or prime mosquito immunity, leading to susceptibility or refractoriness to the parasite in future exposure.

CHAPTER THREE

MATERIALS AND METHODS

All experiments were performed at the Department of Zoology's Animal House in Maseno University, from January 2017 to April 2017, at temperatures of 27°C ± 5°C and 70% ± 5% relative humidity.

3.1. *Anopheles gambiae* rearing

Anopheles gambiae eggs were obtained from The Center for Global Health Research, KEMRI-Kisian, and were kept at the Department of Zoology Animal House, in Maseno University. The eggs, kept in a filter paper in enclosed petri dishes, were added to 1 liter of dechlorinated tap water (tap water left in an open bucket for 48 hours) in plastic bowls of volume 20×15×5 cm^3. The bowls were lined with No. 1 Whatman filter paper (Whatman International Limited, England) to prevent eggs from adhering to the sides of the plastic bowls and drying out. Each bowl was kept at room temperature and given time for egg hatching.

The hatched larvae were fed on Liquifry No. 1 (Interpet Ltd., Dorking, Surrey, UK) (1 g daily) for the first two days and then on grinded cat food (Purina, Go cat®, UK) (1 tablet daily) for the rest of the larval period (Bukhari et al., 2010). The larvae were allowed to mature to the pupal stage, and pupa were removed with a plastic pipette and placed in 300 ml clear plastic cups inside holding cages measuring 30×30×30 cm^3 (Plate 3.1), for further development to adults.

All the adults (in the holding cages) were fed *ad libitum* on 6% glucose water (Bukhari et al., 2010) soaked in cotton wool.

Plate 3.1. Mosquito cages (30×30×30 cm^3) in an insectary at the department of zoology, Maseno University, Kenya. Photo taken by Ong'wen Fedinand, on 20/03/2017.

3.2. *Pantala flavescens* nymph rearing

Pantala flavescens dragonfly nymphs were captured from rice paddies in Ahero Irrigation Scheme, in Ahero, and identified according to Paul & Kakkassery, (2013). Two hundred nymphs of different sizes were collected and transported to the Animal house in Maseno University, where only those nymphs that weighed 2.5 g and above were each kept in a 300 ml clear plastic cup, each with 50 ml dechlorinated tap water. The nymphs that weighed below 2.5 g were kept together in the bowls of volume 20×15×5 cm^3 with 250 ml water, and holding 5 nymphs in each bowl. Thereafter, third instar (1-2 days old- first instar, 3-4 days old- second instar, 5-6 days old- third instar, 7-9 days old- fourth instar) larvae were added in that cup or bowl as food, depending on the nymphs present. The larvae were used as feed to sustain the nymphs awaiting

experimental use. All the uneaten larvae were removed from the cups or bowls once they pupated. All the nymphs used in the experiments weighed 3g ± 1mg each (the weight of an average sized nymph, not too small to withstand new environmental conditions, and not too old to molt into adult before end of experiment). The nymphs were starved for 24 hours before being used in the treatment exposure.

3.3. Determining the efficacy of *Pantala flavescens* nymph against *A. gambiae* larvae

This experimental set up included one control group and one treatment group. In the control group, the larvae were not exposed to *P. flavescens* nymph, while in the treatment group, the larvae in each replicate were exposed to *P. flavescens* nymph. Each group was replicated four times. Each replicate consisted of 30 third instar larvae placed in a bowl with 1 liter of de-chlorinated tap water. In the treatment group, the larvae in each replicate were exposed to one *P. flavescens* nymph kept in the same bowl. In all the groups, the number of missing larvae was recorded separately after every hour for the first 12 hours, and then, after 24 hours in one day. The missing larvae were believed to have been predated on by the nymphs.

3.4. Determining the effect of *P. flavescens* nymph on development rate of *A. gambiae* larvae

This experimental set up included four groups (one control and three treatments). In the control group (Group I), the larvae were not exposed to *P. flavescens* nymph, while in the treatments; Groups II, III and IV, the larvae were exposed to varying numbers of constrained *P. flavescens* nymphs (Table 3.1), constrained by a clear, plastic cup with small holes (small enough to allow water circulation but not larvae to pass through), but in the same bowl with the larvae (as in Plate

3.2). The holes in the plastic cup allowed free movement of water and therefore, the predator and prey were in the same environment. These nymphs were provided with third instar larvae daily as food, to keep them alive during the treatments. Each group was replicated four times with each replicate consisting of 30 first instar (one day old) larvae placed in a bowl with 1 liter water and observed till pupation day. Development time was recorded as the duration in days of development from first instar stage to pupation.

Table 3.1. Number of constrained nymphs exposed to larvae

Experimental Groups	**Number of Constrained nymphs**
Group I (Control)	0
Group II	1
Group III	2
Group IV	4

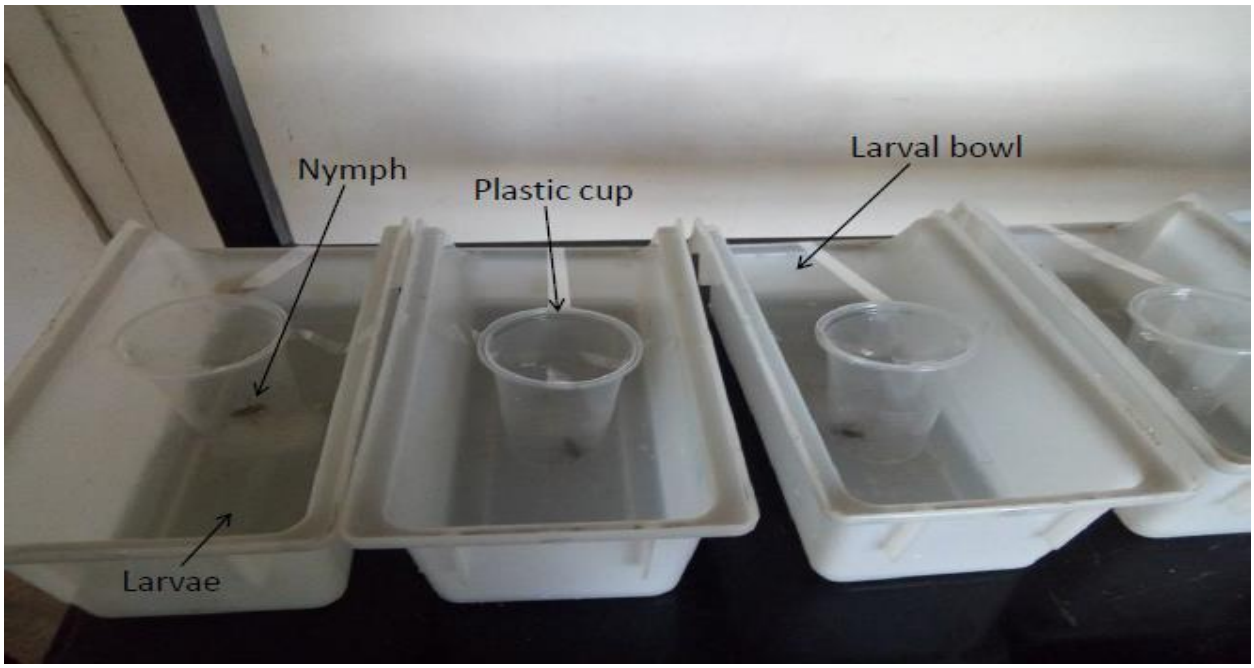

Plate 3.2. Constrained *Pantala flavescens* nymphs. Photo taken by Ong'wen Fedinand, on 20/03/2017.

3.5. Viability and efficacy of *B. bassiana* against *A. gambiae* larvae

3.5.1. Viability test of fungal spores before experimental exposure

Spores of *Beauveria bassiana* (IMI- 391510) were provided by IN2CARE®, Wageningen, The Netherlands. They were stored in a 1 liter, tightly covered plastic container at 4°C in a refrigerator until use. The spores of *B. bassiana* were tested for viability before experiment, according to the procedure by Mnyone et al. (2010) and got 97% germination percentage that was above the required germination percentage of 85% (Mnyone et al. 2010).

3.5.2. Fungal exposure to the experimental groups of larvae

The efficacy of *B. bassiana* against *A. gambiae* larvae was determined by recording the number of dead mosquito larvae after fungal exposure (Bukhari et al., 2010) which leads to fungal infection. There were four groups: Group I (control) and three treatment groups. Each group had four replicates of 30 first instar larvae in a bowl with 1 liter of water. The control group was not exposed to the fungus but the treatment groups were exposed. The treatment larval bowls were transferred to an adjacent room, in the same Animal house for fungal exposure. Fungal exposure was done by dusting weighed spores on the water surface at the start of the experiment in different dosage as shown in table 3.2. After spore dusting, the larval bowls were left for 5 minutes for the spores to settle down. The bowls were then returned back into the rearing room, placed just next to the control bowls and observed for larval death.

Dead larvae were separately recorded daily for the next 9 days, when all the larvae in all the groups had either died or pupated. The amount of food added was adjusted based on the daily mortality and the remaining larval population (Bukhari et al., 2010).

Table 3.2: Fungal dosage used in larvae exposure

Experimental Groups	Fungal dose (mg)
Group I (Control)	0
Group II	3
Group III	6
Group IV	12

3.6. Determining the survival of adult *A. gambiae* exposed to *B. bassiana* after they emerged from water with the predator, with the parasite, or with both predator and parasite.

The stored spores of *B. bassiana* (IMI- 391510) were once more tested for their viability using the protocol by Mnyone et al., (2010) and got 96.7% germination percentage.

3.6.1. The fungal exposure to mosquito larvae and adults

(i) Larval infection

This experimental set up included five groups: one control group and four treatment groups. Each group was replicated four times with each replicate consisting of 30 first instar larvae placed in a bowl with 1 liter of dechlorinated tap water. Experiments 3.4 and 3.5 were used to determine the nymph number (1 nymph), and fungal dose (6 mg),which would not lead to instant larval death, but which were able to lower their survival. *P. flavescens* nymph was constrained in a 300 ml clear plastic cup with small holes, which was in the same larval bowl that contained the larvae (as shown in Plate 3.2). These nymphs were fed on third instar larvae every day to keep them alive during the treatments. Fungal exposure to the larvae was done in a separate room as described earlier then the bowls returned to the experimental room. Dead larvae and pupae were separately recorded daily in all the groups.

All pupae that survived were put into different 300ml transparent plastic cups, labeled according to their groups and replicates with each cup holding the number pupated on that day. The date the pupae were placed in the cups and their number was indicated on the cups, which were then covered with a small piece of the normal mosquito net to prevent the developed adults from escaping. 6% glucose solution soaked in cotton wool was put on the net covering the cup.

Several larvae had to be raised up in bowls holding 30 larvae each to ensure that each group had 3 replicates of 30 female adults being exposed/ infected with the fungus for adult survival study. This was to cater for the high larval mortality in the treatments with fungal pre-exposure.

The number of adults that developed and survived was recorded. Adult fungal infection was only done on live adults that were 2-3 days old. The exposure was as shown in Table 3.3.

Table 3.3. Vector exposure to predator and/or parasite.

Larval Groups	**Exposed at larval mosquito stage to**		**Exposed at adult mosquito stage to**
	P. flavescens	*B. bassiana*	*B. bassiana*
Group I (Control)	No	No	No
Group II	No	No	Yes
Group III	Yes	No	Yes
Group IV	No	Yes	Yes
Group V	Yes	Yes	Yes

(ii) Adult infection

The exposure of the fungus to adult mosquito was also done in the neighboring room to avoid fungal contamination of the control mosquitoes. The mosquitoes were then returned to the rearing room, and placed just next to the control mosquitoes for observation.

When the pupae in the cups had developed into adults, they were transferred into holding cages on that same day and given time to grow till when 2-3 days old. The date and number of emerged

adults in every group and replicate were recorded daily. Females were separated from males by placing a heated water bottle on top of the cage to mimic an animal host. Only females were chosen and once separated by the mimic, they were sucked using an aspirator and transferred into different cages. Three replicates, each with 30 adults were infected with *B. bassiana* fungus using electrostatic net. The net was cut into a marked size of 22 cm×11 cm to fit along the inner surface of a 300 ml plastic cup. The net was then put inside a 20×15×5 cm^3 bowl. Thereafter, 5 g of fungal spores (enough dose to cover the net) were added into the bowl and then covered with its lid. It was shaken thoroughly and then given 5 minutes for the spores to settle down in the bowl. The net, now full of fungal spores on it was removed and coiled within the 300 ml clear plastic cup and held in place by staple pins. The cup was placed inside a different mosquito cage, where exposure/infection was done, just to help recover any mosquito that might escape during transfer from the exposure cups to the holding cups. A single female mosquito was picked up by an aspirator from the holding cage and introduced into the exposure cup, which had the fungus-exposed electrostatic net. The top of the cup was covered with a normal mosquito net and the mosquito was left there for 10 minute exposure time period. After that, it was transferred into a holding cup using a clean aspirator, covered with another net and the date of infection labeled on the cup. The mosquito was given 6% glucose solution soaked in cotton wool, which was placed on top of the covering net, and then monitored till death. The procedure, (some steps in Plate 3.3) was repeated for all the 360 female adults (in all the replicates) in the treatment groups.

(a)

(b)

(c)

(d)

Plate 3.3. Adult mosquito exposure to fungus: Photo by Ong'wen Fedinand, on 20/03.2017:

(a) the cage in which the transfer of adult mosquitoes from infection to holding cups was done; (b) bowl containing the fungal spores and the electrostatic net; (c) the electrostatic net coiled inside the 300 ml transparent cup; (d) adult mosquitoes in labeled cups for observation after infection.

The control mosquitoes were also taken through the electrostatic net exposure, but with a net not exposed to fungal spores. The net used for the control mosquitoes was put inside an empty bowl without the fungus and then shaken.

Mortality of the adult mosquitoes was monitored daily, after which dead ones were removed from each holding cup and confirmed for fungal infection. This was done by dipping cadavers in 70% ethanol to remove external microbiota (which does not affect the internally growing fungus) (Farenhorst et al., 2010), and then incubating them on moist filter paper in sealed Petri dishes at 27 ± 1°C. After 3-5 days mosquito cadavers were examined for fungal sporulation, specifically emerging hyphae, using a dissecting microscope (Farenhorst & Knols, 2010).

(iii) Spore density on electrostatic net

Spore density on the electrostatic net to which the mosquitoes were exposed was calculated. Spore count was done by cutting a 1 cm^2 piece of the electrostatic net, which was placed in the bowl with 5 g fungus then shaken and left for 5 minutes to settle. Thereafter, 0.1% Tween 80 solution was prepared and 8 ml placed in 10 ml vial, which was replicated thrice (Tween 80 removes the fungal spores from the electrostatic net hence they become suspended in the solution). After 5 minutes, when the fungus in the bowl had settled, the net was picked by a pair of forceps and placed in the vial then shaken rapidly. Thereafter, 0.1µl of the solution with suspended fungal spores was taken using a micro-pipette and administered on a haemocytometer then covered with a cover slip. The number of spores lying within the central grid of the haemocytometer were counted at ×100 magnification under a compound microscope and recorded. This was done five times in different zones and the average calculated.

3.7. Statistical analysis

To determine the predation efficacy of *P. flavescens* nymph against *A. gambiae* larvae, Mann-Whitney U Test was used to determine the difference in the mean number of dead (missing) larvae in the control and treatment groups after 24 hours. The lethal median time to death (LT50) was calculated by Probit analysis of the larval survival over time. In order to determine development rate from first instar larvae to the pupal stage of *A. gambiae* reared in the presence of varying densities of *P. flavescens* nymph, Kaplan-Meier analysis was used to determine the mean number of days to pupation and pair wise comparison between the experimental groups. Cox regression was used to determine the difference in the pupation rate of the larvae in the control group and the treatment groups. Cox regression describes the increased or decreased likeliness of an event (in this case development to pupal stage), due to a covariate (presence of varying densities of predator) in terms of hazard ratio (HR). In the efficacy of varying dosage of *B. bassiana* against *A. gambiae* larvae survival, HR was determined by Cox regression. Cox regression was also used to determine the difference in the mortality rate of the larvae in the control group and treatment groups after fungal exposure. In the survival of adult mosquitoes exposed to the fungus after pre-exposure to predator and/or parasite, the lethal median time to death, LT50 (95%CI) was calculated by Kaplan-Meier analysis of the adult survival over time. Cox regression was used to determine the difference in the survival of adult mosquitoes in the control group and treatment groups.

CHAPTER FOUR

RESULTS

4.1. Predation efficacy of *P. flavescens* nymph against *A. gambiae* larvae

In the experiment to quantify predation efficacy of *P. flavescens* nymph, no mortality was recorded in the control group within 24 hours. On the other hand, there was high larval mortality in the treatment group within the first hours (Figure 4.1), with an average predation of 88.33% after 24 hours across all the replicates. In the treatment group, the LT50 was 0.6 (95% CI: 0.25-1.3) hours. There were no dead or injured larvae found in any group, but only missing larvae in the treatment group which had been predated upon.

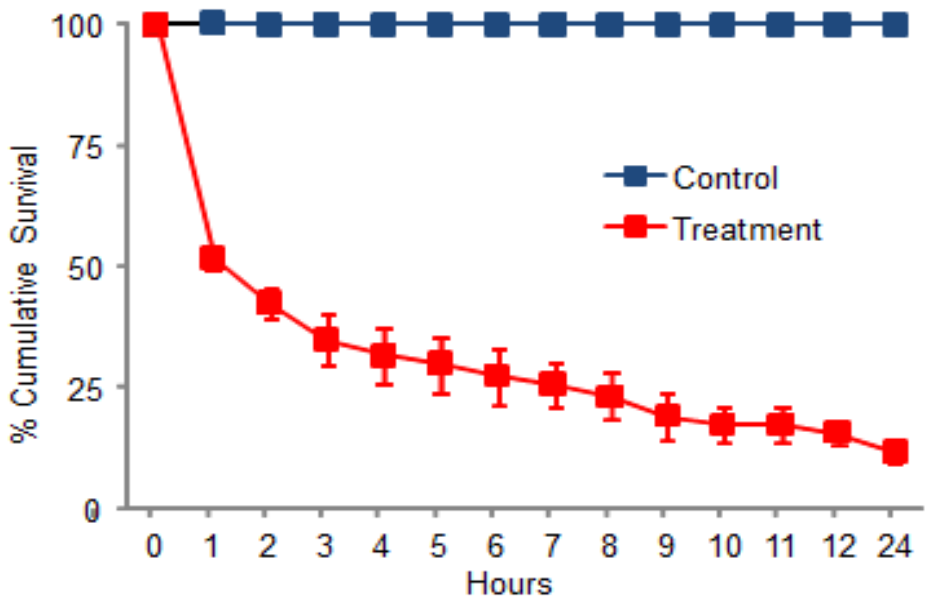

Figure 4.1. Percentage cumulative larval survival

Control group was without *P. flavescens* nymph exposure while the treatment group was exposed to one *P. flavescens* nymph.

There was a significant difference in the mean number of dead larvae between the control and treatment groups at 24 hours (Z=-12.667, P<0.001) (Figure 4.2).

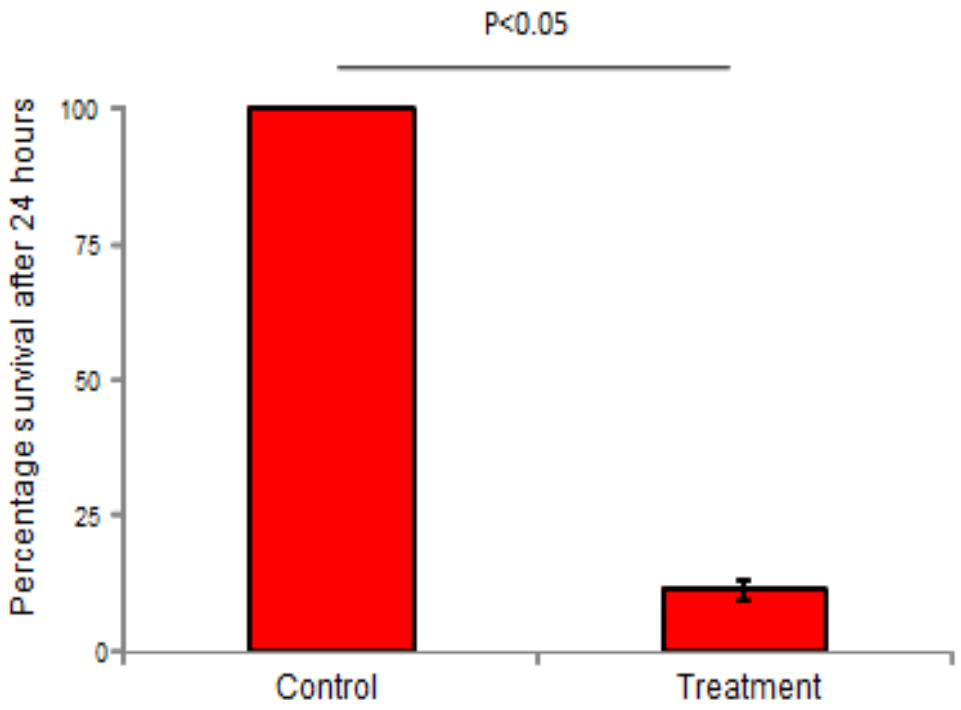

Figure 4.2. Percentage larval survival over time

Mann-Whitney U test on number of surviving larvae at each time point showed significant difference.

4.2. Development rate of *A. gambiae* larvae reared in the presence of varying densities of *P. flavescens* nymph

In the experiment to quantify development rate of *A. gambiae* larvae reared in the presence of varying densities of *P. flavescens* nymph, it was found that the varying predator density has a variable effect on the development rate of *A. gambiae* larvae over time. The larvae in the control group (Group I) took the longest period, a mean of 7.85 (95% CI: 7.8-8.0) days (where 1 day = 24 hours), to develop to pupae. When larvae were exposed to only one nymph (Group II) the mean days to pupation was 7.1 (95% CI, 7.0-7.2), which was the shortest period across all the groups. The larvae exposed to two nymphs (Group III) had a mean of 7.4 (95% CI, 7.3-7.5) days to pupation, while the larvae exposed to four nymphs (Group IV) had a mean of 7.75 (95%CI, 7.6-7.9) days to pupation (Figure 4.3). Kaplan-Meier pairwise comparison showed a significant difference in number of days to pupation between control group larvae exposed to no nymph and the larval groups exposed to one and two nymphs ($P<0.001$), but no difference between the control group larvae and the larval group exposed to four nymphs ($P=0.227$). The larval group exposed to four nymphs was significantly different from the larval groups exposed to one and two nymphs ($P<0.001$ and $P<0.01$ respectively) (Figure 4.3).

The groups that were significantly different are indicated (Kaplan-Meier pair wise comparison).

Cox regression analysis showed that, compared to the control group, the larvae exposed to one nymph were 2.0 times more likely to pupate while the larvae exposed to two nymphs were 1.4 times more likely to pupate (HR values in Table 4.1).

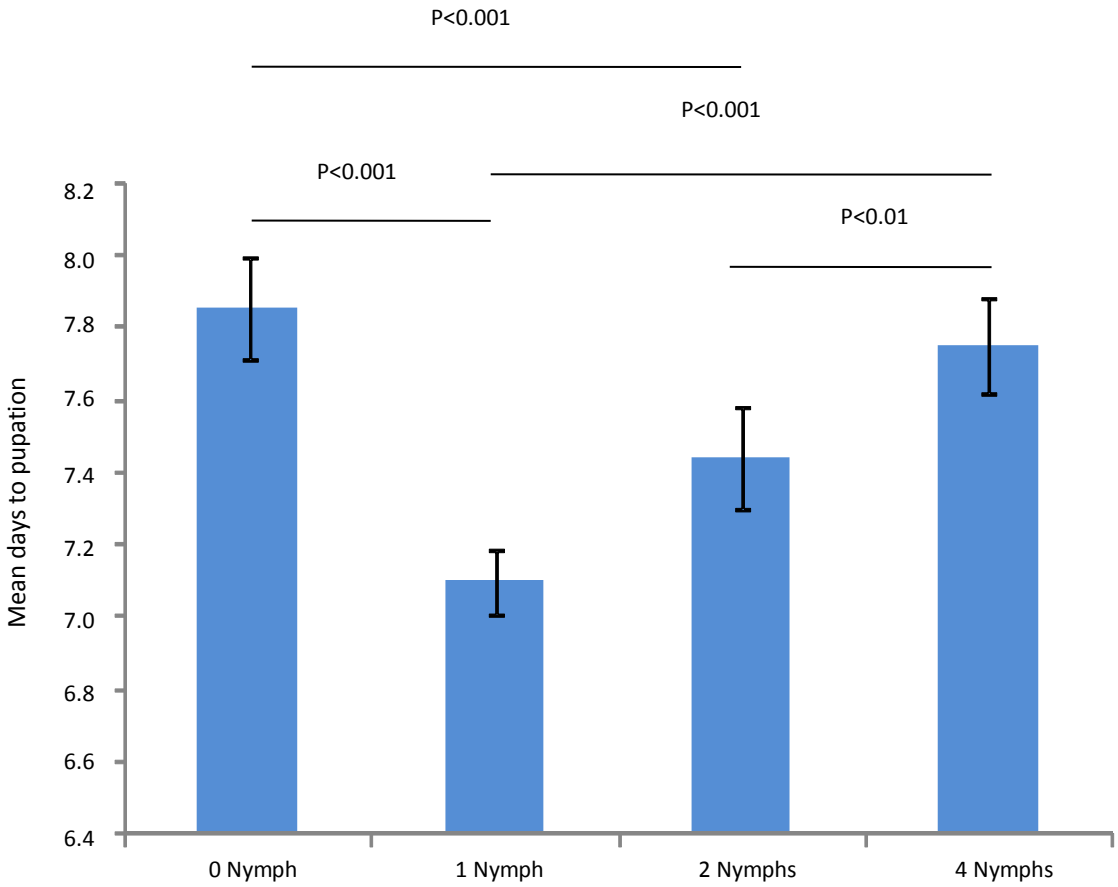

Figure 4.3. Mean days to pupation of larvae exposed to constrained predator

Table 4.1. Hazard ratios of larvae exposed to constrained predator

Group	**No. of nymphs**	**HR**	**95% CI**	**P-value**
Group I	0	1	-	-
Group II	1	2.0	1.5-2.6	**<0.0001**
Group III	2	1.4	1.1-1.8	**0.02**
Group IV	4	1.1	0.8-1.4	0.50

Significant P-values are shown in bold.

4.3. The efficacy of varying dosage of *B. bassiana* against *A. gambiae* larvae

On the efficacy of varying dosage of *B. bassiana* fungus against *A. gambiae* larvae, it was found that all the treatment/exposed groups had lower survival than the control group which was not exposed to the fungus. All the larvae in the treatment groups took a maximum of 9 days (where 1 day = 24 hours) before death or pupation (Figure 4.4).

Cox regression showed a significant difference between larvae in the control group (Group I) and all the larvae in the treatment groups; Groups II, III and IV, ($P<0.000$), exposed to 3 mg, 6 mg and 12 mg of fungus respectively.

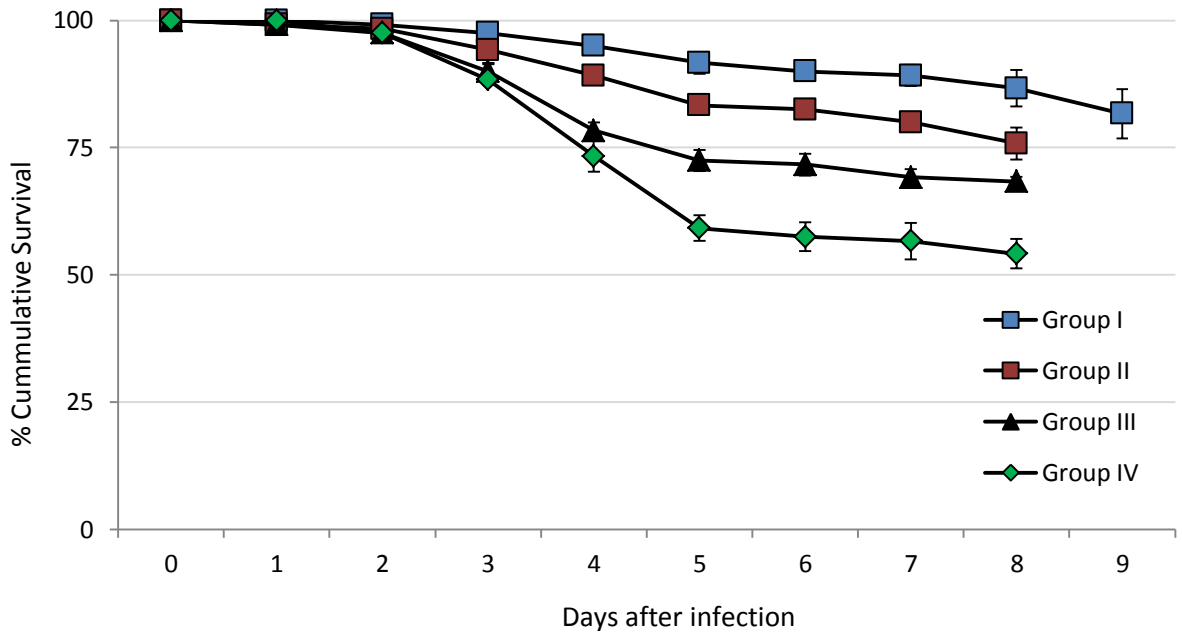

Figure 4.4. Survival of larvae exposed to fungus

Cox regression analysis showed that, compared to the control group, larvae exposed to 3 mg of fungus were 2 times more likely to die than the control larvae, while those exposed to 6 mg of

fungus were 2.5 times more likely to die, and those exposed to 12 mg were 3.5 times more likely to die (HR values in Table 4.2).

Table 4.2. Hazard ratios of experimental groups in fungal efficacy test.

Group	**Fungal dose (mg)**	**HR**	**95% CI**	**P-value**
Group I	0	1	-	-
Group II	3	2.0	1.2-3.3	**0.01**
Group III	6	2.5	1.5-4.2	**0.000**
Group IV	12	3.5	2.2-5.7	**0.000**

All the treatment groups are significantly different from the control group.

4.4. Survival of adult mosquitoes exposed to fungus *B. bassiana* after predator and/or parasite pre-exposure

Kaplan-Meier analysis of survival data on adult mosquito exposure to fungus following larvae pre-exposure to predator and/or fungus indicate that Group I (control- neither pre-exposed nor exposed to any factor) adults had LT50 of 23 days, which is longer than LT50 of all the treatment groups (Table 4.3).

Table 4.3. LT50 values of adult mosquito survival, hazard ratios and P-Values in adult exposure

Group	Larval exposure to		Adult exposure to fungus	LT50 (95%CI) (Days)	HAZARD RATIOS		
	Nymph	Fungus			HR	95% CI	P-value
Group I	No	No	No	23 (20-26)	-	-	-
Group II	No	No	Yes	5 (4.7-5.3)	45.8	17.8-117.7	**<0.001**
Group III	Yes	No	Yes	4 (3.8-4.1)	67.4	26.0-174.2	**<0.001**
Group IV	No	Yes	Yes	5 (4.7-5.3)	50.9	19.8-130.8	**<0.001**
Group V	Yes	Yes	Yes	4 (3.7–4.3)	112.0	43.3-289.5	**<0.001**

LT50 values of adult mosquitoes in the control (without fungal exposure) and treatment groups (with fungal exposure) over time (days). Hazard ratios (HR) and 95% CI indicate the death rate of fungus exposed adult mosquitoes in the experimental groups after predator and/or parasite pre-exposure.

Considering the adult survival over time in days, Group I (control) adults (neither pre-exposed nor exposed to any factor) survived longer than group II (with adults not pre-exposed to any factor), Group IV (with adults pre-exposed to fungus), Group III (with adults pre-exposed to predator) and Group V (with adults pre-exposed to both predator and fungus), with decreasing survival respectively (Figure 4.5).

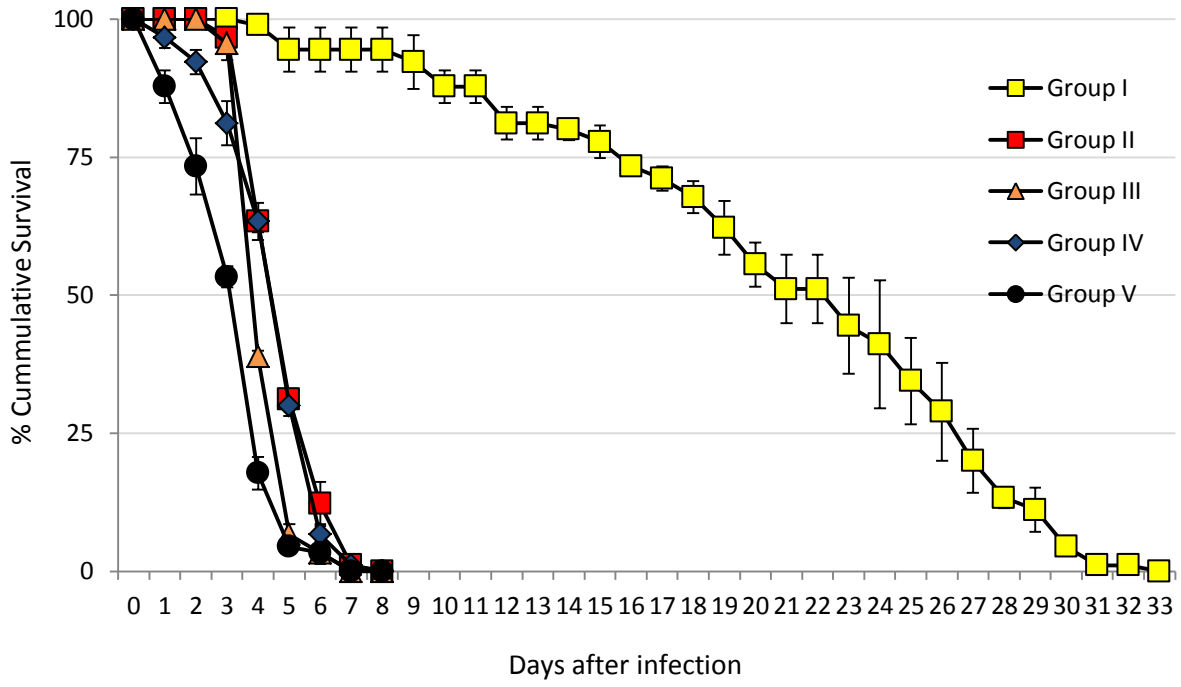

Figure 4.5. Adult mosquito survival curve

On the other hand, Cox regression analysis indicates that all the treatment groups were statistically significant in the test for adult mosquito survival (Table 4.3). Looking at the hazard ratios (Table 4.3), Group I was not exposed to any hazard. Group II was exposed to the least hazard, followed by Groups IV, III, and Group V which was exposed to the highest hazard.

In the examination of mosquito cadavers for fungal sporulation, all the treatment mosquito (exposed to a dose of 3.1 x 10^8 spores of fungus) cadavers were found positive of fungal infection, while control mosquito cadavers were not positive.

CHAPTER FIVE

DISCUSSION

This study aimed at understanding the additive effects of *Pantala flavescens* nymph and *Beauveria bassiana* on *Anopheles gambiae* development and survival. More specifically, it focused on: the predation efficacy of *P. flavescens* nymph against *A. gambiae* larvae; the development rate of *A. gambiae* larvae reared in the presence of varying densities of *P. flavescens* nymph; the efficacy of varying dosage of *B. bassiana* against *A. gambiae* larvae survival; and the survival of adult mosquitoes exposed to the fungus *B. bassiana* following different pre-exposure scenarios.

5.1. Predation efficacy of *P. flavescens* nymph against *A. gambiae* larvae

Results of this experimental work show a high predation efficacy of *P. flavescens* nymph against *A. gambiae* larvae, as an average of 88.33% of third instar larvae were predated on within 24 hours across the replicates. Similarly high predation efficacy of other species of dragonfly nymphs against the prey *Anopheles arabiensis* larvae was reported by Gouagna et al. (2012). The same high predation efficacy was observed by Mikhali (2008) in his study in Vihiga County of Kenya, when he exposed third instar larvae of *A. gambiae* to unspecified species of dragonfly nymphs to determine their predation efficiency. He obtained percentage predation of 95% within 24 hours. It is worth noting that all studies that were reviewed found high predation efficacies of different species of dragonfly nymphs on *Anopheles* mosquito larvae, and no study on the efficacy of *P. flavescens* nymph was found. There is a significant difference between the survival

of *A. gambiae* larvae reared in the absence of *P. flavescens* nymph and those reared in *P. flavescens* nymph presence.

Despite the high predation efficacy reported in this study, it is important to note that the study set up was experimental and so, it is not clear whether similar predation levels would occur *in situ*.

5.2. Development rate of *A. gambiae* larvae reared in the presence of varying densities of *P. flavescens* nymph

In the experiment to determine development rate of *A. gambiae* larvae reared in the presence of varying densities of *P. flavescens* nymph, the larval development rate was shown to increase and then decrease with increase in predator density. It has been shown that presence of predators may have non-lethal (trait mediated) effects on prey that are attributable to intimidation (Preisser & Bolnick, 2008; Werner & Peacor, 2003). The non-lethal effects of predators include inducing changes in development (Lima & Dill, 1990; Lima, 1998; Werner & Peacor, 2003). Larvae exposed to one and two nymphs took fewer days to develop to pupae because predator presence affected their physiology (Stoks et al., 2006) in a way that influenced their energy allocation to favor faster development to escape predation risk.

Larvae reared in the presence of one predator nymph took the least days to pupation because they may have experienced the strongest effects that indicated high predation risk. In predator-prey interaction, prey may be able to detect the presence of predator by their chemical cues (or "kairomones"), which are usually released directly by a potential predator (Beketov & Liess, 2007). Since prey development will be faster under predator exposure if this reduces mortality (Werner & Anholt, 1993), these larvae had to develop faster to escape from predator-induced stress and predation risk. The findings of this study are consistent with studies done by Kweka et

al. (2012) and Ng'habi et al. (2005), who found out that in natural population, shortened pupation time of *A. gambiae* influences predator avoidance. Also, though different species of prey and predator were used, Benard (2004) found out that *Aedes triseriatus* mosquito larvae exposed to the predator *Toxorhynchites rutilis* took shorter time to develop to pupae.

Larvae reared in the presence of two dragonfly nymphs took longer time to pupate than larvae reared in the presence of one dragonfly nymph. The reason could be that these larvae experienced less predator cues compared to those exposed to one nymph. This may have resulted due to the territorial nature and cannibalism of the nymphs which may have invested more on competitive and defensive strategies against themselves. Studies have shown that interspecific and intraspecific competition of ordonate nymph for space and food, and their aggressive interactions lead to inter-ordonate predation and cannibalism (Baker, 1986; Buskirk, 1989; Crowley et al., 1987; Robinson & Wellborn, 1987; Rowe, 1980; Wissinger, 1988, 1989a, 1989b). The predators may have invested more in defensive mechanisms against each other, which may have happened at the cost of predation and prey directed cues. They needed to ensure that they do not become prey to other nymphs. The higher the predator number, the higher the feeling of insecurity and investment in intraspecific defense, and the lower the predator cues against the prey. However, the predators still produced some cues that affected larval physiology (Lima & Dill, 1990; Lima, 1998; Werner & Peacor, 2003), allowing them to invest some energy in their development. This work contrasts the work of Zuharah and others who found out that cues from many predators affected their prey much more than cues from fewer predators (Zuharah et al., 2013). However, their study was on backswimmer predator *Anisops wakefieldi* and its prey *Culex pervigilans*. This shows that increase in predator density may lead to increase or decrease in larval development rate, depending upon the predator species and larval species.

The other possible reason for the positive, then negative relation, whereby larvae exposed to one and two dragonfly nymphs developed faster than the control larvae and those exposed to four nymphs could be reproductive success. The need to achieve reproductive success by developing faster to adults that would escape from predator presence then undergo reproduction was never prioritized by larvae exposed to four nymphs in their energy allocation. They may have realized that death is unavoidable due to high concentration of predator cues. The control larvae also never prioritized reproductive success because they were never threatened by any predation.

Another reason for the development trend seen here, (increase and then decrease in development rate with increasing predator density) could be that, when the concentration of chemical predator cues become too high and are everywhere in the environment, the response to it reduces. The larvae stop responding to the nymphs when the predator cues are too high and everywhere in the environment and develop as if in predator absence. Therefore, predator presence in increasing density resulted to increasing cues being felt by exposed larvae, but with decreasing effects, up to the level of feeling no cues, as if not exposed to predator.

The other reason could be that, larvae exposed to four nymphs may have felt the least predator cues, which could be because of the high density of predators that felt much more threatened amongst themselves. The predators being four in number, and being cannibals may have invested much more in defensive mechanisms, with much concentration on one another and less concentration on prey. The cannibalism effects are supported by the work of Buskirk (1989) when he tested for density-dependent effects of cannibalism on survival and size structure of larvae of the dragonfly *Tramea Carolina* . His results demonstrated that cannibalism was strongly density dependent and may contribute to population regulation of dragonflies. Predator cues produced by these nymphs may have been greatly reduced than those produced by two

nymphs that are in the same ecology. Larvae exposed to four nymphs had the same development rate as control larvae because the predator cues against them were never produced in effective levels, allowing them to develop at a rate like the control larvae. The results of this study are supported by what Roux et al. (2015) found when working on *A. coluzzii* larvae and their backswimmer predator *Anisops jaczewskii*. From their work, though they did not concentrate on predator number or density, predator exposure extended the larval development time. However, they highlighted the importance of considering other environmental factors in the larval ecology, which might have influenced their findings. Predator density may have been one of the influencing factors in their findings, since in this study with same findings, all experiments were done at the same time and under similar environmental conditions. However, it is important to note that no other study has been done on development rate of Kisumu strain *A. gambiae* larvae exposed to varying densities of *P. flavescens* nymph. There is therefore a significant difference in the development rate of *A. gambiae* larvae reared in the presence of varying densities of *P. flavescens* nymph.

5.3. Efficacy of varying dosage of *B. bassiana* against *A. gambiae* larvae

In the experiment to quantify the efficacy of varying dosage of *B. bassiana* fungus against *A. gambiae* larvae survival, it was found that all the fungal doses used affected larvae negatively, and there was a dose-dependent response. The control larvae which were not exposed to the fungal parasite survived for a longer period than the treatment larvae. The results indicate that larval mortality increases with increase in fungal dose. With a dose that contains only few fungal spores (low fungal concentration), larvae will show low mortality due to reduced spore-share per larva (Pelizza et al., 2007). This is because the spores which either contact the skin or are

ingested together with food leading to fungal infection and then mortality will have reduced chances of contact or ingestion, unlike higher spore doses with increased spore-share per larva. The fungal spore concentration is therefore critical in increasing or decreasing the hazard posed to the larvae.

When spores enter the larval body through the mouth or siphon, they mechanically block these passages while a few attach to the interior (Bukhari et al., 2010), as others attach to the larval body surface. The attached spores germinate releasing endotoxins as well as damaging the larval tissues with their vegetative growth (Hegedus & Khachatourians, 1995). In this case there is a whole spectrum of offence that has to be tackled by the larval immune system. The more variable the modes of action, the lower is the probability that resistance will develop against the fungus (Mulla et al., 2003).

There is a high efficacy of this fungus against *A. gambiae* larvae. The findings of this study are consistent with the findings of Bukhari et al. (2010) who found out that there was a difference in the effect of *B. bassiana* fungal concentrations against the exposed *Anopheles gambiae* and *Anopheles stephensi* larvae, with increased dosage leading to increased mortality. However, this contrasts with some studies which indicate that the increase in fungus concentration did not show a proportional increase in larval mortality, which may be due to spore clumping in higher dosages, hence reducing the number of spores available for contact with the larvae, as reported by El et al. (2017), who used doses of 5 mg- 40 mg. Their study was on; the effect of *Metarhizium anisopliae* var acridum and *Beauveria bassiana* on the survival of mosquito larvae of *Anopheles arabiensis* Patton and *Culex quinquefasciatus* Say. Bukhari et al. (2010) also indicated the possibility of spore clumping at high fungal concentrations hence reducing the number of spores available for contact with larvae. The reason could be that as larvae moved

around, they avoided spore clumps because of their big sizes and therefore never came in contact with them. Also, larvae may have chosen to reject the spore mass as food because of large clump sizes (Bukhari et al., 2010) which would be difficult to swallow. However, this was different with the tiny single spores that would have been difficult to avoid contacting as larvae moved by, and also were easily swallowed together with food as larvae fed.

The case of this study may have been different because the fungal doses of 3mg, 6mg and 12mg which were used may not have been able to clump together into big spore clumps. This is because, in relation to fungal doses used, they were spread over a larger water surface area determined by the larval bowl size (20×15 cm^2). This ensured that the spores spread over the water surface were available for larval contact or ingestion, and only few spores formed tiny clumps. The case of this study may have been also different due to the fungal strain used and the mosquito strain too. There is therefore a significant difference in the survival of *A. gambiae* larvae exposed to varying dosage of parasitic fungus *B. bassiana*.

5.4. Survival of adult mosquitoes exposed to *B. bassiana* after pre-exposure

The survival of adult mosquitoes exposed to fungus, after larval pre-exposure to the dragonfly nymph and parasitic fungus confirmed that predators and parasites indeed do influence survival of mosquito (Paaijmans et al., 2007; Aniedu et al., 1993; Service, 1993). Results indicate that adults in the control group died after a longer period of time, LT50 of 23 (range 20-26) days, unlike the adults in the treatment groups that took shorter time, LT50 of 4-5 (range 3.7- 5.3) days.

Adult mosquitoes that were neither pre-exposed to the dragonfly nymph nor fungus, but exposed to fungus experienced high hazard ratio (HR=45.8) than the mosquitoes in the control group.

Adults pre-exposed to dragonfly nymph experienced higher hazard ratio (HR=67.4) than adults in the control group. This is because predator exposure resulted to compromised immunity which led to susceptibility to the fungus in the emerged adults. Also, since there was no pathogenic risk at the larval stage, there was no need for larvae to invest in immunity. This led to quicker development of the fungus in the exposed adults, leading to rapid death. The results of this study are consistent with work done by Meadows (2016), whose results show that when mosquito larval environments of *Culex pipiens* contain monocultures of dragonfly *Aeshna,* adult vectors emerging from these environments are more susceptible to *B. bassiana.*

Adults pre-exposed to fungus experienced higher hazard ratio (HR=50.9), than control adults. Some adults died because fungal pre-exposure infection was carried on from larval through pupal to adult stage during metamorphosis (Bukhari et al., 2010; Lord & Roberts, 1987; Sandhu et al., 1993; Wilson et al., 1990). The larvae were faced by hazards from both the carried on larval infection and adult infection. Since the first fungal exposure led to an infection that had already grown to lethal stages, releasing endotoxins as well as damaging the larval tissues with their vegetative growth (Hegedus & Khachatourians, 1995), it had compromised the immunity of larvae at the point of the subsequent exposure or infection, hence rapid death of the adults exposed to the fungus. However, no study was found that showed mosquito immune compromise by the fungus *B. bassiana*. This study results are not consistent with several studies which show that insects are able to enhance immunity to an infection after first exposure, an advantage that can persist across generations (Contreras-Garduño, et al, 2014; Kurtz, 2005; Little & Kraaijeveld, 2004; Moret, 2006; Rodrigues et al., 2010; Tidbury et al., 2010). However, for the entomopathogenic fungus to kill the mosquito at last, it must overcome its immunity, which

means that there was immune compromise by the first infection at the time of the subsequent infection.

Adults pre-exposed to both dragonfly nymph and fungus experienced the highest hazard (HR=112.0), which was much higher than control adults, hence had the lowest survival.

On the other hand, adults pre-exposed to dragonfly nymph experienced a higher hazard (67.4) than those pre-exposed to fungus (HR=50.9). The reason is that predator pre-exposure resulted to compromised immunity hence susceptibility (Boltaña et al., 2013; Stoks et al., 2006) to the fungus, unlike adults pre-exposed to fungus that may have launched an immune action against the fungus but failed to eliminate it, maybe due to the energy resources required. Fungal pre-exposure compromised vector immunity, but not as much as predator pre-exposure.

Adults pre-exposed to both dragonfly nymph and fungus experienced the highest hazard (HR=112.0), which was higher than adults pre-exposed to either the predator or parasitic fungus. This resulted due to combined predator and parasite effects (additive effects) of compromising mosquito larvae immunity, and the emerged adult mosquitoes were never able to launch an immune attack against the fungus. Due to this, the fungal infection spread rapidly, leading to rapid death of several larvae. Even though different organisms were studied, these findings are consistent with findings of studies done by Boonstra et al. (1998); Joop & Rolff (2004); Rigby & Jokela (2000), who found that predator exposure compromises prey immunity.

Though there was no significant difference in adult mosquito survival after *P. flavescens* and *B. bassiana* pre-exposure to *A. gambiae* larvae as single or combined factors, it was clear that vector pre-exposure to the predator poses a higher hazard (HR=67.4) on the adult mosquito survival than pre-exposure to the fungal parasite (HR=50.9).

The predator and parasite have additive effects and no synergy in the combined predator and parasite effects against the mosquito.

Also, there is a significant difference between the survival of control adult mosquitoes and those exposed to *B. bassiana* after predator and/or parasite pre-exposure.

CHAPTER SIX

SUMMARY, CONCLUSION AND RECOMMENDATIONS

6.1. Summary

In summary, according to this study, the efficacy of *P. flavescens* dragonfly nymph against *A. gambiae* larvae survival was found to be very high. The development rate of *A. gambiae* larvae reared in the presence of varying densities of *P. flavescens* nymph increases with one nymph exposure, but decreases with increase in predator density to two nymph exposure and further decreases with increase in predator number to four nymphs. The efficacy of *B. bassiana* fungus against *A. gambiae* larvae survival was found to be high and increased with increase in fungal dose. The survival of adult mosquitoes exposed to the fungus *B. bassiana* after pre-exposure to predator *P. flavescens* nymph, parasite *B. bassiana*, or both the predator and the parasite was greatly reduced to different extents, depending on the pre-exposed factor. The study results suggest an additive effect of the predator and the parasite on *A. gambiae* development and survival.

6.2. Conclusion

Four conclusions emerged from this study and are as follows;

1. The high predation efficacy of *P. flavescens* dragonfly nymph against *A. gambiae* larvae suggest that the predator could be employed in *A. gambiae* larvae control.
2. The positive and then negative relationship between larval development rate and predator density suggest that, as a biological control agent of *A. gambiae* larvae, *P. flavescens*

dragonfly nymph may not be effective either at high predator density or at low prey density.

3. The high parasitic efficacy of *B. bassiana* fungus (IMI-391510) against Kisumu strain *A. gambiae* larvae survival proves that the fungus can be employed as a biological control agent of the vector, whose mortality increase with increase in fungal dose.
4. The additive effects of *P. flavescens* nymph, and *B. bassiana,* against the survival of adult *A. gambiae* exposed to the fungus *B. bassiana* after pre-exposure to predator and/or parasite greatly reduced mosquito survival to different extents, depending on the pre-exposed factor. This shows that, these factors when combined complement each other and are effective in affecting the malaria vector, hence may be used in control of both the larval and adult stages.

6.3. Recommendations

6.3.1. Recommendations from this study

1. Even though *P. flavescens* nymph was found to have high efficacy against *A. gambiae* larvae, care must be taken to ensure that the nymphs used are averagely of the same younger age/size/weight, for repeatability of objective 1 experiment.
2. Though varying densities of *P. flavescens* nymphs affect the development rate of *A. gambiae* larvae, predator density must be adjusted as a function of *A. gambiae* density to ensure effective larval control.
3. There is a high parasitic efficacy of *B. bassiana* against *A. gambiae* larvae survival. However, an effective dose that does not kill the larvae instantly but lowers their survival

must be identified and used in order to allow the larvae to develop into adults and provide a chance for immune action, and even the effects of a successive infection.

4. Due to the additive effects of the predator and parasite against malaria vector development and survival, the combined factors are able to greatly reduce vector development, survival, population and malaria parasite transmission when employed. It is therefore recommended that the Ministry of Health considers this study application in IVM, given that in regions where dragonfly nymphs are present, adult *A. gambiae* mosquitoes might be more susceptible to parasites like *Plasmodium.*

6.3.2. Recommendations for future studies

1. The efficacy of *P. flavescens* nymph against Kisumu strain *A. gambiae* mosquito should be studied in the field, amidst other natural factors, to find out how they affect its predation efficacy against the vector.
2. Field tests should be carried out to determine whether the relation between larval development rate and predator density observed in the laboratory occurs in the wild.
3. Field tests should be carried out to determine if the positive relation between the fungal dosage of the fungal strain used, and the mortality of Kisumu strain *A. gambiae* mosquito occurs in situ.
4. Predator and parasitic fungus pre-exposure have been shown to influence adult mosquito susceptibility to the fungal parasite. However, future studies should be conducted to test on parasites such as *Plasmodium*, to find out if predator and parasite pre-exposure compromise vector immunity in the same way, to influence effects of a subsequent infection with *Plasmodium* parasite.

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APPENDICES

Appendix i

(a) Mann-Whitney U test results of the efficacy of *P. flavescens* nymph against *A. gambiae* larvae. Control Group was not exposed to *P. flavescens* nymph while the treatment Group was exposed to the nymph for 24 hours.

Test Statistics

	Time
Mann-Whitney U	1140.000
Wilcoxon W	8400.000
Z	-12.667
Asymp. Sig. (2-tailed)	.000

a. Grouping Variable: Treatment

(b) Probit analysis table of parameter estimates of the efficacy of *P. flavescens* nymph against *A. gambiae* larvae. Control Group was not exposed to *P. flavescens* nymph while the treatment Group was exposed to the nymph for 24 hours.

Parameter Estimates

						95% Confidence Interval	
	Parameter	Estimate	Std. Error	Z	Sig.	Lower Bound	Upper Bound
PROBIT[a]	Day	-1.745	.148	-11.799	.000	-2.035	-1.455
	Intercept	-.420	.095	-4.419	.000	-.515	-.325

a. PROBIT model: PROBIT(p) = Intercept + BX (Covariates X are transformed using the base 10.000 logarithm.)

Appendix ii

(a) Kaplan-Meier table of means and medians of the development rate of *A. gambiae* larvae reared in the presence of varying densities of *P. flavescens* nymph. Control (Group I) not exposed to any nymph, while the treatments; Groups II, III and IV exposed to one, two and four nymphs respectively.

Means and Medians for Survival Time

	Mean[a]				Median			
			95% Confidence Interval				95% Confidence Interval	
	Estimate	Std. Error	Lower Bound	Upper Bound	Estimate	Std. Error	Lower Bound	Upper Bound
1	**7.85**	**0.07**	**7.71**	**8**	8.000	.099	7.806	8.194
2	**7.1**	**0.04**	**7.01**	**7.18**	7.000	.031	6.940	7.060
3	**7.44**	**0.07**	**7.3**	**7.58**	7.000	.077	6.849	7.151
4	**7.75**	**0.07**	**7.62**	**7.88**	8.000	.069	7.866	8.134
Overall	7.532	.035	7.463	7.601	7.000	.042	6.918	7.082

a. Estimation is limited to the largest survival time if it is censored.

(b) Cox regression table of variables in the equation of the development rate of *A. gambiae* larvae reared in the presence of varying densities of *P. flavescens* nymph. Control (Group I) not exposed to any nymph, while the treatments; Groups II, III and IV exposed to one, two and four nymphs respectively.

Variables in the Equation

							95.0% CI for Exp(B)	
	B	SE	Wald	df	Sig.	Exp(B)	Lower	Upper
Group			29.818	3	.000			
Group(1)	.695	.138	25.416	1	.000	2.004	1.529	2.625
Group(2)	.324	.135	5.757	1	.016	1.382	1.061	1.801
Group(3)	.090	.133	.459	1	.498	1.094	.843	1.420

Appendix iii

(a) Cox regression table of variables in the equation, showing the efficacy of varying dosage of *B. bassiana* against *A. gambiae* larvae survival. The control (Group I) was not exposed to fungus, while the treatments; Groups II, III and IV were exposed to 3 mg, 6 mg and 12 mg of the fungus respectively.

Variables in the Equation

							95.0% CI for Exp(B)	
	B	SE	Wald	df	Sig.	Exp(B)	Lower	Upper
Group			27.712	3	.000			
Group(1)	.679	.264	6.603	1	.010	1.972	1.175	3.311
Group(2)	.930	.253	13.487	1	.000	2.534	1.543	4.163
Group(3)	1.260	.248	25.730	1	.000	3.526	2.167	5.739

Appendix iv

(a) Kaplan-Meier table of means and medians for survival time, for adult mosquitoes exposed to the fungus *B. bassiana* after they emerged from water with the predator *P. flavescens* nymph, with the parasite *B. bassiana*, and with both the predator and the parasite. Control (Group I) was not exposed to any factor, while the treatments; Groups II, III, IV and V, were exposed at the larval stage to; none of the factor, the predator, fungus, predator and fungus, respectively, then all the developed adults exposed to the fungus.

Means and Medians for Survival Time

	Mean[a]				Median			
			95% Confidence Interval				95% Confidence Interval	
Group	Estimate	Std. Error	Lower Bound	Upper Bound	Estimate	Std. Error	Lower Bound	Upper Bound
1	20.967	.773	19.451	22.482	23.000	1.414	20.228	25.772
2	5.044	.116	4.817	5.272	5.000	.151	4.703	5.297
3	4.444	.082	4.283	4.606	4.000	.091	3.822	4.178
4	4.711	.151	4.415	5.007	5.000	.145	4.716	5.284
5	3.400	.150	3.107	3.693	4.000	.145	3.716	4.284
Overall	7.713	.353	7.021	8.406	5.000	.096	4.812	5.188

a. Estimation is limited to the largest survival time if it is censored.

(b) Cox regression analysis table showing variables in the equation for adult mosquitoes exposed to the fungus *B. bassiana* after they emerged from water with the predator *P. flavescens* nymph, with the parasite *B. bassiana*, and with both the predator and the parasite. Control (Group I) was not exposed to any factor, while the treatments; Groups II, III, IV and V, were exposed at the larval stage to; none of the factor, the predator, fungus, predator and fungus, respectively, then all the developed adults exposed to the fungus.

Variables in the Equation

							95.0% CI for Exp(B)	
	B	SE	Wald	df	Sig.	Exp(B)	Lower	Upper
Group			115.593	4	.000			
Group(1)	3.825	.481	63.208	1	.000	45.846	17.855	117.722
Group(2)	4.210	.485	75.383	1	.000	67.350	26.038	174.210
Group(3)	3.930	.482	66.546	1	.000	50.883	19.795	130.797
Group(4)	4.719	.485	94.827	1	.000	112.014	43.332	289.555

Appendix v

Research approval letter

MASENO UNIVERSITY
SCHOOL OF GRADUATE STUDIES

Office of the Dean

Our Ref: MSC/SC/00081/014

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Date: 23rd May, 2017

TO WHOM IT MAY CONCERN

RE: PROPOSAL APPROVAL FOR ONG'WEN FEDINAND — MSC/SC/00081/014

The above named is registered in the Master of Science programme, in the School of Physical and Biological Sciences, Maseno University. This is to confirm that his research proposal titled "Effect of Dragonfly (Pantala flavescens) Nymph and Fungus (Beauveria bassiana) on Mosquito (Anopheles gambiae) Development and Survival" has been approved for conduct of research subject to obtaining all other permissions/clearances that may be required beforehand.

MASENO UNIVERSITY
DEAN SCHOOL OF GRADUATE STUDIES
23 MAY 2017
P.O. Box PRIVATE BAG, MASENO

Prof. J.O. Agure
DEAN, SCHOOL OF GRADUATE STUDIES

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