Addis Ababa University

Graduate Programme

College of Natural and Computational Sciences

Department of Zoological Sciences (Insect Sciences Stream)

The role of grasses in natural breeding habitats of gravid female *Anopheles* mosquitoes (Diptera: Culicidae) on oviposition preference as well as larval development and survival

By

Yelfwagash Asmare

A dissertation submitted to the Graduate Programme of Addis Ababa University in partial fulfilment of the requirement for the degree of Doctor of Philosophy in Biology (Insect Sciences)

August/ 2017

ADDIS ABABA UNIVERSITY
SCHOOL OF GRADUATE STUDIES

The role of grasses in natural breeding habitats of gravid female *Anopheles mosquitoes* (Diptera: Culicidae) on oviposition preference as well as larval development and survival

By

Yelfwagash Asmare

***A Thesis Presented to the School of Graduate Studies of the Addis Ababa University in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Biology* (Insect Sciences)**

Approved by Examining Board:

Name	Signature
1. Dr. Habte Tekie	(Advisor)
2. Prof. Daniel Boakye	(Examiner)
3. Dr. Melaku Girma	(Examiner)
4. Prof. Abebe Getahun	(Chairman)

Dedication

The thesis is dedicated to my beloved father Asmare Kessete (Baba) and mother Birhane Demeke (Tata) for their prayers, innumerable love and encouragement.

Acknowledgement

First and foremost, I thank Almighty God for His help to undertake this research and for His infinite blessings throughout my life.

I would like to express sincere gratitude to my supervisor, Dr. Habte Tekie, Associate Professor at the Department of Zoological Sciences, Addis Ababa University (AAU), for accepting me as a PhD student and his patience, guidance, encouragement and invaluable advice throughout my time as his student. I am also sincerely thankful to the late Dr. Emiru Seyoum, Associate professor, AAU; may God place his soul in heaven.

I am deeply grateful to Prof. Rickard Ignell, Head of Division of Chemical Ecology, Department of Plant Protection Biology, Swedish University of Agricultural Sciences (SLU), Alnarp, Sweden, for his invaluable support and advice throughout this study as well as in writing the manuscripts to be achieved for publication. In addition, I would like to express my heartfelt thanks to Dr. Sharon Hill, Associate professor at Division of Chemical Ecology, Department of Plant Protection Biology, SLU, Alnarp, Sweden. I am grateful for her support, invaluable comments, and statistical advice. Moreover, I would like to thank Dr. Richard Hopkins, Head of Pest Behaviour, Natural Resources Institute, Faculty of Engineering and Science, University of Greenwich, U.K. for his constructive ideas in my studies. I sincerely thank all members of the chemical ecology group at Alnarp, SLU for their supports with great scientific environment.

In addition, I like to gratefully express my sincere thanks to Prof. Abebe Getahun, Chairman, Department of Zoological Sciences (AAU) for his encouragement and patience to accept my justification for the extension of study period at AAU. I would also like to express my sincere thanks to all staff members and students of the Insect Science stream at the Department of Zoology (AAU) for their support and assistance.

I would like to acknowledge the Swedish Institute Guest Scholarship Program for providing me the scholarship and their financial support which helped me to pursue my work at SLU, Alnarp, Sweden. Without their invaluable support, it would have been impossible to conduct this research.

I would like to express my heartfelt thanks to Debre Markos University (DMU), my employer and sponsor, for granting me the study leave and financial support for my study. I sincerely thank and acknowledge the School of Chemical and Food Engineering, Bahir Dar University (BDU), for their support with laboratory facilities in plant oil extraction for mosquito larval bioassays. I would like to extend my thanks to the Ethiopian Public Health Institute (EPHI) for their generous support in mosquito rearing and laboratory bioassays. I would like to express my appreciation to all staff members at these institutions; special thanks to Dr. Adugna Weyessa at EPHI for his cooperation to provide the latest revised mosquito rearing manual. I am also sincerely thankful for the people in the villages around Lake Tana and Mecha Wereda Agriculture and Rural Development Office in Merawi town for their kind assistance and support during my field visits for sample collection.

I am very grateful to my parents (Asmare and Birhane), brothers (Kassahun, Yohanes and Yacobe), and sisters (Tazash, Shega, and Mintwab) for their love, encouragement and prayers. My family deserves a lot of credit and appreciation. I am extremely grateful to my beloved husband Esubalew Mengistie for his love, understanding, prayers, consistent encouragement and patience to complete my study. I am also thankful to my children (Selamawit, Hanna, and Paulos) for their love that makes me happy and strong.

Abbreviations and Acronyms

ACT	Artemisinin-Based Combination Therapy
BH-660	Bako Hybrid-660
Bti	*Bacillus thuringiensis israelensis*
Bs	*Bacillus sphaericus*
DEET	*N*, *N*-Diethyl-*meta*-toluamide
DDT	Dichlorodiphenyltrichloroethane
IRS	Indoor Residual Spraying
ITNs	Insecticide Treated Nets
IVM	Integrated Vector Management
IMVM	Integrated Malaria Vector Management
LLINs	Long-Lasting Insecticidal Nets
PMD	p-menthane-3, 8-diol
SIT	Sterile Insect Technique
WHO	World Health Organization

Table of Contents

List of Figures

List of Tables

Abstract

The malaria vectors *Anopheles arabiensis* and *Anopheles coluzzii* are widely distributed in sub-Saharan Africa, the region with the highest malaria burden in the tropics. Previous research findings show both positive and negative correlations between *Anopheles* larvae productivity and vegetation in and close to breeding habitats. The aim of the present studies is to determine species specific association between *Anopheles* mosquitoes and grasses in natural habitats. Larval sampling in natural breeding sites associated with grasses was used as a proxy to identify the link between grasses and oviposition preference in *Anopheles* mosquitoes. Grasses have their own impacts on the life cycle and oviposition behavior of mosquitoes and exploiting their potential in vector control is a novel approach.

Oviposition behaviour of *Anopheles* mosquitoes is odour-mediated and behavioural response of *An. arabiensis* and *An. gambiae*/*An. coluzzi* to volatile extracts of grasses using oviposition and attraction bioassays were investigated. The results showed that grass volatiles affect differentially oviposition site selection by *Anopheles* mosquitoes. Both the larval *Anopheles* in natural breeding habitats and gravid female of *An. arabiensis* and *An. coluzzi* in laboratory demonstrated strong preference to Poaceae grasses than Typhaceae and Cyperaceae grasses ($P < 0.05$).

The dietary effects of grasses pollen was studied to test why gravid *Anopheles* mosquitoes and their larvae show preference hierarchy to grasses. The hypothesis that dietary effect of pollen of grasses is based on the quality of pollens and this is characterized by C: N ratio and grain size of pollens. The results showed that there were significant differences between pollen from grasses by their C: N ratio and grain size ($P < 0.0001$), and these differences demonstrated differential effects on development rate and survival of larval *An. arabiensis* ($P < 0.05$). The pollens from all

the grasses enhanced pupation rate more than the nitrogen rich artificial diet TetraMin®. The small grained and carbon rich *Typha latifolia* and *Pennisetum setaceum* pollens resulted in more enhanced development rate of larval *An. arabiensis* than the large grainedcarbon-rich *Zea mays* pollen, nitrogen-rich TetraMin® fish food and *Echnocloa pyramidalis* pollen. *Zea mays* pollen, and TetraMin® fish food equivalently increased survival of larval *An. arabiensis* develop in to adulthood. The effects of grass pollens in larval diets were more pronounced in the females of *An. arabiensis*. This is similar with previous studies, indicate that male anophelines develop faster and emerge sooner than females.

The effect of grasses oils on larval survival was also studied to test why both gravid *Anopheles* mosquitoes and their larvae showed lower preferences to Typhacae and Cyperaceae grasses and especially no larvae was found in natural breeding habitats associated with Cyperaceae. The absence of *Anopheles* larvae in habitats with Cyperaceae grass is due to higher production of natural oil and this could result in mortality of larval *Anopheles* due to toxic nature of Cyperaceae grasses in addition to the physical nature of oils that causes suffocation. The results showed the rhizome sections of *C. papyrus* had significantly higher oil content (2.09 ± 0.23) than that of *T. latifolia* (1.455 ± 0.011) ($P < 0.05$). Oil extracts from both *T. latifolia* and *C. papyrus* significantly increased larval mortality on *An. arabiensis* in a dose dependent manner ($P<0.0001$). No significant differences were observed between oils of the two grass species ($P = 0.624$). In contrast, increasing the doses of the oils had significant positive correlation with larval mortality of the tested mosquito ($P < 0.0001$). Therefore, it is possible to conclude that presence/growing of *C. papyrus* in breeding habitats could reduce *Anopheles* mosquito population due to the larvicidal effect of higher amount of natural oil production by its rhizome.

Generally grasses close to and in breeding habitats are drivers of vector population dynamics and malaria transmission: grasses volatiles modulate oviposion site selection, grasses pollen has larval nutrition input and grasses oils markedly affect larval survival and development of *Anopheles* mosquitoes. The findings in this study indicated that Poaceae and Typhaceae grasses are indicators for the availability of suitable breeding habitats for *Anopheles* mosquito and could help for monitoring malaria vector populations and for planning malaria interventions. In contrast, Cyperaceae grasses can be promising component of integrated malaria vector management as the rhizome had higher amount of oil which could be released directly to the breeding water with larvicidal effects on *An. arabiensis*.

Key words: *Anopheles arabiensis*, *Anopheles coluzzii*, gravid mosquitoes, attraction, oviposition, larval survival, grass volatiles, pollen, oils

Chapter 1. General Introduction

1.1. Malaria transmission trends and interventions

1.1.1. Global malaria disease burden

Malaria is a mosquito vector borne infection and deadly disease affecting humans worldwide. It is a major burden in developing countries in the tropics, particularly sub-Saharan Africa where it has the greatest impact on health and economic development (WHO, 2015a; 2016). This disease causes serious morbidity and mortality in children less than 5 years of age, and pregnant women, as well as travelers from malaria-free areas that lack immunity (WHO, 2014; 2015a; 2015b). About 3.2 billion people are at risk of malaria infection and associated diseases in many countries around the world. Among these, 1.2 billion people are at high risk of morbidity due to infection with malaria (WHO, 2014; 2015a). The estimated number of malaria cases in 2014 were about 198 million with 584 000 deaths, similarly estimated numbers of 214 million malaria cases with 438 000 deaths in 2015 (WHO, 2015b).

1.1.2. Malaria situation in Ethiopia

Ethiopia is one of the countries in Africa with major malaria burden despite considerable efforts that have been made to suppress the level of transmission (FMOH, 2008; 2012). The two predominant causative agents of malaria, *Plasmodium falciparum* Welch and *Plasmodium vivax* Grassi & Filetti are widely distributed in the country and are responsible for 60% and 40% of malaria cases, respectively, posing greatest morbidity and mortality in children under age of 5 years (Alemu *et al*., 2013; Scott *et al*., 2016; Seyoum *et al*., 2016; WHO, 2014; 2016). This is due to the challenges in malaria control including the cost of control programs and drugs

resistance of the parasites (White, 2004; Gueye *et al*., 2014; WHO, 2014; 2015b; 2016) as well as resistance of vectors to insecticides via natural selection pressure due to injudicious and indiscriminate usage (Enayati and Hemingway, 2010; Russell *et al*., 2011; Yewhalaw *et al*., 2011; Protopopoff *et al*., 2013; Killeen *et al*., 2014). Rise in temperature and humidity trigerred by climate change is one of the factors for increaseed malaria transmission intensity in Ethiopia and other tropical coutries due to their favourable effects on the mosquito vector population growth and parasite development (Siraj *et al*., 2014). The presence of *Anopheles arabiesnsis* Patton and *P. falciparum* in different parts of Ethiopia contributes to most of the malaria burden in the country (Abose *et al*., 1998; Hunt *et al*., 1998; Seyoum *et al*., 2016; WHO, 2014; 2016).

1.1.3. Malaria parasites

The most studied causative agents of human malaria are protozoan parasites including *Plasmodium falciparum*, *P. vivax*, *P. Malariae* Grassi & Filetti, and *P. ovale* Stephens (Gilles and Warrell, 1993; Schlagenhauf, 2004; Snow *et al*., 2005; Cox, 2010). Recently, the earlier primate's malaria parasite *Plasmodium knowlesi* Knowles & Gupta is identified in human (Cox-Singh *et al*., 2008). Of these *Plasmodium falciparum* and *P. vivax* are the principal malaria parasites in sub-Saharan Africa and pose greatest health threat in the region (Schlagenhauf, 2004; Snow *et al*., 2005; WHO, 2014). *Plasmodium falciparum* is known to cause cerebral malaria mostly results serious morbidity followed by death (WHO, 2014; 2016).

Optimal temperature, humidity and precipitation in sub-Saharan Africa are suitable conditions for the development of the most important malaria parasite *P. falciparum* in the mosquito vectors (Noden, 1995; Patz *et al*., 2003; Ramasamy and Surendran, 2012). *Plasmodium falciparum* is the predominant malaria parasite in the low land regions of Ethiopia while *P. vivax* is common in the

cooler temperate areas of the country as it is able to complete its sporogonic development in the mosquito vectors at lower temperatures (Scott *et al*., 2016; Seyoum *et al*., 2016; WHO, 2014; 2016).

1.1.4. *Anopheles* mosquitoes and malaria

At present about 537 species of *Anopheles* mosquitoes are recorded worldwide (Harbach, 2013) of which 70 *Anopheles* species have the capacity to transmit human malaria; and 41 species are considered important malaria vectors (Sinka *et al*., 2010; Harbach, 2013). This difference in vectorial capacity among *Anopheles* species is attributed to the innate genetic variation in susceptibility of these *Anopheles* mosquitoes to the *Plasmodium* parasites and their longevity and capacity to support the parasites to the infective sporozoite stage which can invade humans (Blandin *et al*., 2008; Lefèvre *et al*., 2013; Clayton *et al*., 2014).

The *Anopheles gambiae* complex including *Anopheles gambiae sensu stricto* and *An. arabiensis* are the major malaria vectors in sub-Saharan Africa. *Anopheles funestus* Giles, *Anopheles moucheti* Evans, and *Anopheles nili* Theobald are also important malaria vectors in the region (Coetzee *et al*., 1989; Coetzee and Fontenille, 2004; Coetzee *et al.,* 2013). The other members of the *An. gambiae* complex, *Anopheles merus* Donitz and *Anopheles melas* Theobald have localized significance in transmitting malaria in East Africa and West Africa, respectively.

In Ethiopia, *An. arabiensis* is the major malaria vector and *Anopheles pharoensis*, *An. funestus* and *An. nili* are also important malaria vectors in different parts of the country (Abose *et al*., 1998; Hunt *et al*., 1998). The distribution range of these malaria vectors is expanding due to climate change and global warming that brings favourable temperature for their development and survival (Siraj *et al*., 2014).

1.1.5. Factors regulating malaria transmission

Malaria transmission pattern and its burden widely vary in endemic and epidemic prone areas. The physical and biological factors and the interactions between them in the environment affect the biology of malaria parasites and the distribution and population dynamics of mosquito vectors (Patz *et al*., 2003; Minakawa *et al*., 2005; Walton, 2005; Mushinzimana *et al*., 2006; Fillinger *et al*., 2009; Murdock *et al*., 2016).

Environmental factors including temperature, humidity and amount of rainfall are important regulators of the biology of both the vectors and parasites of malaria, and consequently affect the transmission intensity of the disease (Patz *et al*., 2003; Ramasamy and Surendran, 2012; Abiodun *et al*., 2013; Ajayi *et al*., 2010; Yamana and Eltahir, 2013). Murdock *et al*. (2016) reported that increasing temperature as a result of climate change could reduce the vectorial potential of the important vectors *Anopheles gambiae* and *Anopheles stephensi* in high malaria transmission settings. Similarly decreases in temperature negatively impact the populations of *Anopheles* mosquitoes and the development of malaria parasites in the vector, resulting in reduced malaria transmission (Noden, 1995; Kirby and Lindsay, 2009)

Among the biotic factors vegetation and microbial organisms in breeding habitats are sources of larval nutrients (Merritt *et al*., 1992). Vegetation in and close to breeding habitats also provides suitable physical microhabitats (Mushinzimana *et al*., 2006). *Anopheles arabiensis* larvae occur with higher density in microhabitats associated with Poacea grasses than habitats with Typhaceae and Cyperaceae (Bøgh *et al*., 2003; Gouagna *et al*., 2012; Khater *et al*., 2013; Asmare *et al*., 2017). Grasses provide pollen and detritus for mosquito larvae, and for microbial organisms such as bacteria which are parts of mosquito larval food. The pollen from cultivated and wild grasses

supplement the natural food for *Anopheles* larvae in breeding habitats; support the development of mosquitoes and increas malaria transmission (Merritt *et al*., 1992; Ye-Ebyo *et al*., 2000; 2003a; Asmare *et al*., *in press*).

On the other hand, natural enemies including predators and parasites in breeding habitats are larval mortality factors affecting negatively vector *Anopheles* mosquito population and reducing malaria transmission (Kweka *et al*., 2011; Omoya and Akinyosoye, 2013). Moreover, some aquatic grasses in breeding habitats have negative impact on the breeding success of malaria mosquitoes (Goma, 1960a; Fillinger *et al*., 2004; Yohannes *et al*., 2005) which may be due to their higher amount of natural oil production which have potential larvicidal effects to suppress mosquito population, and therefore reduce malaria transmission (Goma, 1960b; Lindsay and Martens, 1998; Mouchet *et al*., 1998).

1.1.6. Emerging approaches in malaria vector management

Prevention and control of malaria transmission using integrated vector management (IVM) and prompt and effective treatment of infection with drugs are the corner stone approaches among malaria interventions (Lehane *et al*., 2004; Smith *et al*., 2011; Kamareddine, 2012; WHO, 2015b). Vector managemet tools to suppress the population of *Anopheles* mosquitoes such as chemical insecticides, biological control, environmental management, and personal protection measures are long time in use to prevent malaria transmission in different malarious regions of the world. With these global efforts, it is currently reported that malaria incidence rates are reduced by 41% between 2000 and 2015 and by 21% between 2010 and 2015 (WHO, 2016). Such suppressed level of malaria incidence has a potential challenge of reemergence and could

be a significant public health threat despite the ongoing control efforts (Ecchoff, 2011; Ouattara and Laurens, 2015).

The re-emergence of malaria in many places is attributed to drug resistance of the parasites (White, 2004; Gueye *et al*., 2014; WHO, 2014; 2015b; 2016) and insecticide resistance of mosquito vectors (Enayati and Hemingway, 2010; Russell *et al*., 2011; Protopopoff *et al*., 2013; Killeen *et al*., 2014; Riveron *et al*., 2015) and their behavioural changes (Ndenga *et al*., 2016; Russell *et al*., 2016). Therefore, understanding ecological significance of biological factors including microbial population, aquatic invertebrate and vertebrate populations and plants in and close to mosquito breeding habitats has crucial input for the development of new vector management options.

Biotic ecological factors like vegetation, natural enemies, micro organisms associated with breeding habitats have significant role in regulating survival and population dynamics of malaria mosquitoes which can be exploited for vector management (Matias and Adrias, 2010; Kweka *et al*., 2011; Ondiaka *et al*., 2015). In addition, vector management targeting host seeking mosquitoes using human odours (Kline, 2006; Matowo *et al*., 2013; Webster *et al*., 2015) and gravid traps using electrical devices (Dugassa *et al*., 2012; 2014; 2016) provide promising insights to exploit physical and chemical signals involved in oviposition site selection by *Anopheles* mosquitoes for malaria vector management.

As biotic components of mosquito breeding habitats, grasses have their own direct influence on the life cycle and behavior of mosquitoes and their potential in vector control remains untapped. Therefore, there is a need to investigate species-specific associations between *Anopheles* mosquitoes and grasses for the development of novel and durable vector management options for

sustainable integrated malaria vector management (IMVM). Recently, behavioural and physiological responses of gravid females in *Anopheles gambiae* and *Anopheles arabiensis* to volatiles from soil infusion (Lindh *et al*., 2015), fungi (Eneh *et al.,* 2016), and from vegetation in breeding habitat especially grasses (Wondwosen *et al*., 2016; 2017; Asmare *et al*., 2017) offer novel approaches for integrated malaria vector management.

1.2. Rationale of the study

Research on the role of biotic factors involved in malaria mosquito feeding and breeding behaviour and their population dynamics is providing new approaches in the management of malaria mosquitoes. Exploiting the ecological interactions in breeding habitats; especially the role of grasses on oviposition behavioral response of gravid female *Anopheles*, as well as the survival and development of their immature stages is crucial for the development of novel effective malaria vector management options. Mass trapping gravid vector mosquitoes which search sites for oviposition using volatiles of grasses is complementary with conventional methods currently in use, indoor residual spraying (IRS) and insecticide treated nets (ITNs) for the control of host seeking adult mosquitoes. Malaria vectors control at the larval stages using natural oils of aquatic grasses in breeding habitats can also be exploited as green insecticides to suppress mosquito populations for integrated malaria vector management. In addition, the role of grass pollen as a supplementary larval diet in breeding habitats aggravating mosquito proliferation need to be investigated for monitoring and intervention.

The present studies investigates the differential effects of grass volatiles on oviposition behavior of the malaria vectors *Anopheles arabiensis* and *Anopheles coluzzii* and evaluates the effect of grass pollen and oils from breeding habitats on *An. arabiensis* larval survival and development,

and thereby contribute towards novel vector management options for malaria control and elimination.

1.3. Objectives of the study

1.3.1. General objective

- To investigate the effects of grasses in natural breeding habitats on oviposition site selection and breeding success to adulthood in *Anopheles* mosquitoes.

1.3.2. Specific objectives

- To determine larval density in breeding habitats with dominant grass species including *Echnocloa pyramidalis*, *E. stagnina*, *Typha latifolia* and *Cyperus papyrus* around the shore area of Lake Tana, Ethiopia.
- To evaluate the effect of volatile extracts of *E. pyramidalis*, *E. stagnina*, *T. latifolia* and *C. papyrus* on ovipositon behaviour of gravid *An. arabiensis* and *An. coluzzii* mosquitoes under laboratory conditions.
- To investigate the dietary effect of pollen collected from *T. latifolia*, *E. pyramidalis, Pennisetum setaceum* and *Zea mays* on survival and development of *An. arabiensis* larvae under laboratory conditions.
- To evaluate the larvicidal effect of oil extracts from *T. latifolia* and *C. papyrus* on *An. arabiensis* larvae under laboratory conditions.

Chapter 2. Literature Review

2.1. Malaria parasite biology and transmission pattern

The sporogonic development of *Plasmodium* species occuring in *Anopheles* mosquito vectors depends on environmental factors such as temperature and relative humidity which determine the longevity of the vectors to support the parasite to the sporozoite stage (Noden, 1995; Patz *et al*., 2003). Moreover, sugar feeding by adult *Anopheles* mosquitoes from sources of plant species can affect a *Plasmodium* parasite development to the infective stage (Hien *et al*., 2016).

Anopheles mosquitoes acquire the gametocyte stages of *Plasmodium* parasites through blood feeding on infected humans (Cox, 2010; Lef `ever *et al*., 2013). Gametocytes picked from an infected person by the female *Anopheles* mosquito with the blood meal reach the midgut and further differentiate in to mature micro and macro gametes (gametogony; Figure 2.1) and fertilization takes place in the lumen of the midgut to form zygotes. The zygotes then develop into motile ookinetes wich penetrate the midgut epithelium and form oocysts on the outer wall of the midgut. Each matured oocyst under goes cell division to form large number of sporozoites (CDC, 2016). The mature oocysts rapture and sporozoites are released into hemocoel from where they migrate in the hemolyph to invade salivary glands of the mosquito. The sporozoites further multiply and stay in the salivary glands of the vector *Anopheles* mosquito and are injected into a new human host with the saliva while the mosquito takes its blood meal (Igweh, 2012; Cox, 2010).

The sporozoites introduced into the human body are carried by blood circulation and establish in the liver tissue. The sporozoites in liver cells mature into schizonts and undergo asexual multiplication which rapture and release large number of merozoites (exo-erythrocytic

schizogony; Figure 2.1). The merozoites join into circulatory system where they attack red blood cells and develop into trophozoites. These trophozoites undergo asexual multiplication and form schizonts in the red blood cells which later rapture to produce large number of merozoites (erythrocytic schizogony; Figure 2.1). The repeated invasion of red blood cells by merozoites and their development in to schizonts cause distruction of red blood cells which is manifested in malaria pathogenesis. At some stage, some of the merozoites develop in to gametocytes in the human host and are picked up by the female *Anopheles* mosquito with the blood meal and the extrincic cycle of the parasite (sporogny; Figure 2.1) continues leading to the spread of malaria in the human population in a given area (Igweh, 2012; CDC, 2016).

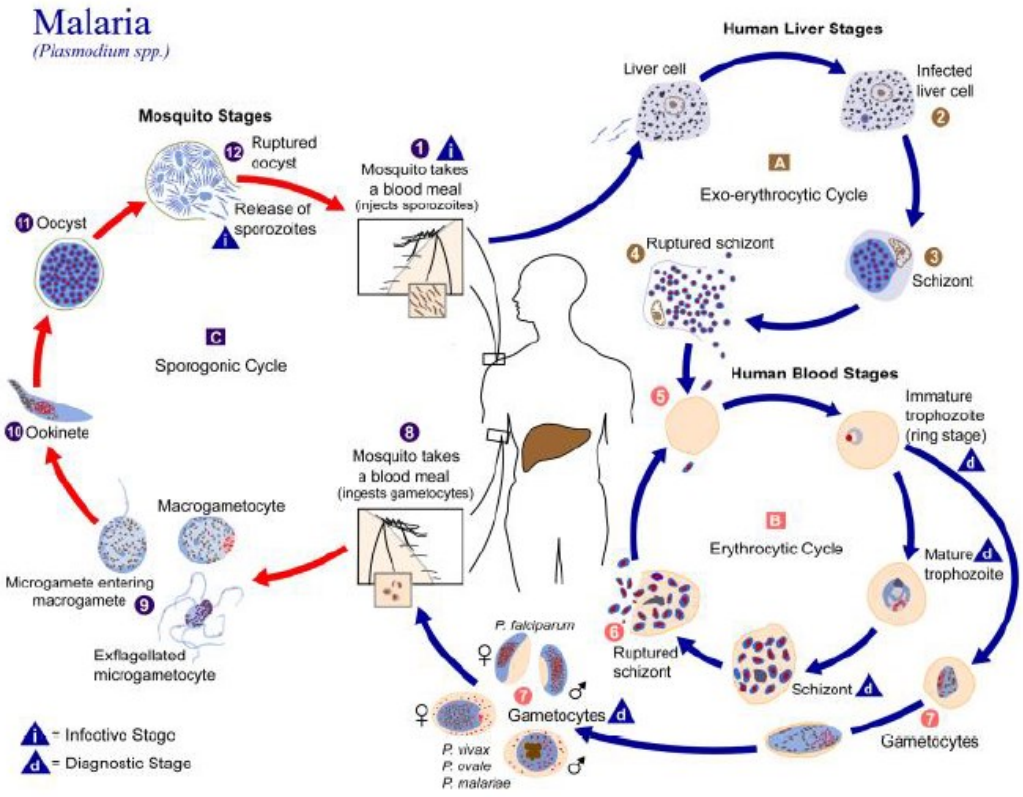

Figure 2.1. Life cycle of malaria parasites (Source: CDC, 2016)

Malaria in humans is mostly caused by the predominant parasites, *Plasmodium falciparum* and *Plasmodium vivax* which result in serious impact on public health in different parts of the world (WHO, 2014; 2016). *Plasmodium falciparum* is the most prevalent malaria parasite in Africa including Ethiopia which in extreme conditions causes a serious form of cerebral malaria, mostly resulting in deaths and often requiring emergency intervention to save the lives of people (FMOH, 2004a; 2004b; WHO, 2014; 2016). Some sporozoites of *Plasmodium vivax*, *P. ovale*, and *P.malariae* do not develop and mature into schizonts, and instead remain latent in the liver casusing relapse some period of time after recovery (Igweh, 2012). This requires the use of tissue schizonticides to destroy the liver stage of the parasite and prevent relapses (Kyu and Fernandez, 2009).

Plasmodium vivax has a wider distribution than *P. falciparum* and is reported in many tropical countries as well as some temperate regions of the world including Ethiopia (Alemu *et al*., 2013; Scott *et al*., 2016; Seyoum *et al*., 2016; WHO, 2014; 2016). The wider distribution of *P. vivax* is associated with its higher capability than *P. falciparum* to withstand lower temperatures for completing the development processes in the mosquito vector to the infective stage. *Plasmodium malariae* and *P. ovale* are among the most studied malaria parasites which have a limited distribution and prevalence and cause a mild form of the illness (Mueller *et al*., 2007). However, these mild parasites with the more serious *P. falciparum* or *P. vivax* co-infections cause severe morbidity which may require effective treatment (Mueller *et al*., 2007; Senn *et al*., 2014; WHO, 2015a).

Malaria transmission intensity and prevalence is influenced by physical and biological factors in the environment which have significant effects on the development of the parasites in the mosquito vectors (Eckhoff, 2011; Ouattara and Laurens, 2015). Favourable temperature, humidity and availability of breeding habitats result in an increase malaria mosquito populations in sub-Saharan Africa (Hay *et al*., 2000; Martin *et al*., 2008; Abiodun *et al*., 2013; Beck-Johnson *et al*., 2013), consequently leading to intensified malaria transmission in the region (WHO, 2014; 2016).

2.2. Biology and life cycle of *Anopheles* mosquitoes

Anopheles mosquitoes undergo holometabolous type of development with the egg stage, four larval intars, pupal stage, and adults (Figure 2.2). Male and female mosquitoes feed on plant sugars, swarm and mate in the proximities of breeding habitats. The female mosquitoes then look for potential hosts and take blood meals required for egg development. Blood fed female

mosquitoes rest for 2-3 days in which the gonotrophic cycle of blood meal digestion and egg maturation is followed by oviposition (Clements, 1992; 1999; Lardeux *et al*., 2008). The gravid females search for breeding sites and choose suitable oviposition sites using combinations of visual and chemical cues (Bentley and Day, 1989; Takken and Knols, 1999; Ogbunugafor and Sumba, 2008; Okal *et al*., 2013). A gravid female *Anopheles* mosquito lays a batch of 50-200 eggs depending on the mosquito species and nutritional status (Clements, 1992; 1999; Lardeux *et al*., 2008). The eggs hatch into first instar larvae and feed on microorganisms, and detritus in the breeding habitats to develop into second instar, third instar, and fourth instar larvae (Merritt *et al*., 1992; Eckhoff, 2011; Rejmánková *et al*., 2013). The fourth instar larvae pupate and later on the adult *Anopheles* mosquitoes emerge from the pupae to continue the life cycle (Clements, 1992; 1999).

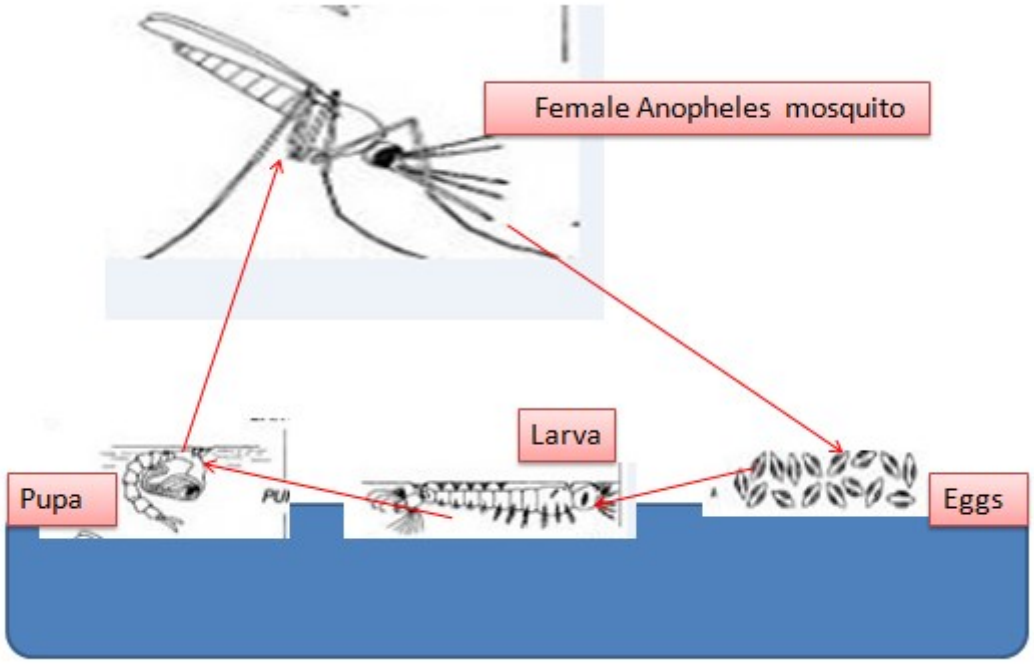

Figure 2.2. Life cycle of *Anopheles* mosquitoes (Adapted from Rejmánková *et al*., 2013; EPHI, 2017)

Adult *Anopheles* mosquitoes require sugar from host plants as source of energy for swarming, mating and searching for blood meals (Manda *et al*., 2007). Host seeking female *Anopheles* mosquitoes take blood meals from vertebrate hosts and undergo blood meal digestion, egg maturation and oviposition to complete the gonotrophic cycle (O'Meara, 1987; Clements, 1992; 1999; Lardeux *et al*., 2008). Both male and female mosquitoes feed on sugar sources after emergence (*e.g.* plant juice, nectar, and honeydew) for their immediate energy requirements (Gary and Foster, 2004; Manda *et al*., 2007; Gary *et al*., 2009).

2.3. Distribution of major malaria vectors in Africa

The distribution of the major malaria vectors in different parts of the world is determined by environmental factors mainly, temperature and rainfall which influence humidity and availability of suitable breeding habitats (Craig *et al*., 1999; Minakawa *et al*., 2002; Kashiwada and Ohta, 2010; Yamana and Eltahir, 2013). Optimum mean temperatures of 27°C ± 3°C and relative humidity of 70 ± 5 % RH (Bayoh and Lindsay, 2003; Murdock *et al*., 2016) and availability of suitable breeding habitats (Ramasamy *et al*., 1992; Kashiwada and Ohta, 2010) favour higher densities of these vector species ofen resulting in increased malaria transmission in different parts of the region (Coetzee *et al*., 1989; Sinka *et al*., 2010; 2012).

Increase in temperature above this optimum range due to global warming decreases the survival of *Anopheles gambiae* and malaria parasite development in this vector which could reduce the burden of malaria in high malaria transmission settings (Bayoh and Lindsay, 2003; Murdock *et al*., 2016). Moreover, changes below these optimal temperature ranges negatively impacts the development of malaria parasites in the vector and reduce densities of the vector mosquitoes with

decreased malaria transmision in the region (Bayoh and Lindsay, 2003; Kirby and Lindsay, 2009).

Among the *Anopheles gambiae* complex sibiling species, *Anopheles gambiae* s.s. *Anopheles coluzzii* Coetzee & Wilkerson, and *Anopheles arabiensis* are efficient malaria vectors in tropical Africa, while *Anopheles bwambae* White, *An. quadriannulatus* Theobald and *Anopheles amharicus* Hunt, Wilkerson & Coetzee are members of the *An. gambiae* complex, but not considered as major vectors of human malaria (Coetzee *et al*., 2004; Hay *et al*., 2010; Coetzee *et al*., 2013). *Anopheles bwambae* is restricted in its distribution, only known to occur in geothermal springs in western Uganda (White, 1985). *Anopheles quadriannulatus* and *An. amharicus* are highly zoophilic in their host preference (Coluzzi, 1984).

Anopheles merus in East Africa and *Anopheles melas* in West Africa are salinity tolerant malaria vectors in the *Anopheles* gambiae complex contributing to the malaria burden in Africa (Coetzee *et al*., 2004; Coetzee *et al*., 2013). These species are not as efficient as *An. gambiae* or *An. arabiensis* in transmitting malaria, and attain dominant vector status at high densities in their respective distribution ranges (Cuamba and Mendis, 2009).

Anopheles funestus, *Anopheles moucheti*, and *Anopheles nili*, are also important malaria vectors in different parts of tropical Africa (Coetzee *et al*., 1989; 2013; Coetzee and Fontenille, 2004). These species show higher preference to feed on humans (Coetzee and Fontenille, 2004).

In Ethiopia, *An. arabiensis, An. pharoensis, An. funestus*, and *An. nili* are important malaria vectors with wide distribution in many parts of the country with *An. arabiensis* as the predominant malaria vector (Abose *et al*., 1998; Hunt *et al*., 1998). *Anopheles arabiensis* is widely distributed in malarious areas of the country and its abundance is associated with

temporary and semi-permanent microhabitats for malaria transmition (Kibret *et al*., 2010; 2014; Stryker *et al*., 2012; Jaleta *et al*., 2013; Asmare *et al*., 2017).

2.4. Ecology and behavior of *Anopheles* mosquitoes

Host seeking, blood feeding, and oviposition behaviours of adult female *Anopheles* mosquitoes are expressed based on their internal physiological states (Takken and Knols, 1999) and are regulated by physical and biological factors in their surrounding environment (Stryker *et al*., 2012). Physical and chemical cues produced by the biological components in and around breeding habitats are indicators for locating resources including sugar source plants (Nikbakhtzadeh *et al*., 2014), blood meal hosts (Takken and Knols, 1999; Takken, and Verhulst, 2013), and oviposition sites (Bentley and Day 1989; Beehler *et al*., 1994) used by *Anopheles* mosquitoes.

Mosquitoes utilize vision to locate distant habitats of some hosts and oviposition sites (Kennedy, 1942; Bidlingmayer, 1994; Beehler *et al*., 1994; Dugassa *et al*., 2012; 2014). As the sugar seeking, host seeking and gravid female *Anopheles* mosquitoes move close to hosts or oviposition sites, olfactory cues become highly important for identifying sugar sources, blood hosts and oviposition sites (Webster and Cardé, 2016). Once mosquitoes identify the location of sugar sources, blood hosts or oviposition sites, they depend on tactile receptor detectable cues including temperature and chemical signals to prefer among the sugar source plants, blood hosts and oviposition sites (Bentley and Day, 1989; Takken and Knols, 1999; Manda *et al*., 2007).

2.4.1. Sugar feeding in *Anopheles* mosquitoes

Adult *Anopheles* mosquitoes are attracted by volatiles from host plants and obtain sugar from nectar, honeydew, rotting fruits and plant juice (Healy and Jepson, 1988; Knudsen *et al*., 1993; Nyasembe *et al*., 2012; Nikbakhtzadeh *et al*., 2014). Sugar feeding plays a significant role in regulating vectorial fitness including longevity, fecundity, flight capacity and resource-seeking behaviours of female *An. gambiae* (Gary and Foster, 2004; Manda *et al*., 2007; Zhu *et al*., 2015).

Studies on *Anopheles* mosquitoes sugar feeding indicate that some plant species reduce the vectorial fitness of mosquitoes in transmission of malaria. For example, *Mangifera indica* fruits and *Thevetia neriifolia* flowers reduce vectorial capacity in laboratory reared *Anopheles coluzzii*. This reduced infective capacity of *Plasmodium* parasite in *An. coluzzii* could be due to their toxic secondary metabolites and/or their lower nutritional quality which needs further studies (Hien *et al*., 2016). Therefore, utilization of plant species which are anti-*Plasmodium* sugar sources in different environmental situations may be a promising option in controlling malaria transmission based on multi trophic interactions of malaria vectors, the *Plasmodium* parasites and the host plant species.

2.4.2. Mating, host-seeking, and blood feeding in *Anopheles* mosquitoes

The newly emerged adult *Anopheles* mosquitoes swarm and mate once in life for insemination of females required for eggs to be fertile (Clements, 1992). Mating behaviour of *Anopheles* mosquitoes is influenced by availability of plant sugars which are used for energy requirements needed for swarming and mating performance (Stone *et al*., 2009; Gary *et al*., 2009). External environmental factors including flight tones, visual cues from the swarm, and chemical cues such as pheromones and kiaromones from vertebrate hosts are important in recognition of potential

mates by male mosquitoes and mating in *Anopheles* mosquitoes (Charlwood *et al*., 2003; Tripet *et al*., 2004; Diabate *et al*., 2011; Diabate and Tripet, 2015).

Female *Anopheles* mosquitoes seek for hosts to take blood meals required for egg maturation and nutritional requirements for their survival and longevity (Scott and Takken, 2012). Host preferences of *Anopheles* mosquitoes vary depending on the avilabilty of vertebrate hosts as sources of blood meals for egg development. Important malaria vectors including *An. gambiae s.s*., *An. coluzzii*, *An. funestus*, *An. moucheti*, and *An. nili* are anthropophilic in their feeding preference (Coetzee and Fontenille, 2004; Scott and Takken, 2012). *An. arabiensis* are opportunistic, in that these mosquitoes feed on both humans and/or cattle depending on availability of the hosts (Dekker and Takken, 1998; Takken and Verhulst, 2013). On the other hand, *Anopheles quadriannulatus* and *An. amharicus* are predominantly zoophilic in their respective range of distribution (Coluzzi, 1984; Dekker and Takken, 1998; Pates, 2002; Takken and Verhulst, 2013).

Anopheles mosquitoes use chemical cues, visual cues, and body heat in locating their blood meal sources (Takken and Verhulst, 2013; McMeniman *et al*., 2014; van Breugel *et al*., 2015). Compounds such as carboxylic fatty acids, lactic acid, ammonia, 1-octen-3-ol, and carbon dioxide (CO_2) that emanate from vertebrate animals attract host seeking mosquitoes (Kline, 2006; Webster *et al*., 2015). Once host seeking mosquitoes locate their preferred host either indoors or outdoors they feed on blood from the hosts. Engorged mosquitoes then search for suitable resting microhabitats to complete the gonotrophic cycle (Clements, 1999; Lardeux *et al*., 2008).

2.4.3. Oviposition site selection by gravid *Anopheles* mosquitoes

Gravid female of mosquitoes search for oviposition sites, discriminate between the available sites and lay their eggs in these suitable site based on physical features and chemical cues from the potential breeding sites (Bentlay and Day, 1998). Research findings indicate that ovipostion site selection by gravid female *Anopheles* mosquitoes is influenced by the ecology of potential breeding habitats which can support the development of their immature stages (Muturi *et al*., 2012; Himeidan *et al*., 2013; Afify and Galizia, 2015; Day, 2016). Ecological factors such as conspecific density, presence of competitors and natural enemies, temperature, age of the habitat, degree of permanency, and the quality and quantity of food resources are regulating oviposition site selection by *Anopheles* mosquitoes. Gravid *Anopheles* mosquitoes detect all these factors using visual, olfactory, gustatory and chemo-tactile cues to discriminate between sites for oviposition (Bentley and Day, 1989; Resetarits and Wilbur 1989; Clements 1999; Reiskind and Wilson, 2004).

Olfactory cues such as pheromones and kairomones released from different biotic components in breeding habitats are used by *Anopheles* mosquitoes to locate the suitabile oviposition sites (Navarro-Silva *et al*., 2009). Oviposition pheromones in conspecific larval habitats at lower densities attract the gravid *Anopheles gambiae* (Munga *et al*., 2006a; Ogbunugafor and Sumba, 2008). On the other, hand oviposition pheromones emanating from higher density of conspecific immature stages act as repellents, since gravid female *An. gambiae* and *Aedes Aegypti* recognizes higher densities of conspecific immatures as competitors (Michaelakis *et al*., 2005; Munga *et al*., 2006a; Ogbunugafor and Sumba, 2008).

Microbial origin olfactory cues released by bacteria and filamentous algae in breeding habitats attract gravid *Anopheles gambiae* and *An. pseudopunctipennis* (Sumba *et al*., 2004; Huang *et al*., 2006; Torres-Estrada *et al*., 2007). Cedrol is a bioactive compound released from soil infusion that attracts gravid *An. gambiae* (Lindh *et al*., 2015). Other compounds such as phenol, 4-methylphenol (*p*-cresol), 4-ethylphenol, indole, and 3-methylindole (skatole) produced from fermenting plant parts and grassses by microbial activities act upon plant materials which facilitates fermentation. The compounds released out from the fermentation process are oviposition attractants and stimulants for several mosquito species (Afify and Galizia, 2015).

Volatile extracts from grass species including *Brachiaria mutica*, *Cynodon dactylon*, *Jouveastra minea*, *Fimbristylis spadicea*, and *Ceratophyllum demersum* attract gravid female of *Anopheles albimanus* at lower doses (Torres-Estrada *et al*., 2005). These volatile extracts are a mixture of terpenoid and alcohol identified as guaiacol, phenol, isoeugenol, longifolene, caryophyllene, phenyl ethyl alcohol, and p-cresol. These findings suggest that volatiles released from live grasses around breeding habitats could be important olfactory cues for oviposition site selection by gravid females *Anopheles* mosquitoes (Himeidan *et al*., 2013). Volatiles from wild Poaceae grasses including *Echinocloa pyramidalis* and *E. Stagnina* showed higher oviposition attractant and/or stimulant effects on gravid females of *An. arabiensis* and *An. coluzzii* compared to those of *Typha latifolia* (Typhaceae) and *Cyperus papyrus* (Cyperaceae) grasses (Asmare *et al*., 2017a). Moreover, volatiles of cultivated Poaceae grass species such as rice (*Oryza sativa*) and maize (*Zea mays*) attract gravid females and/or stimulate oviposition in *An. arabiensis* (Wondwosen *et al*, 2016; 2017).

Volatiles produced by predators and competitors act as kiaromones and deter mosquitoes from ovipositing in habitats where these natural enemies are present (Munga *et al*., 2006a). For

instance, volatiles from the backswimmer *Notonecta maculate* repell and deter ovipositing females of *An. gambiae*, *Culiseta longiareolata*, and *Culex laticinctus* from laying their eggs in and close to natural enemies' habitats which will reduce survival and development success of their offspring (Arav and Blaustein, 2006; Munga *et al*., 2006a; Silberbush *et al*., 2008; 2010). These findings indicate that gravid mosquitoes are highly dependant on olfactory cues to select suitable oviposition site close to nutrient resource microbial populations and vegetation and avoid laying their eggs in habitats with mortality factors and natural enemies to ensure survival and development of their offspring until adult emergence.

2.5. Factors regulating *Anopheles* mosquito populations

2.5.1. Environmental factors

Temperature, relative humidity, and precipitation affect *Anopheles* mosquito population dynamics and malaria transmission intensity (Kashiwada and Ohta, 2010; Ramasamy and Surendran, 2012). The rise in temperature due to global warming has increased the distribution of *Anopheles* mosquitoes and malaria transmission to cooler areas where they have not been observed before. Malaria incidences reported in the highlands of sub tropical and temperate zone areas which previously were free from the disease are triggered by increases in temperature (Ramasamy and Surendran, 2012; Wimberly *et al*., 2012; Beck-Johnson *et al*., 2013).

The range of temperature which favours proliferation of *Anopheles gambiae* is about 24°C-30 °C (Bayoh and Lindsay, 2003; Murdock *et al*., 2016). Even though a limited rise in temperature from the optimal range increases vector population densities, increases above these optimal temperatures could potentially reduce malaria transmission in high transmission settings (Murdock *et al*., 2016) and decreases below the optimal ranges negatively impacts on the

development of malraia mosquitoes (Hay *et al*., 2000; Martin *et al*., 2008; Kirby and Lindsay, 2009).

The amount of precipitation also affects availability of breeding sites and survival of the vector mosquitoes. Increased rainfall optimizes the relative humidity and increases the availability of breeding pools which favour the development and survival of the *An. gambiae* complex mosquitoes and other malaria vectors which leads to increases in mosquito population densities and malaria transmission (Ramasamy *et al*., 1992; Kashiwada and Ohta, 2010; Yamana and Eltahir, 2013). However, excessive rainfall reduces mosquito population by washing away the aquatic stages from breeding habitats (Ajayi *et al*., 2010; Ramasamy and Surendran, 2012).

2.5.2. Biotic resources in *Anopheles* breeding habitats

Microbial fauna/flora as well as aquatic vegetation including algal mats and macrophytes are food resources for mosquito larvae in aquatic habitats. The microorganisms and organic detritus in most natural aquatic habitats provide the bulk of larval food and also contribute to productivity of breeding habitats (Merritt *et al*., 1992). Grasses and other vegetation in and close to breeding habitats increase detritus which provide nutrients for mosquito larvae (Himeidan *et al*., 2013). Moreover, grasses around *Anopheles* mosquitoes breeding habitats provide their pollen and supplement the natural food in the larval habitats (Ye-Ebyo *et al*, 2000; 2003a).

Availability of larval food in breeding habitats is a vital requirement for survival and development of *Anopheles* mosquitoes which determines their larval abundance and vectorial capacity of adult populations of *An. gambiae*, *An. coluzzii*, *An. darlingi*, *and An. stephensi* (Araújo *et al*., 2012; Takken *et al*., 2013; Shapiro *et al*., 2016; Vantaux *et al*., 2016). The quality of larval food also affects larval development rate and flight, reproductive fitness, survival,

longevity and larger body size for adult mosquitoes and determines their vectorial capacity upon emergence (Ye-Ebiyo *et al*., 2000; 2003a; Kivuyo *et al*., 2014; Moller-Jacobs *et al*., 2014; Vantaux *et al*., 2016).

The quality of larval food in breeding habitats is characterized by nutrient content including proteins, carbohydrates, amino acids, vitamins, and minerals (Merritt *et al*., 1992). Other characteristics of food such as particle size affect the rate of ingestion and assimilation in *Anopheles* larvae (Merrit *et al*., 1992); grain size and C: N ratio of grasses pollen affects larval development rate and survival of *Anopheles arabiensis,* which may determine thier vectorial fitness (Asmare *et al*., 2017b).

2.5.3. Biotic mortality factors in *Anopheles* mosquitoes

Predators have significant ecological roles in regulating *Anopheles* mosquitoes populations in breeding habitats and are the major larval mortality factors under natural conditions (Matias and Andrias, 2010; Kweka *et al*., 2011; Omoya and Akinyosoye, 2013). Predacious aquatic insects, nematodes, cyclopoid copepods, and larvivorous fish in breeding habitats play role in reducing mosquito populations (Marten and Reid, 2007; Chandra *et al*., 2008; Kamareddine, 2012). The notonectids including belostomatids and amphibians (Ohba *et al*., 2010), crustaceans (Su and Mulla, 2002), dragonflies and damselflies (Mandal *et al*, 2008; Manna *et al*., 2008), and wolf spiders (Futami *et al*., 2008) are also common predators of mosquito larvae and pupae. These predatory species are frequently encountered in breeding habitats of *An. gambiae s.l.* such as agricultural drainages, rice fields and small water bodies where they play important roles in suppressing populations of *An. gambiae* complex and *An. funestus* in malariuos areas (Minakawa *et al*., 2005).

Entomopathogenic fungi with mosquitocidal activities in the genera *Coelomomyces*, *Culicinomyces*, *Beauveria*, *Metarhizium*, *Lagenidium*, and *Entomophthora* have the potential to reduce the populations of *Anopheles* mosquitoes under natural conditions which can be exploited for malaria vector management (Mnyone *et al*., 2009; Ondiaka *et al*., 2015). Protozoan species such as *Vavraia culicis* and *Nosemia algerae* check mosquito vector populations under natural conditions and can be exploited as biological agents to reduce malaria transmission (Yap, 1985; Kamareddine, 2012). *Bacillus thuringiensis israelensis* (*Bti*) and *Bacillus sphaericus* (*Bs*) are naturally occurring entomopathogenic bacteria with larvicidal effects on *Anopheles* mosquitoes and are used in biological control of mosquito larvae (Charles and Nielsen-LeRoux, 2000; Lacey and Siegel, 2000; Kamareddine, 2012).

Breeding habitats with *Cyperus papayrus* and *Phragmites australis* are found with lower densities of *Anopheles* mosquito larvae (Goma, 1960a; Fillinger *et al*., 2004; Yohannes *et al*., 2005). The possible explanation for reduced densities of the larvae in these habitats is the release of natural oils from these grass species (Lindsay and Martens, 1998; Mouchet *et al*., 1998) which cause suffocation due to surface layer on breeding water (Goma, 1960a) and the larvicidal toxicity (Ameen *et al*., 2011; Kweka *et al*., 2012). This is in contrast to the role of these grasses in providing larval nutrient resources (Merritt *et al*., 1992; Ye-Ebyo *et al*., 2000; 2003a) and creating larval microhabitats with shade from sunlight and optimum water temperature (Manguin *et al*., 1996; Bayoh and Lindsay, 2003; Paaijmans *et al*., 2008; Ndenga *et al*., 2011).

2.6. Vector management in malaria control

Vector management is an effective malaria control approach long time in use to reduce the public health burden, morbidity and mortality in malarious areas in different parts of the world

(WHO, 2014; 2015c). Malaria vector management using chemical insecticides and non chemical control measures such as genetic modifications of mosquito vectors, environmental management and personal protection methods as components of integrated vector management (IVM) are recommended to reduce populations of mosquitoes and their vectorial fitness (Yohanis *et al*., 2005; Corby-Harris *et al*., 2010; Kamareddine, 2012; Alphey *et al*., 2013; WHO, 2015b).

2.6.1. Chemical insecticide-based vector management

Chemical insecticides used for malaria vector control include larvicides applied in breeding habitats and adulticides used as indoor residual spraying (IRS) and insectide treated nets (ITNs). These vector managemet tools are effective conventional options which incorporate utilization of of chemical insecticides (WHO, 2012). Chemical insecticides such as petroleum oils, Paris green (copper acetoarsenite), monolayer surface films, dichlorodiphenyltrichloroethane (DDT), organophosphate formulations, synthetic pyrethroids and insect growth regulators are used to control malaria mosquitoes at the larval stage (WHO, 2005). Chemicals insecticides including dichloro-diphenyl-trichloroethane (DDT), chlordane, toxaphene, dieldrin, aldrin, endrin, heptachlor and mirex are highly persistent and have effects on non-target aquatic organisms, as well as they are with a property for resistance development of mosquito vectors (Fu *et al*., 2003). Instead the less persistent and relatively safe compounds, such as carbamates, pyrethroids, and Temephos are recommended for use as larvicides (WHO, 2006).

Insecticides including pyrethroids, organophosphates and carbamates are used for IRS, whereas only pyrethroids are used for ITNs. Implimentation of IRS for the management of host seeking mosquito vectors protects about 106 million people globally with the 49 million people in Africa from malaria in 2015 (WHO, 2016). Even though implementation of IRS has limitations in

terms of resources and cost in many parts of sub-Saharan Africa, there are evidences for the effectiveness of these control measure in reducing malaria transmission in this region (WHO, 2015c). The utilization of ITNs in combination with IRS is also effective for the control of malaria vector populations (West *et al*., 2014; WHO, 2015c). Scaling up the coverage of these chemical insecticide-based vector management options reduces malaria incidence globally (WHO, 2016).

2.6.2. Non-chemical vector management options

Modifying the environment to make breeding habitats un-suitable affects malaria mosquito larval development and survival which could contribute to reduce adult mosquito populations and intervene with malaria transmission (WHO, 1982; Yohannes *et al*., 2005). Environmental modification activities include flooding or draining of wetlands, controlling water levels in reservoirs, rotating vegetation cover in irrigation and other agricultural fields, and altering water salinity are used and successful measures (WHO, 1982; Rafatjah, 1988). In addition, the growing of shade trees near breeding habitats could reduce breeding water temperature, and consequently reduce the mosquito vectors populations (Rafatjah, 1988; Laumann *et al*., 2010).

Knowledge about interactions of malaria mosquitoes with the environment and community mobilization for environmental modification activities is crucial for effective suppression of mosquito vector population (Knight *et al*., 2003; Yohannes *et al*., 2005). For example, source reduction and destruction of breeding habitat of mosquitoes in collaboration with the community, resulted in reduction of half percentage (50%) of *An. arabiensis* population and malaria transmission in north Ethiopia (Yohannes *et al*., 2005). Moreover, care should be taken to avoid malaria mosquito proliferation due to road and dam construction, and development of irrigation

schemes; these developmental activities should be accompanied with proper design and maintenance to avoid mosquito breeding pools (Knight *et al*., 2003).

Biological control of mosquitoes using the entomopathogenic fungi *Metharhizium anisopliae* and *Beuveria bassiana* as adulticides (Scholte *et al.*, 2003; Mnyone *et al*., 2009; Mouatcho, 2010; Ondiaka *et al*., 2015) and the bacteria *Bacillus thuringiensis israelensis* (*Bti*) and *Bacillus sphaericus* (*Bs*) as larvicides (Lacey, 2007; Kamareddine, 2012) are widely used in integrated vector management to suppress populations of malaria mosquitoes in different transmission settings. Viruses such as *Anopheles gambiae* denso virus (AgDNV) with larvicidal and adulticidal activities could also be considered for management of malaria mosquitoes (Ren *et al*., 2008). The protozoan entomopathogenic species *Vavraia culicis* and *Nosemia algerae* retard the development of *Plasmodium* parasites in the mosquito vector and potentially be exploited for integrated vector management (Yap, 1985). Introduction of *Gambusia affinis* and *Tilapia nilotica* in ponds and reservoirs will benefit communities in reducing malaria mosquito populations in addition to fish farming (Menon *et al*., 1978; Chandra *et al*., 2008).

Personal protection methods including air-conditioning, screens, covering exposed parts of the body during biting periods are environmentally friendly malaria mosquito management methods used to reduce human vector contact (Schoepke *et al*., 1998; WHO, 2015a). Natural or synthetic repellents are applied on skin or cloth and as aerosols within a house for personal protection methods against mosquito bites (Maia and Moore, 2011; Leal, 2014; CDC, 2017). Integrated use of these repellents with ITNs protects people against mosquito bites indoor and outdoor (Mng'ong'o *et al*., 2011; Sangoro *et al*., 2014).

Plant species, *Corymbia citrodora* and *Ocimum suave* volatiles in the smoke of burning as well as fresh leaves have repellent effects against malaria and yellow fever vector mosquitoes (Dube *et al*., 2011); *Eucalyptus camaldulensis* volatiles in the burning smoke have repellent effects on *Anopheles arabiensis* and *Anopheles pharoensis* (Dugassa *et al*., 2009); *Ocimum kilimandscharicum* volatiles in the smoke of burning as well as fresh leaves show repellence activities on *An. arabiensis* and *An. gambiae* (Kweka *et al*., 2008); and mosquitocidal and repellent effects of different plant species (Karunamoorthi *et al*., 2015) provide hints that we have many opportunities to use host seeking vector mosquito repellents.

P-menthane-3, 8-diol (PMD) and Cidriodiol are identified as natural plant derived compounds which have remarkable ability to repel host seeking mosquitoes (Maia and Moore, 2011, CDC, 2017). Even though the synthetic repellent *N, N*-Diethyl-3-methlybenzamide (DEET) is still in use (Leal, 2014), the plant derived repellents are more promising green protectants in terms of reducing the risk of chemical insecticides (Karunamoorthi *et al*., 2015).

2.6.3. Genetic modification in mosquito vector management

Genetic modification on mosquitoes is used to induce sterility and refraction against the pathogens they transmit, to reduce population densities, and vectorial fitness of the mosquitoes (Alphey *et al*., 2010; 2013; Atyame *et al*., 2015; Mains *et al*., 2016). It requires expensive procedures and uses specific technologies including sterile insect technique (SIT) with recombinant DNA technology, irradiation, and infection with *Wolbachia* to produce sterile mosquitoes (Alphey *et al*., 2010; Mains *et al*., 2016). Application of this method may also be constrained by cultural, political, or regulatory issues (Alphey *et al*., 2010; McLean and Jacobs-Lorena, 2016).

Sterile Insect Tecnique (SIT) uses releasing laboratory reared sterile male mosquitoes which have the capacity to compete with fertile wild male mosquitoes for mating in natural conditions. The repeated releases of sterile males of a given mosquito species has the potential to reduce its population densities in successive generations. Inducing sterility in mosquitoes using *Wolbachia* for SIT is a widely used approach in genetic control of mosquito vectors. Successful sterility of males in *Aedes aegypti* and *Culex quinquefasciatus* using *Wolbachia* has been effectively implemented for the control of these mosquitoes (Alphey *et al*., 2013; Atyame *et al*., 2015).

The replacement of wild mosquitoes with transgenic non-biting mosquitoes or mosquitoes that are refractory to the pathogens is another approach involving genetic modification that reduces the vetorial fitness of the mosquito. This approach requires molecular tools that allow gene manipulation (Toure *et al*., 2004; Terenius *et al*., 2008). Genetic modification in mosquitoes for malaria control aims to suppress the mosquito vector populations by transgenic manupliation of inheritable lethal genes and/or by reduction of susceptibility of vectors to the *Plasmodium* parasites resulting in the buildup of refractory mosquitoes in successive generations (Corby-Harris *et al*., 2010; McLean and Jacobs-Lorena, 2016).

2.7. Challenges and perspectives in malaria vector management

Targeting adult stages of mosquito vectors using ITNs and IRS have become less effective due to development of insecticide resistance in *Anopheles* mosquitoes (Enayati and Hemingway, 2010; Yewhalaw *et al*., 2011; Protopopoff *et al*., 2013; Riveron *et al*., 2015) and the behavioural shift of mosquitoes from indoor feeding and indoor resting to outdoor feeding and outdoor resting behaviour in *An. arabiensis* (Russell *et al*., 2011), in *An. gambiae* (Kitau *et al*., 2012; Ndenga *et al*., 2016), and *An. farauti* (Russell *et al*., 2016).

Effective management of malaria mosquitoes by integrating source reduction and application of natural enemies in larval habitats (Sharma *et al*., 1986); community mobilization for the proper utilization of ITNs and larval source management (Yasuoka *et al*., 2006); integrated application of IRS and ITNs (Beier *et al*., 2008) are recorded in many high malaria transmission settings. Moreover, including semiochemicals as a component of integrated mosquito vector management has a potential to enhance sustainable management of malaria vectors. There are success stories of integrated vector management in several countries of the world that involved semiochemicals for malaria suppression (Otieno *et al*., 1988; Barbosa *et al*., 2010; Michaelakis *et al*., 2005). For example, host seeking mosquito trap baited with human foot odours and CO_2 treated with the insecticide pyriproxifen and used with LLINs reduces populations of *An. arabiensis* (Matowo *et al*., 2013).

Further innovations are required to develop novel vector management options to reduce the limitations of chemical insecticides, their ineffectiveness to control mosquito vectors due to resistsnce development and behavioural shift of the mosquitoes, consequently minimize malaria transmission. Knowledge on ecological factors regulating the biology and behaviours of mosquitoes and consequently their population dynamics as well as epidemiological profile of malaria transmission provides opportunities to expoloit their potential for the development of ecologically sound and sustainable interventions. Research on odour-mediated behaviours of malaria mosquitoes using volatiles (Michaelakis *et al*., 2005; Nikbakhtzadeh *et al*., 2014; van Loon *et al*., 2015) can be innovative in the development of new interventions to overcome the challenges related with usage of IRS and ITNs.

Grasses associated with breeding sites are among biological components which have potential roles in regulating behaviours of *Anopheles* gravid females in oviposition site selection, larval

survival and development. For these reasons, the present study is undertaken to determine ecological significance of grasses and their products including volatiles, pollen and natural oils on oviposition site selection, larval survival and development of malaria mosquitoes in breeding habitats.

Chapter 3. The Role of Grass Volatiles on Oviposition Preferences by *Anopheles arabiensis* and *Anophele coluzzii*

3.1. Introduction

Oviposition site selection by gravid *Anopheles* malaria mosquitoes is a key moment in the reproductive success of the individual, and thus the population dynamics of the species (Ndenga *et al*., 2011; White *et al*., 2011). Consequently, the search for an oviposition site has important implications with regard to the control of malaria vectors (White *et al*., 2011). When insects select oviposition sites, they make choices on increasingly fine spatial scales, starting with selecting a habitat in which to search (Hambäck *et al*., 2009; Raffa *et al*., 2016; Webster and Cardé, 2016). In the case of egg-laying mosquitoes, they may have to leave a habitat in which they have been acquiring blood meals, in order to enter a habitat containing egg-laying sites. These habitats differ in physical, chemical and biological characteristics providing cues for the searching female, and directly influencing the distribution, survival and abundance of mosquito larval populations (Gimnig *et al*., 2001; Minakawa *et al*., 2004; Vanwambeke *et al*., 2007; Fillinger *et al*., 2009; Ndenga *et al*., 2012).

The use of an oviposition site by mosquitoes is dependent on their relative position in the landscape (Mushinzimana *et al*., 2006; Vanwambeke *et al*., 2007; Fillinger *et al*., 2009; Ndenga *et al*., 2012), visual cues (Dugassa *et al*., 2012; 2014), water vapour plumes (Okal *et al*., 2013), semiochemical cues associated with water bodies (Rejmánková *et al*., 2005; Sumba *et al*., 2008; Afify and Galizia, 2015; Lindh *et al*., 2015) and the physical parameters of the water (Gimnig *et al*., 2001; Minakawa *et al*., 2004).

The most productive natural larval habitat types for *An. gambiae/An. coluzzii* and *An. arabiensis* are transient puddles (Ndenga *et al*., 2011), often surrounded by short grasses (Minakawa *et al*., 2004; Fillinger *et al*., 2004; 2009). Both of these major vectors in sub-Saharan Africa have also been recorded in more stable water bodies, such as the littoral zone of lakes and in swamps (Fillinger *et al*., 2004; Minakawa *et al*., 2005; Imbahale *et al*., 2011; Ndenga *et al*., 2011; Minakawa *et al*., 2012; Asmare *et al*., 2017). Vegetation often populates these wetland habitats (Fillinger *et al*., 2004; Ndenga *et al*., 2011; Minakawa *et al*., 2012), and *An. gambiae/An. coluzzii* and *An. arabiensis* are commonly found amongst cattails (*Typha spp.*; Typhaceae) and dallis grasses (Paspalum *spp.*; Poaceae) (Bøgh *et al*., 2003; Gouagna *et al*., 2012; Khater *et al*., 2013). In contrast, habitats populated by reeds (*Phragmites spp.*; Poaceae) and papyrus (*Cyperus papyrus*; Cyperaceae) generally produce low numbers of mosquitoes (Goma, 1960b; Fillinger *et al*., 2004; Yohannes *et al*., 2005); probably due to the natural oil production of these species that reduces larval survivorship (Lindsay and Martens, 1998; Mouchet *et al*., 1998). Hence, grasses appear to play an important role in the natural breeding site selection of *An. arabiensis* and *An. gambiae/An. coluzzii*. Yet, the influence of natural grasses and other emergent vegetation on the oviposition site selection by gravid female *Anopheles* mosquitoes is not clearly understood. Moreover, the nature of the volatiles emitted from wild grasses and how they affect the behaviour of *An. arabiensis* and *An. gambiae/An. coluzzii* has not been investigated to date.

The objective of this study was to investigate anopheline larval occurrence and abundance in natural breeding habitats populated by four wild grass species: antelope grass, *Echinochloa pyramidalis* (Poaceae); hippo grass, *Echinochloa stagnina* (Poaceae); common cattail; *Typha latifolia* (Typhaceae); and papyrus reed, *Cyperus papyrus* (Cyperaceae), and to correlate the behavioural response of gravid *An. arabiensis* and *An. coluzzii* to the natural volatiles collected

from these grasses. The implications for anopheline ecology and vector management are discussed.

3.2. Materials and methods

3.2.1. Description of study sites and *Anopheles* larval sampling

Anopheles larval sampling was made in potential breeding habitats at the southern littoral region of, and wetlands adjacent to, Lake Tana, Ethiopia (11°37′ N, 37°21′ E; 1830 m above sea level), (Figure 3.1). The climate of the study area is typical of semi-arid regions close to the equator, with a high diurnal temperature variation between daytime extremes of 30 °C to night time lows of 6 °C, but mainly varies between 20-27 °C. Rainfall may reach up to 2000 mm per year, falling in one rainy season from May to October, with a peak during July–August (Vijverberg *et al*., 2009). During the El Niño event of 2014–2015, this region experienced a severe drought, with an overall reduction in rainfall of on average 50% (FAO, 2016), which had a drastic effect on *Anopheles* mosquito populations. For this reason, larvae of all stages, rather than first instars alone, were collected once in early September and again in late September 2015, in an attempt to sample during the main proliferation period of mosquitoes in the study area. *Cyperus papyrus* and *Typha latifolia* are among the dominant grasses in deep water bodies of the lakeshore, whereas *Echinocloa pyramidalis* and *Echinocloa stagnina* predominantly are found at the edge of the lakeshore or in wetlands adjacent to the lake (Appendices, Plate A).

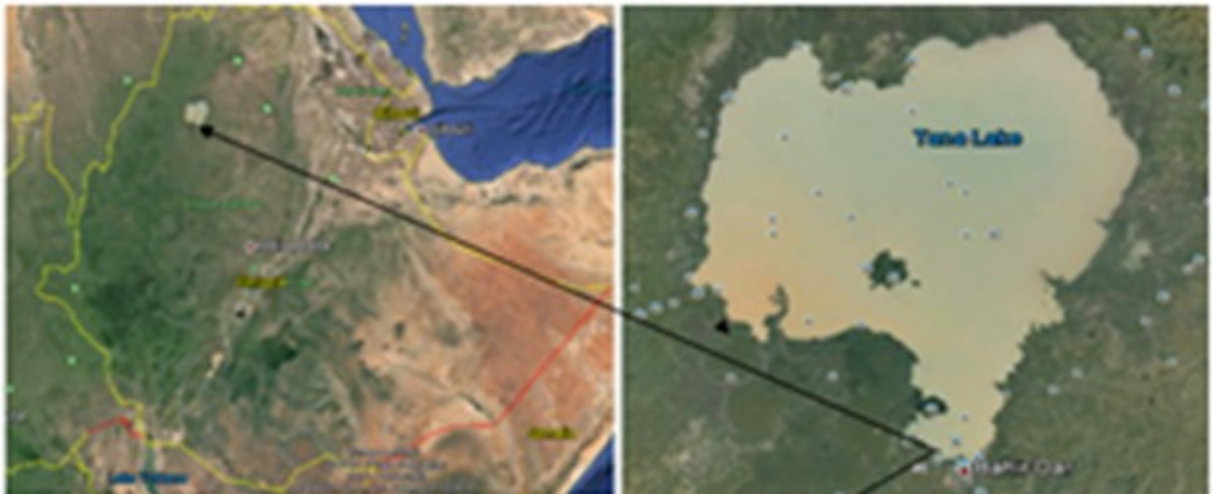

Figure 3.1. Map of study area at the southern gulf of Lake Tana, around Bahir Dar.

In the study area, 10 sub-sites dominated by each individual grass species were selected. In each sub-site, 10 separate samplings of larvae (technical replicates) were made using a standard 350 ml dipper (WHO, 2005). The collected *Anopheles* larvae were counted and recorded for each larval habitat associated with the different grass species. Of the collected larvae, 10 % were preserved in 70 % ethanol for subsequent identification to species using standard PCR analysis (Scott *et al*., 1993).

3.2.2. Headspace odour collection from grasses in breeding habitats

Freshly cut grass (100 g), including the vegetative and reproductive parts (Asmare *et al*., 2017), was enclosed in a Teflon bag (Toppits, Cofresco, Germany). A charcoal-filtered continuous airstream (1 l min^{-1}) was drawn by a Personal Air Sampler (PAS-500, Spectrex, Redwood City, CA, USA) over the grass onto an aeration column for 2 h (Figure 3.2). Aeration columns were made of Teflon tubing (4.5 cm x 3mm i.d.), holding 50 mg Porapak Q (50/80 mesh; Waters Associates, Milford, MA, USA) between glass wool plugs. The columns were rinsed with 1 ml re-distilled n-hexane (Merck, Darmstadt, Germany) before use. Adsorbed volatiles were eluted

with 300 µl re-distilled n-hexane. Odour collections from each grass species were pooled separately and then stored in sealed glass capillary tubes at -20 °C until used for behavioural experiments.

Figure 3.2. Head space grass odour collection using fresh cut grass sample enclosed in a Teflon bag, a Personal Air Sampler with Teflon tubing holding 50 mg Porapak, and a wire guaz bag holding charcoal to filter the air.

3.2.3. *Anopheles* mosquito rearing

Anopheles arabiensis (Dongola strain) and *Anopheles coluzzii* (Suakoko strain) were kept at 27±1 °C, 70± 5 % RH, and at a 12 h: 12 h light: dark photoperiod. Larvae were reared in plastic trays (22 cm × 34 cm × 10 cm) filled with 1 l distilled water, and fed powdered Tetramin® fish food (Tetrawerke, Melle, Germany) daily. Pupae (80-100) were placed in BugDorm-1 insect cages (30 cm × 30 cm × 30 cm; Mega View Science, Taiwan) for adult emergence. Adult

males and females were kept together and provided *ad libitum* access 10 % sucrose solution. For colony maintenance, female mosquitoes were blood fed on de-fibrinated sheep blood via a membrane-feeding system (Discover Workshops, Accrington, UK). Eggs were laid in 30 ml plastic cups (Nolato Hertila, Sweden) filled with distilled water, and then transferred to larval trays for hatching. For experiments, female mosquitoes, 4 days post-emergence, were allowed access to sheep blood (Håtunalab, Bro, Sweden) from an artificial feeder (Hemotek Discovery Workshops, Accrington, UK) for 3 h. The engorged females 6-8 h post-blood meal were then transferred to a new cage until used for experiments to test behavioural responses of gravid females to grass volatiles in light, temperature and moisture regulated experiment rooms in SLU, Alnarp, Sweden.

3.2.4. Wind tunnel attraction bioassay

Attraction of *Anopheles arabiensis* and *Anopheles coluzzii* to the headspace odour extracts of the four grass species was analysed in a wind tunnel assay (Majeed *et al*., 2014) (Figure 3.3). Cotton rolls (DAB Dental AB, Upplands Väsby, Sweden) were used as dispensers, and the amount of extract pipetted onto the dispensers corresponded to the amount of volatiles released during 0.04, 0.4, 4, 10, and 20 min from the individual grass species. An equivalent amount of *n*-hexane was used as a control. Both treatment and control dispensers were suspended from a 5 cm wire coil at the upwind end of the wind tunnel (Appendices, Plate D).

Ten individual female mosquitoes, 48 h post-blood meal, were transferred to a release cage 2 h prior to experiments (Appendices, Plate D). The chambers were then placed in the downwind end of the wind tunnel, where the insects were allowed 5 minutes to adapt, before the butterfly valve of the cage was opened for their release (Figure 3.2). Attraction to either treatment or control was analysed as the proportion of mosquitoes that made source contact within one minute

after release. Each release rate for each grass volatile extract and the control was replicated ten times.

One experiment day was used for each grass volatile, starting with the control (the solvent *n*-hexane) and continued for a given grass volatile from the lower to the higher release rates. The glass made wind tunnel chamber and wire coil which carry the odour dispensers were washed with alcohol and distilled water. Thenafter these materials were placed in oven for 8 h to make them odour free for the next day experiment. The mosquito release cages were also washed and left aeration between each grass volatile experiment day to avoid any odour contamination.

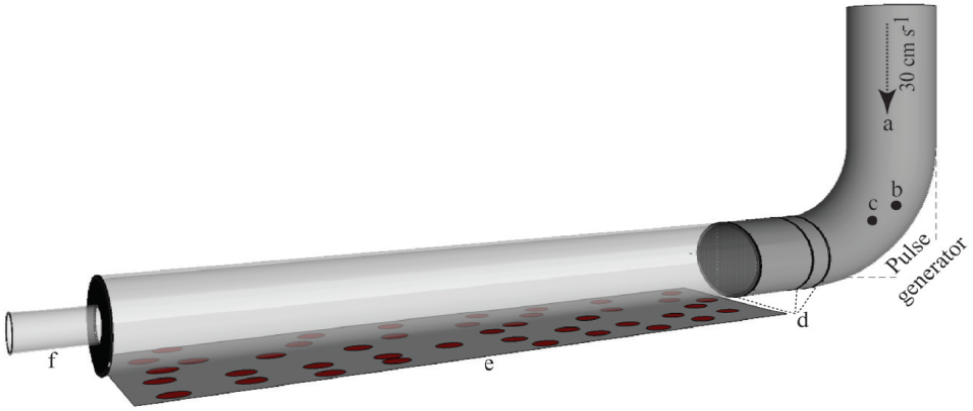

Figure 3.3. Wind tunnel experimental setup for behavioural bioassay: (a) charcoal filtered and humidified air, (b) pressurized air inlet, (c) stimulus inlet into which the odours were blown to travel through (d) stainless steel mesh plume diffusers, (e) and into the glass flight tunnel (f) towards release chamber. (Source: Majeed *et al*., 2014)

3.2.5. Two-choice oviposition bioassay in experimental cages

The oviposition response of *Anopheles arabiensis* and *Anopheles coluzzii* to the volatile extracts of the four grass species was analysed in BugDorm-1 insect cages kept in a climate-controlled room at 25 °C, 70±5 % RH, and at a 12 h: 12 h light: dark photoperiod. Plastic cups (30 ml;

Nolato Hertila) filled with 10 ml distilled water provided the oviposition substrate, and were located in opposite corners of the cage, 2 cm from each wall (Figure 3.4). The treatment cups were conditioned with one of the wild grass volatile extracts, in the same amounts used in the wind tunnel bioassay. An equivalent amount of *n*-hexane was used as a control. Location of treatment and control cups in experiment cage were exchanged in between each experiment (Asmare *et al*., 2017a). Ten mosquitoes, 48 h post-blood meal, were released into the experimental cages at 08:00-10:00, and the number of eggs in the treatment and control cups counted after 48 h. An oviposition index was determined by: (number of eggs laid in treatment cup - number of eggs laid in control cup) / (total number of eggs within the experimental cage). Each release rate of each grass volatile extract was replicated 5 times.

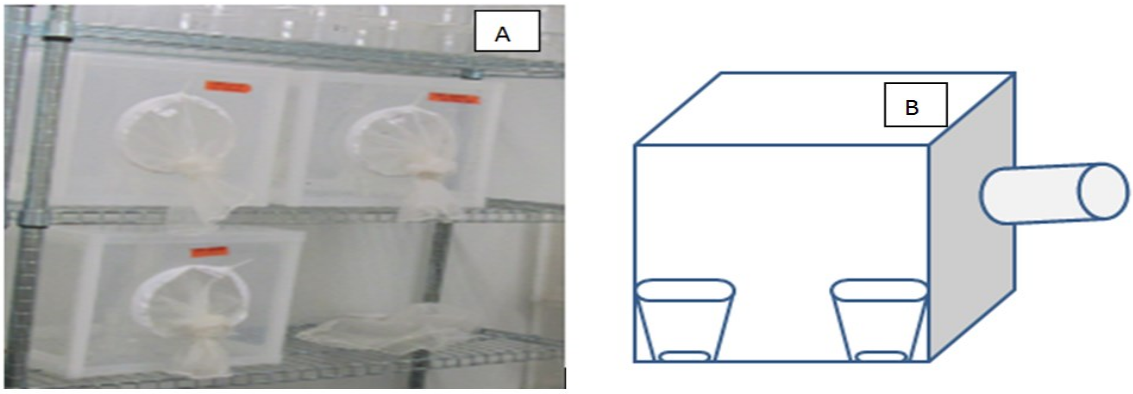

Figure 3.4. Two-choice oviposition bioassay setup in experimental cage: (A) experiment cages arranged on shelf, (B) treatment and control cups arranged in the experiment cage.

3.2.6. Multi-choice oviposition bioassay in semi-field tent experimental setting

Greenhouse cage tents (2 m × 2 m × 2 m; BioQuip, Rancho Domniguez, CA, USA) were used as multi-array bioassays to analyse the oviposition preference of *Anopheles arabiensis* and *Anopheles coluzzii* to the four wild grass volatile extracts and a control (Figure 3.5). The tents

were kept in a greenhouse (Appendices, Plate B) at 27±1 °C, 50±5 % RH, and at a 12 h: 12 h light: dark photoperiod. As above, 30 ml plastic cups filled with 10 ml distilled water provided the oviposition substrate. Treatment cups were conditioned with the four wild grass volatile extracts in an amount corresponding to the volatiles released during 0.4 min from the individual grass species, while the control cup was conditioned with the equivalent amount of *n*-hexane. Treatment and control cups were set up in a 5 x 5 matrix (20 cm between cups) (Appendices, Plate C). The matrix was changed in between replicates (n=10 for each *Anopheles* species) to avoid position effects of the treatments (Asmare *et al*., 2017a). Twenty female mosquitoes, 48 h post-blood fed, were released into the tents, and the number of eggs counted after 24 h. The oviposition response was scored by counting the number of eggs in the treatment and control cups.

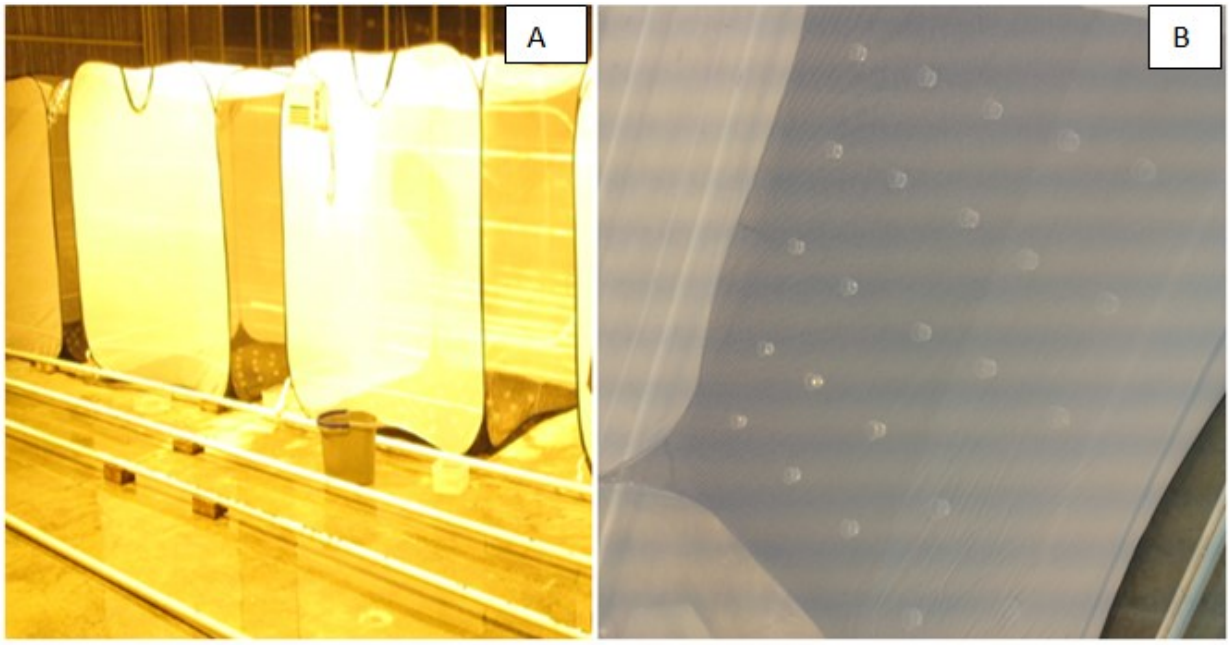

Figure 3.5. Multi-choice oviposition bioassay setup in experimental tent: (A) experiment tents arranged in green house, (B) treatment and control cups arranged in experiment tent.

3.2.7. Statistical analyses

The data from the larval survey were subjected to a univariate general linear model (GLM), using the statistical software IBM SPSS Statistics for Windows, Version 21.0. Significant differences between means were determined at α=0.05 and post hoc multiple comparisons among the grasses were made using the Tukey's HSD test. The behavioural responses of gravid *An. coluzzii* and *An. arabiensis* in the wind tunnel and two-choice oviposition bioassay were analysed using a nominal logistic fit model, in which choice was the dependent variable, weighted by the number of 1) mosquitoes in the attraction assays and 2) eggs laid in the oviposition assays, with dose as the independent fixed effect and replicate as a random effect (JMP® Pro 12.0.1. SAS Institute Inc., Cary, NC, USA). In the tent experiments (5-choice oviposition assays), the number of eggs was used as the weight, the choice as the dependent variable, the grasses and control as the independent fixed effect, and the tent and replicates as the random effects. Here, we report the χ^2 and *P*-value from the likelihood ratio test. Significant differences between the individual doses (wind tunnel and two-choice assays) and grasses (five-choice assay) were determined by odds ratio pair wise comparisons.

3.3. Results

3.3.1. *Anopheles arabiensis* larval density in natural grass habitats

No significant effect of sub-site location was found (F = 0.367, DF_d = 9, DF_n = 3, P = 0.999). Hence, the larval abundance data collected from the breeding habitats associated with each grass species was pooled in subsequent analysis. *An. arabiensis* was the most abundant species comprising more than 40 % of the specimens in the study area, and was the only member of the *An. gambiae sensu lato* complex to be identified following PCR analyses of 48 mosquito larvae.

A significantly higher number of *An. arabiensis* larvae were found in *E. pyramidalis* dominated breeding habitats than in any of the other potential breeding habitats (Figure 3.6). The number of larvae in the remaining habitats also differed significantly, with breeding habitats dominated by *E. stagnina* containing more larvae than breeding habitats dominated by *T. latifolia* (Figure 3.6). Interestingly, no larvae were found in habitats dominated by *C. papyrus* (Figure 3.6).

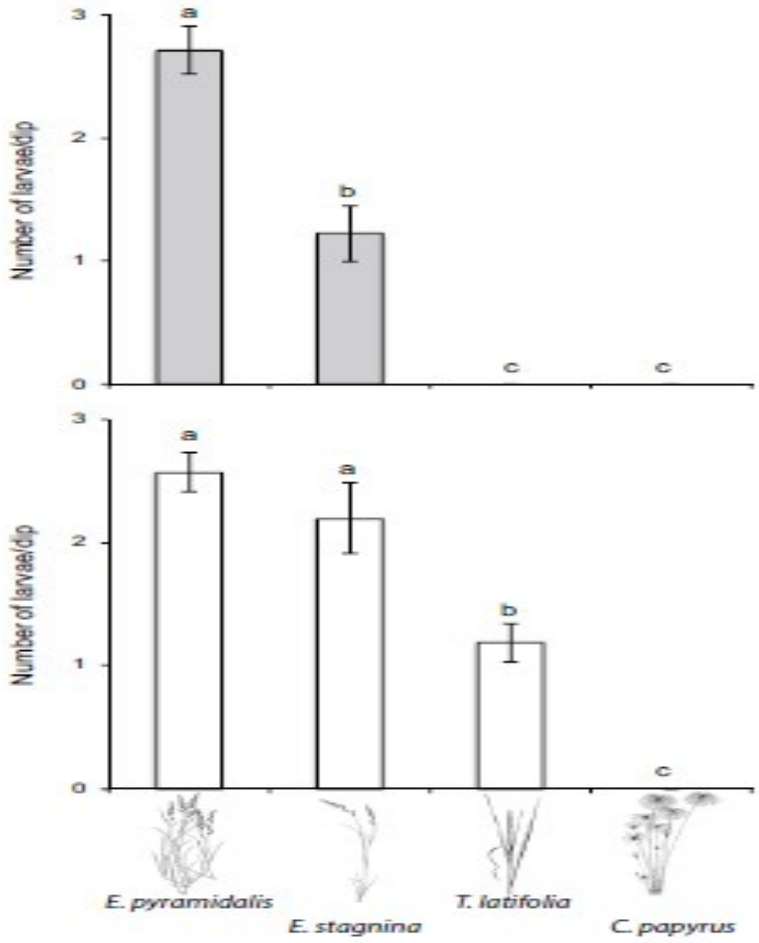

Figure 3.6. *Anopheles* larval density in natural breeding habitats dominated by four grass species, *Echinochloa pyramidalis*, *Echinochloa stagnina*, *Typha latifolia* and *Cyperus papyrus*, in early (top) and late (bottom) September. The mean larval densities with different letter designations are significantly

different from one another (univariate general linear model with a Tukey's post hoc analysis; $P < 0.005$). *Error bars* represent the standard error of the mean.

3.3.2. Attraction of *Anopheles arabiensis* and *Anopheles coluzzii* to grass volatiles

Attraction of *An. arabiensis* and *An. coluzzii* to the grass volatile extracts was dose dependent and differed among the grass species (*An. arabiensis*: Dose, χ^2=34.51, P<0.0001; Grass, χ^2=13.11, P=0.0044; *An. coluzzii*: Dose, χ^2=46.25, P<0.0001; Grass, χ^2=41.46, P<0.0001) (Figure 3.7). No significant difference in the attraction of *An. arabiensis* to the volatile extracts of *E. pyramidalis, E. stagnina* and *T. latifolia* was found, however, the attraction to the *C. papyrus* volatile extract was significantly lower than each of the other grass volatile extracts (Table 3.1). Attraction of *An. coluzzii* to the volatile extracts of either the Poaceae (*E. pyramidalis* and *E. stagnina*), or the Typhaceae (*T. latifolia*) and Cyperaceae (*C. papyrus*), did not differ. The attraction to the volatile extract of the Typhaceae and Cyperaceae, however, was significantly lower than that of the Poaceae (Table 3.1).

Table 3.1. Cross-comparative analysis of the attraction of gravid mosquitoes to volatile extracts of grass species associated with *Anopheles* breeding habitats.

		Attraction			
		An. arabiensis		***An. gambiae/An. coluzzii***	
Level 1	**Level 2**	**Odds Ratio**	***P***	**Odds Ratio**	***P***
E. pyramidalis	*E. stagnina*	0.8361	0.3210	0.8929	0.5313
E. pyramidalis	*T. latifolia*	0.9226	0.6546	2.011	*0.0002*
E. pyramidalis	*C. papyrus*	1.553	*0.0181*	2.431	*<0.0001*
E. stagnina	*T. latifolia*	1.103	0.5802	2.252	*<0.0001*
E. stagnina	*C. papyrus*	1.858	*0.0007*	2.722	*<0.0001*
T. latifolia	*C. papyrus*	1.684	*0.0045*	1.209	0.3436

The analysis is done using a nominal logistic regression model fit $\alpha = 0.05$.

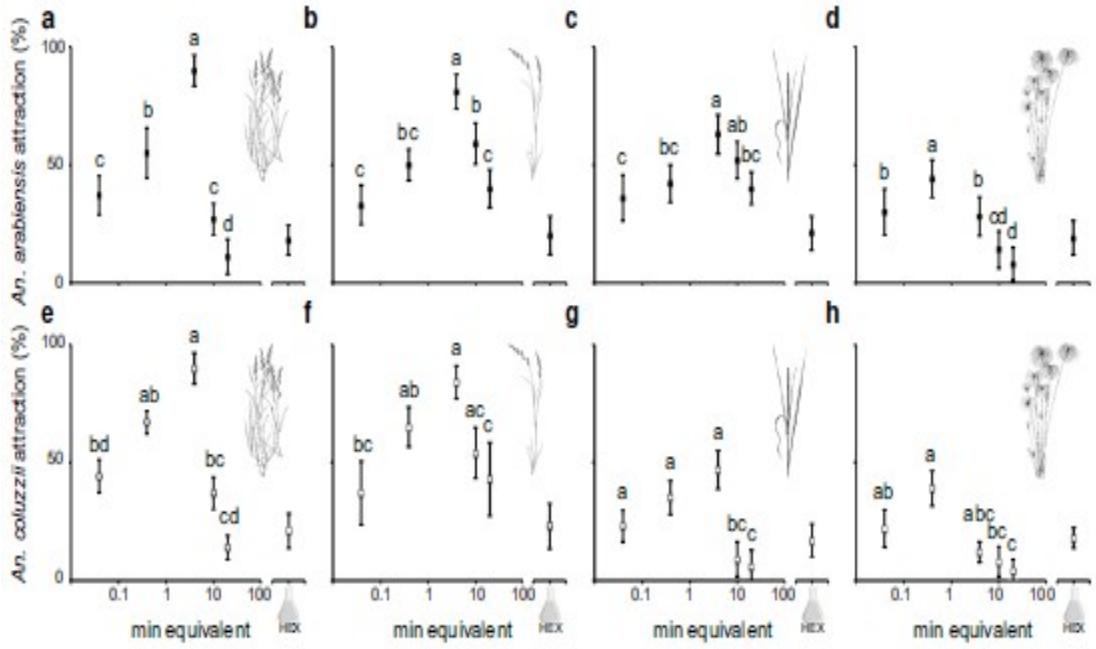

Figure 3.7. Attraction of gravid *Anopheles arabiensis* and *Anopheles coluzzii* to grass volatile headspace extracts, from *Echinochloa pyramidalis* (a, e) *Echinochloa stagnina* (b, f), *Typha latifolia* (**c**, **g**) and *Cyperus papyrus* (d, h), respectively. Release rate of the volatile headspace extracts is given in minute equivalents. The solvent control was hexane (HEX). The mean percent attraction values with the same letters indicate no significant difference from one another (nominal logistic fit model, χ^2 and $P < 0.05$ from the likelihood ratio test, significant differences were determined by odds ratio pairwise comparisons). Error bars represent the standard error of the mean.

3.3.3. Oviposition response of *Anopheles arabiensis* and *Anopheles coluzzii* to grass volatiles

The oviposition response of *An. arabiensis* and *An. coluzzii* to water conditioned with the grass volatile extracts was both dose dependent and differed among the grass species (*An. arabiensis*: Dose, χ^2=94.67, P<0.0001; Grass, χ^2=44.79, P<0.0001; *An. coluzzii*: Dose, χ^2=47.35, P<0.0001; Grass, χ^2=24.29, P<0.0001) (Figure 3.8). No significant differences in oviposition response were found for *An. coluzzii* to water conditioned with volatile extracts of *E. pyramidalis, E. stagnina* and *T. latifolia,* but the oviposition response to these grass extracts were significantly higher than

that observed for the *C. papyrus* volatile extract (Table 3.2). Similarly, for the *C. papyrus* volatile extract, the oviposition response of *An. arabiensis* was significantly lower than to that of all the other grass volatile extracts (Table 2). In contrast to *An. coluzzii*, the volatile extract of *T. latifolia* stimulated a lower oviposition response in *An. arabiensis* than that of *E. pyramidalis* (Table 3.2).

Table 3.2. Cross-comparative analysis of the oviposition response of gravid mosquitoes to volatile extracts of the grasses associated with *Anopheles* breeding habitats.

		Oviposition			
		An. arabiensis		***An. gambiae***	
Level 1	**Level 2**	**Odds Ratio**	***P***	**Odds Ratio**	***P***
E.pyramidalis	*E. stagnina*	0.8425	0.0687	1.005	0.9556
E.pyramidalis	*T. latifolia*	0.7780	*0.0076*	0.8700	0.1261
E.pyramidalis	*C. papyrus*	0.5364	*<0.0001*	0.6800	*<0.0001*
E. stagnina	*T. latifolia*	0.9233	0.3932	0.8657	0.1043
E. stagnina	*C. papyrus*	0.6366	*<0.0001*	0.6766	*<0.0001*
T. latifolia	*C. papyrus*	0.6894	*<0.0001*	0.7816	*0.0067*

The analysis is done using a nominal logistic regression model fit $\alpha = 0.05$.

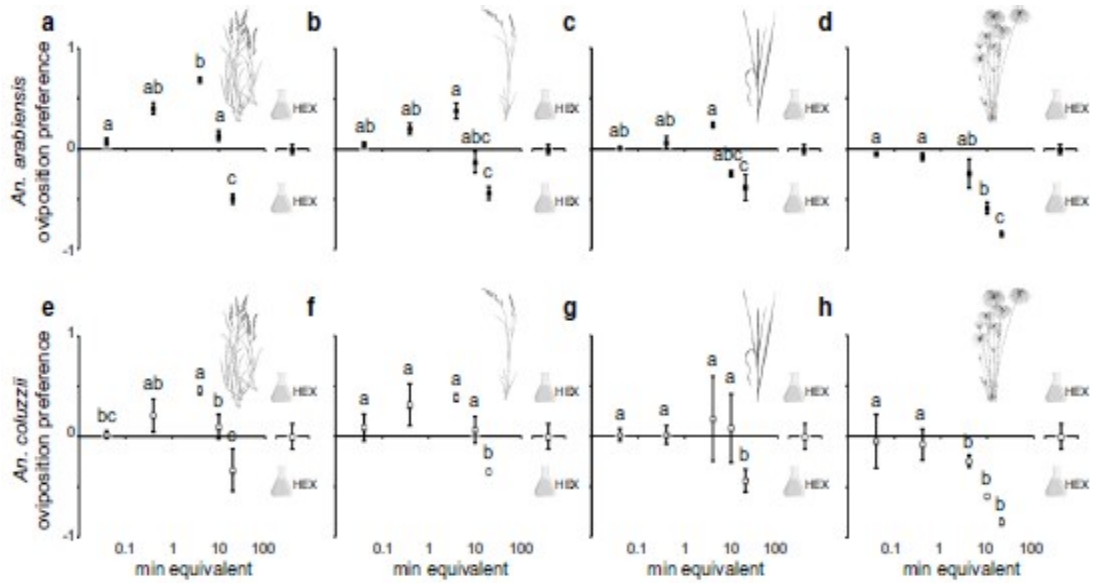

Figure 3.8. Oviposition response of gravid *Anopheles arabiensis* and *Anopheles coluzzii* to grass volatile headspace extracts, from *Echinochloa pyramidalis* (a, e) *Echinochloa stagnina* (b, f), *Typha latifolia* (c, g) and *Cyperus papyrus* (d, h), respectively. Release rate of the volatile headspace extracts is given in minute equivalents. The solvent control was hexane (HEX). Mean oviposition preferences with the same letters indicate no significant difference from one another (nominal logistic fit model, χ^2 and $P < 0.05$ from the likelihood ratio test, significant differences were determined by odds ratio pairwise comparisons). Error bars represent the standard error of the mean.

3.3.4. Tent experiments response of gravid *Anopheles arabiensis* and *Anopheles coluzzii* to grass volatiles

In the five-choice oviposition assay, the overall oviposition preference hierarchy of both *Anopheles* species to the grass volatile extracts was found to be *E. pyramidalis* > *E. stagnina* ≥ *T. latifolia* = *C. papyrus* (Figure 3.9). Water conditioned with volatile extracts of either *E. pyramidalis* or *E. stagnina*, elicited a significantly higher oviposition response than the control in both *An. arabiensis* and *An. coluzzii,* while that of *T. latifolia* elicited a significantly higher response than the control in *An. arabiensis* alone (Figure 3.9). In contrast, the oviposition

response of *An. coluzzii* to water conditioned with volatile extracts of *T. latifolia,* as well as that of *C. papyrus*, was significantly lower than that of the control.

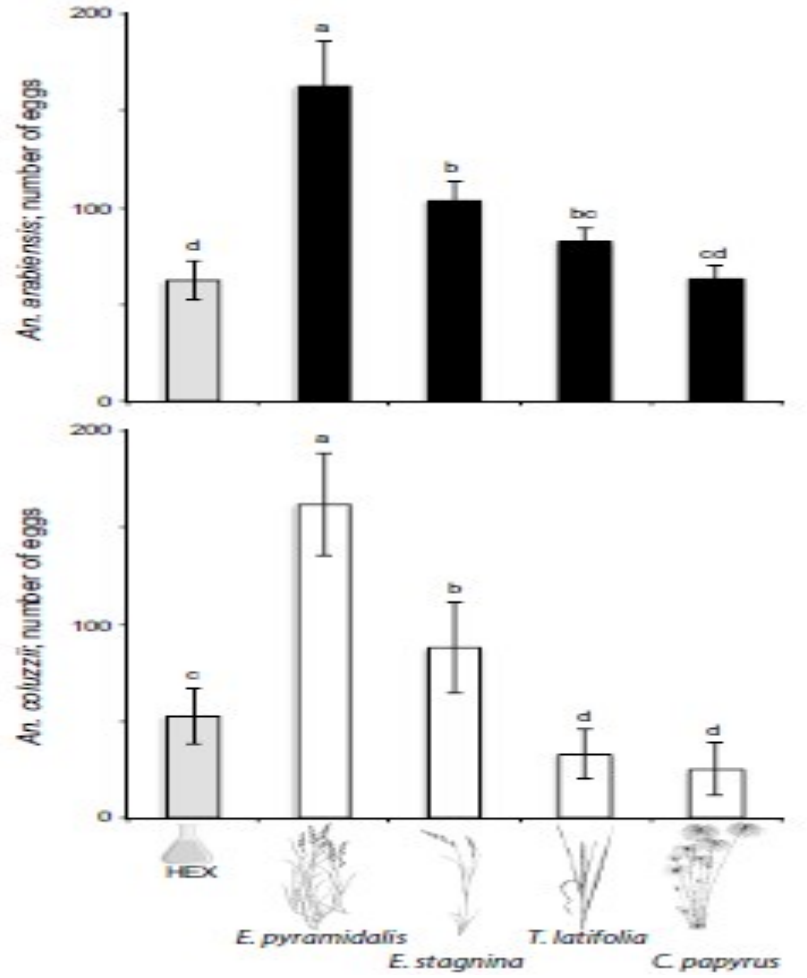

Figure 3.9. Oviposition preference hierarchy of *Anopheles arabiensis* (top) and *Anopheles coluzzii* (bottom) in a multi-choice arena, to water conditioned with volatile headspace extracts of the four grass species, *Echinochloa pyramidalis*, *Echinochloa stagnina*, *Typha latifolia* and *Cyperus papyrus*. Mean numbers of eggs with the same letters indicate no significant difference from one another (nominal logistic fit model, χ^2 and $P < 0.05$ from the likelihood ratio test, significant differences were determined by odds ratio pairwise comparisons). Error bars represent the standard error of the mean.

3.4. Discussion

Field collection of *Anopheles arabiensis* larvae indicated a role for emergent vegetation in the oviposition site selection and survival of malaria mosquitoes. Larval densities were highest in Poaceae-associated habitats, much lower in *Typha*-associated sites and absent from *Cyperus*-associated sites. One potential mechanism regulating the differential distribution of larvae may be an odour-based oviposition site selection preference. Gravid female *Anopheles arabiensis* and *Anopheles coluzzii* were, indeed, found to be differentially attracted to all grass volatile extracts, yet were only stimulated to oviposit on water conditioned with Poaceae volatile extracts. This was further supported by multi-choice oviposition assays revealing that both species demonstrated a preference hierarchy among the grass volatiles, *E. pyramidalis* > *E. stagnina* > *T. latifolia* ≥ *C. papyrus* (Asmare *et al*., 2017). The *Anopheles* larval density variation in grass species associated habitats and differential preferences of gravid females of *Anopheles* mosquitoes to volatiles of the grasses may also reflect the variation between grasses in provision of pollen as sources of nutrients and amount of natural oil production in larval habitats, which may affect larval survival differentially in the various grass-associated habitats.

Emergent vegetation in aquatic habitats are commonly associated with the presence or absence of *An. gambiae/An. coluzzii* and *An. arabiensis* larvae (Fillinger *et al*., 2004; Mwangangi *et al*., 2007; Ndenga *et al*., 2011; Minakawa *et al*., 2012). The observed patterns of association with vegetation are consistent with previous reports (Bøgh *et al*., 2003; Fillinger *et al*., 2004; 2009). In studies in which the vegetation has been characterised, habitats associated with Poacaea generally have a higher *An. gambiae/An. coluzzii* and *An. arabiensis* larval density compared with that of habitats associated with Typhaceae and Cyperacaea (Bøgh *et al*., 2003; Fillinger *et al*., 2004; 2009). While vegetation in these habitats is known to influence characteristics, such as

shading, temperature, water flow, predator abundance and nutrients, they also provide gravid mosquitoes with chemical cues important to habitat selection.

An increasing body of evidence suggests that anopheline mosquitoes make use of olfactory cues as positive indicators for oviposition site selection (Torres-Estrada *et al*., 2005; Lindh *et al*., 2015; Wondwosen *et al*., 2016; 2017). While the focus of this study was not to identify the specific salient volatiles in these attractive and aversive grasses, the behavioural results presented here indicate a strong and robust preference for the headspace extracts of the Poaceae grasses. Interestingly, the majority of the previously identified olfactory cues that drive the oviposition site selection in *An. gambiae s. l.* originate from wild and cultivated grasses of the Poaceae family (Torres-Estrada *et al*., 2005; Asmare *et al*., 2017; Wondwosen *et al*., 2016; 2017). These odours include α- and ß-pinene, 3-carene, caryophyllene, limonene and nonanal (Torres-Estrada *et al*., 2005; Köllner *et al*., 2008; He *et al*., 2010; Lindh *et al*., 2015; Wondwosen *et al*., 2016; 2017), which are not affected by mechanical damage of the plants, but are thought to be constitutively expressed (Xu *et al*., 2002; Yan and Wang, 2006; von Mérey *et al*., 2013). Future work will be aimed at identifying the salient volatiles in the grasses that elicited attraction in gravid anophelines in this study.

The differential preference of gravid *Anopheles* species to the grasses may be due to the nutritive quality variation of grass pollen across species (Schmidt *et al*., 1987; Roulston and Cane, 2000). For example, typha pollen is known to be nutrient poor compared with maize pollen (Schmidt *et al*., 1987; Roulston and Cane, 2000). Nutrients derived from maize pollen have been shown to enhance larval development, adult size and survival (Ye-Ebiyo *et al*., 2000; 20003a).

Females that select larval habitats associated with grasses shedding nutrient rich pollen have the potential to increase their own fitness by providing their offspring with selective advantages, as previously shown for maize pollen. Selective pressures may also be involved in the avoidance of *C. papyrus*-associated habitats by gravid anophelines, as this grass secretes essential oils that create a thin film on the surface of the water, preventing mosquito larvae from breathing (Lindsay and Martens, 1998; Mouchet *et al*., 1998).

3.5. Conclusions

The demonstrated hierarchical preference of gravid *An. coluzzii* and *An. arabiensis* for grasses indicates that vegetation types associated with larval habitats are instrumental in the oviposition choice of the malaria mosquitoes. Current and future analysis of *Anopheles* oviposition ecology associated with grasses is likely to provide key larval habitat characteristics that may be integrated into current methods of larval control. Also, identifying volatile cues from grasses that modulate gravid malaria mosquito behaviours has distinct potential for the development of tools to be used in future monitoring and control methods.

Chapter 4. Grass Pollen Affects Survival and Development of Larval *Anopheles arabiensis* Patton

4.1. Introduction

Nutrients in breeding habitats are essential for the survival and development of mosquitoes (Araújo *et al*., 2012; Takken *et al*., 2013; Moller-Jacobs *et al*., 2014; Shapiro *et al*., 2016). Nutrition during larval development determines the size and metabolic reserves of adult mosquitoes upon emergence (Merritt *et al*., 1992; Ye-Ebiyo *et al*., 2000; 2003a; Kivuyo *et al*., 2014; Moller-Jacobs *et al*., 2014). In addition, and in relation to the body size, the number of eggs produced by female mosquitoes is governed by the size of the preceding blood meal (Lyimo and Takken, 1993; Ameneshewa and Service, 1996; Roitberg and Gordon, 2005). Consequently, the paucity of knowledge concerning the natural diet of malaria mosquito larvae affects efforts to understand the dynamics of vector populations, and thus to effectively plan management programs in sub-Saharan Africa (Merritt *et al*., 1992; Kebede *et al*., 2005; Garros *et al*., 2008).

The most productive natural larval habitat types for *Anopheles arabiensis* Patton, a primary vector of malaria in sub-Saharan Africa, are transient puddles (Ndenga *et al*., 2011), often found amidst wild and domesticated grasses (Bøgh *et al*., 2003; Ye-Ebiyo *et al*., 2003a; Minakawa *et al*., 2004; Mwangangi *et al*., 2007; Fillinger *et al*., 2009; Mwangangi *et al*., 2010; Gouagna *et al*., 2012). Grasses are potential sources of mosquito larval nutrients, derived directly from shed pollen (Ye-Ebiyo *et al*., 2000; 2003a), and indirectly from accumulated detritus and associated microorganisms (Merritt *et al*., 1992; Garros *et al*., 2008). The importance of grasses on the nutritional ecology of malaria mosquitoes is reflected in that gravid *An. arabiensis* are attracted by and discriminate among grass-associated habitats (Wondwosen *et al*., 2016; 2017; Asmare *et*

al., 2017a), in which they lay their eggs and thereby provide their offspring with access to these resources. The pollen from maize enhances larval development, vector density and mosquito longevity (Ye-Ebiyo *et al*., 2000; 2003a). Although feeding on maize pollen is a recent adaptation for *An. arabiensis* (Ye-Ebiyo *et al*., 2000; 2003a), their larvae readily ingest whole pollen grains, suggesting that this behaviour has evolved from feeding on pollens of other grasses which occur in and close to breeding sites as grass pollens are carried by wind to arrive larval habitats.

Particle size is a major factor influencing mosquito larval diet. Mosquito larvae are limited in the particulate matter that they ingest, with the maximum size reported being 50 µm, and most studies reporting that smaller particles are more highly represented (Merritt *et al*., 1992). Morphology and physiology associated with the mouthparts of the larvae limit the size of the diet particle and regulate their feeding behaviors to optimize ingestion and nutrient assimilation rates (Merritt, 1987; Merritt *et al*., 1992).

One of the important nutrient requirements of an insect for its survival and development is the carbon: nitrogen ratio of a diet (Raubenheimer and Simpson, 1995; 1999; Patt *et al*., 2003; Mayntz *et al*., 2005; Raubenheimer *et al*., 2009). While proteins have been found to be essential for growth in mosquito larval diets, carbohydrates have not, although carbohydrates greatly increase larval growth rate within an optimal range (Sneller and Dadd, 1981; Timmerman and Briegel, 1999).

The carbohydrate and protein content of pollen have been the focus of many studies, predominantly in the context of plant-animal interactions (Roulston *et al*., 2000). Pollen is a major food source for many flower visiting insects, such as honey bees and pollen beetles (Cook

et al., 2004; Keller *et al*., 2005). The variable nature of the protein content of pollen among plants (15-50 % of the dry mass) influences plant-insect interactions, with a tendency for insect-pollinated plants to produce pollen richer in protein content (Roulston *et al*., 2000). The protein content of grass pollens investigated, including Cyperaceae, *Typha* and Poaceae, ranges from 17 to 25 % of the dry mass (Roulston *et al*., 2000). Whether the protein content of *Zea mays* pollen is a key driver of the observed enhanced development and survival of *An. arabiensis* larvae (Ye-Ebiyo *et al*., 2000; 2003a), or if carbohydrate content influences these processes, is yet unknown.

In this study, the association between grasses, particularly their pollen, and the malaria vector *An. arabiensis* is further characterized in order to assess the impact of pollen quality on larval development and survival, and thereby infer the effect of a pollen-containing diet on vector dynamics and reproduction. The nutrient composition of grass pollen, specifically the carbohydrate to protein ratio, and pollen grain size, in *Typha latifolia*, *Echinochloa pyramidalis*, *Pennisetum setaceum*, and *Zea mays* differentially affected larval performance. The potential evolutionary impact of the observed association is discussed.

4.2. Materials and Methods

4.2.1. Pollen collection

Pollen was collected from broadleaf cattail, *T. latifolia* L. (Typhaceae), and Antelope grass, *E. pyramidalis* Lam. (Poaceae), at the edge of the shore area of Lake Tana (Chapter 3, section 3.2.1 above), from crimson fountain grass, *P. setaceum* Forssk. (Poaceae), in the adjacent inland area of the lake, and from maize, *Z. mays* L. (BH660 cultivar; Poaceae), in the farm land area of Merawi (11°30′ N, 37°15′ E). The pollens were collected by covering mature flowers with a paper bag and then agitating. The estimated weight of pollen released by individual

inflorescences of *T. latifolia*, E. *pyramidalis*, *P. setaceum*, and *Z. mays* was ca. 0.100 mg, 0.020 mg, 0.025 mg, and 1.500 mg, respectively. The collected pollen was sterilized within 24 h of collection using UV light overnight for ca. 12 h, and then stored on silica gel until used for further experiments. In total, approximately 10 g of pollen grains were collected from each species (Figure 4.1).

Figure 4.1. Store of pollen collected from study grass species associated with *Anopheles* breeding habitats: *Typha latifolia*, *Echinocloa pyramidalis*, *Pennisetum setaceum*, and *Zea mays*.

4.2.2. Pollen nutrient analysis

A combustion CHNS/O analyzer (Flash 2000, Thermo Fisher Scientific, Waltham, MA, USA) was used to determine the carbohydrate and protein content of the grass pollen, according to the manufacturer's protocol. Approximately 5 mg of sample pollen from each grass species was enclosed in tin capsules (8 mm × 5 mm). The capsule was inserted into the combustion chamber using the auto-sampler at a temperature of 1050 ºC. In the presence of a catalyst and in a high

oxygen environment, the temperature was elevated to 1800 °C and all carbon and nitrogen in the samples were oxidized forming carbon dioxide and NO_x.

The gasses were carried by helium through a reduction column where NO_x was reduced to N_2. The gasses were then transported to a gas chromatograph column (CHNS separation column, Thermo Fisher Scientific), where N_2 and CO_2 were separated and then detected by a thermal conductivity detector (TCD). The signal was transferred from the detector via an A/D converter to a computer and analyzed (Eager Exerience, version 1.2, Thermo Fisher Scientific). The nitrogen values were converted to crude protein by a multiplier of 6.25 (Roulston et al., 2000). Thereafter, the C: N ratio of the grass pollen was determined by % C / % N × 6.25.

4.2.3. Pollen grain size analysis

The pollen grain sizes were measured according to Roulston *et al*. (2000). Pollen grains were mounted in paraffin oil and measured at 400 × with an ocular micrometer. Polar axis measurements were taken for all pollen grains. Volumes were calculated using volume equations for spheres ($4/3\pi p^3$). Ten randomly selected grains per slide were measured and these values were averaged for calculating volumes.

4.2.4. Mosquito rearing

Anopheles arabiensis were maintained at 27 ± 2 °C, 80 % relative humidity and at a 12 h: 12 h light: dark photoperiod in the insectary of the Ethiopian Public Health Institute (EPHI), Addis Ababa. Larvae were reared in plastic trays (22 cm × 34 cm × 10 cm) filled with 1 l distilled water and fed powdered Phoenix Koy Pellets (Armitage Pet Products Ltd., Nottingham, UK) daily. Pupae were removed from the rearing trays and placed in insect cages (30 cm × 30 cm ×

30 cm) for adult emergence. Adult males and females were kept together and provided *ad libitum* access to 10 % sucrose solution. For colony maintenance, female mosquitoes were blood fed on a live rabbit at 3- to 4-day intervals. Ethical approval for feeding on live rabbits was obtained from the EPHI Research Ethical Review Committee (EPHI, 2017; FMST, 2014). Eggs were laid in 30 ml plastic cups filled with distilled water, and then transferred to larval trays for hatching. The 3rd instar larval stages of the *An. arabiensis* mosquitoes were used at the start of all experimental bioassays.

4.2.5. Larval development and survival analyses

Development and survival of individual *An. arabiensis* larvae in 150 ml plastic cups (Dongyang City Plastics Co., Shanghai, China) filled with 50 ml of distilled water (Figure 4.2) were investigated using artificial and pollen-containing larval diets. To determine the baseline for comparison across pollen and pollen supplemented diets, the ED_{50} denoting the effective dose for 50 % survival to adulthood on the artificial diet, TetraMin® fish food (Tetrawerke, Melle, Germany), was established using 0.50 mg, 0.75 mg, 1.00 mg and 1.50 mg of the TetraMin® alone (Asmare *et al*., 2017b).

The ED_{50} (0.75 mg) per individual larva in an experiment cup was then used as the dose for each pollen diet, as well as for the TetraMin® control (Appendices A and B). A second control, a double dose (1.50 mg) of the TetraMin® fish food, was also included in the pollen-supplemented diet experiments to ensure one diet with 100 % survival to adulthood. Two sets of experiments were done to determine the dietary effect of the grass pollen alone and the supplementation of the ED_{50} of a standard larval diet with pollen on survival and development of larval *An. arabiensis*. For all experiments, 10 replicates were performed on three separate occasions.

Treatment and control cups were paired, to buffer for any day effect. The number of larvae surviving, pupating and emerging as adults were recorded each day until all larvae had either died or emerged. Of the adults, the number of male and female that emerged were also recorded.

Figure 4.2. Bioassay setup to test the performance of larval *An. arabiensis* with grasses pollen diets.

4.2.6. Data analyses

The C: N ratios and pollen grain sizes were determined to meet the assumption for normal distribution, prior to analysis using a univariate general linear model (GLM; IBM SPSS Statistics for Windows, Version 21.0. Armonk, NY: IBM Corp.). Significant differences between C: N ratios and pollen grain sizes were determined at $\alpha = 0.05$ and *post hoc* comparisons among the grass pollens were made using Tukey's HSD test.

A fully parametric form of survival analysis was used to model the data and to produce a measure of the probable rate of larval mortality, pupation, and adult emergence in 3rd instar *An. arabiensis* larvae fed on treatment and control diets (JMP® Pro 12.0.1. SAS Institute Inc., Cary, NC, USA). The model was based on the Weibull distribution. For both pollen alone diets and supplemented diets, an overall model of the effects of both sex and diet on larval development was performed for pupation and adult emergence; censoring those that died before the end of the experiment. Similarly, an overall model of the effect of diets on larval survival was also performed; censoring those that emerged before the end of the experiment. The effect of sex on larval survival was not assessed. Wald's test was used to determine significant differences between diets based on χ^2 and *P*-values. *A priori* pairwise analyses of the probability models, using non-linear variable slope curve fits (Hill slope, top and bottom of the curve) based on AICc and percent probabilities, were conducted among the pollen diets and the TetraMin® controls (0.75 mg and 1.50 mg, respectively; Prism, version 5a, GraphPad Software, Inc, La Jolla, CA, USA).

4.3. Results

4.3.1. Pollen grain size and nutrient content

All pollen grains were spherical in shape, with smooth surfaces. The pollen grain volume differed significantly between grass species (Figure 4.3A), with *Zea mays* (mean diameter 92.2 ± 1.65 µm) > *Pennisetum setaceum* (47.1 ± 0.57 µm) > *Echinocloa pyramidalis* (42.8 ± 0.84 µm) > *Typha latifolia* (26.5 ± 0.34 µm) ($F = 1033$, $df = 3$, $P < 0.0001$). The C: N ratios of pollen differed between grass species with the pollen C: N ratios of *T. latifolia* > *Z. mays* = *P. setaceum*

> *E. pyramidalis* (Figure 4.3B). *Typha latifolia* had a higher proportion of carbon (52 %), compared with the three other species (44 %).

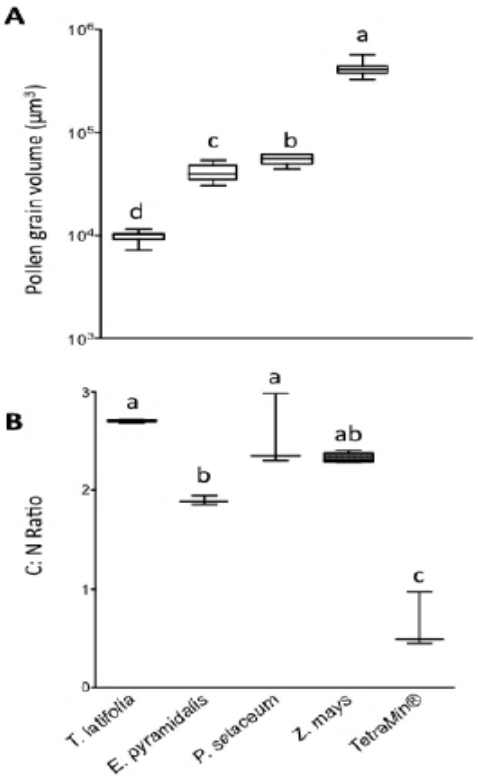

Figure 4.3. Size (A) and carbon to nitrogen (C: N) ratios (B) of *Typha latifolia, Echinochloa pyramidalis, Pennisetum setaceum* and *Zea mays* pollen are described in box plots (whiskers ± maximum and minimum values). The C: N ratio of the TetraMin® control is also shown. Means with different letter designations are significantly different from one another (ANOVA with Tukey's HSD *post-hoc* analysis; $P < 0.05$).

4.3.2. *Anopheles* larval survival

The overall larval survival differed significantly among the diets tested (ANOVA $F = 9.235$, df = 9, $P < 0.0001$). *Post hoc* pairwise comparisons revealed that larval survival on the pollen alone diets was lower than that on the 2 × ED_{50} TetraMin® diet (Figure 4.4A). Larval survival on the

pollen supplemented diets was also lower than the 2 × ED_{50} TetraMin® diet, with one exception. The *Z. mays* pollen supplemented diet did not differ from either the 2 × ED_{50} TetraMin® diet or the other pollen supplemented diets, but the larval survival was significantly higher than on the other pollen alone diets (Figure 4.4A). Interestingly, both *Z. mays* pollen diets did not differ from each other (Figure 4.4A).

There was no overall effect of diet on the probability of larval survival over time in *An. arabiensis* when fed on any of the treatments (1.50 mg) (Wald's $\chi^2 = 2.5808$, $df = 4$, $P = 0.6302$; Figure 4.4B). However, in subsequent pairwise comparisons among each pollen diet and the TetraMin® control, *T. latifolia* pollen was shown to significantly decrease the probability of survival (AICc = 2.935, $P = 81.26$ %), while the probability of survival on the other pollen diets did not differ from the control. *Zea mays* pollen was shown to significantly increase the probability of survival compared to each of the other grass pollens (*E. pyramidalis*, AICc = 16.66, $P = 99.98$ %; *P. setaceum*, AICc = 16.01, $P = 99.97$ %; *T. latifolia*, AICc = 18.89, $P = 99.99$ %). Moreover, the probability of larvae dying when the basic diet, TetraMin® (0.75 mg), was supplemented with pollen (0.75 mg), was found to be significantly different from the overall model ($\chi^{2=}19.14$, $df = 4$,$P = 0.0007$; Figure 4.4C). All of the larvae fed on the 2 × ED_{50} TetraMin® diet survived to adulthood, *i.e.,* larvae had a longer probable life expectancy when feeding on 2 × ED_{50} TetraMin® diet than on grass pollen supplemented diets (*E. pyramidalis*, AICc = 30.28, $P > 99.99$%; *P. setaceum*, AICc = 46.80, $P > 99.99$ %; *T. latifolia*, AICc = 38.17, $P > 99.99$ %; *Z. mays*, AICc = 26.77, $P > 99.99$ %; Figure 4.4C). Only larvae fed on *P. setaceum* supplemented diets were found to have a shorter probable life expectancy than those fed on the 0.75 mg TetraMin® diet (AICc = 22.20, $P > 99.99$ %).

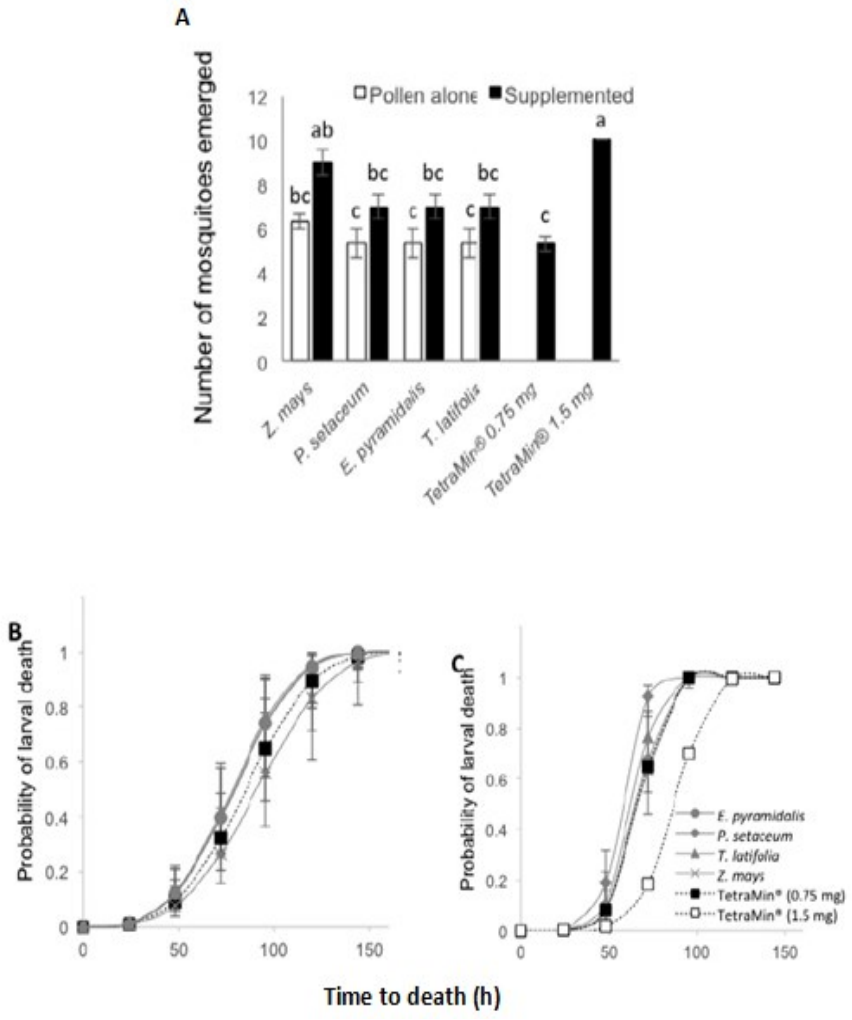

Figure 4.4. The survival to adulthood of *Anopheles arabiensis,* presented as number of mosquitoes emerged (A), and the probable life expectancy of the larvae in response to *Echinochloa pyramidalis*, *Pennisetum setaceum, Typha latifolia* and *Zea mays* pollen alone (B) and pollen supplemented TetraMin® fish food (C) diets. Control diets include the effective dose for 50 % survival (ED_{50}) and 2 × ED_{50} TetraMin® fish food (0.75 mg and 1.5 mg, respectively). The error bars represent the standard error of the mean (A) and whiskers represent the upper and lower limits of variation (B, C).

4.3.3. Effect of pollen on pupation of *Anopheles arabiensis*

The overall model analysis of the effect of grass pollen diets on developmental time from 3rd instar to pupation in *An. arabiensis* showed significant differences between the diets ($\chi^{2=}$ 90.31, df = 14, $P < 0.0001$), with both food ($\chi^{2=}$ 12.92, $df = 4$, P=0.0117) and sex ($\chi^{2=}$ 69.41, $df = 2$, $P < 0.0001$) contributing to the significant deviation from the overall model. As there was no interaction between food and sex ($\chi^{2=}$ 4.723, $df = 9$, $P = 0.7969$), each of these factors were analyzed separately.

This analysis showed that female larvae fed on pollen had a higher probability of a reduced developmental time between 3rd instar and pupation compared to when they were fed on fish food ($\chi^{2=}$ 15.21, $df = 4$, $P = 0.0043$; Figure 4.5A); most 3rd instar female larvae (70-80%) fed on pollen generally pupated by 48 h after the start of the experiment whereas only ca. 30% of the 3rd instar female larvae fed on the fish food were likely to have pupated within this time frame. Moreover, larvae fed on each of the pollens were found to have a take less time from 3rd instar to pupation compared to those fed on the 0.75 mg fish food diet (*E. pyramidalis*, AICc = 84.08, $P > 99.99$ %; *P. setaceum*, AICc = 81.53, $P > 99.99$ %; *T. latifolia*, AICc = 69.33, $P > 99.99$ %; *Z. mays*, AICc = 77.28, $P > 99.99$ %). In contrast, the males maintained a consistent developmental time from 3rd instar to pupation in response to all diets ($\chi^{2=}$ 5.225, $df = 4$, $P = 0.2650$; Figure 4.5C).

When pollen was supplemented on top of the basic fish food diet, the overall model analysis of the effect of combination diets on the development time from 3rd instar to pupation in the mosquito showed significant differences between diets ($\chi^{2=}$ 89.71, $df = 16$, $P < 0.0001$), however there was no significant deviation from the overall model by either food ($\chi^{2=}$ 6.138, $df = 5$,

$P = 0.2930$) or sex ($\chi^{2} = 4.199$, $df = 2$, $P = 0.1225$). No effect of the interaction between food and sex was observed ($\chi^{2} = 11.43$, $df = 9$, $P = 0.2473$). Thus, for combination diets, both females ($\chi^{2} = 9.056$, $df = 4$, $P = 0.1068$; Figure 4.5B) and males ($\chi^{2} = 1.980$, $df = 4$, $P = 0.8520$; Figure 4.5D) maintained a consistent developmental time from 3rd instar to pupation in response to all diets.

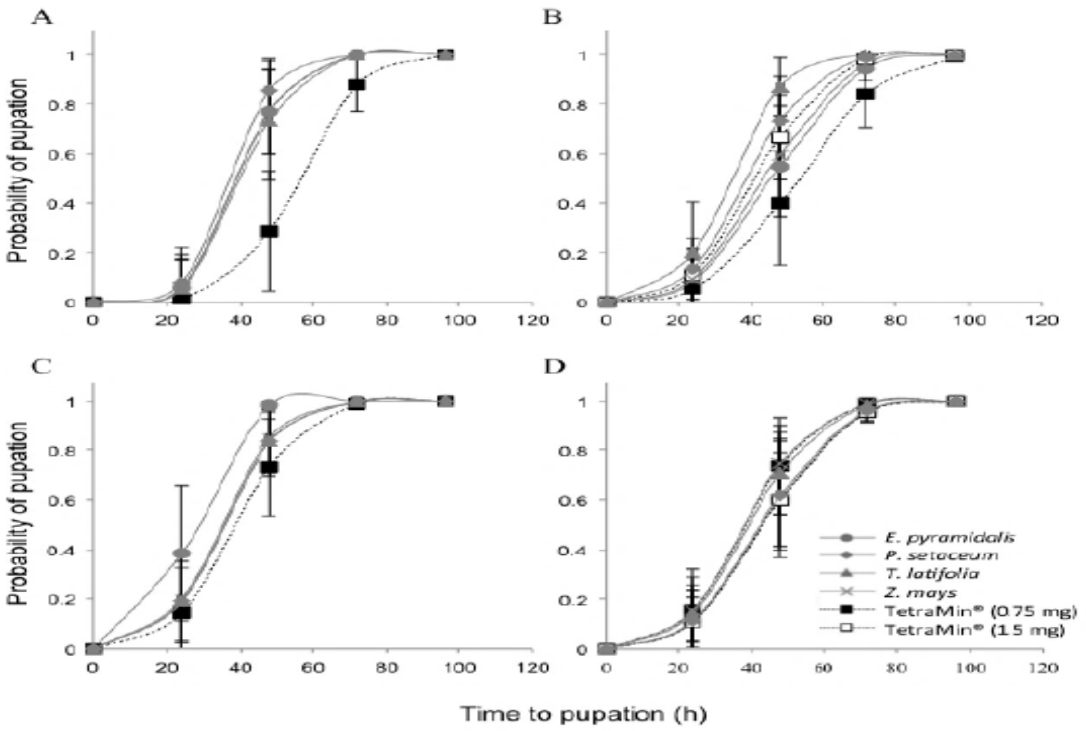

Figure 4.5. The probability of pupation of female (A, B) and male (C, D) mosquitoes over time in response to *Echinochloa pyramidalis*, *Pennisetum setaceum, Typha latifolia* and *Zea mays* pollen alone (A, C) and pollen supplemented TetraMin® fish food (B, D) diets. Control diets include the effective dose for 50 % survival (ED_{50}) and 2 × ED_{50} TetraMin® fish food (0.75 mg and 1.5 mg, respectively). The whiskers represent the upper and lower limits of variation.

4.3.4. Effect of grass pollen in the larval diet on adult emergence

The overall model analysis of the effect of grass pollen diets on development time from pupation to emergence in *An. arabiensis* revealed significant differences between the diets ($\chi^{2=}$24.34, *df* = 14, *P* = 0.0417) with sex contributing significantly to the deviation from the overall model ($\chi^{2=}$15.90, df = 2, *P* = 0.0004), whereas there was no effect of food (χ^{2} =2.53, *df* =4, *P* = 0.6396).

As there was no interaction between food and sex ($\chi^{2=}$4.367, *df* = 8, *P* = 0.8226), each of these factors were analyzed separately. This analysis showed that both female ($\chi^{2=}$4.224, *df* = 4, *P* = 0.3765; Figure 4.6A) and male ($\chi^{2=}$2.870, *df* = 4, *P* = 0.5798; Figure 4.6C) mosquitoes maintained a consistent developmental time from pupation to emergence in response to all diets. Subsequent pairwise analyses revealed that there were no differences in the probability of time to emergence among the pollen diets within each sex (*P* > 0.05). This indicates that the males were emerging as adults sooner than the females, regardless of the diet ingested. Similarly, the overall model analysis of the effect of pollen supplemented diets on the development time from pupation to emergence in the mosquito revealed significant differences between diets ($\chi^{2=}$24.08, *df* = 14, *P* = 0.0449) with sex contributing significantly to the deviation from the overall model ($\chi^{2=}$8.462, *df* = 2, *P* = 0.0145), whereas there was no effect of diet ($\chi^{2=}$5.921, *df* = 4, *P* = 0.2051).

There was no interaction found between food and sex for pollen supplemented diets ($\chi^{2=}$9.022, *df* = 8, *P* = 0.3404). When analyzed separately, the probability of adult emergence in females fed on pollen supplemented diets differed significantly when compared to the overall model ($\chi^{2=}$15.16, *df* = 5, *P* = 0.0097; Figure 4.6B), whereas that of the males did not ($\chi^{2=}$3.993, *df* = 5,

P = 0.5504; Figure 4.6D). Pairwise comparisons among the grass pollens indicated that *T. latifolia* pollen supplemented with Tetramin® emerged as adult females faster than *E. pyramidalis* (AICc = 0.2315, P = 52.89 %) and *Z. mays* (AICc = 0.03921, P = 50.49 %).

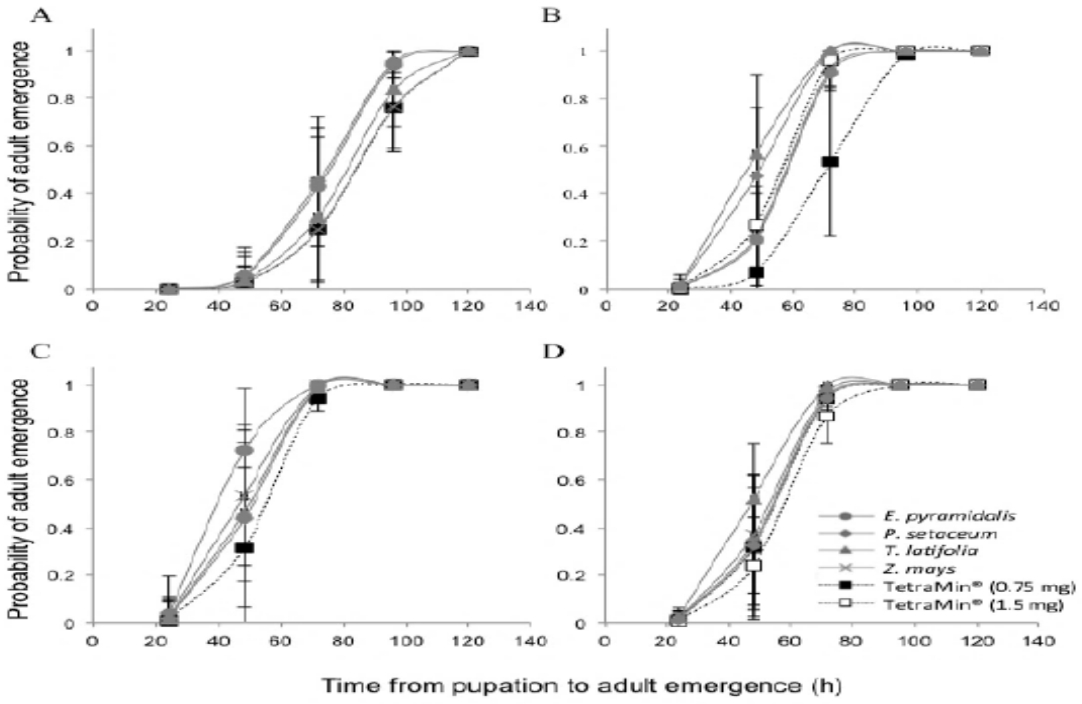

Figure 4.6. The probability of adult emergence of female (A, B) and male (C, D) mosquitoes over time in response to *Echinochloa pyramidalis*, *Pennisetum setaceum, Typha latifolia* and *Zea mays* pollen alone (A, C) and pollen supplemented TetraMin® fish food (B, D) diets. Control diets include the effective dose for 50 % survival (ED_{50}) and 2 × ED_{50} TetraMin® fish food (0.75 mg and 1.5 mg, respectively). The whiskers represent the upper and lower limits of variation.

4.4. Discussion

Pollen is a nutrient rich food source, enhancing growth, development and survival for larvae from many insect orders (Cook *et al*., 2004; Keller *et al*., 2005). Here we show that grass pollen in the diet of *Anopheles arabiensis* larvae significantly affected larval survival, as well as the

time to pupation and adult emergence in females. In line with Ye-Ebiyo *et al*. (2000; 2003a) and Kivuyo *et al*. (2014), *Zea mays* pollen significantly increased larval survival, both alone (equal weight of TetraMin® fish food ED_{50}) and in combination diets (equal weight of TetraMin® fish food $2\times ED_{50}$), compared to the other wild grass pollens alone and combination diets tested. Among the wild grass pollen combination diets *Pennisetum setaceum* pollen demonstrating the shortest time to larval death, the others were able to maintain a similar rate of survival in the larvae (Asmare *et al*., 2017b). TetraMin® fish food $2\times ED_{50}$ demonstrates the highest overall larval survival to adult hood equivallent as in combination diet of *Z. mays* pollen.

Interestingly, time to pupation decreased in females, but not in males that fed on pollen alone when compared to the TetraMin® diet; an effect that was not observed when the pollen was supplemented with TetraMin®. Time to adult emergence in females was differentially affected by feeding on the different pollens, with mosquitoes feeding on *Z. mays* and *Echinocloa pyramidalis* taking a significantly longer time to emerge compared with those feeding on *Typha latifolia* (Asmare *et al*., 2017b). This is in line with previous studies, in which it is demonstrated that male anophelines develop faster and emerge sooner than females (Lehmann *et al*., 2006; Mwangangi *et al*., 2006).

Previous studies indicate that the amount of carbon in the diet of mosquito larvae does not influence the overall survival, but does increase the growth rate (Sneller and Dadd, 1981; Grieco *et al*., 2007). In support of this, the larvae fed on the pollen of all the wild grass species had similar ultimate survival, however *T. latifolia* and *P. setaceum*, those containing the higher relative amounts of carbon, demonstrated shorter times to pupation and adult emergence, and in the case of *P. setaceum*, death.

In line with other studies in mosquitoes (Golberg and De Meillon, 1948; Sneller and Dadd, 1977; 1981; Kivuyo *et al*., 2014), larvae fed on the diet containing higher amounts of nitrogen, in our case TetraMin® fish food (Timmerman and Briegel, 1999; Dennis *et al*., 2010), demonstrated the highest larval survival, but were slower to develop to pupae and to emerge as adults. Thus, these findings indicate that the balance between carbon and nitrogen in the diet plays a significant role in the development and survival of *An. arabiensis* larvae, above and beyond the contribution of each nutrient alone, which is in line with that which has been reported in other insects (Raubenheimer and Simpson 1997; 1998; 1999; Raubenheimer *et al*., 2009).

However, other pollen characteristics, such as size, may also play a role in regulating development and survival. *Zea mays* has a carbon to nitrogen ratio that is similar to *T. latifolia* and *P. setaceum*, however the demonstrated growth rate of larvae fed on this diet was prolonged. This is likely due to the larger size of the *Z. mays* pollen grains, which may reduce the rate of access to the nutrients contained within (Pucat 1965; Merritt *et al*., 1978). However, while *Z. mays* demonstrates a C: N ratio and a pollen size that appear to be that of a lower quality diet (Merritt *et al*., 1992; Young *et al*., 2014), the combination of these factors act synergistically, enhancing the development and survival of the malaria mosquito larvae (Asmare *et al*., 2017b).

Breeding habitats close to maize plantations, where the common food sources, such as detritus and microorganisms, are supplemented with shed pollen, have been shown to increase the survival and development of larval *An. arabiensis*, resulting in increased larval abundance (Ye-Ebiyo *et al*., 2000; 2003a). In addition, the presence of *Z. mays* pollen in the larval diet enhances the size of pupae and emerged adults, as well as prolonging adult survival (Ye-Ebiyo *et al*., 2000; 2003a). This prolific nutrient source provides an abundant source of nitrogen in an

otherwise nitrogen resource poor environment, as the C: N ratios of microorganisms and detritus range from 6 to 9 (Redfield, 1963; Hamilton *et al*., 1992; Baird and Middleton, 2004).

The association between *An. arabiensis* and maize may have evolved out of the fitness benefits gained from feeding on grass pollen. This association is reinforced by the female *An. gambiae sensu lato* preference to select and oviposit in grass habitats (Wondwosen *et al*., 2016; 2017; Asmare *et al*., 2017a), in fact the odours emanating from grass pollen alone are sufficient to attract and stimulate females to oviposit (Wondwosen *et al*., 2017). We argue that the pre-adaptation to be stimulated by (Ye-Ebiyo *et al*., 2003b) and feed on grass pollen by *An. gambiae sensu lato* has been instrumental in establishing and strengthening the association of malaria mosquitoes with grass crops.

Intensified cultivation of these crops has helped to create suitable conditions for increased vector populations, which are linked to greater malaria transmission (Kebede *et al*., 2005). Continued investigation into the natural diet of malaria mosquito larvae will enhance our ability to construct effective Integrated Vector Management programs and to further intervene and suppress disease transmission and to plan and implement improved vector management programs in sub-Saharan Africa.

4.5. Conclusions

Generally grass pollen provides natural dietary input in larval habitats that increase larval survival and development rate. The C: N ratio and grain size of pollens from grasses are the key traits of larval diets to predict survival and development success of malaria mosquito, *An. arabiensis*. Larval *An. arabiensis* fed nitrogen rich TetraMin® and carbon rich *Z. mays* pollen with larger grain demonstrate slower development than that of the other grasses, carbon rich *T.*

latifolia and *P. Setaceum* pollen with smaller grain size. However, TetraMin® and *Z. mays* pollen fed larvae demonstrae longer larval survival and achieve higher adult hood. This indicates the smallest particle size of a diet increase nutrient access rate that accelerates development. Higher survival of larvae on TetraMin® is by its higher nitrogen content where as, on *Z. mays* pollen could be the synergistical effect of two lower qualities (higher carbon content and larger grain). Current and future analysis of natural diets of larval *Anopheles* is likely to provide key larval habitat characteristics to predict the mosquito population dynamics and malaria transmission.

Chapter 5. Effects of Oil Extracts from *Typha latifolia* and *Cyperus papyrus* on Survival of *An. arabiensis* Larvae

5.1. Introduction

Grasses are among the biological characteristics of breeding habitats which determine the abundance of larval stages of *Anopheles* mosquitoes and adult productivity (Minakawa *et al*., 2004; 2012; Yohannes *et al*., 2005; Munga *et al*., 2006b; Mushinzimana *et al*., 2006; Vanwambeke *et al*., 2007; Fillinger *et al*., 2004; 2009; Mwangangi *et al*., 2008; Ndenga *et al*., 2011; 2012; Rejimankova *et al*., 2013). The larvae of *Anopheles coluzzii* and *Anopheles arabiensis* are usually found in breeding habitats with cattails, *Typha spp.* (Typhaceae) and dallis grasses (Paspalum *spp.*; Poaceae) (Bøgh *et al*., 2003; Gouagna *et al*., 2012; Khater *et al*., 2013). On the other hand, the probability of finding anopheline larvae in breeding habitats with reeds such as *Cyperus spp.* (Cyperaceae) and *Phragmites spp.* (Poaceae) grasses is low (Goma, 1960a; 1960b; Fillinger *et al*., 2004; Yohannes *et al*., 2005). This is attributed to the natural oils produced by these grass species which have larvicidal effects (Lindsay and Martens, 1998; Mouchet *et al*., 1998).

In line with the above findings recently *Typha latifolia* (Typhaceae) dominated sites at littoral zone of southern edge of Lake Tana are found with less *Anopheles* mosquito larvae than breeding sites with Poaceae grasses including *Echinochloa pyramidalis* and *Echinochloa stagnina,* and the habitats dominated by *Cyperus papyrus* (Cyperaceae) are found without any *Anopheles* mosquito larvae. Moreover, the gravid females of *Anopheles arabiensis* and *Anophele coluzzii* show lower attraction and oviposition preferences to volatiles of *T. latifolia* and *C. papyrus* in contrast to volatiles of *E. pyramidalis* and *E. stagnina* (Asmare *et al*., 2017a).

Identification of aquatic grass species which have negative effect on breeding success of *Anopheles* mosquitoes is crucial to address the problems in malaria vector interventions (Walton, 2005). Larval control contributes to suppress the populations of *Anopheles gambiae* and *Anopheles arabiensis* and reduce malaria transmission in Africa (Walker and Lynch, 2007), and incorporates source reduction and utilization of chemical and biolarvicides as components of integrated malaria vector management (Yohannes *et al*., 2005; Bukhari *et al*., 2013; WHO, 2006). However, these larval control options have limitations including slow activities of entopmopathogens and development of insecticide resistance in malaria mosquitoes, as well as development of resistance to the toxins of *Bacillus thuringiensis israelensis* and *Bacillus sphaericus* (Lacey, 2007; Wirth, 2010; Cory and Franklin, 2012; Kamareddine, 2012). Moreover, the use of conventional insecticides to target larval stage of mosquitoes introduces risks of toxicity to humans and other non-target organisms in the environment (Amer and Mehlhorn, 2006). The use of plant extracts for mosquito larval control is more ecologically sound and sustainable than the conventional insecticides and the bacterial toxins, and therefore, can be integrated in vector management to reduce malaria transmission.

The factors underlying the relatively lower *Anopheles* larval densities in habitats with *T. latifolia* and the absence of larvae in those with *C. papyrus* necessitate further studies on natural oil content of these grasses and bioactivity of their oil extracts on larval survival in *Anopheles* mosquitoes. Therefore, this study was conducted to evaluate the effects of different concentrations of natural oils extracted from plant parts of the macrophyte species *T. latifolia* (Typhaceae) and *C. papyrus* (Cyperaceae) on survival of larval *An. arabiensis* under laboratory conditions and to assess association of these effects with the amount of natural oil production from the different parts of these macrophytes.

5.2. Materials and Methods

5.2.1. Test plant oil extraction procedures

Natural oils from rhizome and aerial parts of *Typha latifolia* and *Cyperus papyrus* separately were extracted using solvent extraction methods. The parts of these plant species (Figure 5.1A, Figure 5.1B, Figure 5.1C, Figure 5.1D) were collected from their natural habitats at the southern edge of Lake Tana, in sites of *Anopheles* larval sampling (Chapter 3, section 3.2.1 above). The rhizome and areal parts of the test plant species were manually sliced to small sizes separately (Figure 5.1E; Figure 5.1F) and left to dry at room temperature (27-31ºC) for about 10 days under shade (Bream *et al*., 2009). The dried slices of plant materials were crushed into powder (Figure 5.1G, Figure 5.1H) using coffee grinder. Crude oil extraction from the plant materials were made in Bahir Dar University, School of Chemical and Food Engineering, Ethiopia using Soxhlet extractor (Figure 5.2) with an organic solvent, *n*-hexane.

About 20g of a given test plant material was added to the Soxhlet extractor apparatus in one run and subjected to solvent extraction for about 4 hrs. The oil extracts were placed in an oven at 40ºC for 2 hrs to remove excess solvent (*n*-hexane) from the extracted oil samples. The oil extracts were weighted and kept in refrigerator (+4ºC) until use for experiments (Bream *et al*., 2009). The oil content of the test plant materials was determined as percentage yield of oil and calculated as the percent of the ratio of weight of oil to dry weight of the plant materials.

Figure 5.1. Plant material collection and preparation for oil extraction procedure: plant material samples including aerial (A) and rhizome (B) parts of *Cyperus papyrus* and aerial (C) and rhizome (D) parts of *Typha latifolia*, slice of aerial part (E) and slice of rhizome part (F), the dried sample plant materials (G) the powder of plant materials (H).

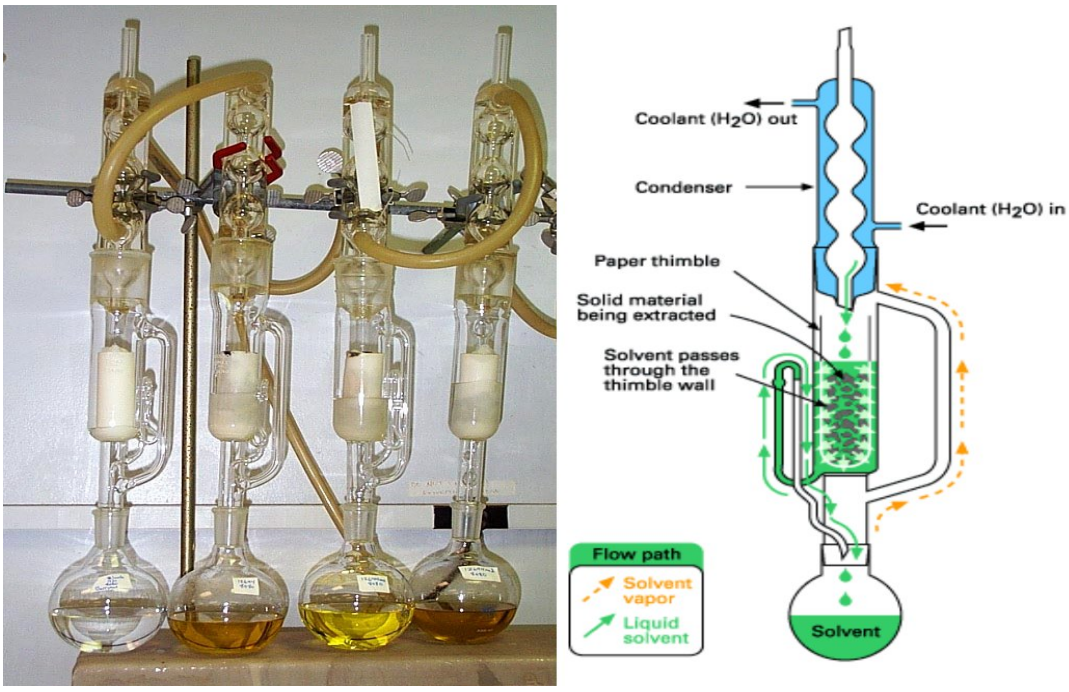

Figure 5.2. Schematic representation of oil extraction apparatus and procedures using Soxhlet extractor (Source: Rassem *et al*., 2016.

5.2.2. Experimental mosquito rearing procedures

Laboratory colonies of *Anopheles arabiensis* were maintained at 27 ± 2 °C, 80 % relative humidity and at a 12 h: 12 h light: dark photoperiod in the insectary of the Ethiopian Public Health Institute (EPHI), Addis Ababa. Larvae were reared in plastic trays (22 cm × 34 cm × 10 cm) filled with 1 l distilled water and fed powdered Phoenix Koy Pellets (Armitage Pet Products Ltd., Nottingham, UK) daily. Pupae were removed from the rearing trays and placed in insect cages (30 cm × 30 cm × 30 cm) for adult emergence. Adult males and females were kept together and provided *ad libitum* access to 10 % sucrose solution. For colony maintenance, female mosquitoes were blood fed on a live rabbit at 3-4 day intervals. Ethical approval for feeding on live rabbits was obtained from the EPHI Research Ethical Review Committee (EPHI, 2017; FMST, 2014). Eggs were laid in 30 ml plastic cups filled with distilled water, and then

transferred to larval trays for hatching. Subsequent identification and separation of each larval stage was made to reduce difficulty for identification of 3rd and 4th stage larvae to transfer the 4th stage larvae from rearing trays to the experimental bioassay cups. The 4th larval stages of *An. arabiensis* mosquitoes were used for experiments to evaluate the bioactivities of the test grass oils.

5.2.3. Bioassays on *Anopheles arabiensis* larval survival with grass oils

Survival of individual *An. arabiensis* larvae in 150 ml plastic cups (Dongyang City Plastics Co., Shanghai, China) filled with 49 ml distilled water were investigated using treatment oils of the grasses and the toxic negative control oil. Initially 10 mg of the crude natural oil extract from aerial and rhizome parts of *Cyperus papyrus* and *Typha latifolia* and the same amount of control oil (refined seed oil of sunflower, *Helianthus annuus*) was dissolved in separate vials in 10 ml of 97% ethanol as solvent (Figure 5.3A). This stock solution of 1 mg oil/ml of solvent (Figure 5.3B) was subjected to a ten-fold serial dilution resulting 0.1 mg/ml, 0.01 mg/ml concentration gradients with a logarithmic scale and used for experiments.

One ml from each concentration gradient of treatment and control oils and 1 ml solvent ethanol (the oil negative control) was directly applied into each cup in the experiment setup (Figure 5.3C). The set of experiment for each treatment and controls with 10 replicates was run on three separate experiment days. Each treatment and control oil at a given concentration gradient with 10 replicates was made on the same day in parallel to buffer day effects. Larval mortality was recorded after 12h, 24h, 48h, 72h and 84h exposure time series and the larvae were maintained without nutritional supplement for this time series in experiment setup.

A 

B 

C 

Figure 5.3. Preparation of oils and experiment cups for bioassay procedure: extracted crude oil (A), oils dissolved in solvent ethanol (B), setup of experiment cups (C).

5.2.4. Statistical analyses

Univariate general linear model (GLM) was applied to analyze the oil content of the grass species and their parts using the statistical software IBM SPSS Statistics for Windows, Version 21.0. Significant differences between means were determined at α=0.05 and post hoc multiple comparisons among the grasses were made using the Tukey's HSD test.

A fully parametric form of survival time to event analysis was used to model the data and to produce a measure of the probability of larval death occurred over time series in *An. arabiensis*

treated with oils over dose series using (SAS (JMP®) Version 10.0.0. SAS Institute University of UK). The model was based on the Weibull distribution for natural oils of the macrophyte species (*Typha latifolia* and *Cyperus papyrus*) and the control refined seed oil of *Helianthus annuus.* Overall model of effects of both type of oils and dose series of oils on larval survival of *An. arabiensis* was run for the time intervals between start of the experiment, i.e. 4th stage larval death as the response (Y) variables; censoring those that emerged before the end of the experiment. Likelyhood ratio test was used to determine significant differences between oils at different doses by their effect on the larval mosquito survival based on χ^2 and *P*-value. Then analysis of time series was made to determine the particular time at which the peak (maximum) larval death resulted by overall effect of oils. Finally univariate general linear model (GLM) was applied to analyze overall larval death by the effect of oils over dose series at the particular time of peak (maximum) larval death using the statistical software IBM SPSS Statistics for Windows, Version 21.0. Significant differences between means were determined at α=0.05 and *post hoc* multiple comparisons among the grasses were made using the Tukey's HSD test.

5.3. Results

5.3.1. Oil contents of Typha latifolia and Cyperus papyrus

The oil contents of the grasses and their parts show some differences (Figure 5.4). The oil content of rhizome part of *Cyperus papyrus* was significantly higher than the oil contents of the aerial part of itself and both rhizome and aerial parts of *Typha latifolia*. The rhizome part of *T. latifolia* was with the lowest oil content of all the plant materials.

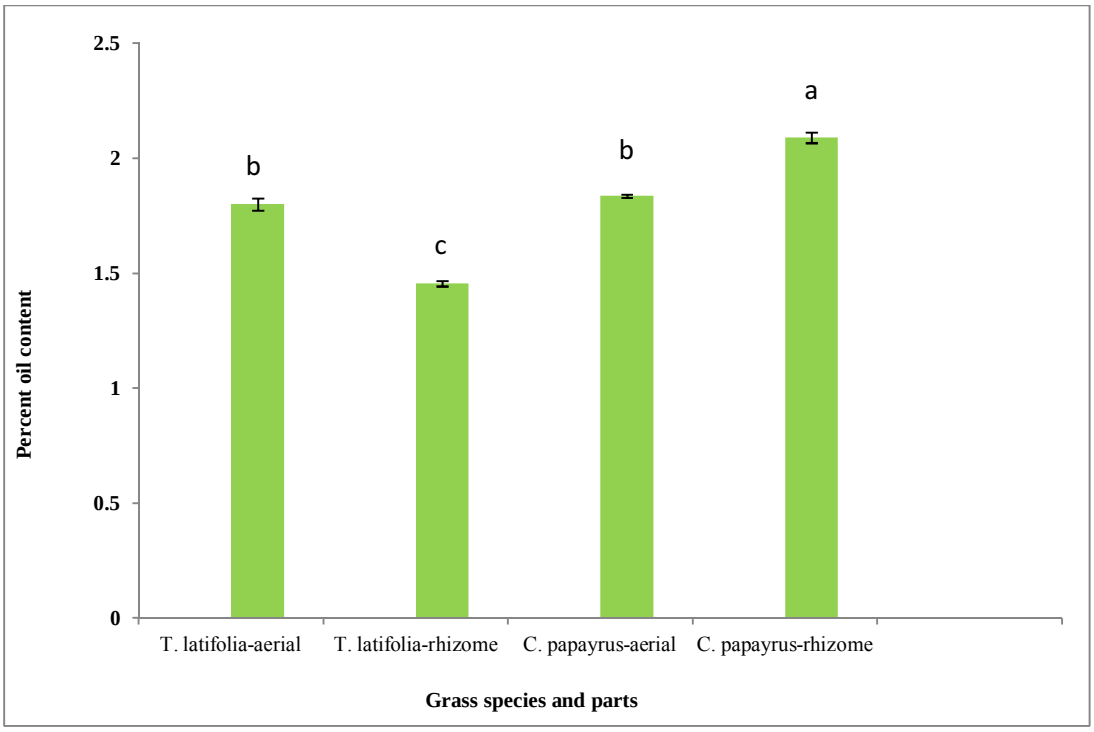

Figure 5.4. Oil contents of grass species and their parts determined as percentage yield of oil which was calculated as the percent of the ratio of weight of oil to dry weight of the plant materials: the oil source grass species are *Typha latifolia* and *Cyperus papyrus.* The parts of the grass species studied separately are aerial and rhizome. The mean oil content with different letters are significantly different from one another (univariate general lenear model with a Tukey's post-hoc analysis; P <0.05). Error bars represent the standard error of the mean.

5.3.2. Effects of oils on survival of larvaae of *An. arabiensis*

There were no overall significant differences between extracted oils from the plant materials, rhizome and aerial parts of *Cyperus papyrus* and *Typha latifolia* as well as the control refined seed oil from *Helianthus annuus* by their effect on survival time of 4th stage larval *An. arabiensis* (DF = 4, χ^2 = 2. 611, P = 0.624). No significant differences between the oils by their increasing effect on survival time of larval *Anopheles arabiensis* over increasing dose series (DF = 4, χ^2 = 0.095, P = 0.998). Significantly higher probabilty of shorter survival time of larval *An.*

arabiensis was shown by increasing the dose of oils (DF = 1, χ^2 = 32. 391, $P < 0.0001$). Based on similar dose dependant effects of all the tested oils on survival time of *An. arabiensis* oil effect analysis over time series was made to find out the time with the peak (maximum) mortality of 4th stage larval *An. arabiensis* and this is 24 h after application of the tested oils on the larvae (Figure 5.5). Further analysis of effect of oil on survival of larvae over dose series at the time of maximum larval mortality (24 h) revealed significantly higher percent mortality of 4th stage larval *An. arabiensis* at 1 ml oil dose than that of 0.1 ml oil, 0.01 ml oil doses and the control ethanol (Figure 5.6).

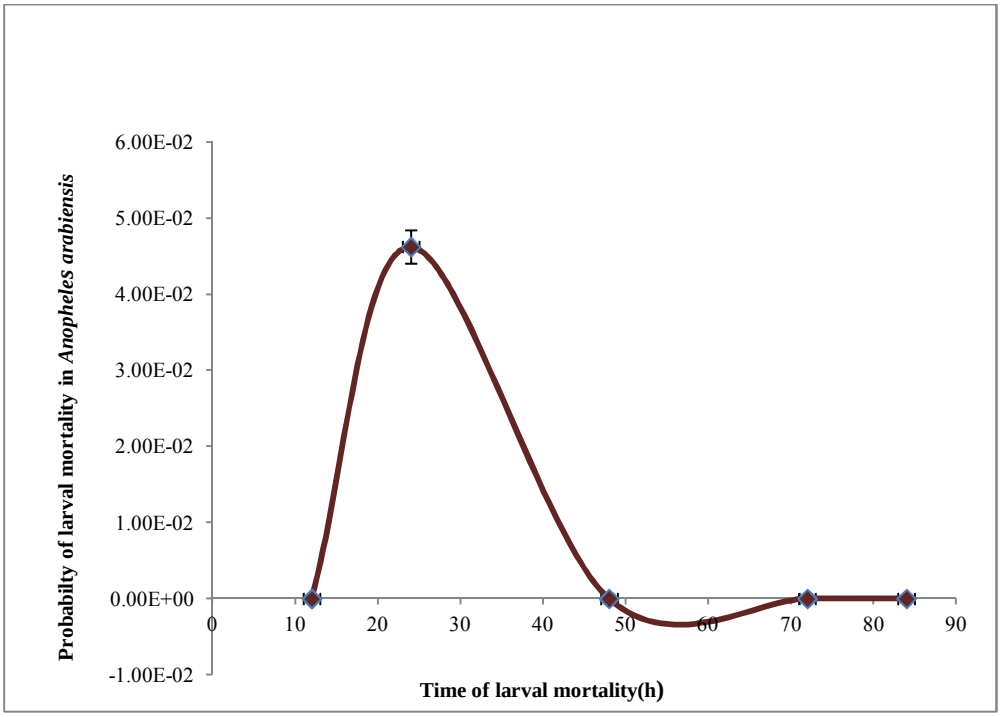

Figure 5.5. Probability of larval mortality in *Anopheles arabiensis* over time series in response to oil under laboratory conditions: Error bars represent the standard error of the mean.

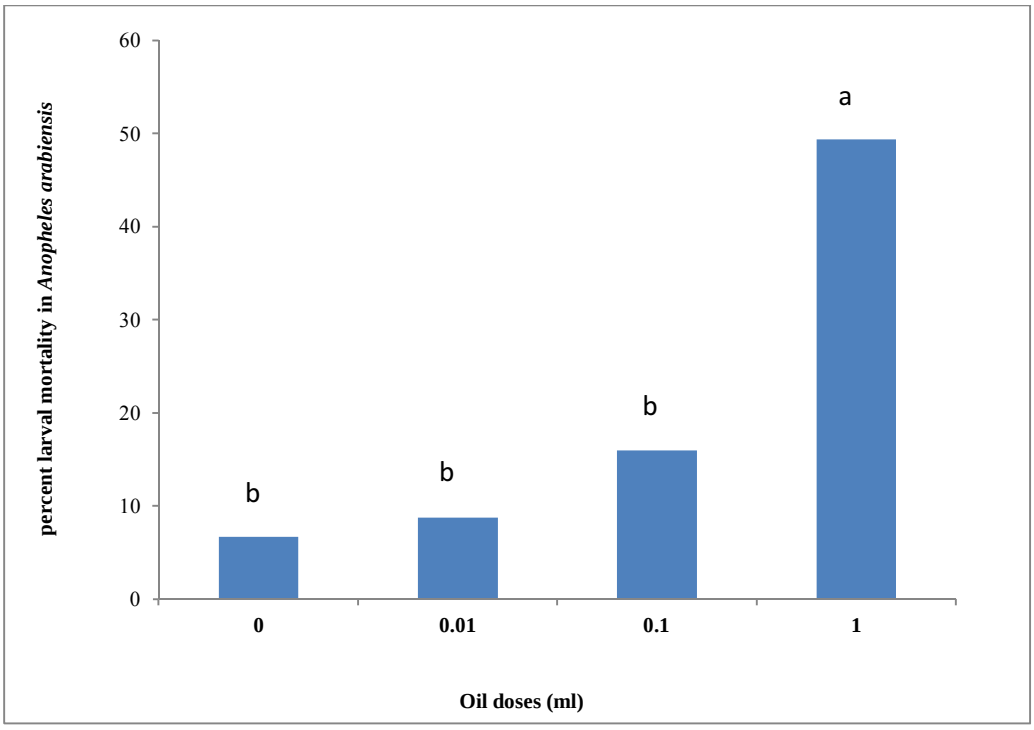

Figure 5.6. Larval mortality of *Anopheles arabiensis* in response to oil dose series at 24 h after application, the time is with the peak (maximum) larval mortality by overall effect of oils. The mean larval mortality with different letters are significantly different from one another (univariate general linear model with a Tukey's post-hoc analysis; $P < 0.05$). Error bars represent the standard error of the mean.

5.4. Discussion

Natural oil content of *Cyperus papyrus* rhizome was significantly higher than that of its aerial part and both the rhizome and aerial parts of *Typha latifolia*. The oil content of *T. latifolia* rhizome was the lowest of all the oil contents of grass species plant materials. Shorter probability of *Anopheles arabiensis* larval survival occurred by increasing the doses of the solvent extracted oils from *Cyperus papyrus* and *Typha latifolia* aerial and rhizome parts as well as the refined seed oil of *Helianthus annuus*. These findings indicate that aquatic grasses have differential amount of natural oil production in natural conditions which causes differential

effects on survival of larval *Anopheles* mosquitoes and therefore results in variation in densities of larval *Anopheles* mosquitoes in breeding habitats.

Previous findings indicated that habitats associated with Typhaceae and Cyperacaea grasses have lower *Anopheles coluzzii* and *Anopheles arabiensis* larval density compared with that of habitats associated with Poacaeae (Bøgh *et al*., 2003; Fillinger *et al*., 2004; Fillinger *et al.*, 2009). Similarly, recent findings indicate that potential breeding habitats of mosquitoes at southern edge of shore area of Lake Tana dominated by different grass species are found with differential larval densities. The *Anopheles* larval density in habitats with Typhaceae is less than the habitats with Poacea grasses and the larvae are not found in habitats with Cyperaceae (Asmare *et al*., 2017a). This variation in preference of *Anopheles* larvae to grasses in field conditions is reflected by differences in preference of gravid female *An. arabiensis* and *An. coluzzii* to volatile extracts from grasses; with lower preference to volatiles of *T. latifolia,* and least preference to *Cyperus papyrus* volatiles in contrast to volatiles of the Poaceae grasses including *Echinochloa pyramidalis* and *Echinochloa stagnina* under laboratory conditions (Asmare *et al*., 2017a).

Oils usually cause suffocation which results in mortality in aquatic stages of mosquito in line with (Goma, 1960b), and this is due to physical nature of oil that creates thin film on water surface (Shuo *et a*l., 2012). The results in this study; increased larval mortality in *An. arabiensis* through increasing the dose of oils including the non-toxic control oil indicated that oils with and/or without toxic nature can result larval mortality due to suffocation. Absence of *Anopheles* larvae in potential breeding habitats dominated by *C. papayrus* could be due to the higher amount of natural oil in its rhizome that is released directly to the breeding water which reduces the probability of larval survival.

Cyperaceae grasses have toxic properties in nature with broad spectrum negative impact on microorganisms (Kakarla *et al*., 2014; Lawal *et al*., 2016). Moreover, essential oil of *C. papyrus* shows lethal effect on the larvae of brine shrimp *Artemia salina* (Ameen *et al*., 2011). Therefore, higher amount of natural oil production by *C. papyrus* under natural conditions could have cumulative effect of both toxicity and suffocation which reduces survival of *Anopheles* larvae and their populations in the area where it is dominant in permanent breeding habitats. The similar effects of oils from *C. papyrus* and T. *latifolia*, and the non-toxic refined seed oil of *H. annuus* on survival of the mosquito larvae under laboratory conditions could be the in-efficient and in-effective property of the Soxhlet extractor with the solvent *n*-hexane to extract toxic nature of *C. papyrus* and may be the other grass, *T. latifolia* (Rassem *et al*., 2016). This needs further studies using other natural oil extraction procedures. The toxic nature of *C. papyrus* oil on microbial populations (Kakarla *et al*., 2014; Lawal *et al*., 2016) may also reduce the natural food resource microbiota in breeding habitats (Merritt *et al*., 1992).

There are reports on toxic effect of oils extracted from plants on survival of larvae in different mosquito species. For example; larvicidal effects of essential oils of the plants in *Guarea* species on *Aedes aegypti* (Magalhães *et al*., 2010), essential oil of Indian borage on *An. gambiae* (Kweka *et al*., 2012), and Mediterranean aromatic plants on *Ae. albopictus* (Conti *et al*., 2010) are well documented. Moreover, extracts from *Phragmites australis* has adult repellent and larvicidal effects against *Cu. pipiens* (Bream, 2009) and extracts from herbal species including *Ferula asafoetida*, *Coriandrum sativum*, and *Trigonella foenum-gracewm* have larvicidal effects on *Aedes aegypti* (Harve and Kamath, 2004). Aquatic herbs like *Cyperus papayrus* with higher amount of natural oils could be used for vector control at the larval stage in natural breeding habitats.

Studies on human safety have reported that species of the grass family Cyperaceae including *C. papyrus* are economically and medically important and their uses include utilization against various disorders such as migraine or abdominal pains or as fumigant and to flavor food is well recorded (Sonwa, 2000; Kakarla *et al*., 2014). Local people in the study area utilize the aerial part of the grass species for manufacturing some local containers, baskets. The rhizome part of the grass species are also utilized for firewood. Recently, the plant has been recommended to be used for ornamental, waste water treatment and ecological remediation, as well as conservation purposes in the study area.

5.5. Conclusions

Both toxic and non-toxic oils could have larvicidal effect on malaria mosquito due to the physical nature that creates thin film on water surface and bring about suffocation. The emergent grass, *C. papyrus* found with higher oil content of its rhizome which has direct contact to the breeding water of *Anopheles* mosquitoes. Therefore, it is possible to recommend *C. papyrus* plantation in permanent breeding habitats in close proximity to villages to suppress *Anopheles* mosquito populations and malaria transmission.

Chapter 6. General Discussion, Conclusions and Recommendations

6.1. General discussion

Anopheles mosquitoes have positive associations with some Poaceae grasses (Bøgh *et al*., 2003; Gouagna *et al*., 2012; Khater *et al*., 2013) as well as negative association with some grasses especially the family Cyperaceae (Goma, 1960a; 1960b; Fillinger *et al*., 2004; Yohannes *et al*., 2005) in breeding habitats. This depends on the species specific characteristics of plants especially their pollen quantity and quality which contributes larval nutrient supplement (Ye-Ebyo *et al*., 2000; 20003a) and natural oil production which has larvicidal effect on mosquitoes (Goma, 1960a; Lindsay and Martens, 1998; Mouchet *et al*., 1998). Gravid female of *Anopheles* mosquitoes discriminate among potential breeding habitats based on olfactory cues produced by biotic components (Torres-Estrada *et al*., 2005; 2007; Himeidan *et al*., 2013; Afify and Galizia, 2015) that directs to habitats with sufficient nutrient and minimum mortality factors (Bentley and Day, 1989). Detail studies in the role of grass volatiles in oviposition site selection by *Anopheles* mosquitoes and the effects of the grasses pollen and oils on *Anopheles* larval survival and development in breeding habitats can contribute for the development of new vector management options to control malaria.

In the present study natural breeding habitats associated with different grasses had differential *Anopheles* larval productivities. The larval density of *Anopheles* mosquitoes in breeding habitats associated with Poaceae grasses, *Echinocloa pyramidalis* and *E. stagnina* is significantly higher than *Typha latifolia*. The special condition in the study site is absence of *Anopheles* larvae in breeding habitats associated with *Cyperus papyrus* (Chapter 3). Volatiles released by emergent grass species differentially attract and stimulate ovipostion by gravid *Anopheles arabiensis* and

Anopheles coluzzii mosquitoes (Chapter 3). Among the study grasses, the volatiles released by Poaceae grasses were significantly more attractive to gravid *An. arabiensis* and *An. coluzzii* as well as stimulative of their ovipostion than that of Typhaceae and Cyperaceae; the lowest preference of gravid female mosquitoes to volatile extracts of Cyperaceae (Chapter 3). The present study results indicate that emergent grass derived olfactory cues play an important role in regulating oviposition site selection by*Anopheles* mosquitoes, and in turn affects larval dispersal in breeding habitats in a given area.

The present findings also indicate that dietary input of grasses pollen as natural larval diets influence population dynamics of malaria mosquitoes. Pollen from all the study grass species including *Typha latifolia*, *Pennisetum setaceum, Echinocloa pyramidalis* and *Zea mays* accelerate pupation. The dietary quality of pollen are characterized by macronutrient balance (C: N ratio) and grain size (Chapter 4). Carbon-rich smaller grained *T. latifolia* and *P. setaceum* pollen increases adult emergence rate more than the nitrogen-rich *E. pyramidalis* pollen and the artificial larval dietTetraMin® and the larger grained carbon-rich *Z. mays* pollen (Chapter 4).

Higher amount of natural oil content is found in *C. papyrus* rhizome (Chapter 5) and increased larval mortality with higher doses of grasses oil under laboratory conditions (Chapter 5) which has direct correlation to the absence of *Anopheles* larvae in natural habitats associated with the grass, *C. papyrus* in the study site (Chapter 3). These findings indicate that grasses with higher amount of natural oils in breeding habitats have significant ecological roles on survival and development of larval *An. arabiensis* and therefore on their populations.

Previous reports show that the two major malaria vectors in sub-Saharan Africa, *An. coluzzii* and *An. arabiensis* have been recorded in stable water bodies, such as the shore area of lakes and in

swamps (Fillinger *et al*., 2004; Minakawa *et al*., 2005; Imbahale *et al*., 2011; Ndenga *et al*., 2011; Minakawa *et al*., 2012). These stable habitats are accompanied with various types of vegetation (Fillinger *et al*., 2004; Ndenga *et al*., 2011; Minakawa *et al*., 2012), and the mosquito species are found in habitats associated with Poaceae and Typhaceae grasses (Bøgh *et al*., 2003; Gouagna *et al*., 2012; Khater *et al*., 2013). In contrast, lower mosquito larval productivity is recorded in *Phragmites* spp. (Poaceae) and *Cyperus papyrus* (Cyperaceae) associated habitats (Goma, 1960; Fillinger *et al*., 2004; Yohannes *et al*., 2005). It has been proposed that due to natural oil production by these grass species that affect survival of the mosquito larvae and reduce larval densities of the mosquito species in the sites (Lindsay and Martens, 1998; Mouchet *et al*., 1998).

Grasses provide pollen and detritus which attract microorganisms (Merritt *et al*., 1992a; Ye-Ebiyo *et al*., 2000; 2003a; Garros *et al*., 2008) all these are potential sources of mosquito larval nutrients in natural breeding habitats. The gravid *An. arabiensis* preference to deposite their eggs close to some Poaceae grasses reflect the potential input of these biotic components in natural larval nutrients (Wondwosen *et al*., 2016; 2017; Asmre *et al*., 2017a; 2017b).

The present findings provide evidence on species specific ecological significance of grasses in regulating *Anopheles* mosquito populations and malaria transmission. Identifying olfactory cues from grasses that modulate the gravid behaviours, dietary input of grasses pollen on larval survival and development, and impact of oil content of emergent grasses on larval survival has distinct potential for monitoring and development of control methods on malaria mosquitoes. This study revealed that there are differences in distributon and abundance of mosquito larvae in natural breeding habitats dominated by grasses species, due to variations in attraction and oviposition of gravid female *Anopheles* mosquitoes to natural grass volatiles; and variations in

dietary input of pollen, and amount of natural oil by different grass species resulting differential effects of grasses on immature stages of *Anopheles* mosquitoes in breeding habitats.

6.2. General conclusions

- The studies verified the role of grass volatiles in oviposition site selection by *Anopheles* mosquitoes and the effects of pollen and natural oil on survival and development of their larvae.
- *Anopheles arabiensis* and *An. coluzzii* are attracted and stimulated for oviposition differentially by volatiles collected from different grass species; gravid *Anopheles* preferences for attraction and oviposition to volatiles of Poaceae is higher than that of Typhaceae and is lowest to volatiles of Cyperaceae. This is also reflected in field condition that the *Anopheles* larvae showed similar pattern of prefrencee to breeding habitats with the grasses
- The amount of pollen production by *Zea mays* is higher than that of all other study grasses including *Pennisetum setaceum*, *Typha latifolia*, and *Echinocloa pyramidalis* and the pollen collected from *Z. mays* demonstrate longer survival and slower development like nitrogen rich artificial diet TetraMin® and *E. pyramidalis* than that of carbon rich and small grain size pollen from *T. latifolia* and *P. setaceum*.
- Rhizome part of *C. papyrus* has higher natural oil content than its aerial part and both the aerial and rhizome parts of *T. latifolia*. The absence of *Anopheles* larvae in natural breeding habitats dominated by Cyperceae along the shore area of southern edge of Lake Tana has direct positive relation with the result of laboratory studies showed that increasing the dose of extracted oils reduces the probability of larval survival in *An. arabiensis*.

6.3. Recommendations

- Further studies on larval productivity of other mosquito vectors such as culicine in potential breeding habitats populated by Poaceae, Typhaceae and Cyperaceae grasses.
- Further studies on chemical analysis of the study grass volatiles and trials on synthetic blends of the volatiles on *Anopheles* mosquitoes and other mosquito species.
- Further studies on bioactivities of rhizome oil from *C. papayrus* on anopheline and culicine mosquitoes using efficient and effective procedures to extract the toxic nature of the macrophyte.
- Emphasis should be taken to monitor malaria transmission and plan interventions in villages close to breeding habitats with natural and domestic Poaceae grasses.
- Plantation of *C. papyrus* in permanent potential mosquito breeding sites can be additional components of IVM for sustainable management of malaria mosquitoes.

References

Abiodun, G.J., Maharaj, R., Witbooi, P., Okosun, K.O. (2013). Modeling the influence of temperature and rainfall on the population dynamics of *Anopheles arabiensis*, *Malar. J.,* **15**:364.

Abose, T., Ye-Ebiyo, Y., Olana, D., Alamirew, D., Beyene, Y., Regassa, L. (1998). *Re-orientation and definition of the role of malaria vector control in Ethiopia*. World Health Organization, Division of Control of Tropical Diseases. Geneva, Switzerland.

Afify, A., Galizia, C.G. (2015). Chemosensory cues for mosquito oviposition site selection, *J. Med. Entomol.*, **52**:120-130.

Ajayi, M.B., Adeleke M.A., Idowu E.T., Awolola, T.S. (2010). Surveillance of mosquitoes vectors in Ajumoni Estate Ogun State, Nigeria, *Ann. Biol. Res*., **1**:16-19.

Alemu, K., Worku, A., Berhane, Y. (2013). Malaria infection has spatial, temporal, and spatiotemporal heterogeneity in unstable malaria transmission areas in Northwest Ethiopia, *PLoS One* **8**: e79966.

Alphey, L., Benedict, M., Bellini, R., Clark, G.G., Dame, D.A., Service, M.W., Dobson, S.L. (2010). Sterile-Insect methods for control of mosquito-borne diseases, *Ann. Vector-Borne Zoonotic Dis*., **10**:295-311.

Alphey, L., McKemey, A., Nimmo, D., Neira Oviedo, M., Lacroix, R., Matzen, K. and Beech, C. (2013). Genetic control of *Aedes* mosquitoes*, Pathog. Glob. Health*, **107:**170-179.

Ameen, O.M., Usman, L.A., Oladosu, I.A., Olawore, N.O., Ogunwande, I.A. (2011). Bioactivity of rhizome essential oils from two varieties of *Cyperus articulatus* (L.) grown in Nigeria, using brine shrimp (*Artemia salina*) lethality tests, *J. Med. Plant Res*., **5**:1031-1033.

Ameneshewa, B., Service M.W. (1996). The relationship between female body size and survival rate of the malaria vector *Anopheles arabiensis* in Ethiopia, *Med. Vet. Entomol*., **10**:170-172.

Amer, A., Mehlhorn, H. (2006). Larvicidal effects of various essential oils against *Aedes*, *Anopheles*, and *Culex* larvae (Diptera, Culicidae), *Parasitol. Res.,* **99**:466-472.

Araújo, M.daS., Gil, L.H.S., e-Silva, A.deA. (2012). Larval food quantity affects development time, survival and adult biological traits that influence the vectorial capacity of *Anopheles darlingi* under laboratory conditions, *Malar. J*., **11**:261.

Arav, D., Blaustein, L. (2006). Effects of pool depth and risk of predation on oviposition habitat selection by temporary pool dipterans, *J. Med. Entomol*., **43:**493- 497.

Asmare, Y., Hill, S.R., Hopkins, R.J. Tekie, H., Ignell, R. (2017). The role of grass volatiles on oviposition site selection by *Anopheles arabiensis* and *Anopheles coluzzii*, *Malar. J*., **16**:65.

Asmare, Y., Hopkins, R.J. Tekie, H., Hill, S.R., Ignell, R. (*in press*). Grass pollen affects survival and development of larval *Anopheles arabiensis* Patton, *J. Insect sci.*

Atyame, C.M., Cattel, J., Lebon, C., Flores, O., Dehecq, J-S., Weill, M., Gouagna, L.C., Tortosa, P. (2015). *Wolbachia*-based population control strategy targeting *Culex quinquefasciatus* mosquitoes proves efficient under semi-field conditions, *PLoS One*, **10**:e0119288.

Baird, M.E., Middleton, J.H. (2004). On relating physical limits to the carbon: nitrogen ratio of unicellular algae and benthic plants, *J. Marine Syst.,* **49**:169-175.

Bale, J.S., Van Lenteren, J.C., Bigler, F. (2008). Biological control and sustainable food production, *Philos. Trans. R. Soc. Lon. Biol. Sci*., **363:**761-776.

Barbosa, R.M.R., Regis, L., Vasconcelos, R. and Leal, W.S. (2010). *Culex* mosquitoes (Diptera: Culicidae) egg laying in traps loaded with *Bacillus thuringiensis* variety *israelensis* and baited with skatole, *J. Med. Entomol.*, **47:**345-348.

Barik, T.K., Sahu, B., Swain, V. (2009). A review on *Anopheles culicifacies*: from bionomics to control with special reference to Indian subcontinent, *Acta. Trop,* **109**:87-97.

Bayoh, M.N., Lindsay, S.W. (2003). Effect of temperature on the development of the aquatic stages of *Anopheles gambiae* sensu stricto (Diptera: Culicidae), *Bull. Entomol. Res.*, **93**:375-381.

Beck-Johnson, L.M., Nelson, W.A., Paaijmans, K.P., Read, A.F., Thomas, M.B., Bjørnstad, O.N. (2013). The effect of temperature on *Anopheles* mosquito population dynamics and the potential for malaria transmission, *PLoS One*. **14:**79276.

Beehler, J.W., Millar, J.G., Mulla, M.S. (1994). Protein hydrolysates and associated bacterial contaminants as oviposition attractants for the mosquito *Culex quinquefasciatus*, *Med. Vet. Entomol.*, **8**:381-385.

Beier, J.C., Keating, J., Githure, J.I., Macdonald, M.B., Impoinvil, D.E. and Novak, R.J. (2008). Integrated vector management for malaria control, *Malar. J.* **7:**1.

Bentley, M.D., Day, J.F. (1989). Chemical ecology and behavioral aspects of mosquito oviposition, *Annu. Rev. Entomol.*, **34:**401-421.

Bidlingmayer, W.L. (1994). How mosquitoes see traps: role of visual responses, *J. Am. Mosq. Control Asso.*, **10**:272-279.

Blandin, S.A., Marois, E., Levashina, E.A. (2008). Antimalarial Responses in *Anopheles* gambiae: from a complement-like protein to a complement-like pathway, *Cell Host Microbe Rev.*, **3**:364-374.

Blaustein, L., Kiflawi, M., Eitam, A., Mangel, M., Cohen, J.E. (2004). Oviposition habitat selection in response to risk of predation in temporary pools: mode of detection and consistency across experimental venue, *Oecologia*, **138**:300-305.

Bøgh, C., Clarke, S.E., Jawara, M., Thomas, C.J., Lindsay, S.W. (2003). Localized breeding of the *Anopheles gambiae* complex (Diptera: Culicidae) along the River Gambia, West Africa, *Bull. Entomol. Res*., **93**:279-287.

Bream, A.S., Hassan, M.I., Fouda, M.A., El-Sheikh, T.M. (2009). Toxicity and repellent activity of *Phragmites australis* extracts against the mosquito vector *Culex pipiens*, *Tunisian J. Plant Protec*., **4**:157-172.

Bugoro, H., Hii, J., Russell, T.L., Cooper, R.D., Chan. B.K.K., Iro'ofa, C., *et al*. (2011). Influence of environmental factors on the abundance of *Anopheles farauti* larvae in large brackish water streams in Northern Guadalcanal, Solomon Islands, *Malar J*., **10**:262.

Bukhari, T., Takken, W., Koenraadt, C.J. (2013). Biological tools for control of larval stages of malaria vectorsa, *Biocontrol Sci. Technol*., **23:**987-1023.

CDC. (2016). Laboratory identification of parasitic diseases of public health concern, public health image liberary (PHIL), malaria illustration, DPDx–parasites and health – malaria (*Plasmodium* spp.) (https://www.dpd.cdc.gov/dpdx/HTML/Malaria.htm).

CDC. (2017). Citriodiol, a natural active ingredient for insect repellents, Citriodiol® (https://www.citrefine.com/citriodiol). Citrefine International Ltd.

Chandra, G., Bhattacharjee, I., Ghosh, A., Chatterjee, S.N. (2008). Mosquito control by larvivorous fishes, *Indian J. Med. Res*., **127:**13-27.

Charles, J.F., Nielsen-LeRoux, C. (2000). Mosquitocidal bacterial toxins: diversity, mode of action and resistance phenomena, *Mem. Inst. Oswaldo Cruz.,* **95**:201-206.

Charlwood, J.D., Thompson, R., Madsen, H. (2003). Observations on the swarming and mating behaviour of *Anopheles funestus* from southern Mozambique, *Malar. J.,* **2**:2.

Charlwood, J.D., Braganca, M. (2012). The effect of rainstorms on adult *Anopheles funestus* behavior and survival, *J. Vector. Ecol*., **37**:252-256.

Craig, M.H., Snow, R.W., le Sueur, D. (1999). A climate-based distribution model of malaria transmission in Sub-Saharan Africa, *Parasitol. Today*, **15**:3.

Clayton, A.M., Dong, Y., Dimopoulos, J. (2014). The *Anopheles* innate immune system in the defense against malaria infection, *J. Innate. Immun*., **6**:169-181.

Clements, A.N. (1992). *The Biology of Mosquitoes*: *development, nutrition and reproduction*. Vol.1, London School of Hygiene and Tropical Medicine, Chapman and Hall, UK: pp. 509.

Clements, A.N. (1999). *The biology of mosquitoes*: *sensory reception and behavior*. Vol. 2, London School of Hygiene and Tropical Medicine, CABI Publishing, UK: pp. 752.

Coetzee, M. (1989) Comparative morphology and multivariate analysis for the discrimination of four members of the *Anopheles gambiae* group in southern Africa, *Mosq. Syst.*, **21**:100-116.

Coetzee, M., Fontenille, D. (2004). Advances in the study of *Anopheles funestus*, a major vector of malaria in Africa, *Insect Biochem. Mol. Biol*., **34**:599-605.

Coetzee, M., Hun, R., Wilkerson, R., Torre, A., Coulibaly, M., Besansky, N.J. (2013). *Anopheles coluzzii* and *Anopheles amharicus*, new members of the *Anopheles gambiae* complex, *Zootaxa*, **3619**:246-274.

Conti, B., Canale, A., Bertoli, A., Gozzini, F., Pistelli, L. (2010). Essential oil composition and larvicidal activity of six Mediterranean aromatic plants against the mosquito *Aedes albopictus* (Diptera: Culicidae), *Parasitol. Res*., **107**:1455-1461.

Cook, S.M., Murray, D.A., Williams, I.H. (2004). Do pollen beetles need pollen? The effect of pollen on oviposition, survival, and development of a flower feeding herbivore, *Ecol. Entomol*., **29**:164-173.

Corby-Harris, V., Drexler, A., Watkins de Jong, L., Antonova, Y., Pakpour, N., Ziegler, R., *et al.* (2010). Correction activation of Akt signaling reduces the prevalence and intensity of malaria parasite infection and lifespan in *Anopheles stephensi* mosquitoes, *PLoS Pathog*, **6**:e1001003.

Cory, J.S., Franklin, M.T. (2012). Evolution and the microbial control of insects, *Evol. Appl.* **5:**455-469.

Cox, F.E.G. (2010). History of the discovery of the malaria parasites and their vectors, *Parasit. Vectors*, **3**:5.

Cox-Singh, J., Davis, T.M., Lee, K.S., Shamsul, S.S., Matusop, A., Ratnam, S., et al. (2008). *Plasmodium knowlesi* malaria in humans is widely distributed and potentially life threatening, *Clin. Infect. Dis.*, **46**:165-171.

Day, J.F. (2016). Mosquito Oviposition Behavior and Vector Control, *Insects*, **7**:65.

Dekker, T., Takken, W. (1998). Differential responses of mosquito sibling species *Anopheles arabiensis* and *An. quadriannulatus* to carbon dioxide, a man or a calf, *Med. Vet. Entomol.*, **12**:136-40.

Dennis, C.A., MacNeil, M.A., Rosati, J.Y., Pitcher, T.E., Fisk, A.T. (2010). Diet discrimination factors are inversely related to δ15N and δ13C values of food for fish under controlled conditions, *Rapid Commun. Mass Sp*., **24**:3515-3520.

Diabate, A., Yaro, A.S., Dao, A., Diallo, M., Huestis, D.L. and Lehmann, T. (2011). Spatial distribution and male mating success of *Anopheles gambiae* swarms, *Evol. Biol*., **11:**184.

Diabate, A., Tripet, F. (2015). Targeting male mosquito mating behaviour for malaria control, *Parasit. Vectors*, **8:**1-13.

Dube, F.F., Tadesse, K., Birgersson, G., Seyoum, E., Tekie, H., Ignell, R. and Hill, S.R. (2011). Fresh, dried or smoked? Repellent properties of volatiles emitted from ethnomedicinal plant leaves against malaria and yellow fever vectors in Ethiopia, *Malar.J.* **10:**1-14.

Dugassa, S., Medhin, G., Balkew, M., Seyoum, A. and Gebre-Michael, T., (2009). Field investigation on the repellent activity of some aromatic plants by traditional means against *Anopheles arabiensis* and *Anopheles pharoensis* (Diptera: Culicidae) around Koka, central Ethiopia, *Acta Trop.*, **112:**38-42.

Dugassa, S., Lindh, J.M., Torr, S.J., Oyieke, F., Lindsay, S.W., Fillinger, U. (2012). Electric nets and sticky materials for analysing oviposition behaviour of gravid malaria vectors, *Malar. J*., **11**:374.

Dugassa, S., Lindh, J.M., Torr, S.J., Lindsay, S.W., Fillinger, U. (2014). Evaluation of the influence of electric nets on the behaviour of oviposition site seeking *Anopheles gambiae sensu stricto*, *Parasit. Vectors*, **7**:272.

Dugassa, S., Lindh, J.M., Lindsay, S.W., Fillinger, U. (2016). Field evaluation of two novel sampling devices for collecting wild oviposition site seeking malaria vector

mosquitoes: oviart gravid traps and squares of electrocuting nets, *Parasit. Vectors*, **9**:272.

Eckhoff, P.A. (2011). A malaria transmission-directed model of mosquito life cycle and ecology, *Malar. J.*, **10**:303.

Eisele, T.P., Larsen D.A., Walker, N., Cibulskis, R.E., Yukich, J.O., Zikusooka, C., Steketee, R.W. (2012). Estimates of child deaths prevented from malaria prevention scale-up in Africa 2001-2010, *Malar. J.*, **11**:93.

Enayati, A. Hemingway, J. (2010). Malaria management: past, present, and future, *Annu. Rev. Entomol*., **55:** 569-591.

Eneh, L.K., Saijo, H., Borg□Karlson, A-K., Lindh, J.M., Rajarao, G.K. (2016). Cedrol, a malaria mosquito oviposition attractant is produced by fungi isolated from rhizomes of the grass *Cyperus rotundus, Malar. J.,* **15**:478.

EPHI. (2017). *Anopheles mosquito rearing and insectary handling guideline*. Ethiopian Public Health Institute. *Addis Ababa, Ethiopia.*

FAO. (2016). *FAO in Ethiopia*. FAO El Niño response plan, Rome, Italy.

Fillinger, U., Sonye, G., Killeen, G.F., Knols, B.G.J., Becher, N. (2004) The practical importance of permanent and semipermanent habitats for controlling aquatic stages of *Anopheles gambiae sensu lato* mosquitoes: operational observation from a rural town in western Kenya, *Trop. Med. Int. Health*, **9**:1274-1289.

Fillinger, U., Sombroek, H., Majambere, S., Van, L.E., Takken, W. (2009). Identifying the most productive breeding sites for malaria mosquitoes in the Gambia, *Malar J.*, **8**:62.

FMOH. (2008). *Ethiopia national malaria indicator survey*. Addis Ababa, Ethiopia, pp. 1-98.

FMST. (2014). *National research ethics review guideline, 5th edition.* Federal Ministry of Science and Technology. Addis Ababa, Ethiopia.

Fu, J., Mai, B., Sheng, G., Zhang, G., Wang, X., an Peng, P., *et al*. (2003). Persistent organic pollutants in environment of the Pearl River Delta, China: an overview, *Chemosphere*, **52**:1411–1422.

Futami, K., Sonye, G., Akweywa, P., Kaneko, S., Minakawa, N. (2008). Diving behavior in *Anopheles gambiae* (Diptera: Culicidae): avoidance of a predacious wolf spider (Araneae: Lycosidae) in relation to life stage and water depth, *J. Med. Entomol*., **45**:1050-1056.

Garros, C., Ngugi, N., Githeko, A.E., Tuno, N., Yan, G. (2008). Gut content identification of larvae of the *Anopheles gambiae* complex in western Kenya using a bar coding approach, *Mol. Ecol. Res.*, **8**:512–518.

Gary, R.E., Foster, W.A. (2004). *Anopheles gambiae* feeding and survival on honeydew and extra-floral nectar of peridomestic plants, *Med. Vet. Entomol.*, **182**:102-107.

Gary, R.E., Cannon, J.W., Foster, W.A. (2009). Effect of sugar on male *Anopheles gambiae* Giles (Diptera: Culicidae) mating performance, as modified by temperature, space, and body size, *Parasit. Vectors*, **2**:19.

Gillies, H.M., Warrell, D.A. (1993). *Bruce-Chewatt's Essential Malariology*, 3rd edition. Arnold, London.

Gimnig, J.E., Ombok, M., Kamau, L., Hawley, W.A. (2001). Characteristics of larval anopheline (Diptera: Culicidae) habitats in Western Kenya, *J. Med. Entomol.*, **38**:282-288.

Golberg, L., Demeillon, B. (1948). The nutrition of the larva of *Aedes aegypti* L4 protein and amino acid requirements, *Biochem. J*., **43**:379-387.

Goma L.K.H. (1960a). The swamp-breeding mosquitoes of Uganda: records of larvae and their habitats, *Bull. Entomol. Res*., **51**:77-94.

Goma L.K.H. (1960b). Experimental breeding of *Anopheles gambiae* Giles in papyrus swamps, *Nature*, **187**:1137-1138.

Gouagna, L.C., Rakotondranary, M., Boyer, S., Lempérière, G., Dehecq, J-S., Fontenille, D. (2012). Abiotic and biotic factors associated with the presence of *Anopheles arabiensis* immatures and their abundance in naturally occurring and man-made aquatic habitats, *Parasit. Vectors*, **5**:96.

Grieco, J.P., Rejmánková, E., Achee, N.L., Klein, C.N., Andre, R., Roberts, D. (2007). Habitat suitability for three species of *Anopheles* mosquitoes: Larval growth and survival in reciprocal placement experiments, *J. Vector Ecol*., **32**:176-187.

Gueye, C.S., Newby, G., Hwang, J., Phillips, A.A., Whittaker, M., Mac Arthur, J.R., *et al*. (2014). The challenge of artemisinin resistance can only be met by eliminating *Plasmodium falciparum* malaria across the Greater Mekong subregion, *Malar. J*. **13:**286.

Hambäck, P.A, Björkman, M., Rämert, B., Hopkins, R.J. (2009). Scale-dependent responses in cabbage herbivores affect attack rates in spatially heterogeneous systems, *Basic Appl. Ecol*., **10**:228-236.

Hamilton, S.K., Lewis, W.M., Sippel, S.J. (1992). Energy sources for aquatic animals in the Orinoco River floodplain: Evidence from stable isotopes, *Gecol.* **89**:324-330.

Harbach, R.E. (2013). The Phylogeny and Classification of *Anopheles*. In: Manguin, S. (Eds), *Anopheles mosquitoes - New insights into malaria vectors.* InTech Publisher, Croatia, pp. 3-55.

Harve, G., Kamath, V. (2004). Larvicidal activity of plant extracts used alone and in combination with known synthetic larvicidal agents against *Aedes aegypti*, *Ind. J. Exp. Boil*., **42**:1216-1219.

Hay, S.I., Omumbo, J.A., Craig, M.H., Snow, R.W., (2000). Earth observation, geographic information systems and *Plasmodium falciparum* malaria in sub-Saharan Africa, *Adv. Parasitol*. **47:**173-215.

Healy, T.P., Jepson, P.C. (1988). The location of floral nectar sources by mosquitoes: the long-range responses of *Anopheles arabiensis* Patton (Diptera: Culicidae) to *Achillea millefolium* flowers and isolated floral odour, *Bull. Entomol. Res*. **78**:651-657.

Hien, D.F.dS., Dabiré, K.R., Roche, B., Diabaté, A., Yerbanga, R.S., Cohuet, A., *et al*. (2016) Plant-Mediated effects on mosquito capacity to transmit human malaria, *PLoS Pathog*, **12**:e1005773.

Himeidan, Y.E., Temu E.A., El Rayah, E.I.A, Munga, S., Kweka, E.J. (2013). Chemical cues for malaria vectors oviposition site selection: challenges and opportunities, *J. Insects*, **685182**:9.

Huang, J., Miller, J.R., Chen, S.C., Vulule, J.M. (2006). *Anopheles gambiae* (Diptera: Culicidae) oviposition in response to agrose media and cultured bacteria volatiles, *J. Med. Entomol.*, **43**:498-504.

Hunt, R.H., Coetzee, M., Fettene, M. (1998) The *Anopheles gambiae* complex: a new species from Ethiopia, *Trans R. Soci. Trop. Med. Hyg.*, **92**:231-235.

Imbahale, S.S, Paaijmans, K.P., Mukabana, W.R., van Lammeren, R., Githeko, A.K., Takken, W.A. (2011). Longitudinal study on *Anopheles* mosquito larval abundance in distinct geographical and environmental settings in western Kenya, *Malar. J.*, **10**:81.

Jaleta, K.T., Hill, S.R., Seyoum, E., Balkew, M., Gebre-Michael, T., Ignell, R., Tekie, H. (2013). Agro-ecosystems impact malaria prevalence: large-scale irrigation drives vector population in western Ethiopia, *Malar. J.*, **12**:350.

Igweh, J.C. (2012). Biology of malaria parasites. In: Omolade, O. (Ed.), *Malaria parasites.* InTech Publisher, ISBN: 978-953-51-0326-4, pp. 11-37.

Kashiwada, M., and Ohta, S. (2010). Modeling the spatio-temporal distribution of the *Anopheles* mosquito based on life history and surface water conditions, *Open Ecol. J.*, **3:**29-40.

Kakarla, L., Allu, P.R., Rama, C., Botlagunta, M. (2014). A review on biological and chemical properties of *Cyperus* Species, *Res. J. Pharm. Biol Chem. Sci.*, **5**:1142**.**

Kamareddine, L. (2012). The biological control of the malaria vector, *Toxins,* **4**:748-767.

Karunamoorthi, K. (2015). Research on mosquitocidal properties of plants: a call for enduring collaborative bridge between the scientific laboratories and the society, *Med. Aromat. Plants*, **4:**165.

Kebede, A., McCann, J.C., Kiszewski, A.E., Ye-Ebiyo. Y. (2005). New evidence of the effects of agro-ecologic change on malaria transmission, *Am. J. Trop. Med. Hyg.*, **73**:676-680.

Keller, I., Fluri, P., Imdorf, A. (2005). Pollen nutrition and colony development in honey bees, *Bee World*, **86**:3-10.

Kennedy, J.S. (1942). On water-finding and oviposition by captive mosquitoes, *Bull. Entomol., Res.*, **32**:279-301.

Khater, E.I., Sowilem, M.M., Sallam, M.F., Alahmed, A.M. (2013). Ecology and habitat characterization of mosquitoes in Saudi Arabia*, Trop. Biomed.*, **30**:409-427.

Kibret, S., Alemu, Y., Boelee, E., Tekie, H., Alemu, D., Petros, B. (2010). The impact of a small-scale irrigation scheme on malaria transmission in Ziway area, central Ethiopia, *Trop. Med. Int. Health*, **15**:41-50.

Kibret, S., Wilson, G.G., Tekie, H., Petros, B. (2014). Increased malaria transmission around irrigation schemes in Ethiopia and the potential of canal water management for malaria vector control, *Malar. J.*, **13**:360.

Killeen, G.F., Seyoum, A., Gimnig, J.E., Stevenson, J.C., Drakeley, C.J., Chitnis, N. (2014). Made-to-measure malaria vector control strategies: rational design based on insecticide properties and coverage of blood resources for mosquitoes, *Mala. J.*, **13:**1.

Kirby, M.J., Lindsay, S.W. (2009). Effect of temperature and inter-specific competition on the development and survival of *Anopheles gambiae sensu stricto* and *An. arabiensis* larvae, *Acta Tropica*, **109**:118-123.

Kitau, J., Oxborough, R.M., Tungu, P.K., Matowo, J., Malima, R.C., Magesa, S.M., *et al*. (2012). Species shifts in the *Anopheles gambiae* complex: do LLINs successfully control *Anopheles arabiensis*, *PLoS One*, **7:** e31481.

Kivuyo, H.P., Mbazi, P.H., Kisika, D.S., Munga, S., Rumisha, S.F., Urasa, F.M., Kweka, E.J. (2014). Performance of five food regimes on *Anopheles gambiae sensu stricto* larval rearing to adult emergence in insectary, *PLoS One*, **9**: e110671.

Kline, D.L. (2006). Traps and trapping techniques for adult mosquito control, *J. Am. Mosq. Control Assoc.*, **22:**490-496.

Knight, R.L., Walton, W.E., O'Meara, G.F., Reisen, K.W, Wass, R. (2003). Strategies for effective mosquito control in constructed treatment wetlands, *Ecol. Eng*. **21**:211-232.

Knudsen, J.T., Tollsten, L., Bergstrom, G. (1993). Floral scents a checklist of volatile compounds isolated by head-space techniques, *Phytochem*., **33:**253-280.

Köllner, T.G., Held, M., Lenk, C., Hiltpold, I., Turlings, T.C., Gershenzon, J., Degenhardt, J. (2008). A maize (E)-β-caryophyllene synthase implicated in indirect defense responses against herbivores is not expressed in most American maize varieties, *The Plant Cell*, **20**:482-94.

Kweka, E.J. Mosha, F., Lowassa, A., Mahande, A.M., Kitau, J., Matowo, J., *et al*. (2008). Ethnobotanical study of some of mosquito repellent plants in north-eastern Tanzania, *Malar. J*., **7**:152.

Kweka, E.J., Zhou, G., Gilbreath, T.M., Afrane, Y., Nyindo, M., Githeko, A.K, Yan, G. (2011). Predation efficiency of *Anopheles gambiae* larvae by aquatic predators in western Kenya highlands, *Parasit. Vectors*, **4**:128.

Kweka, E., Senthilkumar, A., Venkatesalu, V. (2012). Toxicity of essential oil from Indian borage on the larvae of the African malaria vector mosquito, *Anopheles gambiae*, *Parasit. Vectors*, **5**:277.

Kyu, H.H., Fernandez, E. (2009). Artemisinin derivatives versus quinine for cerebral malaria in African children: a systematic review, *Bull. World Health Org.*, **87**:896-904.

Lacey, L.A., Siegel, J.P. (2000). Safety and ecotoxicology of entomopathogenic bacteria. In: Charles, J.F., Delécluse, A., Nielsen-LeRoux, C. (Eds), *Entomopathogenic bacteria: from laboratory to field application*. Kluwer Academic Publisher, Dordrecht, pp. 253-273.

Lacey, L.A. (2007). *Bacillus thuringiensis* serovariety israelensis and *Bacillus sphaericus* for mosquito control, *Bull. Ame.Mosq. Control Assoc*., 23:133-163.

Lardeux, F.J., Tejerina, R.H., Quispe, V., Chavez, T. (2008). A physiological time analysis of the duration of the gonotrophic cycle of *Anopheles pseudopunctipennis* and its implications for malaria transmission in Bolivia, *Malar. J.*, **7**:141.

Laumann, V., Weber, C., Sommer, U. (2010). *Environmental strategies to replace DDT and control malaria*, 2nd edition, Hamburg, pp. 14.

Law, J.H., Regnier, F.E. (1971). Pheromones, *Annu. Rev. Biochem.* **40**:533-548.

Lawal, O.A., Ogunwande I.A., Opoku, A.R., Oyedeji, A.O. (2016). Chemical composition and anti-bacterial activity of essential oils from the rhizomes of *Cyperus papyrus* L. grown in South Africa, *Bull. Latino Am. Caribe. Pl*., **15**:136-143.

Leal, W.S. (2014). The enigmatic reception of DEET: the gold standard of insect repellents, *Curr. Opin. Insect Sci.*, **6:**93-98.

Lefèvre, T., Vantaux, A., Dabiré, K.R., Mouline, K., Cohue, A. (2013). Non-genetic determinants of mosquito competence for malaria parasites, *PLoS Pathog*., **9**:e1003365.

Lehane, M.J., Aksoy, S., Levashina, E. (2004). Immune responses and parasite transmission in blood-feeding insects, *Trends Parasitol*., **20**:433-439.

Lehmann, T., Dalton, R., Kim, E.H., Dahl, E., Diabate, A., Dabire, R., Dujardin, J.P. (2006). Genetic contribution to variation in larval development time, adult size, and longevity of starved adults of *Anopheles gambiae*, *Infect. Genet. Evol*., **6**:410-416.

Lindh, J.M., Okal, M.N., Herrera-Varela, M., Borg-Karlson, A.K., Torto, B., Lindsay, S.W., Fillinger, U. (2015). Discovery of an oviposition attractant for gravid malaria vectors of the *Anopheles gambiae* species complex, *Malar. J*. **14:**119.

Lindsay, S.W., Martens, W.J. (1998). Malaria in the African highlands: past, present and future, *Bull. World Health Org.*, **76**:33-45.

Lyimo, E., Takken, W. (1993). Effects of adult body size on fecundity and the pre-gravid rate of *Anopheles gambiae* females in Tanzania, *Med. Veter. Entomol*., **7**:328-332.

Maia, M.F., Moore, S.J. (2011). Plant-based insect repellents: a review of their efficacy, development and testing, *Malar. J.*, **10**:1.

Majeed, S., Hill, S.R., Ignell, R. (2014). Impact of elevated CO_2 background levels on the host-seeking behaviour of *Aedes aegypti, J. Exp. Biol*., **217**:598-604.

Magalhães, L.A.M., Lima, M. da P., Marques, M.O.M., Facanali, R., Pinto, C. da S., Tadei, W.P. (2010). Chemical Composition and larvicidal activity against *Aedes aegypti* larvae of essential oils from four *Guarea* species, *Molecules*, **15**:5734-5741.

Mains, J.W., Brelsfoard, C.L., Rose, R.I., Dobson, S.L. (2016). Female adult *Aedes albopictus* suppression by *Wolbachia*-infected male mosquitoes, Sci. Rep., **6**:33846

Manna, B., Aditya, G., Banerjee, S. (2008). Vulnerability of the mosquito larvae to the guppies (*Poecilia reticulata*) in the presence of alternative preys, *J. Vector Borne Dis*., **45**:200-206.

Manda, H., Gouagna, L.C., Nyandat, E., Kabiru, E.W., Jackson, R.R., Foster, W.A., *et al*. (2007). Discriminative feeding behaviour of *Anopheles gambiae sensu stricto* on endemic plants in western Kenya, *Med. Vet. Entomol*., **21:**103-111.

Mandal, S.K., Ghosh, A., Bhattacharjee, I., Chandra, G. (2008). Biocontrol efficiency of odonate nymphs against larvae of the mosquito, *Culex quinquefasciatus* Say, *Acta Trop*., **106**:109-114.

Manguin, S., Roberts, D.R., Peyton, E.L., Rejmankova, E., Pecor, J. (1996). Characterization of *Anopheles pseudopunctipennis* larval habitats, *J. Am. Mosq. Control Assoc*., **12**:619-625.

Marten, G.G., Reid, J.W., (2007). *Cyclopoid copepods*, *J. Am. Mosq. Control Assoc*., **23**(2):65-92.

Martin, V., Chevalier, V., Ceccato, P.N., Anyamba, A., De Simone, L., Lubroth, J., *et al*. (2008). The impact of climate change on the epidemiology and control of Rift Valley Fever, *Rev. Sci. Tech. Off. Int. Epi.* **27:** 413-426.

Matias, J.R., Adrias, A.Q. (2010). The use of annual killifish in the biocontrol of the aquatic stages of mosquitoes in temporary bodies of fresh water; a potential new tool in vector control, *Parasite Vectors*, **3**:46.

Matowo, N.S., Moore, J., Mapua, S., Madumla, E.P., Moshi, I.R., Kaindoa, E.W., *et al*. (2013). Using a new odour-baited device to explore options for luring and killing outdoor-biting malaria vectors: a report on design and field evaluation of the Mosquito Landing Box, *Parasit. Vectors*, **6:**137.

Mayntz, D., Raubenheimer, D., Salomon, M., Toft, S., Simpson, S.J. (2005). Nutrient-specific foraging in invertebrate predators, *Sci*. **307**:111-113.

McLean, K.J., Jacobs-Lorena, M. (2016). Genetic control of malaria mosquitoes, *Trends Parasitol*., **32**:174-176.

McMeniman, C.J., Corfas, R.A., Matthews, B.J., Ritchie, S.A., Vosshall, L.B. (2014). Multimodal integration of carbon dioxide and other sensory cues drives mosquito attraction to humans, *Sci. Direct*, **156**:1060-1071.

Menon P.K.B; Rajagopalan, P.K. (1978). Control of mosquito breeding in wells using *Gambusia affinis* and *Aplocheilus blockii* in Pondichery town, *Indian J. Med. Res*. **68:**927-933.

Merritt, R.W., Mortland, M.M., Gersabeck, E.R., Ross, D.H. (1978). X-ray diffraction analysis of particles ingested by filter-feeding animals, *Entomol. Exp. Appl*., **24**:27-34.

Merritt, R.W. (1987). Do different instars of *Aedes triseriatus* feed on particles of the same size, J. *Am. Mosq. Control Assoc.*, **3**:94-96.

Merritt, R.W., Dadd, R.H., Walker, E.D. (1992). Feeding behavior, natural food, and nutritional relationships of larval mosquitoes, *Annu. Rev. Entomol.*, **37**:349-376.

Michaelakis, A., Mihou, A.P., Couladouros, E.A., Zounos, A.K., Koliopoulos, G. (2005). Oviposition responses of *Culex pipiens* to a synthetic racemic *Culex quinquefasciatus* oviposition aggregation pheromone, *J. Agric. Food Chem.,* **53**:5225-5229.

Minakawa, N., Sonye, G., Mogi, M., Githeko, A., Yan, G. (2002). The effects of climatic factors on the distribution and abundance of malaria vectors in Kenya, *J. Med. Entomol*., **39**:833-841.

Minakawa, N., Sonye, G., Mogi, M., Yan, G. (2004). Habitat characteristics of *Anopheles gambiae sensu sstricto* larvae in a Kenyan highland, *Med. Vet. Entomol.*, **18**:301-305.

Minakawa, N., Munga, S., Atieli, F., Mushinzimana, E., Zhou, G., Githeko, A.K., Yan, G. (2005). Spatial distribution of anopheline larval habitats in Western Kenyan highlands: effects of land cover types and topography, *Am. J. Trop. Med. Hyg.*, **73**:157-165.

Minakawa, N., Dida, G.O., Sonye, G.O., Futami, K., Njenga, S.M. (2012). Malaria vectors in Lake Victoria and adjacent habitats in Western Kenya, *PLoS One*, **7**:e32725.

Mng'ong'o, F.C., Sambali, J.J., Sabas, E., Rubanga, J., Magoma, J., Ntamatungiro, A.J, *et al*. (2011). Repellent plants provide affordable natural screening to prevent mosquito house entry in tropical rural settings results from a pilot efficacy study, *PLoS One,* **6:**25927.

Mnyone, L.L., Kirby, M.J., Lwetoijera, D.W., Mpingwa, M.W., Knols, B.G.J., Takken, W., Russell, T.L. (2009). Infection of the malaria mosquito, *Anopheles gambiae*, with two species of entomopathogenic fungi: effects of concentration, co-formulation, exposure time and persistence, *Malar. J.,* **8**:309.

Moller-Jacobs, L., Murdock, C.C., Thomas, M.B. (2014). Capacity of mosquitoes to transmit malaria depends on larval environment, *Parasit. Vectors*, **7**:593.

Mouatcho, J.C. (2010). *The use of entomopathogenic fungi against Anopheles funestus Giles* (*diptera: culicidae*), PhD Thesis, Johannesburg, South Africa. pp. 17-30.

Mouchet, G., Manguin, S., Sircoulon, G., Laventure, S., Faye, O., Onapa, A.W., *et al*. (1998). Evolution of malaria in Africa for the past 40 years: impact of climatic and human factors, *J. Am. Mosq. Cont. Assoc*., **14**:121-130**.**

Mueller, I., Zimmerman, P.A., Reeder, J.C. (2007). *Plasmodium malariae* and *Plasmodium ovale* the 'bashful' malaria parasites, *Trends Parasitol.*, **23**:278-283.

Munga, S., Minakawa, N., Zhou, G. Barrack, O-OJ., Githeko, A.K., Yan, G. (2006a). Effects of larval competitors and predators on oviposition site selection of *Anopheles gambiae* sensu stricto, *J. Med. Entomol.*, **43**:221-224.

Munga, S., Minakawa, N., Zhou, G., Mushinzimana, E., Barrack, S., Githeko, A.K., Yan, G. (2006b). Association between land cover and habitat productivity of malaria vectors in western Kenyan highlands, *Am. J. Trop. Med. Hyg.*, **74**:69-75.

Murdock, C.C., Sternberg, E.D., Thomas, M.B. (2016). Malaria transmission potential could be reduced with current and future climate change, *Sci. Report,* **6**:27771.

Mushinzimana, E., Munga, S., Minakawa, N., Li, L., Feng, C.C., Bian, L., *et al*. (2006). Landscape determinants and remote sensing of anopheline mosquito larval habitats in the western Kenya highlands, *Malar. J*., **5**:13.

Muturi, E.J., Allan, B.F., Ricci, A.J. (2012). Influence of leaf detritus type on production and longevity of container-breeding mosquitoes, *Am. Environ. Entomol. Soc.,* **41**:1062-1068.

Mwangangi, J.M., Muturi, E.J., Shililu, J., Muriu, S.M., Jacob, B., Kabiru, E.W., Mbogo, C.M., Githure, J., Novak, R. (2006). Survival of immature *Anopheles arabiensis* (Diptera: Culicidae) in aquatic habitats in Mwea rice irrigation scheme, central Kenya, *Malar. J*., **5**:114.

Mwangangi, J.M., Mbogo, C.M., Muturi, E.J., Nzovu, J.G., Githure, J.I. (2007). Spatial distribution and habitat characterization of *Anopheles* larvae along the Kenyan coast, *J. Vector Borne Dis.*, **44**:44-51.

Mwangangi, J.M., Muturi, E.J., Shililu, J., Muriu, S.M., Jacob, B., Kabiru, E.W. *et al*. (2008). Contribution of different aquatic habitats to adult *Anopheles arabiensis* and *Culex quinquefasciatus* (Diptera: Culicidae) production in a rice agroecosystem in Mwea, Kenya, *J. Vector Ecol*., **33**:129-138.

Mwangangi, J.M., Shililu, J., Muturi, E.J., Muriu, S., Jacob, B., Kabiru, E.W. *et al*. (2010). *Anopheles* larval abundance and diversity in three rice agro-village complexes Mwea irrigation scheme, central Kenya, *Malar. J*., **9**:228.

Navarro-Silva, M.A., Marques, F.A., DuqueL, J.E. (2009). Review of semiochemicals that mediate the oviposition of mosquitoes: a possible sustainable tool for the control and monitoring of culicidae, *Braz. J. Entomol.*, **53**:1-6.

Ndenga, B.A., Simbauni, J.A., Mbugi, J.P., Githeko, A.K., Fillinger, U. (2011). Productivity of malaria vectors from different habitat types in the western Kenya highlands, *PLoS One* **6**:e19473.

Ndenga, B.A., Simbauni, J.A., Mbugi, J.P., Githeko, A.K. (2012). Physical, chemical and biological characteristics in habitats of high and low presence of anopheline larvae in western Kenya highlands, *PLoS One*, **7**:e47975.

Ndenga, B.A., Mulaya, N.L., Musaki, S.K., Shiroko, J.N., Dongus, S., Fillinger, U. (2016). Malaria vectors and their blood-meal sources in an area of high bed net ownership in the western Kenya highlands, *Malar.J.l*, **15**:76.

Nikbakhtzadeh, M.R., Terbot, J.W., Otienoburu, P.E., Foster, W.A. (2014). Olfactory basis of floral preference of the malaria vector *Anopheles gambiae* (Diptera: Culicidae) among common African plants, *J. Vector Ecol*. **39:**372-383.

Noden, B.H., Kent, M.D., Beier, J.C. (1995). Impact of variations in temperature on early *Plasmodium falciparum* development in *Anopheles stephensi*, *Parasitol*., **111**:539-545

Nyasembe, V.O., Teal, P.E., Mukabana, W.R., Tumlinson, J.H., Torto, B. (2012). Behavioural response of the malaria vector *Anopheles gambiae* to host plant volatiles and synthetic blends, *Parasit. Vectors*, **5:**234.

Ogbunugafor, C.B., Sumba, L. (2008). Behavioural evidence for the existence of a region-specific oviposition cue in *Anopheles gambiae sensu stricto*, *J. Vector Ecol*., **33:**321-324.

Ohba, S.-Y., Kawada, H., Dida, G.O., Juma, D., Sonye, G., Minakawa, N., Takagi, M. (2010). Predators of *Anopheles gambiae sensu lato* (Diptera: Culicidae) larvae inwetlands, Western Kenya: confirmation by polymerase chain reaction method, *J. Med. Entomol*., **47**:783–787.

Okal, M.N., Francis, B., Herrera-Varela, M., Fillinger, U., Lindsay, S.W. (2013). Water vapor is a pre-oviposition attractant for the malaria vector *Anopheles gambiae sensu stricto*, *Malar. J*., **12**:365.

Okumu, F.O., Kiware, *S.S.*, Moore, S.J., Killeen, G.F. (2013). Mathematical evaluation of community level impact of combining bed nets and indoor residual spraying upon malaria transmission in areas where the main vectors are *Anopheles arabiensis* mosquitoes, *Parasit. Vectors*, **6:**17.

O'Meara, G.F. (1987). Nutritional ecology of blood feeding deptera, In: Slansky, F., Rodrguez, J.C. (eds.), *Nutritional ecology of insects, mites and spiders*, New York: Wiley, pp.741-764.

Omoya, F.O., Akinyosoye, F.A. (2013). Evaluation of the potency of some entomopathogenic bacteria isolated from insect cadavers on *Anopheles arabiensis* Giles (Order: Dipthera; Family: *Culicidae*) mosquito larvae in Nigeria, *Afr. J. Microbiol. Res.*, **7**:4877-4881.

Ondiaka, S.N., Masinde, E.W., Koenraadt, C.J., Takken, W., Mukabana, W.R. (2015). Effects of fungal infection on feeding and survival of *Anopheles gambiae* (Diptera: Culicidae) on plant sugars, *Parasit. Vectors*, **8:**1-11.

Ouattara, A., Laurens, M.B. (2015). Vaccines against malaria, *Clin. Inf. Dis.*, **60**:930-936.

Paaijmans, K.P., Jacobs, A.F.G., Takken, W., Heusinkveld, B.G., Githeko, A.K., Dicke, M., *et al*. (2008). Observations and model estimates of diurnal water temperature dynamics in mosquito breeding sites in western Kenya, *Hydrol. Processes*, **22**:4789-4801.

Pascual, M., Ahumada, J.A., Chaves, L.F., Rodo, X., Bouma, M. (2006). Malaria resurgence in the east African highlands: temperature trends revisited, *Proc. Nat. Acad. Sci*., **103**:5829-5834.

Pascual, M., Bouma, M.J. (2009). Do rising temperatures matter, *Ecol.*, **90**:906-912.

Pates, H. (2002). *Zoophilic and anthropophilic behaviour in the Anopheles gambiae complex*. PhD Thesis. London School of Hygien and Tropical Medicine, U.K. pp. 206.

Patt, J.M., Wainright, S.C., Hamilton, G.C., Whittinghill, D., Bosley, K., Dietrick, J., Lashomb, J.H. (2003). Assimilation of carbon and nitrogen from pollen and nectar by a predaceous larva and its effects on growth and development, *Ecol. Entomol*., **28**:17-728.

Patz, J., Githeko, A.K.M., McCarty, J.P., Hussain, S., Confalonieri, U., de Wet, N. (2003). *Climate change and infectious diseases*, In: McMichael, A., Campbell-Lendrum, D., Corvalan, C., Ebi, K., Githeko, A., *et al*. (eds.), *Climate change and human health*: *risks and responses*, World Health Organization, Geneva, Switzerland.

Paucat, A.M. (1965). The functional morphology of the mouth parts of some mosquito larvae, Quest, *Entomol*. **1**:199-220.

Pouniotis, D.S., Proudfoot, O., Minigo, G., Hanley, J.L., Plebanski, M. (2004). Malaria parasite interactions with the human host, *J. Postgrad. Med.*, **50**:30-34.

Protopopoff, N., Matowo, J., Malima, R., Kavishe, R., Kaaya, R., Wright, A., *et al*. (2013). High level of resistance in the mosquito *Anopheles gambiae* to pyrethroid insecticides and reduced susceptibility to bendiocarb in north-western Tanzania, *Malar. J*. **12:**149.

Rafatjah, H.A. (1988). Malaria vector control: environmental management. In: Wernsdorfer, W.H., McGregor, I., (eds.), *Malaria: principles and practices of malariology*. Churchill Livingstone. UK: Edinburgh, pp.1135-1172.

Raffa, K.F., Andersson, M.N., Schlyter, F. (2016). Host selection by bark beetles: playing the odds in a high-stakes game, *Adv. Insect Physiol*., **50**:1-74.

Ramasamy, R., Ramasamy, M.S., Wijsundera, D.A., Dewit, I., Wijesundera, A.P., Dewit, I., *et al*. (1992). High seasonal malaria transmission rates in the intermediate rainfall zone of Sri Lanka, *Ann. Trop. Med. Parasitol.* **86:**591-600.

Ramasamy, R., Surendran S.N. (2012). Global climate change and its potential impact on disease transmission by salinity-tolerant mosquito vectors in coastal zones, *Front. Physiol*., **3**:198.

Rassem, H.H.A., Nour, A.H., Yunus, R.M. (2016). Techniques for extraction of essential oils from plants: a review, *Aust. J. Basic Appl. Sci*; **10**: 117-127.

Raubenheimer, D., Simpson, S.J. (1995). Constructing nutrient budgets, *Entomol. Exp. Appl*., **77**:99-104.

Raubenheimer, D., Simpson, S.J. (1997). Integrative models of nutrient balancing: application to insects and vertebrates, *Nut. Res. Rev*., **10**:151-179.

Raubenheimer, D., Simpson, S.J. (1998). Nutrient transfer functions: the site of integration between feeding behavior and nutritional physiology, *Chemoecol.* **8**:61-68.

Raubenheimer, *D*., Simpson, S.J. (1999). Integrating nutrition: a geometrical approach*, Neth. Entomol. Exp. Appl.*, **91:**67-82.

Raubenheimer, D., Simpson, S.J., Mayntz, D. (2009). Nutrition, ecology and nutritional ecology: toward an integrated framework, *Funct. Ecol*., **1**:4-16.

Redfield, A.C., Ketchum, B.H., and Richards, F.A. (1963). *The influence of organisms on the composition of sea water*. In: M.N. Hill and J. Wiley (eds.), *The sea ideas and observations on progress in the study of the seas*. Vol. 2., pp. 26-77.

Reiskind, M.H., Wilson, M.L. (2004). *Culex restuans* (Diptera: Culicidae) oviposition behavior determined by larval habitat quality and quantity in southeastern Michigan, *J. Med. Entomol.*, **41**:179-186.

Rejmánková, E., Higashi, R., Grieco, J., Achee, N., Roberts, D. (2005). Volatile substances from larval habitats mediate species-specific oviposition in *Anopheles* mosquitoes, *J. Med. Entomol*., **42**:95-103.

Rejmánková, E., Grieco, J., Achee, N., Roberts, D.R. (2013). Ecology of larval habitats. In: Manguin, S. (Eds), *Anopheles mosquitoes - New insights into malaria vectors.* InTech Publisher, Croatia, pp. 397-446.

Ren, X., Hoiczyk, E., Rasgon, J.L. (2008). Viral paratransgenesis in the malaria vector *Anopheles gambiae*, *PLoS Pathog*, **4**:1-8.

Resetarits, W.J., Wilbur, H.M. (1989). Choice of oviposition site by *Hyla chrysoscelis*: role of predators and competitors, *Ecol*., **70**:220-228.

Riveron, J.M., Chiumia, M., Menze, B.D., Barnes, K.G., Irving, H., Ibrahim, *S.S.*, *et al*. (2015). Rise of multiple insecticide resistance in *Anopheles funestus* in Malawi: a major concern for malaria vector control, *Malar. J.* **14:**1-9.

Roitberg, B.D., Gordon, I. (2005). Does the *Anopheles* blood meal - fecundity curve, curve, *J. Vector Ecol*., **30**:83-86.

Roulston, T.H., Cane, J.H., Buchmann, S.L. (2000). What governs protein content of pollen: pollinator preferences, pollen pistil interactions, or phylogeny? *Ecol. Monogr*., **70**: 617-643.

Russell, T.L., Govella, N.J., Azizi, S., Drakeley, C.J., Kachur, S.P., Killeen, G.F. (2011). Increased proportions of outdoor feeding among residual malaria vector populations following increased use of insecticide-treated nets in rural Tanzania, *Malar. J.*, **10**:80.

Russell, T.L., Beebe, N.W., Bugoro, H., Apairamo, A., Chow, W.K., Cooper, R.D., *et al*. (2016). Determinants of host feeding success by *Anopheles farauti, Malar. J*., **15**:152.

Sangoro, O., Kelly, A.H., Mtali, S., Moore, S.J. (2014). Feasibility of repellent use in a context of increasing outdoor transmission: a qualitative study in rural Tanzania, *Malar. J.* **13:**347.

Schlagenhauf, P. (2004): Malaria from pre-history to present. *Infect. Dis. Clin. N. Am.*, **18**:189-205.

Schmidt, J.O., Thoenes, S.C., Levin, M.D. (1987). Survival of honey bees *Apis mellifera* (Hymenoptera: Apidae), fed with various pollen sources, *Ann. Entomol. Soc. Am*., **80**:176-183.

Schoepke, A., Steffen, R., Gratz, N. (1998). Effectiveness of personal protection measures against mosquito bites for malaria prophylaxis in travellers, *J. Travel. Med*. **5**:188-192.

Scholte, E.J., Njiru, B.N. Smallegang, R.C. Takken, W., Knols, B.G.J. (2003). Infection of malaria (*Anopheles gambiae sensu stricto*) and filariasis (*Culex quinquefasciatus*) vectors with the entomopathogenic fungus *Metarhizium anisopliae, Malar. J*., **2:**29.

Scott, J.A., Brogdon, W.G., Collins, F.H. (1993). Identification of single specimens of the *Anopheles gambiae* complex by the polymerase chain reaction, *Am. J. Trop. Med. Hyg.,* **49**:520-529.

Scott, T.W., Takken, W. (2012). Feeding strategies of anthropophilic mosquitoes result in increased risk of pathogen transmission, *Trends Parasitol*., **28:**114-121.

Scott, C.A., Yeshiwondim, A.K. Serda, B., Guinovart, C., Tesfay, B.H., Agmas, A., *et al*. (2016). Mass testing and treatment for malaria in low transmission areas in Amhara Region, Ethiopia, *Malar. J*., **15**:305.

Senn, H., Alattas, N., Boggild, A.K., Morris, S.K. (2014). Mixed-species *Plasmodium falciparum* and *Plasmodium ovale* malaria in a paediatric returned traveler, *Malar. J.*, **13**:78.

Service, M.W. (1996). Studies on sampling larval population of *Anopheles gambiae* complex, *Bull. World Health Org.*, **45**:169-80.

Seyoum, D., Kifle, Y.G., Rondeau, V., Yewhalaw, D., Duchateau, L., Rosas‐Aguirre, A., Speybroeck, N. (2016). Identification of different malaria patterns due to *Plasmodium falciparum* and *Plasmodium vivax* in Ethiopian children: a prospective cohort study*, Malar. J*., **15**:208.

Shapiro, L.L.M., Murdock, C.C., Jacobs, G.R., Thomas, R.J., Thomas, M.B. (2016). Larval food quantity affects the capacity of adult mosquitoes to transmit human malaria, *Proc Biol Sci. R. soc. USA*, **283**:20160298.

Sharma, V.P., Sharma, R.C. and Gautam, A.S. (1986**).** Bio-environmental control of malaria in Nadiad, Kheda district, Gujarat, *Indian J. Malariol*. **23:**95-117.

Shuo, W., Wei, Q., Youyuan, D. (2012). Separation of oil phase from dilute oil/water emulsion in confined space apparatus, *Chin. J. Chem. Eng.*, **20**:239-245.

Silberbush, A., Blaustein, L. (2008). Oviposition habitat selection by a mosquito in response to a predator: are predator-released kairomones air-borne cues? *J. Vector Ecol.*, **33**:208-211.

Silberbush, A., Markman, S., Lewinsohn, E., Bar, E., Cohen, J.E., Blaustein, L. (2010). Predator-released hydrocarbons repel oviposition by a mosquito, *Ecol. Letters*, **13**:1129-1138.

Sinka, M.E., Bangs, M.J., Manguin, S., Coetzee, M., Mbogo, C.M., Hemingway, J., *et al*. (2010). The dominant *Anopheles* vectors of human malaria in Africa, Europe and the Middle East: occurrence data, distribution maps and bionomic précis, *Parasit. Vectors*, **3**:117.

Sinka, M.E., Bangs, M.J., Manguin, S., Rubio-Palis, Y., Chareonviriyaphap, T., Coetzee, M., *et al*. (2012). A global map of dominant malaria vectors, *Parasit. Vectors*, **5**:69.

Siraj, A.S., Santos-Vega, M., Bouma, M.J., Yadeta, D., Carrascal, D.R., Pascual, M. (2014). Altitudinal changes in malaria incidence in highlands of Ethiopia and Colombia, *Science,* **343**:1154-1158.

Smith, T.A., Chitnis, N., Briët, O.T., Tanner, M. (2011). Uses of mosquito-stage transmission-blocking vaccines against *Plasmodium falciparum*, *Trends Parasitol*., **27**:190-196.

Sneller, V.P., Dadd, R.H. (1981). Interaction of amino acids and glucose on growth of *Aedes aegypti* (Diptera: Culicidae) in a synthetic rearing medium, *J .Med. Entomol*., **18**:235-239.

Snow, R.W., Guerra, C.A., Noor, A.M., Myint, H.Y., Hay, S.I. (2005). The global distribution of clinical episodes of *Plasmodium falciparum* malaria, *Nature*, **434**:214-217.

Sonwa, M.M. (2000). *Comparative study of the oils of Cyperus alopecuroides*, *Cyperus papyrus*, *and Cyperus rotundus*, PhD Dissertation. Hamburg, Germany. pp 54-60.

Stone, C.M., Taylor, R.M., Roitberg, B.D., Foster, W.A. 2(009). Sugar deprivation reduces insemination of *Anopheles gambiae* (Diptera: Culicidae), despite daily recruitment of adults, and predicts decline in model populations, *J. Med. Entomol*., **46**:1327-1337.

Stone, C.M., Foster, W.A. (2013). Plant-sugar feeding and vectorial capacity, In: Takken W, Koenraadt C.J.M., (eds.), *Ecology of parasite-vector interactions*. Wageningen Academic Publishers, Vol. 3., pp. 35-79.

Stryker, J.J., Bomblies, A. (2012). The impacts of land use change on malaria vector abundance in a water-limited, highland region of Ethiopia, *Ecol. Health*, **9**:455-470.

Su, T., Mulla, M.S. (2002). Spatial occurrence and hatch of field eggs of the tadpole shrimp *Triops newberryi* (Notostraca: Triopsidae), a potential biological control agent of immature mosquitoes, *J. Vector Ecol*., **27**:128-137.

Sumba, L.A., Guda, T.O., Deng, A.L., Hassanali, A., Beier, J.C., Knols, B.G.J. (2004). Mediation of oviposition site selection in the African malaria mosquito *Anopheles gambiae* (Diptera: Culicidae) by semiochemicals of microbial origin, *Int. J. Trop. Insect Sci*., **24**:260-265.

Sumba, L.A., Ogbunugafor, C.B., Deng, A.L., Hassanali, A. (2008). Regulation of oviposition in *Anopheles gambiae sensu sstricto*: role of inter- and intra-specific signals, *J. Chem. Ecol.,* **34**:1430-1436.

Takken, W., Knols, B.G.J. (1999). Odour mediated behavior of Afro tropical malaria mosquitoes, *Annu. Rev. Entomol.*, **44**:57.

Takken, W., Smallegange, R.C., Vigneau, A.J., Johnston, V., Brown, M., Mordue-Luntz, A.J., Billingsley, P.F. (2013). Larval nutrition differentially affects adult fitness and

Plasmodium development in the malaria vectors *Anopheles gambiae* and *Anopheles stephensi*, *Parasit. Vectors*, **6**:345.

Takken, W., Verhulst N.O. (2013). Host preferences of blood-feeding mosquitoes, *Annu. Rev. Entomol.*, **58**:433-453.

Terenius, O., Marinotti, O., Sieglaff, D., James, A.A. (2008) Molecular genetic manipulation of vector mosquitoes, *Cell Host Microbe,* **4**:417-423.

Timmerman, S.E., Briegel, H. (1999). Larval growth and biosynthesis of reserves in mosquitoes, *J. Insect Physiol*., **45**:461-470.

Torres-Estrada, J.L., Meza-Alvarez, R.A., Cibri´an-Tovar, J. (2005). Vegetation-derived cues for the selection of oviposition substrates by *Anopheles albimanus* (Diptera: Culicidae) under laboratory conditions, *J. Am. Mosq. Control Assoc.*, **21**:344-349.

Torres-Estrada, J.L., Meza- ´ Alvarez, R.A., Cruz-L´opez, L., Rodr´ıguez, M.H., Arredondo-Jim´enez, J.I. (2007). Attraction of gravid *Anopheles pseudopunctipennis* females to oviposition substrates by *Spirogyra majuscula* (Zygnematales: Zygnmataceae) algae under laboratory conditions, *J. Am. Mosq. Control Assoc.*, **23**:18-23.

Toure, Y.T., Oduola, A.M.J., Morel, C.M. (2004). The *Anopheles gambiae* genome: next steps for malaria vector control, *Trends Parasitol*., **20**:143-149.

Tripet, F., Dolo, G., Traore, S., Lanzaro, G.C. (2004). The "wingbeat hypothesis" of reproductive isolation between members of the *Anopheles gambiae* complex (Diptera: Culicidae) does not fly, *J. Med. Entomol*., **41:**375-84.

van Breugel, F., Riffell, J., Fairhall, A., Dickinson, M.H. (2015). Mosquitoes use vision to associate odour plumes with thermal targets, *Curr. Biol.* **25:**2123-2129.

van Loon, J.J., Smallegange, R.C., Bukovinszkiné-Kiss, G., Jacobs, F., De Rijk, M., Mukabana, W.R., *et al*. (2015). Mosquito attraction: crucial role of carbon dioxide in formulation of a five-component blend of human-derived volatiles, *J. Chem. Ecol.* **41:**567-573.

Vantaux, A., Lefèvre, T., Cohuet, A., Dabiré, K.R., Roche, K., Roux, O. (2016). Larval nutritional stress affects vector life history traits and human malaria transmission *Sci. Rep*., **6**:36778.

Vanwambeke, S.O., Somboon, P., Harbach, R., Isenstadt, M., Lambin, E.F., Walton, C., *et al*. (2007). Landscape and land cover factors influence the presence of *Aedes* and *Anopheles* larvae, *J. Med. Entomol*., **44**:133-144.

Vijverberg, J., Sibbing, F.A., Dejen, E. (2009). Lake Tana: source of the Blue Nile. In: Dumont HJ, (ed.), *The Nile*: *Monographiae Biologicae*, Netherlands Springer, Vol. 89., pp. 163-192.

Vythilingam, I., Lim, Y.A.L., Venugopalan, B., Ngui, R., Leong, C.S., Wong, M.L., *et al*. (2014). *Plasmodium knowlesi* malaria an emerging public health problem in Hulu Selangor, Selangor, Malaysia (2009–2013): epidemiologic and entomologic analysis, *Parasit. Vectors*, **7**:436.

Walker, K., Lynch, M. (2007). Contributions of *Anopheles* larval control to malaria suppression in tropical Africa: review of achievements and potential, *Med. Vet. Entomol.*, **21**:2-21.

Walton, W. (2005). Protocol for mosquito sampling for mosquito best management practices on State of California-managed wildlife areas, developed for the integrated pest management committee of the mosquito and vector control association of California, Sacramento, USA.

Webster, B., Lacey, E.S., Cardé, R.T. (2015). Waiting with bated breath: opportunistic orientation to human odor in the malaria mosquito, *Anopheles gambiae*, is modulated by minute changes in carbon dioxide concentration, *J. Chem. Ecol*., **49:**55-66.

Webster, B., Cardé, R.T. (2016). Use of habitat odour by host-seeking insects, *Biol. Rev. Camb. Philos. Soc.*, **92**:1241-1249.

West, P.A., Protopopoff, N., Wright, A., Kivaju, Z., Tigererwa, R., Mosha, F.W., *et al*. (2014). Indoor residual spraying in combination with insecticide-treated nets compared to insecticide-treated nets alone for protection against malaria: a cluster randomised trial in Tanzania, *PLoS Med*., **11:**1001630.

White, G.B. (1985). *Anopheles bwambae* sp.n., a malaria vector in the Semliki Valley, Uganda, and its relationships with other sibling species of the *An. gambiae* complex (Diptera: Culicidae), *Syst. Entomol*., **10**:501-522.

White, N.J. (2004). Antimalarial drug resistance, *J. Clin. Inv.*, **113**:1084-1092.

White, M.T, Griffin, J.T, Churcher, T.S., Ferguson, N.M., Basáñez, M.G, Ghani, A.C, *et al*. (2011). Modelling the impact of vector control interventions on *Anopheles gambiae* population dynamics, *Parasit. Vectors*, **4**:153.

WHO. (1982). *Manual on Environmental Management for mosquito control with special emphasis on malaria vectors*. World Health Organization, Geneva, Switzerland.

WHO. (2005). *Guidelines for laboratory and field testing of mosquito larvicides*. World Health Organization, Geneva, Switzerland.

WHO, (2006). *Pesticides and their application: for the control of vectors and pests of public health importance*, 6th edition. World Health Organization, Geneva, Switzerland.

WHO. (2012). *Global plan for insecticide resistance management in malaria vectors*. World Health Organization, Geneva, Switzerland.

WHO. (2014). *World Malaria Report*. World Health Organization, Geneva, Switzerland.

WHO. (2015a). *Global technical strategy for malaria (2016-2030)*. World Health Organization, Geneva, Switzerland.

WHO. (2015b). *World Malaria Report*. World Health Organization, Geneva, Switzerland.

WHO. (2016). *World Malaria Report*. World Health Organization, Geneva, Switzerland.

Wimberly, M.C., Midekisa, A., Semuniguse, P., Teka, H., Henebry, G.M., Chuang, T.W., Senay, G.B. (2012). Spatial synchrony of malaria outbreaks in a highland region of Ethiopia, *Trop. Med. Int. Health*, **17:**1192-1201.

Wirth, M.C., Walton, W.E., Federici, B.A. (2010). Evolution of resistance to the *Bacillus sphaericus* Bin toxin is phenotypically masked by combination with the mosquitocidal proteins of *Bacillus thuringiensis* subspecies *israelensis*, *Environ. Microbiol*. **12:**1154-1160.

Wondwosen, B., Birgersson, G., Seyoum, E., Tekie, H., Torto, B., Fillinger, U., Hill, S.H., Ignell, R. (2016). Rice volatiles lure gravid malaria mosquitoes, *Anopheles arabiensis*, *Sci. Rep.*, **6**:37930.

Wondwosen, B., Hill, S.R., Birgersson, G., Seyoum, E., Tekie, H., Ignell, R. (2017). A (maize)ing attraction: gravid *Anopheles arabiensis* are attracted and oviposit in response to maize pollen odours, *Malr. J.*, **16**:39.

Xu, T., Zhou, Q., Xia, Q., *et al.* (2002). Effects of herbivore-induced rice volatiles on the host selection behavior of brown planthopper, *Nilaparvata lugens*, *Chin. Sci. Bull.*, **47**:1355-1360.

Yamana, T.K., and Eltahir, E.A.B. (2013). Incorporating the effects of humidity in a mechanistic model of *Anopheles gambiae* mosquito population dynamics in the Sahel region of Africa, *Parasit. Vectors*, **6**:235.

Yan, Z.G., Wang C.Z. (2006). Similar attractiveness of maize volatiles induced by *Helicoverpa armigera* and *Pseudaletia separate* to the generalist parasitoid *Campoletis chlorideae, Ent. Exp. Appl.*, **118**:87-96.

Yap, H.H. (1985). Biological control of mosquitoes, especially malaria vectors, *Anopheles* species. *Southeast Asian J. Trop. Med. Public Health,* **16:**163-172.

Yasuoka, J., Levins, R., Mangione, T.W., Spielman, A. (2006). Community-based rice ecosystem management for suppressing vector anophelines in Sri Lanka, *Trans R Soc. Trop. Med. Hyg*., **100:**995-1006.

Ye-Ebiyo, Y., Pollack, R.J., Spielman, A. (2000). Enhanced development in nature of larval *Anopheles arabiensis* mosquitoes feeding on maize pollen, *Am. Soc. Trop. Med. Hyg*., **63**:90-93.

Ye-Ebiyo, Y., Pollack, R.J., Kiszewski, A., Spielman, A. (2003a). Enhancement of development of larval *Anopheles arabiensis* by proximity to flowering maize *(Zea mays)* in turbid water and when crowded*, Am. J. Trop. Med. Hyg*., **68**:748-752.

Ye-Ebiyo, Y., Pollack. R.J., Kiszewski, A., Spielman. A. (2003b). A component of maize pollen that stimulates larval mosquitoes (Diptera: Culicidae) to feed and increases toxicity of microbial larvicides, *J. Med. Entomol*., **40**:860-864.

Yewhalaw, D., Wassie, F., Steurbaut, W., Spanoghe, P., Bortel, V.W., Denis L.W., *et al*. (2011). Multiple insecticide resistance: an impediment to insecticide-based malaria vector control program, *PLoS One*, **6:**e16066.

Yohannes, M., Haile, M., Ghebreyesus, T.A, Witten, K.H., Getachew, A., Bayass, P, *et al*. (2005). Can source reduction of mosquito larval habitat reduce malaria transmission in Tigray, Ethiopia? *Trop. Med. Int. Health*, **10**:1274-1285.

Young, G.B., Golladay, S., Covich, A., Blackmore, M. (2014). Stable isotope analysis of larval mosquito diets in agricultural wetlands in the coastal plain of Georgia, U.S.A., *J. Vector Ecol*. **39**:234-456.

Zhu, L., Qualls, W., Marshall, J.M., Arheart, K.L., DeAngelis, D.L., McManus, J.W., *et al*. (2015). A spatial individual based model predicting a great impact of copious sugar sources and resting sites on survival of *Anopheles gambiae* and malaria parasite transmission, *Malar J*., **14**:59.

Appendices

Appendix A. Average adult emergence of larval *Anopheles arabiensis* fed on the basic diet fish food over dose series.

Percent adult emergence				
Dose	0.5 mg	0.75 mg	1 mg	1.5 mg
Day I average	40	50	60	100
Day II average	40	50	70	100
Day III Average	50	60	80	100
All day average	43.333	53.333	70	100
Standard Error	1.05409	1.054093	1.82574	0

Appendix B. Linear regression showing effects of amount of larval diet (Tetramin® fish food) on adult emergence in *Anopheles arabiensis* under laboratory condition.

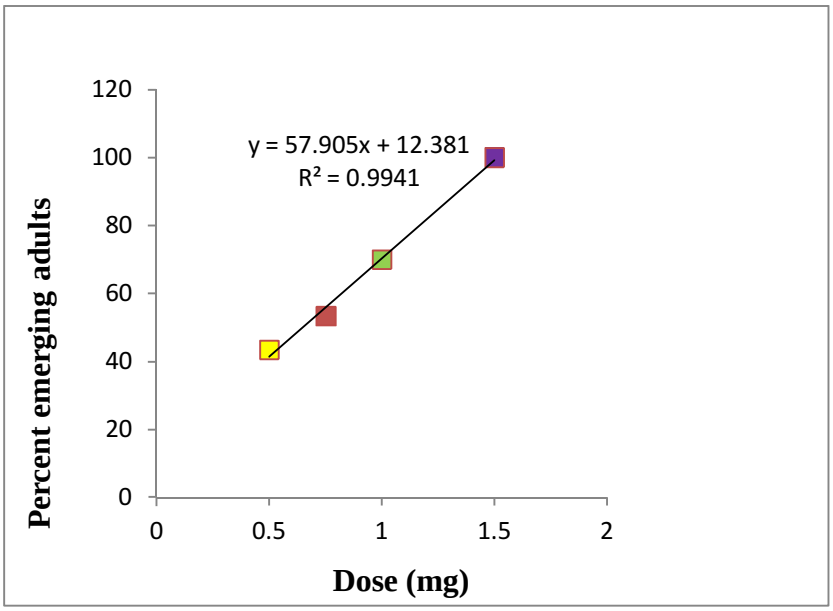

Plate A. larval sampling in natural breeding habitats dominated by grasses.

Plate B. Multichoice oviposition bioassay setup: experiment tent arrangement in temperature and moisture regulated green house.

Plate C. The treatment and control cups arranged in experimental tent for testing oviposition preferences of *An. coluzzii* and *An. arabiensis* to grass volatiles.

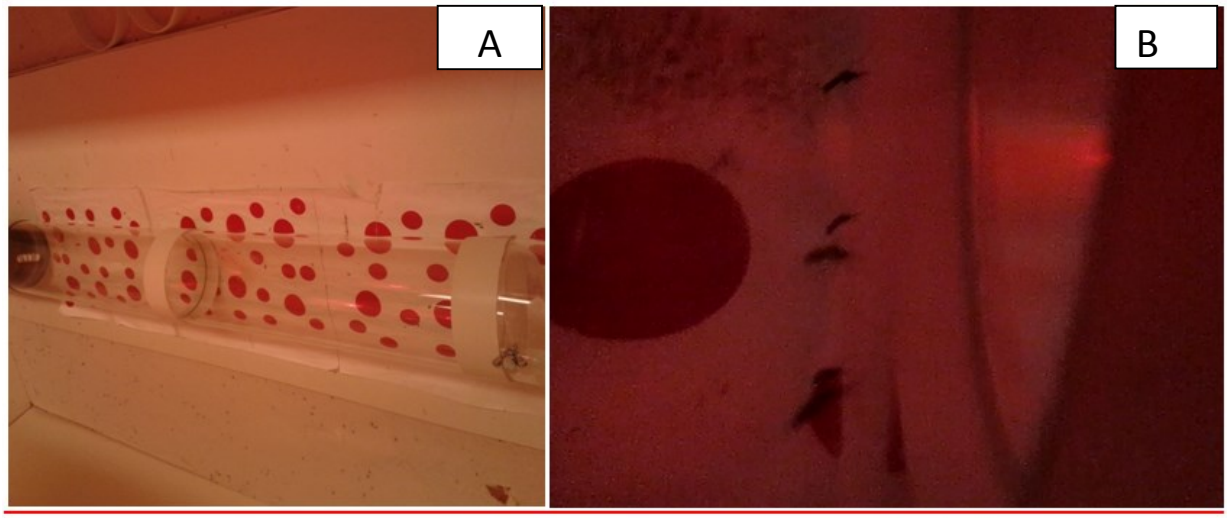

Plate D. wind tunnel, attraction bioassay setup (A), gravid mosquitoes flying towards attractant grass volatile (B).