Addis Ababa University

Graduate Programme

Species Composition, Distribution and Ecology of *Anopheles* Mosquitoes in Relation to Malaria Transmission and Control in Dembiya District, Northwestern Ethiopia

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A Dissertation Submitted to the Graduate Programme of the Addis Ababa University for the partial fulfillment of the requirements for the Degree of Doctor of Philosophy in Zoological Sciences (Insect Sciences)

May 2022

Addis Ababa, Ethiopia

Addis Ababa University

Graduate Programme

I, the undersigned, declare that this thesis entitled as "**Species Composition, Distribution and Ecology of *Anopheles* Mosquitoes in Relation to Malaria Transmission and Control in Dembiya District, Northwestern Ethiopia**" is my original work, and it has never been presented anywhere else. All sources of material used in this thesis are duly acknowledged.

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Abstract

Malaria is an important vector borne disease transmitted by the infective bite of female *Anopheles* mosquitoes in malaria endemic areas in Ethiopia. Malaria vector control requires field and clinical data on malaria transmission and ecology of local vectors. A six-year retrospective malaria data set from health facilities was analyzed to determine trends in malaria prevalence in the two malaria-prone areas of Dembiya District, Northwestern Ethiopia. A cross-sectional parasitological study was conducted to determine the prevalence of malaria during the peak transmission season in the two Kebeles of Dembiya district.

A longitudinal entomological study on the species composition and ecology of adult and immature *Anopheles* mosquitoes was conducted from June 2018 to May 2019. Larvae and pupae of *Anopheles* mosquitoeswere collected from different mosquito breeding habitats using a 350 ml standard dipper, and physicochemical characteristics of the larval breeding habitats were measured in conjunction with larval sampling. CDC light traps, pyrethrum spray catches (PSCs) and artificial pit shelters were used to collect host seeking and resting *Anopheles* mosquitoes from indoors and outdoors. Using morphological keys, collected *Anopheles* mosquitoes were identified to the species level and *An. gambiae s.l* (*sensu lato*) were further identified to sibling species using polymerase chain reaction (PCR). Enzyme-linked immunosorbent assay (ELISA) was used to examine the blood meal source of blood fed *Anopheles* mosquitoes, and to detect

Plasmodium species using circum-sporozoite proteins (CSP). A WHO test tube bioassay was used to assess the susceptibility status of *Anopheles arabiensis* to four insecticides such as pyrethroids, deltamethrin, bendiocarb and fenitrothion.

Malaria is endemic in the area according to retrospective malaria data from health facilities. Over the past six-years, the overall prevalence of malaria cases was 22.4% (484/2157). *Plasmodium falciparum* was responsible for 75.1 % (367/484) of the malaria cases in the study area, while *P. vivax* was responsible for 18.2% (88/484) the malaria cases. The remaining 5.9% (29/484) were mixed infections. Malaria parasites were found in 3.5% (26) of 735 blood smears stained with 3% geimsa and microscopically examined slides. *Plasmodium falciparum* and *P. vivax* were responsible for 65% (17) and 19% (5/26) of the malaria infection, respectively, with 15% (4/26) being mixed infections. Males (18/382; 4.7%) were 2.6 times more likely to be infected with malaria than females (8/353; 2.3%) (AOR = 2.6; 95% CI: 1.041- 6.412; p= 0.041).

A total of 1,629 *Anopheles* larvae and 185 pupae were collected from different breeding habitats. Eight species of *Anopheles* mosquitoes were identified from female mosquitoes that emerged from field collected larvae and pupae, including *Anopheles arabiensis*, *An. pharoensis*, *An. coustani*, *An. christyi*, *An. squamosus*, *An. demeilloni*, *An. danicalicus* and *An. cinereus*. *Anopheles arabiensis* (59.2%) was the most common followed by *An. pharoensis* (35.3%).

Anopheles mosquitoes belonging to 11 species were identified from 2,055 field collected adult specimens during this study: *An. pharoensis, An. arabiensis*, *An. coustani*, *An. demeilloni*, *An. cinereus, An. funestus, An. ardensis,* and *An. squamosus* were identified from both Guramba Bata and Arebiya study sites, whereas *An. garnhami, An. christyi* and

An. nili were identified only from Guramba Bata. *Anopheles pharoensis* was the dominant species identified in both Arebiya and Guramba Bata study sites, accounting for 46.4% (953/2,055), while *An. Arabiensis* was also relatively dominant in both study sites (38.3%; 776/2055).

Anopheles larvae were more abundant in drainage canals (14.7 ± 3.5 larvae/dip) than in other types of breeding habitats such as river side pools (2.0 ± 0.9), hoof prints (3.0 ± 1.2), swamps (3.8 ± 1.2), and puddles (2.7 ± 2.7) ($F_{8,99} = 9.85$; $p<0.001$). The presence or absence of *Anopheles* larvae was associated with physical characteristics of larval breeding habitats such as turbidity (mid turbid) (AOR = 66.03; 95% CI: 2.01-2168.24, $p= 0.019$) and presence of grass (AOR= 12.62; 95% CI: 1.29-122.78, $p= 0.029$).

The mean outdoor density of *Anopheles* mosquitoes collected with a CDC light trap (4.8 ± 1.8 mosquitoes/trap/night) was slightly higher than mean indoor density of *Anopheles* mosquitoes (4.3 ± 1.7 mosquitoes/trap/night) in Arebiya study site. Similarly, in Guramba Bata, the mean density of outdoor *Anopheles* mosquito collected with CDC light trap (8.1 ± 2.6 mosquitoes/trap/night) was higher than indoor *Anopheles* mosquito density (5.5 ± 1.7 mosquitoes/trap/night). The human blood indices (HBI) of indoor and outdoor hosts seeking *An. arabiensis* were 17.4% and 15.3%, respectively. The overall sporozoite rate of *An. arabiensis*, *An. pharoensis* and *An. coustani* was 0.3%, 0.9% and 5.9%, respectively. Whereas, the annual Entomological inoculation rate (EIR) of outdoor hosts seeking *An. arabiensis*was 4.7 infective bites/person/year. *Anopheles arabiensis* was resistant to deltamethrin and permethrin.

In conclusion, the dominant *Anopheles* vector species in the study area were *An. arabiensis* and *An. pharoensis*.The season, and type and physicochemical characteristics

of the breeding habitats, influenced the distribution of *Anopheles* mosquitoes. *Anopheles arabiensis*, *An. pharoensis* and *An. coustani* showed relatively strong exophilic, exophagic and zoophilic tendencies in the study area, which was likely influenced by decades of indoor malaria interventions with IRS and ITNs. *Anopheles arabiensis* has developed resistance to pyrethroids and deltamethrin that have been used over the years. As a result, malaria control and elimination programmes should target outdoor biting and resting *Anopheles* mosquitoes with appropriate resistance management measures.

Keywords: Malaria, larvae, behaviour, susceptibility, *Plasmodium vivax*, *Plasmodium falciparum*, *Anopheles arabiensis*, *Anopheles pharoensis,* Dembiya*,* blood meal index, entomological inoculation rate.

Dedication

This PhD thesis is dedicated to my mother Yeshalem Sertsu, for her unreserved support and love, and to my family.

Acknowledgments

First and foremost, I would like to express my gratitude to Dr. Habte Tekie, Department of Zoological Sciences, Addis Ababa University (AAU), who has assisted me from the conception of this study to its completion. He has continuously followed up on my work and provided constructive comments as well as financial assistance. I would like to thank Dr. Yitbarek W/Hawariat, Department of Zoological Sciences, AAU, for accepting me as a PhD student, as well as for all the help, constructive feedback and friendly attitudethroughout this research. I appreciate his constant supervision and financial assistance. I would also like to extend my thanks to Dr. Sisay Dugassa, the Aklilu Lemma Institute of Pathobiology, Addis Ababa University, for his constant supervision throughout this study. I appreciate his constructive comments, financial support and laboratory facilitation for Enzyme Linked Immunosorbent Assay (ELISA) and Polymerase Chain Reaction (PCR) laboratory work.

I also want to thank Mr. Sitotaw Yalew, Sister Senayt Alemnew, Animut Shiferaw, Destaw Minale and Sister Toyba Ahmed fromKoladiba Town Health Center. The workers at the Guramba Bata and Arebiya health center, as well as everyone else in the two communities who assisted me with sample collection are also thanked.

I sincerely thank Dr. Bezawork Afework, Head of the Department of Zoological Sciences and Professor Abebe Getahun (the former head of the department) for facilitating official works. I would like to thank Mr. Yohannes Negash and Mr. Wossen Sisay for their assistance during ELISA and PCR work at Aklilu Lemma Institute of Pathobiology. My appreciation also goes to Dr. Yared Debebe, Dr. Desta Ejeta, Alemnesh Hailemariam, Yosef Baraki, Meseret Muche, Gizachew Teshome, and Kalkidan Degefaw for their

technical assistance and constructive feedback. Acknowledged institutions include the Aklilu Lemma Institute of Pathobiology, Addis Ababa University, Woldia University, Koladiba City Health Office, and Biodefense and Emerging Infections Research (BEI) for using their resources. Finally, I want to express my warmest affection to my family (mother, brothers, sisters, and my wife) for their unreserved love, supportand a gift of education. Finally, thank Lord Jesus for all the blessings!

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List of Abbreviations and Acronyms

AChE	Acetylcholinesterase
ACT	Artemisinin based combination therapy
ANOVA	Analysis of variance
AOR	Adjusted odds ratio
BBI	Bovine blood index
CDC	Center for Disease Prevention and Control
CI	Confidence interval
COR	Corrected odds ratio
CSA	Central Statistical Agency
CSP	Circum- sporozoite proteins
DALY	Disability Adjusted Life Year
DDT	Dichlorodiphenyl trichloroethane
DNA	Deoxyribonucleic acid
dsRNA	Double-stranded RNA
EIR	Entomological inoculation rate
ELISA	Enzyme-linked immunosorbent assay
FMoH	Federal Ministry of Health
HBI	Human Biting Index
HEGs	Homing endonuclease genes
IRS	Insecticide residual spray
ITNs	Insecticide treated nets
Kdr	Knockdown resistance

LLINs	Long lasting insecticidal nets
OPs	Organophosphates
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PMI	President's Malaria Initiative
PSC	Pyrethrum spray catch
RDT	Rapid Diagnostic Test
RIDL	Release of Insects carrying a Dominant Lethal Gene
RNA	Ribonucleic acid
RNAi	Ribonucleic acid interference
SIT	Sterile Insect Technique
WHO	World Health Organization

Chapter 1: General Introduction

1.1. Overview of the global malaria burden

Malaria is a vector-borne disease caused by protozoa (*Plasmodium* species) which is transmitted by an infective bite of female *Anopheles* mosquitoes (Service, 2012). Globally, a total of 241 million of malaria cases and 627, 000 deaths were reported in 2020, which represents about 14 million more cases and 69,000 more deaths compared to 2019 (WHO, 2021). Of which, the highest burden of malaria is recorded in sub-Saharan Africa, where about 95% of all malaria cases and 96% of all deaths is recorded in 2020 (WHO, 2021). This towering of malaria case and death in sub-Saharan Africa and across the Globe is due to COVID-19 pandemic, which disrupts the malaria prevention and control services (Heuschen *et al*., 2021; WHO, 2021; Heuschen *et al*., 2022).

Five species of *Plasmodium* parasites: *Plasmodium falciparum, P. vivax, P. ovale*, *P. malariae* and *P. knowlesi* (zoonotic) are proved to cause human malaria (Singh and Daneshvar, 2013). From which, *P. falciparum* is the most prevalent malaria parasite in the WHO African Region, accounting for 99.7% of estimated malaria cases in 2017, as well as in the WHO regions of Southeast Asia (62.8%), the Eastern Mediterranean (69%) and the Western Pacific Regions (71.9%). *Plasmodium vivax* is the predominant parasite in the WHO region of the Americas, representing 74.1% of malaria cases (WHO, 2018).

Worldwide, there are more than 400 different species of *Anopheles* mosquitoes of which around 30 are the major malaria vectors (WHO, 2018). In sub-Saharan Africa, there are over 140 species of *Anopheles* mosquitoes, of which less than 20 are incriminated as a malaria vector (Afrane *et al*., 2016). In Ethiopia *Anopheles arabiensis* (a member of

An.gambiae s.l complex) is the predominant vector and *An. pharoensis*, *An. funestus* and *An. nili* serve a minor role in malaria transmission (FMoH, 2017).

Over the past two decades, effective use of core vector management strategies (insecticide-treated nets (ITNs) and indoor residual spray (IRS)), and antimalarial drugs led to a significant reduction in the Global malaria prevalence (Bhattarai *et al*., 2007; WHO, 2021). Similarly, in sub-Saharan Africa an amplified distribution of the aforementioned malaria intervention strategies have had a significant effect on rolling back malaria prevalence (Badmos *et al*., 2021). However, poor socioeconomic status of the people, suitability of the environment for rapid development of *Anopheles* mosquitoes and *Plasmodium* parasites, development of insecticide resistance malaria vectors and drug resistant *Plasmodium* parasites, hindered malaria control programme in sub-Saharan Africa (Badmos *et al*., 2021).

In Ethiopia, the key malaria intervention strategies depends on the use of long- lasting insecticide treated bed nets (LLINs), indoor residual spray (IRS), rapid diagnostic test (RDT), and effective case management using artemisinin based combination treatment (Jima *et al*., 2010). However, the national malaria control and elimination strategy is challenged by the development of behavioural and molecular resistance of malaria vectors in different locality (Taffese *et al*., 2018). Therefore, a continuous monitoring of malaria status, vector ecology, behaviour and insecticide susceptibility status of the primary vectors is important to design effective malaria control and elimination strategy.

1.2. Statement of the problem and justification

In Ethiopia, areas below 2000 m are considered as malarious and targeted to receive malaria intervention strategies such as long lasting insecticide-treated nets (LLINs), indoor residual spray (IRS), rapid diagnostic tests (RDTs) and effective case management with artemisinin-based combination therapy (ACT) (Jima *et al*., 2010). In the country, the major scale up of malaria intervention such as wider distribution of RDTs, active case detection, LLINs, and IRS began in 2005 (WHO, 2017). The scale up distribution of malaria intervention strategies led to a significant reduction of malaria in Ethiopia (Taffese *et al*., 2018). Considering these achievements the government has set an ambitious goal to eliminate malaria by 2030 from the country (FMoH, 2017).

Despite the increased implementation of malaria intervention strategies in Ethiopia malaria remains one of the leading public health problems, where more than 68% of the population is still at risk of malaria infection (FMoH, 2017). This could be because of the development of insecticide resistance (behavioural and molecular) in the major malaria vectors. In addition, it may be related to the behavioural change of the host or due to environmental modification, which enhances malaria transmission. Studies indicated that in different part of Ethiopia, *An. arabiensis* has developed resistance to a number of insecticides including DDT, permethrin, deltamethrin, and malathion (Hemingway and Ranson, 2000; Yewhalaw *et al*., 2011; Balkew *et al*., 2012). A shift in biting activities of *An. arabiensis* from late to early evening hours before people retire to bed was also reported in the country (Kibret and Wilson, 2016). Furthermore, the manipulation of the environment for agricultural activities, water reservoirs and electric dame also intensified

malaria case in Ethiopia (Yewhalaw *et al*., 2009; Kibret *et al*., 2010; Yewhalaw *et al*., 2013; Kibret *et al*., 2014; Kibret *et al*., 2017).

In 1952/53 Dembiya district experienced one of the most dreadful malaria epidemics, which wiped out more than 7000 people and caused a significant economic damage (Ayele, 2017). In the district vector control strategies using DDT have been implemented starting from 1959 (Tulu, 1996). Similar to other malaria endemic areas of the country, the scale up distribution of vector control strategy including case diagnosis and management, distribution of long-lasting insecticidal nets (LLIN) and indoor residual spraying (IRS) resulted a significant reduction of malariain the district (Toyama *et al*., 2016). Despite, a reduction in malaria prevalence, it remains one of the leading causes of morbidity in the district (Alemu *et al*., 2012). Limited reports are available on the malaria transmission dynamics in Dembia, irrespective of the long term and increased implementation of malaria intervention strategies.

Thus, this study was designed to assess malaria prevalence and associated risk factors, which contributed for malaria transmission in the two Kebeles of Dembiya district. The dissertation also discussed the species composition, ecology, and behaviour of *Anopheles* mosquitoes in the study area. In addition, this study addressed the physicochemical characteristics of potential breeding habitats of *Anopheles* mosquitoes and insecticide susceptibility status of *An. arabiensis* in malaria endemic Kebeles of Dembiya district.

1.3. Significance of the study

The results of this study will have a paramount importance in designing effective vector control strategies considering the local vector ecology, behaviour and susceptibility status. Similarly, the result of this study will also provide important information to design vector control strategies targeting the potential breeding habitat of *Anopheles* mosquitoes by disrupting the vector life cycle.

1.4. Objectives of the study

1.4.1. General objectives

- The general objective of this study was to determine the species composition, ecology and behaviour of *Anopheles* mosquitoes in relation to malaria transmission and control in Dembiya district, northwestern Ethiopia.

1.4.2. Specific objectives

- To assess the prevalence of malaria and the associated risk factors in selected Kebeles of the study area.
- To assess the spatio-temporal distribution and physicochemical characteristics of *Anopheles* mosquitoes breeding habitat.
- To evaluate the species composition, distribution and behaviour of adult *Anopheles* mosquitoes (blood feeding, host seeking, and resting).
- To determine insecticide susceptibility status of *An. arabiensis*.

Chapter 2: Literature Review

2.1. Bionomics and behaviour of *Anopheles* mosquitoes

Anopheles mosquitoes are the most important insect groups because they are vectors of protozoan, viral, and nematode parasites (Service, 2012). From which malaria is one of the most important public health diseases transmitted by the infective bite of female *Anopheles* mosquitoes. The life history and behavior of *Anopheles* mosquitoes determine their vectoral capacity (Massey*et al*., 2016). Therefore, understanding the life cycle, biting time, biting location, resting place, and host preference of *Anopheles* mosquitoes is crucial for design effective vector control strategies.

The most important part of *Anopheles* mosquito's life cycle such as egg-laying, larval and pupal development and adult emergence take place in the aquatic environment (Oyewole *et al*., 2009). The abundance, type, and distribution of *Anopheles* mosquitoes breeding habitats determine the abundance of adult *Anopheles* mosquitoes and hence malaria transmission (Rejmánková *et al*., 2013). Therefore, a study, on the identification and characterization of *Anopheles* mosquitoes breeding habitat is important to design effective vector control strategies.

Anopheles funestus and *An. gambiae s.s* are highly susceptible to indoor-based control strategies such as IRS and LLINs because of their anthropophilic, endophilic, and endophagic behavior (Sinka *et al*., 2010). On the contrary, malaria vectors with opportunistic behaviour such as *An. arabiensis* are not easily controlled by indoor based vector control strategies (Sinka *et al*., 2010). This suggests a vector control strategy should consider vector ecology, feeding, resting, mating and reproduction behaviour. For

instance vector control strategies which solely depend on indoor based control strategies in areas where *An. arabiensis* is dominant wouldn't be enough to control malaria. Starting from the next section, we will discuss the oviposition behaviours, life cycle, and other most important behaviour of *Anopheles* mosquitoes in relation to vector control strategies.

2.1.1. Oviposition and life cycle of *Anopheles* mosquitoes

Egg, larvae, pupae, and adult are part of *Anopheles* mosquitoes life cycle (Figure 2.1). Developmental period of the aquatic stages such as egg, larvae and pupae lasts 5-14 days, depending on the species and the ambient temperature. In dry seasons, the number and size of larval habitats significantly reduce and contribute to a low population of adult *Anopheles* mosquitoes (Minakawa *et al*., 1999; Himeidan *et al*., 2009; Govoetchan *et al*., 2014). Therefore, targeting the immature stages of *Anopheles* mosquitoes during the dry season when the number of potential breeding habitat is low, which could result a significant reduction of vector density. An adult female *Anopheles* mosquito lays 50-200 eggs per oviposition, singly on water (Service, 2012). The eggs are boat shaped and have floats on either side. Eggs hatch into larvae within 2-3 days, although hatching may take up to 2-3 weeks in colder climates. The larvae feed on algae, bacteria, and other microorganisms in the surface microlayer (Emidi *et al*., 2017). The larvae will develop in to pupae after going through a four subsequent developmental stages or larval instars. The pupa is a comma-shaped, non- feeding developmental stage of *Anopheles* mosquitoes. After a few days as a pupa, the dorsal surface of the cephalothorax splits and the adult mosquito emerges (Williams and Pinto, 2012).

Chemical cues and some physical factors direct the oviposition site selection of adult females *Anopheles* mosquitoes (Himeidan *et al*., 2013). Considering this, novel gravid *An. arabiensis* attractants were developed from rice (Wondwosen *et al*., 2016), maize (Wondwosen *et al*., 2017), sugar cane (Wondwosen *et al*., 2018) and other grass volatiles (Asmare *et al*., 2017). These products were effective at laboratory and semi fieldconditions, however, a further evaluation of their effectiveness on field scale is waiting for an additional study. Understanding the ecology and physicochemical characteristics of *Anopheles* mosquitoes breeding habitat is important to design effective malaria intervention strategies by interrupting the vector life cycle.

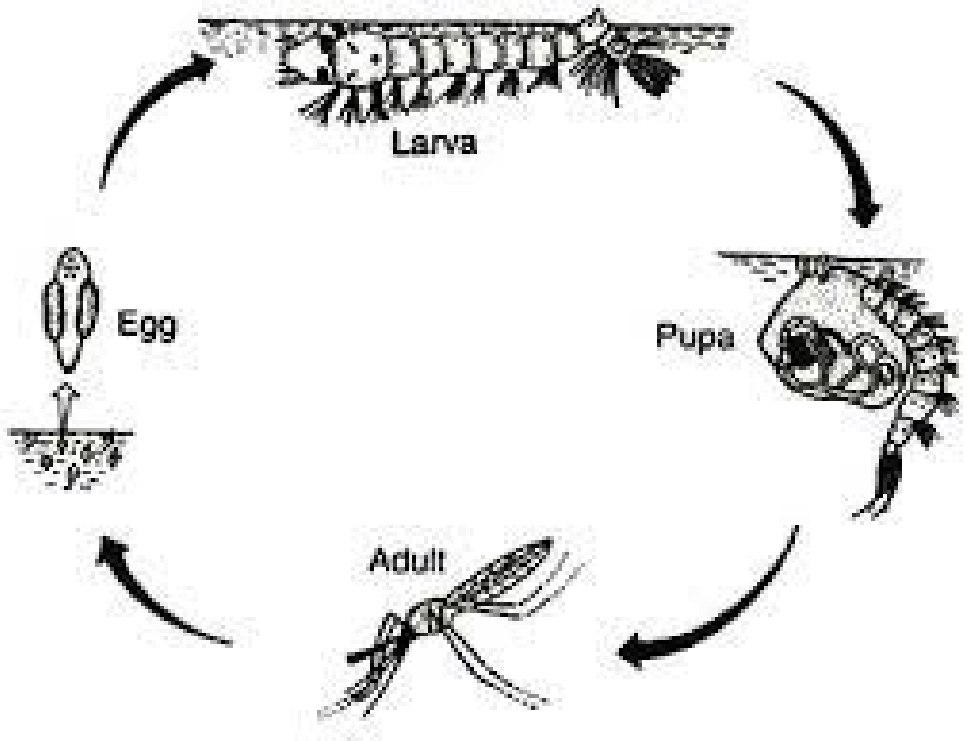

Figure 2.1. A diagrammatic representation of *Anopheles* mosquitoes life cycle (Source: https://www.researchgate.net/publication/329466124).

2.1.2. Mating behaviour of *Anopheles* mosquitoes

Anopheles mosquitoes reproduce sexually to maintain the continuation of their generation. The newly emerged male mosquitoes are unfit for coupling with a female, as the external genitalia require a morphological change. The inversion of the terminalia within the first 24 hr following emergence enable males ready for mating (Clements, 1999). Females are ready to mate almost after they emerge from the pupal stage. Normally, female *Anopheles* mosquitoes mate after taking a blood meal, which is essential for the development of her egg (anautogenous development) (Clements, 1999; Service, 2012). However, few mosquito species can develop the first batch of egg without blood meal (autogenous) (Service, 2012). Mating in mosquitoes usually take place in swarms. Markers such as woodpiles, trash piles, wells, footpaths and grasses marked the male *Anopheles* mosquitoes swarming station (Diabate and Tripet, 2015). At short distance male mosquitoes use acoustic to locate the swarm and females are guided to the swarm by olfactory and visual cues (Takken, 1999). A complete understanding of *Anopheles* mosquitoes mating behaviour is important to develop novel way of trapping and mass killing of males or females and to design vector control strategies using a sterile insect release technique, release of insect caring dominant lethal genes, and other genetic techniques.

2.1.3. Blood feeding, gonotrophic cycle, and longevity of *Anopheles* mosquitoes

Female *Anopheles* mosquitoes require blood meal for their metabolic, egg development and maturation (Service, 2012).When female *Anopheles* mosquitoes find a blood meal from the suitable host, they will rest indoor or outdoor for 2-3 days to digest the blood in

the tropics and 7-14 days in temperate areas before they lay their egg in aquatic environment. Following a blood meal the abdomen of female *Anopheles* mosquitoes becomes dilated, red and later it will be turned in to darker red. As the blood is digested the abdomen of female mosquito becomes whitish posteriorly and dark radish anteriorly and they are called half graved. Gradually, the female mosquitoes fully digest the blood meal and the abdomen becomes dilated and whitish and they are called fully gravid (Service, 2012).

The fully gravid female *Anopheles* mosquitoes will search for a suitable larval habitat to deposit the matured egg. This process of searching a suitable vertebrate host, blood ingestion, digestion, maturation of the ovaries and egg-laying is repeated several times and is called gonotrophic cycle (Detinova, 1962). Each gonotrophic cycle lasts about 2-4 days for *An. gambiae*, but its length depends on factors such as breeding site availability, season, number of previous gonotrophic cycles and temperature (Williams and Pinto, 2012). In the field only a small percentage of females of *An. gambiae* survive for more than three or five gonotrophic cycles (Clements and Paterson, 1981).

The blood feeding behaviour of *Anopheles* mosquitoes is determined by the availability and accessibility of vertebrate host (Takken and Verhulst, 2013). In addition, the *Plasmodium* parasites manipulate the blood seeking pattern and probing frequency of female *Anopheles* mosquitoes (Schwartz and Koella, 2001). Fore instance, *Plasmodium* infected female *Anopheles* mosquitoes tends to show increased blood feeding and host probing frequency to ensure its transmission to the next suitable host (Smallegange *et al*., 2013).

Longevity describes the physiological age of *Anopheles* mosquitoes. *Anopheles* mosquitoes which live long until the *Plasmodium* parasite complete its extrinsic life cycles are potential vector of malaria (Matthews *et al*., 2020). The physiological age of *Anopheles* mosquitoes is determined by dissecting their ovaries and grouping them in to nulliparous (young) and parous (old) (Detinova, 1962). Nulliparous female mosquitoes have a coiled trachiolar skein because they have not laid their first batch of egg (are not infective). Nevertheless, parous female mosquitoes have a stretched trachiolar skien because they have laid their first batch of egg and they could transmit malaria. Vector control strategies such as LLINs and IRS are primarily designed to reduce the longevity of indoor host seeking and resting *Anopheles* mosquitoes, respectively (Sharp *et al*., 1990; WHO, 2006; WHO, 2007; Abose*et al*., 2015; WHO, 2019).

2.1.4. Host preference of *Anopheles* mosquitoes

Host preference is defined as the choice of a particular vertebrate host as a food source, over the other species equally available in the area (Boreham and Garrett-Jones, 1973). Different *Anopheles* mosquito species demonstrate preferences for feeding on animals (zoophily) or on humans (anthropophily); however, females may take a blood meal from a non-preferred host when these are present in the area (Chaves *et al*., 2010). Host preference of Anophelines mosquitoes can be estimated by using the human blood index (HBI) or bovine blood index (BBI). Human blood index (HBI) represents the proportion of *Anopheles* mosquitoes fed on human blood meal (Garrett-Jones, 1964). Whereas, bovine blood index (BBI) represents the proportion of *Anopheles* mosquitoes fed on bovine (Garrett-Jones, 1964). Additionally, forage ratio (FR) is also important to determine the host preference of *Anopheles* mosquitoes in a given locality (Hess *et al*.,

1968). The forage ratio (FR) indicates the percent of blood engorged *Anopheles* mosquitoes which have fed up on either humans or bovine over the percent which either human or cattle comprises in the area (Hess *et al*., 1968).

Host preference of *Anopheles* mosquitoes affects the life history and transmission of *Plasmodium* parasites, as certain groups of animals such as cattle's are dead end for malaria parasites (Takkenand Verhulst, 2013). A continuous monitoring of the host preference of *Anopheles* mosquitoes is important to understand the malaria epidemiology, vectoral capacity of *Anopheles* mosquitoes and to measure effectiveness of vector control programme (Mathenge *et al*., 2001; Massebo *et al*., 2015).

2.1.5. Host seeking and resting behaviour *Anopheles* mosquitoes

The species of *Anopheles* mosquitoes that frequently enter houses to feed are called endophagic in their feeding habits, whereas those that bite their hosts outside houses are called exophagic (Service, 2012). Females are attracted to hosts by various stimuli emanating from their breath or sweat, such as carbon dioxide, lactic acid, octenol, as well as body odors and warmth (McMeniman *et al*., 2014; Raji and DeGennaro, 2017). Additionally, vision could also play a great role in host orientation (van Breugel *et al*., 2015). The host seeking behaviour of *Anopheles* mosquitoes determine the effectiveness of the implemented vector control strategies. A long term implementation of indoor vector control strategies such as IRS and LLINs induce the behavioural shift of *Anopheles* mosquitoes from indoor host seeking to outdoor host seeking tendency (Russell *et al*., 2011; Kibret and Wilson, 2016; Loha *et al*., 2019). Therefore, an

additional vector control strategies, which target exophagic *Anopheles* mosquitoes, is important to control residual malaria transmission.

After blood-feeding, female *Anopheles* mosquitoes rest in order to digest the blood meal and mature their eggs. Depending on the environmental temperature *Anopheles* mosquitoes rest either inside or outside houses for 2-3 days after they take a blood meal from humans, or other hosts, to shelter during digestion of the blood-meal (WHO, 2018). Some species of *Anopheles* mosquitoes showed an increased outdoor resting tendency following the implementation of indoor vector control strategies such as IRS, which makes indoor based vector control programme difficult (Pates and Curtis, 2005). Therefore, a continuous evaluation of the mosquito's choice of post-feeding resting site has major implications in selecting appropriate vector control strategies (Pates and Curtis, 2005).

2.2. Ecology, distribution and behaviour of malaria vectors in Africa

A total of 140 *Anopheles* mosquito species have been recorded in Africa, of which eight of them are considered as effective malaria vectors (Gillies and de Meillon, 1968; Gillies and Coetzee, 1987). The Sub-Saharan Africa region is home for a highly efficient malaria vectors such as *An. gambiae s.l* complex members and *An. funestus* complex (Gillies and de Meillon, 1968; Coluzzi, 1999) (Figure 2.2). *Anopheles gambiae* complex includes nine sibling species such as *An. gambiae s.s* (sensu stricto). Giles (molecular S form), *An. coluzzi* Coetzee and Wilkerson (molecular M form), *An. arabiensis* Patton, *An. quadriannulatus* Theobald, *An. melas* Theobald (West Africa salt breeder), *An. merus* Dönitz (East Africa salt breeder), *An. bwambae* White, *An. comorensis* Brunhes, and *An.*

amharicus Hunt (recently described in Ethiopia). Of which *An. gambiae s.s*, *An. coluzzi* and *An. arabiensis* are an important malaria vectors in Africa (Figure 2.2). The rest six species has no or limited role as a malaria vector because of restricted geographical distribution or zoophilic behaviour (Coetzee *et al*., 2013).

Anopheles gambiae s.s and *An. coluzzi* distribute in the west to east central Africa and south to Angola, however the first also extends across Africa continent to Madagascar (Coetzee *et al*., 2013). *Anopheles gambiae s.s* is one of the most efficient malaria vectors in the world (Coetzee *et al*., 2000). *Anopheles gambiae* is separated in to a five chromosomal diversity such as Forest, Bamako, Savanna, Mopti, and Bissaw and two molecular forms M (*An. coluzzi*) and S (*An. gambiae s.s.*) (Torre *et al*., 2001; Coetzee *et al*., 2013). This vector is relatively a long lived species with a short larval development period (approximately six days from egg to adult under optimal environmental conditions). The larvae of *An. gambiae* s.s inhabit in sunlit, shallow, temporary bodies of fresh water such as ground depressions, puddles, pools and hoof prints and manmade habitats such as rice fields (Minakawa *et al*., 2004). The breeding habitats of *An. gambiae s.s* are often characterized by clear, turbid or polluted water with no vegetation or sparsely vegetated habitats (Gillies and de Meillon, 1968; Edillo *et al*., 2002).

Anopheles gambiae s.s is dominantly endophagic and endophilic species (Degefa *et al*., 2017; Akogbéto *et al*., 2018). However, other studies showed no difference in indoor and outdoor host seeking and resting density of *An.gambiae s.s* (Robert *et al*., 2006). The highly endophilic and endophagic behaviour of *An. gambiae s.s* led to a significant reduction of the vector density in areas where there is an increased implementation of indoor based vector control strategies (Bayoh *et al*., 2010).

Anopheles arabiensis is an important malaria vector in sub-Saharan Africa covering over 70% of the region (Coetzee *et al*., 2000; Coetzee *et al*., 2013). This vector is considered as a species of dry, savannah environments and sparse woodland, though some species live in forested areas with a recent history of land disturbance or clearance. *An. arabiensis* prefers to breed in temporary, small, sunlit, clear and shallow fresh-water pools (Gimnig *et al*., 2001; Himeidan and Rayah, 2008). *Anopheles arabiensis* is described as a zoophilic, exophagic and exophilic species when compared to *An. gambiae* and *An. funestus* (Fontenille *et al*., 1997; Hadis *et al*., 1997; Tirados *et al*., 2006). However, this species also showed an anthropophilic (Fornadel *et al*., 2010), endophilic (Fornadel *et al*., 2008) and endophagic behaviours in different regions depending on location, host availability and the local genotype (Main *et al*., 2016). For instance, a population of *An. arabiensis* in western Africa are highly anthropophilic, endophilic and endophagic, whereas those in the east are highly zoophilic and exophilic (Sinka *et al*., 2010). In addition, its opportunistic behaviour, the host seeking, and resting behaviour of *An. arabiensis* varied following the initiation of vector control strategies (Reddy *et al*., 2011). *Anopheles arabiensis* showed a more exophilic and exophagic tendency in relation to the implementation of indoor vector control strategies such as IRS and LLINs in the different part of the world (Bayoh *et al*., 2010; Benelli and Beier, 2017).

Funestus group comprises a morphologically similar sibling species such as *An. funestus*-like, *An. aruni*, *An. confusus*, *An. parensis*, *An. vaneedeni*, *An. longipalpis* type C, *An. leesoni*, *An. longipalpis* type A, *An. rivulorum*, *An. rivulorum*-like, *An. brucei*, and *An. fuscivenosus* (Gillies and De Meillon, 1968; Dia *et al*., 2013; Harbach, 2013). From which *An. funestus* is the only malaria vector, widely distributed across sub-Saharan

Africa (Gillies and Coetzee, 1987). *An. funestus* usually breed in a large, permanent or semi-permanent body of fresh water with emergent vegetation, such as swamps, large ponds and lake edges (Gillies and De Meillon, 1968). In some localities, *An. funestus* larvae are associated with rice cultivation, exhibiting higher densities in older, maturing fields (Ijumba and Lindsay, 2001).

Anopheles funestus is a highly efficient malaria vector because of its relatively high longevity, human host blood preference and late night biting behaviour (Gillies and de Meilloni, 1968; Coetzee and Fontenille, 2004). *An. funestus* is almost exclusively anthropophilic and preferentially rests indoors, making it very susceptible to control by residual house spraying (Russell *et al*., 2011; Lwetoijera *et al*., 2014). However, zoophilic type of *An. funestus* has been reportedin east Senegal (Dia *et al*., 2013).

Anopheles moucheti has two morphological forms such as *An. moucheti* and *An. m. nigeriensis*. *An. moucheti* is widely distributed across West and central Africa. It is important malaria vectors in equatorial forests, in a village situated along slow moving rivers or streams (Gillies and de Meillon, 1968; Ayala *et al*., 2009). The larvae are found with edges of large, slow flowing river, and often associated with turbid water (Ayala *et al*., 2009). *An. moucheti* is a highly anthropophilic, endophilic and endophagic vector (Antonio-Nkondjio *et al*., 2006).

Forest populations of *An. nili* are highly anthropophilic and endophagic, whereas the savanna populations are more exophilic and exophagic (Antonio-Nkondjio *et al*., 2006; Awono-Ambene *et al*., 2009). The resting habits of *An. nili* are described either indoor or outdoor (Gillies and de Meillon, 1968). The *An. nili* complex includes *An. carnevalei*, *An. nili*, *An. ovengensis* and *An. somalicus*, of which *An. nili* is the most important malaria

vector (Harbach, 2013). *Anopheles nili* is widely distributed across most of West, Central and East Africa that have proved highly efficient in malaria transmission (Gillies and de Meillon, 1968; Gillies & Coetzee, 1987; Ayala *et al*., 2009). The larvae of *An. nili* are often found in vegetation along the edges of streams and large rivers (Fontenille and Simard, 2004).

Anopheles stephensi is an efficient malaria vector in south East Asia and parts of the Arabian Peninsula (Sinka *et al*., 2011). In Africa, the first report of *An. stephensi* is come from Djibouti in 2013 (Faulde *et al*., 2014) and later in Ethiopia in 2016 (Carter *et al*., 2018). The occurrence of *An. stephensi* is associated with outbreak of urban *P. falciparum* malaria in Djibouti (Faulde *et al*., 2014). The breeding habitat of *An. stephensi* includes artificial containers, and collection of water associated with artificial containers (Thomas *et al*., 2016). In addition, in rural areas the larvae of *An. stephensi* breeds in fresh water pools, stream margins and local water storage containers (Sinka *et al*., 2011). Additional studies are required about the vectoral role, geographical distribution, host seeking and resting behaviour, host preference and breeding habitat type of *An. stephensi* in the Horn Africa.

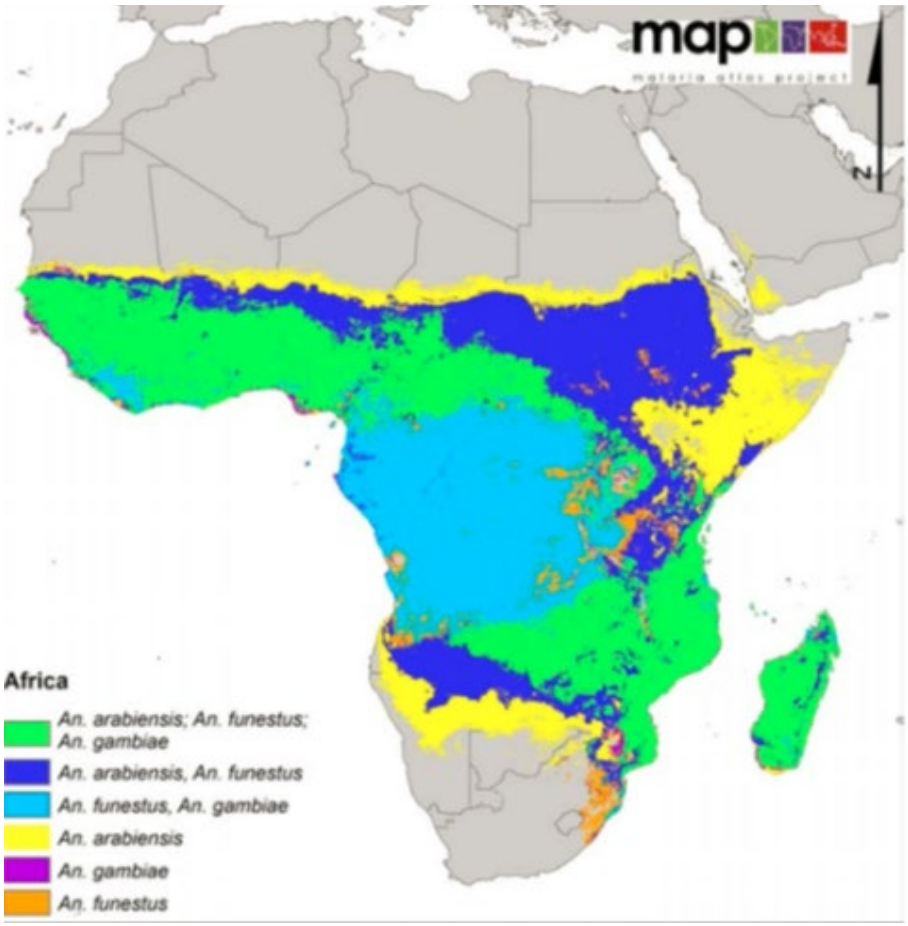

Figure 2.2. A regional map of Africa showing the distribution of dominant *Anopheles* mosquito species (Sinka *et al*., 2012).

Secondary vectors of malaria include those species that play minor part in malaria transmission. *Anopheles* mosquitoes including *An. rivolorum Leeson, An. pharoensis* Theobald*, An.coustani* Laveran, *An. ziemanni* Grtunburg, and *An.squamosus* Theobald are among secondary vectors in Africa (Afrane *et al*., 2016). They are widely distributed to south, east and West Africa (Figure 2.3). Secondary vectors such *An. pharoensis*, *An. ziemanni*, *An. coustani*, *An. squamosus* are usually exophilic (outdoor resting) and exophagic (outdoor biting) (Kawada *et al*., 2012). Therefore, they play an important role

in sustaining malaria transmission after the main malaria vectors have been reduced by IRS and LLINs (Antonio-Nkondjio *et al*., 2006).

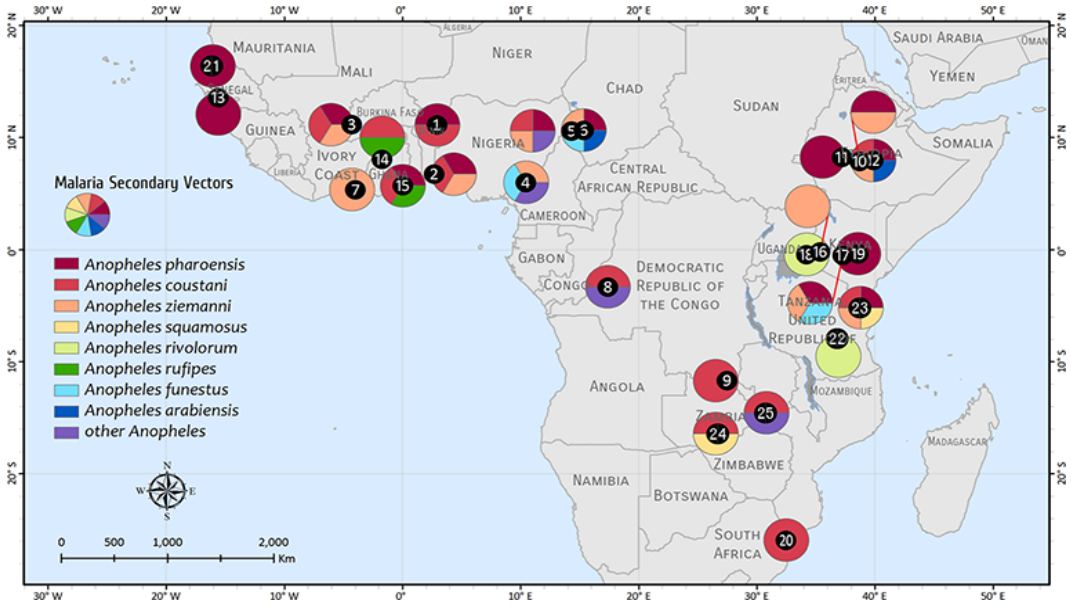

Figure 2.3. Map showing distribution of secondary malaria vectors in sub-Saharan Africa (Afrane *et al*., 2016).

2.3. Ecology, distribution and behaviour of *Anopheles* mosquitoes in Ethiopia

More than 44 species of *Anopheles* mosquitoes are documented in Ethiopia (FMoH, 2014) (Figure 2.4). Few species are incriminated as primary and secondary vectors of malaria, while most species are considered as non-vectors. *Anopheles arabiensis*, a member of the *An. gambiae* complex, is the primary malaria vector in Ethiopia, whereas *An. funestus, An. pharoensis,* and *An. nili* serve as a secondary vector (Gillis and Coetzee, 1987).

Anopheles arabeisnsis and *An. amharicus* are the only member of *An. gambiae s.l* identified from Ethiopia (Hunt *et al*., 1998; Coetzee *et al*., 2013). *Anopheles amharicus* are not known to transmit malaria, because they are highly zoophilic (Fettene *et al*., 2004).

The feeding, host seeking and resting behaviourof *An. arabiensis* is explained by zoophilic/anthropophilic, endophagic/exophagic, and endophilic/exophilic, respectively in different part of Ethiopia. For instance, in southwestern Ethiopia, *An. arabiensis*, showed preferences of bovine blood meal over human irrespective of higher human population size (Habtewold *et al*., 2001; Massebo *et al*., 2013a; Massebo *et al*., 2015; Getachew *et al*., 2019). However, in central Ethiopia (Kibret *et al*., 2010) and east, south and west Ethiopia (Hadis *et al*., 1997) a higher proportion of *An. arabiens is* showed high anthropophilic behaviour.

The *Plasmodium* parasite infection rate of *An. arabiens is* varied in different locality of Ethiopia. In central Ethiopia the overall *P. falciparum* sporozoite rate of *An. arabiensis* was 1.8% (Kibret *et al*., 2010). In southern Ethiopia, the overall *Plasmodium* infection rate of *An. arabiensis* was 0.38%, with a respective *P. falciparum* and *P.vivax* entomological inoculation rate (EIR) of 17.1 and 2.5 infective bites/person/year (Massebo *et al*., 2013B). Similarly, in south western Ethiopia the *P. vivax* and *P. falciparum* sporozoite rate of *An. arabiensis* was 0.46% and the overall EIR was 5.3 ib/p/yr (Abraham *et al*., 2017). In Ghibe, the overall EIR rate for *P. falciparum* and *P. vivax* was 8.4 and 5.4 ib/p/y, respectively (Getachew *et al*., 2019).

Anopheles funestus is a highly endophilic, endophagic and anthropogenic malaria vector, mainly distributed along the swamps of the Baro and Awash rivers and shores of lakes in

Tana in the North and the Rift Valley areas (FMoH, 2014). A detailed study on the *Plasmodium* parasite infectivity rate of *An. funestus* is limited in Ethiopia. Studies from central Ethiopia showed that *An. funestus* had the overall *Plasmodium* parasite infectivity rate and EIR of 4.5% and 47.8 ib/p/yr, respectively (Kibret *et al*., 2017).

In Ethiopia, the behaviour of *An. pharoensis* is explained by more exophagic (Kibret *et al*., 2010), exophilic (Kibret and Wilson, 2016) and zoophagic behaviour (Massebo *et al*., 2015). However, anthropophilic nature of *An. pharoensis* was reported from central Ethiopia (Kibret *et al*., 2010). The overall *P. falciparum* sporozoite rate of *An. pharoensis* was 0.59% in central Ethiopia (Kibret *et al*., 2010). The annual EIR of *An. pharoensis* in the lowland and midland dam village of Ethiopia was 33.3 ib/p/y and 5.0 ib/p/y respectively (Kibret *et al*., 2017). *Anopheles nili* was rarely found resting indoors despite the high densities found biting indoors, it has an exophilic behaviour in lowland areas of western Ethiopia (Krafsur, 1970).

Anopheles stephensi is recently confirmed *Anopheles* mosquito species in Kebri Dahar town, eastern Ethiopia (Carter *et al*., 2018). Currently, an increasing number of *An. stephensi* was reported additional sites from eastern Ethiopia (Balkew *et al*., 2020).). A larger proportion of *An. stephensi* were positive for *P. vivax* (Balkew *et al*., (2021) and they showed a strongly zoophagic tendency (Balkew *et al*., 2020). Further studies are required about the ecology, distribution, behaviour (feeding, host seeking, and host preference) and vectoral capacity of *An. stephensi*.

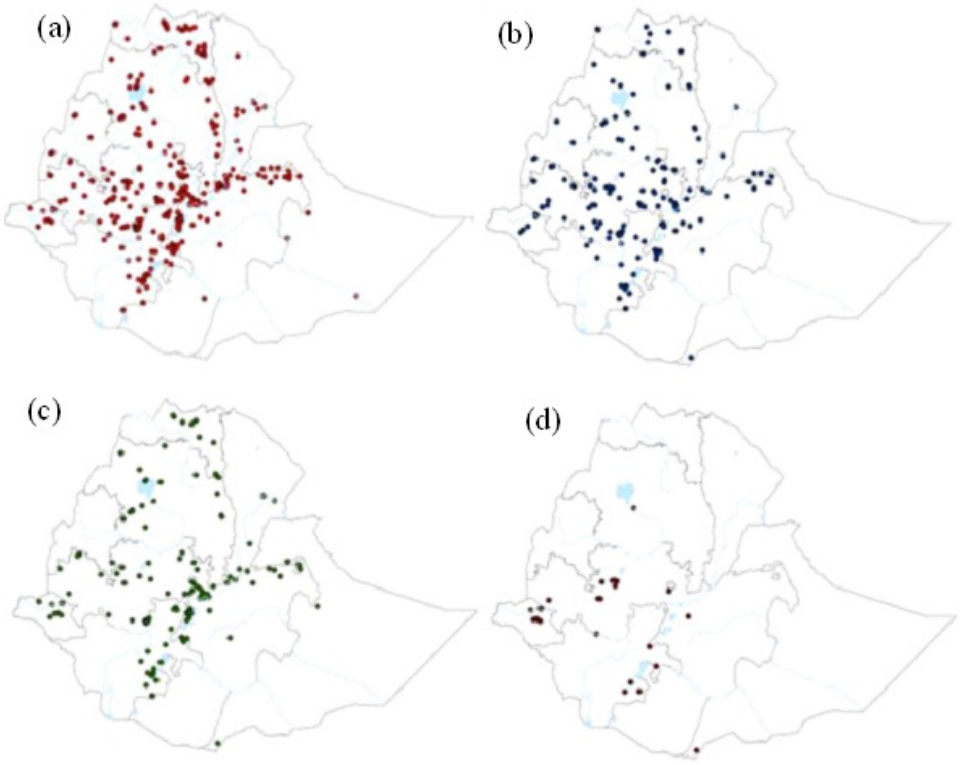

Figure 2.4. Distribution of *An. arabiensis* (a). *An. funestus* (b). *An. pharoensis* (c) and *An. nili* (d) in Ethiopia (based on data collected from 1966-2013). (Source: FMoH, 2014)

2.4. Status of malaria transmission in Ethiopia

In Ethiopia more than 60% of the population and 68% of the total land mass is at risk of malaria infection (FMoH, 2017). The diverse ecology of the country supports a wide range of malaria transmission intensities starting from low transmission areas in the highland regions and semi-arid regions to a highly endemic region in the low land regions (PMI, 2019). Areas with altitude bellow 1000 masl in Gambela, Benishangul-Gumuz and western low land areas of Oromia, Amhara and Tigray and South nation, Nationalities and Peoples are at high risk of malaria (FMoH, 2020). These areas are characterized by

hot and humid climate favorable for the development of malaria parasite and vector. A malaria epidemic is seasonal almost every year in the midlands (Weyna Dega) of Ethiopia (1500 - 2400 m. a. s. l) (FMoH, 2020). Eastern part of the country such as Afar and Somali, malaria is restricted in areas near to the river only because these areas are characterized by high temperature and low humidity, which is not favorable for the vector reproduction (FMoH, 2020). Similarly, central highland areas of the country are free from malaria because of low temperature, which limits the development of *Plasmodium* parasite and the vector (EPHI, 2016) (Figure 2.5).

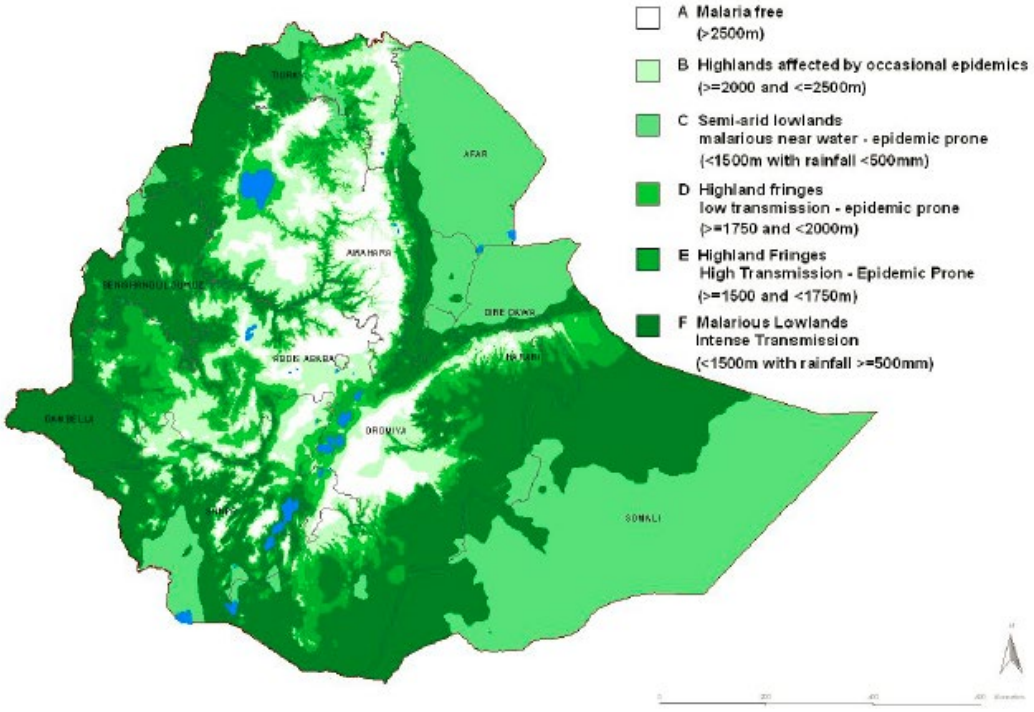

Figure 2.5. Malaria risk stratification in Ethiopia using annual parasite incidence per 1,000 populations (FMoH, 2017).

2.4. Malaria vector control options

Integrated vector control methods incorporating multiple approaches is an important part of malaria intervention strategy across the world (WHO, 2012). Several strategies have

been designed and put into place to reduce the mosquito population, and several others are currently being investigated as possible solutions for rendering the mosquito vector less competent to transmit malaria. Among these strategies are environmental management, biological methods, indoor residual sprays, insecticide treated bed nets, and genetic approaches.

2.4.1. Non-chemical malaria vector control

2.4.1.1. Environmental management

Environmental management involves environmental modifications, environmental manipulation, and modification of human habitation and behaviour (WHO, 1980; WHO, 1982).This method limited mosquito population density through draining, land leveling, filling, changing water levels in reservoirs, flushing streams or canals, and changing water salinity of potential breeding sites. In addition, building human settlements away from potential *Anopheles* mosquito breeding habitats is important to reduce a human vector contact.The diverse ecology and breeding habitat typeS of *Anopheles* mosquitoes make this method difficult to implement.

2.4.1.2. Biological control

Biological control uses a biological agent such as larvivorous fish, bacterial pathogens (*Bacillus thuringiensis israelensis* and *B. sphaericus*) and funguses to reduce vector density (Fillinger *et al*., 2003; Russell *et al*., 2003). A larvivorous fish *Gambusia affinis* is used as a biological control in different part of the world, but its negative impact on native fauna has discouraged its future use (Walker and Lynch, 2007).Therefore, currently native larvivorous fish species are used to control *Anopheles* mosquito larvae.

B. thuringiensis israelensis and *B. sphaericus* are environmentally friendly *Anopheles* mosquito control alternatives, because the toxins they produce are non-toxic to other species (Walker and Lynch, 2007). Additional type of microbial approach is the use of entomopathogenic fungi such as *Beauveria bassiana* and *Metarhizium anisopliae* against *Anopheles* mosquito adult (Blanford *et al*., 2005; Scholte *et al*., 2006). Wolbachia bacteria were also used for *Anopheles* mosquito control programme because they can induce cytoplasmic incompatibility (CI), parthenogenesis, feminization, and male-killing of their host (adult *Anopheles* mosquitoes) (Werren *et al*., 2008).

2.4.1.3. Genetic control

Genetic malaria vector control includes release of sterile insect techniques (SIT), release of insects carrying a dominant lethal gene system (RIDL), Homing Endonucleaus Gene (HEGs), and RNA interference (RNAi) (McGrawand O'Neill, 2013). Release of sterile insect techniques (SIT) is based on mass rearing, radiation mediated sterilization, and release of a large number of male insects into a given target area (Wilke and Marrelli, 2012). Therefore, any successful mating with the sterile insect will result in no offspring, which gradually eliminate or decline the vector population (Wilke *et al*., 2009). Rerelease of insects carrying a dominant lethal genetic system (RIDL) is based on a lethal gene (RIDL) that acts late in development would prevent mosquitoes from becoming adults, the only harmful life stage, yet enable them to survive and compete at the larval stage, when density-dependent competition occurs (Thomas *et al*., 2000; Helinski *et al*., 2008).

Homing endonucleaus genes (HEGs) are selfish genetic elements that were discovered in bacteria. Released males carrying HEGs mate with wild-type female mosquitoes and produce offspring that contain the HEG. Homing endonucleaus genes can be designed to

target vector competence genes, leading to pathogen-resistant female mosquitoes or they can be engineered to suppress vector population by targeting gens to induce sterility, reduction in survival or sex ratio distortion (Windbichler *et al*., 2011). RNA interference (RNAi) is a biological process in which RNA molecules inhibit gene expression or translation, by neutralizing targeted mRNA molecules. It involves manipulating the path way to suppress a given target gene by virtue of introducing a complementary double stranded RNA (dsRNA) to mosquito cells, suppression of gene products involved in key physiologies that impact mosquitoes survival, fecundity, behaviour, or vector status (Airs and Bartholomay, 2017).

2.4.2. Chemical insecticides for malaria vector control

2.4.2.1. Chemical larviciding

Chemical larviciding involves the application of chemical insecticides to reduce vector population by killing the larvae (WHO, 2018). A range of chemical larvicides such as petroleum oil, a poisonous paris green (copper acetoarsenite) and temphos (organophosphate) has been used as *Anopheles* larvae control for over a century (Walker and Lynch, 2007). Furthermore, a monomolecular surface film (MMF) is developed to replace petroleum oil as a larvicides and pupicides (Nayar and Ali, 2003). Unlike the other chemical larvicides, MMF is relatively safe to non-target organism and to the environment (they are biodegradable). Implementation of MMF on rice paddies in Kenya has led to a 93.2% reduction in emergence of adult Anopheline and 69.5% reduction in emergence of Culicines adult (Bukhari *et al*., 2011). Similarly, a laboratory based study indicates that, the implementation of MMF at a standard dose of 1 mL/m^2 resulted in a

significant mortality of pupae and larvae of *Ae. aegypti* and *An. minimus* (Sukkanon *et al*., 2016). However, environmental challenges such as rain fall, wind and vegetation could compromise the success of MMF (Levy *et al*., 1981).

2.4.2.2. Indoor residual spray (IRS)

The indoor residual spraying is the application of long acting chemical insecticides on walls, eaves and ceilings of houses to reduce the life span of indoor resting *Anopheles* mosquitoes (WHO, 2006). Four insecticide classes used for IRS are pyrethroids, organochlorines (DDT, dichlorodiphenyltrichloroethane), organophosphates, and carbamates. Globally, the distribution of IRS in malaria endemic countries has declined from 5.8% in 2010 to 2.6% in 2020 (WHO, 2021). Indoor residual spray (IRS) led to the elimination or reduction of *An. funestus*, one of the major malaria vectors and *An. gambiae s.s*, which rests and bites mostly indoors (Padonou *et al*., 2012). *Anopheles arabiensis*, which does not rest indoors as much as *An. gambiae s.s*, is less affected by IRS, even at high coverage levels, and is responsible for low levels of transmission and seasonal increases and outbreaks (Hansford, 1972; Sharp *et al*., 1990). An increased development of resistant species of *Anopheles* mosquitoes towards different class of insecticides put the global effort of malaria control at risk.

2.4.2.3. Insecticide treated bed nets (ITNs)

Insecticide-treated bed nets have been regarded as an excellent tool to reduce malaria transmission in highly endemic countries, especially by reducing child mortality and morbidity (WHO, 2007). The efficacy of pyrethroid treated bed nets is well known from its implementation in Asia, where it has successfully helped control malaria transmission

(Hung *et al*., 2002). Globally, 2.3 billion ITNs has been distributed from 2004 - 2020, of which 86% (2 billion) was distributed in sub-Saharan Africa (WHO, 2021). Accordingly, the households with one ITNs increased from 5% in 2000 to 65% in 2020 (WHO, 2021). However, in order to achieve a greater efficacy in Africa, insecticide treated bed nets need to be widely distributed among the population, and insecticide reimpregnation services have to be provided at a relatively low cost if continuous protection is to be maintained (Curtis *et al*., 2000). The development of insecticide resistant malaria vectors become a major challenge in malaria control and elimination programme.

2.4.3. Major vector control strategies in Ethiopia

In Ethiopia, malaria control programme was initiated in 1959, as a malaria eradication service with the aim to eradicate malaria in 1980 (WHO, 2007). During this period, indoor residual spray using DDT and case treatment using chloroquine were the major malaria intervention strategies in the country (WHO, 2007). However, considering that malaria eradication is not feasible during the specified period, the national malaria eradication programme has been changed in to malaria control programme in 1977, with the aim to reduce mortality, morbidity and inability to work (FMoH, 2014). Similar with malaria eradication services, malaria control programme was also based on IRS using DDT and case treatment. Nevertheless, the increasing malaria mortality led to a new plan of malaria control (FMoH, 2014).

Using a fund obtained from US presidents malaria initiative (PMI), UNICEF, WHO and the government of Ethiopia, case treatment using effective drugs, IRS (DDT) and ITNs were used as malaria intervention starting from 2000 (FMoH, 2009). Insecticide treated

nets (ITNs) were introduced in 1997/1998 in northwestern Tigray (FMoH, 2004; Aregawi *et al*., 2014). In 2010, an ambitious national malaria strategy was launched with the aim to eliminate malaria in low malaria transmission areas by 2015, and from Ethiopia by 2020 (FMoH, 2010). Currently, federal government of Ethiopia has set a goal to eliminate malaria from the country by 2030 (FMoH, 2020).

In Ethiopia, starting from 2009, DDT was replaced with deltamethrin due to a wide spread development of insecticide resistance, since then different class of insecticides has been used (Yewhalaw *et al*., 2011). Similarly, chloroquine was replaced by sulfadoxine-pyrimethamine (SP) after 1998 for the treatment of uncomplicated *P. falciparum* due to widespread decline in the efficacy of CQ in the country (Alene and Bennett, 1996). Later on, the parasite developed resistance to SP drugs, and the drug was replaced with artemisinin-based combination therapy (ACT) as a first line treatment for *P. falciparum* in 2004 (Jima *et al*., 2005). The use of RDT for improved diagnosis as well as the use of long-lasting insecticidal nets (LLINs) was started in 2004 (FMoH, 2010). Furthermore, in Ethiopia, larval control mainly depends on environmental management, nevertheless temephos is also used as larvicides in areas with limited and known larval habitats (FMoH, 2014).

In Ethiopia, a major scale-up of malaria intervention strategies was began in 2005 with a wide distribution of rapid diagnostic tests (RDTs), ACTs, LLINs and IRS. Between 2005 and 2007, ITN coverage increased 15-fold (over 95% of the nets in Ethiopia are long-lasting insecticidal nets) (Jima *et al*., 2010). In the country 68% of households in malarious areas were protected by at least one ITN and/or IRS and ITN use by children under five and pregnant women increased to nearly 45% (Jima *et al*., 2010). These

increased distribution of malaria intervention strategies led to a significant reduction of malaria in Ethiopia (Taffese et al., 2018; FMoH, 2020). However, the development of insecticide and drug resistant malaria vectors and *Plasmodium* parasites has challenged the national malaria elimination programme (FMoH, 2020).

2.5. Insecticide resistance in malaria vectors

Insecticide resistance is the ability of mosquitoes to survive exposure to a standard dose of insecticide; this ability may be the result of physiological or behavioural adaptation (WHO, 2016). It is the result of the selection of a genetic modification in one or several genes occurring by migration and/or mutation. Currently, the dominant malaria vectors have developed resistance to major class of chemical insecticides used for vector control such as pyrethroids, DDT, carbamates and organophosphates which poses a serious threat to achieving the malaria control programme (Corbel and N'Guessan, 2013). Surveillance of the type and frequency of resistance in *Anopheles* mosquitoes is important to design effective malaria vector control programme.

2.5.1. Mechanisms of insecticide resistance

The various mechanisms that enable insects to resist the action of insecticides can be grouped into four distinct categories such as, target-site resistance, metabolic resistance, reduce penetration and behavioural avoidance (Corbel and N'Guessan, 2013) (Figure 2.6).

A. Metabolic resistance

Metabolic resistance appears because of the over expression of detoxifying enzymes such as esterase's, cytochrome P450 monooxygenases and glutathione-S-transferases, which

enables resistant mosquitoes to metabolize or degrade insecticides before they are able to exert a toxic effect (Hemingway and Ranson, 2000). Over expression of detoxifying enzymes can occur as a result of gene amplification (e.g. duplication) or due to changes in either transacting regulator elements or in the promoter region of the gene (Guillemaud *et al*., 1997; Hawkes and Hemingway, 2002). Metabolic resistance mechanisms have been identified in mosquito populations for all major classes of insecticides currently used for vector control, including organochlorine, organophosphates, carbamates, and pyrethroids (Corbel and N'Guessan, 2013).

B. Target-site resistance

Insecticides such as organophosphates (OP), carbamate, dichlorodiphenyl trichloroethane (DDT), and pyrethroids target a specific site of the insect. The modification of insecticides site of action in resistant strains of insects make the insecticides unable to bind effectively. This change of the sensitivity of insecticide target regions due to non-silent point mutations is called target-site resistance (WHO, 2016). Insensitive acetylcholinesterase, the GABA receptor mutation, and mutations in the voltage-gated sodium channel are the major type of target site resistance (Hemingway *et al*., 2004).

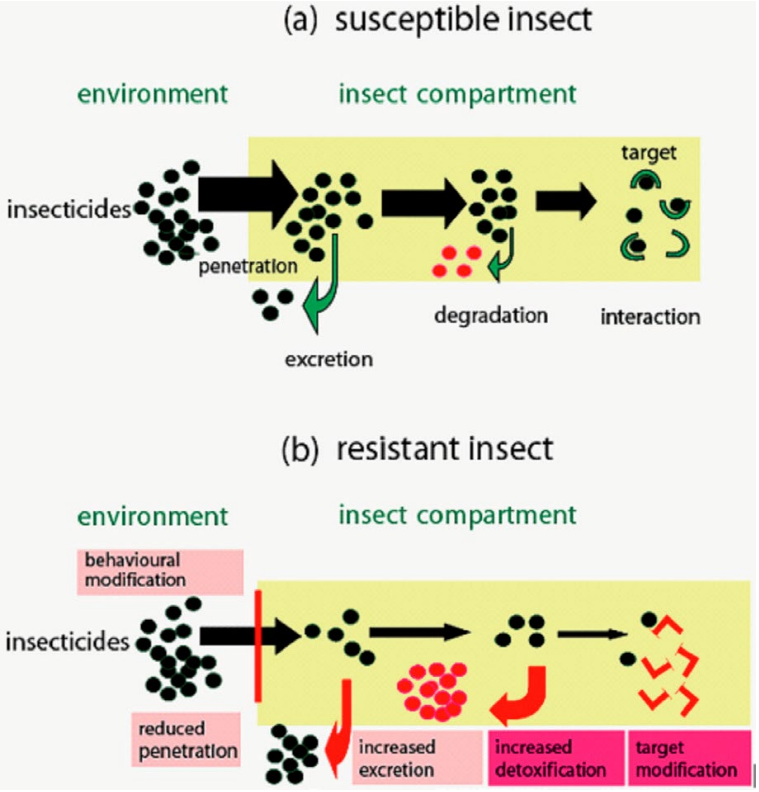

Figure 2.6 Behavioural and physiological mechanism of insecticide resistance in malaria vectors (a) Susceptible (b) Resistant (Lapied *et al*., 2009)

Insensitive acetylcholinesterase: Acetylcholinesterase (AChE) in the nerve synapses of insect is the target site for OP (organophosphate) and carbamate insecticides (Hemingway *et al*., 2004). Acetylcholinesterase is important enzyme to terminate nerve impulses by catalyzing the hydrolysis of neurotransmitter acetylcholine. Organophosphate and carbamate insecticides inhibit the activity of AChE by phosphorylating or carbamylating its active site. Several mutations in the gene encoding for an acetylcholinesterase have been found in insects which result in reduced sensitivity to inhibition of the enzyme by these insecticides (Weill *et al*., 2003; Alout *et al*., 2009). Two sequences of AChE genes (ace-1 and -2) were identified from the *An. gambiae* genome (Holt *et al*., 2002). Organophosphate and carbamate resistance is highly distributed in major malaria vectors in Africa (Figure 2.8). In Ethiopia limited reports are

available about the status of insensitive acetylcholinesterase in major malaria vectors (Yewhalaw *et al*., 2011).

Knockdown resistance (kdr): It is caused by genes involving a mutation in the voltage gated sodium channel, which is the target site of pyrethroids and organochlorine compounds (e.g. dichlorodiphenyl trichloroethane, DDT). The channel comprises four domains (I-IV), each consists six transmembrane helices (S1 – S6) (Figure 2.7). Pyrethroids modify the voltage sensitive sodium channel which, results in the channel opening at the resting potential and causes paralysis and death of the insect due to repetitive discharge in motor and sensory axon (Hemingway *et al*., 2004).

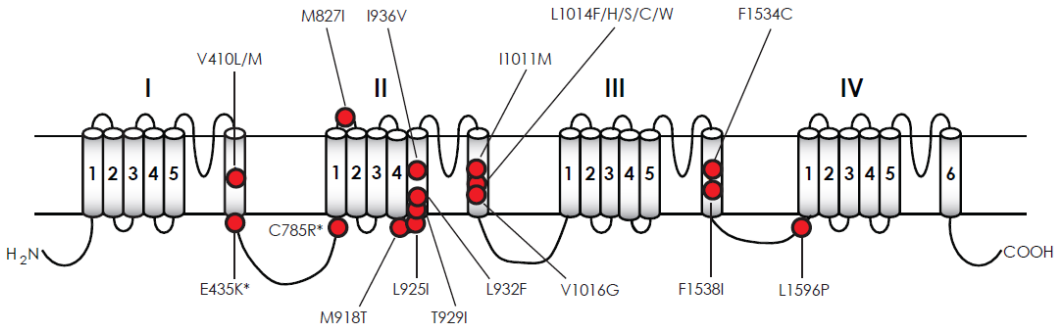

Figure 2.7. Topology of mutation in the voltage-gated sodium channel (Dong *et al*., 2014).

In *An. gambiae s.s* two mutations at the domain II of the voltage-gated sodium channel gene have been associated with resistance to DDT and pyrethroids (Martinez-Torres *et al*., 1998; Ranson *et al*.,2000). This type of resistance is widely distributed in Africa. The first mutation, West African *kdr* (L1014F), involves a nucleotide change resulting in the substitution of leucine residue (TTA) to a phenylalanine (TTT). West African *kdr* (L1014F) is widespread in West Africa at variable frequencies (Fanello *et al*., 2003;

Yawson *et al*.,2004). The second mutation, East African *kdr* (L1014S), consists of a leucine (TTA) to serine (TCA) substitution at the same codon and was originally described in Western Kenya (Ranson *et al*.,2000). West African kdr (L1014F) was reported from south western Ethiopia (Yewhalaw *et al*., 2011), Northern Ethiopia (Fettene *et al*., 2013) and villages in central, northern and south west Ethiopia (Balkew *et al*.,2012). Further more, West African kdr (L1014F) was detected in Cx. pipiens and An. arabiensis in western Ethiopia (Carter *et al*., 2022).

C. Reduced penetration of insecticides

This type of resistance involves the modification of the insect cuticle or the digestive tract lining to prevent the penetration of insecticides towards the target site (WHO, 2016). This type of resistance is also known as cuticular resistance and known to affect a broad range of insecticides. Additional studies are required to identify the effect of reduced insecticide penetration on the malaria dynamics in Ethiopia.

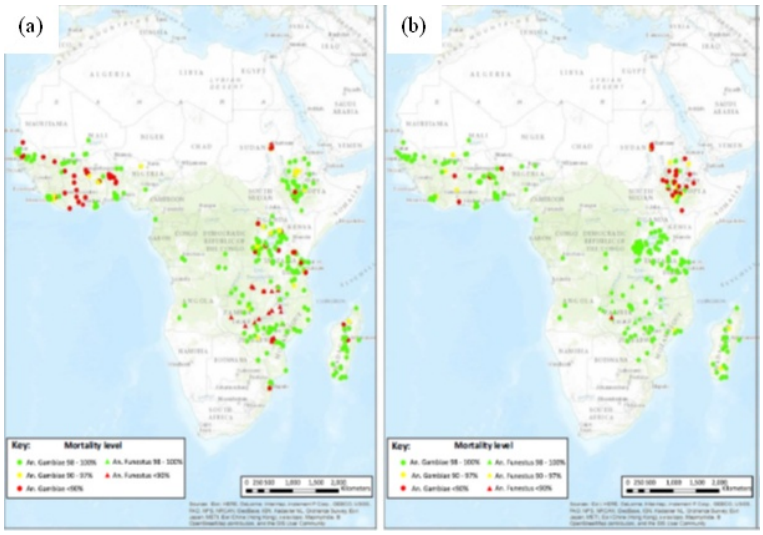

Figure 2.8. The distribution of Carbamate (a) and Organophosphate (b) resistance in Africa. (Source; Ranson & Lissenden, 2016).

D. Behavioural resistance

Behavioural resistance is a modification of insect behaviour in response to a prolonged exposure to insecticides that helps to avoid the contact and/or lethal effects of the insecticides (WHO, 2016).This type of resistance can be divided in to direct contact excitation (irritancy) and non-contact spatial repellency when insects move away from the insecticide treated area before making any direct contact. Change in species composition from a highly endophilic and endophagic *An. gambiae s.s* to a relatively exophilic and exophagic *An. arabiensis* in response to increased coverage of LLINs and IRS has been reported in Senegal (Sougoufara *et al*., 2016). In Kenya, the endophilic *An. gambae s.s* showed exophilic behaviour in relation to the increased use of ITN (Githeko *et al*., 1996). In Ethiopia, a completely exophilic tendency of *An. Arabiensis* was reported in response to an increase in universal coverage of vector control strategies (IRS and LLINs) (Mendis *et al*., 2000; Kibret and Wilson, 2016). These behavioural shifts make this vector not readily available to indoor control methods (IRS and ITNs) (White, 1974; Coluzzi *et al*., 1979; Ameneshewa and Service, 1996; Loha *et al*., 2019). Therfore, a continuous monitoring of the local vector behaviour is vital to achieve the desired malaria control and elimination programmes.

2.5.2. Vector resistance management strategies

Vector resistance management with appropriate vector control relies on having the necessary entomological information (i.e. susceptibility status of vectors to insecticides) (WHO, 2018). A team of expertise organized by World Health Organization in 2010 has designed the global plan for insecticide resistance management strategies (IRM) in

malaria vector (WHO, 2011). This strategy includes rotation of insecticides, mosaic spraying, use of interventions in combination and mixture (WHO, 2012).

Rotation involves the practice of spraying insecticides with different mode of actions in rotation. For example, in areas where there is kdr resistance, the use of DDT or pyrethroid will be less effective, however, the use of carbamate or organophosphate can be effective. Rotation delays the emergence of resistance by removing selection pressure (Nauen, 2007).

Mosaic spraying mainly involves the use of insecticides with different mode of action at the same village. Similar with rotation mosaic approach is used to delay the emergence of resistance by removing the selection pressure (WHO, 2012).

A mixture involves co-formulating two insecticides of different classes (different modes of action). A mixture may be the best way to ensure that insects which survive exposure to one insecticide are killed by the other. The rationale behind mixture is directly killing resistant vector species (WHO, 2011).

Combined intervention uses different insecticide classes in different forms within a house. The best example of combined intervention is using carbamets for IRS and pyrethroids for LLINs. This is a currently applied technique and result a significant reduction of malaria prevalence. This strategy directly kills the resistant vector species which survive the first insecticide by the other class (WHO, 2011).

Chapter 3. Malaria prevalence and associated risk factors in Dembiya district, Northwestern Ethiopia

3.1. Introduction

Malaria remains one of a global public health problem affecting an estimated 219 million individuals in 2017, of which more than 92% were reported from a WHO Africa region (WHO, 2018). From the total global burden of malaria more than 80% were recorded from fifteen countries in sub-Saharan Africa and India (WHO, 2018). In Ethiopia, 4,231,328 malaria cases and 9,433 malaria related deaths have been reported in 2020 (WHO, 2021). More than half of the population in the country (60%) lives in malarious areas, and an estimated 68% of the total population is at risk of malaria infection (FMoH, 2011; PMI, 2019). The transmission of malaria in Ethiopia is seasonal and unstable, and it varies with altitude and rainfall. In most parts of the country, peak malaria transmission occurs after the main rainy season (July to September). In addition, many areas experience a second minor malaria transmission period following a short rainy season from February to March (FMoH, 2011; PMI, 2019). Most of the malaria transmissions in Ethiopia occurs in areas below 2000 m.a.s.l, but endemic regions greater than 2000 m are also documented (Ghebreyesus *et al*., 2000; Woyessa *et al*., 2004).

Plasmodium falciparum and *P. vivax* are the dominant malaria parasite species in Ethiopia, which are responsible for 60% and 40% of malaria cases, respectively (FMoH, 2017; Yalew *et al*., 2017). However, *P. vivax* may be more dominant in different localities of the country with cooler climates (Deress and Girma, 2019). In Ethiopia, *An.*

arabiensis is the primary vector of malaria, whereas *An. pharoensis*, *An. funestus* and *An. nili* are secondary vectors in different parts of the country (FMoH, 2014).

The government of Ethiopia has made a massive scale-up of malaria control interventions starting from 2005 including diagnostic testing, rapid case treatment using artemisinin-based combination therapy (ACT), insecticide-treated bed nets (ITNs), and indoor residual spraying (IRS) (FMoH, 2011; Aregawi *et al*., 2014; Yimer *et al*., 2015; Abose *et al*., 2015). For instance, the proportion of individuals living in malarious areas protected by LLINs increased from nearly zero in 2005 to 51% in 2011, similarly the IRS coverage increased from 10% in 2007 to 38% in 2011 (Taffese *et al*., 2018). This has led to a significant reduction of malaria mortality and morbidity in the country (Aregawi *et al*., 2014; Taffese *et al*., 2018). Based on malaria control achievements obtained in the past years the government of Ethiopia has set a goal to eliminate malaria from the country by 2030 (FMoH, 2017). However, the progress towards malaria elimination is hampered because of the widespread drug resistance by the parasites, and insecticide resistance in the vectors (Taffese *et al*., 2018). This calls for repeated malaria prevalence studies in such areas with high vector control interventions, to design additional malaria control and prevention technologies (FMoH, 2017; Gosling *et al*., 2020).

Dembiya district is one of malaria-endemic area in Ethiopia with long standing implementation of malaria interventions strategies (Tulu, 1996). Over the years, malaria treatment and control measures have resulted in a significant reduction of malaria in the district (Toyama *et al*., 2016). Despite of this considerable progress in malaria control, the disease is still a public health problem in the district (Alemu *et al*., 2012; Agegnehu *et al*., 2018). This suggests that a continuous study of the status of malaria prevalence and

its determinants in the district are important to design and implement evidence based malaria prevention and control strategies. Therefore, this study aimed to evaluate the retrospective and present trend of malaria transmission, and identify socio-economic factors for malaria transmission in selected Kebeles of Dembiya district, Northwestern Ethiopia.

3.2. Material and Methods

3.2.1. Description of the study area

This study was conducted in Dembiya district, located in the north Gondar Administrative Zone of Amhara Regional State. The district is located at 12°39' N and 37°09' E. Kola Diba is the district's town, located 750 km north of Addis Ababa and 35 km southwest of Gondar. The southern part of the district is bordered by Lake Tana. The district has 45 Kebeles (Kebeles is the lowest administrative unit in Ethiopia). The population of Dembiya district was estimated to be approximately 271,000 in 2007, of which 50.9% (138,000) were male and 49.1% (133,000) were females (CSA, 2007). The majority of the population (91%) lives in rural areas, with the majority working in agriculture; the remaining 9% live in urban areas. The district has 49,528 rural households with an average household size of 4.3 people (CSA, 2007).

The elevation of the Dembiya district is between 1500 to 2600 m above sea-level. The agro-ecology of the district is midland (woyna-dega) with a respective mean annual minimum and maximum temperature of 11^0C and 32^0C and the mean annual rainfall that ranges from 995 to 1175 mm. Information obtained from the district Agricultural Bureau indicated that the respective proportion of areas considered as plain, mountainous, valleys, and wetland is 87%, 5%, 4.8%, and 3.2%. Out of the total area of the district, 31% is cultivated land, 16% is none cultivable land, 5.6% forest and bush, 12.8% grazing, 8.1% is covered with water, 20.2% swamp and 4.3% is residential areas. The district receives bimodal rainfall, with the short rainy season from March to May and the main rainy season from June to September.

The major crops grown in the district includes teff (*Eragrostis teff*), maize (*Zea mays)*, barley (*Hordeum vulgare*), red highland sorghum (*Sorghum bicolor*), and finger millet (*Eleusine Coracana*). Besides, legumes and pulses such as chickpeas (*Cicer arietinum*) and cowpeas (*Vigna unguiculata*) are also grown in the district. They also grow some cash crops like red papper (*Capsicum annuum* Group), niger seed (*Guizotia abyssinica*), fenugreek (*Trigonella foenum-graecum*), black cumin (*Nigella sativa*), white cumin (*Cuminum cyminum*), and rice (*Oryza sativa*) with a limited number of farmlands.

Guramba Bata ($12^0$21' N and $37^0$20' E, altitude < 2000m.a.s.l.) located 7 km from Kola Diba town. A seasonal river persists until the end of December serving as one of tributaries to Lake Tana. Guramba Bata has one health post and one health center, 1113 households with 6008 inhabitants (2974 are male and 3034 are females) in 2017/18 (unpublished Health Office Report) (Figure 3.1).

Arebiya ($12^0$20'N and $37^0$22' E, altitude < 2000m.a.s.l.) is located 17 km away from Kola Diba town. The Megech River is one of the most important rivers serving as a water source during a dry season and drains into Lake Tana. Within 1976 households, the locality has a total of 8632 inhabitants (4298 are male and 4384 are females) in 2017/18. There is one health post in the locality (Health Office Report, Unpublished data) (Figure 3.1).

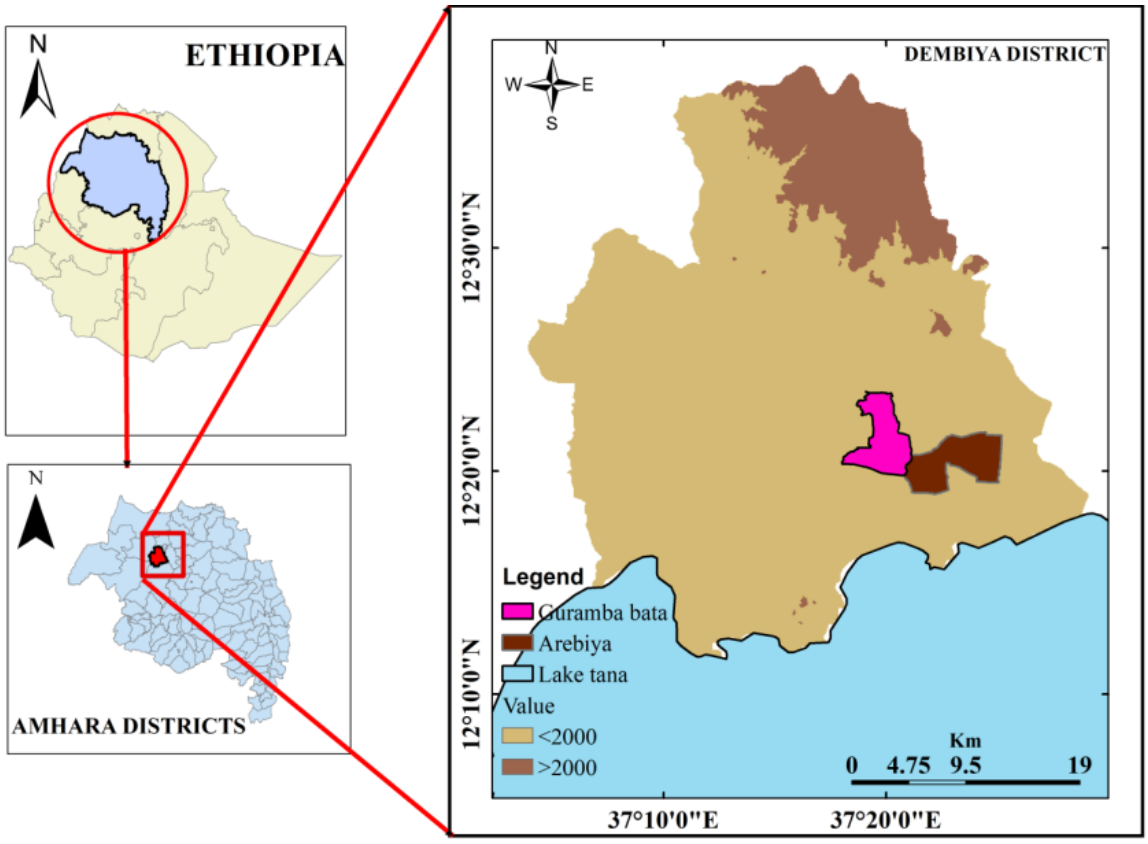

Figure 3.1 Map of the study area.

3.2.2. Study Design

A retrospective study was conducted to determine the six-year (2012 to 2017) of malaria prevalence by reviewing reports at Guramba Bata health center and Arebiya health post. A cross-sectional parasitological survey was also conducted in the two study Kebeles of Dembiya district (Guramba Bata and Arebiya) following the end of the long rainy season (October to November, 2018). The two Kebeles were selected based on their long history of implementing vector control strategies such as IRS and LLINs (Unpublished Health Office Report).

3.2.3. Retrospective malaria data collection

To assess the retrospective trend of malaria prevalence in the study areas implementing IRS and LLINs vector intervention measures, a six-year malaria retrospective data (2012-2017) was obtained from Guramba Bata health center and Arebiya health post. The retrospective malaria prevalence data in the two Kebeles were recorded from microscopic and RDT techniques which were implemented in the health facilities to confirm the presence of *Plasmodium* parasite in the blood samples.

3.2.4. Sample size determination for active case detection

The sample size formula necessary for estimating a population proportion of a small finite population was used to determine the sample size (Israel, 1992).

$$n=\frac{\boldsymbol{no}}{1+\frac{n0-1}{N}}$$

Where n is the minimum sample size for a small population and n_o is the sample size for a larger population, N is the population size (N for Guramba Bata= 6008 and N for Arebiya= 8632) and n_o is calculated using a single point proportion formula. i.e.

$$no = \frac{z^2 * p(1-p)}{d^2}$$

Where, p= prevalence of intestinal parasites (50%), d= margin of error (0.05); Z= standard score corresponds to 1.96.

$$no=\frac{1.96^2 0.5(1-0.5)}{0.05^2}= 385$$

$$n_2=\frac{385}{1+\frac{385-1}{6008}} = 365$$

and

$$n_2=\frac{385}{1+\frac{385-1}{8632}} = 370$$

Where n_1 is the sample size for Guramba Bata study site and n_2 is the sample size for Arebiya study site.

3.2.5. Blood sample collection and prevalence study

Blood samples were taken from 365 individuals from Guramba Bata and 370 individuals from Arebiya study sites. These individuals were randomly selected from 160 households, considering 4.3 average persons to household of Amhara region (CSA, 2007).

Thick and thin smears from finger-prick blood samples were prepared from a total of 735 individuals by well-trained laboratory technicians, from randomly selected households at the end of the rainy season (October - December, 2018). All thin smears were air dried and fixed with methanol in the field. Both thick and thin blood smears were stained with 3% Giemsa solution for 30 minutes in staining jars in the laboratory. The stained slides were rinsed with tap water and placed in an upright position to dry. The stained thick and thin films were examined with 100x oil immersion objective under a light microscope. The thick blood smear samples were first examined for the presence of *Plasmodium* parasites to determine whether the sample is positive or negative. When samples were positive, thin blood smears were examined for species identification (Warrell & Gilles, 2017).

3.2.6. Socioeconomic survey

A structured questionnaire was prepared to collect information about socio-economic data of the study participants while taking blood samples (Appendix 1 and Appendix 2). Questionnaires were filled by field assistants in consultation with the head of a household during blood sample collection.

3.2.7. Ethics approval and consent to participate

Ethical approval and consent to participate Ethical clearance was obtained from Addis Ababa University, institutional ethical review board of the College of Natural and Computational Sciences (Ref No CNSDO/692/10/2018). Written consent was obtained from the head of the household and other study participants before sampling. Individuals proved to be positive for malaria during blood film examination were treated with anti-malarial drug prescribed by physicians.

3.2.8. Statistical analysis

The data on retrospective and prospective prevalence of malaria parasites in the two study sites, different age groups, sexes, years and species type were entered using Microsoft excel data sheet and were analyzed using SPSS version 20 (Armonk, NY: IBM Corp). Chi squire test was used to compare the difference in frequency of malaria prevalence between independent variables (sex, Kebeles, and age). Association between independent variables with dependent variables was analyzed using bivariate logistic regression analysis. Multivariate logistic regression was used to analyze the relative contribution of each independent variable to the dependent variable at $p \leq 0.05$.

3.3. Results

3.3.1. Socio-demographic data

Blood samples for microscopic examination were collected from 735 randomly selected individuals from the two study Kebeles of which 50.3% (n= 370) were from Arebiya and 49.7% (n= 365) were from Guramba Bata. Males comprised 52% (n= 382) while females were 48% (n= 353) of individuals in the sample (Table 3.1). The age groups, below 15, 5-9, 10-14, and above 15 accounted for 7.3% (n= 54), 18.9% (n= 139), 17.8% (n= 131), and 55.9% (n= 411) of the study participants, respectively. The majority of the study participants were farmers (86.7%; n= 637) and the rest (13.3%; n= 98) were merchants. Most of the study participants (45.9 %; n= 341) were not educated. All study participants were from rural areas (Table 3.1).

Table 3.1. Socio-demographic data of the study participants in the two Kebeles of Dembiya district, Northwestern Ethiopia (Values in parenthesis are percentages).

Variables	**Study sites**		**Total (%)**
Sex	Arebiya	Guramba Bata	
Male	200(54.1)	182 (49.9)	382 (52)
Female	170 (45.9)	183 (51.8)	353 (48)
Total	370 (50.3%)	365 (49.7%)	735
Age			
<5	30 (8.1)	24 (6.6)	54 (7.3)
5-9	76 (20.5)	63 (17.3)	139 (18.9)
10-14	74 (20)	57 (15.6)	131 (17.8)
≥15	190 (51.4)	221 (60.5)	411 (55.9)
Total	370	365	735
Occupation			
Farmer	332 (89.7)	305 (83.6)	637 (86.7)
Merchant	38 (10.3)	60 (16.4)	98 (13.3)
Total	370	365	735
Educational status			
No formal education	146 (39.5)	195 (53.4)	341 (46.4)
Primary school attendees	119 (32.2)	104 (28.5)	223 (30.3)
Secondary school attendees	71 (19.2)	48 (13.2)	119 (16.2)
More than secondary	34 (9.2)	18 (4.9)	52 (7.1)
Total	370	365	735 (100)

3.3.2. Retrospective trends of malaria prevalence

Out of 2,157 individuals who visited the two health facilities seeking treatment and suspected to have malaria, 22.4% (n= 484) were positive for malaria parasites (Table 3.2). Microscopic and RDT results indicated that 19.4% (n=281) individuals in Arebiya and 28.7% (n= 203) individuals in Guramba Bata were infected with malaria parasites during the six-year period (2012-2017). From the total malaria prevalence *P. falciparum* accounts 75.8% (n=367/484), whereas *P. vivax* and mixed accounts 18.2% (n=88/484) and 5.9% (n=29/484), respectively.

There were significant differences in malaria cases among the age groups in both health facilities (Arebiya (χ^2=111.8, df =3, p=0.000); Guramba bata (χ^2=231.7, df =3, p=0.000)). Malaria was more prevalent in individuals between the 18-64 age groups in both health facilities. Malaria parasites were detected in 28.5% (n= 226) individuals in Arebiya health post, and 67.1% (n= 143) individuals in Guramba Bata Health center in the 18-64 age group (Table 3.2). On the other hand, relatively low number of malaria cases was recorded in the 6-17 years age groups (7.2% in Arebiya Health post and 9.3% in Guramba Bata health center) (Table 3.2). The difference in malaria cases between sexes were statistically significant in both Arebiya health post (χ^2= 102.3, df =1, p=0.000) and Guramba Bata study sites health center (χ^2= 21.7, df = 1, p= 0.000). Higher malaria cases were recorded in males (27.3% and 35.9%, in Arebiya and Guramba Bata, respectively) than in females during the six-year period in both health facilities (Table 3.2). Furthermore, *P. falciparum* was detected in individuals of all age groups, but it was predominant in individuals between the 18-64 years age group (23.3% and 53.3% in

Arebiya and Guramba Bata respectively). *Plasmosium vivax* was frequently recorded in children less than 5 years of age group in both study Kebeles (Table 3.2).

The lowest malaria cases in Arebiya health post (10.9%; n= 31) were recorded in 2016, while the highest malaria cases (41.9%; n= 72) were encountered in 2017 (Figure 3.2). Similarly, lower malaria cases (11.6%; n= 11) were detected in 2016 with highest (47.4%; n= 72) malaria cases were recorded in 2017 in Guramba Bata health center (Figure 3.2).

Plasmodium falciparum was the predominant species in the study sites during the six year period (2012-2017) (Figure 3.3) with the highest *P. falciparum* malaria cases (35.8%; n= 116) recorded in 2017. *Plasmodium vivax* and mixed infections were recorded in relatively lower magnitude in both sites during the six-year period (Figure 3.3).

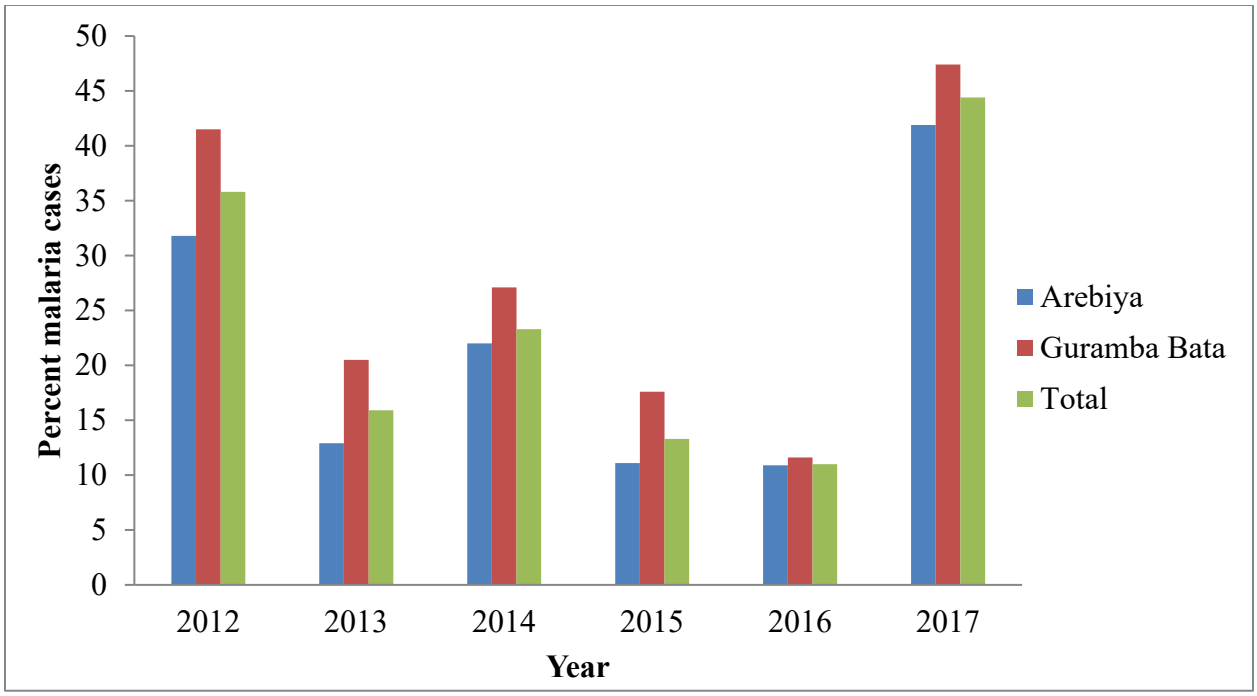

Figure 3.2. Six-year annual percent malaria cases reported in Arebiya and Guramba Bata (2012 - 2017).

Table 3.2. A six years retrospective trend of malaria cases in the two Kebeles of Dembiya district (2012- 2017).

No. of malaria parasite positive cases (values in parenthesis are %malaria cases)										
Study sites			Arebiya					Guramba Bata		
Age	No. Examined	P.f (%)	P.v (%)	Mixed (%)	Total +ve (%)*	No. Examined	P.f (%)	P.v (%)	Mixed (%)	Total +ve(%)**
< 5	63	2(3.2)	4(6.3)	0	6(9.5)	66	9(13.6)	8(12.1)	0	17(25.8)
6-17	553	23(4.2)	17(3.1)	0	40(7.2)	418	23(5.5)	8(1.9)	8(1.9)	39(9.3)
18-64	792	187(23.6)	27(3.4)	12(1.5)	226(28.5)	213	114(53.5)	20(9.4)	9(4.2)	143(67.1)
>65	41	9(21.9)	0	0	9(21.9)	11	0	4(36.2)	0	4(36.4)
Total	1449	221(15.3)	48(3.3)	12(0.8)	281(19.4)	708	146 (20.6)	40 (5.6)	17 (2.5)	203(28.7)
Sex	No. Examined	P.f (%)	P.v (%)	Mixed (%)	Total +ve (%)ǂ	No. Examined	P.f (%)	P.v (%)	Mixed (%)	Total +ve (%)ǂǂ
Male	931	207 (22.2)	36 (3.9)	11 (1.2)	254 (27.3)	398	110 (27.6)	23 (5.8)	10 (2.5)	143 (35.9)
Female	518	14 (2.7)	12 (2.3)	1 (0.2)	27 (5.2)	310	36 (11.6)	17 (5.5)	7 (2.3)	60 (19.4)
Total	1,449	221 (15.3)	48 (3.3)	12 (0.8)	281 (19.4)	708	146 (20.6)	40 (5.6)	17 (2.4)	203 (28.7)

*= χ^2=111.8, df =3, p=0.000; ** = χ^2=231.7, df =3, p=0.000; ǂ = χ^2= 102.3, df =1, p=0.000; ǂǂ = χ^2= 21.7, df = 1, p= 0.000

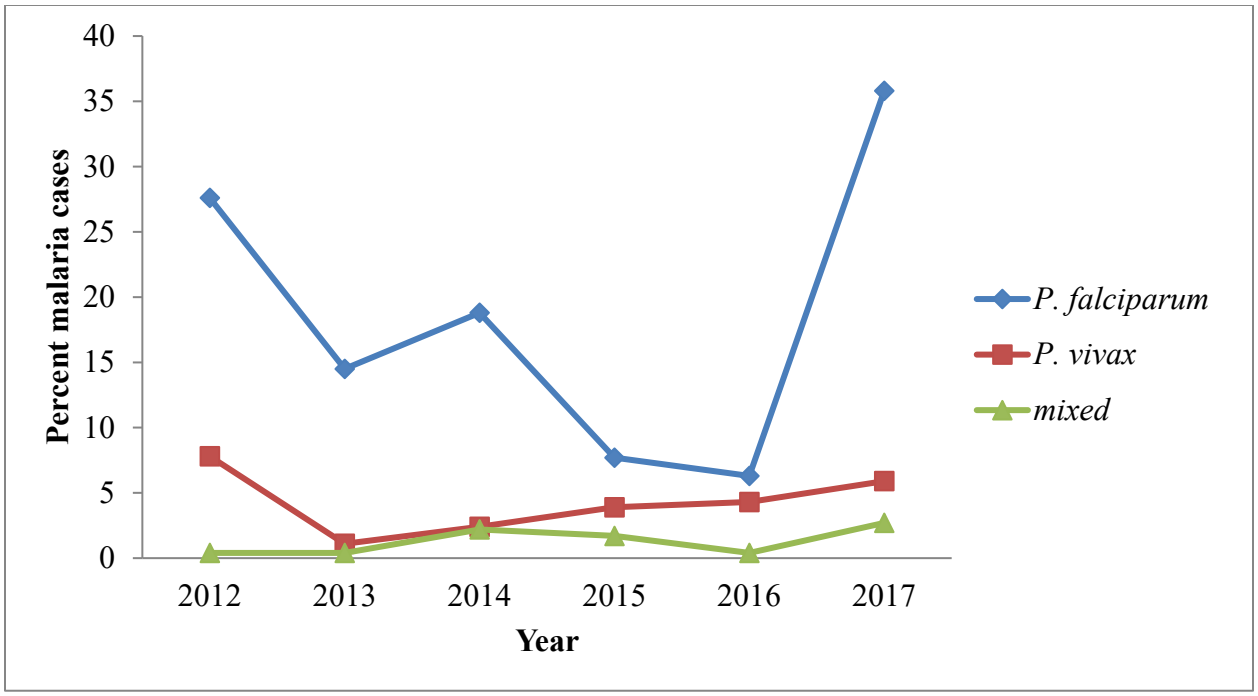

Figure 3.3. Six-year annual species specific prevalence of malaria parasites reported in Arebiya and Guramba Bata (2012 - 2017).

3.3.3. Prevalence of malaria parasites from blood sample examination

Out of the total 735 thick and thin blood smears taken from individuals who participated in the study, 3.5% (n= 26) were positive for malaria parasites. The results from the cross sectional survey indicated that there was no statistically significant difference in percent malaria prevalence between the two Kebeles (χ^2= 0.06, df =1, p=0.814). The prevalence of malaria infection in Guramba Bata and Arebiya study areas were 3.8% (n= 14) and 3.2% (n= 12), respectively (Table 3.3). *Plasmodium falciparum* was the predominant malaria parasite (65.3%, n= 17/26) in the study area, followed by *P. vivax* (19.2%, n=5/26), and mixed infections (15.4%, n= 4/26).

The frequency of malaria infection among the age groups was statistically significant in Arebiya study site (χ^2= 8.3, df =3, p=0.040) (Table 3.3). Malaria was more prevalent in

the age group > 15 years old at this study site (5.8%). Whereas, in Guramba Bata study site the age group > 15 were more infected with malaria (5%) than the other, but it was not statistically significant (χ^2=2.32, df =3, p= 0.509) (Table 3.3). Males were more infected with malaria in Arebiya (5%) and the difference in malaria case between sexes were statistically significant (χ^2 = 4.3, df =1, p= 0.039) (Table 3.3). Similarly, in Guramba Bata study sites males were more infected with malaria (4.4%) than females, though the difference was not statistically significant (χ^2= 0.31, df = 1, p= 0.579) (Table 3.3).

Table 3.3. Prevalence of malaria from the cross-sectional study in two Kebeles of Dembiya district (October-November, 2018).

Number and proportions of microscopic malaria parasite positive blood samples											
Study sites				Arebiya					Guramba Bata		
Age	No. Examined	P.f(%)	P.v(%)	Mix (%)	Total +ve (%)*	Age	No. Examined	P.f (%)	P.v(%)	Mix(%)	Total +ve(%)**
< 5	30	0	0	0	0	< 5	24	1(4.2)	0	0	1(4.2)
5-9	76	0	0	0	0	5-9	63	1(1.6)	0	0	1(1.6)
10-14	74	1(1.4)	0	0	1(1.4)	10-14	57	0	1(1.8)	0	1(1.8)
≥15	190	6(3.2)	2(1.1)	3(1.6)	11(5.8)	≥15	220	8(3.6)	2(0.9)	1(0.5)	11(5)
Total	370	7(1.9)	2(0.5)	3(0.8)	12(3.2)	Total	365	10(2.7)	3(0.8)	1(0.3)	14(3.8)
Sex	No. Examined	P.f (%)	P.v (%)	Mix (%)	Total +ve (%)ǂ	Age	No. Examined	P.f (%)	P.v (%)	Mix (%)	Total +ve (%)ǂǂ
Male	200	6 (3)	1 (0.5)	3 (1.5)	10 (5)	Male	182	4 (2.2)	3 (1.6)	1 (0.5)	8 (4.4)
Female	170	1 (0.6)	1 (0.6)	0	2 (1.2)	Female	183	6 (3.3)	0	0	6 (3.3)
Total	370	7 (1.9)	2 (0.5)	3 (0.8)	12 (3.2)	Total	365	10 (2.7)	3 (0.8)	1 (0.3)	14 (3.8)

* = χ^2= 8.3, df =3, *p*=0.040; ** = χ^2=2.32, df =3, *p*= 0.509; ǂ = χ^2 = 4.3, df =1, *p*= 0.039; ǂǂ = χ^2= 0.31, df = 1, *p*= 0.579

3.3.4. Malaria risk factor analysis

Bivariate and multivariate analysis indicated that risk factors such as sex, age, outdoor activity in the evening, awareness about malaria transmission, the frequency of LLIN distribution, and application of IRS were significantly associated with malaria prevalence (p<0.05). However, respondent's occupation, educational level, the last time respondents received IRS were not significantly associated with malaria transmission (p>0.05; Table 3.4).

Males were 2.6 times more likely to be infected with malaria than females (AOR = 2.6, 95% CI: 1.04, 6.41) and individuals with high outdoor activity were 16.4 times more vulnerable than individuals with limited outdoor activities (AOR= 16.4, 95% CI: 1.82, 147.85). Respondents who are not aware of malaria transmission and control were highly infected with malaria than those who were aware of it (AOR=0.3, 95% CI: 0.12-0.82). The last time respondents received LLINs (before a year) was associated with a low level of malaria prevalence in the study area (Table 3.4).

Table 3.4. Bivariate and multivariate analysis of factors associated with malaria infection in the study sites, Dembiya district.

Variables	Category	Total Examined	Positive (%)	OR (95% CI)		
				COR	AOR	*p* value
Sex	Female	353	8 (2.3)	1	1	
	Male	382	18(4.7)	2.13(0.92-4.97)*	2.58(1.04-6.41)	0.041
Age	<5	54	0	1	1	
	5-9	139	1(0.7)	0.38(0.02-6.252)*	0.31 (0.02-5.27)	0.417
	10-14	131	3(2.3)	0.82(0.07-9.26)	0.53 (0.04-6.48)	0.617
	≥15	413	22(5.3)	2.99(0.39-22.69)	2.15(0.27-16.92)	0.466
Occupation	Farmer	637	25(3.9)	3.96(0.53-29.58)	4.16(0.49-35.22)	0.191
	Merchant	98	1(1)	1	1	
Educational status	No education	341	14 (4.1)	1.07(0.24-4.85)	1.19(0.24-5.93)	0.837
	Primary	223	6 (2.7)	0.69(0.14-3.53)	0.87(0.16-4.74)	0.873
	Secondary	119	4 (3.4)	0.87(0.15-4.90)	1.58(0.25-10.02)	0.629
	> Secondary	52	2 (3.8)	1	1	
Outdoor activity	Yes	571	25 (4.4)	7.46(1.00-55.50)*	16.42(1.82-147.85)	0.013
	No	164	1 (0.6)	1	1	
Awareness about transmission	Yes	441	12 (2.7)	1	1	

	No	286	14 (4.9)	1.87(0.85-4.11)*	3.17(1.22-8.24)	0.018
Period of receiving last IRS	<6 month	541	20 (3.7)	1	1	
	6-12 month	194	6 (3.1)	0.83(0.33-2.10)	1.98(0.66-5.91)	0.221
Period of receiving last LLIN	<6month	16	3 (18.8)	1	1	
	6-12 month	15	4 (26.7)	1.58(0.288-8.61)	3.32(0.47-23.74)	0.231
	>1 year	704	19 (2.7)	0.12(0.032-0.46)*	0.12(0.02-0.57)	0.008

*: Indicates significant values at $p \leq 0.05$

3.4. Discussion

This study evaluated the six-year retrospective malaria prevalence data from health facility records in the Guramba Bata and Arebiya Kebeles, where vector control strategies such as IRS and LLINs have been used for more than a decade. A snapshot cross-sectional malaria survey was also conducted to determine the level of malaria transmission and the malaria parasites that prevail in the study area. The result of this study showed that malaria is still one of the most important causes of morbidity in the study area. In addition, it was evident that people's outdoor activities during the night, the community's low knowledge level about malaria control and prevention and history of receiving LLINs were determining factors which affect malaria transmission in the study area. These imply that malaria elimination programmes need to focus on improving knowledge of the community about malaria prevention and control strategies and look for additional vector control strategies targeting outdoor malaria transmissions in the study area.

The overall prevalence of malaria cases detected in the retrospective study was 22.4% (n=484) with percent malaria cases peaking towards 2017 (44.4%), albeit of the ongoing IRS and LLINs malaria vector control strategies implemented in the study area. This high prevalence of malaria suggests the need for additional malaria intervention strategies to achieve the intended goal of malaria elimination in the study area. A relatively higher percent of malaria cases were reported from a similar retrospective study in the nearby Kola Diba health centre (39.6%) (Alemu *et al*., 2012), and Serbo health center (43.8%) (Karunamoorthi and Bekele, 2009). However, this result is higher than a retrospective

study conducted in Metema hospital (Ferede *et al*., 2013) and Kombolcha (Gebretsadik *et al*., 2018), where the prevalence was 17%, and 7.5% respectively.

The trend of the six-year retrospective data indicated that malaria prevalence varied from year to year, with relatively lower malaria cases recorded in 2013, 2015 and 2016. The reduced number of malaria cases during these years could be associated with the accumulated effect of scaled-up malaria intervention strategies in the study area. In contrast to this trend, a relatively higher percentage malaria cases were reported in 2017 (44.4%). The main reason for an increased trend of malaria in 2017 may be the change in *Anopheles* mosquito behaviour favoring outdoor biting and resting tendency or due to the development of insecticide resistant vector species or drug resistant *Plasmodium* parasites. In addition it could also be associated with low level of knowledge or perception of the community about malaria prevention and control strategies. This is in agreement with the existing scenarios advocating that malaria remains a public health problem in Ethiopia even though intensive vector intervention strategies were implemented (Benelli and Beier, 2017).

The overall prevalence of malaria from this cross-sectional study was 3.5% (26/735) and *P. falciparum* was the predominant malaria parasite, this is comparable with the 3.9% prevalence reported from a cross-sectional study conducted in Hawasa town (Alemu, 2006). The result of this prevalence is lower than the 5.3% prevalence of malaria reported in Gondar Town (Tilye and Deressa, 2007), the 5.2% malaria prevalence from Jimma town (Alemu *et al*., 2011B), and 22.1% prevalence among children's less than five years in Arba Minch Zuria (Abossie *et al*., 2020). This difference could be attributed to the

variation in intensity of vector control strategies, altitude, microclimate, habitat modifications, and community awareness about malaria prevention and control methods.

The species specific prevalence in this study showed that *P. falciparum* was the dominant, whereas *P. vivax* and mixed prevalence holds the second and third position respectively. This is in line with the fact that *P. falciparum* is the dominant parasite in many parts of Ethiopia with altitude below 2000 m a.s.l (PMI, 2015). Similar trend of *Plasmodium* parasite distributions were reported from Gilgel-Gibe (Yewhalaw *et al*., 2009) and children from Northern Ethiopia (Ghebreyesus *et al*., 1999), migrant laborers from Northwestern Ethiopia (Aschale *et al*., 2018) and from patients attending Chagni health center (Belay *et al*., 2021). Retrospective studies from Kola Diba health center (Alemu *et al*., 2012), Serbo health center (Karunamoorthiand Bekele, 2009), Metema hospital (Ferede *et al*., 2013), Kombolcha (Gebretsadik *et al*., 2018), and Tselemti Woreda (Shiferaw *et al*., 2018) and a survey from different part of Ethiopia (Abose*et al*., 2003) also support this study finding. However, reports showed that *P. vivax* was the dominant cause of infection in some part of Ethiopia (Alemu *et al*., 2011A; Alemu *et al*., 2011B; Woyessa *et al*., 2012; Tadesse *et al*., 2018). This variation could be associated with the difference in altitude of the study areas, where *P. falciparum* is dominant in lowland areas (bellow 2000 m) and study period (*P. vivax* is dominant during the dry season), the emergence of drug resistant *P. vivax* to chloroquine, and the relapsing nature of *P. vivax* (Deress and Girma, 2019; FMoH, 2020).

The retrospective and prospective studies indicated that malaria infections were more prevalent in males than in females. Similar studies indicated that males are more infected with malaria than females in different part of Ethiopia (Alemu *et al*., 2014; Tesfahunegn

et al., 2019; Agegnehu *et al*., 2018; Dufera *et al*., 2020) and in Kenya (Sultana *et al*., 2017). It was presumed that individual behaviours, environmental and socio-economic factors contribute to transmission of malaria in Ethiopia (Deressa *et al*., 2007; Graves *et al*., 2009). Likewise, malaria was more prevalent in individuals above the age group of ≥15. This is in agreement with a retrospective study conducted in Kombolcha (Gebretsadik *et al*., 2018) and Kola Diba health centers (Alemu *et al*., 2012). Males at these productive ages are actively involved in outdoor activities such as agriculture and cattle herding in the evening which makes them vulnerable to outdoor *Anopheles* mosquito biting. Males usually spend the night outside the house tending cattle in the study sites. These outdoor activities at night were predictors associated with malaria transmission in the study areas. Similarly, different reports indicated that outdoor activities in the evening contributed to high malaria transmission (Graves *et al*., 2009; Agegnehu *et al*., 2018) mainly due to the fact that individuals with outdoor activities are exposed to outdoor biting by *Anopheles* mosquitoes (Kibret *et al*., 2010).

The current study showed that poor awareness and knowledge about malaria prevention and control contributed to the prevailing malaria transmission in the study area. Similar reports indicated that awareness about malaria was associated with malaria transmission in Ethiopia (Agegnehu *et al*., 2018) and Kenya (Sultana *et al*., 2017). This urges for a continuous need to educate and increase awareness of the local communities about malaria transmission towards improved malaria prevention and control strategies.

This study assesses malaria prevalence using both retrospective and prospective data (cross-sectional study). Because of a resource limitation we were unable to conduct a longitudinal study, which is best study design for this type of study.

3.5. Conclusions

In conclusions, despite the long standing implementation of vector control strategies such as LLINs and IRS in Dembiya district, malaria remains one of the most important health problems in the community. The total prevalence of malaria parasites from a retrospective and prospective study was 22.4% and 3.8% respectively, where *P. falciparum* was found to be the dominant *Plasmodium* species in the study area. Factors such as sex, low knowledge level about malaria prevention and control, outdoor activity during the evening and history of access to LLINs are risk factors for the existing malaria prevalence in the study area. Therefore, the national malaria elimination programme should incorporate additional malaria prevention and control strategies targeting outdoor malaria transmission. Moreover, community health education package about malaria prevention and control should be a part of malaria elimination strategy in the study area.

Chapter 4: Habitat characterization and spatial distribution of *Anopheles* mosquito larvae in Dembiya district, Northwestern Ethiopia

4.1. Introduction

Mosquitoes are responsible for the transmission of different types of diseases such as malaria, yellow fever, dengue, and West Nile fever (Alonso *et al*. 2011; Braack *et al*. 2018). Malaria is a life-threatening infectious disease caused by protozoan parasites of the genus *Plasmodium* that are transmitted through the bites of infected female *Anopheles* mosquitoes. Globally, an estimate of 241 million malaria cases and 627,000 deaths were reported in 2020, of which 95% of malaria cases and highest proportion of deaths were reported from a WHO Africa region (WHO, 2021). In this region 80% of all malaria deaths are recorded in children's under the age of five. From the total malaria case across the globe 55% is recorded in sub-Sahara Africa (WHO, 2021). In Ethiopia, more than 60% of the population is at risk of malaria infection and 68% of the country's landmass is malarious (FMoH, 2014). The diverse ecology and favorable environmental conditions of the country supported the rapid development of *Anopheles* mosquitoes and *Plasmodium* parasites (Taffese *et al*. 2018).

There are more than 400 different species of *Anopheles* worldwide, of which around 30 are known malaria vectors (WHO, 2018). In Ethiopia, *An. arabiensis* is the primary malaria vector, whereas *An. funestus*, *An. pharoensis* and *An. nili* are secondary vectors (Gillies and Coetzee, 1987). The most important stage of *Anopheles* mosquito life cycle such as egg-laying, larval and pupal development, and adult emergence takes place in

aquatic environment (Oyewole *et al*., 2009). Different species of *Anopheles* mosquitoes have their own preferred aquatic habitats. For instance, *An. arabiensis* prefers to breed in temporary, small, sunlit, clear and shallow fresh-water pools (Gimnig *et al*., 2001; Edillo *et al*., 2006; Himeidan & Rayah, 2008).

The abundance and distribution of *Anopheles* mosquito breeding habitats are key factors that determine the density of adult *Anopheles* mosquito populations and transmission of malaria parasites (Carter *et al*., 2000; Rejmánková *et al*., 2013). Several environmental characteristics like climate, physical, chemical and biological conditions of the breeding habitats have effects on the development and survival of the *Anopheles* mosquito larvae (Meretaet *al*., 2013; Roux and Robert, 2019). Anthropogenic factors like agricultural expansion, construction of dams and urbanization affects the diversity and distribution of *Anopheles* mosquito breeding habitat and larval development (De Silva and Marshall, 2012). Climate change associated with these anthropogenic effects favors rapid development of *Anopheles* mosquitoes and *Plasmodium* parasite in areas with antecedently low malaria transmission (Alonso *et al*., 2011).

In Ethiopia, malaria control programme mainly depends on the management of clinical malaria cases or control efforts targeting adult mosquitoes by selective indoor residual spray (IRS) and insecticide-impregnated bed nets (LLINs) (FMoH, 2014). However, these strategies are challenged by the development of drug resistant *Plasmodium* parasites and insecticide resistant vector species (Messengeret *al*. 2017; Taffese *et al*. 2018; Lohaet *al*. 2019). Considering this, interests are rising to use larval source management as a part of integrated vector management in the country (Gari and Lindtjørn, 2018; Asale *et al*. 2019). Nevertheless, our knowledge about mosquitoes

breeding habitat diversity, distribution and characteristics is limited and insufficient to design effective malaria intervention strategies by larval control (Rejmánková *et al*., 2013).

Therefore, the aim of this study was to assess the species composition, distribution and ecology of *Anopheles* mosquito larvae and pupae in two malaria endemic Kebeles of Dembiya district, northwestern Ethiopia. The result of this study will provide important information to design effective vector control strategies through larval source management and larviciding.

4.2. Materials and Methods

4.2.1. Description of the Study area

The study was conducted in Arebiya and Guramba Bata Kebeles, and the detailed description of the areas has been given in chapter three of this dissertation.

4.2.2. Study design

A longitudinal study design was implemented to assess the ecology, breeding habitat type and species composition of *Anopheles* mosquitoes in selected Kebeles of Dembiya district. This two study sites were selected based on their high level of malaria endemicity, implementation of vector control strategies (IRS and LLINs) for a long time and accessibility.

4.2.3. Collection of *Anopheles* larvae and pupae

The larvae and pupae of *Anopheles* species were collected from different breeding sites such as drainage canals, artificial pit shelters, hoof prints, and rain pools (Figure 4.1 (a),

(b), (c), (d)). The larval collection was performed for two consecutive days per month for 12 months. Before sampling, habitats were inspected for the presence or absence of *Anopheles* larvae and pupae. When *Anopheles* larvae were present, repeated dips were taken using standard WHO dippers (350 ml capacity) depending on the size of each larval habitat (WHO, 1975). For small habitats like hoof prints, samples taken with droppers from several sites were pooled to get appropriate larval sampling (Soleimani-Ahmadi *et al*. 2014). Sampling was performed in the morning (09:00-12:00) or in the afternoon (14:00-17:00) for about a maximum of 30 minutes depending on the size of each larval breeding habitat.

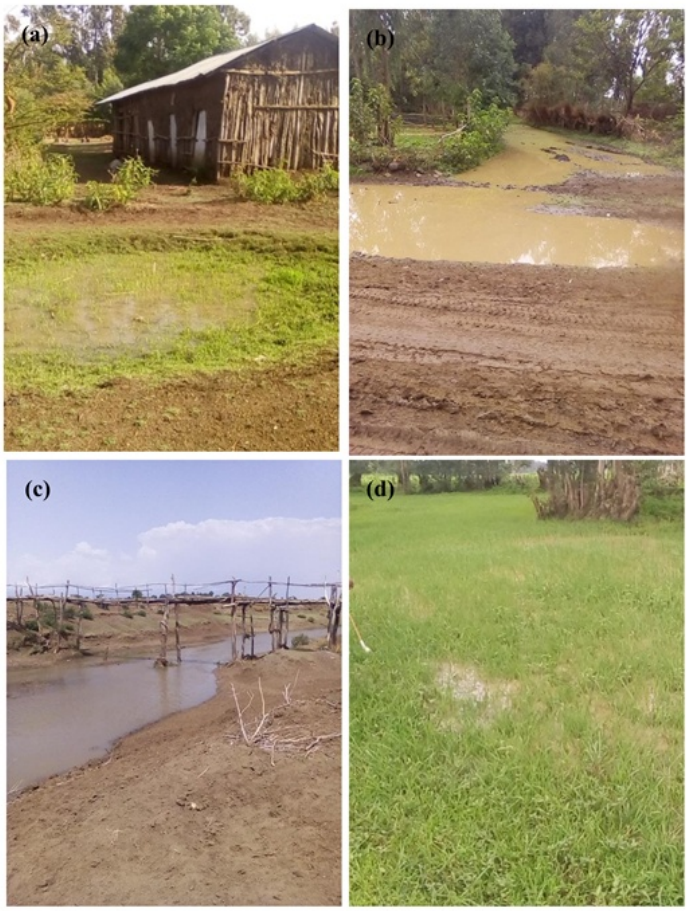

Plate 4.1. *Anopheles* mosquito potential breeding habitats: Artificial pit (a) Drainage canals (b) River pools (c) Swamp (d).

4.2.4. Species Identification

Anopheles larvae and pupae were sorted out from *Culicines* using a hand lens (10X) based on gross morphological characteristics (Verrone, 1962; Gillies and Coetzee, 1987). The 3rd and 4th instar *Anopheles* larvae, and *Anopheles* pupae collected from each type of breeding habitat, were transferred to separately labeled mosquito breeders (BioQuip, Dimensions: 7-3/4″ (195 mm) high x 3-5/8″ (92 mm) diameter) and reared in the field lab based on WHO guidelines (WHO, 1975). The adult *Anopheles* that emerged from field collected larvae and pupae were used for species identification based on morphological characteristics (Verrone, 1962; Gillies and Coetzee, 1987). Morphologically identified adult *An. gambiae s.l* was individually preserved in Eppendorf tubes with silica gel and cotton for further molecular identification.

4.2.5. Species identification using PCR

Preserved adult *An. gambiae s.l* specimens from field collected and reared larvae and pupae were further identified to sibling species using a ribosomal DNA polymerase chain reaction (PCR) by including the primers for *An. gambiae s.s*, *An. arabiensis*, *An. quadriannulatus*, *An. amharicus, An.merus* and *melas* (Scott *et al*., 1993).Extraction of template DNA from adult *An. gambiae sl.* was based on a protocol developed by Collins *et al.* (1987). The ribosomal region targeting specific single nucleotide polymorphisms (SNPs) for *An*. *gambiae s.l* complex was amplified in a multiplex reaction as described Wilkins *et al*., (2006). Agarose gel prepared by following Wilkins *et al*., (2006) protocol were used for gel-electrophoresis and a MultiDoc-ItTM Imaging System-Masterflex computer software was used to detect DNA fragments and capture photo of the DNA bands (the full protocol is presented under appendix 3 of this dissertation).

4.2.6. Physico-chemical characteristics of larval habitat

Physical characteristics of the breeding habitat, including water depth, turbidity, vegetation, presence of algae, bottom substrate, habitat stability, lotic or lentic water, sunlight intensity and its distance from a nearby house, habitat type were measured and recorded (Minakawa *et al*. 1999).

Average water depth was taken after measuring the depth from different spots using a ruler and classified into two categories (≥0.5 m, and ≤0.5 m). Turbidity was determined after observing the water sample within a glass test tube and classified as low, medium and turbid. The vegetation type was classified as no vegetation, and grass-based on personal observation. Presence or absence of algae was also documented based on personal observation. Similarly, the bottom substrate type is classified as muddy, clay, silt, and sandy, based on observation. A habitat was considered temporary if water persists for ≤2 weeks and permanent if the water persists for ≥ 2 weeks. Whether the water is flowing or not was determined based on observation. Light intensity was visually categorized as sunlit if the habitat received full sunlight that could occur throughout the day otherwise it was considered as shaded. Distance to the nearby house was measured in meter and classified as, 1. ≤100 m, 2.101 to 200 m, 3.201 to 300 m, 4. 301 to 500 m.

Chemical characteristics of the breeding habitats (positive or negative for *Anopheles* larvae or pupae) such as temperature, pH, and conductivity were measured at the field with HANNA© HI 98130 Combo pH & EC tester (Hanna Instruments Inc., Kehl am Rhein, Germany) with the probes placed 2 to 3 cm below the water surface.

4.2.7. Data analysis

The density of *Anopheles* larvae and pupae were expressed as the total number of *Anopheles* larvae per total number of dips taken. After checking for normality, all dependent variables were $\log_{10}(x+1)$ transformed, and subjected to statistical analysis. Since the data's were found to be normally distributed after transformation, parametric test such as one-way analysis of variance (ANOVA) and student t-test (for independent variables with two categories) were used to analyze differences in mean larval densities among breeding habitat types and other environmental variables. When significant differences were observed in one-way ANOVA, means were separated using Tukey's HSD (Tukey's Honestly Significant Difference) test at $\alpha=0.05$.

Multiple logistic regression analysis was used to detect the best predictor environmental variables associated with the presence or absence of *Anopheles* larvae (Sattler *et al*. 2005). The percentages of species composition of *Anopheles* mosquitoes collected from each breeding habitat was calculated (number of *Anopheles* mosquitoes species * 100/ total number of species identified). Pearson correlation analysis was used to assess the relationship between larval densities and chemical characteristics (pH, temperature, and conductivity). The data were analyzed using SPSS version 20 (Armonk, NY: IBM Corp), $p \leq 0.05$ were considered as significant.

4.3. Results

4.3.1. Breeding habitat types and abundance of *Anopheles* larvae and pupae

During the one-year study period, a total of 108 potential larval habitats (60.2% from Arebiya and 39.8% from Guramba Bata) were assessed for the presence of *Anopheles* larvae. From the total sampled potential habitats, only 41 were positive for *Anopheles* larvae and pupae (Table 4.1). From which, the predominantly encountered larval habitats were rain pools (17.6% (n= 19)), and river pool (17.6% (n= 19)). More than 80% of the larval breeding habitats were recorded during the long rainy season. Whereas, during the dry season, the distribution of *Anopheles* larvae and pupae were restricted to water pools at riversides, pits dug for plastering a house, and temporary habitats around the hand pump water well.

The highest mean densities of *Anopheles* larvae were recorded from breeding sites in drainage canals (14.7 ± 3.5 larvae/dip), followed by abandoned burrow pits dug for plastering houses (8.8 ± 3.1 larvae/dip), swamps (3.8 ± 1.2 larvae/dip), and hoof prints (3 ± 1.2 larvae/dip). In addition, *Anopheles* larval densities were also recorded from puddles (2.7 ± 2.7 larvae/dip), riverside water pools (2.0 ± 0.9 larvae/dip), and tyre tracks (0.4 ± 0.4 larvae/dip) (Table 4.1). The difference in mean densities of *Anopheles* larvae and pupae among habitats was statistically significant ($F_{(8, 99)}= 9.85$,p=0.000) ($F_{(8, 99)}= 3.46$, p= 0.001), respectively (Table 4.1).

Table 4.1. Distribution of *Anopheles* mosquito larvae and pupae in different types of breeding habitats in the two Kebeles of Dembiya district (June 2018 to May 2019).

Habitat type	No. of breeding sites surveyed (%)	No. of dips	No. of larvae collected	Mean (larvae/dips) ± se	No. of pupae collected	Mean (pupae/dips) ± se
Riverside	19 (17.6)	99	279	2.0 ± 0.9^{ab}	48	0.3 ± 0.1^{ab}
Burrow pits	14 (12.96)	70	421	8.8 ± 3.1^{bc}	49	0.99 ± 0.4^{ab}
Drainage canals	12 (11)	48	566	14.7 ± 3.5^{c}	59	1.6 ± 0.6^{b}
Tyre tracks	8 (7.4)	24	9	0.4 ± 0.4^{a}	3	0.1 ± 0.1^{a}
Hoof prints	7 (6.5)	19	63	3.0 ± 1.2^{ab}	5	0.2 ± 0.2^{ab}
Swamps	14 (12.96)	56	205	3.8 ± 1.2^{ab}	9	0.2 ± 0.1^{ab}
Rain pools	19 (17.6)	69	30	0.2 ± 0.2^{a}	0	0.0 ± 0.0^{a}
Puddles	7 (6.5)	33	56	2.7 ± 2.7^{a}	12	0.6 ± 0.6^{ab}
Streams	8 (7.4)	30	0		0	
Total	108(100%)	448	1629		185	

The mean larval and pupal densities with different letter designations in a column are significantly different with Tukey's HSD post hoc analysis at $\alpha=0.05$.

4.3.2. Species composition and monthly distribution of *Anopheles* larvae and pupae

The species composition of *Anopheles* mosquitoes identified from the two study sites during the study period is presented in Table 4.2. A total of 1,629 *Anopheles* larvae and 185 pupae were collected from the two Kebeles. From the total collected immature stages of *Anopheles* mosquitoes, 52.3% (852) larvae and 65.9% (122) pupae were from Arebiya and 47.7% (777) larvae and 34.1% (63) pupae were from Guramba Bata. The difference in mean *Anopheles* larval and pupal density between the two study sites were not statistically significant ($t_{(106)} = -0.454$, $p= 0.651$) and ($t_{(106)} =0.70$, $p= 0.485$) respectively.

From the total collected *Anopheles* larvae and pupae, 835 females and 788 males have successfully emerged into adults. The rest 191 larvae and pupae were not able to emerge to adult. Therefore, only 835 female *Anopheles* mosquitoes were subjected to species identification based on morphological features. All *An. gambiae s.l* samples used for species identification using PCR were found to be *An. arabiensis*. Eight species of *Anopheles* such as *An. arabiensis*, *An. pharoensis*, *An. coustani*, *An. christyi*, *An. squamosus*, *An. demeilloni*, *An. danicalicus*, and *An. cinereus* were identified (Table 4.2). Of these, *An. arabiensis* (59.2%) was the dominant species followed by *An. pharoensis* (35.3%) and *An. coustani* (2.99%), while the least common species were *An. dancalicus* and *An. cinereus* (Table 4.2).

Monthly distribution of *Anopheles* larvae and pupae showed that, the highest density of *Anopheles* larvae in Arebiya was collected in June, September, October, and May

(Figure 4.2 (a)). In the meantime, less density of *Anopheles* larvae was recorded during July and August, which is corresponding to pick monthly rainfall, which creates unstable larval breeding habitat due to over flooding. Similarly, in Guramba Bata high larval density was recorded starting from June, August, September, and October (Figure 4.2 (b)). However, the density of *Anopheles* larval density sharply declined in both study areas during a dry season (January, February, and March) (Figure 4.2 (a) & (b)).

Table 4.2. Species composition and abundance of *Anopheles* mosquitoes collected from the two Kebeles of Dembiya district (June 2018 to May 2019) (Values in parenthesis are percentages)

Species	**Arebiya**	**Guramba Bata**	**Total**
An. arabiensis	297	197	494 (59.2)
An. pharoensis	113	182	295 (35.3)
An. christyi	6	2	8 (1)
An. squamosus	3	0	3 (0.4)
An. coustani	9	16	25 (2.99)
An. demeilloni	2	4	6 (0.7)
An. dancalicus	0	1	1 (0.1)
An. cinereus	0	3	3 (0.4)
Total	430 (51.5)	405 (48.5)	835 (100)

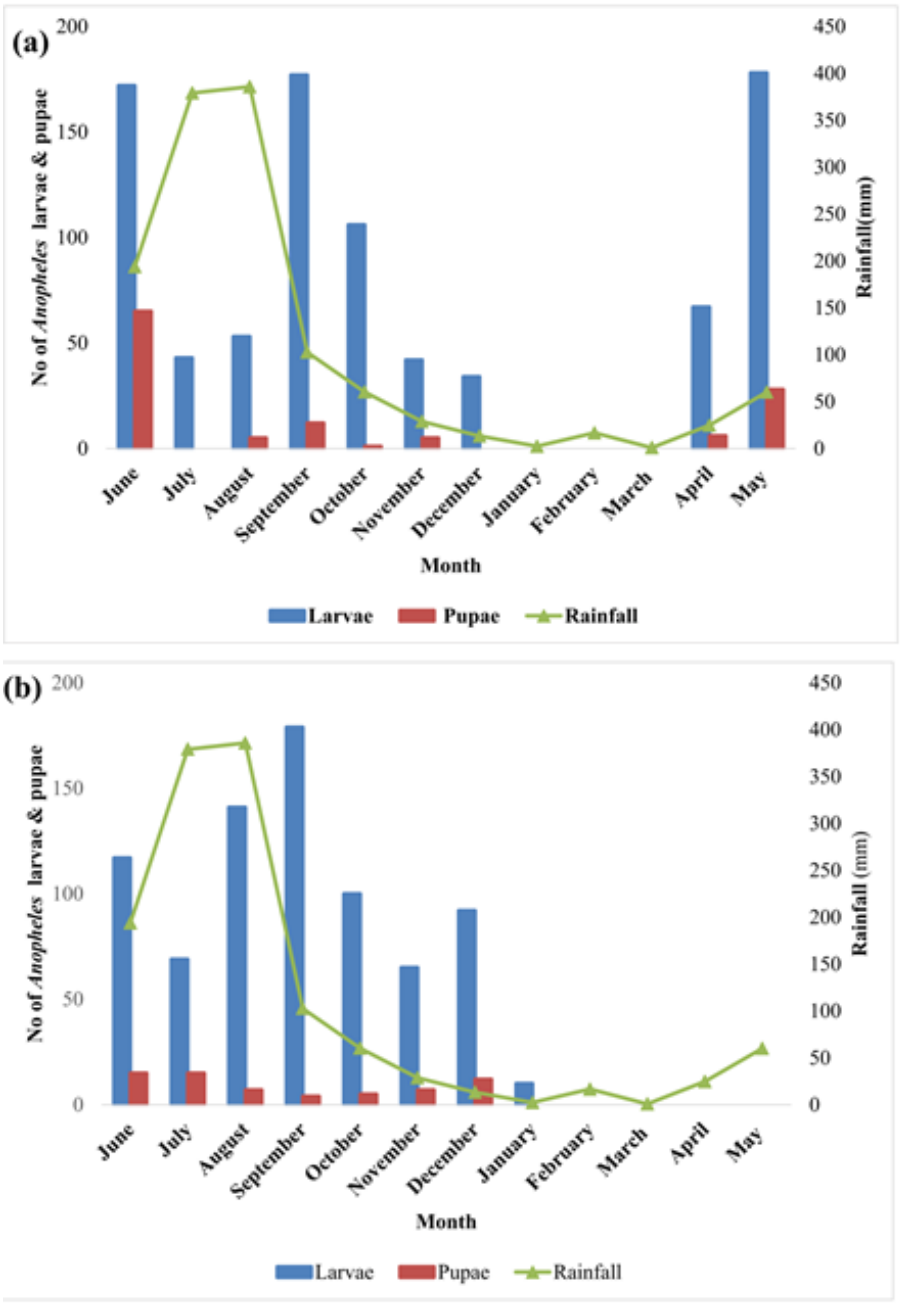

Figure 4.1. Monthly distributions of *Anopheles* mosquito larvae and pupae in Arebiya (a) Guramba Bata (b) study sites from June 2018-May 2019.

4.3.3. Species specific monthly distribution of *Anopheles* mosquitoes

The species-specific monthly distribution of the dominant *Anopheles* mosquitoes is indicated in Figure 4.3 (a) & (b). The highest density of *An. arabiensis* in Arebiya was recorded during June (16.5%), September (9.1%), and May (21.6%) (Figure 4.3 (a)). Similarly, in Guramba Bata the highest density of *An. arabiensis* was recorded during June (12.8%), July (10.4%), and September (12.3%), but its number sharply declined after the end of the long rainy season (Figure 4.3 (b)). The number of *An. pharoensis* reached its peak around the end of the main rainy season in the two study areas (Figure 4.3(a) & (b)).

4.3.4. *Anopheles* species specific breeding habitat types

The associations of species-specific *Anopheles* larvae in different breeding habitat types are presented in Table 4.3. Larvae of *An. arabiensis* were collected from drainages, burrow pits, water pools at riversides, tyre tracks, hoof prints, and puddles. High number of *An. arabiensis* was collected near the edge of a small temporary and permanent habitat with still and mid turbid water, grass, and full sunlight access. *Anopheles pharoensis* was collected from a wide range of permanent habitats such as riverside water pools, swamps, and grassy burrow pits. The breeding habitats of *An. pharoensis* were relatively turbid, full of vegetation and partial sunlight access. *Anopheles coustani* was collected from permanent habitats such as riverside water pools, burrow pit, and swamps. The most common breeding sites of this species were usually permanent habitats with relatively turbid water, vegetation, and partial sunlight access.

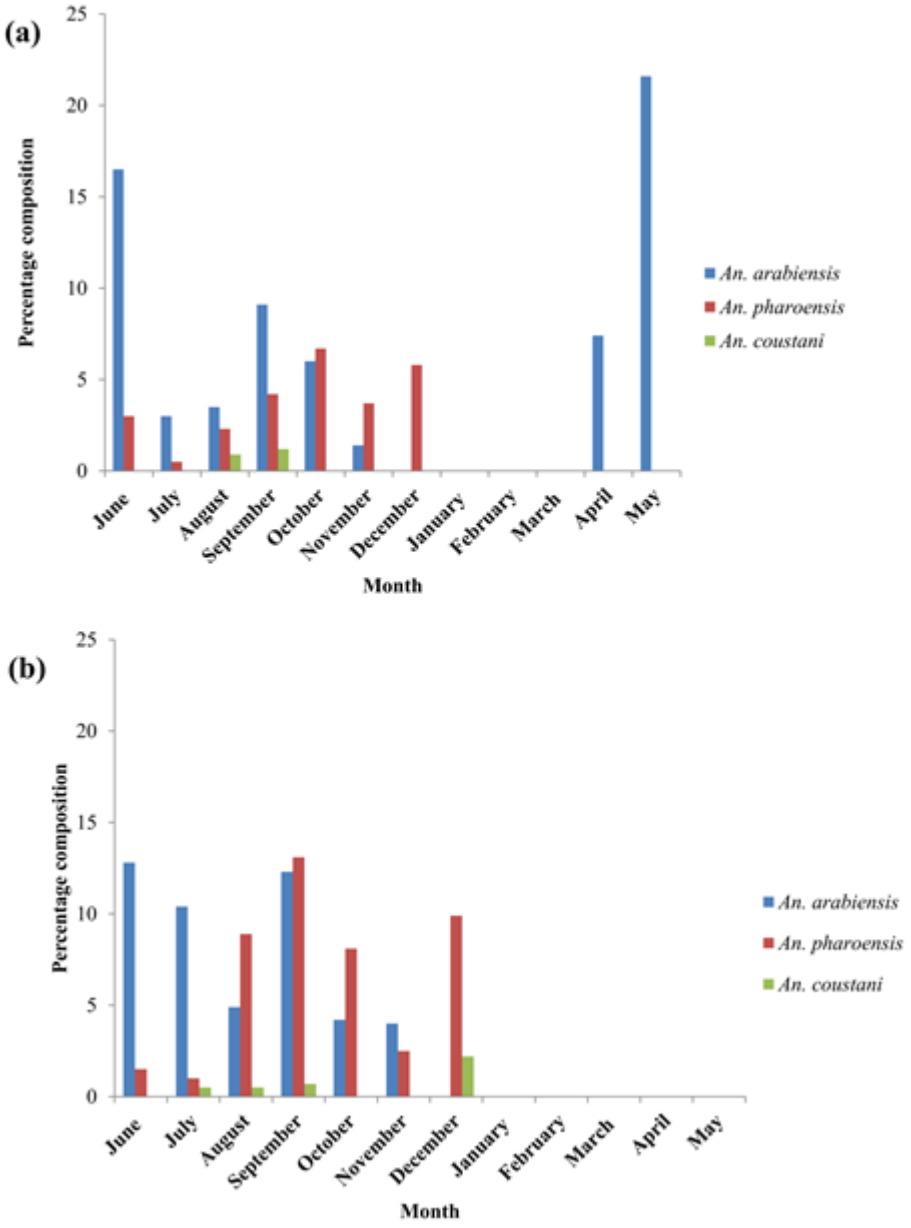

Figure 4.2. Monthly distribution of dominant species *Anopheles* mosquito in Arebiya (a) and Guramba Bata (b) study sites from June 2018-May 2019**.**

Table 4.3. Species-specific spatial distribution of *Anopheles* mosquitoes in the two Kebeles of Dembiya district (June 2018-May 2019) (Values in parentheses are the percentages).

	Anopheles mosquito larval breeding habitat types								
Species	Riverside	Burrow pit	Drainage canal	Tyre track	Hoof print	Swamp	Rain pool	Puddles	Total
An. arabiensis	78 (15.8)	148 (29.9)	174 (35.2)	5 (1)	26 (5.3)	26 (5.3)	11 (2.2)	26 (5.3)	494 (59.2)
An. pharoensis	83 (28.1)	69 (23.4)	64 (21.7)	-	-	79 (26.8)	-	-	295 (35.3)
An. coustani	9 (36)	5 (20)	4 (16)	-	-	7 (28)	-	-	25 (2.9)
An. christyi	1 (12.5)	5 (62.5)	1 (12.5)	-	-	1 (12.5)	-	-	8 (0.96)
An. demeilloni	-	2 (33.3)	4 (66.7)	-	-	-	-	-	6 (0.7)
An. squamosus	-	2 (66.7)	-	-	-	1 (33.3)	-	-	3 (0.4)
An. cinereus	3 (100)	-	-	-	-	-	-	-	3 (0.4)
An. dancalicus	-	-	1 (100)	-	-	-	-	-	1 (0.1)
Total	174 (20.8)	231 (27.7)	248 (29.7)	5 (0.6)	26 (3.1)	114 (13.7)	11 (1.3)	26 (3.1)	835 (100)

4.3.5. Association of *Anopheles* larval densities with physical characteristics of larval breeding habitat types

The associations of mean larval densities with physical variables of the breeding habitat types are presented in Table 4.4. The densities of *Anopheles* larvae were significantly associated with shallow depth (≤0.5 m), medium turbidity, availability of grasses, muddy bottom substrates, distance to the nearest house (≤100 m), and presence of algae ($p \leq 0.05$).

A bivariate analysis showed that average depth (COR=2.54; 95% CI: 0.98-6.6, $p \leq 0.05$) turbidity (COR=28.4; 95% CI: 3.61-224.27, $p \leq 0.05$), vegetation (COR=17.82; 95% CI: 5.62-56.54, $p \leq 0.05$), distance to the nearest house (COR= 32.7; 95% CI: 4.15-257.27, $p \leq 0.05$) and presence of algae (COR=4.92; 95% CI: 1.99-12.11, $p \leq 0.05$) were significantly associated with the presence or absence of *Anopheles* larva (Table 4.5).

The final model for the parameters associated with presence or absence of mosquito larvae showed that turbidity (AOR = 66.03; 95% CI: 2.01-2168.24, $p = 0.019$) and vegetation cover (AOR= 12.62; 95% CI: 1.29-122.78, $p = 0.029$) were key physical factors which determine the presence or absence of *Anopheles* mosquitoes larvae (Table 4.5).

Table 4.4. Association of physical characteristics of breeding habitat types with *Anopheles* larval densities.

Physical factors	Variables	Densities of *Anopheles* larvae		
		Mean ± se	*p*	F
Average depth	≤0.5 m	5.2±1.01	0.040	2.144
	≥0.5 m	1.8±0.57		
Turbidity	Low	0.63±0.45	0.000	19.899
	Med	6.96±1.24		
	High	0.55±0.37		
Vegetation	No vegetation	0.69±0.43	0.000	-4.89
	Grass	7.44±1.29		
Bottom substrate	Muddy	6.74±1.08	0.000	8.231
	Clay	0.44±0.34		
	Sandy	3.21±3.21		
Water current	Stagnant	4.69±0.96	0.180	-1.35
	Flowing	2.34±0.92		
Light intensity	Full sunlight	4.28±0.81	0.687	0.404
	Shaded	3.57±1.84		
Distance	≤100m	7.04±1.22	0.000	7.76
	100 -200m	0.53±0.53		
	201-300m	0.00±0.00		
	301 - 400m	0.17±0.17		
Algae	Present	10.16±2.16	0.000	5.66
	Absent	1.74±0.40		
Water persistency	Temporary	2.6±0.70	0.99	1.66
	Permanent	5.15±1.18		

Table 4.5. A bivariate and multivariate analysis of association of physical factors of breeding habitats with presence or absence of *Anopheles* mosquito larvae.

Factors	Variables	OR (95% CI)		*p*-value
		COR	AOR	
Average depth	≤0.5	2.5 (0.98-6.60)*	0.4 (0.04-4.32)	0.450
	≥0.5	1	1	
Turbidity	Low	3.6 (0.35-37.36)	22.5 (0.66-774.51)	0.084
	Med	28.4 (3.61-224.27)*	66 (2.01-2168.24)	0.019
	High	1	1	
Vegetation	No vegetation	1	1	
	Grass	17.8 (5.62-56.54) *	12.6 (1.29-122.78)	0.029
Bottom substrate	Muddy	21.7 (2.64-177.76)	8.6 (0.24-308.82)	0.238
	Clay	0.00 (0.00)	0.00 (0.00)	0.997
	Sandy	1	1	
Water current	Stagnant	0.36 (0.12-1.05)	0.1 (0.01-1.29)	0.077
	Flowing	1	1	
Light intensity	Full sunlight	2.1 (0.76-5.79)	2.21 (0.25-19.92)	0.479
	Shaded	1	1	
Distance	≤100m	32.7 (4.15-257.27)*	27.99 (0.65-1215.17)	0.083
	100 -200m	1.39 (0.08-23.71)	0.99 (0.01-132.14)	1.00
	201-300m	0.00	0.00	
	301 - 400m	1		
Algae	Present	4.92 (1.99-12.11)*	6.94 (0.78-62.09)	0.083
	Absent	1		
Water persistency	Temporary	1.15 (0.51-2.61)	1.51 (0.23-9.99)	0.67
	Permanent	1		
Surface debris	Low	1.81 (0.54-6.08)	7.90 (0.53-117.17)	0.133
	Medium	1.11 (0.34-3.68)	0.723 (0.07-7.89)	0.791
	High	1	1	

* indicates statistically significant values at $p = 0.05$

4.3.6. Correlation of chemical characteristics of larval habitat with *Anopheles* density

The total density of *Anopheles* larvae was positively correlated with temperature (r= 0.331 and *p*= 0.013) and pH (r= 0.697 and *p*= 0.00). However, the density *Anopheles* larvae was negatively correlated with conductivity (r= -0.321, *p*=0.016) (Table 4.6).

Table 4.6. Correlation of chemical characteristics of breeding habitat with density of *Anopheles* mosquito larvae.

Chemical factors	Total larval density
Temperature (°C)	0.331*
pH	0.697**
Conductivity (µS/cm)	-0.321*

**. Correlation is significant at the 0.01 level

*. Correlation is significant at the 0.05 level

4.4. Discussion

This study showed that the spatio-temporal distribution and species composition of *Anopheles* mosquito larval density were greatly affected by the physicochemical characteristics of the breeding habitat and the rainfall pattern of the Kebeles. Describing larval habitat characteristics in terms of environmental attributes and identifying relationships between breeding habitat and larval density is important to develop novel methods of vector control by targeting the aquatic stage of *Anopheles* mosquitoes in areas with high vector intervention strategies.

In this study, rain pools, river pools, burrow pit, swamp, drainage canal, tyre track, hoof print, puddle, and stream were the identified larval breeding habitats, of which rain pools and river pools were the dominantly observed breeding habitats. Rain during rainy period produces many rain pools and river edges, which are potential sites for larval development. In concurrent with this study, *Anopheles* mosquitoes prefer to breed at the edges of rivers and streams, in temporary rain pools, ponds, dams, drainage ditches, burrow pits, rice fields, swamp margins, roadside puddles, and in tree holes close to human dwellings (Shililu *et al*., 2003; Yohannes *et al*., 2005; Omlin *et al*., 2007). In addition, similar habitat types were recorded in previously studies elsewhere in Ethiopia (Meretae*t al*., 2013) and Kenya (Imbahale *et al*., 2011).

The distribution of *Anopheles* larvae during the dry season was limited to water pools at river sides, pits dug for plastering a house, drainage canals and water pools around hand pump water well. This is because during the dry season the water will be confined to temporary habitats such as river edges, burrow pits, drainage canals and swamps, which

are important for the breeding of *Anopheles* mosquitoes. Similarly, studies showed that number and size of *Anopheles* reproduction habitats are reduced during the dry season (Animut and Negash, 2018). This restricted distribution of *Anopheles* mosquito breeding habitat during the dry season makes them more vulnerable for larval management. Larval management during a dry season is less coasty than management during a wet season in areas where dry season is associated with limited breeding habitats (WHO, 2013). Dry season larval management will hamper the exponential reproduction rate of *Anopheles* larvae at the end of long rainy season hence it will limit malaria transmission (Animut and Negash, 2018). This approach could be effective to reduce malaria transmission associated with insecticide resistant malaria vectors and outdoor host seeking mosquitoes, as it manages the immature stage (egg, larvae and pupae) confined in a small aquatic environments (Killeen *et al*., 2002).

The results of this study indicated that the density of *Anopheles* larvae varies among habitat types, where a significantly higher density was recorded from water pools at drainage canal and the grassy edge of burrow pits. This larval density variation in the different habitats could be explained by the spatiotemporal differences in food resource and predation pressure in different habitats and the complex interaction between physicochemical factors such as water turbidity, depth, temperature, salinity, and dissolved oxygen (Mala and Irungu, 2011; Kipyab *et al*., 2015;Roux and Robert, 2019).

Anopheles mosquito species such as *An. arabiensis*, *An. pharoensis*, *An. coustani*, *An. christyi*, *An. squamosus*, *An. demeilloni*, *An. danicalicus*, and *An. cinereus* were identified from the two study areas. This result is in line with finding from the west Gojam zone (Animut and Negash, 2018), south-central Ethiopia (Animut *et al*., 2012),

and central Ethiopia (Kenea *et al*., 2011). The highest density of *Anopheles* larvae was recorded during the rainy seasons. The reason is that rainfall and humidity strongly affect the availability of larval habitat, *Anopheles* species and distribution (Imbahale *et al*., 2011).

Anopheles arabiensis was the dominant species identified in the study area, similar to report from southwest Ethiopia (Getachew *et al*., 2020), Addis Zemen, south Gondar, (Kindu *et al*., 2018), south-central Ethiopia (Animut *et al*., 2012), and western Kenya (Kweka *et al*., 2012). The highest density of *An. arabiensis* was recorded in June and September when the rainy season starts and retreats. Similar observations showed that populations of *An. arabiensis* usually increase as the rains withdraw (Getachew *et al*., 2020). The result of this study also indicated that *An. arabiensis* was largely distributed in small temporary habitats with still and less turbid water and grasses and full sunlight access. The reason for this could be the presence of less larval predation pressure in small temporary habitats than permanent habitats and temporary habitats with full sunlight access provides a warmer water, which resulted in a high algal density (source of food for larvae) and rapid development of larvae to pupae (Gimnig *et al*., 2002). In concurrent with this study, *An. arabiensis* was reported to breed in small, temporary habitats with algae such as footprints, rain pools, puddles, tyre tracks, and garden wells (Mattah *et al*., 2017). These species were also identified from temporary habitats such as swamps, irrigation canals, sand pools, canal leakage pools, water harvesting pools, and brick-making pits in central Ethiopia (Kenea*et al*., 2011).

Anopheles pharoensis was dominantly found in aquatic habitats with turbid, full of vegetation and partially sunlight. Different reports also supported this observation, in

which the density of *An. pharoensis* is higher in aquatic habitats with floating vegetation and with shady condition (Teklu *et al*., 2010).

Physical characteristics of *Anopheles* mosquito breeding habitat such as low turbidity and the presence of grass were the associated with the presence or absence of *Anopheles* mosquito larvae in this study. In addition, the density of *Anopheles* mosquito larvae was positively correlated with temperature and pH and negatively correlated with water conductivity. Conductivity measures the amount of inorganic matters and ions in water, therefore, as turbidity of the water increases due to flooding conductivity also increase proportionally, which in turn affects the development of larval population (Edillo*et al*., 2006). Low water turbidity and full sunlight access increase the water temperature and hence leads to a rapid larval development (Paaijmans *et al*. 2010). This result coincides with previous works conducted in the highlands of Ethiopia (Dejenie *et al*., 2011), Kenya (Minakawa *et al*., 1999), and Tanzania (Emidi *et al*., 2017). Furthermore, grasses could provide a hiding place for *Anopheles* larvae to avoid predation in the breeding habitats.

Following the habitat characteristics and density *Anopheles* mosquito's larvae/pupae for one year is the strength of this study. However, the use of the same rainfall data for the two study sites is the limitation of this study, which could bias the result. Additionally, we were not able to identify emerged male *Anopheles* mosquitoes and dead immature stages in to species level.

4.5. Conclusions

Anopheles mosquitoes such as *An. arabiensis*, *An. pharoensis*, *An. coustani*, *An. christyi*, *An. squamosus*, *An. demeilloni*, *An. danicalicus*, and *An. cinereus* were identified during this study, of which *An. arabiensis* was the dominant species. Breeding habitats such as drainage canals and burrow pits served as a potential reproduction site for *Anopheles* species in the study area. The distribution of *Anopheles* mosquito larvae is greatly affected by the physicochemical characteristics of the breeding habitats such as water turbidity, vegetation cover, pH, temperature, conductivity and season. A potential breeding habitat, such as drainage canals and burrow pits should be targeted for malaria vector control by using larvicides and source reduction during the dry season as a part of integrated vector management in the study area. Further study on identification of the natural predators of *Anopheles* mosquito's laravae/pupae in the study areas is recommended.

Chapter 5: Species composition, monthly distribution and behaviour of adult *Anopheles* mosquitoes in Dembiya district, northwestern Ethiopia

5.1. Introduction

Malaria is one of the leading public health problems in Ethiopia. Three quarter of the country's land mass and 68% of the total population is at risk of malaria infection (PMI, 2015). The two species of *Plasmodium* parasites such as *P. falciparum* and *P. vivax* are responsible for 60% and 40% of the total malaria cases in Ethiopia, although their relative composition varies across different Kebeles (Alemu *et al*., 2011A; Tesfaye *et al*., 2011; Alemu *et al*., 2012). *Anopheles arabiensis*, is the primary vector of malaria in Ethiopia, whereas other species such as *An. funestus*, *An. pharoensis* and *An. nili* are considered as secondary malaria vectors (FMoH, 2014).

In Ethiopia, the major malaria intervention strategies are prompt case treatment using artemisinin based combination therapy, and vector control strategies such as long lasting insecticide-treated bed nets (LLINs) and indoor residual spray (IRS) (Yimer *et al*., 2015). Long-lasting insecticide treated bed nets (LLINs) reduce malaria transmission by killing or blocking *Anopheles* mosquitoes that attempt to take a blood from human. Whereas, IRS kills and reduces longevity of *Anopheles* that rests on insecticide treated surfaces such as walls and other structures (WHO, 2014).

The scale up of malaria intervention strategies in Ethiopia was started since 2005 (Taffese *et al*., 2018). During post intervention period (2006 - 2011), the proportion of

population at malaria risk protected by LLINs is increased by 51%, IRS coverage increased by 35%, and active case treatment exceeded 87% when compared to pre intervention period (before 2005) (Aregawi *et al*., 2014). Because of this increased distribution, malaria inpatient cases and death in all age groups were reduced by 54% and 68%, respectively in 2011 than pre intervention period (2001–2005) (Aregawi *et al*., 2014).

Despite of reduction in overall malaria prevalence, malaria control is challenged by the development of insecticide resistance, shift in vector species composition and increasing vector behavioural change (Rowland *et al*., 2001; Yewhalaw *et al*., 2011; Kiware *et al*., 2012; Iwashita *et al*., 2014; Killeen *et al*., 2014). Recent reports from Ethiopia indicated that *An. arabiensis* was resistant to major class of insecticides, such as DDT, permethrin, deltamethrin, and malathion (Alemayehu *et al*., 2017; Mekuriaw *et al*., 2020). Additionally, this vector showed an increased outdoor biting and resting tendency and a shift in biting hour from late in the evening to early evening before people retire to bed has also been reported in the country (Kibret *et al*., 2016).

Entomological indicators including entomological inoculation rate (EIR), vector longevity, feeding preferences, the susceptibility of the vector to the parasites, and biting behaviour of *Anopheles* mosquitoes are important to determine the vectoral capacity of *Anopheles* mosquitoes and malaria transmission intensity (Garrett-Jones, 1964; Hess *et al*., 1968; Beier *et al*., 1987; Beier *et al*., 1988; Lines *et al*., 1991; Drakeley *et al*., 2003). Highly antropophilic *Anopheles* mosquito's species with high EIR, parasite permissibility, and longevity are considered as important vectors of malaria. Hence, it is

important to assess these entomological indicators in order to achieve the desired malaria control and elimination strategies in malaria endemic areas.

Dembiya is malaria endemic area in Ethiopia with a long history of implementing vector control strategies (Tulu, 1996). The trend of malaria infection in this district has been significantly reduced after the increased implementation of malaria intervention strategies (Toyama *et al*., 2016). However, malaria remains a series public health challenge in various Kebeles of the district (Alemu *et al*., 2012). Limited studies are available on the species composition, ecology, and behaviour of the local malaria vectors in the District. Therefore, this study aimed to assess the species composition, distribution and behaviour of *Anopheles* mosquitoes in malarious Kebeles of Dembiya district. The result of this study will help to design vector control strategy considering their behaviour and ecology.

5.2. Material and Methods

5.2.1. Description of the Study area

This study was conducted in the two malarious Kebeles of Dembiya district (Guramba Bata and Arebiya). The areas were briefly described in chapter three of this thesis.

5.2.2. Study design

A longitudinal study design was implemented to assess the ecology, breeding habitat type and species composition of *Anopheles* in two selected Kebeles of Dembiya district. This two study sites were selected based on their high level of malaria endemicity, implementation of IRS and LLINs for long time and accessibility.

5.2.3. Survey of host animals as blood meal sources

Information about the total population number living in the two study sites was obtained from the health center. Similarly, the available number of potential hosts such as bovines (cattle, goats), dogs, and chickens in the two study sites were collected from the local agricultural offices (unpublished).

5.2.4. Indoor and outdoor host seeking mosquito collection

Adult *Anopheles* mosquito collection was carried out for one year starting from Jun 2018 to May 2019 (Figure 5.1 (a), (b), (c), (d)). Indoor and outdoor host seeking mosquito collection was performed using CDC light traps (John W. Hock Ltd, Gainesville, FL., USA). For indoor host seeking *Anopheles* mosquito collection, a total of five CDC light traps were installed near to bed at a height of 1.5 m from 18:00 to 06:00 h in five randomly selected houses from each locality for two consecutive nights per month for each village (Figure 5.1 (a)). For outdoor host seeking *Anopheles* mosquito collection, five CDC light traps were installed near to animal enclosure in five randomly selected households from each locality (Figure 5.1 (b)). The same houses were used for adult mosquito collection through the year.

5.2.5. Indoor and outdoor resting mosquito collection

Indoor resting *Anopheles* mosquito collections were performed using a pyrethrum spray catches (PSCs) from another ten randomly selected houses from each locality starting from 06:30 to 09:30 h (Figure 5.1 (c)). Before PSC is implemented all food items, feeding utensils and small animals were evacuated from houses, and all openings and eaves of windows and doors were sealed. The floors were covered with white sheets

before spraying houses with a bygone aerosol (SC. Johnson & Son. Inc, USA). Fifteen minutes after spraying, knocked down *Anopheles* mosquitoes were collected by using forceps, paper cups, and a torch light (WHO, 1995). In addition, mouth aspirators were used to collect indoor resting mosquitoes (such as walls, ceilings, underneath of household furniture, and on materials hung on the walls).

Additional five houses from each locality were randomly selected for outdoor resting mosquito collection using artificially constructed pit shelters (constructed in the back yard of each selected house). The pit shelters have a depth of 1.5 m and with an opening of 1.2 m x 1.2 m. In each shelter four cavities with a horizontal depth of 30 cm were dug on each side (Figure 5.1 (d)). Mouth aspirators were used to collect resting mosquitoes after covering the mouth with untreated bed net. Collection was done two times per a month in the morning from 6:30 am to 10:00 am. Mouth aspirators were also used to collect outdoor resting mosquitoes from various outdoor possible mosquito resting sites fromeach locality (ground holes, tree holes, open cattle sheds and among vegetation). The collection was performed two times per a month for 30 minutes in each possible resting site.

Plate 5.1 Sampling of adult *Anopheles* mosquito using different collection techniques; Outdoor CDC LT (a) Indoor CDC LT (b) PSC (c) Pitshelter (d)

5.2.6. Species identification of *Anopheles* mosquitoes

Identification of all collected adult *Anopheles* mosquitoes into species level was conducted based on morphological key described by Gillies and Coetzee, (1987). Female *Anopheles* mosquitoes were further classified as unfed, blood fed, half-gravid and gravid. Morphologically identified *An. gambiae sensu lato* (*s.l*) and female

Anopheles mosquitoes were kept in a labeled 1.5 ml Eppendorf tube with cotton wool over silica gel desiccant. All collected mosquito specimens were kept at room temperature (25^{O}C) for later mosquito processing.

5.2.7. Species identification using an rDNA–polymerase chain reaction (PCR)

The protocol used for the identification of the sibling species of *An. gambiae s.l* using an rDNA–polymerase chain reaction (PCR) has been described in chapter four of this thesis (the full protocol is presented on appendix 3 of this dissertation).

5.2.8. Enzyme-Linked Immunosorbent Assay (ELISA) for Blood meal analysis

Blood meal source of engorged female *Anopheles* mosquitoes were examined using direct ELISA techniques using bovine and human antibodies with little modification (Beier *et al*., 1988). The abdomens of freshly fed female *Anopheles* mosquitoes were ground in 100 µl phosphate buffered saline (PBS), which was further diluted by adding 100µl PBS. A 100 µL of prepared samples were added to each well and incubated for 3 hr at room temperature. The incubated mixture was washed twice with PBS- tween 20. This was followed by addition of 50 µl host specific conjugate of bovine or human diluted 1:2000 (or 1:250 for bovine) in 0.5% boiled casein containing 0.025% Tween 20 to each well and incubated for additional 1 hr at room temperature. After 1 h, wells were washed three times with PBS-Tween 20, and 100 µL of ABTS peroxidase substrate was added to each well. Absorbance at 405 nm was determined with an ELISA reader 30 min after the addition of substrate. The result was interpreted as positive if the absorbance value exceeded the mean plus three times the standard deviation of the four negative

controls (unfed laboratory colony of *An. arabiensis*). Human blood obtained from humans (volunteer), and cow blood obtained from abattoirs was used as a positive control.

5.2.9. Enzyme-Linked Immunosorbent Assay (ELISA) for *Plasmodium* parasite detection

Circum-sprozoite (CSP) detection of the parasite within mosquito gut was performed based on a protocol developed by Beier *et al*. (1987). The head and thorax of *Anopheles* mosquitoes were ground in labeled 1.5 ml centrifuge tube using a pestle by adding a 50 µl of grinding buffer. The grinding pestle was washed twice with 100 µl of grinding buffer catching the rinses in the tube containing the mosquitoes triturate until the final volume reached 250 µl.

A 50 µl of *P. falciparum*, *P. vivax* 210 and *P. vivax* 247 capture monoclonal antibody (mAb) solution was placed in each well of separate plates assigned for each species. The plates were covered and incubated for 30 minutes at room temperature. The well contents were aspirated and banged on a paper towel five times. Each well were filled with 200 µl blocking buffer (BB) solution and incubated for one hour at room temperature. Well contents were aspirated and banged five times on a paper towel. A 50 µl mosquito sample, a positive control (*P. falciparum*, *P. vivax*-210 and *P. vivax*-247) and a negative control of unfed *An. arabiensis* from an established colony were added in each respective plate wells. The plates were covered and incubated at room temperature for 2 hours, and well contents were aspirated, banged on a paper towel and washed two

times using 200 µl PBS-Tween-20. The well contents were aspirated and banged on a paper towel with each wash.

A 50 µl peroxidase labeled conjugate solutions of *P. falciparum*, *P. vivax*-210 and *P. vivax*-247 were added to each well to the respective plates and incubated for one hour at room temperature. The plates were washed thrice with 200µl PBS-Tween-20 after the well contents are aspirated and banged on a paper towel. A 100 µl ABTS substrate solution was added in each well and the covered plates were incubated 30 minutes at room temperature. Finally, the plates were read at 405 nm absorbance using ELISA plate reader. The sample was considered as positive if the sample absorbance value is above the two times mean absorbance value of negative samples.

5.2.10. Data analysis

The density of *Anopheles* mosquitoes were calculated as a number of female *Anopheles* mosquito/trap/night for each collection method. All dependent variables were checked for normality and $\log_{10}(x+1)$ transformed when it didn't conform to normality. Student t test was used to compare mean *Anopheles* mosquito density between study Kebeles and indoor and outdoor locations. One-way analysis of variance (ANOVA) was used to compare the mean density among the different species of *Anopheles* mosquitoes. When significant differences were observed in one-way ANOVA, means were separated using Tukey's HSD (Tukey's Honestly Significant Difference) test at $\alpha=0.05$. The data were analyzed using SPSS version 20 (Armonk, NY: IBM Corp).

Human blood index (HBI) was estimated as a number of *Anopheles* mosquitoes fed on human blood meal over the total *Anopheles* mosquitoes tested for blood meal origin (Garrett-Jones, 1964). Similarly, bovine blood index (BBI) was estimated as a number of *Anopheles* mosquitoes fed on bovine blood meal over the total *Anopheles* mosquitoes tested for blood meal origin (Garrett-Jones, 1964). Mixed blood meal was included in calculating human blood index and bovine blood index. The relative feeding preference or forage ratio (FR) of *Anopheles* mosquitoes were calculated by dividing the percent of blood engorged *Anopheles* mosquito which have fed up on either humans or bovine to the percent which either human or cattle comprises in the area (Hess *et al*., 1968). If the FR is one (near 1) the host is neither preferable nor avoided by the local vector; If FR is significantly > 1, the host is preferred by the vector and if it is less than 1, the host is not preferable (Hess *et al*., 1968).

The sporozoite rate was calculated as the proportion of *Anopheles* mosquitoes positive for (*P. vivax* or *P. falciparum)* CSPs over the total number of *Anopheles* mosquito tested for CSPs. Annual entomological inoculation rate (EIR) for *Anopheles* mosquito was calculated from mosquito collection by CDC light trap using the formula, 1.605 × (Number of CSP positive ELISA results from CDC light traps/no. mosquitoes tested) × (No. mosquitoes collected from CDC light traps/No. trap-nights) × 365 (Lines *et al*., 1991; Drakeley *et al.,* 2003).

5.3. Results

5.3.1. Availability of *Anopheles* mosquitoes alternate hosts

There were a good number of cattle, goats, sheep and chickens which can potentially serve as putative blood source for *Anopheles* mosquitoes. Accordingly, from the total potential host animal populations, 19.9% were cattle and 23.6% were chicken (Table 5.1).

Table 5.1. Number of alternative blood meal sources in the study sites, Dembiya district, Northwestern Ethiopia (Unpublished data, Dembiya district Agricultural Bureau).

No	Number	Percentage
Cattle	6,980	19.9
Goat	39	0.1
Sheep	4,334	12.4
Donkey	756	2.2
Chickens	8,275	23.6
Human	14,640	41.8
Total	35,024	100

5.3.2. Species composition and monthly distribution of *Anopheles* mosquitoes

During a one year study period (Jun 2018 - May 2019) a total of 2,055 female *Anopheles* mosquitoes belonging to 11 species were collected (Table 5.2). From which, 56.6% (n= 1,164) were collected from Guramba Bata and 43.3 (n= 891) were from Arebiya Kebele. The difference in mean number of *Anopheles* mosquitoes between the two study sites was statistically significant ($t_{(679)} = -1.983$, p= 0.048). *Anopheles arabiensis*, *An. pharoensis*, *An. coustani*, *An. demeilloni, An. cinereus, An. ardensis, An. squamosus* and *An. funestus*

were identified from Arebiya study site. On the other hand, *An. arabiensis*, *An. pharoensis*, *An.coustani*, *An. demeilloni*, *An. garnhami, An. christyi, An. cinereus, An. funestus, An. ardensis, An. squamosus,* and *An. nili* were identified from Guramba Bata (Table 5.2). In this study, *An. pharoensis* was the predominant species identified in both Arebiya and Guramba Bata study sites, accounting for 46.2% (n=412) and 46.5% (n=541) of the total species, respectively. The second dominant species was *An. arabiensis* with a respective composition of 42.3% (n= 377) and 34.3% (n=399) in Arebiya and Guramba Bata study sites (Table 5.2). There was a significant difference in *Anopheles* mosquitoes species mean number in Arebiya ($F_{(7, 22)} = 13.317$; $p = 0.000$) and Guramba Bata ($F_{(10, 33)} = 6.011$; $p = 0.000$) (Table 5.2).

The density of *Anopheles* mosquitoes showed a steady increment starting from June to September in the two study sites; however, it significantly declines after the end of long rainy season (Figure 5.2 (a) and (b)). The highest density of indoor and outdoor host seeking *Anopheles* mosquitoes in Arebiya was recorded in September (12.20 and 12.80 mosquitoes /LT/night respectively). The density showed a slow increment starting from May in this study area (Figure 5.2 (a)). In Guramba Bata the highest density of indoor and outdoor host seeking *Anopheles* mosquitoes were recorded in August (12.3 and 13.8 mosquitoes/LT/night, respectively) and September (7.2 mosquitoes/LT/night and 15.9 mosquitoes /LT/night, respectively) (Figure 5.2 (b)).

Table 5.2. Species composition and abundance of *Anopheles* mosquito using different adult mosquito collection methods in the two study sites of Dembiya district, Northwestern Ethiopia (June 2010-March 2011).

Study site	**Species**	CDC Light Trap		Mouth Aspirator		PSC		PitShelter		Total		Mean ± se
		no.	%	no.	%	no.	%	no.	%	no.	%	
Guramba Bata	*An. arabiensis*	227	27.3	38	29.9	70	79.5	64	54.2	399	34.3	1.9±0.2[a]
	An. pharoensis	381	45.8	88	69.3	18	20.5	54	45.8	541	46.5	1.9± 0.3[a]
	An. coustani	146	17.6	1	0.8	-		-		147	12.6	0.6± 0.5[ab]
	An. demeilloni	27	3.2	-		-		-		27	2.3	0.4± 0.4[b]
	An. garnhami	1	0.1	-		-		-		1	0.1	0.1 ± 0.1[b]
	An. christyi	14	1.7	-		-		-		14	1.2	0.3 ± 0.3[b]
	An. cinereus	5	0.6	-		-		-		5	0.4	0.2 ± 0.2[b]
	An. funestus	8	0.9	-		-		-		8	0.7	0.2 ± 0.2[b]
	An. ardensis	12	1.4	-		-		-		12	1	0.3 ± 0.3[b]
	An. squamosus	9	1.1	-		-		-		9	0.8	0.3 ± 0.3[b]
	An. nili	1	0.1	-		-		-		1	0.1	0.1 ± 0.1[b]
	Total	831	100	127	100	88	100	118	100	1164		100
Arebiya	*An. arabiensis*	207	36.3	45	36.9	63	72.4	62	55.9	377	42.3	1.9 ± 1.1[a]
	An. pharoensis	264	46.2	77	63.1	24	27.6	47	42.3	412	46.2	1.8 ± 0.2[a]
	An. coustani	73	12.8	-		-		2	1.8	75	8.4	1.2 ± 0.7[ab]
	An. cinereus	5	0.9	-		-		-		5	0.6	0.2 ± 0.2[b]
	An. demeilloni	3	0.5	-		-		-		3	0.3	0.2 ± 0.2[b]
	An. ardensis	14	2.5	-		-		-		14	1.6	0.3 ± 0.3[b]
	An. squamosus	2	0.4	-		-		-		2	0.2	0.1 ± 0.1[b]
	An. funestus	3	0.5	-		-		-		3	0.3	0.2 ± 0.2[b]
	Total	571	100	122	100	87	100	111	100	891		100

CDC: Center for disease control; PSC: Pyrethrum Spray Catches

Means followed by the same letter in the row are not significantlydifferent from each other at $p \leq 0.05$

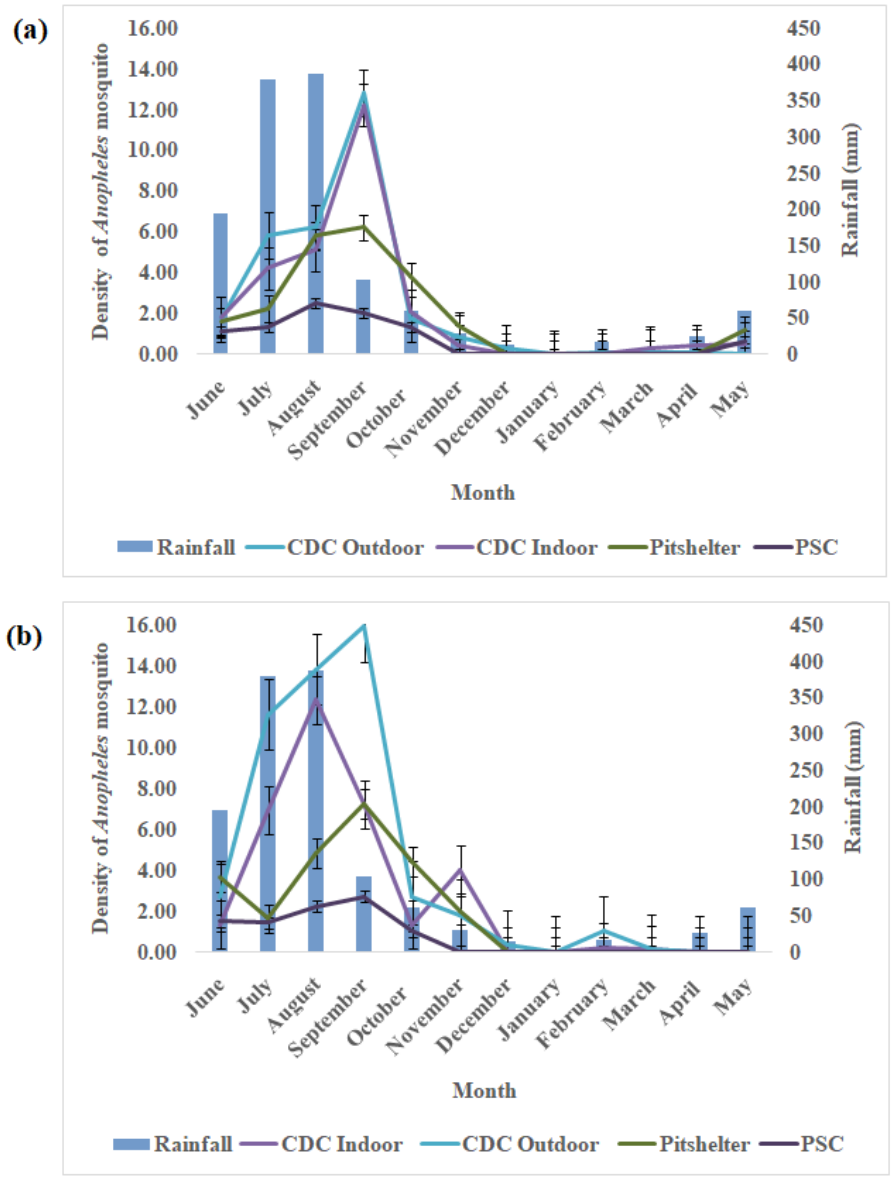

Figure 5.1. Monthly distribution of *Anopheles* mosquito in Arebiya (a) and Guramba Bata (b) study sites (June 2010-March 2011).

5.3.3. Indoor and outdoor *Anopheles* mosquito density

The indoor and outdoor host seeking and resting densities of *Anopheles* mosquitoes in different locations of the study sites is presented in Table 5.3 and Table 5.4. In Arebiya, a slightly high mean density of outdoor host seeking *Anopheles* mosquitoes (4.8 ± 1.8 mosquitoes/trap/night) were collected than indoor (4.3 ± 1.7 mosquitoes/trap/night),

however the difference was not statistically significant (t_{10}= 0.196, *p*=0.849) (Table 5.3). Similarly, non-significant difference was observed between the indoor and outdoor density of host seeking *An. arabiensis* (t_{10}= 0.188, *p*= 0.855), *An. pharoensis* (t_{10}= -0.121, *p*= 0.906) and *An. coustani* (t_{10}= -1.224, *p*= 0.249) in Arebiya (Table 5.3). The density of outdoor resting *An. arabiensis* was higher than indoor resting in this study site, but the difference was not statistically significant (t_{10}= -1.366, *p*= 0.202). Likewise, the outdoor resting density of *An. pharoensis* was higher than the indoor resting density, though the difference was not statistically significant (t_{10}= -1.614, *p*= 0.138) (Table 5.4).

In Guramba Bata, a relatively higher density of outdoor (8.1 ± 2.6 mosquitoes/trap/night) host seeking *Anopheles* mosquitoes were collected than indoor (5.5 ± 1.7 mosquitoes/trap/night), but the difference was not statistically significant (t_{10}= -0.623, *p*=0.547) (Table 5.3). The outdoor host seeking density of *An. arabiensis* in this study site was higher than the indoor density, but it was not statistically significant (t_{10}= -0.855, *p*= 0.412) (Table 5.3). The density of indoor and outdoor host seeking *An. pharoensis* was comparably equal (t_{10}= 0.116, *p*= 0.910) (Table 5.3). The outdoor density of host seeking *An. coustani* was significantly higher than indoor host seeking density (t_{10}= -2.637, *p*= 0.025) (Table 5.3). In Guramba Bata, the density of outdoor resting *An. arabiensis* was higher than indoor resting; however, the difference was not statistically significant (t_{10}= -0.904, *p*= 0.387) (Table 5.4). Significantly, higher density of outdoor resting than indoor resting *An. pharoensis* was recorded during this study (t_{10}= -2.812, *p*= 0.018) (Table 5.4).

Table 5.3. Indoor and outdoor density of host seeking *Anopheles* mosquitoes in the two study sites of Dembiya district, northwestern Ethiopia (June 2018-March 2019)

		Collection site and method		
Site	Species	CDC indoor (mean ± se)	CDC outdoor (mean ± se)	*p*. value
Arebiya	*An. arabiensis*	1.8 ± 0.7	1.4 ± 0.4	0.855
	An. pharoensis	2.2 ± 1.0	2.2 ± 0.9	0.906
	An. coustani	0.3 ± 0.1	0.96 ± 0.5	0.249
	Total density	4.3 ± 1.7	4.8 ± 1.8	0.849
Guramba	*An. arabiensis*	1.3 ± 0.494	2.2 ± 0.703	0.412
Bata	*An. pharoensis*	3 ± 0.997	3.1 ± 1.25	0.910
	An. coustani	0.4 ± 0.3	1.96 ± 0.8	0.025
	Total density	5.5 ± 1.7	8.1 ± 2.6	0.547

Table 5.4. Indoor and outdoor resting densityof *Anopheles* mosquitoes in the two study sites of Dembiya district, northwestern Ethiopia (June 2018-March 2019).

		Collection site and method		
Study site	Species	PSC (mean ± se)	Pit shelter (mean ± se)	*p*. value
Arebiya	*An. arabiensis*	0.96 ± 0.3	1.93 ± 0.4	0.202
	An. pharoensis	0.4 ± 0.2	1.6 ± 0.6	0.138
	Total	0.73 ± 0.3	1.85 ± 0.7	0.219
Guramba Bata	*An. arabiensis*	1.2 ± 0.3	2 ± 0.7	0.387
	An. pharoensis	0.3 ± 0.2	1.9 ± 0.6	0.018
	Total	0.73 ± 0.3	1.96 ± 0.7	0.241

5.3.4. Abdominal status of indoor and outdoor*Anopheles* mosquitoes

From the total indoor and outdoor host seeking *Anopheles* mosquitoes, 50.5% and 63.9%, respectively were unfed. From which, about 58.6% of indoor host seeking and 67.9% of outdoor host seeking *An. arabiensis* were unfed. Similarly, the dominant number of indoor and outdoor host seeking *An. pharoensis* was unfed (46.8% indoor and 57.4% outdoor, respectively) (Table 5.5).

From the total indoor and outdoor resting *Anopheles* mosquitoes the dominant number (53.2% and 68.3% respectively) were freshly fed. More than half of indoor and outdoor resting *An. arabiensis* was freshly fed. Additionally, 48.2% and 70.9% indoor and outdoor resting *An. pharoensis* were freshly fed, respectively (Table 5.6).

Table 5.5. Abdominal status of host seeking *Anopheles* mosquitoes in the study area, Dembiya district, northwestern Ethiopia. (Values in parenthesis are percentages)(June 2018-March 2019).

	CDC-Light Trap Indoor					CDC-Light Trap Outdoor				
Species	Unfed	Freshly Fed	Half Gravid	Gravid	Total	Unfed	Freshly Fed	Half Graveid	Gravid	Total
An. arabiensis	130 (58.6)	82 (36.9)	5 (2.3)	5 (2.3)	222	144 (67.9)	52 (24.5)	8 (3.8)	8 (3.8)	212
An. pharoensis	146 (46.8)	125 (40.1)	30 (9.6)	11 (3.5)	312	191 (57.4)	134 (40.2)	7 (2)	1 (0.3)	333
An. coustani	20 (48.8)	18 (43.9)	1 (2.4)	2 (4.9)	41	126 (70.8)	48 (26.9)	4 (2.2)	-	178
An. cinereus	3 (50)	3 (50)	-	-	6	3 (75)	-	1 (25)	-	4
An. demeilloni	2 (12.5)	13 (81.3)	1 (6.3)	-	16	5 (35.7)	8 (57.1)	1 (7.1)	-	14
An. ardensis	-	-	-	-	-	20 (76.9)	6 (23.1)	-	-	26
An. squamosus	-	-	-	-	-	7 (63.6)	4 (36.4)	-	-	11
An. funestus	1 (20)	4 (80)	-	-	5	3 (50)	3 (50)	-	-	6
An. garnhami	1 (100)	-	-	-	1	-	-	-	-	-
An. christyi	3 (75)	1 (25)	-	-	4	9 (90)	1 (10)	-	-	10
An. nili	1 (100)	-	-	-	1	-	-	-	-	-
Total	307(50.5)	246(40.5)	37 (6.1)	18(2.9)	608	508 (63.9)	256(32.2)	21(2.6)	9 (1.1)	794

Table 5.6. Abdominal status of resting *Anopheles* mosquitoes in the study area, Dembiya district, northwestern Ethiopia. (Values in parenthesis are percentages)(June 2018-March 2019).

Collection methods	Status	*An. arabiensis*	*An. pharoensis*	*An. coustani*	Total
Indoor (PSC and Mouth Aspirator)	Unfed	6 (3.6)	2 (2.4)	-	8 (3.2)
	Freshly Fed	93 (55.7)	41 (48.2)	-	134 (53.2)
	Half Gravid	46 (27.5)	28 (32.9)	-	74 (29.4)
	Gravid	22 (13.2)	14 (16.5)	-	36 (14.3)
Total		167	85	-	252
Outdoor (Pit shelter and Mouth Aspirator)	Unfed	4 (2.3)	-	-	4 (0.99)
	Freshly Fed	113 (64.6)	158 (70.9)	3 (100)	274 (68.3)
	Half Gravid	40 (2.3)	49 (21.97)	-	89 (22.2)
	Gravid	18 (10.3)	16 (7.2)	-	34 (8.5)
Total		175	223	3	401

PSC: Pyrethrum Spray Catches

5.3.5. Blood meal sources and host preference

A total of 552 *Anopheles* mosquitoes were subjected to blood meal source analysis using a direct ELISA. The result indicated that, of the total tested *Anopheles* mosquitoes 5.3% (n=29), 42.5% (n=235), 5.8% (n=32) and 46.4% (n=256) had a blood meal origin of human, bovine, mixed and unknown, respectively (Table 5.7 & 5.8).

Anopheles arabiensis collected using indoor and outdoor CDC light trap had a low human blood index (17.4%, and 15.3%, respectively). On the other hand, *An. arabiensis* collected using indoor and outdoor CDC light trap had a relatively high bovine blood index (50% and 20.3%, respectively). Similarly, *An. pharoensis* collected using indoor and outdoor CDC light trap exhibited a high bovine blood index (60.5% and 55.5%, respectively) than human blood index (10.5% and 10%, respectively) (Table 5.7).

Resting *Anopheles* mosquitoes collected using pit shelters, indoor mouth aspirator, outdoor mouth aspirator and PSC had a higher bovine blood index than human blood index (Table 5.8). The human blood index of *An. arabiensis* collected using pit shelters; indoor mouth aspirator, outdoor mouth aspirator and PSC were 7.3%, 0%, 12.5% and 8.3%, respectively. Whereas, the bovine blood index of *An. arabiensis* collected using pit shelter, indoor mouth aspirator, outdoor mouth aspirator and PSC was 41.5%, 27.3%, 62.5% and 37.5%, respectively (Table 5.8).

The bovine blood index of *An. pharoensis* collected using pit shelters, indoor mouth aspirator, outdoor mouth aspirator, and PSC were 50%, 0%, 50% and 0%, respectively. However, none of indoor and outdoor resting *An. pharoensis* analyzed for blood meal were positive for a human blood (Table 5.8).

Table 5.7. Blood meal sources of host seeking *Anopheles* mosquitoes in the study area, Dembiya district, northwestern Ethiopia. (Values in parenthesis are percentages)

Species	CDC Indoor					CDC Outdoor				
	No	HBI (%)	BBI (%)	MB (%)	Un (%)	No	HBI (%)	BBI (%)	MB (%)	Un (%)
An. arabiensis	46	0.17(17.4)	0.5(50)	0.04(4.3)	0.3(32.6)	59	0.15(15.3)	0.2(20.3)	0.03(3.4)	0.67(67.8)
An. pharoensis	152	0.1(10.5)	0.6(60.5)	0.07(7.9)	0.36(36.8)	110	0.1(10)	0.55(55.5)	0.05(5.5)	0.4(40)
An. coustani	15	0.06(6.7)	0.6(60)	0.06(6.7)	0.4(40)	34	0.14(14.7)	0.55(55.9)	0.11(11.8)	0.41(41.2)
An. cinereus	5	-	0.2(20)	-	0.8(80)	1	-	-	-	1
An. demeilloni	6	0.16(16.7)	0.16(16.7)	-	0.66(66.7)	13	-	0.38(38.5)	-	0.61(61.5)
An. funestus	3	0.33(33.3)	0.3(33.3)	-	0.3(33.3)	4	-	0.5(50)	0.25(25)	0.5(50)
An. chrysti	1	-	-	-	1	2	0.5(50)	0.5(50)	0.5(50)	0.5(50)
An. ardensis	-	-	-	-	-	5	-	-	-	1
An. sqaumosus	-	-	-	-	-	3	-	0.66(66.7)	0.3(33.3)	0.33(0.3)
Total	228	0.11(11.8)	0.56(56.6)	0.06(6.6)	0.38(38.2)	231	0.12(12.1)	0.44(44.2)	0.06(6.5)	0.5(50)

HBI: Human blood index; BBI: Bovine blood index, Un: Unknown; MB: Mixed Blood

Table 5.8. Blood meal sources of resting *Anopheles* mosquitoes in the study area, Dembiya district, northwestern Ethiopia. (Values in parenthesis are percentages).

Collection method	Location	Species	No. analyzed	HBI (%)	BBI (%)	MB (%)	Un (%)
Pit shelter	Outdoor	*An. arabiensis*	41	0.07(7.3)	0.4(41.5)	0.05(4.9)	0.56(56.1)
		An. pharoensis	2	-	0.5(50)	-	0.5(50)
Mouth Aspirator	Indoor	*An. arabiensis*	11	-	0.27(27.3)	-	0.7(72.7)
		An. pharoensis	2	-	-	-	1
	Outdoor	*An. arabiensis*	8	0.12(12.5)	0.6(62.5)	-	0.25(25)
		An. pharoensis	2	-	0.5(50)	-	0.5(50)
		An. coustani	1	-	-	-	1
Pyrethrum	Indoor	*An. arabiensis*	24	0.08(8.3)	0.37(37.5)	-	0.54(54.2)
Spray catches		*An. pharoensis*	2	-	-	-	1
		Total	93	0.06(6.5)	0.38(38.7)	0.02(2.2)	0.56(56.9)

HBI: Human Blood Index, BBI: Bovine Blood Index; MB: Mixed Blood, Un: Unknown

5.3.6. Foraging ratio of *Anopheles* mosquitoes

The result indicated that *An. arabiensis*, *An. pharoensis*, *An. coustani,* and *An. funestus* show strong relative feeding preference of bovine blood over a human blood. *An. arabiensis* showed a 6 times strong preference of bovine blood than human blood. The relative bovine feeding preference of *An. pharoensis* and *An. funestus* was 15 and 3 times higher than the human blood respectively. In this study a bovine blood preference of *An. coustani* was 9 times higher than human blood (Table 5.9).

Table 5.9. Foraging ratio of *Anopheles* mosquitoes in the study area, Dembiya district, northwest Ethiopia.

Species	%HB	%HP	Human FR	%BB	%BP	Bovine FR
An. arabiensis	12.3	41.8	0.3	37.6	19.9	1.9
An. pharoensis	10.0	41.8	0.2	57.4	19.9	2.9
An. coustani	12	41.8	0.3	56	19.9	2.8
An. funestus	28.6	41.8	0.7	42.9	19.9	2.2

%HB: Percent Human blood; %HP: Percent human in population; Human Forage ratio (FR) = %HB/ %HP; Bovine Forage ratio (FR) = %BB/ %BP

5.3.7. Sporozoite rate in *Anopheles* mosquitoes

A total of 792 female *Anopheles* mosquitoes, which belong to 9 species such as *An. arabiensis* (n=335), *An. pharoensis* (n=332), *An. coustani* (n=68), *An. ardensis* (n=10), *An. cinereus* (n=11), *An. demeilloni* (n=21), *An. funestus* (n=7), *An. squamosus* (n=4) and *An. christyi* (n=4) were tested for the presence of circum-sporozoite protein (CSP) in their salivary gland (presence of *P. falciparum*, *P. vivax* 210, and *P. vivax* 247 CSPs). From the species analyzed for CSP, 9 specimens (*An. arabiensis* (n=1)*, An. coustani* (n=4), *An. pharoensis* (n=3) and *An. squamosus* (n=1) collected using CDC light trap were positive for CSP. From the total CSP, *An. coustani* (n= 4), *An. pharoensis* (n= 1) and *An. arabiensis* (n= 1) were positive for *P. vivax 210.* In addition, *An. pharoensis* (n= 2) and *An. squamosus* (n= 1) were positive for *P. vivax* 247. None of the analyzed *Anopheles*species were found to be positive for *P. falciparum* CSP (Table 5.10).

The sporozoite rate of indoor and outdoor CDC collected *An. arabiensis* was 0 and 0.9% respectively, whereas the overallsporozoite rate was 0.3%. The overall, indoor and outdoor sporozoite rate of host seeking *An. pharoensis* was 0.9%, 1.6%, and 0% consecutively. The sporozoiterate of indoor and outdoor CDC collected *An. coustani* was 6.7% and 6%, respectively which results an overall sporozoite rate of 5.9%. The sporozoite rate of indoor and outdoor CDC collected *An. squamosus* was 0 and 25% respectively (Table 5.10).

Table 5.10. Sporozoite rate in *Anopheles* mosquitoes in the study area, Dembiya district, northwestern Ethiopia

Species	Type of CSPs	Indoor			Outdoor		
		CDC-LT	PSC	MA	CDC-LT	PS	MA
An. arabiensis	No of tested	89	37	20	108	63	18
	No of Pv (210) +ve (%)	-	-	-	1(0.9)	-	-
	No of Pv (247) +ve (%)	-	-	-	-	-	-
An. pharoensis	No of tested	182	4	4	127	7	8
	No of Pv (210) +ve (%)	1(0.5)	-	-	-	-	-
	No of Pv (247) +ve (%)	2(1.1)	-	-	-	-	-
An. coustani	No of tested	15	-	-	50	1	2
	No of Pv (210) +ve (%)	1(6.7)	-	-	3(6)	-	-
	No of Pv (247) +ve (%)	-	-	-	-	-	-
An. squamosus	No of tested	-	-	-	4	-	-
	No of Pv (210) +ve (%)	-	-	-	-	-	-
	No of Pv (247) +ve (%)	-	-	-	1(25)	-	-
Total	No of tested	286	41	24	289	71	28
	No of Pv (%)	4 (1.4)	-	-	5 (1.7)	-	-

CDC-LT: CDC-Light trap; PS: Pit shelter; MA: Mouth Aspirator; PSC: Pyrethrum Spray Catch; Pv: *Plasmodium vivax*.

5.3.8. Entomological inoculation rate by *Anopheles* mosquitoes (EIR)

The estimated annual entomological inoculation rate (EIR) of *Anopheles* species collected using CDC light trap from the two Kebeles of Dembiya is indicated in Table 5.11. The annual EIR of *An. arabiensis* collected outdoor using CDC light trap was 4.7 infective bites/person/year (ib/p/year) (Table 5.11). The annual EIR of *An. pharoensis* collected indoor was 12.1 ib/p/year (Table 5.11). The annual EIRs of indoor and outdoor

CDC collected *An. coustani* were 6.9 and 25.7 ib/p/year, respectively. Outdoor CDC collected *An. squamosus* had annual EIR of 7.2 ib/p/year (Table 5.11).

Table 5.11. Annual Entomological inoculation rate (EIR) of *Anopheles* mosquitoes in the study area, Dembiya district, northwestern Ethiopia.

Species	Variables	CDC-Light Trap	
		Indoor	Outdoor
An.arabiensis	SR	-	0.9
	EIR	-	4.7
An.pharoensis	SR	1.6	-
	EIR	12.1	-
An.coustani	SR	6.7	6
	EIR	6.9	25.7
An.squamosus	SR	-	25
	EIR	-	7.2
Overall	SR	1.4	1.7
	Annual EIR	20.8	32.67

SR: Sporozoite Rate; EIR: Entomological Inoculation Rate

5.4. Discussion and Conclusions

5.4.1. Discussion

The result of this study showed that *An. pharoensis* was the dominant species in the two malaria endemic Kebeles of Dembiya district. Similarly, *An. pharoensis* was the predominant species in the irrigated village of central Ethiopia during the dry season (Kibret *et al*., 2010). The presence of cattle near the households may contribute to a high density of more zoophilic vectors such as *An. pharoensis (*Zeru *et al*., 2020*)*. In addition, the less endophilic and endophagic behaviour of *An. pharoensis* could make them less susceptible to indoor based control strategies (Degefa *et al*., 2017). Besides, the presence of suitable breeding habitats of *An. pharoensis* near human dwellings could also be the reason for its high density in the study areas. *Anopheles arabiensis* was the second dominant vector identified during this study. Similarly, this vector was reported as a second dominant vector in south-central Ethiopia (Kenea *et al*., 2016). Even though, *An. arabiensis* was the second dominant vector during this study, its percentage composition was comparable with studies from different parts of Ethiopia (Massebo *et al*., 2013a; Getachew *et al*., 2019).

The mean density difference between indoor and outdoor host-seeking *An. arabiensis* was insignificant in the two study sites. Likewise, a study conducted in Kenya showed that density difference between indoor and outdoor seeking *An. gambiae s.l* was not significant (Degefa *et al*., 2017). At the same time, the exophagic density of *An. arabiensis* in this study was higher than the outdoor host-seeking density of *An. arabiensis* from other parts of Ethiopia (Taye *et al*., 2006) and Kenya (Ogola *et al*.,

2017). In concurrent with this study, the outdoor density of host-seeking *An. gambiae s.s* and *An. melas* was high following the initiation of vector control strategies in Equatorial Guinea (Reddy *et al*., 2011). This increased outdoor host-seeking density of *An. arabiensis* could be attributed to the long-term implementation of vector control strategies (LLINs and IRS) which induce exophagic and exophilic tendencies or the difference in the level of IRS and LLINs coverage (Kibret and Wilson, 2016). This result could also be associated with the availability of other alternative hosts such as cattle's outdoor (Massebo *et al*., 2015).

In addition, in this study the difference between the mean density of indoor and outdoor resting *An. arabiensis* was not significant in the two study Kebeles. Similarly, a study from Kenya (Degefa *et al*., 2017) and North Cameroon (Ekoko *et al*., 2019) showed an equal density of indoor and outdoor resting *An. gambiae s.l.* However, the density of outdoor resting *An. arabiensis* recorded during this study is still high, which could be associated with, insecticide induced avoidance of the vector contact with insecticide-treated surfaces and rapid exit from the house.

Comparably an equal density of outdoor and indoor host-seeking *An. pharoensis* was recorded during this study. Similarly, equal density of indoor and outdoor host-seeking *An. pharoensis* was recorded in Kenya (Degefa *et al*., 2017). A relatively high endophagic tendency of *An. pharoensis* recorded in this study could be associated with the presence of cattle shelters near to human residences or cattle sharing humans' houses during the night. An experimental study conducted in southwest Ethiopia proved that *An.*

pharoensis was more prevalent indoors when a calf was present either inside, or adjacent to a tent relative to a tent without a calf present (Zeru *et al*., 2020).

The blood meal source preference of *Anopheles* mosquitoes determines their vectoral role and affects vector the efficacy of vector control strategies. During this study, the HBI of host-seeking *Anopheles* mosquitoes collected indoors was comparable with the outdoor HBI. Additionally, the BBI of indoor host-seeking *Anopheles* mosquitoes was higher than BBI index of outdoor host-seeking *Anopheles* mosquitoes. Consistently, the bovine blood index (BBI) for *An. arabiensis* was significantly higher in indoor collected populations (71.8%) than in outdoors (41.3%), and the human blood index (HBI) did not differ significantly between the two populations in Kenya (Muriu *et al*., 2008). This high bovine blood index and mixed feeding behaviour of indoor collected Anopheles mosquitoes could be due to interrupted feeding, response to increased vector control strategies, and the location of cattle close to human dwellings, or cattle sharing people houses (Ndenga *et al*., 2016). Briefly, the physical barriers provided by the bed net and its repellency effect (pyrethroid - treated bed nets), may force female *Anopheles* mosquitoes to seek blood from unpreferred hosts such as cattle, which share the humans' house at night. Hence, treating livestock with insecticides and constructing a separate cattle shade is vital to control zoophilic malaria vectors.

A high proportion of *Anopheles* mosquitoes blood meal source was unidentified during this study. Similarly, a study conducted in southwestern Ethiopia indicated that a large proportion of *An. arabiensis* had an unidentified blood meal source (Massebo *et al*., 2013). The result could be associated with a limited number of antibodies used during this study, which might not be enough to identify other available blood sources in the

area. Furthermore, the low sensitivity of ELISA to distinguish blood meal origin from different species may result in an overestimation of the unknown blood meal source (Ngo and Kramer, 2003). Therefore, it is crucial to use a variety of antibodies for the ELISA test, and a highly accurate technique such as PCR should be used, to identify the blood meal sources of *Anopheles* mosquitoes.

The relative feeding preference result of this study indicated a strong zoophilic tendency of *An. arabiensis*. Similarly, the zoophilic tendency of *An. arabiensis* was reported from southwest Ethiopia (Massebo *et al*., 2015) and a similar proportion of *An. arabiensis* that fed on humans and bovine were reported from south-central Ethiopia (Animut *et al*., 2013). Differently, the anthropophilic nature of this vector was reported from Konso district in southern Ethiopia (Tirados *et al*., 2006). Previous works before the scaleup of vector control strategies from east, south, and west Ethiopia also indicated that *An. arabiensis* were more anthropophilic (Hadis *et al*., 1997). Even though, the vector shows a zoophilic tendency in this study, appreciably high HBI of *An. arabiensis* was recorded from both indoor and outdoor collected vector specimens suggesting the opportunistic behaviour of this vector.

Anopheles pharoensis collected indoors and outdoor has had a strong zoophilic tendency in agreement with previous works from south-central Ethiopia (Animut *et al*., 2013). Because cattle share a people's house during the night the indoor BBI of *An. pharoensis* was higher than the outdoor. In addition, a meaningful number of HBI of *An. pharoensis* was recorded indicating that *An. pharoensis* have opportunistic feeding behaviour.

Malaria case reduction is achieved only when the annual EIR of principal malaria vectors is less than 1 ib/p/year (Beier *et al*., 1999). In this study, the overall annual EIR of *An. arabiensis* collected using an outdoor CDC light trap was 4.7 *P. vivax* ib/p/year. This result indicates there is a high outdoor malaria transmission in the study area, and additional vector control strategies are compulsory to avert malaria transmission. A relatively higher EIR of *An. arabiensis* were reported from southwest Ethiopia (5.3 infection bites/person/eight months) (Abraham *et al*., 2017), south-central Ethiopia (33 and 14.5 *P. vivax* ib/p/year year one and two respectively) (Animut *et al*., 2013), and south-western Ethiopia (Massebo *et al*., 2013). The variation could be due to the difference in the number of *Anopheles* mosquitoes tested for CSP, and the level of malaria endmicity.

In addition, in this study *An. coustani, An. pharoensis* and *An. squamosus* were positive for *Plasmodium* circum-sporozoite protein. The result suggests that these vectors could play a vital role in maintaining malaria transmission in the area when primary malaria vectors are suppressed with indoor based vector control strategies such as LLINs and IRS. The EIR of *An. pharoensis* collected from indoor CDC light traps were 12.1 ib/p/year. This result is higher when compared with the EIR of *An. pharoensis* in south-central Ethiopia (0 and 2.3 *P. vivax* ib/p/year for years one and two, respectively) (Animut *et al*., 2013).

Interestingly, the EIRs of indoor and outdoor CDC light traps collected *An. coustani* were 6.9 and 25.7 ib/p/year, respectively, regardless of their zoophagic behaviour. Previous studies also detected a *Plasmodium* CSP and comparably high EIR in *An. coustani* in Ethiopia (Yewhalaw *et al*., 2014) and Kenya (Mwangangi *et al*., 2013). This suggests that

there might be a change in physiology and biting behavior of *An. coustani* in the study area, and it needs a further research. A report by Durnez *et al*., (2011) indicated that there is also a possibility of false positivity in ELISA results, which could lead to an overestimation of EIR in zoophagic *Anopheles* mosquitoes like *An. coustani*. Therefore, it is pertinent to conduct a further investigation into the vectorial role of *An. coustani, An. pharoensis,* and *An. squamosus* using PCR.

5.4.2. Conclusions

In this study, 11 species of *Anopheles* mosquitoes including *An. pharoensis, An. arabiensis*, *An. coustani*, *An. demeilloni*, *An. cinereus, An. funestus, An. ardensis,* and *An. squamosus* were identified from Arebiya and Guramba Bata study sites, Dembiya district. Whereas, *An. garnhami, An. christyi* and *An. nili* were identified only from Guramba Bata study site. *Anopheles arabiensis* and *An. pharoensis* were the dominant vector species identified in the two study sites. Equal density of indoor and outdoor host-seeking and resting *Anopheles* mosquitoes was recorded in this study. *Anopheles arabiensis*, *An. pharoensis, An. coustani,* and *An. squamosus* showed a strong zoophilic tendency. A circum-sporozoite protein of *P.vivax* was detected from speciemens of *An. arabiensis, An. pharoensis, An. coustani*, and *An. squamosus*. The annual outdoor EIR of *An. arabiensis* was high, indicating that outdoor malaria transmission is a problem in the study area. The detection of *P. vivax* CSP in specimens of *An. pharoensis, An. coustani*, and *An. squamosus* suggests their role as a secondary malaria vector in the two study areas; however, it needs further investigation using more accurate techniques such as PCR. Therefore, additional vector control strategies such as larval source management, odor-baited mosquito traps, improving house conditions, separating cattle shade, zooprophylaxis, and treating livestock with insecticides should be a part of integrated vector management in the study area.

Chapter 6: Insecticide susceptibility status of *Anopheles arabiensis* in the Dembiya district, northwestern Ethiopia

6.1. Introduction

Malaria is one of the world's most important public health problem responsible for nearly 229 million outpatient cases and 409, 000 deaths in 2019 (WHO, 2020). From which more than 94% outpatient malaria cases (215 million) and 95% malaria deaths were reported from WHO African region (WHO, 2020). In Ethiopia, malaria is responsible for an average of 2,614,852 cases and 5,626 deaths (WHO, 2020). Currently, malaria control and elimination programme is challenged by lack of effective vaccines, the spread of parasite resistance to antimalarial drugs, and mosquito resistance to insecticides (Moyes *et al*., 2020).

In Ethiopia vector control strategy primarily depends on the use of indoor residual spray (IRS) and insecticide treated bed nets (ITNs) (FMoH, 2010). Ethiopia has made a progress in reducing malaria prevalence after the increased distribution of IRS and LLINs starting from 2005 (FMoH, 2017). Following the scale up distribution of malaria intervention strategies, Ethiopia has showed 57% and 54% decline in malaria incidence and mortality respectively between 2015 and 2018 (WHO, 2018). Based on these achievements the government of Ethiopia has set a nation wide malaria elimination goal by 2030 (FMoH, 2020). However, the effectiveness of IRS and LLINs is currently compromised because of widespread emergence of insecticide resistance in major malaria vectors, which threatens malaria elimination and control programme in Ethiopia (Asale *et al*., 2014; Yewhalaw *et al*., 2011).

Insecticide resistance is the ability of mosquitoes to survive exposure to a standard dose of insecticides which could be due to physiological or behavioural adaptation (WHO, 2016). In Ethiopia, *An. arabiensis* has developed resistance to different classes of insecticides such as DDT (organochlorine), malathion (organophosphate), bendiocarb and propoxur (carbamate), alpha-cypermethrin, cyfluthrin, deltamethrin, etofenprox, lambda-cyhalothrin and permethrin (pyrethroids) (Balkew*et al*., 2003;Yewhalaw *et al*.,2011; Balkew *et al*.,2012; Messenger *et al*., 2017). Moreover, in southern, southwestern and central Ethiopia, West African kdr alleles (L1014F-kdr) were detected from a larger proportion of *An. arabiensis* tested with a frequency ranged from 95% to 100% (Balkew *et al*., 2010; Yewhalaw *et al*., 2011).

Dembiya district is one of malaria prone area in northwestern Ethiopia. Indoor residual spray (IRS) has been implemented as the major vector control strategy starting from 1956 (Tulu, 1996). In addition, as a part of the nationwide malaria elimination programme a larger proportion of the society in the district were protected with LLINs (unpublished health office report). Even though the malaria case showed a sharp decline in the district, the disease is still a public health problem responsible for a larger proportion of outpatient cases (Alemu *et al*., 2012). This continued public health importance of malaria in the district could be attributed to the development of insecticide resistance in the local malaria vector. Limited published reports are available about the insecticide susceptibility status of the *An. arabiensis* towards different classes of insecticides in the district. Therefore, this study was designed to assess the susceptibility status of *An. arabiensis* towards different class of insecticides in selected Kebeles of Dembiya district. The result

of the study will provide base line information to design effective vector control strategies.

6.2. Material and Methods

6.2.1. Description of the Study area

The study was conducted in selected Kebeles of Dembiya district that have a long history of implementing vector control strategies (IRS and LLINs). The study area is described in detail in chapter 4 of this thesis.

6.2.2. Collection and processing of mosquitoes for susceptibility test

During June to July of the study period, *Anopheles* mosquito larvae/pupae collected by dipping from rock holes, irrigation channels, artificial breeding sites, ponds, stream margins, river basin, swamps, reservoirs and rain pools from the two villages. The larvae were reared to adults in the laboratory under standard conditions (25 ± 2^0C and relative humidity of 80%) (WHO, 1995). The larvae were not given any additional food, but, the newly emerged adults were givena 10% sucrose solution. A 3-5 day blood unfed female *Anopheles* mosquitoes were tested for insecticide resistance using a World Health Organization insecticide susceptibility test-kit and standard procedures (WHO, 2016).

6.2.3. Insecticide susceptibility test procedures

Morphologically identified *An. arabiensis* batches of 25 unfed, 3-5 days old adult females were exposed to filter papers impregnated with 1% fenitrothion (organophosphate), 0.1% bendiocarb (carbamate), 0.75% permethrin and 0.05% deltamethrin (pyrethroids). The knockdown were recorded after 10, 15, 20, 30, 40,

50, and 60 minutes and up to 2 hrs for fenitrothion. An equal numbers of mosquitoes were exposed to control papers impregnated with olive oil (organophosphate/carbamate control), and silicone oil (pyrethroid control). After one hour of exposure (two hours for 1% fenitrothion), mosquitoes were transferred into holding tubes and given a 10% sucrose solution with cotton pads. Mortality was recorded after 24 hours.

6.2.4. Data analysis

Percentage mortality of the control and exposure tube was calculated following a WHO guidline (WHO, 2016). If the control mortality is between 5% and 20%, the exposure mortality was corrected by using Abbott's formula. If the control mortality is > 20 the experiment was discarded (WHO, 2016). If mortality rate is less than 90%, the insect population is classified as "resistant" but, if the mortality rate between 90% and 97% the population were considered as "suspected resistant" and finally it is classified as "susceptible" if the mortality is > 98% (WHO, 2016). A probit analysis was implemented to calculate the KDT_{50} and KDT_{90} of the respective insecticides. SPSS, version 20was used for data analysis (Armonk, NY: IBM Corp).

$$Control\ mortality\ (C) = \frac{Number\ of\ dead\ mosquitoes}{Total\ number\ of\ mosquito\ applied\ to\ control\ tubes}$$

$$Exposure\ mortality(E) = \frac{Number\ of\ dead\ mosquitoes}{Total\ Number\ of\ mosquito\ applied\ to\ exposure\ tube}$$

$$Corrected\ exposure\ mortality\ (\%) = \frac{E - C}{100 - C} * 100$$

6.3. Results

6.3.1. Insecticide susceptibility status of *An.arabiensis* in the study area

A total of 1000 female *An. arabiensis* mosquitoes from the two Kebele swere exposed to four insecticides such as bendiocarb (0.1%) (Olive oil), fenitrothion (Olive oil) (1%), deltamethrin (silicone oil control) (0.05%), and Permethrin (0.75%) (Silicone oil). Fenitrothion caused 100% mortality of *An. arabiensis* at both study sites. Similarly, in Guramba Bata and Arebiya study sites, bendiocarb resulted in an average mortality of 97.3% and 100%, respectively. Deltamethrin and permethrin caused a mortality rate of less than 90% on average (Figure 6.1). For the control, the average percentage mortality of *An. arabiensis* was less than 5%.

In the Guramba Bata and Arebiya study sites, the KDT_{50} o fenitrothion was 119.3 and 100.7 minutes, respectively. The KDT_{50} of bendiocarp in Guramba Bata was 45.7 minutes and 44.9 minutes in Arebiya. Deltamethrin and permethrin KDT_{50} and KDT_{90} were not achieved within one hour in either of the two study site (Table 6.1).

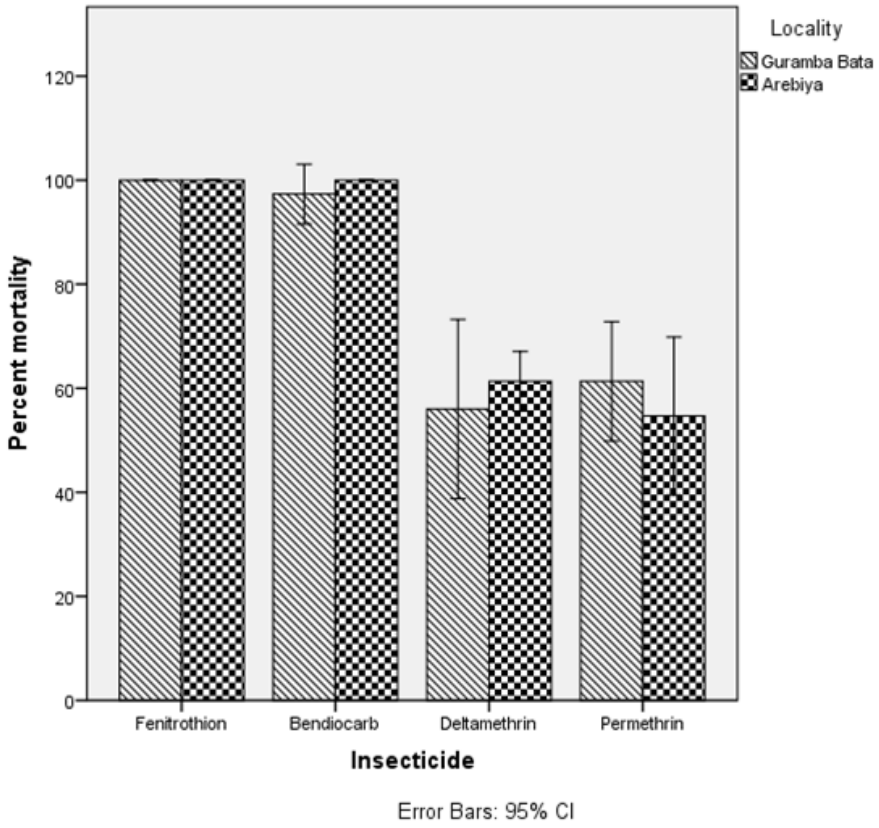

Figure 6.1. Percentage insecticide susceptibility status of *An. arabiensis* in the two study sites.

Table 6.1. The KDT_{50} and KDT_{90} of different class of insecticides against *An.arabiensis*.

Kebele	Insecticide	N	KDT_{50} (95% CI)	KDT_{90} (95% CI)
Arebiya	Fenitrothion	60	100.7 [87.3-137.5]	170.9.1[128.7-349.7]
	Bendiocarb	60	44.9 [38.9-57.1]	79.7 [61.2-142.7]
	Deltamethrin	60	Not observed	Not observed
	Permethrin	60	Not observed	Not observed
Guramba Bata	Fenitrothion	60	119.3 [102.6-167.7]	205.7 [152.4-443.4]
	Bendiocarb	60	45.7 [41.0-54.5]	69.3 [57.2-109.6]
	Deltamethrin	60	Not observed	Not observed
	Permethrin	60	Not observed	Not observed

KDT: Knockdown Time

6.4. Discussion and Conclusions

6.4.1. Discussion

This study evaluated the susceptibility status of *An. arabiensis* to different classes of insecticides such as fenitrothion (organophosphate), bendiocarb (carbamate), and permethrin and deltamethrin (pyrethroids) in Dembiya district northwestern Ethiopia. The result showed that *An. arabiensis* collected from the field was resistant to deltamethrin and permethrin but, susceptible to fenitrothion and bendiocarb in Arebiya and Guramba Bata study sites. Similarly, *An. arabiensis* population in southwestern Ethiopia were resistant to deltamethrin and permethrin (Yewhalaw *et al*., 2011), to DDT, malation and propoxure in Northern Ethiopia (Christian *et al*., 2013), *An. arabiensis* populations in central, western, south-western and southern Ethiopia were also resistant to DDT and deltamethrin (Alemayehu *et al*., 2017).

Development of insecticide resistance in *An. arabiensis* species is not uncommon in Ethiopia, which could be linked to the long-term use of this insecticide for IRS and LLINs. A nationwide survey in Ethiopia confers that *An. arabiensis* have developed resistance towards DDT and pyrethroids, which is consistent with increased use of DDT for IRS and pyrethroids for LLINs (Messenger *et al*., 2017). The long-term use of insecticides may have created a selective pressure that favors the evolution and survival of resistant strains of *Anopheles* mosquitoes (WHO, 2016).

6.4.2. Conclusions

Anopheles arabiensis was resistant to deltamethrin and permethrin in both study sites of Dembiya district. The development of insecticide resistance could jeopardize the area's ongoing malaria control and elimination programme. The policy makers are encouraged to use integrated vector management approach, rather than solely depending on insecticide-based vector control strategies, and to implement different vector resistance management strategies. A further researche on the insecticide resitance mechanism of of the local malaria vector is recomended.

Chapter 7: General Conclusions and Recommendations

7.1. Conclusions

In this study, the species composition, ecology and behaviour of *Anopheles* mosquitoes were evaluated in malaria endemic Kebeles of Dembiya district which has a long history of implementing vector control strategies, northwestern Ethiopia. Accordingly, the result indicated that malaria remains a problem in the district despite the long standing malaria control and elimination strategies implemented.

Malaria was found to be 3.5% prevalent, with *P. falciparum* being the most common parasite found. Malaria transmission in the study area is strongly associated with factors such as outdoor activity and sex (being male).

The two dominant vectors identified from the larval and adult *Anopheles* mosquito specimens collected from the two study sites were *An. arabiensis* and *An. pharoensis*. Drainage canals and burrow pits had the highest density of *Anopheles* mosquito larvae and pupae. The presence or absence of *Anopheles* mosquito larvae were related to water turbidity (mid turbidity) and grass presence. Correlation analysis revealed that pH and temperature were positively correlated to the density of *Anopheles* mosquito larvae.

Following the long rainy season and thereafter in short rainy season, the highest density of *Anopheles* species larvae and pupae were recorded. In the study sites, the density of outdoor host seeking and resting *Anopheles* mosquitoes was comparable with the density of indoor host seeking and resting *Anopheles* mosquitoes. *Anopheles arabiensis, An. pharoensis,* and *An. coustani* were found to have azoophilic tendency. *Plasmodium vivax* protein was found in *An. arabiensis, An. pharoensis, An. coustani*, and *An. squamosus*.

7.2. Recommendations

A further study about the impact of migration of daily laborers from the nearby malarious (Ethio-Sudan border) area on the local malaria dynamics of the study sites is recommended. This could be crucial to design malaria control and elimination strategies considering malaria infected individuals from those areas. On the other hand, further studies bout the identification of natural predators of *Anopheles* mosquito larvae/pupae, the type and frequency of resistance mechanism of *An. arabiensis* are recommended. Moreover, study is required to investigate the vectoral role of *An. pharoensis*, *An. squamosus* and *An. coustani* in the study area. Integrated vector control strategies targeting outdoor host seeking and resting *Anopheles* mosquitoes and potential reproduction sites should be incorporated to achieve the intended goal of malaria control and elimination programme in the study areas.

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Appendices

Appendix 1

Ethics approval and consent to participate

Ethics approval and consent to participate Ethical clearance was obtained from Addis Ababa University, institutional ethical review board of the College of Natural and Computational Sciences (Ref No CNSDO/692/10/2018). Written consent was obtained from the head of the household and other study participants before sampling. Individuals proved to be positive for malaria during blood film examination were treated with anti-malarial drug prescribed by physicians

English version of questionnaires and consents

Dear participant

My name is Mihretu Tarekegn Nigatu, I am a Ph.D student at Addis Abba University college of natural science. I am working a Ph.D research entitled as **"Species Composition, Distribution and Ecology of *Anopheles* Mosquitoes in Relation to Malaria Transmission and Control in Dembiya district, Northwestern Ethiopia"** which can help in designing effective malaria control strategy**.** As a part of the project I am evaluating the factor which contributed for the prevalence of malaria in fogrea woreda.Therefore, I kindly request you to fill this questionnaire which is designed to point out potential malaria risk factors in this area. Thank you!!!

I. Socio-demography of the participant (Household head)

1. Age __

	Age					
	0-5	6-12	13-17	18-64	≥65	Total
Number						

2. Sex: 1. Male 2. Female

3. Educational level: 1. No education 2. Primary 3.Secondary 4. More than secondary

4. Location: 1. Rural 2. Urban

5. Occupation: 1. Farmer 2. Employee 3.Merchant 5. If any_____

6. Number of individuals living in this house:

7. Your monthly income. Specify_____

II. Factor associated with prevalence of malaria

1. Do you have any idea how malaria is transmitted? 1. Yes 2. No

2. If your answer for number for question 1 is yes, how it is transmitted?

4. Roofing material: 1. Iron sheet 2. Concrete 3.Grass 4. If any other____

5. House wall: 1. Mud 2. Wood/timber 3. Cement 4. Stones 5. Other_____

6. Number of rooms: 1. 1 2. 2 3.>3

7. Number of windows: 1. 1 2. 2 3.3 4.>3

8. Presence of window screening: 1. Yes 2. No

9. Number of doors: 1. 1 2. 2 3. 3 4.>3

10. Presence of door screening: 1. Yes 2. No

11. Type of domestic animals in the compound: 1. sheep 2. Goat 3.Cattle 4.Donkey 5. Horse 6.Chicken 7. Other _____

12. Major dusk outdoors activities: 1. Farming 2. Fetch water 3. Keeping livestock 4. Other___

13. Did your house receive indoor residual spraying in the previous years? 1. Yes 2. No

14. If you answer for Q No 15 is yes, when is the last time of IRS spray? 1. <6 months 2. 6-12 months 3. >a year

15. When did you receive an insecticide impregnated bed net? 1. <6 months 2.6-12 months 3.>a year

16. How often did you use LLINs? 1. Not used 2. Always 3. Sometimes

17. How many LLINs are available in the room? _____

Consent form for CDC light trap mosquito collection

Name of the participant _____ Age_____ Sex_____

Collector name_____ Site /Village_____

As a study participant the researchers have informed me about the study objective "**Species Composition, Distribution and Ecology of *Anopheles* Mosquitoes in Relation to Malaria Transmission and Control in Dembiya district, Northwestern Ethiopia".** The study is important for the development of effective malaria control strategy in relation with irrigation activity in the study area. To achieve the goal of the research I have been informed that the mosquito collection will be carried out in my home for one year twice a month. The procedure involves setting CDClighttrapcatches in one of our bed room near to our bed for the whole night to collect the incoming mosquitoes.Mosquitoes caught will be collected by collectors early in the morning and will be kept in the vials with silica gel. Next day collected mosquitoes will be transported for further identification and processing. I am well informed that my families will not involve inmosquitocollection

Effect

Despite of discomfort effect of mosquito collection during the morning, they informed me that therewill benoriskson my family members in allowing researchers to collect mosquitoes' by light trap in my home.

Benefit

As far as the benefit is concerned, there will be no direct benefit I can get from the research, but the research will be helpful to understand mosquito dynamics, behaviour and density which would help to designing effective preventive and control measures for major malaria disease.

Incentives:

No incentives will be given for me except acknowledgment for letting my house mosquito collection.

Confidentiality:

Finally, the information collected from this research project will bekept confidential. Information will not have name on it, but a number assigned to it instead.

Consent form for blood sample collection

Name of the participant _____ Age_____ Sex_____

Lab. technician Name_____Site /Health center _____

As a study participant the researchers have informed me about the study objective "**Species Composition, Distribution and Ecology of *Anopheles* Mosquitoes in relation to Malaria Transmission and Control in Dembiya district, Northwestern Ethiopia".** The study is important for the development of effective malaria control strategy in relation with irrigation activity in the study area. To achieve the goal of the research I and my family members have been informed to give a drop of blood sample. They informed me blood samples will be collected by experienced health expertise based on the established aseptic procedure by finger priker. They also told me that if positive result is observed treatment will be given for free to my family as prescribed by physicians. Following the information I have got, I have agreed to participate on this study with my family members. The laboratory result will be confidential.They gave me enough time to think over it before I signed this informed consent. Therefore after I take the

aforementioned information, I willingly gave my informed consent to involve in the study.

Full Name	**Signature**	**Date**
Participant _____	_____	_____
Investigator _____	_____	_____

Appendix 2

Amharic version of questionnaire and Consents

2.1 የተወደዳችሁ የዚህ ጥናት ተሳታፊዎች

እኔ አቶ ምህረቱ ታረቀኝ በአዲስ አበባ ዩንቨርሲቲ ፡ በተፈጥሮሳይንስ ትምህርት ፋኩልቲ ስር በዞሎጂ ትምህርት ክፍል በኢንሴክት ሳይንስ የሶስተኛ ዲግሪ በመስራት ላይ እገኛለሁ፡፡በአሁኑ ሰአት የመመረቂያ ፁሁፌን "**Species Composition, Distribution and Ecology of *Anopheles* Mosquitoes in Relation to Malaria Transmission and Control in Dembiya district, Northwestern Ethiopia**" በሚል ርእስ በመስራት ላይ እገኛለሁ፡፡ጥናቱ ሲጠናቀቅ የወባ በሽታን ለመከላከል ትልቅ ጠቀሜታ ይኖረዋል፡፡በአሁኑ ሰአት የጥናቱ አካል የሆነዉን በናንተ አካባቢ ለወባ በሽታ መስፋፋት ምክንያት የሆኑትን ችግሮች ለመለየት ያመቸን ዘንድ ይህንን መጠይቅ አዘጋጂቻለሁ ፡፡ ስለሆነም እርስዎ ጥያቄዎችን አንብበዉ ታማኝና ግልፅ ሆነዉ መልስዎትን እንዲሰጡ ስል በትህትና እጠይቃለሁ፡፡ለሚያደርጉልኝ ትብብር በቅድሚያ ከልብ አመሰግናለሁ!

I. Socio-demography of the participant (Household head)

1. እድሜ __

2. ፆታ ሀ. ወንድ ለ. ሴት

3. የትምህርት ደረጃ: ሀ. አልተማርኩም ለ. የመጀመሪያ ደረጃ ሐ. ሁለተኛ ደረጃ መ. ከፍተኛ ትምህርት

4. የመኖሪያ አካባቢ: ሀ. ከተማ ለ. ገጠር

5. የስራ ዓይነት: ሀ. ግብርና ለ. ተቀጣሪ ሐ. ነጋዴ መ. ሌላ_____

7. በቤት ዉስጥ የሚኖሩ የቤተሰብ ብዛት

እድሜ						
	0-5	6-12	13-17	18-64	≥65	Total
ብዛት						

7. ወርሓዊ ገቢ_____

II. Factor associated with prevalence of malaria

1. የወባ በሽታ በምን እንደሚተላለፍ ይዉቃሉ? ሀ. አዎ ለ. አላዉቅም

2. የጥያቄ ቁጥር 1 መልስዎ አዎ ከሆነ በምን አንደሚተላለፍ አብራራ/ሪ?_____

4. የቤትዎ ጣራ ክዳን የተሰራበት: ሀ. ቆርቆሮ ለ. ሲሚንቶ ሐ. የሳር መ. ሌላ

5. የቤትዎ ወለል ከምን የተሰራ ነዉ:ሀ. አፈር ለ. ጣዉላ ሐ. ሲሚንቶ መ. ሌላ

6. የቤትዎ የክፍል ብዛት :ሀ. 1 ለ. 2 ሐ. >3

7.የመስኮት ብዛት: ሀ. 1 ለ. 2 ሐ. 3 መ. >3

8. የመስኮት ስክሪን አለ ወይ: ሀ. አወ ለ. የለም

9. የቤትዎ በር ብዛት: ሀ. 1 ለ. 2 ሐ. 3 መ. >3

10. የበር ስክሪን አለወይ: ሀ. አለ 2. የለም

11. በግቢዎ ያለዉ የቤት እንስሳ አይነትና ብዛት:

የቤት እንስሳ አይነት							
	በግ	ፍየል	ከብቶች	አህያ	ፈረስ	ዶሮ	ሌላ
ብዛት							

12. ከቤት ዉስጥ የሚከዉኑት የስራ አይነት: ሀ. ግብርና ለ. ዉሀመቅዳት ሐ. የቤት እንስሳዉችን መጠበቅ መ. ሌላ_____

13. ባለፉት አመታት የርስዎ ቤት ላይ የወባ መካላከያ የኬሚካል ርጭት ተካሂደል ወይ? ሀ. አወ ለ. አልተረጨም

14. ለጥያቄ ቁጥር 15 የሰጡት መልስ አወ ከሆነ፣ለመጨረሻ ግዜ የተረጨዉ መች ነበር? ሀ. <6 ወር ለ.6-12 ወር 3.ከአመት በላይ ሆኖታል

15. በካሚካል የተነከረ አጎበር ከወሰዱ ስንት ግዜ ሆኖዎታል? 1. <6 ወር 2. 6-12 ወር 3. ከአመት በላይ

16. በኬሚካል የተነከረዉን አጎበር ምን ያህል ይጠቀሙታል? ሀ. አልጠቀመዉም (ለምን_____) ለ. ሁልጊዜ ሐ. አንድ እንድ ጊዜ

17. በቤትዎ ስንት በኬሚካል የተነከረ አጎበሮች አሉ? _____

2.2 በCDC light trap የወባ ትንኝ ለመሰብሰብ የሚያስችል የስምምነት ሰነድ

ስም _____ እድሜ_____ ፆታ_____

ሰብሳቢዉ ስም _____ ቀበሌ_____

እንደጥናቱ ተሳታፊነቴ ፤ ጥናቱን የሚያከናዉነዉ ተመራማሪ ፤ የጥናቱን ማለትም "**Species Composition, Distribution and Ecology of *Anopheles* Mosquitoes in relation to Malaria**

Transmission and Control in Dembiya district, Northwestern Ethiopia”. አላማ አስረድቶኛል ። በዚህም መሰረት ጥናቱ ቢሰራ የወባ በሽታን ለመከላከል ጥሩ ግብአት እነደሚሆን ስለተረዳሁ ፡ ለጥናቱ የሚስፈልጉትን ነብሳቶች ቤቱ ዉስጥና ዉጭ ለአንድ አመት (በወር ሁለት ጊዜ) በሚተከሉ መሳራያወች እንዲሰበስብ ተስማምቻለሁ ። በመሳሪያዉ የተያዙትን ነብሳት በባለሞያዉ አማካኝነት ለጥናት በጥዋት እነደሚሰበሰቡም አዉቃለሁ።.በዚህ ጥናትም ምንም አይነት የቤተሰቤ አባል እንደማይሳተፍም አረጋግጠዉልኛል።

የጥናቱ የጎንዮሽ ጉዳት

የተሰበሰቡትን የወባ ትንኞች በጥዋት ሲወሰዱ ከሚፈጥረዉ ረብሻ ዉጭ ፡ ጥናቱ ቤት ዉስጥ ሲሰራ ምንም አይነት ጉዳት አያስከትልም።

ጥቅሙ

ከዚህ ጥናት ምንም አይነት ቀጥተኛ ጥቅም አላገኝም ። ነገር ግን ጥናቱ የወባን በሽታ በሓገር አቀፍ ደረጃ ለመከላከል ይጠቅማል።

ማበረታቻ

ጥናቱ ሲጀመርም ሆነ ሲጠናቀቅ ከምስጋና ዉጭ ምንም አይነት ማበረታቻ አይገኝበትም ።

ሚስጥራዊነት:

ከቤቱ የሚሰበሰቡት መረጃወች ሚስጥራዊ ይሆናሉ ። መረጃወች ስሰበሰበም የመለያ ቁጥር እንጂ ምንም አይነት የመለያ ስም አይኖረዉም ።

<u>የደም ናሙና መዉሰጃ ስምምነት</u>

ደም የሚወሰድበት ሰዉ መለያ_____ እድሜ_____ ፆታ___

የላብራቶሪ ባለሞያዉ ስም _____ጤና ጣቢያ _____

በዚህ ጥናት “**Species Composition, Distribution and Ecology of *Anopheles* Mosquitoes in Relation to Malaria Transmission and Control in Dembiya district, Northwestern**

Ethiopia” ተሳታፊ በሞሆኔ ስለጥናቱ አላማ ተነግሮኛል ስለሆነም የዚህ ጥናት ለማካሄድ ያመች ዘንድ እኔና ቤተሰቦቸ የደም ናሙና ለመስጠት ተስማምተናል **::** ይህን ዉሳኔ እንድወስን በቂ ጊዜ ተሰጥቶኛል::

ሙሉ ስም ***ፊርማ*** ***ቀን***

ተሳታፊ_____ _____ _____

እጥኝ _____ _____ _____

Appendix 3:

Molecular technique used to identify sibling species of *Anopheles gambiae* s.l.

Extraction of mosquito DNA

Extraction of template DNA from adult *An. gambiae s.l* was based on a protocol developed by Collins *et al.* (1987). The mosquito leg was ground in a 1.5 mL Eppendorf tubes containing 50µL grind buffer (0.08M sucrose, 0.05% SDS, 5% 0.1 M tris-HCL pH 9, 0.08M NaCl, 0.06M EDTA pH8.0) with a sterile blue Konte's pestle until no recognizable parts remain. The grinding pestle was then rinsed with additional 50µL of grind buffer grind buffer within the tube. The ground product was incubated at 65°C for 30 minutes. A 13 µL of 8M ice cold potassium acetate was added and incubated on ice for 30m to precipitate the mosquito parts and insoluble and denatured proteins. Thesolution was centrifuged at maximum speed of 13,200 revolutions/minute at room temperature for 20 minutes. The formed supernatant was transferred to new 1.5 µL Eppendorf tube and 200 µl of cold 100% ethanol was added, mixed well and left at room temperature for 5 minute. Centrifuge this ethanol DNA precipitate with a maximum speed of 13,200 revolutions/minute at room temperature for 20 minutes to pellet the DNA. The ethanol was carefully removed and the DNA pellet was rinsed with 200 µL of 70% ethanol and then the ethanol was removed with pipetting. The DNA sediment was air-dried for 30 minutes at room temperature. The DNA pellet was dissolved in 100µl sterilized water with gentle tapping of the tube to allow the DNAs to re-suspend for amplification process.

DNA amplification

The ribosomal region targeting specific single nucleotide polymorphisms (SNPs) for *An.gambiae s.l* complex was amplified in a multiplex reaction following a method developed by Wilkins *et al*., (2006). A master mix containing 2.5 µL of 10x reaction buffer with 15µL $MgCl_2$, 0.5 µL each of 10 mM dNTPs, 0.5 µL 25mM $MgCl_2$, 1 µL of each primers Universal (UN), *An.arabiensis* (AR), *An.quadranulatus/amharicus* (AQ), *An.gambiae* s.s (GA), and*An.merus/melas* (ME), 5 µL PCR water and 0.1 µL Tag DNA polymerase in a 0.5 microfuge tube. A template DNA of 1 µL was added in to the master mix and the plates were well covered and spin down in micro centrifuge at maximum speed for two to three minutes and amplified. PCR reaction conditions were conducted in a thermocycler programmed for 34 cycles at a denaturation temperature of 95^{o}C for 30s, an annealing 50^{o}C for 30s, an extension 72^{o}C for 30s. Final elongation temperature of 72^{o}C for 5 min and a temperature of 4^{o}C hold for cooling.

Gel electrophoresis

Agarose gel was prepared by following Wilkins *et al*., (2006). A 1.5g of agarose powder was added to 100ml of 1xTAE buffer and mixed in a microwavable flask. The the agarose solution was heated in microwave for two minutes and allowed to cool. Then after a 10 µl gel red was added in to the agarose gel and mixed thoroughly. Agarose gel was powered in to gel tank and left for 30 until it was completely solidified. Gel tray was placed in to gel tank and the gel was completely covered with 1xTAE buffer. A 2µl of 6x loading dye was added for each sample on a parafilm and 10 µl DNA ladder was added on the first well. A 10 µl PCR amplified sample was mixed with a loading dye on a parafilm and loaded in to the corresponding well. The positive and negative ion

electrodes were connected to a power bank and the gel was allowed to run at voltage of 90 volt, current of 400amper for 90 minutes until it reaches the middle of the try.

The gel was transferred in to the middle of the UV Transilluminator machine screen and covered with the camera box. A Multi Doc-ItTM Imaging System-Masterflex computer software was used to detect DNA fragments and capture photo of the DNA bands. The visualization of the gel electrophoresis result was determined based on the DNA size of the band. The DNA fragments were interpreted by using bands of the markers on the first and the last lane of the gel. *An. gambiae sl.* sibling species were identified by comparing the DNA band with already known molecular weight ladder bands. DNA fragments having 464 bp, 529 bp, 637 bp and 388 bp were determined to be *An. gambiae s.s* Giles/*An. coluzzi* Coetzee and Wilkerson*, An. melas* Theobald/*An. merus* Donitz and *An. quadirianulatus* Theobald*, An. amharicus* Hunt, Wilkerson and Coetzee and *An. arabiensis* Patton respectively.

Appendix 4

Photo of DNA bands taken from the camera