Malaria Vectors and Transmission Intensity in an Epidemic Prone Area of Bure District, West Gojam, Northwestern Ethiopia

By

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Declaration

I, Tilahun Adugna Wassie, declare that this thesis is my original work, has not been presented for a degree in any other University or Institution and that all sources of material used for the thesis have been rightfully acknowledged.

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ADDIS ABABA UNIVERSITY

SCHOOL OF GRADUATE STUDIES

COLLEGE OF NATURAL SCIENCES

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Dedications

This work is dedicated to my beloved parents, Adugna Wassie and Teguda Molla and my lovely wife Astede Dreso and my daughter, Bruketayet Tilahun.

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Abbreviations and Acronyms

ACT	Artemisinin Based Combination Therapy
ANOVA	Analysis of Variance
APSs	Artificial Pit Shelters
BBI	Bovine Blood Index
CDC	Center for Disease Control and Prevention
CHP	Center for Health Protection
DALYs	Disability-Adjusted Life Years
DDT	Dichloro-Diphenyl-Trichloroethane
DNA	Deoxyribonucleic Acid
EC	Ethiopian Calendar
EIR	Entomological Inoculation Rate
ELISA	Enzyme-Linked Immunosorbent Assay
FI	Feeding Index
FR	Forage Ratio
GDP	Gross Domestic Product
HBI	Human Blood Index
HEGs	Homing Endonuclease Genes
HRB	High Resolution Blend
HSD	Honestly Significantly Differently
IPM	Integrated Pest Management
IPTp	Intermittent Presumptive Treatment in Pregnancy
IRS	Indoor Residual Spraying
IVM	Integrated Vector Management

LLINs	Long Lasting Insecticidal Nets
LTs	Light Traps
m.a.s.l	Meters Above Sea Level
MoH	Ministry of Health
NGOs	Non Governmental Organizations
NIAID	National Institute of Allergy and Infectious Disease
PCR	Polymerase Chain Reaction
PMI	President's of Malaria Initiative
PSCs	Pyrethrum Spray Catches
RBM	Roll Black Malaria
RDTs	Rapid Diagnostic Tests
RIDL	Release of Insects Carrying a Dominant Lethal
s. l.	sensu lato
SNNPR	South Nation Nationalities People Regional State
SPSS	Statistical Package for the Social Sciences
s. s.	sensu stricto
SSA	Sub-Saharan Africa
UNICEF	United Nations Children's Fund
WHO	World Health Organization

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Abstract

Malaria Vectors and Transmission Intensity in an Epidemic Prone Area of Bure District, West Gojam, Northwestern Ethiopia

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Malaria is one the leading health problem in Ethiopia. In the past few decades' places located above 2,000 meters elevation were considered as malaria free areas. However, the major malaria epidemics were seen up to 3,000 meters due to climate and land-use changes (ecological changes). Currently, due to the mass distribution of long lasting insecticide treated nets (LLINs) and nationwide implementation of Artemisinin Based Combination Therapy (ACT) and case management, the reduction of malaria morbidity and mortality have observed throughout Ethiopia. Regardless of these tremendous efforts, still malaria is one of the health problems of some highland parts of the country. In the study area, there was no any information on the diversity, abundance and spatio-temporal distribution of both the adult and larva of *Anopheles* mosquitoes. Hence, the objective of this study was aimed to investigate the species composition and abundance, blood feeding pattern, parous rates, longevity, entomological inoculation rates of *Anopheles* mosquitoes in Bure district, Northwestern Ethiopia. Similarly, species composition and distribution of the larva of *Anopheles* were studied and habitats were identified and characterized in each month. Entomological study of *Anopheles* mosquitoes was conducted longitudinally in each month, from July 2015 to June 2016 in three villages, Bukta, Workimdr and Shnebekuma. Adult *Anopheles* mosquitoes were collected by Light Traps, Pyrethrum Spray Catches and Artificial Pit Shelters. Identification of *Anopheles* species was done using morphological keys and species-specific polymerase chain reaction. Parous rates were determined by dissecting of the abdomen of unfed mosquitoes and then longevity was calculated from parous rates (p) using $d = \frac{1}{-lnP}$. Blood meal origins and circumsporozoite proteins were analyzed by Enzyme-Linked Immuno Sorbent Assay (ELISA). The human blood index (HBI) was calculated as the proportion of the mosquitoes fed on human blood meal of the total mosquitoes tested. Sprorozoite rate was expressed as the proportion of mosquitoes with Circum-sporozoite protein (CSP) to the total numbers of mosquitoes tested. Larvae were

collected from different breeding habitats using 350ml white dipper (and graduated pipette) and habitats were characterized; and morphologically 3rd and 4th stages of these larvae were identified to species level using keys from July 2015 - June 2016. Independent-Samples T-Test and One-way- ANOVA were applied to evaluate mosquitoes densities difference between villages and species, indoor and outdoor host seeking mosquitoes, overall human and bovine blood indices and environmental variables. Nine adult species were identified (*Anopheles demeilloni*, *An. arabiensis*, *An. funestus*, *An. coustani*, *An. squamosus*, *An. cinereus*, *An. pharoensis*, *An. rupicolus*, and *An. natalensis*). Of 4703, *An. demeilloni* (50.7%, n = 2383) was the most dominant than others ($p < 0.05$). Spatially, the largest numbers of adult *Anopheles* mosquitoes were collected in non-irrigated village (Shnebekuma) (2.39 ± 0.10 adults/CDC-LT) than irrigated (Bukta) (1.35 ± 0.11 adults/CDC-LT) ($p < 0.05$). The overall (single plus mixed) HBIs of *An. arabiensis*, *An. funestus*, *An. coustani*, *An. squamosus* and *An. cinereus* were equal to the overall BBIs of the correspondent species ($p > 0.05$). The CSP-ELISA test revealed that only sporozoite infected *Anopheles* mosquitoes were found only in non-irrigated villages (Shnebekuma and Workmidr). Overall sporozoite rates of *An. funestus*, *An. coustani*, *An. arabiensis* for *P. vivax* and *P. falciparum* were 0.57%, 0.23% and 0.2%, respectively. Morphologically, seven species of *Anopheles* larvae were identified (*An. demeilloni*, *An. gambiae* s.l, *An. funestus* s.l, *An. coustani* s.l, *An. squamosus*, *An. cinereus* and *An. pharoensis*). Of 3490, *An. demeilloni* (34.8%) was the most dominant than others ($p < 0.05$). The density of *Anopheles* larva was statistically insignificant in the three villages ($p > 0.05$). In conclusion, three of the most important malaria vectors of Ethiopia were identified; *An. arabiensis*, *An. funestus* and *An. pharoensis.* Most of the adults and sporozoite infected *Anopheles* mosquitoes were found only in non-irrigated villages. All these results implying that, irrigation activity has not any influence on the abundance, composition of *Anopheles* mosquitoes and prevalence of malaria in the three villages. Generally, vectors infection and malaria transmission rates were very low. Thus, breeding habitat management must be in incorporated into control program and applied throughout the year together with LLINs and IRS, especially for non irrigated villages.

Key Words: Bure district, *An. coustani*, irrigation, sporozoite rate, larval habitats, IRS.

Chapter 1: General Introduction

1.1 General Background

Malaria is a complex disease caused by protozoan parasites belonging to the genus *Plasmodium* that the mosquitoes transmit through blood feeding (Heggenhougen *et al*, 2003; Ramirez *et al*., 2009). It is one of the most important vector born diseases that cause morbidity and mortality throughout the world (Paaijmans, 2008; Ebi, 2009) and one of the major diseases of poor people in developing countries (Wimberly *et al*., 2012a).

Annually, worldwide cases of acute illness due to malaria are estimated to 300-500 million, and much of the burden was laid on Africa children (Walker, 2002; Ebi, 2009) and pregnant women (Heggenhougen *et al.*, 2003; Dawit *et al*., 2013). According to Heggenhougen *et al*. (2003), from 80% of the malaria load in the world, Africa accounted for 90 % due to *Plasmodium falciparum* (RBM, 2004). This parasite caused approximately 1-million deaths (RBM, 2004) and over 200 million clinical events among the people of Africa each year (Snow *et al*., 1999).

Based on World Health Organization (WHO) estimate, at global level, in 2009, nearly half of the world's population (WHO, 2011); in 2010 and 2011, 3.3 billion people; and in 2012, about 207 million were at risk of malaria infection, but most of the risk load was in area of sub-Saharan Africa (SSA) (Nevill, 1990; WHO, 2011 & 2012a) which accounted for about 90% (UN Millennium Project, 2005a). In 2013, 1.2 billion populations were at high risk**,** 198 million cases and 584, 000 deaths were recorded (WHO, 2014). In 2015, globally the average estimated malaria deaths were 429, 000 from 212 million new cases (WHO, 2017); however, most of these deaths occurred in

the African region (92%) (WHO, 2016b). Recently, the burden of malaria has been aggravated by both resting and feeding behaviors changes (Boreham & Garrett-Jones, 1973) and insecticides resistance capacity of the vectors (Okwa, 2012; Jindal *et al*., 2014). In parallel, malaria parasites (e.g., *Plasmodium falciparum* and *P. vivax*) have developed resistance to anti-malarial drugs (Aminake & Pradel, 2013; Sinha *et al*., 2014; Nordqvist, 2015).

Regardless of all these burdens and challenges, malaria burden has began to decline starting from 2000, which resulted in incidence rate fall of 37% and death rate decline by 60% globally (WHO, 2016b). Between 2001 and 2013, an estimated 4.2 million lives (WHO, 2014) were saved as a result of proper administration of anti-malaria drugs (Kendall, 2012) and massive scale-up of long lasting insecticides treated nets (LLINs) (Killeen *et al*., 2007; Kendall, 2012). Of these figures, 4.1 million (97%) lives were saved in the under-five age group in SSA (WHO, 2014). Similarly, WHO (2017) reported the cut of new malaria cases and death rates by 21% and 29%, respectively worldwide between 2010 and 2015. In SSA, tremendous reduction of malaria burden is found due to scaling up of prevention, diagnosis, and treatment in an integrated manner (Prudhomme *et al*., 2010).

1.2 Statements of the Problem and Rationale of the Study

In Ethiopia, malaria is one of leading health problems (Midekisa *et al*., 2012). The disease happened in both low and highlands up to 2,500 meters above sea level (m.a.s.l) (Zhou *et al*., 2004; Graves *et al*., 2009). Amhara region is one of the highland parts of Ethiopia with an elevation ranging from 506 to 4,517 m.a.s.l (Midekisa *et al*., 2012). Based on altitude, the region encompasses both stable (below 1,500 meters) and unstable (between 1,500 to 2,300 meters) malaria transmission (Kiszewski &

Teklehaimanot, 2004). In 2013, high incidence of malaria was observed in 30 districts of the region. Of these, Bure ranked seventh from all surveyed areas in the region (Amhara Health Regional State Bureau, Unpub., 2013). Among other districts in the region, Bure is unique in many respects and almost all of the villages (lowest administrative kebeles areas) are malarious. This is highly connected with the occurrence of many water resources, presence of very conducive temperature and agricultural activities in the district. Therefore, occurrence of breeding habitats (Matthys *et al*., 2006; Kreuels *et al*., 2008) and conducive temperature (Bayoh & Lindsay, 2003; Impoinvil *et al.*, 2007), and agricultural activities (Kebede *et al*., 2005; Jaleta *et al*., 2013) have played paramount role not only for the vector dynamics, but also for the parasites sovereignty in the vectors' body.

In Africa, malaria intervention has been made through a rapid scale-up of LLINs (Noor *et al.*, 2009), followed by the scale-up of indoor residual spraying (IRS) (Pluess *et al*., 2010); however, these interventions are usually given without detail understanding of the distribution, species composition and behavior of the local vectors (Sinka *et al*., 2010). Being this, currently these practices have become one of the leading causes for the development of resistance by the vectors (Boreham & Garrett-Jones, 1973; Jindal *et al*., 2014). Usually, the biology of each species is unique in many ways, such as the development of larvae habitats, behavior of the adult mosquito, susceptibility to *Plasmodium* parasites and the ability to transmit these parasites (Oaks *et al*., 1991). Therefore, study on the biology and the ecology of any vector before the administration of any intervention is a prerequisite for the successful management of malaria disease.

In Bure district, there was no documented data with regard to mosquito biology, ecology and population dynamics. So far, intervention activity has applied without any

study of the bionomics of vectors in the district. Therefore, study on "***Malaria Vectors and Transmission Intensity in an Epidemic Prone Area of Bure District, Northwestern Ethiopia***" was very vital, timely and appropriate to generate reliable data to produce everlasting solution to control malaria in Bure and its surrounding districts. This study was designed to answer the following research questions:

1) What are the compositions and the dynamics of *Anopheles* mosquitoes?
2) What are the blood meal sources of the vectors?
3) What is the longevity, sporozoite and entomological inoculation rates of the vector?
4) Which habitat types favor the development of *Anopheles* larva?

1.3 Objectives of the Study

1.3.1 General Objective

To assess malaria vectors and transmission intensity in an epidemic prone area of Bure district.

1.3.2 Specific Objectives

1) To know malaria vectors species composition and dynamic in the study sites,
2) To determine the feeding behavior and blood meal source of *Anopheles* species,
3) To investigate parous rate and longevity of the vectors,
4) To estimate infective and entomological inoculation rates of the vectors,
5) To determine the occurrence and dynamic of *Anopheles* larvae species, and
6) To describe the characteristics and types of larval habitats.

Chapter 2: Review of Related Literature

2.1 Trends of Global Malaria Burden

Malaria is one of the most important vector born diseases that cause morbidity and mortality throughout the world (Ebi, 2009; Wimberly *et al*., 2012a) and remains one of the three (others HIV/AIDS and tuberculosis) most essential tropical infectious diseases in humans worldwide (Satoskar *et al.,* 2009; Kendall, 2012).

Annually, malaria alone is expected to kill between 1.1 - 2.7 million people throughout the world (Srivastava, 2009). Similarly, between 300 - 500 million episodes are expected at global level (Bloland, 2001; RBM, 2004). Of these figures, most of them were children under five years of age (Ebi, 2009; Dawit *et al.,* 2013) and pregnant women (Heggenhougen *et al.,* 2003; Dawit *et al*., 2013); even the health problem was much worsen in SSA (RBM, 2004; Wimberly *et al*., 2012a & 2012b).

At global status, in 2008, one fifth of the world's population was at risk of malaria, 247 million episodes and one million deaths were recorded (Okwa, 2012). In 2009, the number of malaria cases and deaths were 225 million and 781, 000, respectively (WHO, 2010a). In 2010, 216 million cases of malaria were recorded, of these 655,000 estimated persons were died due to *Plasmodium falciparum* (WHO, 2011). In 2012, an estimated of 627,000 people died (WHO, 2013a); and in 2013, 1.2 billion population were at high risk, 198 million cases and 584,000 deaths were recorded (WHO, 2014). In 2015, globally the average estimated malaria deaths were 429, 000 from 212 million new cases (WHO, 2017); however, most of these deaths occurred in the African region (92%), followed by the South-East Asia Region (6%) and the Eastern Mediterranean

region (2%), almost all (99%) these deaths resulted from *Plasmodium falciparum* (WHO, 2016b).

The burden of malaria is not limited to morbidity and mortality; its socioeconomic impacts are very tremendous. In general, malaria is a disease of poverty and causes of poverty (Mendis *et al*., 2001; Ricci, 2012) and most of the malaria burden has been seen in poor countries (Teklehaimanot & Mejia, 2008). Therefore, the disease affects the poor's by exacerbating inequity in health and impeding development (Okwa, 2012). Individual and country development impediment are directly and indirectly attached with medical costs, absenteeism from work for self or child care, failure to open new areas for agriculture, tourism, and effects on fertility, population growth, saving and investment (Wentworth, 2010; Okwa, 2012); lost of productivity, time spent seeking treatment and funeral ceremony, poor cognitive development. Besides, it has great social impacts, incapable to practice in social development tasks and cultural roles and beliefs (Jones & Williams, 2004; Ricci, 2012).

However, the burden of malaria has began to decline starting from 2000 onward (WHO, 2011; WHO & UNICEF, 2015), where incidence rates estimated to fall by 37% and death rates by 60% globally (WHO & UNICEF, 2015; WHO, 2016b). Between 2001 and 2013, an estimated 4.2 million lives (WHO, 2014) were saved as a result of a scale-up of malaria interventions including administration of anti-malaria drugs (Kendall, 2012) and LLINs (Killeen *et al*., 2007; Kendall, 2012). Of these figures, 4.1 million (97%) lives were saved in the under-five age group in SSA (WHO, 2014). In the same years, WHO (2017) also reported the reduction of malaria case incidence by 21% and mortality rate by 31% in SSA.

2.2 Malaria Situations in Africa

According to WHO (1999) and Heggenhougen *et al*. (2003), from 80% of the malaria in the world, Africa accounted for 90%; of which, 90% of global mortality was in SSA due to *Plasmodium falciparum* (Okwa, 2012) and it caused approximately one million deaths and over 200 million clinical events among the people in each year. Based on WHO estimate, in 2009, nearly half of the world's population (WHO, 2011); in 2010 and 2011, 3.3 billion people; and in 2012 about 207 million were at risk of malaria infection globally; but most of the risk load was in SSA (WHO, 2011 & 2012a) which accounted about 90% (UN Millennium Project, 2005a). In 2010, from 216 million malaria cases in the world, 81% of cases and 91% of death were recorded in SSA and most of them were children under five (86%) (WHO, 2011 & 2012a).

Furthermore, insect-borne diseases cause significant economic losses in countries where they are endemic through lost of productivity and healthcare expenditure (Elden *et al*., 2010; Brown, 2011). In Africa, malaria alone can decrease gross domestic product (GDP) by as much as 1.3% in countries with high levels of transmission (Wentworth, 2010; Okwa, 2012). The economic consequences of malaria related diseases are higher and contribute to more than 10% of DALYs (Disability-Adjusted Life Years) in malaria endemic region. Generally, the attributed factors for much burden were linked with the occurrence endemic parasite (*P. falciparum*) (Teklehaimanot & Mejia, 2008), ideal climatic condition, and the wide spread of the most effective and difficult to control malaria vectors, *Anopheles gambiae* Giles, *An. arabiensis* Patton and *An. funestus* Giles (Kiszewski *et al*., 2004; WHO, 2006). Moreover, the anthropophily and high longevity nature of both *An. gambiae* s.l and *An.*

funestus were the other reasons for a great stability of malaria in Africa (Coosemans *et al*., 1992).

However, from 2000 onward (WHO, 2011) malaria mortality has declined by 33% in the WHO African region, due to the implementation of active control interventions, including wider usage of LLINs and IRS, and implementation of intermittent presumptive treatment in pregnancy (IPTp) (WHO, 2009 & 2010a). Since 2005, Steketee & Campbel (2010) reported the declination of over 20% in all-causes of childhood mortality in Africa through case management and the practice of the above interventions. WHO (2016b) also reported the declination malaria deaths in children aged under 5 years and the number was estimated to have decreased by 60% since 2000 and by 29% since 2010. Besides, in 36 African countries, between 2001 and 2010, it was possible to have saved the life of 842,800 individuals under-five and estimated to have reduced malaria mortality by 24% in year 2010 due to the scale-up of LLINs (Korenromp, 2012). In SSA, between 2001 and 2015, 663 million malaria cases were prevented due to malaria control interventions, it is estimated that 69% were due to use of LLINs, 21% due to artemisinin based combination therapy (ACT) and 10% due to IRS (WHO, 2016a). President's of Malaria Initiative also indicated the presence of reduction in malaria cases and mortality rate for those PMI focus countries in SSA, form 2010 to 2015 (PMI, 2017). In the same years, WHO (2017) also reported the reduction of malaria case incidence by 19% and mortality rate by 29% in SSA.

In particular to Ethiopia, malaria is one the leading health problem of the country (Midekisa *et al*., 2012; Carter Center, 2013; PMI, 2014). Three-fourths (75%) of the total area of country is malarious and more than two-thirds (approximately 68%) of the total populations live below 2,000 meters of altitude, and therefore, it is considered as

malarious risk area (Pennas & Girma, 2012; Ayele *et al*., 2012). Due to these reasons, malaria is the leading health problem of the country. However, recently the area coverage has not been revised in connection with changes such as urbanization, irrigation or dam, or land use and covers change (MoH, 2010).

Historically, Ethiopia had numbers of malaria epidemic cases. Epidemics occurred in the country in various years were, including 1958, 1987-1988, 1991, 1992 and 1998 - 1999, 2003, 2004 and 2005 (Fontaine *et al*., 1961; Kiszewski & Teklehaimanot, 2004). However, epidemics between 1953 and 1958 (Lindsay & Martens, 1998), in 1958 (Fontaine *et al*., 1961) and in 1998 (Connor *et al.*, 2010) had devastation effects. Between 2001 and 2005, the average annual figure of malaria case has increased from around 5 million to almost double at 9.5 million per year (PMI, 2008; Pennas & Girma, 2012). Finally, from 2004 - 2005 malaria was reported as the primary cause of health problems in Ethiopia, accounted for 17% of outpatient visits, 15% of hospital admissions, and 29% of inpatient deaths (MoH, 2005; Pennas & Girma, 2012) and the disease causes death of 70,000 individuals each year (Adugna, 2008). Entirely, all these epidemics were majorly caused by climatic incidents (Confalonieri *et al*., 2007; NIAID, 2001) together with low level of immunity, population movement, altered patterns of agriculture, prevalence of drug and pesticide resistance (Heggenhougen *et al*., 2003; Ebi, 2009), failure of malaria control programs, degradation of public health infrastructure and socioeconomic factors (UN Millennium Project, 2005b; Eskindir & Markussen, 2010).

Regardless of the above factors, from 2007 onward impacts of malaria have been reduced due to the implementation of massive intervention by the Ethiopian Government in 2005 and 2006, which was assisted by NGOs (PMI's, RBM, and Global

Fund). According to recent malaria assessment made in Ethiopia, malaria admission and death have been decreased by 54% and 55%, respectively, as compared to baseline period of 2001-2004 and reduction is still continued (MoH, 2010). Similarly, the findings of Otten *et al*. (2009), Shargie *et al*. (2008 & 2010), and Jima *et al*. (2010) confirmed this reality too. Otten and colleagues showed the reduction outpatients malaria by 81% and inpatients by 70% and deaths by 79% in 2007 as compared with baselines years from 2001-2006 due to mass distribution of LLINs and nationwide implementation of ACT. Jima and colleagues also indicated that the declination of malaria inpatient admissions by 2 to 3-fold from 2005 to 2009. WHO (2015a) reported that malaria case incidence was decreased 50 - 75% in 2000 - 2015 in Ethiopia. Moreover, in Tigray region, Ethiopia, Gerensea & Teklay (2017) reported the reduction of malaria morbidity by 33% and mortality by 43% between 2011/12 to 2014/15. Toyama *et al*. (2016) also described the presence of a sharp decline of malaria cases in Amhara region (Bure-Zuria, Dembia, and Mecha districts) due to case management, massive distribution of LLINs and the application of IRS. As a result, to date some African countries included malaria elimination target in their national malaria control program (Ranson, 2014), such as Ethiopia, Madagas-car, Senegal, Zambia, and Zimbabwe and Zanzibar (PMI, 2017).

2.3 Highland Fringes of Malaria

Previously, mostly malaria transmission was occurred in an altitude below 2,000 m.a.s.l; however, currently this situation has changed and epidemics are raised to 2,500 meters of the East African Highlands (Zhou *et al*., 2004; Senay & Verdin, 2005). Altitude above 2,000 meters are expected to be malaria free due to the presence of low temperature that cannot support the growth of mosquito and its fitness, and sporozoite

in mosquito's body (Abeku *et al*., 2003; Tchuinkam *et al*., 2010). However, East African highlands, including the high lands in western Kenya (Hay, *et al*., 2002a & 2002b), Ethiopia, Tanzania (Tesi, 2011; Wimberly *et al*., 2012b), Rwanda, Madagascar and Uganda (Mouchet *et al*., 1998) have been experienced a continuously malaria recurrence (Shanks *et al*., 2005; Checchi *et al*., 2006), majorly due to the results of environmental (climate changes including increased temperature, unusually high rainfall with extended periods) (Delacollette, 2004; Alonso *et al*., 2010; Tesi, 2011), biological (presence of efficient highland vectors with sporozoite infection and human migration-movement of infected individual from low to highland, population growth), and socioeconomic factors (transportation and housing, land clearance/ deforestation, irrigation and dams, and deteriorating health systems due to war, civil conflicts, and declining resources) (Alonso *et al*., 2010; Tesi, 2011). Besides, topography, human settlement pattern, drug resistance, and changes in *Anopheles* vector populations **(**Zhou *et al*., 2004; Shanks *et al*., 2005), the reduced efficacy of LLINs, insecticide resistance and lack of proper use of LLINs (Protopopoff *et al*., 2008; Atieli *et al*., 2011; Zhou *et al*., 2011) and global warming (Jetten *et al*., 1996; Siraj *et al*., 2014). In these highlands, the dwellers have had very low immunity and malaria epidemics have caused significant human mortality (Alonso *et al*., 2010) in both children and adults (Fontaine *et al*., 1961).

In Ethiopia, malaria often was occurred below 2,000 m.a.s.l (Lindsay & Martens, 1998; Woyessa *et al*., 2002). Above 2, 000 meters altitude were considered malaria free areas (Rishikesh, 1966; MoH, 2012); but the major malaria epidemics (due to *Plasmodium falciparum* and *P. viva*x) were seen up to 2,400 m.a.s.l in Oromia, Amhara, and Tigray regions (Ayele *et al*., 2012; Midekisa *et al*., 2015), even up to 3,000 meters (Graves *et*

al., 2009). This was probably due to movement malaria infected people from lowland to highland areas.

In Oromia, Amhara, and Tigray regions of Ethiopia, the numbers of mortality and morbidity cases were very high. In 1953, in highlands region of Amhara, epidemics were expected to kill 7, 000 people; 1958 the epidemic affected 3 million people and led to 150,000 deaths (Fontaine *et al*., 1961). In 1958, around Akaki and along the Addis-Jima road, the epidemics were covered from 1,200 to 2,200 meters elevation. The other most serious outbreak in the country was in 1998, more than 403 localities in Tigray; 1,544 localities with 3.4 million people in Amhara; 812 localities with more than 1.2 million people in Oromia; 300 localities with more than 216,317 clinical cases in SNNPR were seriously affected and high mortality rate were recorded too (Connor *et al.*, 2010). Moreover, sever epidemics were observed in 2003 (Hay, 2003) with a total of 50 epidemics at elevations of from 1,500 to 2,500 meters in Amhara and SNNP regions (Kiszewski & Teklehaimanot, 2004). All these epidemics were caused by the presence of high rainfall for an extended period, optimum temperature and relative humidity (Verdin, 2005; Mbogo, 2012) and land-use changes (Zhou *et al*., 2004; Siraj *et al*., 2014).

2.4 Distributions of Human *Plasmodium* Species in Africa

Malaria remains undeniable one of the most important tropical infectious diseases in humans worldwide. It is caused by single-celled parasites of the genus *Plasmodium* (protozoan) (Bannister & Sherman, 2009), which are transmitted from one human to another by the biting of female *Anopheles* mosquitoes (Carter & Mendis, 2002; Heggenhougen *et al*., 2003). There are five *Plasmodium* species that affect human health: *Plasmodium falciparum, P. vivax, P. malariae, P. ovale*, and *P. knowlesi*

(Kantele & Jokiranta, 2011; Varnado *et al*., 2012; Obare *et al.*, 2013); but *P. knowlesi* is very less common as compared to the other parasites, and commonly called primate malaria and widely distributed in Malaysia and other Southeast Asian countries (Cox-Singh *et al*., 2008; Kantele & Jokiranta, 2011).

Plasmodium ovale is naturally distributed in SSA (Morrow, 2007) and the islands of the western Pacific (Mueller *et al*., 2007); however, currently many findings indicated that this parasite is found through the world (in Asia & South America) (Collins & Jeffery, 2005; Mueller *et al*., 2007) and the highest disease burden is occurred in SSA (Morrow, 2007; Obare *et al*., 2013). *P. falciparum* is found in tropic and sub-tropic regions; but *P. malariae* has worldwide distribution (Morrow, 2007), though the incidence burden is very minimum (Oaks *et al*., 1991). *P. vivax* is distributed in widest geographic location (in tropics, subtropics, and temperate regions) (Mendis *et al*., 2001; Assafa *et al.*, 2006), but highly dominant in the horn of Africa (including Djibouti, Eritrea, Ethiopia, Somalia, and Sudan) (Snow & Omumbo, 2006).

Totally, respect to predominance, only *P. falciparum* and *P. vivax* have worldwide distribution (Mendis *et al*., 2001) and the most common species in SSA, but only *P. falciparum* is definitely the most important and responsible for most severe illness and deaths (Snow *et al.*, 2001; Kantele & Jokiranta, 2011) and bears over 90% of the global burden (Snow & Omumbo, 2006). On the other hand, *P. vivax* accounts for more than half of all malaria cases outside SSA (Okwa, 2012).

In Ethiopia, there are four types of *Plasmodium* species: *P. falciparum*, *P. vivax*, *P. malariae* and *P. ovale* (Krafsur & Armstrong, 1982), but *P. falciparum* and *P. vivax* are the most important parasites (MoH, 2005; Mbogo, 2012; Pennas & Girma, 2012) and found almost all parts of the country, extended to 3,000 m.a.s.l. *P. ovale* was found

in different parts of Ethiopia, in Arba Minch (in 1966), in Gambella and Humera (in 1968) (Collins & Jeffery, 2005), and in North Gondar (Alemu *et al*., 2013a); however, currently rarely revealed. *P. malariae* was first detected in 1938 in south Omo district (Archetti, 1940) and then in Gambella between 1967-1969 (Krafsur & Armstrong, 1978 & 1982), and was comprised less than 1% of all cases (Fisiha, 2002).

Generally, from 2004 - 2005, the average annual figure of malaria case had increased from around five million to almost double at 9.5 million (PMI, 2008; Pennas & Girma, 2012) and these figures were fully attributed only by *P. falciparum* and *P. vivax*, which accounted 60% and 40% of the malaria cases, respectively (Deressa *et al*., 2003a; Ketema *et al*., 2011). However, to date proportions of the causative agents varies from place to place and from season to season (Yewhalaw *et al*., 2009; Carter Center, 2013) due to differences in altitude, rainfall, temperature, population movement (Carter Center, 2013), host and vector characteristics, and change in health care infrastructure (Alemu *et al*., 2011; Carter Center, 2013). For example, WHO (2011) reported that *P. falciparum* and *P. vivax* account for 70% and 30% of all laboratory confirmed cases, respectively in Ethiopia. Five years retrospective data (between July 2003 - June 2008) reported by Karunamoorthi and Bekele (2012) in Oromia region Ethiopia, showed that *P. falciparum* contributes 62.4% and *P. vivax* 37.3% of malaria cases.

On the other hand, in recent years, the prevalence of *P. vivax* exceeded *P. falciparum* in some parts of the country, in Jima (Alemu *et al*., 2011) and in Dill town (Molla & Ayele, 2015). Tesfaye *et al*. (2012) showed the dominant of *P. vivax* by 62.5% in the highland fringe of Butajira. Tefera (2014) and Gari *et al*. (2016) also reported that *P.vivax* (70.41%, 84.6%) was dominant than *P. falciparum* (23.08%, 15.4%) in patients attending at Hallaba health center and blood taking samples from people of Adim Tullu

district, Ethiopia, respectively. Generally, WHO (2016b) described four countries accounted for 81% of estimated deaths due to *P. vivax* (India, Indonesia and Pakistan); Ethiopia was the fourth countries and shared 12%.

2.5 Malaria Vectors in Africa: Composition and Ecology

Composition: Mosquitoes are small to medium-sized insects which have only two wings, a long slender body, long legs and long needle-shaped mouthparts/the proboscis/ as long as the length of the palps in the adult stage (Rozendaal, 1997; Harbach, 2007). They are flies, belong to the family Culicidae, order Diptera, class Insecta, phylum Arthropoda (Rueda, 2008). So far, over 3,500 mosquitoes have been recorded worldwide (Fang, 2010; Harbach & Kitching, 2016). They grouped into forty genera (Harbach, 2004) and a single family called the Culicidae (Manguin & Boete, 2011). This family divided into two subfamilies, the Anophelinae and Culicinae. Subfamily Anophelinae has three genera (*Anopheles* Meigen, Bironella Theobald and Chagasia Cruz) (Harbach, 2004 & 2007), but only the genus of *Anopheles* is involved in malaria transmission from human to human (Harbach, 2004; Varnado *et al*., 2012).

From over 3,500 well recognized species of mosquitoes, about 537 species are *Anopheles* (Harbach, 2013) and only between 70-80 is known to transmit human malaria worldwide (Robert *et al*., 2011). Out of them, 41 are considered as dominant vector species/species complexes and capable of transmitting malaria at higher level (Hay *et al*., 2010; Sinka *et al*., 2012).

In Africa, there are 140 *Anopheles* species, but only 20 are known to transmit malaria to human (Hay *et al*., 2000; Sinka *et al*., 2012). Of them, about 7 species namely *Anopheles arabiensis*, *An. funestus*, *An. gambiae*, *An. melas*, *An. merus*, *An. moucheti* and *An. nili* have been reported as the dominant vector species (Hay *et al*., 2010; Sinka

et al., 2012). In addition to these, some mosquitoes, such as *An. pharoensis* in Egypt (Kawada *et al*., 2012; Snow *et al*., 2015) and Ethiopia (Kibret *et al*., 2008 & 2017; Animut *et al*., 2013a), *An. hancocki* in Cameroon, *An. mascarensis* in south-east Madagascar, *An. rivulorum* in Tanzania (Wilkes *et al*., 1996) and *An. bwambae* in Uganda (Snow *et al*., 2015) are considered as secondary vectors and have increased malaria burden by extending the malaria transmission period (Awono-Ambene *et al*., 2004). Others species such as *An. coustani* (Bekele *et al*., 2012; Kawada *et al*., 2012; Mwangangi *et al*., 2013), *An. marshalli* (Steyn, 1946), *An. mascarensis* (Fontenille & Campbell, 1992), and *An. squamosus* (Gillies, 1964) were reported as vectors in various Africa countries.

In Ethiopia, over 42 species of *Anopheles* were identified (Hay, 2003; Gaffigan *et al*., 2013). Of these, the major malaria vector is *An. arabiensis* (Krafsur & Armstrong, 1978; White *et al*., 1980, Balkew *et al*., 2006) whereas *An. pharoensis*, *An. funestus* and *An. nili* are secondary vectors (Krafsur & Armstrong, 1978; MoH, 2010). But, recently, Kelel (2010), Yewhalaw *et al.* (2014) and Degefa *et al.* (2015) reported *Plasmodium* infected *An. coustani* in Jima zone, Ethiopia; therefore this species could be the other possible secondary malaria vector in Ethiopia.

Ecology: Ecology of *Anopheles gambiae* complex: *An. gambiae* groups are composed of at least eight species (Harbach, 2004): *An. arabiensis*, *An. gambiae* s.s, *An. melas*, *An. merus*, *An. moucheti*, *An. quadriannulatus*-A, *An. coluzzii* and *An. amharicus* (Coetzee *et al*., 2013). However, the most efficient vectors in Africa are *An. gambiae* s.s, *An. arabiensis* and *An. coluzzii* (Hunt *et al*., 1998; Sinka *et al*., 2012; Harbach, 2013). *An. gambiae* s.s: the adults of these species are usually predominant in humid forest environments (Gillies & Wilkes 1965; Coetzee *et al*., 2000) and highest densities usually are registered during (Onyabe & Conn, 2001) and after the rainy season

(Awolola *et al*., 2002; Cohuet *et al*., 2004) because the increased rainfall increases the numbers of breeding habitats and provides sufficient nutrients for the larvae (Koenraadt *et al*., 2004). Grass which is found near to the breeding sites also have played great role for the newly emerged adult and gravid mosquitoes as a shade and resting places. *An. arabiensis* Patton: this adult mosquito is common in dry and savannah environment (Awolola *et al*., 2002; Sinka *et al*., 2010), sparse woody and cleared land (Coetzee *et al*., 2000) and more common in arid and urban SSA's environment (Gillies & Wilkes 1965; Gillies & Coetzee, 1987), where an increasing proportion of resides (Jones *et al*., 2012).

Ecology of *An. funestus* Giles groups: These groups are widespread throughout SSA and Madagascar (Fontenille & Simard, 2004; Dia *et al*., 2013). They are composed of ten species: *An. funestus* s.s., *An. rivulorum* Leeson, *An. leesoni* Evans, *An. vaneedeni* Gillies & Coetzee, *An. parensis* Gillies, *An. confusus* Evans & Leeson, *An. aruni* Sobti, *An. fuscivenosus* Leeson, *An. brucei* Service (Gillies & Coetzee, 1987) and *An. rivulorum*-like species (is present in Burkina Faso and Cameroon) (Cohuet *et al*., 2003; Spillings *et al*., 2009). However, *An. funestus* is the only efficient vector in African (Gillies & Coetzee, 1987; Kamau *et al*., 2003). Additionally, *An. rivulorum* was reported as a minor vector in Tanzania (Cohuet *et al*., 2003) and Kenya (Kawada *et al*., 2012). *An. funestus* commonly found in dry season, e.g., in forest savannah (in Cameron) (Cohuet *et al*., 2004), in humid savannah (in Burkina Faso) (Dabire *et al*., 2007) and dry savannah (in Gahana) (Dadzie *et al*., 2013) and it reaches peak density in the early dry season (Cohuet *et al*., 2003).

Ecology of *An. pharoensis*: Geographically, *An. pharoensis* is found mainly in west and east Africa (Kibret *et al*., 2009). It is a major transmitter in arid and semiarid regions

with permanent and shaded water bodies (Gillies & Coetzee, 1987) including in marshes, ponds, rice fields, lakeshores, large vegetated swamps, old water lands and often extensive wet season flooding and floating vegetation.

Ecology of *An. nili* groups: This group consists of four species including *An. nili* s.s, *An. somalicus*, *An. carnevalei* (Brunhes *et al*., 1999) and *An. ovengensis* (Awono-Ambene *et al*., 2004). Except *An. somalicus*, the rest members are known by transmitting malaria in SSA (Antonio-Nkondjio & Simard, 2013). *An. nili* s.s has a wide geographic distribution in tropic Africa, mainly in humid savannas areas (Fontenille & Loclzouarn, 1999; Okara *et al*., 2010) and is one of among the most important malaria vectors in SSA. Specially, it is found in forested and degraded forest areas (Antonio-Nkondjio *et al*., 2002; Awono-Ambene *et al*., 2009) across most of west, central and east Africa (Gillies & Coetzee, 1987; Ayala *et al*., 2009).

Ecology of *An. moucheti* groups: *An. moucheti* groups are comprised three spices: *An. moucheti moucheti* s.s, *An. moucheti nigeriensis* and *An. bervoetsi* (Fontenille & Simard, 2004). The adult is found in forested and degraded forest areas of equatorial Africa (Fontenille & Simard, 2004; Awono-Ambene *et al*., 2009). *Anopheles melas* Theobald and *An. merus*: both are saltwater species and their distribution is limited in brackish water breeding sites of the west and east coast of Africa, respectively (Lanzaro & Lee, 2013; White *et al*., 2013). *An. coluzzii* is found widely spread throughout SSA (Coetzee *et al*., 2013). The adult is commonly found around freshwater habitats and present all year round in the dry savannahs of West Africa. This species breed in man-made permanent aquatic habitats (e.g., rice irrigation schemes) (Wondji *et al*., 2005; Costantini *et al*., 2009).

2.6 Factors Determining Vector Distribution and Malaria Transmission

2.6.1 Factors Determining Adult Vectors Distribution

Anopheles mosquitoes are occurred worldwide heterogeneously in tropics and temperate regions. However, they are absent only from a few islands, the majority of the Pacific Islands, including the New Zealand, Fiji and New Caledonia (Harbach, 2013) and in the Antarctic (Rueda, 2008; Manguin & Boete, 2011; CDC, 2015). They are found as high as 6,000 m.a.s.l in mountainous regions and as deep as 1,250 meters (below sea level) in caves and mines (Manguin & Boete, 2011). The distribution of mosquito species differs within the environment conditions (Forstinus *et al*., 2015), and depends on altitude and bioclimatic structures (Mwagangi *et al*., 2007b). Moreover, each vector species has its own specific ecological requirements and tolerance capacity towards deviations (Ayala *et al*., 2009; Dotson, 2010; Pennas & Girma, 2012).

Therefore, identification of the ecological requirements of malaria vectors is very basic to design the most appropriate control strategy (Service, 1989). The ecological factors including temperature, rainfall, relative humidity and altitude are the determined factors for the existence of each specific vector (Snow & Omumbo, 2006; Patz *et al*., 2008) as well as their presence and abundance in a given area (Maxwel *et al*., 2003). Generally, *Anopheles* mosquito's biological (Oaks *et al*., 1991), habits and habitats are species-specific (Varnado *et al*., 2012) and they can live in a variety of habitats such as fresh water, brackish water, or any water (clear, turbid or polluted), except in marine habitats with high-salt concentrations (Rueda, 2008).

Temperature is one of the most main environmental factors that decide the distribution and rate of development (Bayoh & Lindsay, 2004; Snow & Omumbo, 2006; Stresman, 2010), and the survival rate of the larvae and adults (Christiansen-Jucht *et al*., 2014),

and the sporogonic cycles in mosquito's body (Mouchet *et al*., 1998). Adult vectors need 21-31°C atmospheric temperature (Martens *et al*., 1995); however, extreme high temperature beyond 30°C make adult mosquitoes less productive and if it reaches from 40°C to 42°C, total death will happen (Snow & Omumbo, 2006; Tesi, 2011). For parasite development, the optimum range of temperature is between 25°C and 30°C. The minimum and the maximum temperature for the survival for *Plasmodium falciparum* is 18°C and 40°C, respectively (Snow & Omumbo, 2006; Stresman, 2010).

Rainfall is the second primary environmental factors that limit the vectors densities by determine the number and the productivity of the breeding sites (Mouchet *et al*., 1998). These nutrient provided habitats are important to lay eggs and for completing the life cycle of mosquitoes. Therefore, the presence and quality of larval habitats are the principal determinants of adult mosquito abundance and distribution (Zimmerman, 1992; Mwangangi *et al*., 2007b). Mosquito uses various water resources, canal leakage pools and irrigated field puddles (due to irrigation plans) (Appawu *et al*., 2004; Kibret *et al*., 2009; Jaleta *et al*., 2013), shoreline of small and large dams reservoir (Keiser *et al*., 2005; Dejenie *et al*., 2011; Yewhalaw *et al*., 2013) for egg lay.

Furthermore, the reservoir water and rainfall help to increase the survival and longevity of the adult by increasing the moisture (over 60%) to the surrounding area in which adult is found (Jawara *et al*., 2008; Snow & Omumbo, 2006). Low air moisture content (35-50%) reduced the survival capacity of the adult mosquito (Umaru & Akogun, 2016). Thus, mostly adult mosquito like optimum humidity between 60 - 80% (Snow & Omumbo, 2006; Dotson, 2010) and this humidity help to elongate the adult survival rates and fully determines the total egg production and the stability of the vector population (Milby & Reisen, 1989). Other factors, such as altitude, deforestation,

urbanization etc have their own contributions on the abundence and the composition of mosquitoes. At lower altitude, density and species composition of malaraia vectors are high (Lindblade *et al*., 2000) due to having an increased temperature. On the other hand, because of the presence of decreased temperature at higher altitude, the density and the diversity of mosquitoes are low (Kulkarni *et al*., 2006). At higher elevation, temperature become lower and retarded the metabolism rate of both the adult and the larva and finally this lower temperature kill all stages of mosquitoes (Patz *et al*., 2008). Because of this reason, many malaria episodes are documented less than 1,500 meters than higher elevation (Brooker *et al*., 2004; Ernst *et al*., 2006; Hernandez-Avila *et al*., 2006; Kazembe, 2007).

Land cover changes and deforestation helped efficient malaria vectors to colonized new areas due to the availability of sun-exposed temporal and permanent potential breeding habitats (Mouchet *et al*., 1998). The augmented temperature helps to increase growth rate and survivorship of the larvae (Minakawa *et al*., 2004 & 2005; Munga *et al*., 2006). Due to these reasons, currently *An. gambiae* s.s and *An. funestus* invaded and reinvaded the highland areas in western Kenya (Chen *et al*., 2006; Imbahale *et al*., 2011; Kweka *et al*., 2011) and an increased risk of malaria transmission was observed (Antonio-Nkondjio *et al*., 2005; Guerra *et al*., 2006; Vittor *et al*., 2009). The positive roles of deforestation on the rate of larvae and pupae development and malaria prevalence have documented (Tuno *et al*., 2005; Afrane *et al*., 2006; Munga *et al*., 2007; Wang *et al*., 2016). Urbanization has positive effectes on the distribution and abundance of adult mosquitoes by creating breeding habitats (Gimnig *et al*., 2001). Because of formation of proliferation sites, large number of *An. gambiae* s.s in Nigeria (Awolola *et al*., 2007) and *An. gambiae* s.l in Ghana were collected (Chinery, 1984).

Other factors such as chemical properties of habitats (Mutero *et al*., 2004, Mwangangi *et al*., 2007b) and pollution are regulated the dynamics of the vector population positively and negatively. Additionally, biological factors such as predators, parasitoids, certain bacteria and fungi are known by minimized the abundance of the adult vectors by reduced the larvae densities (Chase & Knight, 2003; Mwangangi *et al*., 2007b). Housing condition and intervention methods also affect the dynamic of the adults. Higher density of indoor-resting *An. arabiensis* was found in open eaves houses than closed eaves in Ethiopia (Animut *et al*., 2013b). Interventions through LLINs, IRS and larval control (environmental management) have reduced mosquito's densities and their daily survival (Walker & Lynch, 2007; Eckhoff, 2011; Atieli *et al*., 2011).

2.6.2 Ecology and Growth Determining Factors of *Anopheles* Larvae

2.6.2.1 *Anopheles* Mosquito Larvae: Biology and Ecology

Mosquito larval habitats are the places where eggs are laid, larvae hatch and change into instars, then become pupate and adults (Gimnig *et al*., 2002; Paaijmans *et al*., 2008; Eckhoff, 2011). *Anopheles* mosquitoes undergo complete metamorphosis which included four stages (Egg → larva → pupa → adult) (Ramirez *et al*., 2009). Except the adult stages, all are exclusively aquatic (Rozendaal, 1997; Renchie, 2007; Rueda, 2008). In tropic climates, eggs hatch within 2-3 days (Rozendaal, 1997; Varnado *et al*., 2012), but in colder temperate hatching may not occur until after 2-3 weeks (WHO, 2005), depending on the ambient temperature condition (Beck-Johnson *et al.*, 2013). The best favorable water temperature for egg hatching is between 24°C and 30°C (Bayoh & Lindsay, 2003; Impoinvil *et al*., 2007).

Anopheles larvae have three separated body regions: head, thorax and abdomen. They can be differentiated from the other aquatic insects by being legless and having a

bulbous thorax (i.e., wider than both the head and the abdomen). The head part contains a pair of antennae and a pair of compound eyes. Prominent mouth-brushes are present in most species and serve to sweep water contain of minute food particles into the mouth. *Anopheles* larvae have four active larval instars and all stages require water to develop. Therefore, no mosquito larva can withstand desiccation (Rozendaal, 1997; Renchie, 2007; Rueda, 2008; Dotson, 2010). As compared to other larval forms of subfamilies Culicidae, the larvae of subfamily Anophelinae do not have a siphon and breathe via spiracles (Varnado *et al*., 2012). In general, the larva needs about 4-7 days in tropics (between 24°C and 30°C) to transform into pupae stages, if no shortages of food (Rozendaal, 1997).

Anopheles larvae tend to feed at the air/ water interface or on the bottom while *Culex* and *Aedes* typically feed throughout the water column (Rueda, 2008; Dotson, 2010). They feed on microorganisms (Varnado *et al*., 2012) such as yeasts, bacteria and fungus (Rozendaal, 1997; Renchie, 2007), algae (Gimnig *et al*., 2002), corn pollen (Hamoudi & Sachs, 1999; Kebede *et al*., 2005), detritus (particulate organic matters) (Gimnig *et al*., 2002) and other organic matters such as dead invertebrates.

Finally, the full grown the 4th larval instars change into comma-shaped pupal stages. Pupae do not feed but spend most of their time at the surface of water to take-in air through the respiratory trumpets (Renchie, 2007; Rueda, 2008). If disturbed, they swim up and down in the water in a jerky fashion. In the tropics weather, this stage needs 1- 4 days to convert into adult stages (Rozendaal, 1997; Varnado *et al*., 2012). Under good conditions, the entire period from egg to adult takes about 7-13 days (Rozendaal, 1997; Coetzee *et al*., 2000; Phasomkusolsil *et al*., 20013); however, another laboratory and field studies indicated that the whole life cycle took 9.8 to 23.3 and 8 to 22 days,

respectively (Okogun, 2005). After the adult female get maturation, she under goes a mate and feed blood meal, and then become gravid (fully well developed eggs) (Rozendaal, 1997). Finally, the gravid female searches suitable larval habitats (Paaijmans & Thomas, 2011) and lays individual eggs approximately 50 - 200 (depending on the species) small brown or blackish boat shaped on the water surface in every few days at night time (Ramirez *et al*., 2009).

In tropical areas, adult mosquitoes live on average 1-2 week, whereas in temperate countries their longevity is about 3- 4 weeks. Under the most favorable conditions (in the tropics), the average lifespan of female *Anopheles* mosquitoes is 3 - 4 weeks and continues to lay eggs throughout their life time and most will lay 1–3 batches of eggs, though some may lay as many as six/ seven batches (Charlwood *et al*., 2000) and/ or over ten cycles (Gillies & Wilkes 1965). The number of batches/ gonotrophic cycles depends on the size of the vectors (Gimnig *et al*., 2002; Lyimo *et al*., 2012), mosquito species (Briegel, 1990), availability of blood meals (Lyimo *et al*., 2012) and sugar or plant nectars, applying intervention (LLINs & IRS) (Eckhoff, 2011), availability of breeding site (Okogun, 2005), number of previous gonotrophic cycles and temperature (WHO & UNICEF, 2005).

Mosquitoes breeding habitat: Breeding habitat of *Anopheles* mosquitoes are species specific (Ramirez *et al*., 2009; Forstinus *et al*., 2015) and survive in large varies of habitats (Washburn, 1995). Breeding sites varies from place to place (Rozendaal, 1997; Dotson, 2010) including both natural (in more) and anthropogenic sites (Gimnig *et al*., 2002; Silver, 2008; Manguin & Boete, 2011), permanent or small or large, freshwater (Rueda, 2008) or saltwater (Varnado *et al*., 2012). Generally, the productivity (quality) of breeding habitats is measured by the total numbers of eggs deposited at first, enlargement, development rate, survival of the mosquito immature and the quality of

the resulting adults (Ye-ebiyo *et al*., 2003). Thus, they are said to be the most important inputs to adult distribution, abundance and fitness of the adults (Chen *et al*., 2006; Forstinus *et al*., 2015).

The larvae of *An. gambiae* s.s are preferred to breed inside sunny and clear water, usually having a small depressions like foot or hoof prints, periphery of holes and burrow pits, roadside puddles due to tyre-tracks, irrigation ditches and other man-made shallow water bodies (Gimnig *et al*., 2001; Chen *et al*., 2006), rice field, dam and irrigation canals (O'Connor, 1967; Mouchet *et al*., 1998). They also reproduce inside polluted water that loaded with organic matter (Castro *et al*., 2010), in flood plains, and in pools of water along river/ lake shores particularly when there are fluctuations in water level (Minakawa *et al*., 2008). Additionally, grass growing in the larval habitats have been played a great role than other vegetation types or open habitats (Fillinger *et al*., 2004; Imbahale *et al*., 2011) in protecting mosquito larvae from being swept by running water (Paaijmans *et al*., 2007) or from predator attacks; however, Gimnig *et al*. (2001) reported the absence of *An. gambiae* s.s larvae in aquatic vegetation or surface films.

The larval of *An. arabiensis* more prefer to small, temporary, sunlit, clear and shallow fresh water pools (Gimnig *et al*., 2001; Chen *et al*., 2006; Sinka *et al*., 2010), although it is able to utilize many varieties of habitats such as slow flowing, shaded streams, large and small natural and man-made habitats (Sinka *et al*., 2010). Sometime, it is found in turbid waters (Gimnig *et al*., 2001), brackish habitats (Bogh *et al*., 2003), irrigated rice fields (their density reach peak when the plants are too small) (Sinka *et al*., 2010; Mwangangi *et al*., 2007a) and in fish ponds (Sinka *et al*., 2010); dam and irrigation canals (O'Connor, 1967; Mouchet *et al*., 1998); rain pools, brick making pits

and various pools, including canal leakage pools and water harvesting pools (Kibret *et al*., 2010 & 2012); however, absent in habitats with aquatic vegetation or surface films (Gimnig *et al*., 2001).

The larvae of *An. funestus* breed in relatively shaded, permanent clean fresh water bodies with aquatic vegetation (O'Connor, 1967; Lindsay & Martens, 1998; Gimnig *et al*., 2001), in artificial semi-permanent breeding habitats (due to urbanization) (Robert *et al*., 2003) with floating or emerging vegetation, in permanent swamps with emergent vegetation and reaches its peak density in the early dry season (Gillies & Wilkes, 1965; Cohuet *et al*., 2004). The natural breeding sites encompasses edges of swamps, weedy and grassy parts of rivers, streams, furrows, ditches and ponds (Dia *et al*., 2013); whereas artificial breeding including rice fields, wells and domestic water-containers (Ibrahim *et al*., 2005) and seepage pools at dam base and reservoir shoreline puddles (Kibret *et al*., 2012). Mostly, they are absolutely absent in open water areas and rarely found in habitats covered by a film and thick stands of aquatic vegetation (Gimnig *et al*., 2001).

Anopheles pharoensis larvae breed in swamps, irrigation canals, rain pools, brick making pits and various pools (canal leakage pools and water harvesting pools) (O'Connor, 1967; Kibret *et al*., 2008 & 2010; Kenea *et al*., 2011) and streams (Animut *et al*., 2012).

2.6.2.2 Factors Determining *Anopheles* Mosquito Larva Abundance and Distribution

Productivities of mosquito breeding habitats are determined by the interaction and the combination of both biotic and abiotic factors (Service 1977); the combined effects of

these factors determine the densities and rate of mosquito larva development (Washburn, 1995; Chase & Knight, 2003; Stresman, 2010).

Temperature is one of the most main environmental factors that limit the distribution and rate of development of the vectors (Bayoh & Lindsay, 2004; Snow & Omumbo, 2006; Stresman, 2010). Usually, the favorable water temperatures for egg hatching and larvae development is between 24°C and 30°C (Bayoh & Lindsay, 2003; Impoinvil *et al*., 2007); however, extreme cold and hot temperature increased chance of mortality and reduced rate of survival (Bayoh & Lindsay, 2004). The laboratory work indicated that high larval (including pupae) mortality rate of *An. gambiae* s.s when temperatures were below 18 °C and above 32 °C (Bayoh & Lindsay, 2004).

Rainwater is another very essential environmental factor that helps to increase the larval density (Koenraadt *et al*., 2004) by increasing the numbers and the productivity of breeding sites (Mouchet *et al*., 1998). Without rainfall, mosquito cannot lay eggs at all (Snow & Omumbo, 2006); thus, if water source is available yearly, mosquito breeds through the year (Matthys *et al*., 2006; Kreuels *et al*., 2008).

Mosquito larvae do feed various types of food resources, such as algae and pollen of maize (Hamoudi & Sachs, 1999; Ye-Ebiyo *et al*., 2003). Using these types of food resources, large numbers of larvae and large sized adults of *Anopheles* (e.g., *An. arabiensis*) were collected in their habitats (Hamoudi & Sachs, 1999; Ye-ebiyo *et al*., 2000). Generally, pollen from maize farm has increased vector density and size, mosquito longevity, and malaria infection rate (Ye-ebiyo *et al.*, 2000 & 2003; Kebede *et al*., 2005). Agricultural fertilizer [$(NH4)_2SO_4$, NH_4-N] has increased the density of mosquito too, both at normal agricultural practice (Mutero *et al*., 2004) and in the laboratory conditions (Duguma & Walton, 2014). Therefore, the larval dietary affected

larval survival, development, size, reproductive success and survival of adult mosquitoes (Moller-Jacobs *et al*., 2014).

Predators, competitors, cannibalism, pathogens, crowding, poor water and food quality have negative influence on the density of the larvae and the rate of larval development (Okogun, 2005; Mwangangi *et al*., 2007b; Eckhoff, 2011). For example, competition within the larval environment have reduced food availability and caused the larval mosquitoes to either intake less food or spend more energy foraging for food, result in lower density of the larvae, a reduced adult size, survivorship and fecundity (Gimnig *et al.*, 2002; Knight *et al*., 2004).

Land use and land cover changes have played a greater role for larval densities and malaria prevalence. Deforestation, swamp drainages for agriculture, excavation of lands, and vegetation clearance are known by increase and extend the larval habitats of malaria vectors (Minakawa *et al*., 2005; Guerra *et al*., 2006; Vittor *et al*., 2009). Especially, larval habitats formed by deforestation, canopy and vegetation clearance are sun exposed, rich in nutritional status and known by change the microclimate of the local larval habitats (e.g., have higher temperature and low humidity); therefore, this climate helps to increase not only the larval survivorship and growth rate (Minakawa *et al*., 2004 & 2005; Munga *et al*., 2006), but also good for prolonging adult vector survival and parasite development (Minakawa *et al.*, 2004; Zhou *et al*., 2004). Clearing forest canopy cause to increase algal contents (Munga *et al*., 2006) and these algae become sources of food for the immature (Kaufman *et al*., 2006).

2.7 Behavior of Adult Malaria Vectors

Understanding the biology and behavior of *Anopheles* mosquitoes can help to understand how malaria is transmitted and can aid in designing appropriate control

strategies (Gatton *et al.*, 2013). Each species of *Anopheles* has its own flight range, feeding, biting, host preference and host selection behavior (Becker *et al*., 2010; Varnado *et al*., 2012). Behaviors such as flight, host seeking and feeding generally takes place more in a warm environment.

2.7.1 Dispersal Habits: Dispersal refers to a goal-oriented flight of *Anopheles* mosquitoes from one place to another. It depends on the number of factors, including the productivity and the patterns of distribution of vector breeding sites and human habitations on the ground, the effective dispersal range of the vector mosquitoes radiating from it (Carter *et al*., 2000), wind speeds and directions, and features of the surrounding (including the ground surface, the vegetation and the fauna). Most of the flights of adult mosquitoes are within 1-km radius (Le-Menach *et al*., 2005), but capable of flying up to 3 kilometers (Walker, 2002). Generally, the maximum flight ranges of African vector appears to be approximately 2-3 km whereas about or more 5 km in the Americas (Carter *et al*., 2000).

2.7.2 Mating Habits: Newly emerged adult *Anopheles* mosquito is not sexually matured. Male mosquitoes require about 24 hours (Takken *et al*., 2007), but *An. gambiae* s.s and *An. arabiensis* males will sexually active 48h post-emergence with peak mating activity 3-7 days post emergence (Howell & Knols, 2009). This duration of time requires for their terminalias (genitalia) have rotated (180 C°) and mature and antennal fibrillae are mature enough to become erect and detect females (Howell & Knols, 2009). On the other hand, female mosquitoes need 48 - 72 hours before they become receptive to males; usually prior to blood feeding in the wild (Howell, 2010). After maturation, opportunities of males to have mate are limited by several factors. First, sugar feeding is important to both male survival and mating ability. Due to their

small energy reserves relative to females, newly emerged males must locate a carbohydrate source in order to survive and sexual maturity (Howell & Knols, 2009). Generally, mating is occurred during the early evening and is believed to occur primarily in swarms. That is, *Anopheles* male mosquitoes aggregate just before dusk and commence swarming at the onset of sunset. And then, both males and females meet within the swarm. *Anopheles* males can mate several times and may reach to three times in his lifetime (Howell & Knols, 2009) whereas mostly females mate once in her life time (Oaks *et al*., 1991; Becker *et al*., 2010). Some female mates before taking a first blood meal, and then lay the first batch of eggs (Autogenous), but in several of them may blood-feed prior to mating.

2.7.3 Host Feeding Preferences: Host preference /seeking/ is associated with mosquito ability to select appropriate vertebrate blood host species (Boreham & Garrett-Jone, 1973; Takken & Verhulst, 2013) using olfactory receptors (Takken & Knols, 1999; Olanga *et al*., 2010), visual and thermal stimuli (Becker *et al*., 2010). It is genetically determined characteristic of the mosquito (Pates, 2002; Main *et al*., 2016). Mosquito host selection ability is regulated by lots of factors, including host odors and variability of odors between humans (Lindsay *et al*., 2002; Mukabana *et al*., 2002) or animals, carbon dioxide and heat emitted from the animal or person (Rozendaal, 1997; Dekker *et al*., 1998). For example, *An. gambiae* s.s is highly attracted by human skin odor and mixture of body odor, heat and moisture (Mukabana *et al*., 2002; Olanga *et al*., 2010).

Additionally, host choices and subsequent feeding success depend on host availability (Becker *et al*., 2010; Main *et al*., 2016) including host accessibility, density, vulnerability/ host defense mechanisms, host size, and proximity to mosquito habitats (Lyimo & Ferguson, 2009; Okwa, 2012), environmental factors, flight behavior and

feeding periodicity of the mosquitoes (Molaei *et al*., 2008). Within species, variation of host choice is common and depending on season, microhabitat, foraging experience, local host availability and host defense mechanisms (Lyimo & Ferguson, 2009; Becker *et al*., 2010). Intervention through LLINs, IRS, and space spraying determine the successful feeding and oviposition nature (Eckhoff, 2011).

2.7.4 Biting Habits: Many of *Anopheles* species have a peak of night biting activity (WHO, 1982) and feed between the hours of dusk and dawn when the air is more humid and warm (Varnado *et al*., 2012). Among those nocturnal or crepuscular species (Rueda, 2008), few species of mosquitoes frequently enter houses to feed and this feeding habit is endophagic; whereas others bite their hosts outside houses are said to be exophagic (WHO, 2005). Usually mosquitoes prefer to bite the lower extreme human body parts (e.g., lower leg, ankles and feet) than other body parts during sit time either indoors or outdoors of the house (e.g., *An. arabiensis*, *An. gambiae* s.s and *An. funestus*) (Dekker *et al*., 1998; Braack *et al*., 2015).

Mosquito biting power is managed with intrinsic factor that to be attracted by one host and depend on mosquito host preference (Lindsay *et al*., 2002) and ability to bite a specific host (i.e., biting efficiency and biting effectiveness) (Boreham & Garrett-Jones, 1973). Furthermore, house proximity to larval habitats (Smith *et al*., 1995), well built house (Takken & Verhulst, 2013) and having personal protection (Lindsay *et al*., 2002) vary biting rates. Usually, people live in houses found close to larval habitats experienced higher biting rates than constructed further away from breeding sites (Smith *et al*., 1995). Well screening windows and doors, closed eaves, netted and insect-screen ceilings reduced rate of biting as compared with unscreened houses

(Lindsay *et al*., 2003; Kirby *et al*., 2009). People with well personal protection had shown very minimum biting rate too (Lindsay *et al*., 2002).

2.7.5 Blood Meal Sources: Mosquito blood meal sources determine their own feeding rates, adult survival, fecundity, hatching rates, and developmental times (Phasomkusolsil *et al*., 2013). Only female *Anopheles* mosquito takes blood (as a source of protein) meals from humans, birds, mammals, or other vertebrates animals (Rueda, 2008; Dotson, 2010; Forstinus *et al*., 2015) for egg development (Briegel, 1986; Lyimo & Takken, 1993).

Individual species of *An. gambiae* complex differs in host-feeding preferences (Shiff, 2002 in Ramirez *et al*., 2009; Kulkarni *et al*., 2006). Many species bite humans; a few feed on humans in preference of other animals, but others never bite humans at all. Species that usually feeds on humans are anthropophagic (e.g., *An. gambiae* s.s, *An. funestus* and *An. nili*) (Kiswewski *et al.*, 2004; Pates & Curtis, 2005); whereas others mainly feed on animals, zoophagic (e.g., *An. pharoensis*) (Kawada *et al*., 2012; Killeen *et al*., 2013). However, currently *An. gambiae* s.s, *An. arabiensis*, *An. funestus*, *An. coustani* and *An. melas* found to feed blood of both humans, goats, calves, chickens, cows, dogs and goats and equines (Bogh *et al*., 2001; Muriu *et al*., 2008; Lyimo *et al*., 2012), is depend on availability of host types.

2.7.6 Resting Habits: Female mosquitoes seek resting places to digestion their blood-meals. Some species rest inside houses for blood-meal digestion and maturation of the ovaries, endophilic while others take a rest outdoors, exophilic (WHO, 2005; Pates & Curtis, 2005; Paaijmans & Thomas, 2011). *An. gambiae* s.s and *An. funestus* are naturally endophilic species (Pates & Curtis, 2005); whereas *An. arabiensis* is exophilic (Takken & Lindsay, 2003).

Indoor resting mosquitoes highly depend on indoor temperature. This temperature is strongly depending on season, altitude, its surroundings (Afrane *et al*., 2006 & 2008), the nature of the building structure (Okech *et al*., 2004; Atieli *et al*., 2009), the number of occupants and whether people burn wood indoors (Bockarie *et al*., 1994). In most cases, endophilic resting behavior allowing relatively longer survival rates than for exophilic adult mosquitoes, and this endophilic habits increase the tendency to feed on humans repeatedly (WHO, 2006). On the other hand, outdoor resting mosquitoes seek shelter in a varieties of environments, such as under the eaves of huts, in dry pots, canal water pipes, undersides of bridges, at bases of trees, in tree holes, heaps of fallen leaves, cracks and crevices of brick pits, cracks and holes in the ground, small edge under rocks, granaries, etc (Haridi, 1972; Githeko *et al*., 1996). All these sites are likely to be heavily shaded (Haridi, 1972), and have their own specific microclimate temperature (Meyer *et al*., 1990). However, currently some mosquitoes have changed their natural endophilic habit to exophilic due to DDT spraying programs (e.g., *An. gambiae* s.s, *An. funestus* and *An. sacharovi*) (Rajaonarivelo *et al.*, 2004; Pates & Curtis, 2005).

2.8 Entomological Inoculation Rate

Entomological inoculation rate is an ideal indicator of malaria transmission. It is calculated as the product of the mosquito biting rate and the sporozoite rate (Smith *et al*., 1995 & 2004). Therefore, EIR is considered a more direct measure of transmission intensity, but it differs widely in between villages and in the same locality (Obala *et al*., 2012). The dynamics of EIR is governed by the fluctuation in vector densities and their sporozoite rates (Krufsur & Armstrong, 1978), and influenced by temperature, altitude, rainfall, and urbanization (Obala *et al*., 2012). EIR values also are used to quantify the

impact of vector control measures (LLINs, IRS, and source reduction on malaria transmission) (Shaukat *et al*., 2010), malaria endemicity (Burkot & Graves, 1995) and the risk of epidemic development (Onori & Grab, 1980). Therefore, effective vector control measures directly decrease malaria incidence (sporozoite rates) and then EIRs (Smith & McKenzie, 2004; Killeen & Smith, 2007; Shaukat *et al*., 2010). The human landing catch (HLC), Centers for Disease Control (CDC) and pyrethrum spray catches (PSCs) have used to estimate the EIR. In malaria endemic areas of Africa, EIRs range from 1 to >1000 infective bites per year (ib/p/year) (Beiee *et al*., 1999); however, Trape & Rogier (1996) have been reported between 0.01 and 1000 ib/p/year. In Ethiopia, EIRs varies from zero in most non-irrigated villages (e.g., *An. arabiensis*, *An. pharoensis* and *An. funestus*) (Woyessa, 2001; Aklilu, 2008; Tesfaye *et al*., 2011; Bekele *et al*., 2012 & 2013; Gari *et al*., 2016; Kenea, 2016; Taye *et al*., 2016) to 102 ib/p/y (eg., *An. arabiensis*) (Jelata *et al*., 2013) and 129 ib/p/y (eg., *An. arabiensis*) (Kibret *et al*., 2017) in irrigated villages. Kibret and others found 47.8 ib/p/y for *An. funestus* in irrigated villages. In Ethiopia; however, mostly the EIRs of *An. arabiensis* is less than 20 ib/p/y (Animut *et al*., 2013a; Massebo *et al*., 2013a; Getachew, 2017; Ejeta, 2017).

2.9 Malaria Control Strategies and Their Challenges

2.9.1 Malaria Control Strategies

Malaria vectors are prevented and controlled mainly based on three fundamental methods: environment managements, biological, and chemical methods (Pates & Curtis, 2005; WHO, 2013a). Integrated vector management (IVM) and zooprophylaxis (WHO, 1982, 1995 & 2012b) are also the others environmental friendly vector control options. Currently, releasing engineered mosquitoes (CHP, 2004; Ramirez *et al*., 2009; Godfray, 2012) is incorporated. Out of these intervention, Ethiopia is practicing mass

distribution of LLINs and application of IRS (for adult vectors), environmental management (for larval stages) (Carter Center, 2013) and case detection and prompt treatment using ACT (Otten *et al*., 2009).

2.9.1.1 Environment Managements: It helps to control the larvae of malaria vectors through environmental modification, environmental manipulation (Mutero *et al.*, 2012; WHO, 2013c; Forstinus *et al*., 2015) and modification (manipulation) human habitation or behavior (Walker, 2002). I) Environment modification is a permanent alteration to the environment, including landscaping, surface water drainage, filling and land recovery, coverage of water storage containers with mosquito-proof lids or permanent blocks and coverage of the water surface with a material opaque to mosquitoes, land leveling and transformation and impoundment restrictions (CHP, 2004; Keiser *et al*., 2005). II) Environmental manipulation, is a way of making temporarily unfavorable breeding grounds for vectors including water salinity changes, stream flushing, regulation of water level in reservoir, drainage or flooding swamp, vegetation removal (Keiser *et al*., 2005; WHO, 2012b). III) Manipulation of human habitation or behavior has helped to reduce man-vector- pathogen contact. It is a ways of make settlements away from vector sources, having mosquito proofing houses, usage of personal protection and hygiene measures against vectors, waste water and excreta disposal away from house, removing vegetables from around house, improved house construction (Ibrahim *et al*., 2005; Keiser *et al*., 2005).

2.9.1.2 Biological Control: Biological control helps to control targeted mosquitoes by utilize natural enemies and biological toxins to achieve effective vector management (Walker, 2002; WHO, 2013c). Some of the proven and effective natural enemies against mosquito larvae are larvivorous fish (*Gambusia affinis* and *Poecilia reticulata*)

and other fish like *Aphanius dispar*, *Aplocheilus blocki*, *Cyprinus carpio*, *Ctenopharyngodon idella*, *Tilapia* spp, *Catla catla*, *Labeo rohita*, and *Cirrhinus mrigala* (Walker, 2002; Juliano, 2007; WHO, 2013c). In addition to fish, predatory insect larvae (dragonfly, Toxorhynchites mosquitoes), copepods, nematode worms (*Romanomermis culcivorax*), fungi, and bacterial larvicides (*Bacillus thuringiensis* israelensis-Bti and *Bacillus sphaericus*-Bs) (Manguin & Boete, 2011; WHO, 2013c), birds, bats, newts, beetles, larvae of tabanides, nymphs of damsel, mayflies, and protozoan (CHP, 2004) and Azolla from aquatic plant are very effective to control larval and/or pupa stages of the mosquito (Walker, 2002; WHO, 2013c).

2.9.1.3 Chemical Control: Chemical control is a way of control *Anopheles* mosquitoes using insecticide impregnated materials (e.g., tents, blankets, plastic sheets, top sheets, clothing, curtains, hammock nets, fly curtains, LLINs), spray of insecticides and larvicides (Wentworth, 2010; WHO, 2013b & 2013c). The attack of *Anopheles* mosquitoes can be prevented by using chemical repellants, including oil of N, N-diethyl -3- methylbenzamide (DEET), Citronella Oil, Neem oil, Lavender oil, Pepperminal oil, etc (Geetha & Roy, 2014). Moreover, prevention has been made by taking available drugs called chemoprophylaxis, such as Chloroquine, Proguanil, Pyrimethamine, Mefloquine and Doxycycline. Larvicide chemicals such as organophosphate temephos, Paris-green, synthetic pyrethroids are very effective to apply in breeding sites to control the adult vectors (CHP, 2004; WHO, 2013b). Totally, LLINs and IRSs are the mainstay of malaria vectors control programme because they are highly effective, have a relatively low cost, and their production and distribution can be rapidly scaled up.

2.9.1.3.1 Management Options of Insecticide Resistance *Anopheles* Mosquitoes: Insecticide resistance mosquitoes are managed by rotations, combined intervention, mosaics, and mixtures of insecticides (WHO, 2012b; Corbel & N'Guessan, 2013). I) Rotation of insecticide, it is the application of two or more insecticides with different mechanisms of action from one year to the next (Cloyd, 2011). II) Combination of intervention, the way of controlling the vector populations using a combination of two vector control tools, such that a mosquito that survives contact with one (e.g. LLIN) is exposed to the other one (e.g., IRS) (WHO, 2012b; Corbel & N'Guessan, 2013). III) Mosaic spraying, characterized by the use of one insecticide in one geographic area and a different molecule in neighboring areas; like combination of interventions, the mosquitoes are exposed to more than one insecticide type. IV) Mixtures, it is way of two or more insecticides classes are mixed to make a single product or formulation (Cloyd, 2011; WHO, 2012b).

2.9.1.4 Genetic Engineering Techniques: To date, malaria vectors can be controlled by genetic engineering means (CHP, 2004). Some of the most important experiences are releasing of transgenic vectors that carried a dominant lethal gene (Asokan, 2007; Alphey *et al*., 2008; Alphey, 2014), using engineered Wolbachia bacteria (Brelsfoard & Dobson, 2009), and homing endonuclease genes, HEGs (Deredec *et al*., 2008).

2.9.1.4.1 Release of Insects Carrying a Dominant Lethal (RIDL): It is a kind of genetic sterilization, achieved by releasing engineered male insects that carrying a dominant lethal gene that would mate with wild females and all progeny would inherit the lethal gene and consequently die before reaching adult stage (Alphey *et al.*, 2008**)**. Using this technique, *An. stephensi stephensi* in Indian cities (Ramachandra-Rao, 1984) and *An. arabiensis* in southern-Nigerian cities (Kristan *et al.*, 2003) were possible to control. RIDL approaches also can make mosquitoes unable to transmit diseases where it may

be possible to develop resistant mosquitoes to over-express genes involved in deactivate parasites in the insect stomach or salivary glands (Asokan, 2007).

2.9.1.4.2 Engineered *Wolbachia* Bacteria: *Wolbachia* (*Wolbachia pipientis)* is a wide group of obligatory intracellularly maternally transmitted bacteria (Beech *et al*., 2012) belonging to the alphaproteobacteria (Bourtzis, 2007). These bacteria mainly live in reproductive and other body tissue and organs (Dobson *et al*., 1999; Agersew, 2015) including in the cytoplasmic vacuoles of insects, isopods, mites and nematodes (Townson, 2002).

Now a day, genetically modified *Wolbachia* bacteria are the best candidates to control disease transmission rate through *Anopheles* mosquito. Once in reproductive organ, the bacteria cause a numbers of reproductive alterations including cytoplasmic incompatibility (CI, males sterility), thelytokous parthenogenesis (- is exclusively production of female offspring by infected female), feminization (-is the conversion of genetic male into functional female) and male killing (- is the death of male embryos during early development) (Hurst *et al*., 2000; Bourtzis, 2007; Engelstadter & Telschow, 2009). The success of these techniques was proved for the first by Bian *et al.* (2013) in *An. stephensi* and by Bourtzis (2007) in *An. gambiae*. Moreover, once in mosquito bodies, engineered bacteria start to produce effectors molecules that kill the *Plasmodium* parasite or inhibit its development; and adapt to live in the mosquito mid-gut successfully compete with the mosquito's endogenous microbiota (Riehle *et al*., 2007).

2.9.1.4.3 Engineered *Metarhizium anisopliae*: *Metarhizium* (*Metarhizium anisopliae*) is genetically modified fungi, play pivotal role to control malaria vectors via paratransgenic approaches (Riehle *et al*., 2007; Wilke & Marrelli, 2012).

Paratransgenic approach is a way of delivering genetically transformed bacterial flora or fungus native to disease-transmitting vectors to export molecules that interfere with pathogen transmission. Engineered molecules affect the host's ability to transmit the pathogen and decrease pathogen transmission without adverse effects on vectors themselves (Hurwitz *et al*., 2011). Mostly, the fungal species have the advantage of surviving in the environment for months as spores and can infect mosquitoes directly through the cuticle, unlike bacteria which need to be ingested to infect mosquitoes (Thomas & Read, 2007). Furthermore, genetically transformed *Metarhizium* is very effective and can express the effectors molecule by producing the antimicrobial toxin (scorpion), which interfere with *P. falciparum* development, reducing mosquito infectivity (Fang *et al*., 2011).

2.9.1.4.4 Homing Endonuclease Gene's (HEGs): It is a type of selfish genetic element that exists naturally in fungi, bacteria, and plants, but has not been found in insects (David *et al*., 2013). This gene can copy itself and promotes its movement from one allele to the other by creating a double strand break at a specific long target site in an allele that does not have the HEGs (Yahara *et al*., 2009). Theoretically, a HEG linked to a desired trait and can spread rapidly through a population. It may induce mortality when an individual is homozygous, thereby benefiting from on the HEG's ability to drive itself into a population while simultaneously suppressing the mosquito population (Deredec *et al.*, 2011).

There are two ways HEGs are currently being researched to suppress mosquito populations: I) by disrupting female fertility genes, i.e., the HEG may insert itself into a vital fertility gene, thereby disrupting the gene and killing the developing offspring (Burt, 2003); or II) by creating male-biased sex ratios, the HEG carried on the Y-

chromosome would cleave and disrupt the X- chromosome (but not insert itself in this case), causing a greater number of males in the population. Therefore, implementation of either HEGs method could suppress *An. gambiae* populations, ultimately reducing malaria transmission (Windbichler *et al*., 2007).

2.9.1.5 Integrated Vector Management (IVM): It is "a rational decision-making process targeting the global targets set for vector-borne disease control, by making vector control more efficient, cost effective, ecologically sound and sustainable" (Beier *et al*., 2008; Mutero *et al*., 2012). IVM activities minimizing environmental pollution by using different control strategies in relation to local vector bionomics including use to mechanical, cultural, biological and chemical control in together (Alston, 2011; Carvalho *et al*., 2014). In African, some countries have registered successful results after implementing IVM, such as Kenya (Fillinger *et al*., 2009; Mutero *et al*., 2015), Zambian (Chanda *et al*., 2008) and Tanzania (De-Castro *et al*., 2004). They showed markedly reducing malaria-related morbidity and mortality with better protection of the environment. Therefore, IVM is so important not only to manage insecticide resistance malaria mosquitoes, but also for the safety of the environmental (Beier *et al*., 2008; Chanda, 2015).

2.9.1.6 Zooprophylaxis: It is purposeful use of livestock for the diversion of host seeking disease carrying insects from humans to animals (domestic or wild animals) result in reduction malaria transmission and incidence (WHO, 1982 & 1995). It is divided into active, passive, combination and insecticide zooprophylaxis. Active zooprophylaxis is a reduction in malaria or human biting by a deliberate deployment of domestic animals as a barrier between mosquitoes breeding sites and human settlement; whereas passive zooprophylaxis is the occur of unexpected reduction in malaria because of the normal presence of livestock (Bouma & Rowland, 1995; Bogh *et al*.,

2001). Combination zooprophylaxis is helped to reduce the disease risks by using LLINs and IRS being integrated with livestock placed in a separate shed in order to induce a push-pull effect. Here, the LLINs and IRS are used as pushing factor, whereas domestic animals used as pulling factor (Kaburi *et al.*, 2009; Iwashita *et al*., 2014). Insecticide zooprophylaxis, is the way of killing blood feeding mosquitoes using insecticide (e.g., Entomopathogenic fungi, *Metarhizium anisopliae* and *Beauveria bassiana*) treated animals (Bogh *et al*., 2001; Lyimo *et al*., 2012). Here insecticide (usually used deltamethrin) is applied to the hair and skin of domestic livestock, such as cattle, goats and sheep, using a sponge or animal dip. Then, mosquitoes pick up a lethal dose of insecticide when they attempt to feed on a treated animal (WHO, 2005).

However, the purpose of zooprophylaxis as a diversion of host seeking disease vectors is debatable, because increasing number of animals improve availability of blood meals, which in turn increase mosquito survival and population density; thus, it may actually increase malaria transmission, zoopotentiation (Bogh *et al*., 2001 & 2002; Saul, 2003). Usually, zooprophylaxis is not working for highly anthropophilic species (e.g., *An. funestus*), whereas effective for those zoophilic and opportunistic species (Donnelly *et al*., 2015). Zooprophylaxis is the best environmental tool for vector-borne diseases management and control (Ault, 1994). It has good potential to be a component of IVM strategies due to its non-chemical nature, optimal combination with bed nets, potential social desirability and minimal financial requirements (Donnelly *et al*., 2015).

Generally, the effectiveness zooprophylaxis depends on several factors including: I) the dominant vector is highly zoophilic and livestock are kept away from human sleeping residence during peak vector activity, II) the use LLINs and IRS mandatory, including socio-economic factors, III) the distance from human dwelling to the breeding sites of

mosquitoes should be far, and IV) the indoor and outdoor host seeking behavior of the vector should be known to know the exact host preference (Donnelly *et al*., 2015; Asale *et al*., 2017).

2.9.2 Challenges of Malaria Vector Control Strategies

Recently, control of malaria vectors and treatment of malaria cases have become challenging. Some of the vectors have changed their resting behaviors and became exophily (WHO, 2006) and others developed insecticides resistance capacity (Antonio-Nkondjio *et al*., 2009; Okwa, 2012). In nature, endophilic species like *An. gambiae* s.s (Githeko *et al.*, 1996; Faye *et al.*, 1997) and *An. funestus* became exophily (Rajaonarivelo *et al.*, 2004) while endophagic and endophilic *An. sacharovi* became exophily and zoophagic (Boreham & Garrett-Jones, 1973) due to DDT spraying programs. On the other hand, exophilic species of *An. arabiensis* (Rajaonarivelo *et al*., 2004; Tirados *et al*., 2006) has become endophily (Dukeen & Omer, 1986; Faye *et al*., 1997). Other vectors have developed resistance to various insecticide chemicals (WHO, 2012b; Jindal *et al*., 2014). *An. arabiensis* resisted to DDT, malathion, permethrin and pyrethroids (Matowo *et al*., 2010; Fettene *et al*., 2013; Alemayehu *et al*., 2017) and *An. funestus* resisted to permethrin and DDT (WHO, 1992; Hunt *et al*., 2010).

Generally, almost the four classes of insecticides (organophosphates, organochlorines, carbamates and pyrethroids) have faced resistance (WHO, 2012b; Jindal *et al*., 2014; Chanda, 2015) and occurrence of resistance reported approximately two thirds of countries with ongoing malaria transmission has been occurred (WHO, 2012b). After 1947, more than 100 mosquitoes' species are reported as resistant to one or more insecticides; however, over 50 of the mosquitoes are *Anopheles* (Hemingway & Ranson, 2000). Again, from 2010 onward, insecticide resistance in malaria vectors has

been reported in 53 of 65 reporting countries around the world. Of these, 41 have reported resistance to two or more insecticide classes (WHO, 2014), but the most commonly reported resistance is pyrethroids, the most frequently used insecticide in malaria vector control (WHO, 2014; PMI, 2014). To date, 64 countries have documented presence of resistance to at least one insecticide in major malaria vectors world-wide (Chanda, 2015).

Moreover, this new behavior is caused by the frequent use of LLINs (e.g., durable linings, eaves curtains) (Lindsay *et al.*, 1998; Chandre *et al*., 2010), IRS (Gatton *et al*., 2013), the misuse and overuse of pesticides in farming systems (WHO, 2006; Soko *et al*., 2015) and climate change (Soko *et al*., 2015). Therefore, the overall resistance to insecticides by the vectors is expected to threaten disease control activity and in-turn lead to necessitate of the development of other new anti-vector chemicals and strategies to control resistant mosquito populations in particular and other mosquitoes as a whole.

The challenges of malaria control are not limited with vectors. Malaria parasites have developed resistance to anti-malarial drugs; these made more worsen and greatest challenges to malaria control today (Bloland, 2001; Okwa, 2012). Until now, three *Plasmodium* species, i.e., *P. falciparum*, *P. vivax* and *P. malariae* have been developed resistance to various anti-malaria drugs (WHO, 2010b; Jindal *et al*., 2014) and this resistance character is seen in various part of the world. *P. falciparum* has resisted to almost all drugs, including Chloroquine, Sulfadoxine (Fansidar), Mefloquine, Quinine, Halofantrine and Artemisnin (Dondorp *et al*., 2011; Sinha *et al*., 2014). Similarly, *P. vivax* has developed resistance against to Chloroquine and Primaquine (Dao *et al*., 2007), Amodiaquine, Mefloquine and Fansidar (Eyasu, 2015). *P. malariae* is resistance to Chloroquine (Maguire *et al*., 2002). Besides, the presence of asymptomatic (not

detectable by microscopy or RDT, but detectable by molecular techniques, like PCR) nature of malaria infection (Aminake & Pradel, 2013) and the negative test result of *P. falciparum* in human body (Kokwaro, 2009) are the other challenges to control the disease. WHO (2015b) incorporated lack of sustained international and domestic financing as a challenges of malaria control prior to biological challenges. Totally, the resistance capacity of the parasites to drugs is due to misdiagnosis of malaria, improper use of medications, use of fake and low quality of malaria drugs, drug interactions, poor or erratic absorption, lack of resistance surveillance (Bloland, 2001; Kendall, 2012), genetic mutation, use of low drug concentration and poverty (do not have the financial means to prevent or treat the disease) (Aminake & Pradel, 2013; Sinha *et al*., 2014; Eyasu, 2015).

Generally, drug resistance by malaria parasites can managed by establishing well follow-up practices and patient compliance, reducing overall drug pressures (decreasing using anti-malaria drug via using improved malaria diagnostic techniques), improving the way drugs are used, and administration of combination therapy (combination of anti-malarial drugs) (Bloland, 2001; Eyasu, 2015), applying nanotechnology (to determine the exact dosage of anti-malaria drugs), stem cell treatment (to produce erythrocytes with modified haemoglobin alternatives that are associated with protection from malaria), using anti-malarial peptides (having of natural or synthetic origin) (Sinha *et al*., 2014) and vaccines development from sporozoite, merozoite, and gametocyte or any other source (Krogstad, 1996). Moreover, implementation of appropriate policies (regulation of drug supply management as well as rigid penalties) and development of new drugs to control resistance are other best measures (Aminake & Pradel, 2013).

2.9.2.1 Insecticide Resistant Mechanisms of *Anopheles* Mosquitoes

There are four major types of insecticide resistance exist in *Anopheles* mosquito (WHO, 2012b; Corbel & N'Guessan, 2013).

2.9.2.1.1 Target Site Resistance: The main targets of insecticides are receptors or enzymes of the nervous system, such as acetylcholinesterase (AChE), the voltage-dependent sodium channel (CNaVdp), and the receptor of γ-aminobutyric acid. Mutation of these targets is a very effective resistance mechanism, inducing cross-resistance to all insecticides acting on the same target (Sokhna *et al*., 2013). Currently, insecticides such as malathion, fenitrothion, propoxur, dieldrin, DDT and pyrethroid are not effective and not used longer to kill malaria vectors (Liu *et al*., 2006; Etang *et al*., 2007). For example, *An. arabiensis* is against to the four classes of insecticide using target site resistance mean (WHO, 2012b).

2.9.2.1.2 Metabolic Resistance: It is the most common resistant mechanism in insects (Bloland, 2001), occurs when increased levels or modified activities of an enzyme system (e.g., esterases, cytochrome P450 monooxygenases, and glutathione S-transferases) cause an early deactivation of the insecticide before it reaches its target in the mosquito (Matowo *et al*., 2010; Gatton *et al*., 2013). This kind of resistance is observed by *An. gambiae* (Ranson *et al*., 2002), *An. funestus*, (Hargreaves *et al*., 2000) and *An. arabiensis* (Hamusse *et al*., 2012), usually after DDT sprays. Recently, in Ethiopia *An. arabiensis* was found against to DDT and pyrethroids (Alemayehu *et al*., 2017) due to metabolic and target-site mutation mechanisms (Messenger *et al*., 2017).

2.9.2.1.3 Cuticular Resistance: It is a modification in the composition or structure of the mosquito's cuticle that hinders the permeability of the insecticide, leading to a poor absorption and reduced efficiency. For example, *An. funestus* is resisted to pyrethroid due to the presence of thicken cuticle (Wood *et al*., 2010).

2.9.2.1.4 Behavioral Resistance: It is the result of a continuous exposure to a particular insecticide; mosquitoes may modify their feeding and breeding behaviors so as to avoid the lethal effects of the insecticide (Gatton *et al*., 2013). Behavioral resistance could be mobility and immobility (Lockwood *et al*., 1984). Both mechanisms allow insects to avoid contact with the toxic product or limit the duration of this contact (Sokhna *et al*., 2013). All these kinds of behavior change are the result of intensive indoor use of insecticides, but there is currently insufficient data to assess whether these behavioral avoidance traits are genetic or adaptive responses (Bogh *et al*., 1998). The presence of behavioral resistance is proven in various vector groups, e.g., *An. gambiae* s.l and *An. funestus* due to a regular use of impregnated bed-nets (Bogh *et al*., 1998), *An. farauti* (Mouchet *et al*., 2008) and *An. arabiensis* due to DDT spraying (Hamusse *et al*., 2012) and *An. gambiae* s.s and *An. arabiensis* due to high LLINs coverage (Kitau *et al*., 2012).

Chapter 3: General Materials and Methods

3.1 Description of the Study District

The study was carried out in Bure district, Northwest of Ethiopia from July 2015 to June 2016. Geographically, Bure district is situated at an altitude ranging from 700 (Blue Nile gorge) - 2,350 m.a.s.l (Woinma-ambaye sub-district). The district is surrounded by Awi and Sekela districts in the North, Denbecha district and Oromia zone in the South, Wemberma district in the West, and Jabi-tehnan district in the East. The district is 410 Km away from Addis Ababa towards northern parts and 152 Km away from Bahir Dar with the West (Fig. 3.1). Currently, it encompasses 28 kebeles (the smallest administrative unit in Ethiopia), out of which 20 from rural and 8 from urban kebeles (Sub-districts). The district had 185, 265 populations; out of these 92,384 were males and 92, 881 were females. From all populations in the district, 67,719 people were found in town and 117, 546 were in the rural (Bure District Administrative Office, Unpub. Report, 2016).

In terms of socioeconomic, the majority (85%) of the populations are farmers who grow maize, teff (*Eragrostis teff*), pepper, potatoes, wheat, millets, followed by bean & pea, sunflower, niger, spices, vegetations and others (e.g., fruits and vegetables such as lemon, oranges, banana, avocado, sugarcane, cabbage, etc) during rainy season, and the rests are merchants (6.8%) and others (NGO's, civil servant) (8.2%). Additionally, small holder farmers and urban inhabitants used irrigation to grow vegetables and potatoes. Most of the farmers are superfluous harvester. Regarding animal husbandry, cattle, sheep, chickens, mules and donkeys are reared by the farmer. Dog was common in most houses. Additionally, both modern and traditional bee-keepers were available. The majority of the populations in the district live in houses made of mud and

corrugated iron roofs (Bureau District Agricultural & Rural Developmental Office, Unpub. Report, 2016; Personal Obser.).

Forest coverage of Bure district is very minimal as compared with the total land cover of the district. The district covers about 70, 647 hectares (ha) of land; out of these 16.4% were (11572 ha) forests. The main components of trees in the studied villages were *Eucalyptus saligna*, Broad-Leaved Croton (*Croton macrostachyus* Hochst), Podocurpus, Cordia, Albizia, Juniper, Granville, Acacia species and Ficus species.

The majority of Bure district has sub-tropical zone (Woina-Dega) climate with annual mean minimum and maximum temperature of 9.9°C and 29.2 °C, respectively and 2,000 mm mean annual rainfall range being 1,350 - 2,500mm. The major rainy season of the district is from July to September and small amount is obtained from May to June and from October to December. The rest of the months (January - April) are dry seasons (Midekisa *et al*., 2015). The moisture content (humidity) of the district was not indicated due to the absence of data from Bahir Dar Metrological Agency.

The study was conducted in three rural villages: Bukta, Workmidr and Shnebekuma, from July 2015 - June 2016. The three villages were selected purposefully due to various reasons. Based on district health officers, these villages are more malarious than others. Preliminary survey indicated that, Bukta was a village where both traditional and modern irrigation practiced, and there were potential breeding habitats, such as springs and marshes; whereas Shnebekuma was non-irrigated village and agriculture was based on rain only. It had various water resources, including small streams, marshes and ditch. The third village was Workmidr, non-irrigated and agriculture was based on rain only, but marshland and small artificial pits (for nursery purpose) were available. Besides, these three villages are very close to the main road

route for electric accesses to recharge secondary cells of CDC-LTs. Between villages, the distance difference ranged from 7 - 20.5 km. Therefore, to meet the intended objectives, these villages were selected.

Bukta village: This village is found 8 km away from Bure town. Its geographic coordination is 10° 43.734' N and 037° 06.555' E with an elevation of 2,157 m.a.s.l at the center of the village. The village had 100 households with a total of 403 inhabitants. The village has one annually flowing stream, called 'Adafita' and two marshlands. Marshlands were fully covered with small length (short) grasses for some months (July to November) and important for grazing purpose. Moreover, these marshlands were among the most water supplier to the river through the year. The village residents have used traditional irrigation for many decades and modern irrigation since 2010. The dam was built 2010 to irrigate 40 hectares and to benefit about 132 farmers. The river water was stopped its natural flowing route at the end of December for irrigation purpose. Followed this, due to seepage/ leakage and presence of spring waters along the riverbank, many stream pockets were present beneath both the traditional and modern dam.

The village farmers are majorly known by cultivating coffee beans, avocado, banana, sugar cane, citrus fruits, spices, red onion, potatoes, cabbages, beets and tomato during dry seasons using irrigation. Though both the three study villages are surplus harvesters, farmers in Bukta village are very rich (as compared with the rest study villages) due to the production of the aforementioned extra crops during the dry season.

Workmidr village: It is found about 12.5 km away from Bure town and located at 10° 37.376' N and 037° 02.392' E. This village is located at an elevation of 2,024 m.a.s.l at the center of the village. The village had 167 households and 542 inhabitants. Near to

the village, one highly extended marshland was found. It was covered by long emerged grass throughout the year. The marshland water drift very slowly throughout the year and used for animal drinking, and the grasses used for animal fodder. The dried grasses (thatch) obtained from marshes also used for making a roof for cultural granary house, latrine and kitchen and to make house utensils and mattress. Besides marshland, manmade-pits were found, which were used for nursery such as peppers and eucalyptus trees.

Shnebekuma village: This village is found 8.5 km away from Bure town with 10° 38.932' N and 037° 02.251' E geographical coordinates. It is located at 2,025 m.a.s.l at the center of the village. The village had about 136 households and 542 occupants. In this village, one highly extended and three small marshlands and one small stream were found. The name of the large marshland is 'Demebek', the whole part was highly covered with thick long emerged grasses. Both the small and the large marshlands have large quantity of water that flow slowly throughout the year and give an immunes role to the community. These water resources served as a source of water for both animal and human. Marshlands grasses were used for grazing and animal fodder, and for roof making as described above. The name of the river is 'Wiynetu', except the head of the river, there were no any riverine trees, but intermittently emergent grass were grown across the stream. The stream water was stopped flowing at the end of November due to the reduction of water from the stream head following the coming dry season. As a result, many stream pockets were formed in the gorge.

Generally, in the course of this survey, bed nets were distributed for the three villages, once per year. It was distributed on the first week of September 2014 and 2015. Specially, during September 2015, one bed net for two individuals was given by the government. According to the national spraying operation guidelines (MoH, 2012),

anti-malaria chemical spraying (IRS) (Deltametrin) was administered to the three villages on the first week of September 2014 and September 2015 (Bure District Health Office, Unpub. Report, 2016; Personal Com.).

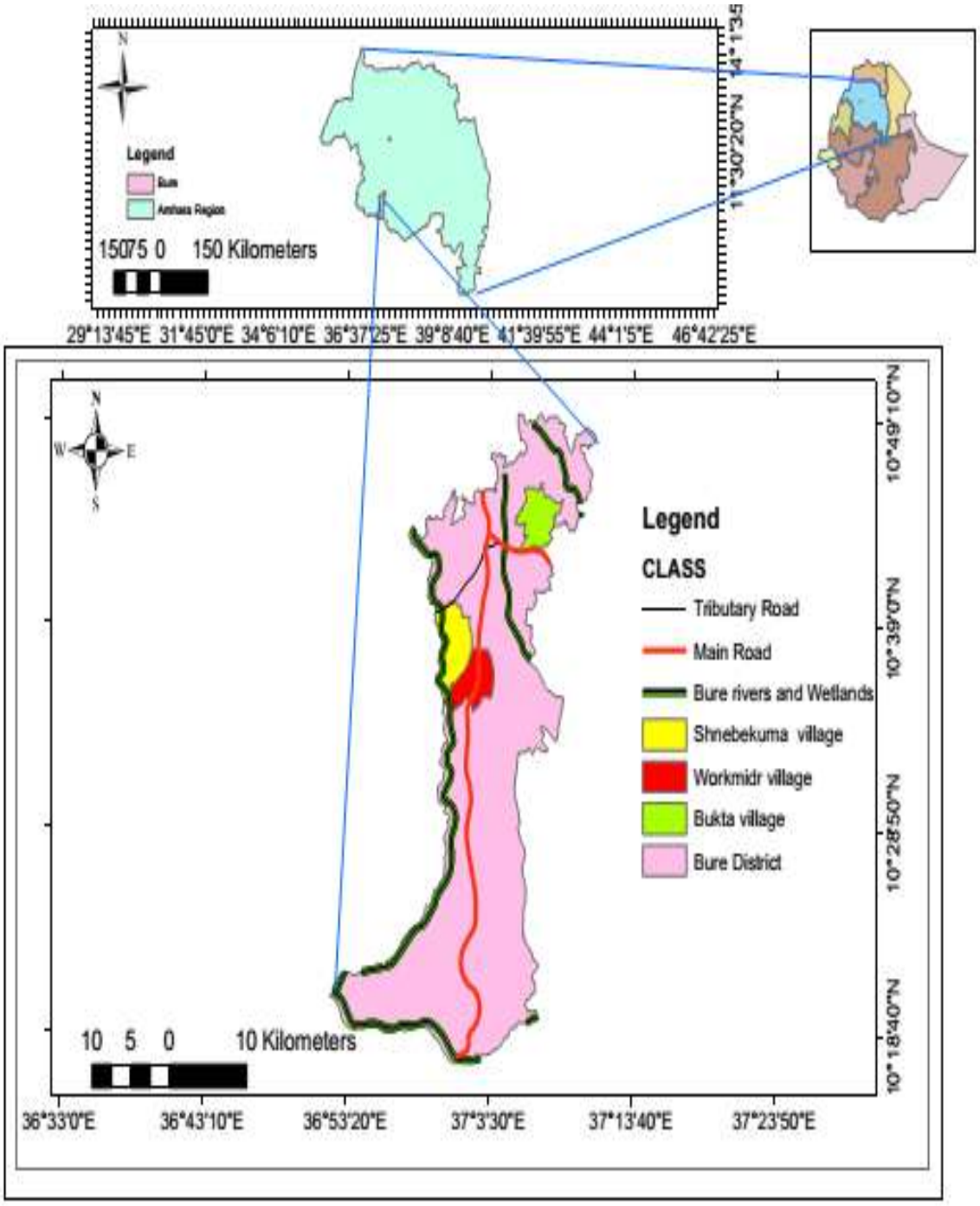

Figure 3.1: Map of Bure district

3.2 Entomological Data Collection, Processing and Identification

3.2.1 Adult Mosquito Collection, Processing and Identification

Adult *Anopheles* mosquitoes were sampled longitudinally from July 2015 - June 2016, after permission was obtained (Ethical clearance) from various Institutes. Entomological surveys were conducted monthly in each village, for one consecutive year using Center for Disease Control and Prevention Light Trap (CDC-LT), Pyrethrum Spray Catches (PSCs) and Artificial Pit- Shelters (APSs).

Indoor adults of *Anopheles* mosquitoes were collected from 6:00 PM (sunset) to 6:00 AM (sunrise) by using CDC miniature light traps (Model 512; J. W. Hock Co., Atlanta, USA) once per month per house (Lines *et al*., 1991; Mboera *et al*., 1998). In the same trends, outdoor adult's mosquitoes were collected by CDC-LTs from 06:00PM to 06:00AM hrs once per month.

Indoor-resting mosquitoes were collected in the mornings from 6:00 AM to 2:30 AM hrs using PSCs technique in every month at once. Collection was made based on WHO (2003 & 2013b) procedures using white floor sheets, hand lenses, Baygon, small petri-dishes, paper cups with net covers, forceps, cotton wool, and a torch. Additionally, outdoor-resting mosquitoes were collected in the morning from 6:30 AM - 7:30 AM hrs from artificially made pit shelters (1.5 m depth, 1.0 m width and 1.2 m length) using a handheld mouth aspirator, paper cup with net covers, cotton wool, torch and pencil (WHO, 1975). The number of human occupants and other potential hosts in each surveyed house during the previous night were recorded. Moreover, the house condition of each surveyed house was recorded; including the types of house, type of wall, numbers of LLINs used and spray status.

After collection (CDC-LTs, PSCs and APSs), mosquitoes were sorted to genus *Anopheles* and *Culex* using morphological futures (Verrone, 1962a) and finally male *Anopheles* and *Culex* were discarded after counted. Then, genus *Anopheles* was identified and counted based on morphological features (Verrone, 1962a; Gillies & Coetzee, 1987; Glick, 1992). The identified female *Anopheles* species were preserved in labeled vials (eppendorf) using desiccant (silica gel) for polymerase chain reaction (PCR) analysis and blood host preference studies. The abdomen of all unfed females was dissected and their respective head thoraxes were placed in labeled eppendorf with desiccant to determine sporozoite rates.

Moreover, morphologically identified and preserved *An. gambiae* sibling specie were further identified into species level using PCR (Wilkins *et al*., 2006; DNeasy Blood and Tissue Kits, 2011). The details are described in chapter four. Both blood digestion stages and parous rates were determined by WHO (2003 & 2013b). Identification of blood meal sources and infectious (*Plasmodium falciparum* and *P. vivax*) female *Anopheles* mosquitoes (head-thorax) using enzyme linked immune-sorbent assay (ELISA) were made based on Beier *et al*. (1988) and Wirtz (2016), respectively. The details of blood and sporozoite ELISA are described in chapter five and chapter six, respectively.

3.2.2 Mosquito Larva Collection, Identification and Characterization of Breeding Habitats

Mosquito larva sampling, identification and habitat characterization were made longitudinally from July 2015 - June 2016 in each month in Bukat, Workmidr and Shnebekuma villages. Samples were collected using a standard 350 ml white dipper (Clarke Mosquito Control Products, Roselle, IL) from large breeding habitats. 5ml graduated pipettes was used to collect samples from small breeding habitats (WHO,

2003 & 2013b). In each large breeding habitat types, 10-dips were taken; whereas in small breeding habitats the taken samples were pooled into 10-dips volumes. After collection, mosquito larvae in a white enamel sorting tray were sorted, counted and identified to genus of *Anopheles* and *Culex*; and then *Culex* and 1st and 2nd stages of *Anopheles* larvae were discarded. Dipper, funnel, sieve, large white tray, pipette, specimen tubes (larval vials), 75% alcohol, cotton wool, a pencil, and labeling paper were used for collection, sorting and packing purpose (WHO, 2003). Finally, only 3rd and 4th instars of genus *Anopheles* were transferred to labeled vial using a plastic pipette, and then immediately 75% ethanol alcohol added to kill and preserve them. Soon, morphological identification at species level was carried out in their respective breeding habitats based on key guidelines (Verrone, 1962b; Gillies & Coetzee, 1987). Each types of habitat were characterized by using standard keys made by Minakawa *et al*. (1999), Sattler *et al*. (2005), Mwangangi *et al.* (2007a), Majambere *et al*. (2008), and Liu *et al*. (2012). The details of sampling, identification and characterization techniques are explained in chapter seven.

3.3 Data Analysis

Entomological data were entered in to Microsoft Excel 2007 and analyzed using SPSS version-20 soft-ware packages (SPSS Inc, Chicago, IL). Before analysis was conducted, data were cleaned and transformed into Log10 (x +1) in SPSS. The presence of variation in densities between adults of *Anopheles* and *Culex*, between indoor and outdoor host seeking adults *Anopheles*, between parous and nonpaorous rates of *Anopheles*, between larvae of *Anopheles* and *Culex* were tested using independent-samples T-test ($p < 0.05$). Variations between species (both adult and larval species) and species among villages, and larval density among environmental factors (characteristics) of the larval habitats were analyzed using one way analysis of variance (ANOVA) ($p < 0.05$). Significant means (in ANOVA) were separated using Tukey test (HSD). For each environmental variable, simple correlation between larval

abundance and individual parameters were first checked ($p < 0.05$) and only significant associations were further examined by multiple step-wise linear regressions to determine the best predictor variables associated with relative abundance of the larval species of *Anopheles*. All statistical analyses were performed at the 5 % significance level. Parous rate, daily survival, infectivity rates, HBI and BBI were calculated based on WHO (2003 & 2013b). EIR was calculated based on Drakeley *et al*. (2003). The presence of variation between HBI and BBI in each *Anopheles* species was checked using independent sample T-test ($p < 0.05$).

3.4 Ethical Clearance

Adult *Anopheles* mosquitoes were sampled longitudinally from July 2015 - June 2016. Before mosquito collections, permission written document (Ethical clearance) was obtained from Addis Ababa University Ethical Committee (Ethical Clearance Reference No.: CNSDO/382/07/15), Amhara Health Regional Bureau (Permission Reference No.: H/M/TS/1/350/07) and the Head of the Bure District Health Office (Permission Reference No.: BH/3/519L/2). Additionally, verbal consent was obtained from the head of each household.

Chapter 4: Species Diversity, Abundance and Distribution of Anophelines Mosquitoes in Bure District, Northwestern Ethiopia

4.1 Introduction

Malaria is a complex disease caused by protozoan parasites (genus *Plasmodium*) and transmitted by blood feeding infectious mosquitoes (Heggenhougen *et al*., 2003; Ramirez *et al*., 2009). Over 3,500 mosquitoes have been recorded worldwide (Rueda, 2008, Fang, 2010; Harbach & Kitching, 2016); however, about 537 species are *Anopheles* (Harbach, 2013) and only 70-80 is known to transmit human malaria worldwide (Robert *et al*., 2011). Of these, 41 are considered to be dominant vector species (Hay *et al*., 2010; Sinka *et al*., 2012) and capable of transmitting malaria by large. In Africa, there are 140 *Anopheles* species, but only twenty are known to transmit malaria to human (Hay *et al*., 2000; Sinka *et al*., 2012). In Ethiopia, over 42 species of *Anopheles* were recorded (Hay, 2003; Gaffigan *et al*., 2013), but the major malaria vector is *Anopheles arabiensis* (O'Connor, 1967; White *et al*., 1980; Balkew *et al*., 2006) while *An. pharoensis*, *An. funestus* and *An. nili* are reported as secondary vectors (O'Connor, 1967; Krafsur & Armstrong, 1978; Fisiha, 2002).

In Ethiopia, malaria disease is the leading health problem of the country (Carter Center, 2013; PMI, 2014) because three-fourth (75%) of the total area of the country is malarious and more than two-third (approximately 68%) of the total populations live below 2,000 m.a.s.l (Pennas & Girma, 2012; Ayele *et al*., 2012). However, many studies have confirmed the occurrence of malaria disease up to 2,500 m.a.s.l in many highland fringes of Ethiopia (Negash *et al*., 2005; Graves *et al*., 2009), such as in the outskirts of Addis Ababa (Woyessa *et al*., 2004), in Amhara region (Mecha, Dera,

Fogera and Ankesha district) (Tamiru *et al.*, 2014; Lake *et al*., 2016) and in SNNPR (highland fringe of Butajira) (Guthmann *et al*., 2007; Tesfaye *et al*., 2011 & 2012).

Amhara region is one of the highland parts of Ethiopia where malaria is a common disease (Alemu *et al*., 2013b; Tamiru *et al.*, 2014; Lake *et al*., 2016). However, entomological monitoring in the Amhara region is incomplete (Vajda & Webb, 2017) and little information is known about the dynamics of malaria vectors and transmission intensity in the region (Ndenga *et al*., 2006). Bure district is one of the malarious areas in Amhara region, where there was no any study conducted on the diversity, abundance and spatio -temporal distribution of *Anopheles* mosquitoes.

Therefore, study on the species compositions, dynamics, distributions of mosquitoes at the local level (Grillet, 2000; Coetzee, 2004) and how they differs each other biologically (Ramirez *et al*., 2009; Eckhoff, 2011) can help to design and apply appropriate control measures including integrated vector control strategies to defeat malaria and necessary to develop early warning systems for predicting malaria epidemics (Kulkarni *et al*., 2006; Ramirez *et al*., 2009; Kihadye *et al*., 2010). Hence, this study was aimed to assess the diversity, abundance, and the distribution of *Anopheles* mosquitoes in Bure district, Northwestern Ethiopia. Knowledge obtained in this study will serve as base-line data for developing appropriate malaria control strategies.

4.2 Materials and Methods

4.2.1 Description of the Study Area

The study was conducted in Bure district, northwestern of Ethiopia, which is located at the distance of 410 km from Addis Ababa. The detail descriptions of the three study villages and designs are described in chapter three (Section 3.1).

3.2.2 Entomological Data Collection, Processing and Identification

Adult *Anopheles* Mosquito Collection: Longitudinal entomological surveys were conducted monthly in three villages (Bukta, Workmidr and Shnebekuma) from July 2015 to June 2016 using CDC-LTs, PSCs and APSs. In each village, 9 houses for CDC-LTs and 10 houses for PSCs were randomly selected and scattered in near to the breeding sites, in the middle and periphery sides of village. In parallel, 27 CDC miniature light traps were prepared to collect outdoor host seeking mosquitoes for the three villages, each had 9-CDC-LTs. Additionally, six APSs were prepared in three villages to collect outdoor resting mosquitoes, each village had two.

Indoor adult (host-seeking) *Anopheles* mosquitoes was collected from 6:00 PM (sunset) to 6:00 AM (sunrise) in 9-fixed houses by using CDC miniature light traps (Model 512; J. W. Hock Co., Atlanta, USA) once per month per house. A CDC-LT with a lid was hanged at the height of 1.5 meter above the floor beside the untreated bed net in which an adult human volunteer slept under the bed net. All occupants in the houses were left to use untreated nets prior to the start of the entomological survey (Lines *et al*., 1991; Mboera *et al*., 1998). Similarly, outdoor adult mosquito was collected by 9-CDC-LTs from each village, which hanged on tree branches 4 - 5 meters (depending on the size of compound) away the same house used for indoor mosquito collection from 06:00AM to 06:00PM hrs. The number of human occupants and other potential hosts in each surveyed house during the previous night were recorded. Moreover, the house condition of each surveyed house was recorded; including the types of houses, type of wall, numbers of LLINs used and spraying status.

Indoor-resting mosquitoes were sampled in the mornings from 6:00 AM to 8:30 AM hrs from 10 randomly selected houses by using PSCs technique in every month at once per house. Spray sheet was constructed from white cotton sheets (sizes: 2 x 1 m, 2 x 2 m and 2 x 3 m) that can cover all the inside area of each sprayed houses. Before spray, all people, all food items, house utensils and all animals were removed from the house, and all openings and eaves of windows and doors were closed with pieces of cloth. Then, the floor and furniture were covered with white sheets, the windows and door closed before spraying with a chemical Baygon aerosol (SC. Johnson & Son. Inc, USA). Finally, one operator walked outside of the house by spraying baygon on open spaces and holes in the walls to prevent from mosquitoes escaping; and the other was entered in the room and closed the door and moved in a clockwise direction and applied spray towards the ceiling until the room was filled with fine smog (WHO, 2003 & 2013b).

After spraying, the operator was left the room quickly and closed the door for 15 minutes. After 15-minutes, collection was begun by opening the door from the doorway by picked up the sheets one at a time and carried the sheet outside. Collection and examination of the mosquitoes were made outside in daylight. For PSCs purpose, white floor sheets, hand lenses, Baygon (composition, 0.1% Prallethrin, 0.1% Permethrin and 98.8 % ww Inert Ingredients; Batch No: 173P06/2014; Registration No: 171-2-809), small petri-dishes, paper cups with net covers, forceps, cotton wool, and a torch were used. The number of occupants who slept and the types of animals in each house in the previous night were recorded. Moreover, the house condition of each surveyed house was recorded, including types of house, types of wall, no of LLINs used and spraying status (WHO, 2003 & 2013b).

Outdoor-resting mosquitoes were collected from artificially made pit shelters by using a handheld mouth aspirator, paper cup with net covers, cotton wool, torch and pencil. Pit shelters were constructed under the shade of various dense shrub trees 10 - 15 meters away from the resident villages. APSs had 1.5 m depth, 1.0 m width and 1.2 m length (1.5 m × 1.0 m × 1.2 m). Approximately 0.5 m from the bottom of each pit-shelter, a 30-cm horizontal deep cavity was prepared in each of the four sides (WHO, 1975). Collection was made from 6:30 AM - 7:30 AM hrs. Before collection begins, the mouth of each pit shelter was covered with insecticide untreated white net to prevent mosquitoes from escaping and for visibility purpose. Resting mosquitoes were collected for about 10 - 20 minutes in each pit.

Adult *Anopheles* mosquito species identification: Mosquitoes collected by CDC-LTs, PSCs and APSs were sorted into genus *Anopheles* and *Culex* (Verrone, 1962a). *Culex* and male *Anopheles* were discarded after recorded. Finally, the genus *Anopheles* was identified and counted based on their own morphologically features to each *Anopheles* species using taxonomic keys (Verrone, 1962a; Gillies & Coetzee, 1987; Glick, 1992).

Molecular identification of *An. gambiae complex*: Morphological identified and individually preserved *An. gambiae* sibling species (from CDC-LTs and PSCs), which were in fridge at Addis Ababa University Insect Laboratory taken into Jima University for further analysis using PCR. DNA was extracted from individual preserved *An. gambiae* complex species based on DNeasy Blood and Tissue Kits (2011). Extraction procedures as followed:

1) All legs and wings of mosquito were removed and placed in a 1.5ml centrifuge tube, and then 180 µl buffer ATL (Lot No: 154016195; Maf No: 1614758) was added. Following this, 20 µl Proteinase-K (Lot No: 154016946; Maf No: 1017738) was added

and mixed using vortex properly (Analog Vortex Mixer) and incubated at 56°C until lysed completely. Finally, the samples were vortex for 15 seconds and centrifuged by eppendrof centrifuge machine (Centrifuge Mode: 5430) to 5 minutes at 300 x g (190 rpm). 2) About 200 µl buffer AL (Lot No: 154012765, Germany) was added and samples mixed, and incubated at 56°C for 10 minutes. 3) About 200 µl ethanol absolute (C_2H_5OH) (CAS: 64-17-5, Batch No: 99025001, Maf Date: 10 may 2016, Exp Date: 10 May 2018) was added and mixed using vortex machine. 4) All the samples from each tube were transferred into a DNeasy-Nini-spin column placed into 2ml collection tubes. Transferred samples were centrifuged for 1-minute at 6000 x g (8000 rpm) and finally the flow-through and collection tubes were discarded. 5) Spin column was placed in a new 2ml collection tube and 500 µl buffer AW1 (Lot No: 148050747, Maf No: 1014797) was added and centrifuged for 1-minute at 6000 x g (8000 rpm). Finally, both the flow-through and collection tubes were discarded, and spin column was transferred into a new collection tubes. 6) About 500 µl buffer-AW2 (Lot No: 148047724, Maf No: 1014577) was added into a transferred spin column and centrifuged for 3-minutes at 20,000 x g. 7) Both flow-through and collection tubes were discarded, and spin column was transferred into a new 1.5ml micro-centrifuge tube. 8) About 200 µl buffer-AE (Lot No: 148051542, Maf No: 1014591) was eluted the DNA to the center of the spin column membrane. After completion of elution /wash/, spin column was discarded and DNA- solution in 1.5ml micro-centrifuge tube was incubated for 1-minute at room temperature. Finally, after incubation, centrifuge was made for 1-minute at 6000 x g; and each sample was transferred into separated PCR-tube. All centrifuge steps were performed at 20°C.

DNA amplification: After completion of DNA extraction, reaction mix was prepared. It was composed of 19µl PCR mix and 1µl DNA sample. The procedures were: 1) PCR-

mix was prepared by mixing both 10µl Dream Taq Geeen PCR-mater-mix (2X) (No: K1081, Lot No: 00355703), 2µl Primers and 7µl PCR Nuclease free water. Dream Taq Geeen PCR-mater-mix was a ready to use solution, composed of Dream Taq-DNA-polymerase, optimized Dream Taq Green-Buffer (2X), $MgCl_2$ and dNTP (dATP, dCTP, dGTP and dTTP). Primers were four in numbers, 0.5µl Gambiae-AG (*An. gambiae*), 0.5µl Gambiae-AR (*An. arabiensis*) (Lot No:9021486), 0.5µl Gambiae-Q specie (*An. quadriannulatus*) and 0.5µl Gambiae-UN (common to all species of the *An. gambiae* complex). 2) Before preparation of reaction mix, properly labeled thin-walled PCR-tube was placed in ice box, and master-mix was vortex gently. About 10µl master-mix, 2µl primer and 7µl PCR Nuclease free water and 1µl DNA sample were added into PCR-tube. 3) Finally, homogenized samples were placed in PCR machine (Primer Thermo Cycler) (Product code: 155Wmax; 3PRimBASE/02) for amplification. Primer Thermo Cycler was used for DNA amplification and done according to the instruction given by the manufacturer. The program had denaturation, annealing and extension, and happened respectively. Initial denaturation was made at 95°C for 3 minutes (1cycle), secondary denaturation at 95°C for 30 seconds, annealing at 90°C for 30 seconds, and elongation at 72°C for 1-minute for 40 cycles, and final elongation at 72°C for 15 minutes for 1-cycle. The total number cycles for 2 ° denaturation, annealing and 1° elongation were 40 (Wilkins *et al*., 2006).

Gel electrophoresis: After completion of DNA amplification, agarose gel was prepared as described by Wilkins *et al*. (2006). It was composed of TAE buffer (Tri acetate buffer) (Cat No: 719204, Carton No: 351-009-491), agarose, 3:1 HRB (High Resolution Blend) (Code: E776-250G, Lot No: 0462C036), and ethidium bromide (Lot No: NL 1636731, Product No: 17896). Procedures were: 1) About 75ml of 1x TAE buffer was poured in a beaker and then 1.5 gram agarose was added. The beaker placed

in Panasonic oven (NN-SM332W) for 2 minutes until agarose was completely dissolved. 2) Soon, about 12µl EDTM bromide (dying chemical used to bind DNAs and allow the DNA to be seen) was added into a boiled solution and homogenized. Homogente solution poured into the gel-casting that had comb, and allowed to say at room temperature for 30-minutes to solidify. 3) Finally, the prepared agarose gel (2%) was placed in gel-electrophoresis apparatus (MINI GEL-II Complete Electrophoresis System, Cat. No.700-0003). 4) About 10µl PCR amplified DNAs, 10µl negative (Distilled water) and 10µl positive control (laboratory strains, *An. arabiensis*), and 10µl the PCR low ladder were loaded into the agarose gel hole according to Gel-setup form. 5) Gel-electrophoresis apparatus adjusted to 70 voltages for one hour in dark room to separate amplified from non-unamplified fragments. At the end, agarose-gel was placed on UVP (Photo Doc-It- imaging system or UV- Trans-illuminator) to see the nature of the bands. Those mosquitoes that remained unamplified (without any band on the gel) were tested three times, in an independent manner.

PCR 100bp low ladder (pH = 7.5 - 8) (Cat No: P1473) had 10 bands, ranging 100-1000bp in exact 100bp spaced /ladder/ recombinant repeats (Appendix 4a and 4b). It was prepared from 2µl Gel loading buffer (Cat No: G2526), 5µl dd-free water and 5µl standard PCR-ladder. Out of 12 µl prepared PCR ladder, only 10µl was used for electrophoresis purpose.

4.3 Data Analysis

Anopheles mosquito data was entered in to excel computer program for cleaning and analyzed using SPSS version-20 (SPSS, Inc., Chicago, IL, USA). Before any analysis, non-normalized data was transformed [log10(x+1)]. Mean variations between adults of *Anopheles* and *Culex*, between indoor and outdoor host seeking adults mosquitoes were

tested using Independent- samples T-test ($p < 0.05$). Variation in mean densities between adult species and species among villages were analyzed using one way analysis of variance (ANOVA) ($p < 0.05$). Significant means (ANOVA) were separated using Tukey test (HSD). Simple descriptive statistics (count, percentage, tables, and figures) were used to assess different variables. Moreover, *Anopheles* mosquitoes biting densities (host seeking) across villages were calculated as the sum of each species female *Anopheles* caught in the village during the 12-month sampling period divided by the total number of CDC-LTs for night-biting mosquitoes in each village (mosquito/CDC-LTs/night). Similarly, indoor and outdoor mosquito biting densities were calculated as the sum of the female *Anopheles* catches caught in the villages during the 12-months sampling period divided by the total number of CDC-LTs for night-biting mosquitoes in the villages (Okello *et al*., 2006). All statistical analyses were performed at the 5 % significance level.

4.4 Results

4.4.1 Diversity and Abundance of Adult *Anopheles* Mosquitoes

A total of 11,625 female mosquitoes were collected in Bure district using all collection methods. Of these, 59.5% (n= 6922) belonged to the genus *Culex*, while the rest 40.5% (n = 4703) were from the genus *Anopheles*. The proportions of the two genera have not shown statistical significant difference ($t = 1.165$; $df = 22$; $p = 0.257$). Morphologically, nine species of the genus *Anopheles* were identified in the three villages, belonged to *Anopheles demeilloni* (50.7%), *An. gambiae* s.l (16.0%), *An. funestus* (13.6%), *An. coustani* (12.9%), *An. squamosus* (5.0%), *An. cinereus* (1.5%), *An. pharoensis* (0.32%), *An. rupicolus* (0.006%) and *An. natalensis* (0.002%). The majority of *Anopheles* mosquitoes were collected by CDC-LTs (99.6%) as compared to PSCs (0.4%) and APSs (0.0%) (Table 4.1).

Table 4.1: Composition and relative abundance of *Anopheles* mosquitoes in three sites by collection method

Study Sites		*Anopheles* Species, No (%)									
	Methods	*An.ar*	*An. ph*	*An. fu*	*An. co*	*An. sq*	*An. ci*	*An.dem*	*An.rup*	*An.nata*	Total
Bukta	CDC-LTs	72 (100)	3 (100)	53 (100)	103 (99)	22 (100)	45 (90)	51 (100)	1 (100)	0	350 (98.3)
	PSCs	0	0	0	1(1.0)	0	5(10)	0	0	0	6(1.7)
	APSs	0	0	0	0	0	0	0	0	0	0
	Total	72 (100)	3 (100)	53 (100)	104 (100)	22 (100)	50 (100)	51 (100)	1 (100)	0	356 (100)
Workmidr	CDC-LTs	90 (98.9)	3 (100)	34 (100)	113 (100)	17 (100)	1 (100)	211 (100)	2 (100)	0	471 (99.8)
	PSCs	1(1.1)	0	0	0	0	0	0	0	0	1(0.2)
	APSs	0	0	0	0	0	0	0	0	0	0
	Total	91 (100)	3 (100)	34 (100)	113 (100)	17 (100)	1 (100)	211 (100)	2 (100)	0	472 (100)
Shnebekuma	CDC-LTs	588 (100)	9 (100)	544 (98.4)	389 (100)	196 (100)	15 (83.3)	2121 (100)	0	1 (100)	3863 (99.7)
	PSCs	0	0	9(1.6)	0	0	3(16.7)	0	0	0	12(0.3)
	APSs	0	0	0	0	0	0	0	0	0	0
	Total	588 (100)	9 (100)	553 (100)	389 (100)	196 (100)	18 (100)	2121 (100)	0	1 (100)	3875 (100)
Total CDC-IN-LTs		343	10	261	225	80	30	828	0	1	1778
Total CDC-OUT-LTs		407	5	370	380	155	31	1555	3	0	2906
Overall of each species, by LTs, No (%)		750 (99.9)	15 (100)	631 (98.6)	605 (99.8)	235 (100)	61 (88.4)	2383 (100)	3 (100)	1 (100)	4684 (99.6)
Overall of each species, by PSCs, No (%)		1(0.1)	0(0.0)	9(1.4)	1(0.2)	0	8(11.6)	0	0	0	19 (0.4)
Overall of each species, by APSs (No %)		0	0	0	0	0	0	0	0	0	0
All total, No (%)		751 (100)	15 (100)	640 (100)	606 (100)	235 (100)	69 (100)	2383 (100)	3 (100)	1 (100)	4703 (100)
Overall % species		16	0.32	13.6	12.9	5	1.5	50.7	0.006	0.002	100

Note: An.ar = *An.arabiensis*, An.ph = *An.pharoensis*, An.fu = *An.funestus*, An.co = *An.coustani*, An.squ = *An.squamosus*, An.ci = *An.cinereus*, An. dem = *An.demeilloni*, An.rup = *An.rupicolus* and *An.nata* = *An.natalensis*

As compared with methods of collection, nine species were identified using CDCD-LTs; however, PSCs (n = 4) and APSs (n= nil) had the least numbers of species (Table 4.1). From all species, the most prominent was *An. demeilloni*, followed by *An. gambiae* s.l, *An. funestus* and *An. coustani*; whereas *An. pharoensis*, *An. cinereus*, *An. rupicolus* and *An. natalensis* were the least representatives (one-ANOVAs: $F_{8, 99} = 24.593$, $p < 0.0001$) (Table 4.2).

Table 4.2: Mean comparison of *Anopheles* mosquitoes by sites and by species (Adults/CDC-LT/night) (Mean = M and Std. Error SE)

Sites	**M ± SE**	**P-value**
Bukta	1.35 ± 0.11^{b}	0.001
Workmidr	1.28 ± 0.20^{b}	
Shnebekuma	2.39 ± 0.10^{a}	
***Anopheles* Species**	**M ± SE**	**P-value**
An. arabiensis	0.31 ± 0.04^{b}	0.001
An. pharoensis	$0.01 \pm 0 .01^{d}$	
An. funestus	0.25 ± 0.05^{bc}	
An. coustani	0.23 ± 0.06^{bc}	
An. squamosus	0.12 ± 0.04^{cd}	
An .cinereus	0.04 ± 0.01^{d}	
An. demeilloni	0.61 ± 0.07^{a}	
An. rupicolus	0.001 ± 0.002^{d}	
An. natalensis	0.001 ± 0.001^{d}	

Note: Means of each species followed by the same letter (s) are not significantly different from each other ($p < 0.05$, Tukey HSD)

A total of 66 specimens (8.8%, 66/751) of *An. gambiae* s.l were further identified to species by PCR techniques, of which 63 (95.5%) were successfully amplified and identified to *An. arabiensis*. Only three (4.5%) specimens which were checked for three

times were not amplified. Hence, all *An. gambiae* s.l complex collected in this study were *An. arabiensis* (Appendix 4a and 4b).

4.4. 2 Spatio -Temporal Dynamics of *Anopheles* Mosquitoes in Three Villages

Spatially, the largest numbers of pooled adult *Anopheles* were collected in two non-irrigated villages, Shnebekuma (n = 3875) and Workmidr (n = 472) than irrigated village, Bukta (n = 356) (Table 4.1 and Figure 4.1). Analysis of ANOVA indicated the presence of significant mean variation in overall *Anopheles* mosquito's densities between the three villages ($F_{2, 33} = 19.202$, $p < 0.0001$) (Table 4.2).

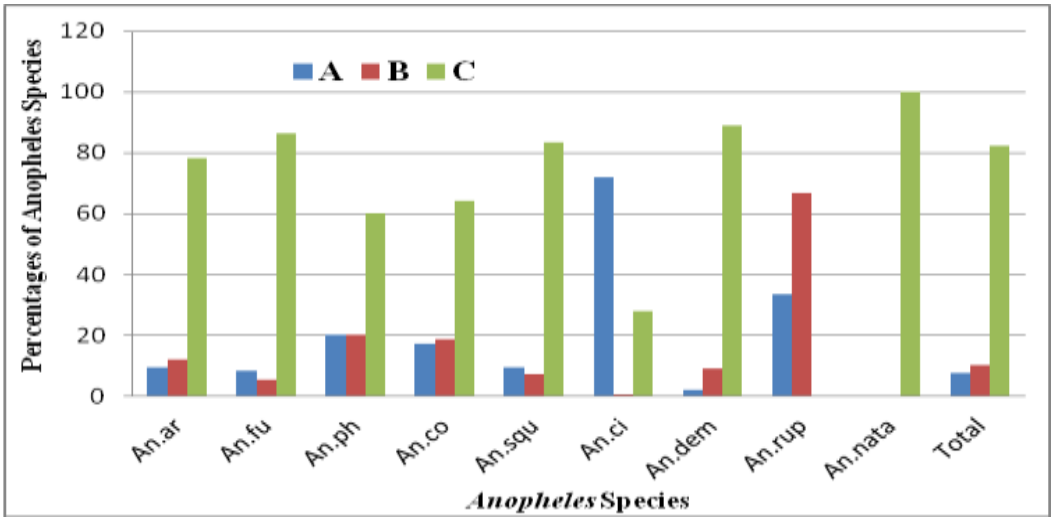

Figure 4.1: Percentage of *Anopheles* species per villages. A= Bukta (irrigated), B = Workmidr (nonirrigated) and C = Shnebekuma (nonirrigated). An.ar = *An. arabiensis*, An. fu = *An. funestus*, An.ph = *An. pharoensis*, An.co = *An. coustani*, An.squ = *An. squamosus*, An.ci = *An. cinereus*, An.dem = *An. demeilloni*, An. rup = *An. rupicolus* and An. nata = *An. natalensis.*

Mean comparison of each *Anopheles* mosquito across villages is described in Table 4.3. Highest proportions of *An. arabiensis*, *An. funestus*, *An. squamosus* and *An. demeilloni* were recorded from Shnebekuma than other villages ($p < 0.05$). In Bukta, only *An. cinereus* was dominant as compared with other villages (72.5%) ($p < 0.05$). *An.*

pharoensis, *An. rupicolus* and *An. natalensis* were very scarce and not distributed uniformly in the three villages; however, the rests species were highly persistent in all villages. *An. rupicolus* was only found in Bukta and Workmidr; on the other hand *An. natalensis* was only recognized in Shnebekuma village (Figure 4.1).

Table 4.3: Mean comparison of each *Anopheles* species in three villages (Adults/CDC-LT/night) (at 2 and 33 degree of freedom)

Species	Mean ± Std. Error			P-value
	Bukta	Workmidr	Shnebekuma	
An. arabiensis	0.73 ± 0.10^{b}	0.79 ± 0.13^{b}	1.55 ± 0.12^{a}	0.001
An. pharoensis	0.06 ± 0.05	0.06 ± 0.05	0.15 ± 0.071	0.438
An. funestus	0.51 ± 0.13^{b}	0.45 ± 0.11^{b}	1.48 ± 0.11^{a}	0.001
An. coustani	0.73 ± 0.16	0.49 ± 0.12	1.11 ± 0.20	0.082
An. squamosus	0.28 ± 0.11^{b}	0.27 ± 0.09^{b}	1.01 ± 0.12^{a}	0.001
An. cinereus	0.47 ± 0.12^{a}	0.03 ± 0.03^{b}	$.25 \pm 0.09^{ab}$	0.005
An. demeilloni	0.60 ± 0.10^{b}	0.90 ± 0.20^{b}	2.13 ± 0.10^{a}	0.001

Note: Means of each species followed by the same letter (s) are not significantly different from each other ($p < 0.05$, Tukey HSD).

The overall abundance of *Anopheles* mosquitoes across months is described in Figure 4.2. Seasonal variation of *Anopheles* mosquitoes was observed. The largest overall *Anopheles* densities were collected during small rainy month (October, 20 adult/CDC-LT/ month) followed by main rainy season (September, 11.4 adult/CDC-LT/month). The least overall densities were collected during short (small) rainy season (May, 1.8 adult/CDC-LT/month). The densities of *Anopheles* mosquitoes were started increasing from June and reached to its peck densities on October. After November, the mean monthly density showed irregular reduction through the dry and short rainy seasons,

except on March. Generally, most of the adults were collected in wet season (main rainy and short rainy season) than dry season (Figure 4.2).

As described in Figure 4.2, the density of each *Anopheles* species across months showed similar pattern as the overall densities of *Anopheles* mosquitoes. *An. demeilloni* was dominant through the study period. Highest densities of *An. demeilloni* (10.2 adult/ CDC-LT/month) and *An. funestus* (4.35 adult/ CDC-LT/ month) were recorded after the main rainy season (October) while the least densities of these species were recorded during small rainy season, May (0.54 adult/ CDC-LT/month), and June (0.19 adult/ CDC-LTs/month), respectively.

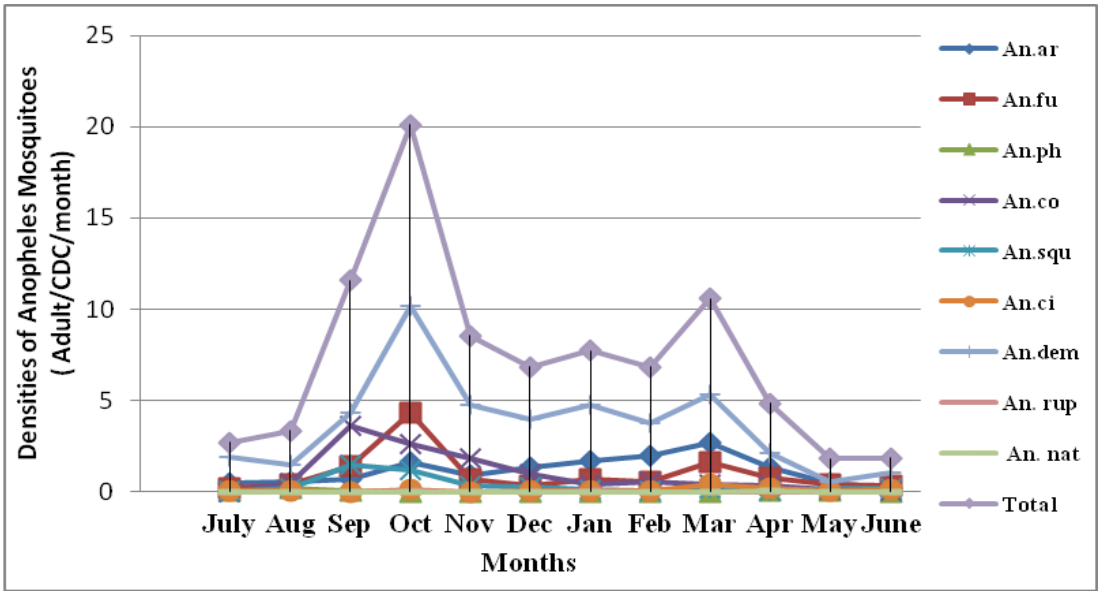

Figure 4.2: Densities of adult *Anopheles* mosquitoes across months (CDC-LT only). An.ar = *An. arabiensis*, An. fu = *An. funestus*, An.ph = *An. pharoensis*, An.co = *An. coustani*, An.squ = *An. squamosus*, An.ci = *An. cinereus*, An.dem = *An. demeilloni*, An. rup = *An. rupicolus* and An. nat = *An. natalensis*. Main rainy season = July - Sep; Small rainy = Oct – Dec and May - June; and Dry = Jan – April.

Different from *An. demeilloni* and *An. funestus*, peaked density of *An. arabiensis* was observed during dry month (March, 2.69 adult/CDC-LT/month) while minimum

density was collected during small rainy month, June (0.19 adult/CDC-LT/month). Densities of *An. coustani* and *An. squamosus* were maximum (3.63 adult/CDC-LT/month, 1.48 adult/ CDC-LT/month) in main rainy month (September), but minimum densities were recorded during July (zero) and February (0.04 adult/ CDC-LT/month), respectively. Similar to *An. arabiensis*, peak *An. cinereus* density was documented during March (0.41 adult/ CDC-LT/month), but no any *An. cinereus* was collected on September and November. In most surveyed months, the densities of *An. pharoensis*, *An. rupicolus* and *An. natalensis* were very low; especially *An. natalensis* was caught only during April (0.02 adult/ CDC-LT/month).

4.4.3 Comparison of Mean Densities of Indoors and Outdoors Adult *Anopheles* Mosquitoes from CDC-LTs Collection

Overall proportion of indoor and outdoor adult *Anopheles* mosquitoes from CDC-LTs by species is presented in Figure 4.3. The average proportion of outdoor *Anopheles* mosquitoes (62.0%) was higher than indoor proportion (38.0%); though statistically insignificant ($p > 0.05$). Similarly, each species of *Anopheles* mosquito showed similar trend, mean indoor was equal to outdoor density ($p > 0.05$) (Table 4.4). In general, the result of this study revealed that the absence significant difference between indoor and outdoor mean densities of each species.

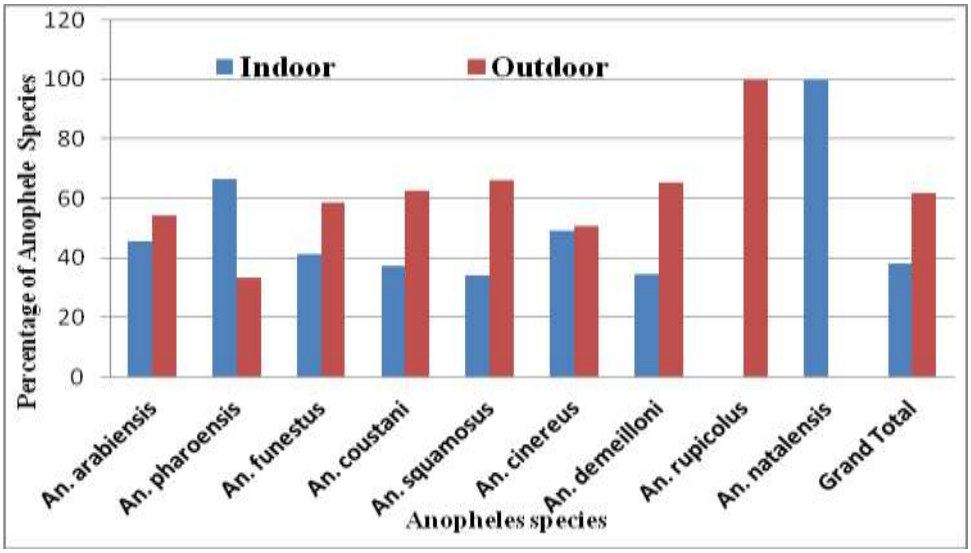

Figure 4.3: Percentage of indoor and outdoor *Anopheles* mosquitoes by species using light traps

Table 4.4: Mean densities of indoor and outdoor *Anopheles* mosquitoes by sites and by species(mosquitoes/CDC- LT/night) (degree of freedom = 22)

Sites	Indoor M ± SE	Outdoor M ± SE	P (2-tailed)
Grand Total	0.77 ± 0.06	0.88 ± 0.10	0.330
Bukta	0.38 ± 0.07	0.33 ± 0.07	0.602
Workmidr	0.43 ± 0.10	0.36 ± 0.09	0.627
Shnebekuma	1.06 ± 0.08	1.24 ± 0.12	0.211
Species	**M ± SE**	**M ± SE**	**P(2-tailed)**
An. arabiensis	1.12 ± 0.21	1.26 ± 0.25	0.681
An. pharoensis	0.04 ± 0.03	0.01 ± 0.00	0.274
An. funestus	0.23 ± 0.04	0.26 ± 0.06	0.733
An. coustani	0.19 ± 0.19	0.26 ± 0.25	0.408
An. squamosus	0.09 ± 0.02	0.14 ± 0.05	0.339
An. cinereus	0.04 ± 0.01	0.04 ± 0.02	0.991
An. demeilloni	0.51 ± 0.06	0.67 ± 0.09	0.166
An. rupicolus	0.00 ± 0.00	0.004 ± 0.01	0.168
An. natalensis	0.001 ± 0.01	0.00 ± 0.00	0.328

Note: Independent-samples t-test significance at $p < 0.05$.

4.5 Discussion

The overall aim of this study was to examine the diversity, abundance and distribution of *Anopheles* mosquitoes in the three villages. A total of nine *Anopheles* species composed of *An. arabiensis, An. funestus, An. pharoensis, An. coustani, An. squamosus, An. cinereus, An. demeilloni, An. rupicolus* and *An. natalensis* were identified in highland fringe of Bure district. Of these species, *An. arabiensis* is the main malaria vector of Ethiopia (Fontaine *et al*., 1961; White *et al*., 1980; Balkew *et al.*, 2006), was also among others recorded in such high altitude area (range 2,000 - 2,157 m.a.s.l) with a large proportion (16%, n= 751) next to *An. demeilloni* (50.7%). This is in agreement with Woyessa (2001), Woyessa *et al.* (2004), and Dejenie *et al.* (2012) reports, where *An. arabiensis* found from the outskirts of Addis Ababa and Tigray region (2,110 m.a.s.l and 2,170 m.a.s.l). Similarly, Animut *et al*. (2013a) and Tesfaye *et al.* (2011) found *An. arabiensis* at an altitude of 2,200 m.a.s.l and 2,280 m.a.s.l in south-central and southern Ethiopia, respectively. Moreover, this result is in agreement with other studies conducted in the highland of Kenya (Chrispinus *et al*., 2011; Mulambalah *et al*., 2011; Kweka *et al*., 2015).

This study discovered the secondary malaria vectors of Ethiopia, *An. funestus* and *An. pharoensis* (Krafsur & Armstrong, 1978; Fisiha, 2002). *An. funestus* was the third abundant vector (13.6%, n= 640) next to *An. arabiensis*, which was recorded in the higher altitude. Previously, O'Connor (1967) has documented *An. funestus* in Gojam (Amhara region). However, it is inconsistent with other reports from Ethiopia and elsewhere, who recorded this species below 2,000 m.a.s.l (Ototo *et al*., 2015; Daygena *et al*., 2017; Degefa *et al*., 2017). The occurrence of this species up to 2,157 m.a.s.l is attributed by the presence of increased temperature as a result of climate change, land

cover changes and deforestation (Afrane *et al*., 2006 & 2012; Kweka *et al*., 2015). *An. pharoensis* was among the least prevalent species in the present study and its occurrence at higher altitude has reported in various highland parts of Ethiopia. Woyessa (2001) and Woyessa *et al*. (2004) reported *An. pharoensis* from the periphery of Addis Ababa (2,110 m.a.s.l). Recently, Animut *et al*. (2013b) obtained *An. pharoensis* in Wurib at 2,200 m.a.s.l, in southern central Ethiopia.

Anopheles coustani was another mosquito recorded at 2,157 m.a.s.l. This observation is in line with Woyessa (2001), Woyessa *et al*. (2004) and Chrispinus *et al.* (2011), who found *An. coustani* in highlands of Ethiopia and Kenya. *An. coustani* is a known malaria vector in Kenya (Mulambalah *et al*., 2011; Mwangangi *et al*., 2013; Ogola *et al*., 2017) and the possible vector in Ethiopia (Kelel, 2010; Yewhalaw *et al.*, 2014; Degefa *et al*., 2015). The later scholars have detected sporozoite infected *An. coustani* in southern central Ethiopia (Jima zone). Moreover, in the present survey, *An. coustani* was not only found in larger numbers (n = 606), but also found infected with sporozoite (as described in chapter six). Therefore, all these results suggesting the necessity of regular monitoring of both the adults and larvae of *An. coustani* before any significant impact happens. *An. squamosus* was another species found in higher elevation. O'Connor (1967), Shililu *et al.* (2003), Chrispinus *et al.* (2011) and Mulambalah *et al*. (2011) recorded this species in the highland parts of Ethiopia, Eretria and western Kenya, respectively.

Our study detected other potential malaria and non-malaria vectors such as *An. demeilloni*, *An. cinereus*, *An. rupicolus*, and *An. natalensis* at higher altitude (2,000 - 2,157 m.a.s.l). *An. demeilloni* was the leading dominant *Anopheles* species (50.7%) as compared with all identified species in this study area. Similar to this finding, Animut

et al. (2013a), Gone *et al*. (2014) and Daygena *et al*. (2017) recorded *An. demeilloni* between 2,200 m.a.s.l to 2,650 m.a.s.l in southern central Ethiopia. Mulambalah *et al*. (2011) and Dejenie *et al*. (2012) obtained *An. demeilloni* as a secondary prominent species next to *An. Christyi* and *An. arabiensis* in western highlands of Kenya and in Ethiopia, respectively. Moreover, previous studies made in Ethiopia (over 2,200 m.a.s.l) (Ovazza & Neri, 1955) and in Eretria (over 2,000 m.a.s.l) (Shililu *et al*., 2003) showed the presence of this species too. In current study, presence sporozoite infected *An. demeilloni* was not checked; however, lately Conn *et al*. (2016) and Daygena *et al.* (2017) detected sporozoite infected *An. demeilloni* in the high lands of western Kenya and highland of Dirashe district, southern Ethiopia, respectively. Therefore, all these evidences implying that, *An. demeilloni* would be the possible malaria vector in Ethiopia and needs serious attention.

Anopheles cinereus was another species identified at 2,157m.a.s.l. Similarly, various studies (Animut *et al*., 2013a; Gone *et al*., 2014; Daygena *et al*., 2017) conducted in Ethiopia showed the occurrence of *An. cinereus* between 2,170 - 2,560m.a.s.l. Other findings have also indicated the occurrence of *An. cinereus* between 1,950 - 2,170 m.a.s.l in Ethiopia and Eretria (Woyessa, 2001; Shililu *et al*., 2003). Moreover, Shililu *et al*. (2003 & 2004) detected *P. falciparum* infected *An. cinereus* in Eretria; therefore, all these evidences indicate *An. cinereus* as a potential malaria vector in Ethiopia, and hence this species needs regular entomological monitoring in space and time; especially in Bukta because very large proportion was found in this irrigated village.

The other species this study discovered were *An. rupicolus* (0.06%, n=3) and *An. natalensis* (0.02%, n =1). The presences of *An. rupicolus* above 2,000 m.a.s.l have reported by Ovazza & Neri (1955) and Shililu *et al.* (2003) in Ethiopia and Eretria,

respectively. Previously, O'Connor (1967) has recorded *An. natalensis* in Gojam (Amhara region), Ethiopia. Recently, Getachew (2017) was collected both *An. rupicolus* (n = 40) and *An. natalensis* (n = 1) at an altitude ranged 1,500 -1,800m.a.s.l in southern western Ethiopia. Therefore, these two species found both in low and highland areas.

Overall, the proportion of *An. demeilloni*, *An. arabiensis*, *An. funestus*, *An. coustani*, and *An. squamosus* were very high in this study as compared with other reports (Woyessa *et al*., 2004; Dejenie *et al*., 2012; Animut *et al*., 2013b), which is due the presence of high temperature as a result of climate change (Afrone *et al*., 2006) and the expansion of maize cultivation which favored the positive growth of the larvae (Ye-ebiyo *et al*., 2003; Kebede *et al*., 2005; Minakawa *et al*., 2005). Moreover, the occurrence of breeding habitats through the dry months contributed a great role for the presence of very large proportions of *Anopheles* mosquitoes in this study.

The largest proportions of *Anopheles* mosquitoes were collected by LTs (99.6%) than PSCs (0.4%) and APSs (0.0%). The effectiveness of CDC-LTs over the other methods is in agreement with Mala *et al*. (2011b), Massebo *et al*. (2013a) and Animut *et al*. (2013a); over 50% of *Anopheles* mosquitoes trapped by CDC-LTs. However, it contradicts Jaleta *et al*. (2013) report, who collected relatively larger numbers of *An. arabiensis* using APSs (35.4%) than PSCs (33.3%) and light traps (31.3%) in western Ethiopia.

The proportions of outdoor host seeking *Anopheles* were higher than indoor though their log-mean densities were insignificant ($p > 0.05$). Similarly, mean monthly indoor and outdoor densities of the seven species were not statistically different ($p > 0.05$). Proportion of outdoor collected *An. arabiensis* was equal to indoor. This kind of host

seeking behavior was observed in various parts of Ethiopia and Kenya (Olanga *et al*., 2015; Kenea *et al.,* 2016; Taye *et al*., 2016). However, it contradicts other reports that conducted in Ethiopia and Nigeria (Woyessa *et al*., 2004; Oyewole *et al*., 2007; Taye *et al*., 2017); *An. arabiensis* showed equal indoor and outdoor host seeking density. On the other hand, Kerah-Hinzoumbe *et al*. (2009), Lelisa *et al*. (2017) and Ogola *et al*. (2017) found high density of indoor host seeking *An. arabiensis* than outdoor Ethiopia and various parts of Africa.

The density of indoor and outdoor host seeking *An. funestus* was the same. Olanga *et al*. (2015) observed similar trend. However, Kerah-Hinzoumbe *et al*. (2009), Degefa *et al*. (2017) and Ogola *et al*. (2017) collected predominantly indoor than outdoor host seeking of *An. funestus* in various parts of Africa. Contradictory to these reports, Taye *et al.* (2006), Kenea *et al*. (2016), and Kibret *et al.* (2017) found more outdoor than indoor host seeking density of *An. funestus* in various parts of Ethiopia. The density of indoor and outdoor host seeking of both *An. pharoensis* and *An. coustani* was equal, which were different from other findings that conducted in Ethiopia and Kenya (Taye *et al*., 2016 & 2017; Lelisa *et al*., 2017; Degefa *et al*., 2017; Ogola *et al.,* 2017). Generally, this study indicated that these vectors were active both in and outside of house. Moreover, the indoor host seeking density of these mosquitoes implying that these species against to LLINs and IRS.

Anopheles mosquitoes were collected in three villages in various land use areas. The largest proportions of adults of *Anopheles* were collected in non-irrigated villages, Shnebekuma (82.4%) and Workmidr (10.0%) than irrigated (Bukta) (7.6%) village. This trend was true for all species, except for *An. cinereus.* In Ethiopia and Africa,

Mboera *et al*. (2010), Dejenie *et al*. (2012) and Kibret *et al.* (2017) collected large numbers of *Anopheles* in irrigated than non-irrigated villages.

In the present study, presence of extremely high proportion of mosquitoes in non-irrigated village was caused by the presence more productive breeding habitats throughout the study period, that allowed the mosquitoes to reproduce annually (Mwangangi *et al*., 2012) than irrigated village. Altitudinal difference (temperature variation) could be the other possible source of variation (Animut *et al*., 2013a; Kibret *et al*., 2017). In Tanzanian, Maxwell *et al.* (2003) was collected 12 times greater *Anopheles* densities in the lowland than highland area. Similarly, Kulkarni *et al*. (2006), Animut *et al*. (2013a) and Kibret *et al*. (2017) were found higher proportion of *Anopheles* mosquitoes in lowland than highland altitude villages in Africa.

Moreover, the lower densities of *Anopheles* in irrigated villages (and areas near a dam) was due to a greater wealth created in the community via irrigation, which helped to construct good houses, result in prohibition of the mosquitoes to enter in the house; thereby minimum numbers of mosquitoes were collected. Many studies have proved the purpose of well constructed houses in reducing the abundances mosquito in the house (Konradsen *et al.*, 2003; Atieli *et al*., 2009; Njie *et al*., 2009). In irrigated village (Bukta), the health center was established at the center of the settlement (inhabitants) than non-irrigated villages. Being very near, the villagers may have better knowledge about malaria control and prevention (Sissoko *et al*., 2004).

Distribution of *Anopheles* mosquitoes was found to be linked with surveyed seasons. Overall *Anopheles* mosquitoes density peaked after the end of the main rainy season (October, 20.1 adult/CDC-LTs/month) and reached peck level on October. This finding agrees with Kibret *et al*. (2017) report, who found higher density of *Anopheles*

mosquito between October and November in Ethiopia. However, it contradicts in various studies (Babatunde, 2009; Taye *et al*., 2016; Abraham *et al*., 2017), peak mosquito density was shown during the main rainy season, from August to September and during dry season (January). In our study, peaked density of mosquitoes in October was due to the presence of non-fluctuated water level of breeding sites together with elevated temperature; these provide very conducive breeding ground for *Anopheles* mosquitoes. In this study, new LLINs were distributed to all villages between 1- 7/ 09/ 2015 (from 26/12/2008 – 3/13/2008 E.C). In the same period, IRS was applied (Bure District Health Office, Unpub. Report, 2016; Personal Com.), but had less effect.

4. 6 Conclusions

In conclusion, the current study recorded the largest proportions of *Anopheles* mosquitoes in non-irrigated villages than the irrigated village throughout the year. This finding documented the most important malaria vectors of Ethiopia, *An. arabiensis* and *An. funestus* in all surveyed months and other potential vectors. The density of both indoor and outdoor host seeking mosquitoes was equally. Generally, irrigation has not any influence on the densities of mosquitoes. Therefore, the presence of *An. arabiensis*, *An. funestus* and other potential vectors throughout the year warrants the top priority of breeding habitat management, parallel to using LLINs and IRS. Environmental management must be applied throughout the year, especially in non-irrigated villages. The presence of insecticide resistant mosquitoes must be checked and other alternative measures should be in place because the density of mosquitoes was not decreased after the application of IRS and the use of new LLINs. Health education on malaria should be given strictly.

Chapter 5: Determination of Blood Meal Sources and Feeding Behavior of Anophelines Mosquitoes in Bure District, Northwestern Ethiopia

5.1 Introduction

The blood feeding behavior of malaria vectors is an important parameter in malaria epidemiology (Garrett-Joneset *et al*., 1980). This behavior can influence vectorial potential (Koutsos *et al*., 2007) and depending on the vertebrate host groups with which the mosquito makes contact and influence the spatial distribution of a disease (Dye & Hasibeder, 1986; Richards *et al*., 2006). The most successful malaria vectors feed commonly on humans and secondarily on cattle and other domestic animals depending on host availability (Garrett-Joneset *et al*., 1980).

The Human Blood Index (HBI) represents the proportion of blood meals derived from humans in mosquito vectors. It may be used to estimate the human biting habit, an important component of vectorial capacity and helps to substitute to measure malaria transmission (Richards *et al*., 2006; Lardeux *et al*., 2007; Gunathilaka *et al*., 2016). HBI is a measure of one aspect of the feeding pattern (performance) of a mosquito population and the probable incidence of new infections from any case of malaria that may be present in an area or be imported after the parasite reservoir is depleted (Bruce-Chwatt *et al*., 1966).

The study of host-feeding patterns is an essential part of understanding the epidemiology of disease transmitted by arthropods (Kay *et al.*, 1979; Ree *et al.*, 2001), which is influenced by several factors, including the intrinsic host preference of the species, nutritional requirement, host availability, vector density, social and cultural

practices of the human population (Kay *et al*., 1979). Host preference studies have also been used to monitor the effectiveness of vector control programmes by observing a reduction in blood-feeding behaviour, and have served as evidence of control failure (Mathenge *et al*., 2001; N'Guessan *et al*., 2007; Kariuki *et al*., 2013). *Anopheles* exhibits a wide range of host preferences, such as humans, cattle, sheep, horses, pigs, dogs, cats, birds, reptiles and other mammals (Garcia-Rejon *et al*., 2010; Gunathilaka *et al*., 2016). Those mosquitoes usually feed on humans are anthropophagic; whereas others feed mainly on other mammals, zoophagic. However, others are opportunistic; feeding on both humans and other animals (Takken & Lindsay, 2003; Rueda, 2008). Usually, animal feeding vectors are known by suppressing the human blood meal source and reducing the level of infection in the local vector population (Seyoum *et al*., 2002; Iwashita *et al*., 2014).

However, HBI results do not always reflect host preference (Boreham & Garrett-Jones 1973). Therefore several authors proposed different indices to separate preferential versus opportunistic feeding patterns of mosquitoes (Hess *et al*., 1968, Kay *et al*., 1979). The forage ratio (FR), which is measures of host selection patterns, i.e., quantifies vector selection of a particular vertebrate host rather than other available hosts (Boreham & Garrett-Jones 1973). FR is helped to show the attribute of only one host preference (Kay *et al*., 1979) and it does not require a full host census (Zimmerman *et al*., 2006). The other parameter is the feeding index (FI), which helps to compare the observed proportion of blood feeds on one host with another host divided by the expected comparative proportion of feeds on the two hosts (Kay *et al.,* 1979; Richards *et al*., 2006).

Generally, the knowledge of the HBI, blood-feeding preferences and pattern of a mosquito species provides insight into its vector potential (Dye & Hasibeder 1986;

Richards *et al*., 2006) and the epidemiology of diseases transmission (Ree *et al*., 2001; Puente *et al*., 2013) and allows to design and implement efficient strategies for vector control (Garrett-Jones, 1964; Bruce-Chwatt *et al*., 1966; Garcia-Rejon *et al*., 2010). In our study, the HBI, FR and host preferences (HFI) of *Anopheles* mosquitoes were not known so far. Therefore, this study was conducted to determine the abdominal status, HBI, FR and HFI of *Anopheles* mosquitoes in Bure district. Knowledge obtained in this study will help to developing appropriate malaria control strategies.

5.2 Materials and Methods

5.2.1. Descriptions of the Study Area

The study was carried out in Bure district, Northwest of Ethiopia from July 2015 to June 2016. The detail descriptions of the study villages and designs are presented in chapter three (Section 3.1 and 3.2).

5.2.2 Survey of Vertebrate Hosts

Humans and domestic animals (Checken and mammals) census report were obtained by interviewing the family leaders during house to house survey at the district (in the three study villages). The number of humans and domestic vertebrates in neighboring houses were not counted. Potential blood hosts for *Anopheles* from PSCs were not included due to the presence of very low numbers engorged *Anopheles*.

5.2.3 Adult *Anopheles* Mosquitoes Collection and Identification

Adult *Anopheles* mosquitoes were collected using CDC-LTs, PSCs and APSs from July 2015 to June 2016. Description of study designs and procedures are presented in chapter three (section 3.2.2) and chapter four (section 4.2.2). Morphological identification was carried out by keys made by Verrone (1962a), Gillies & Coetzee

(1987) and Glick (1992). Molecular identification of *An. gambiae* s.l was approved by PCR as described in chapter four (section 4.2.2).

5.2.4 Determination of Blood Digestion Stages of *Anopheles* Mosquitoes

The abdominal conditions of *Anopheles* were determined as the result of blood digestion and ovarian development by a standard key into unfed (the abdomen is flattened), freshly fed (abdomen appears bright or dark red from the blood in the midgut and the ovaries occupy only a small area at the tip of the abdomen), half-gravid (blood is dark in colour, and occupies three to four segments on the ventral surface and ovaries occupy most of the abdomen) and gravid (blood is reduced to a small black patch on the ventral surface or may be completely digested and ovaries occupy all the rest of the abdomen) (WHO, 2003 & 2013b). At the end, freshly fed and half gravid of the *Anopheles* was taken into Jima University for blood ELISA test.

5.2.5 Identification of the Blood Meal Sources of *Anopheles* Mosquitoes

Enzyme Linked Immune Sorbent Assay (ELISA) technique is the best and widely used technique that detects *Plasmodium* circumsporozoite protein (CSP) from sporozoites in the thoracic salivary glands and to determine infection rates in mosquitoes (Beier *et al*.,1986; Fontenille *et al*., 2001). The origin of blood meals from all freshly fed and few half-gravid females collected using CDC-LTs were identified as human and bovine using ELISA as described by Beier *et al*. (1988). From all collected species, only freshly blood fed and half-gravid of *An. arabiensis*, *An. funestus*, *An. coustani*, *An. squamosus* and *An. cinereus* were determined using peroxidase-conjugates for human and phosphatase-conjugated goat anti-bovine IgG for bovine.

The procedures were: 1) Only the abdomen of freshly blood fed and half-gravid *Anopheles* species were cut with sterile forceps and placed into labeled eppendrof tube, separately. 2) After adding of 100 µl PBS (phosphate-buffered saline, pH 7.4), the abdomen was crashed using electrical pestle. Finally the pestle was rinsed with 100 µl PBS to have a total of 200 µl final volumes. 3) Then 100 µl of homogenate, 100 µl positive (human and animal serums, at 1/ 10 in PBS) and 100 µl negative (from a laboratory colony of *An. arabiensis* adults not fed with blood) as a control were added in 96-ELISA plates according to the prepared ELISA-sheet. 4) Plates were covered (to avoid contamination and evaporation) and incubated (helps marker becomes attached to well surface) at room temperature for 2 hours. After incubation, the wells contents were discarded and banged five times on the tissue paper (to avoid the remained content) and washed (ELISA-washer) three times with 200µl PBS-Tween-20 (to remove unattached marker). 5) About 50µl human peroxidase conjugate (1° attaches to sample) (Lot No: 023M4782; Batch No: 023M4782; Product No: A0170) was added; plates were covered and incubated for one hour at room temperature. 6) Plates were washed by ELISA-washer three times with 200µl PBS-Tween-20, and 100µl of ABTS [2, 2'-azino-bis (3- ethylbenzothiazoline-6-sulphonic acid] was added in each plate/ well and incubated for 30-minutes for human blood detection. ABTS peroxidase substrate was prepared from peroxidase of substrate solution-A (Lot No: 080775; Product Code: 50-64-00) and from peroxidase substrate solution-B (Lot No: 080831; Product Code: 50-65-00). 7) For Bovine blood sources, 50µl bovine phosphatase conjugate (Lot No: 062M4761V/ Sigma-aldrich.com) was added, then covered and incubated for one hour at room temperature. 8) The wells were washed three times with 200µl PBS-Tween-20 by ELISA-washer and 100µl of pNPP (Cat No: 0421-01; Lot No: H4014-VG96) substrate was added in each plate and incubated for one hour. 9) Finally, positive

samples, included positive control were changed into blue green colors for human blood (peroxidase) and dark yellow reactions (phosphatase) for bovine blood (visually seen). Immediately, using ELISA reader, the value of each plate was determined at 405 nm wave length. Samples were considered positive if absorbance values exceeded two times the mean of three negative controls, blood of unfed mosquitoes.

5.3 Data Analysis

The human blood index (HBI) and bovine blood index (BBI) were calculated as the proportion of the mosquitoes fed on either human or bovine blood meals out of the total blood meals determined (Garrett-Jones, 1964), respectively. Mixed (human + bovine) blood meals were added to the number of human and bovine blood meals when calculating the overall HBI and BBI (Papp *et al*., 2011; Mala *et al*., 2011b). Presences of significant difference between HBI and BBI were checked by Independent-Samples T-test ($p < 0.05$). Variation among potential blood meal sources (host types) for *Anopheles* was separated by one way ANOVA. Mean variation (in ANOVA) was separated by Tukey test (HSD) ($p < 0.05$). Before applying mean comparison, normality of blood meal sources (host types), HBI and BBI were checked and data were transformed [log 10(x + 1)]. Besides, descriptive statistic (counts, percentages, tables and graphs) was used to present the frequency of blood meal sources, HBI and BBIs. Every statistical test was made at 0.05 significant values.

Foraging ratio (FRs) was performed in order to obtain the proportion of blood meals occurring for human and cattle only. FRs were calculated as the percent of female *Anopheles* mosquitoes (five species) containing blood of a particular host divided by the percent of the total available host population represented by particular host (Hess *et al*., 1968) as described with the equation below:

$$FR: (NAE/NTE)/(NAP/NTP)$$

Where, FR: foraging ratio of *Anopheles* species,

NAE: number of engorged female mosquitoes containing blood from host-1

NTE: total number of engorged females,

NAP: number of hosts of type one in the collection area, and

NTP: total number of hosts of all types in the collection area.

Foraging rations of 1.0 indicated neither a selective bias nor avoidance of a particular host animal (opportunistic = equally feeding); FRs significantly > 1.0 indicated a selective bias, and values < 1.0 indicated avoidance of a host in favor of other available hosts (Hess *et al*., 1968; Kay *et al*., 1979). However, in our study, the percentage of the FRs was calculated for only human and cattle and comparison was made between the two hosts. The host preference indices (HFI), which is defined as the observed proportion of feeds on one host (human) with respect to another (cattle) divided by the expected comparative proportion of feeds on these two hosts (human plus cattle), was calculated using the formula described by Kay *et al.* (1979) and Richards *et al*. (2006). The formula was:

$$\mathbf{HFI} = (\mathbf{Nx}/\mathbf{Ny})/(\mathbf{Ax}/\mathbf{Ay})$$

Where ‘Nx’ and ‘Ny’ are the mean numbers of blood meals taken from hosts ‘x’ and ‘y’ per study site, respectively, and ‘Ax’ and ‘Ay’ are the mean numbers of hosts ‘x’ and ‘y’ per study site, respectively. An index of 1.0 indicates equal feeding on the two hosts. An HFI > 1 indicate that host ‘x’ is preferentially fed upon, whereas a value < 1 indicated that host ‘y’ is preferentially fed upon (Richards *et al*., 2006). HFIs were calculated for each pair of hosts (Humans: Cattle) (Kay *et al*., 1979).

5.4 Results

5.4.1 Blood Digestion Status of Female *Anopheles* Mosquitoes

The blood digestion stage of each adult female *Anopheles* mosquito species is presented in Table 5.1 and Figure 5.1. Of 4,684 collected *Anopheles* mosquitoes (light traps only), the largest proportions were unfed (69.5%), followed by fed (24.4%), gravid (3.9%), and half-gravid (1.9%). This trend was similar for each species too, except for *An. rupicolus* and *An. natalensis.*

Overall, relatively higher numbers (n= 638) of fed *Anopheles* mosquitoes were collected from indoor than outdoor collection (n =507) using CDC-LTs. Overall outdoor HG was 56.7%, greater than indoor collection (43.3%). Collection methods comparison indicated that over 95% of unfed, fed, and half-gravid were collected by CDC-LTs while the remainder was trapped by PSCs. However, APT was not effective to catch at least one *Anopheles* specimen (Table 5.1). Because of the unsuccessfulness of both PSCs and APSs, the degrees of exophily and endophily behavior were not determined. For blood ELISA test, only 609 specimens (only by light traps) belonging to *An. arabiensis* (n = 208), *An. funestus* (n= 213), *An. coustani* (n= 122), *An. squamosus* (n= 54) and *An. cinereus* (n= 12) were analyzed.

Table 5.1: Percentage of blood digestion stages of female *Anopheles* mosquitoes by collection methods, from July 2015 - June 2016

Feeding Status	Blood Digestion Stages			Blood Digestion Stages by collection methods			
	Indoor	Outdoor	Total No (%)				
	LT	LT		LTs	PSCs	APSs	Total (%)
UF	1014	2253	3267 (69.5)	3267	9	0	3276 (69.7)
F	638	507	1145 (24.4)	1145	8	0	1153 (24.5)
HG	39	50	89 (1.9)	89	0	0	89 (1.9)
G	87	96	183(3.9)	183	2	0	185(3.9)
All total	1778	2906	4684 (100)	4684	19	0	4703(100)

Note: Unfed (UF), Fed (F), Half-gravid (HG) and Gravid (G)

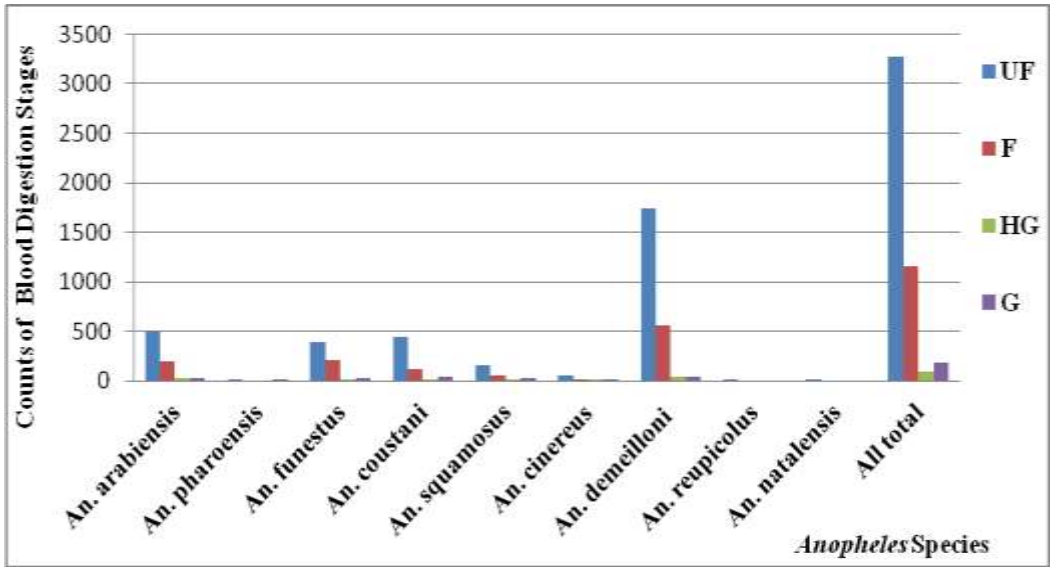

Figure 5.1: Proportion of blood digestion status of each *Anopheles* species, from July 2015 - June 2016. Unfed (UF), Fed (F), Half-gravid (HG) and Gravid (G)

5.4. 2 Compositions and Abundances of Potential Blood Meal Hosts for *Anopheles* Mosquitoes

During the course of this study, totally 3,803 hosts were registered in all surveyed houses in the three villages while CDC-LTs were set annually. Of these hosts, Bukta accounted for 39.2%, Shnebekuma for 33.0% and Workmidr for 27.8%. Hosts were composed of bovine (40.0%), humans (37.7%), sheep (16.0%), chickens/ hens (4.0%), donkeys (0.8%), mules (0.7%) and dogs (0.7%) in 324 surveyed houses (Table 5.2). Statistically, human and cattle hosts were equal and significantly higher than the other hosts ($F_{6, 77} = 160.863$; $p < 0.001$) (Table 5.3).

Table 5.2: Proportion of vertebrate hosts species identified in Bure district, from July 2015 - June 2016

Hosts	Sites			Total (%)
	Bukta	Workmidr	Shnebekuma	
	No (%)	No (%)	No (%)	
Human	477 (32.0)	472 (44.7)	486 (38.7)	1435 (37.7)
Bovine	490 (32.9)	454 (42.9)	576 (45.9)	1520 (40.0)
Sheep	323 (21.7)	114 (10.8)	173 (13.8)	610 (16.0)
Donkey	20 (1.3)	11 (1.0)	0 (0.0)	31(0.8)
Mule	28 (1.9)	0 (0.0)	0 (0.0)	28 (0.7)
Checken (fowl & cock)	151(10.1)	0 (0.0)	0 (0.0)	151(4.0)
Dog	0 (0.0)	7 (0.7)	21(1.7)	28 (0.7)
Total	1489 (100)	1058 (100)	1256 (100)	3803 (100)
Percentage	39.1	27.8	33.0	3803 (100)

Table 5.3: Mean densities of vertebrate hosts species in Bure District, July 2015 - June 2016

Hosts	M ± SE
Human	4.419 ± 0.07^{a}
Bovine (cattle)	4.681 ± 0.100^{a}
Sheep	1.881 ± 0.093^{b}
Donkey	0.166 ± 0.045^{b}
Mule	0.180 ± 0.046^{b}
Hen	1.417 ± 0.252^{b}
Dog	0.2658 ± 0.05973^{b}

5.4.3 Behavior of Feeding Preference and Blood Meal Indices of *Anopheles* Mosquitoes

Table 5.4 shows the blood meal origins and HBI of *Anopheles* mosquitoes from three collection sites using CDC-LTs. Overall 609 *Anopheles* mosquitoes (fed and HG) belonged to five species (*An. arabiensis, An. funestus, An. coustani, An. squamosus,* and *An. cinereus*) were tested by ELISA. Of these, 575 specimens were (n= 575 from CDC-LTs) positive for blood antigen of the host's blood and the rests specimens had unidentified blood meals (n = 34). Of 575 positive samples, the largest proportions (57.6 %) were from indoor collections than outdoor-collections (42.4 %) (Table 5.4).

From 208 tested of *An. arabiensis*, only 94.7% were positive (n = 124 for LTs-indoor and 73 for Outdoor) for blood antigen of the host's blood and the remainder was unknown (5.3%, n= 11). Out of these positive blood meals, 97% had mixed blood meal (human and bovine origin). The overall (single plus mixed) HBI of *An. arabiensis* was 91.8%, of these 62.9% were from indoor and 37.1% were outdoor LTs. The average

(single plus mixed) BBI of *An. arabiensis* was 94.7%, of these 62.9% were outdoor and 37.1% were from indoor LTs. Overall HBI and BBI had not showed any statistical significant difference between them ($p > 0.05$) (Table 5. 5).

The result of this study revealed that from 213 total tested *An. funestus*, only 95.8% of them were positive for blood meal-ELISA while 4.2% had unidentified blood meals. Most of the positive blood samples (57.4%) were from indoor collection and 42.6% were from outdoor collection. Out of the total positive specimens, 95.6% had mixed blood meal (human and cattle). The overall (single plus mixed) HBI of *An. funestus* was 91.5%, which was slight less than the overall BBI (95.8%). However, overall HBI and BBI had not showed any statistical significant difference between them ($p > 0.005$). A total of 122 specimens of *An. coustani* were tested for blood-ELISA. The majority of them (92.6%) were positive for their blood antigen of the hosts. From all positive samples, 53.1% were from indoors and 46.9% were outdoor collection. Totally, out of all positive specimens, the largest proportions had mixed blood meals (93.8%) and the rest had bovine (4.4%) and human blood meals only (1.8%). The overall HBI (88.5%) was lower than BBI (91%) of *An. coustani*; though statistically insignificant ($p > 0.05$).

Table 5.4: Results of enzyme-linked immune sorbent assay tests for identification of blood meal sources, HBI and BBI of five *Anopheles* species (only by CDC-LTs)

Tested Species	**No Tested**	Tested positive # (%)	# Un (%)	Single		# Mixed Blood %	CDC-LTs		Overall (single + Mixed)	
				Human, # %	Bovine, # %		In # (%)	Out # (%)	# HBI (%)	# BBI (%)
An. arabiensis	208	197 (94.7)	11(5.3)	0	6 (3)	191 (97)	124 (62.9)	73 (37.1)	191 (91.8)	197 (94.7)
An. funestus	213	204 (95.8)	9 (4.2)	0	9 (4.4)	195 (95.6)	117 (57.4)	87 (42.6)	195 (91.5)	204 (95.8)
An. coustani	122	113 (92.6)	9(7.4)	2(1.8)	5 (4.4)	106 (93.8)	60 (53.1)	53 (46.9)	108 (88.5)	111(91)
An. squamosus	54	51 (94.4)	3(5.6)	0	12 (23.5)	39 (76.5)	24 (47.1)	27 (52.9)	39 (72.2)	51 (94.4)
An. cinereus	12	10 (83.3)	2(16.7)	0	0	10 (100)	6(60)	4 (40)	10 (83.3)	10 (83.3)
All Total	609	575 (94.4)	34(5.6)	2 (0.3)	32(5.6)	541 (94.1)	331(57.6)	244 (42.4)	544 (89.3)	574(94.3)

Note: Overall HBI (Human blood index) and BBI (Bovine Blood Index) were calculated as the number of mosquitoes positive for human and Bovine (including mixed blood meal) blood divided by the total number tested species respectively. Un, as unidentified blood meals.

Fifty four *An. squamosus* were analyzed for blood-ELISA, only 94.4% were positive and the rest (5.6%) had unidentified blood meals sources. Of these positive samples, 47.1% were from indoors and 52.9% were outdoor collection; however, the majority of them (76.5%) had mixed blood meal. The overall BBI (94.4%) was higher than HBI (72.2%); however, statistically insignificant ($p > 0.05$). From twelve tested specimens of *An. cinereus*, 83.3% of them were positive and all of them had mixed blood meals (100%). Of these, the majority (60%) of them were from indoors as opposed to outdoor (40%) collection. The overall HBI and BBI of *An. cinereus* were similar (83.3%). Generally, the result of finding revealed that all the five test species took blood either host was available; though the overall bovine blood was greater. This study had one limitation, unable to examine other possible blood hosts which were registered during this survey using ELISA.

Table 5.5: Overall mean comparison of HBI with BBI of the five *Anopheles* species in Bure district, using CDC-LTs (from July 2015 - June 2016)

Tested Species	Percentage of overall HBI/BBI		M ± SE	P (2-tailed)
An. arabiensis	Overall HBI	91.8	1.04 ± 0.14	0.961
	Overall BBI	94.7	1.05 ± 0.14	
An. funestus	Overall HBI	91.5	1.11 ± 0.09	0.868
	Overall BBI	95.8	1.34 ± 0.09	
An. coustani	Overall HBI	88.5	0.76 ± 0.15	0.966
	Overall BBI	91.0	0.75 ± 0.15	
An. squamosus	Overall HBI	72.2	0.47 ± 0.12	0.746
	Overall BBI	94.4	0.53 ± 0.13	
An. cinereus	Overall HBI	83.3	0.18 ± 0.22	1.000
	Overall BBI	83.3	0.18 ± 0.22	

Note: degree of freedom = 22 and $p < 0.05$

5.4.4 Seasonal Blood Meal Distributions of *Anopheles* Mosquitoes (results of ELISA)

Seasonality of the overall (single human/ bovine blood meals plus mixed blood meal) blood meal taken from five *Anopheles* mosquitoes is presented in Figure 5.2a and 5.2b. All these species exhibited considerable seasonal variation. The proportion of *An. arabiensis* that had blood meals from human in each month was ranged from nil (May) to 26.6% (March). Similarly, this species didn't feed bovine blood in May (nil), but maximum proportion was acquired during March (25.8%). Log mean showed no any statistical difference between the choice of the two blood sources across months ($t = -0.05$; $df = 22$; $p = 0.961$). *An. funestus* was acquired the least and largest proportions of human (2.1%, 30.6%) and bovine blood meals (2%, 25.8%) in December and October, respectively. However, the choice between the two blood sources was insignificant ($t = -0.168$; $df = 22$; $p = 0.868$). *An. coustani* obtained the highest proportion of blood meals (25.9%) from human and bovine during September, but no human blood during July and May at all. The log mean blood meals taken from the two hosts had not significant difference ($t = 0.043$; $df = 22$; $p = 0.966$). *An. squamosus* was another mosquito that had blood meals from human and bovine. Human and bovine blood meals were ranged from nil (February, March, and April) to 35.1% and 9.5% (September), respectively; however, statistically insignificant ($t = -0.326$; $df = 22$; $p = 0.746$). *An. cinereus* was the last adult females without any human or bovine blood mean in September, November, December, February, May and June, but had 40% mixed blood meal during March. Statistically, the choice of hosts between human and cattle were not different ($t = 0.000$; $df = 22$; $p = 1$).

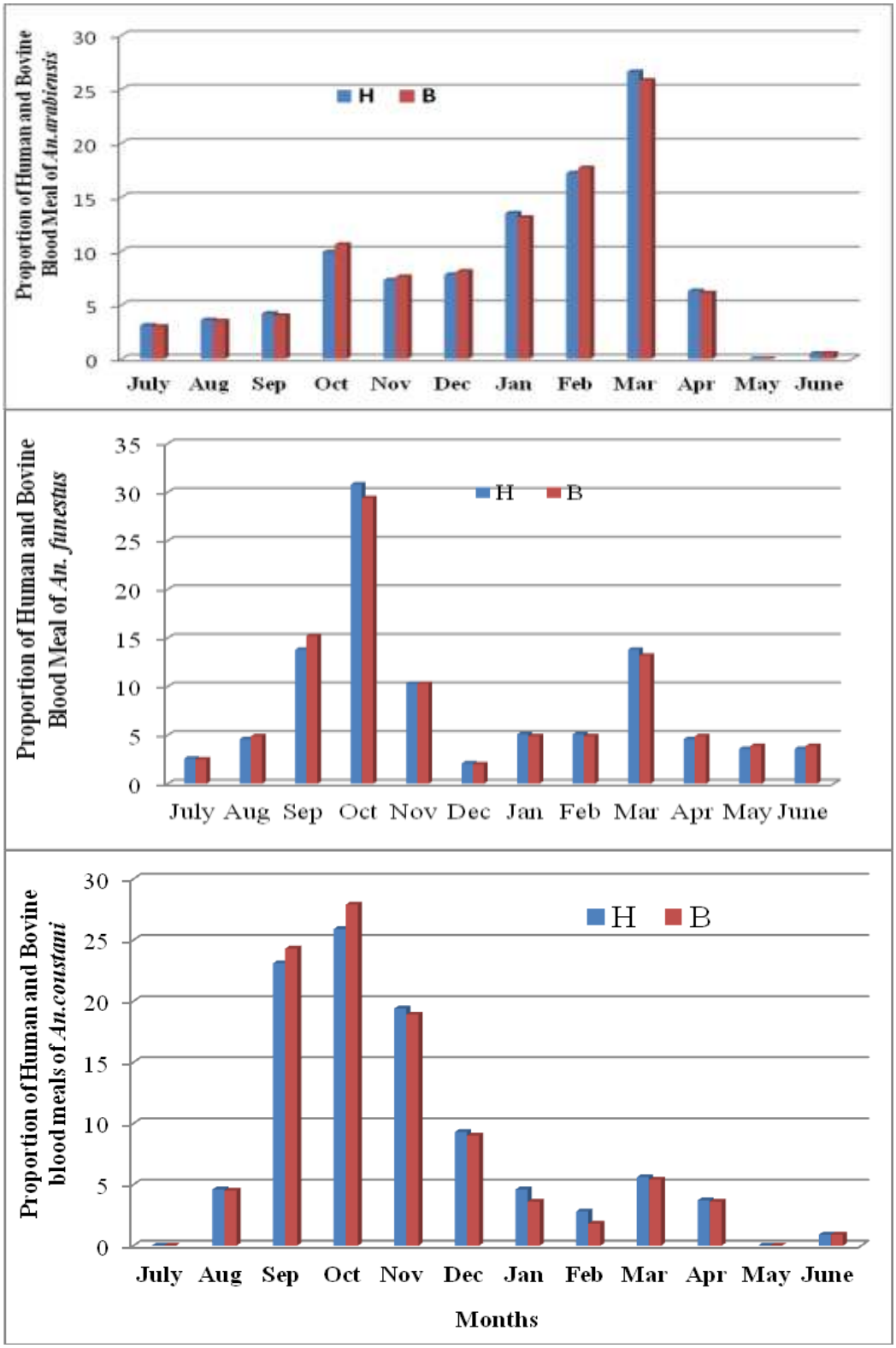

Figure 5.2a: Comparison of mammalian-derived blood meal sources of the three engorged *Anopheles* mosquitoes (Human with bovine blood). H, as Humans blood and B, as Bovine blood

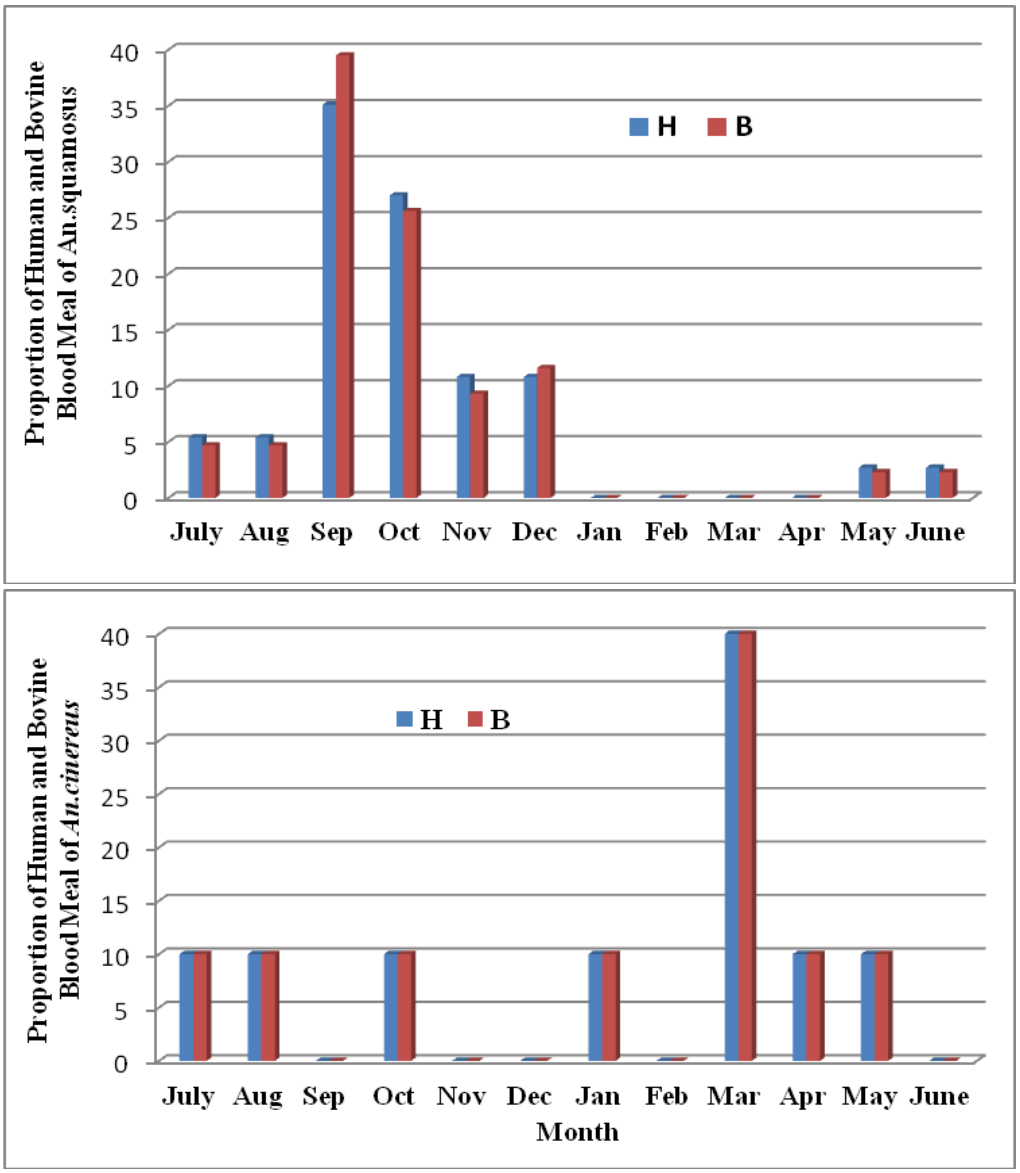

Figure 5.2a: Comparison of mammalian-derived blood meal sources of the two engorged *Anopheles* mosquitoes. Main rainy season = July - Sep; Small rainy = Oct – Dec and May – June; and **Dry** = Jan – April.

5. 4. 5 Foraging Ratio and Host Preference Index of *Anopheles* Mosquitoes

Foraging ratio values and feeding preference index (host feeding index) of *Anopheles* mosquitoes are presented in Table 5.6. The FR for a human was slightly > 1.0 for the five *Anopheles* species. Similarly, the FR for cattle was slightly > 1.0 for the five *Anopheles* species. Therefore, within this very narrow difference, it is difficult to

indicate their blood meal choices. Calculation of the HFI for each pair of vertebrate hosts revealed that both blood meals were preferred by both species, except for *An. squamosus* (Table 5.6).

Table 5.6: Foraging Ratios (FR) and Host Preference Index (HPI) of five *Anopheles* species in Bure, from July 2015 – June 2016

Species	Total FR		HPI
	Human	Bovine	Human: Bovine
An. arabiensis	1.88	-	1.03
	-	1.84	
An. funestus	1.88	-	1.01
	-	1.86	
An. coustani	1.82	-	1.03
	-	1.77	
An. squamosus	1.49	-	0.81
	-	1.84	
An. cinereus	1.71	-	1.06
	-	1.62	

5.5 Discussion

In this study, over 95% of *Anopheles* mosquitoes were collected by CDC-LTs. This is identical with other findings that, more mosquito vectors were trapped while host seeking than resting (Mala *et al*., 2011b; Massebo *et al*., 2013a; Mayagaya *et al*., 2015). From 4,703 total collected mosquitoes, the largest proportions (69.7%) were unfed. Consistent with our study, Fornadel *et al*. (2010) in Zambia, Bashar *et al*. (2012) in Bangladesh and Getachew (2017) in Ethiopia collected most of the unfed *Anopheles* mosquitoes using CDC-LTs. These unfed mosquitoes are stimulated and attracted by light generated by incandescent bulbs from light-traps (Costantini *et al*., 1998; Fornadel

et al., 2010). As a result, mosquitoes were caught while searching for their blood meals before they took blood. However, contradicts to this study, Animut *et al.* (2013a) collected large numbers of fed *Anopheles* species than unfed using light traps in Ethiopia. The catches of higher numbers of fresh fed mosquitoes using light traps are due to recapturing of mosquitoes after repeatedly feeding of their hosts (Norris *et al.*, 2010).

In the present study, the majority (97%) of *An. arabiensis* had mixed blood meal and the largest proportions (62.9%) of this blood meal were from LTs-indoors than outdoor collection (37.1%). The mixed blood meal proportion of *An. arabiensis* in this study was larger when compared to other reports. For example, Massebo *et al.* (2013b) reported 65%, Animut *et al*. (2013a) reported 13.2%, Yewhalaw *et al*. (2014) reported 1.6%, Getachew (2017) reported 4.4%, and Ngom *et al*. (2013) reported 14.4%. The highest proportion of mixed feeding associated with mixed dwellings (humans and cattle) nature of the sites (Bruce-Chwatt *et al*., 1966; Hadis *et al*., 1997). The proportions of indoor mixed feeding were higher for all species is due to occurrence of very high disturbances (Boreham & Garrett-Jones, 1973) or climatic factors (Hadis *et al*., 1997; Bashar *et al*., 2012). The overall (single plus mixed) HBI of *An. arabiensis* was 91.8%, is similar to Fornadel *et al.* (2010) report, who obtained 94% HBI in Zambia; however, higher than other findings in Ethiopia. Massebo *et al.* (2013b) found 75%, Animut *et al*. (2013a) found 47.6%, Yewhalaw *et al*. (2014) found 11.2%, Getachew (2017) found 54.4% and Kibret *et al*. (2017) found 76.9%. Higher overall HBI is probably associated with the inappropriate use LLINs or ignorance of other personal protective equipments by the communities and the closeness of breeding habitats to the settlement.

The result of this study demonstrated that most (95.6%) of *An. funestus* had mixed blood meal (humans and cattle) than single bovine blood meal sources. The absence of single human blood contradicts to other studies by Mbogo *et al*. (1993), Mwangangi *et al*. (2003) and Tanga *et al*. (2011), who reported extremely high single HBI for *An. funestus* in Kenya (100%, 86.3%) and Cameroon (98%), respectively. Mbogo *et al*. (1993) and Mwangangi *et al*. (2003) did get any cattle blood fed of *An. funestus* at all (n= 454). Therefore, this result suggesting that *An. funestus* may changes its blood meal sources from human to cattle. In our study, the overall (single plus mixed) HBI (91.5%) and BBI (95.8%) of *An. funestus* were greater than as compared to other findings that made in Kenya (HBI = 25.2%; BBI = 57.7%) (Muriu *et al.*, 2008) and in Ethiopia (HBI = 86.0%; BBI =14.3%) (Kibret *et al*., 2017).

The blood ELISA result of *An. coustani* indicated that, the majority (92.8%) of them had mixed blood meals, and the rest had only bovine (4.4%) and human blood meals (1.8 %). Though proportionally different, Muriu *et al.* (2008) reported 71.4% and 5.4% of *An. coustani* fed on human and bovine blood only in Kenya, respectively. In Ethiopia, Getachew (2017) also reported *An. coustani* with 33.3% human and 66.7% (2/3) bovine blood meals. On the contrary, Yewhalaw *et al*. (2014) didn't get any *An. coustani* with human blood in Ethiopia. Moreover, as described in chapter six, *An. coustani* was found infected by *P. vivax.* Therefore, all these results implying that *An. coustani* may involve in malaria transmission in the district. Prevention of mosquitoes through IRS and LLINs should be included *An. coustani*.

Totally, this study indicated the presence of high overall HBI (88.5%) and BBI (91.0%) of *An. coustani* as compared to other findings; Getachew (2017) reported 33.3% (HBI) and 67.7% (BBI) and Kibret *et al*. (2017) reported 38.5% (HBI) and 71.7% (BBI) in Ethiopia. In this findings, even though statistical insignificant, the proportion of the overall BBI was higher than HBI of *An. coustani*. This is in lines with Getachew (2017)

and Kibret *et al*. (2017) reports, as explained above. In Kenya, Muriu *et al.* (2008) reported highest overall BBI (74.4%) than HBI (8.4%) of *An. coustani* too.

Similarly, *An. squamosus* showed similar blood preference as like that of *An. arabiensis*, *An. funestu* and *An. coustani* (HBI equal BBI). Almost the majority (72.2%) of them had mixed blood meal and the remainder had single BBI (23.5%). However, the FI of *An. squamosus* indicated that this species preferred bovine than human blood meal. The host preference index also showed that *An. squamosus* preferred more bovine than human blood source. Different from all blood ELISA tested species, the overall proportion of HBI and BBI of *An. cinereus* was exactly equal (83.3%). This species showed flexible feeding behavior, either zoophilic or anthropophilic. This species had only mixed blood meals (cattle and humans) and most of them (60%) were from indoors than outdoor (40%). Different from the current study, Animut *et al*. (2013a) reported higher proportion single bovine blood meals (52.5%) and very minimum proportion of human blood (20.3%) and mixed blood (15.3%) of *An. cinereus* in Ethiopia. However, the allover HBI and BBI of *An. cinereus* were very larger as compared to Animut *et al.* (2013a) result, who found 35.6% HBI and 67.8% BBI. This result revealed that *An. cinereus* showed flexible feeding behavior, both zoophilic and anthropophilic and may involve in disease transmission.

Generally, from 609 ELISA tested *Anopheles* mosquitoes, only 5.6% had unidentified blood meals, composed of *An. arabiensis* (n= 11), *An. funestus* (n= 9), *An. coustani* (n= 9), *An. squamosus* (n= 3) and *An. cinereus* (n= 2). These unidentified specimens were considered as a limitation because they were not tested further by ELISA for other blood meal choices of *Anopheles* mosquitoes. These vectors might be fed blood of other animals (Sheep, Donkeys, Mules, Hens and Hens) which were accessed in the study area. The presence of domestic animals alone or in together with humans has

been associated with a decrease in malaria transmission rates due to ‘zoophilic deviation’ (Bruce-Chwatt *et al*., 1966; Seyoum *et al*., 2002). As a result, animals are considered as dead end of the malaria pathogens and serve as zooprophylaxis for zoophagic vectors, for these five species in this study.

In general, all blood ELISA tested *Anopheles* mosquitoes (except *An. cinereus*) showed slightly higher overall BBI over HBI. The possible explanation for such occurrence is that, in our study area animals were kept indoors at night, and both humans and cattle were lived in the same house, due to these reasons mosquitoes first encounter animals freely while searching for blood meal sources. In this study, the government has used to distribute LLINs and applying IRS once a year. The deterrent effect of LLINs and IRS make man less readily available than his cattle; therefore, mosquito forced to bite more cattle (Bruce-Chwatt *et al*., 1966; Takken & Verulst, 2013) whilst live in the same house. Besides, our animal census survey indicated that, the proportions of cattle (51.4%) were higher than humans (48.6%); this may the other causes for the occurrence of high average BBI over HBI.

This study also revealed that, the proportions of indoor mixed blood of all the five species were higher than outdoor mixed blood meal, which may suggest that the vectors were interrupted while feeding outdoors on cattle and moved into houses to complete their feeding in a single night or on consecutive nights (Boreham & Garrett-Jones, 1973; Norris *et al*., 2010; Bashar *et al*., 2012). The presence of appropriate indoor micro-climate than outdoor could be other possible reason for the occurrence of such greater proportion indoor of mixed blood meal (Afrane *et al*., 2006; Paaijmans & Thomas, 2011). In this study sites, houses were traditional (made of mud) and served as cooking, sleeping and tethering livestock, result in increased indoor temperature. Hence, this microclimate attracts more mosquitoes and provides more access to blood meal sources. The presence of higher proportions of mixed blood meals (humans and

others) are very important than single human meals because mixed feed tends to diminish the density of gametocytes in the mosquito stomach, thereby reducing the chance of fertilization of the female gamete and reduce the chances of malaria vector becoming infected (Burkot *et al.*, 1988; Muriu *et al.*, 2008; Bashar *et al*., 2012; Ngom *et al.*, 2013).

Generally, the FRs and HFIs results showed that except for *An. squamosus*, the rest species preferred equally the blood meals of human and cattle.

5.6 Conclusions

The proportion of mixed blood meals of *An. arabiensis*, *An. funestus*, *An. coustani*, *An. squamosus* and *An.cinereus* were higher than the proportion single blood (humans or cattle only) of these species, respectively. Presence of higher mixed blood meals is acting as a barrier to malaria transmission (Bruce-Chwatt *et al*., 1966; Seyoum *et al*., 2002). Therefore, intentionally keeping cattle near or inside human habitations are very crucial in order to divert mosquitoes and to reduce the rate of malaria transmission in the study villages (Burkot *et al*., 1988), especially, combination of zooprophylaxis is applicable. *An. coustani* had single human blood meal and this species might be linked with malaria transmission. So, controlling activities should be considered this species too. Similarly, the presence of higher proportions of overall HBI indicated the necessity of studying the practices and efficacy of LLINs of the community.

Chapter 6: Parous Rates, Longevity, *Plasmodium* Infection and Entomological Inoculation Rates of Anophelines Mosquitoes in Bure District, Northwestern Ethiopia

6.1 Introduction

Malaria is transmitted by the blood feeding of infectious female *Anopheles* mosquitoes (Koutsos *et al*., 2007; Sinka, 2013) with a complex parasite life cycle, which depends on both humans and mosquitoes (Garrett-Jones *et al*., 1980; Bannister & Sherman, 2009; Ndoen *et al*., 2012). In Africa, malaria transmission is varied within districts, between seasons (Appawu *et al*., 2004; Sissoko *et al.*, 2004; Mboera *et al*. 2007), between houses (Rwegoshora *et al*., 2007) and within agricultural zones (Sissoko *et al*., 2004).

In Ethiopia, malaria is transmitted by a leading malaria vector, *Anopheles arabiensis* (White *et al*., 1980, Balkew *et al*., 2006) and by secondary vectors (*An. pharoensis*, *An. funestus* and *An. nili*) (Fisiha, 2002; MoH, 2010). Several factors have reported to influence the vectorial role of mosquitoes in disease transmission including vector density and heterogeneity, resting and blood-feeding behavior, host preference, longevity, susceptibility to Plasmodium infection, micro-ecological, socioeconomic factors, poor environmental sanitation and environmental modification (Cohuet *et al*., 2010; Ndoen *et al*., 2012; Mboera *et al*., 2015; Molina-Cruz *et al*., 2016). Therefore, a full understanding of these factors is a precondition in planning effective vector control measures (Adeleke *et al*., 2010).

The intensity of malaria transmission is measured by human blood frequency, parous rates, daily survival rate, sporozoite rate, and entomological inoculation rates (EIRs) (Kelly-Hope & McKenezie, 2009; Charlwood *et al*., 2011; Obala *et al*., 2012). Female

parous status is a key index of vector competence, adult vector longevity, recruitment rate of adults, and the length of a gonotrophic cycle (Tsuda *et al*., 1991; Smith & McKenzie, 2004). Of these, age and the ability of vectors to survive are among the important factors in the epidemiology of vector-borne diseases (Smith & McKenzie, 2004; Uttah *et al*., 2013), which depends on proportion of mosquitoes that have ever fed on a human. Usually, older females also have higher exposure rates to malaria parasites during the previous human blood meal. Therefore, changes in the parous rate reflect many aspects of the population changes (Tsuda *et al*., 1991).

Entomological inoculation rate is an ideal indicator of malaria transmission, that helps relate both the human-biting activity of the *Anopheles* vectors and the risk to humans of malaria infection (Smith *et al*., 1995 & 2004). Therefore, EIR is considered a more direct measure of transmission intensity, but it differs widely in between villages and in the same locality (Obala *et al*., 2012). The dynamics of EIR is governed by the fluctuation in vector densities and their sporozoite rates (Krufsur & Armstrong, 1978); which again influenced by temperature, altitude, rainfall, and urbanization (Obala *et al*., 2012). EIR values also are used to quantify the impact of vector control measures (Coosemans *et al*., 1992), such as LLINs, IRS, and source reduction on malaria transmission (Shaukat *et al*., 2010), malaria endemicity (Burkot & Graves, 1995) and the risk of epidemic development (Onori & Grab, 1980). Therefore, effective vector control measures directly decrease malaria incidence (sporozoite rates) and then EIRs (Smith & McKenzie, 2004; Killeen & Smith, 2007; Shaukat *et al*., 2010). Generally, understanding the dynamics of malaria transmission in a population is very significant; it provides an insight into the magnitude of the problem and helps to describe when and where the greatest risk occurs. Hence, it helps to facilitate the development of appropriate control strategies (Smith *et al*., 2004, 2005, Hay *et al*., 2008).

Malaria is obviously a serious health problem in Amhara region (Wimberly *et al*., 2012b; Alemu *et al*., 2013a; Midekisa *et al*., 2015). Bure district is one of the major malaria focus areas in the region known by the high epidemic which has claimed many lives (Kebede *et al*., 2005; Amhara Health Bureau, Unpub, 2014; Kassa *et al*., 2015). Recently made epidemiological studies indicated that prevalence of malaria has showed sharp reduction in Bure surrounding districts (Toyama *et al*., 2016). However, entomological monitoring is incomplete in Amhara region (Vajda & Webb, 2017) and totally absent in Bure district in particular. Therefore, the present study was designed to investigate the parous rates, longevity, sporozoite and entomological inoculation rates of *Anopheles* species in Bure district, Northwestern Ethiopia. Studying the entomological parameters (parous rate, longevity, sporozoite and entomologic inoculation rates) of *Anopheles* mosquitoes will help to measure the impact of the ongoing (existed) vector control measures (such as LLINs, IRS and source reduction) and to assess the malaria transmission intensity in the district. Moreover, the obtained data serve as a base line data for both this study district and other surround areas.

6.2 Materials and Methods

6.2.1 Description of the Study Area

The study was conducted in Bure district, Northwest of Ethiopia. This study was conducted in three villages, Bukta (irrigated village), Workmidr (rain fed = non-irrigated) and Shnebekuma (rain fed = non-irrigated village). The details of the three villages are described in chapter three (section 3.1).

6.2.2 Adult *Anopheles* Mosquitoes Collection and Identification

Adult *Anopheles* mosquitoes were collected using CDC-LTs, PSCs and APSs from July 2015 to June 2016. Description of study designs and procedures are presented in

chapter three (section 3.2.2) and chapter four (section 4.2.2). Morphological identification was made by keys of Verrone (1962a), Gillies & Coetzee (1987) and Glick (1992). Molecular identification of *An. gambiae* s.l was carried out using PCR as described in chapter four (section 4.2.2).

6.2.3 Determination of Parous Rate and Longevity of *Anopheles* Mosquitoes

Female *Anopheles* mosquitoes (*An. arabiensis, An. funestus, An. coustani, An. squamosus, An. cinereus* and *An. pharoensis*) collected by CDC-LTs were analyzed for parous rate determination after dissecting the ovary. Dissection was carried out based on WHO (2003 & 2013b) protocol. After dissection, the ovaries were allowed to dry, examined and then classified as parous (those *Anopheles* mosquitoes that have taken a blood meal at least once and laid eggs at least once and as a result the tracheoles have become stretched out) or nulliparous (*Anopheles* mosquitoes that have not taken a blood meal yet and have not laid eggs and as a result have females in which the ovaries have coiled tracheolar skeins) under a compound microscope using the 10x objective, and further confirmed using the 40x objective. For dissection purpose, dissecting microscope, compound microscope, dissecting needles, fine forceps, slides, dropper and distilled water were used.

Parous rate was determined as the proportion of parous mosquitoes (*An. arabiensis, An. funestus, An. coustani, An. squamosus, An. cinereus* and *An. pharoensis*) to the total mosquitoes dissected (WHO, 2003 & 2013b). Longevity of the above six species were determined by using parous rates of the correspondent species. It was expressed as the cube root of the proportion of parous rate of each species considering three days for its gonotrophic period (Davidson, 1954) as described by Ree *et al*. (2001).

6.2.4 Determination of *Plasmodium* Infection and Entomological Inoculation Rates

To determine infection rate, the heads and thoraces of *An. arabiensis*, *An. funestus*, *An. coustani*, *An. squamosus* and *An. cinereus* females were tested by ELISA for CSP of *P. falciparum*, *P. vivax*-210 and *P. vivax*-247 based on Wirtz (2016) procedures.

Before directly running ELISA procedures, all necessary inputs were prepared and labeled, including phosphate buffered saline/ PBS (Sigma No: D5773), blocking buffer/BB, Grinding buffer/ GB (IGEPAL CA-630, Cat No: 198596, Lot No: 4908k) and Washing solution/ PBS-Tween-20 (BP337-100, Lot No: 121051, CAS 905-64-5 polysorbate). The preparation of each working solution was performed based on Wirtz (2016). Blocking buffer was made from 0.5% Casein (Code: E666-500G, Lot No: 1612C497, Cat No: 900-71-9), 0.1NaOH and Red phenol sodium salt (Cat No: 102604, Lot No: R27115); Grinding buffer from igepal CA-630 and BB; washing solution from Tween-20 and PBS.

Procedures in brief were: 1) the dried "head-thorax" portion of individual female *Anopheles* was placed in 1.5ml eppindorff tube; 50µl of grinding buffer (GB) was added and grinding was made using electrical-pestle 2) After completion of grinding, the pestle was rinsed with two 100 µl volumes of GB, to have 250 µl final volumes. 3) Then, 50µl of the capture monoclonal antibodies (MAb) of *P. falciparum* (Cat No: 37-00-24-2; Lot No: 2013pCAP), *P. vivax* (*P.v*-210, Lot No: 060415), and *P. vivax* (*P.v*-247, Lot No: 030276) were added to labeled 96-wells of separate polyvinyl chloride microtiter plates in duplicates (six in number). And then, the wells covered and incubated at room temperature for 30 minutes. 4) After incubation, well contents were removed, banged five times on paper towel, and then 200 µl BB was added again in each well, covered and incubated for one hour to block the remaining active binding

sites. 5) Well contents were then aspirated and banged five times on paper towel, and 50 µl of each mosquito triturate (including positive and negative control) was added according to the prepared sporozoite ELISA-sheets, and covered and placed to incubate for two hours. For positive controls, *P.f* (fragmented stock solution) for *P.f*-wells, *P.v*-210 (fragmented stock solution) for *P.v*-210-wells and *P.v*-247 (fragmented stock solution) for *P.v*-247-wells and for negative control unfed laboratory reared species were used. 6) After 2 hrs of incubation, the mosquito triturates were aspirated and the wells were washed twice with 200 µl PBS-Tween-20 using ELISA-Washer /well washer (Model No: BioTek-WLx 50). 7) Then, 50µl of peroxidase-linked MAbs of *P.f* (Cat No: 37-00-24-3; Lot No: 2013PfHRR), *P.v*-210 (Cat No: 37-00-24-3; Lot No: 060414) and *P.v*-247 (Cat No: 37-00-24-4; Lot No: 030246) were added to respective wells of the microtiter plate, then covered and incubated for one hour. 8) After one hrs incubation, the plates were washed three times with 200 µl PBS-Tween-20 using ELISA-Washer and 100 µl substrate solutions were added in single well and covered and incubated for thirty minutes. Substrate solutions was a mixture of ABTs peroxidase substrate solution-A (Lot No: 080775; Product Code: 50-64-00) and Peroxidase substrate solution-B (Lot No: 080831; Product Code: 50-65-00). 9) Finally, after 30 minutes incubation, absorbance values at 405 nm were recorded from the ELISA plate reader (BioTek-ELx800). Those samples considered positive if the mean absorbance values of the duplicate assays exceeded twice the mean of the three negative controls as recommended by Wirtz (2016).

6.3 Data Analysis

Data were entered and cleaned using 2007-Microsoft Excel and analyzed using SPSS software package version-20.0 (SPSS, Chicago, IL, USA). Parous rate, longevity and sporozoite rate were calculated based on WHO (2013b). Parous rate was estimated as

the number of mosquitoes with parous ovaries divided by number of female dissected multiplied by 100. The probability of survival of *An. arabiensis*, *An. funestus*, *An. coustani*, *An. squamosus* and *An. cinereus* through one-day is equivalent to the cube root of the proportion of parous females in the population sample; because there was no direct observation of the gonotrophic cycle (gc), the age estimation of these species was made on a 'gc' value of 3-days (Davidson, 1954). Therefore, the probability of surviving one day (denoted as p) for the above five species was estimated as (Ree *et al*., 2001; Ndoen *et al*., 2012):

$P = \sqrt[3]{Proportion\ of\ Parous}$; Whereas, mosquito life expectancy (longevity as d) was calculated using the formula proposed by Warrell & Gilles (Ree *et al*., 2001; Ndoen *et al*., 2012):

$$d = \frac{1}{-lnP}.$$

Sporozoite rate was estimated as the number of mosquitoes with sporozoites divided by number of females examined multiplied by 100 (WHO, 2013b). Using sporozoite rate, annual and monthly EIRs were estimated as described Drakeley *et al*. (2003). The annual EIRs were estimated by standard method: 1.605 (no. of ELISA positive from CDC-LTs/ no. ELISA tested) X (no of *An. arabiensis/ funestus/ coustani* collected from CDC-LTs/ Total no. number of CDC-LTs used) x 365 days. Monthly EIR was estimated by standard method: 1.605 (no. ELISA positive from CDC-LTs/ Total no. ELISA tested used) x (no. *An. arabiensis/ funestus/ coustani* collected from CDC-LTs/ Total no. number of CDC-LTs) x no. days per month. Independent-samples T-test was carried out to check the presence of difference between parous and nonporous rates. One way- ANOVA was applied to confirm the presence of parous between species and sporozoite rates difference between villages ($p < 0.05$). Before running independent-

samples T-test and ANOVA, normality of data was first checked and transformed [log10(x+1)]. When significant differences were observed in ANOVA, the Tukey test (HSD-Test) was used to separate the means ($p < 0.05$).

6.4 Results

6.4.1 Parous Rates of *Anopheles* Mosquitoes

The results of ovarian dissection for parous rates and mean comparison of parous rates of six *Anopheles* species are shown in Table 6.1 and 6.2. During this study period, a total of 952 unfed host-seeking *Anopheles* mosquitoes (*An. arabiensis*, n = 341; *An. funestus*, n = 239; *An. coustani*, n = 252; *An. squamosus*, n = 87; *An. cinereus*, n = 24; and *An. pharoensis*, n = 9) were dissected for parous rate determination; the ovarian dissection indicated that, the overall parous rate (51.3%, n= 488) was higher than nulli-parous rate (48.1 %, n= 446), though statistically insignificant (t = 1.604; df = 10; p = 0.14).

Table 6.1: Parous rates, daily survival and longevity of *Anopheles* species caught by LTCs in Bure area, July 2015 June 2016

Species	**# Un**	**# D**	**# P**	**% P**	**DS**	**Age (Days)**
An. arabiensis	498	341	177	51.9	0.804	4.6
An. pharoensis	9	9	4	44.4	0.763	3.7
An. funestus	382	239	151	63.2	0.858	6.5
An. coustani	436	252	106	42.1	0.749	3.5
An. squamosus	153	87	39	44.8	0.765	3.7
An. cinereus	41	24	6	25.0	0.630	2.2
All Total	1478	952	488	51.3	0.800	4.5

The average parous rate of *An. arabiensis* was 52.0%, ranged from 34.6% in Bukta to 57.9% in Shnebekuma (Table 6.2). Higher parous rate recorded in Shnebekuma than other villages ($F_{2, 33} = 6.974$; $p = 0.003$). Parous rate was varied and peak parous was recorded in March (69.0%) and October (63.0%) (Figure 6.1). The overall parous rate of *An. pharoensis* was 44.4.0%, ranged from nil in Workmidr to 66.7% in Shnebekuma; however, statistically insignificant ($F_{2, 33} = 1.094$; $p = 0.347$). Monthly parous rate of *An. pharoensis* was highly varied, between 100% (1/1) (July) and nil (all months, except July and June). The average parous rate of *An. funestus* was 63.2%, ranged from 25.0% in Workmidr to 69.3% in Shnebekuma. Even though statistically insignificant ($F_{2, 33} = 2.75$, $p = 0.079$), the highest parous rate (69.3%) of *An. funestus* was observed in non-irrigated village, Shnebekuma than others (Figure 6.1 and 6.2) (Table 6.3).

Table 6.2: Parous rates, daily survival and longevity of six *Anopheles* species in three villages

	Bukta				Workmidr				Shnebekuma			
	# D	% P	DS	L	# D	% P	DS	L	# D	% P	DS	L
An. ar	52	34.6	0.70	2.8	49	40.8	0.74	3.3	240	57.9	0.83	5.5
An. ph	4	50.0	0.79	4.3	2	0	0	0	3	66.7	0.87	7.4
An. fu	17	29.4	0.67	2.5	17	25.0	0.62	2.2	205	69.3	88.5	8.2
An. co	40	27.5	0.65	2.3	67	52.2	0.81	4.6	145	41.4	0.75	3.4
An. sq	6	33.3	0.69	2.7	13	30.8	0.68	2.5	68	48.5	0.79	4
An. ci	22	27.3	0.65	2.3	2	0	0	0	0	0	0	0

Note: An.ar = *An. arabiensis*, An.ph = *An. pharoensis*, An. fu = *An. funestus*, An.co = *An. coustani*, An.sq = *An. squamosus*, An.ci = *An. cinereus*, Un = unfed, D= No of dissected, P = % Parous rate, DS = Daily survival and, L = Longevity.

Peak parous rate of *An. funestus* was recorded only in March (83.0%). The overall parous rates for *An. coustani*, *An. squamosus* and *An. cinereus* were 42.0%, 44.8% and 25.0%, respectively. The overall parous rates of *An. coustani* and *An. squamosus* did not vary significantly between villages. However, the parous rate of *An. cinereus* showed variation among villages, and the highest rate recorded in Bukta (63.0%) ($F_{2, 33}$ = 5.044, p = 0.012). Monthly mean parous rates of three species (*An. coustani*, *An. squamosus* and *An. cinereus*) were highly varied, peak host seeking habit of *An. coustani*, *An. squamosus* and *An. cinereus* were 94.0% in October, 73.0% in October and 100.0% in May, respectively (Figure 6.1 and 6.2).

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Table 6.3: Mean densities of parous rates of six *Anopheles* species across three villages

Villages	*Anopheles* Species	M ± SE	P-values
Bukta	*An. arabiensis*	0.0713 ± .020[b]	0.003
Workmidr		0.102 ± .023[b]	
Shnebekuma		0.179 ± .020[a]	
Bukta	*An. pharoensis*	0.0502 ± .034	0.347
Workmidr		0.000 ± .000	
Shnebekuma		0.0398 ± .028	
Bukta	*An. funestus*	0.052 ±.019	0.079
Workmidr		0.033 ±.018	
Shnebekuma		0.371 ±.197	
Bukta	*An. coustani*	0.059±.079	0.867
Workmidr		0.058±.089	
Shnebekuma		0.076±.101	
Bukta	*An. squamosus*	0.029±.019	0.118
Workmidr		0.024±.017	
Shnebekuma		0.084±.028	
Bukta	*An. cinereus*	0.042±.0187[a]	0.012
Workmidr		0.000± 0.00[b]	
Shnebekuma		0.000± 0.00[b]	

Note: Means of each species followed by the same letter (s) are not significantly different from each other (P < 0.05, Tukey HSD)

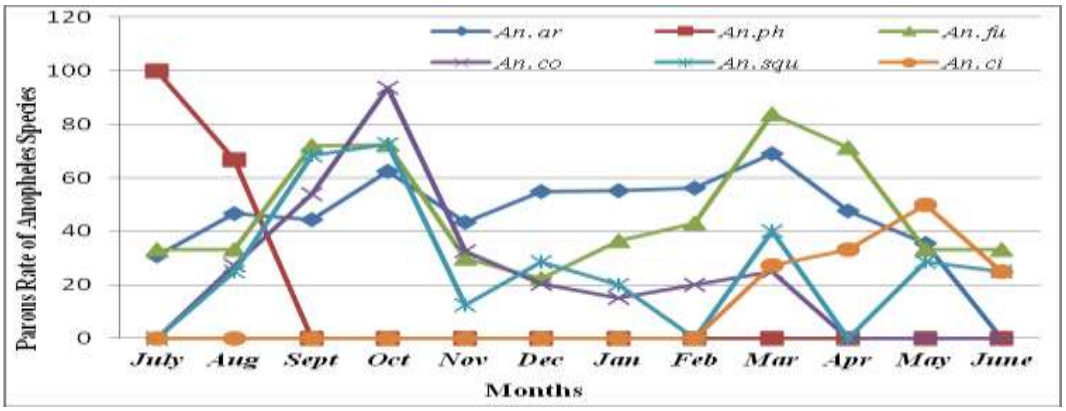

Figure 6.1: Overall monthly parous rate of six *Anopheles* species in Bure district. **(**An.ar =*An. arabiensis*, An. fu = *An. funestus*, An.ph = *An. pharoensis*, An.co = *An. coustani*, An.squ = *An. squamosus*, An.ci = *An. cinereus*, An.dem = *An. demeilloni*)

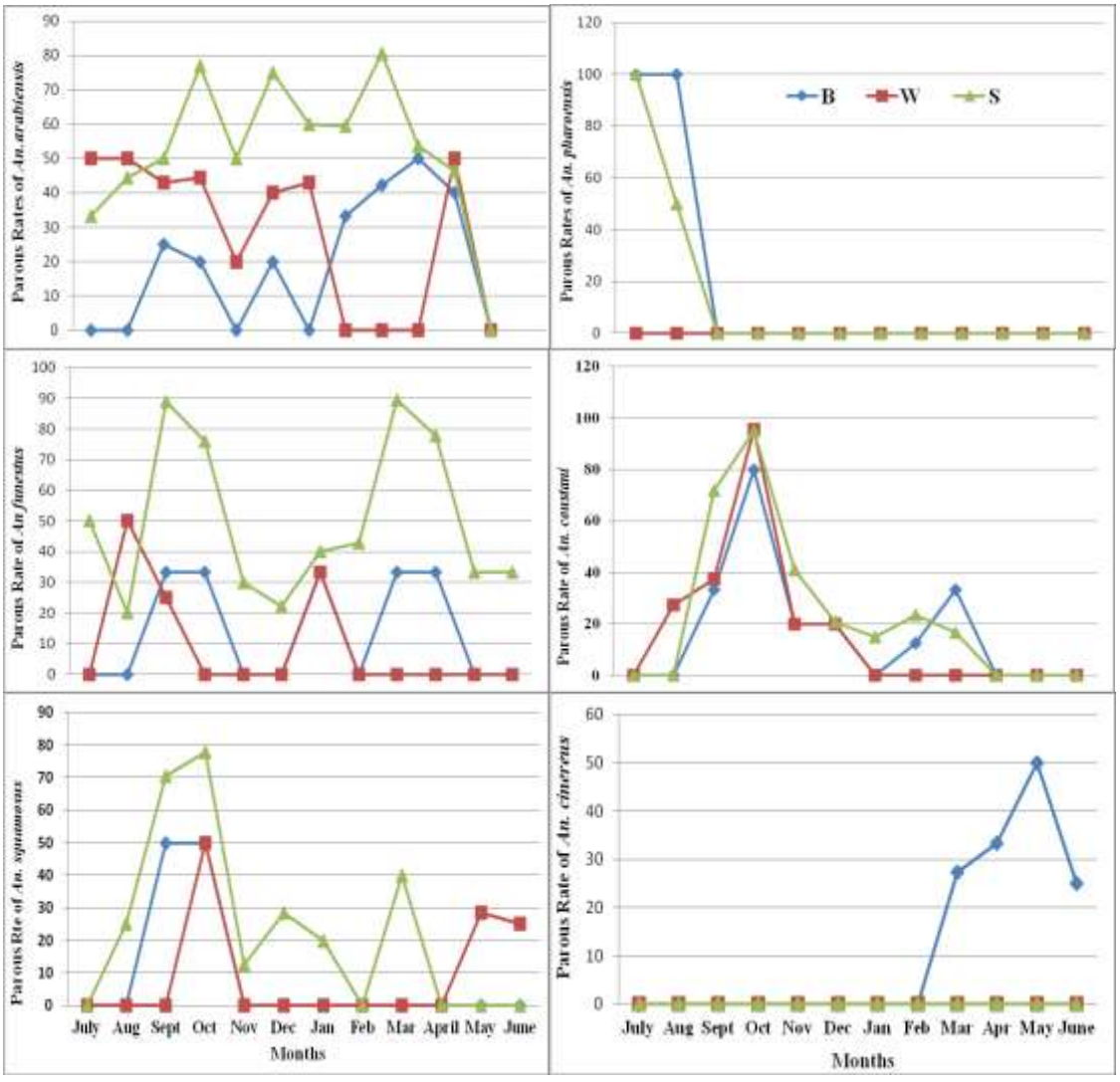

Figure 6.2: Monthly parous rates of six *Anopheles* species in three various ecological villages. B= Bukta, W= Workmidr, S = Shenbekuma

6.4.2 Longevity of *Anopheles* Mosquitoes

Table 6.1 and 6.2 shows the average longevity of six *Anopheles* species. Age variation was observed among each species and the same species in various ecological villages, ranged from 2.2 days (*An. cinereus*) to 6.5 days (*An. funestus*). The overall age of *An. arabiensis* was 4.6 days (ranged 2.8 to 5.5 days). Average longevity of *An. pharoensis*, *An. funestus*, *An. coustani*, *An. squamosus* and *An. cinereus* were 3.7 days (ranged from 0 - 7.4), 6.5 days (ranged from 2.2 - 8.2), 3.5 days (ranged from 2.3 - 4.6), 3.7 days (ranged 2.5 - 4) and 2.2 days (ranged from 0 - 2.3), respectively. In general, except age of *An. coustani* in Workmidr and *An. cinereus* in Bukta, the rest species had relatively highest longevity in Shnebekuma than other villages.

6.4.3 Sporozoite Rates of *Anopheles* Mosquitoes

The distribution of *Plasmodium* species (by vectors) and annual sporozoite rate of *Anopheles* species (collected only by using light traps) are shown in Table 6.4. In total, 1,474 *Anopheles* mosquitoes were analyzed for *Plasmodium* Circumsporozoite-Proteins (CSPs), out these 8 were from PSCs and 1,466 were from light traps, comprised 488 *An. arabiensis*, 350 *An. funestus*, 436 *An. coustani*, 152 *An. squamosus* and 40 *An. cinereus*. Eight specimens from PSCs were not positive samples (*Plasmodium* species) and not included in sporozoite rate and EIR calculation. Of the five tested species, sporozoites were only detected from *An. arabiensis*, *An. funestu* and *An. coustani*, belonged to two *Plasmodium* species, *P. falciparum* (25%) and *P. vivax* (75%). Of 1,466 specimens of *Anopheles* mosquitoes, only 4 specimens were positive for CSPs. Negative specimens (n= 192) for CSP species (*An. squamosus* and *An. cinereus*) were excluded for sporozoite rate and EIR calculation.

A single *An. arabiensis* and *An. coustani* were positive for *P. falciparum* and *P.vivax*, respectively and two *An. funestus* specimens were positive for *P. vivax* (not confirmed by PCR). The overall *Plasmodium* infective rate (*P. falciparum* and *P. vivax*) was 0.314% (4/1274). Infection rate of *An. arabiensis*, *An. funestus*, and *An. coustani* was (0.2 %, 1/488), 0.57% (2/350) and (0.23%, 1/436), respectively. Among the two variants of *P. vivax*, *P.v*-247 was detected from *An. funestus* and *P.v*-210 was from *An. coustani*. These results implied that these two species are responsible for malaria transmission in the area; however, *An. coustani* is needed further confirmation (Table 6.4).

Annual sporozoite rates of the three species showed different in the three villages (Table 6.4). All sporozoite infected *Anopheles* mosquito specimens were collected only from non-irrigated villages. In Shnebekuma *An. arabiensis* had 0.3%, *An. funestus* and *An. coustani* accounted 0.32% and 0.34%, respectively. In Workmidr, *An. funestus* accounted 5.6%. Monthly sporozoite rate distribution was varied (Table 6.5). Infected *Anopheles* mosquitoes were collected in July, December and January 2016. Higher sporozoite rate of *An. arabiensis* was recorded (7.14%, 1/14) in main rainy season (July) than all other seasons (nil). On the contrary, the largest sporozoite rate of *An. funestus* was 9.1% (1/11) and 4.76% (1/21) in small rainy season (December) and in dry seasons (January), respectively. Similarly, the sporozoite rate of *An. coustani* was 2.7% (1/37) and high during small rainy season (December) than all other seasons (nil).

Table 6.4: CDC light trap based assessment of sporozoite rates and EIRs of the three positive *Anopheles* species in the three villages

Sites	CSP-Positive Species	No of Tested Species	*P.f*, No (%)	*P.v*-247, No (%)	*P.v*-210, No (%)	Total No (%)	EIR
Shnebekuma	*An. arabiensis*	369	1(0.27)	0	0	1(0.3)	4.3
Workmidr		63	0	0	0	0	0
Bukta		56	0	0	0	0	0
Total		488	1(0.20)	0	0	1(0.20)	1.4
Shnebekuma	*An. funestus*	312	0	1(0.32)	0	1(0.3)	4.7
Workmidr		18	0	1(5.6)	0	1(5.6)	5.1
Bukta		20	0	0	0	0	0
Total		350	0	2 (0.57)	0	2(0.57)	3.3
Shnebekuma	*An. coustani*	297	0	0	1(0.34)	(0.34)	3.6
Workmidr		72	0	0	0	0	0
Bukta		67	0	0	0	0	0
Total		436	0	0	1(0.23)	1(0.23)	1.3
All Total		1274	1(0.08)	2(0.16)	1(0.08)	4(0.31)	5.7

Note: CSP = circumsporozoite protein; *P.f = Plasmodium falciparum*; *P.v = P. vivax*

6.4.4 Entomological Inoculation Rates of *Anopheles* Mosquitoes

Estimate annual and monthly EIRs of *An. arabiensis*, *An. funestsu* and *An. coustani* from CDC-LTs in three villages are shown in Table 6.3 and 6.4. The overall estimated EIR of *Anopheles* mosquitoes was 5.7 infectious bites /person /year (ib/p/y) for both *P. falciparum* and *P. vivax.* In particular, the overall annual *P.v*EIR of *An. funestus* was 3.3 ib/p/y, Workmidr had the highest (5.1 ib/p/y) than Shnebekuma (4.7 ib/p/y) and Bukta villages (0 ib/p/y). The overall annual *P.f*EIR of *An. arabiensis* was 1.4 ib/p/y, Shnebekuma had the highest (4.3 ib/p/y) than Workmidr (0 ib/p/y) and Bukta (0 ib/p/y) villages. The overall annual *P.f*EIR of *An. coustani* was 1.3 ib/p/y, Shnebekuma had the highest (3.6 ib/p/y) than Workmidr (0 ib/p/y) and Bukta (0 ib/p/y) villages.

Seasonal variation of EIR is shown in Table 6.4. Highest monthly estimated EIR of *An. arabiensis* was 1.6 ib/p/month during the main rainy month (July). Monthly estimation

of EIR of *An. funestus* was highest (1.7 ib/p/month) in dry season (January) followed by small rain season, December (1.3 ib/p/month). Monthly EIR of *An. coustani* was 1.3 ib/p/month and detected only in the small rainy season (December).

Table 6.5: Monthly CDC light trap based assessment of sporozoite and entomological inoculation rates of the three *Anopheles* species in Bure area

Months	CSP-positive species	No Tested	*P.f* No (%)	*P.v*-247, No (%)	*P.v*-210 No (%)	Total (%)	EIR
July 2015	*An. arabiensis*	14	1 (7.14)	0	0	1 (7.14)	1.6
December 2015	*An. funestus*	11	0	1 (9.1)	0	1 (9.1)	1.3
January 2016		21		1 (4.8)	0	1 (4.8)	1.7
December 2015	*An. coustani*	37	0	0	1 (2.7)	1 (2.7)	1.3

Note: Sporozoite infected mosquitoes were not detected during August, September, October, November, February, March, April, May and June; therefore, sporozoite rates and EIRs were zero, and are not included in the table.

6.5 Discussion

During this study period, six species included *An. arabiensis*, *An. funestus*, *An. coustani*, *An. squamosus*, *An. cinereus*, and *An. pharoensis* were dissected for parous rate determination. The parous rate of *An. arabiensis* (52%) in our study was lower than the 73.2% (Taye *et al*., 2006), the 68% (Massebo *et al*., 2013a), the 68.8% (Kenea *et al*., 2016), the 80% (Gari *et al*., 2016), the 57% and the 64.5% (Taye *et al*., 2016 & 2017) that reported in various parts of Ethiopia. The lower parous rate could be due to the influence of LLINs and IRS (Smith & McKenzie, 2004; Killeen & Smith, 2007). Currently, the distribution of LLINs is reached one for two individuals in the present

study area (Bure District Health Office, Unbub. Report, 2016; Personal Obser.). However, it is larger than the 2.3% (Woyessia, 2001), the 28.5% (Kibret *et al*., 2008) and the 33.4% (Aklilu, 2008) parous rates of *An. arabiensis* that reported in various parts of Ethiopia. This high proportion of parous rate might be connected with the closeness of the breeding habitats and the settlements result in better access to the hosts. In this study, all the three villages are found less than one kilometer radius to the breeding habitats.

The highest parous rate of *An. arabiensis* was 57% in non-irrigated village than irrigated village. This is probably due to the closeness of the breeding habitats and the occurrence of very large numbers of this species throughout the year, which was associated with breeding habitats. The presence of better precaution in irrigated village and presence of day-to-day supervision by the health expert could play a great role for such variation. Parous rate of *An. arabiensis* was peak in March (69.0%) and October (63.0%). In parallel, Kibret *et al*. (2009) reported higher parous rates of *An. arabiensis* during the dry months (March). In this study, the increased parous rate during October indicated that the working intervention had less effect to this mosquito population because reduction of parous rate was not observed immediately after the distribution of LLINs and application of IRS on the first week of September in the district, i.e., 1 - 7/ 09/ 2015 (from 26/12/2008 – 3/13/2008 E.C).

This study revealed that the overall parous rate of *An. pharoensis* was 33%, which was higher as compared to the 0% (Woyessia, 2001), the 28.2% (Aklilu, 2008) and the 16% (Gari *et al*., 20016) reports from different parts of Ethiopia. The closeness of the breeding sites to villages might be played a great role for such difference. However, it was very lower than the 71.7% (Dia *et al*., 2008), the 41.4% (Kibret *et al*., 2008) and the 60% (Taye *et al*., 2017) of the parous rates of *An. pharoensis* that reported from

Ethiopia and Africa. The lower parous rate could be due to the trapped of very few numbers of *An. pharoensis* throughout the study year in the three villages, only collected from July to September.

In our study the average parous rate of *An. funestus* (63%) was higher as compared to the 37% documented in Senegal (Dia *et al*., 2008) and the 48.7% documented in Mozambique (Charlwood & Tomas, 2011). The higher parous rate in this survey might be connected with the closeness of the breeding habitats and the dweller, result in better access to the hosts. The average parous rate of *An. coustani* was 42.0%, which was greater than the 7.5% report in Zeway Ethiopia (Kibret *et al*., 2008). The average parous rates for *An. squamosus* and *An. cinereus* were 45.0% and 25.0%, respectively. Monthly parous rates of *An. funestus*, *An. coustani*, *An. squamosus* and *An. cinereus* were highest in March, October, October and May, respectively. Therefore, these results indicated these species were gonotrophically older and could complete the life cycle of malaria parasite for malaria transmission. On the other hand, the annual average parous rates of *An. arabiensis*, *An. pharoensis*, *An. coustani* and *An. cinereus* were lowest, which showed these species were not old enough to transmit the disease efficiently throughout the year.

The present study demonstrated the occurrence of age variations among each species and the same species in various ecological villages. The overall longevity of *An. arabiensis* was 4.6 days, ranged from 2.8 days (Bukta) to 5.5 days (Shnebekuma). The difference between the two villages was due the presence of significantly higher numbers of *An. arabiensis* and sufficient moistures throughout the year in Shnebekuma. Generally, in this study the overall longevity (4.5 days) of *An. arabiensis* is not comparable with Gari *et al*. (2016) and Taye *et al*. (2016 & 2017) findings, who

documented between 7 to 25 days, 5.3 and 6.9 average days of *An. arabiensis* (*An. gambiae* s.l) in different parts of Ethiopia. This variation was linked with the mass emergence of *An. arabiensis* and the impacts of LLINs and IRS. Therefore, in our study district the average longevity of *An. arabiensis* was not sufficient to complete the life cycle of malaria parasite for malaria transmission throughout the year because *P. falciparum* is required from 12-14 days at 25°C and between 22-23 days at 20°C. Similarly, *P.vivax* is required 9 - 10 days at 25°C and 16 - 17 days at 20°C to complete their life cycle in mosquitoes (WHO, 1975).

In this study, the overall longevity of *An. pharoensis* was 2.7 days (ranged 0 - 7.4), which is larger than 1.6 days (ranged from 0-1.8 days) reported in Ethiopia (Gari *et al*., 2016). On the other hand, Taye *et al*. (2017) reported 5.9 average days (ranged from 3.3 - 8.6 days) of *An. pharoensis* in Ethiopia. The low life expectancy of *An. pharoensis* in this study was due to the collection of small proportion of *An. pharoensis*, which was only from July to September. Totally, the average annual longevity of *An. arabiensis*, *An. pharoensis*, *An. funestus*, *An. coustani*, *An. squamosus* and *An. cinereus* were very lowest for the completion of the sporogonic cycle in their bodies (WHO, 1975).

The present survey revealed that the overall annual CSP rate of all the *Anopheles* species (*P. falciparum* and *P. vivax*) was 0.31%, which is almost comparable to 0.33% that reported in Eritrea (Shililu *et al*., 2003). However, it was very higher than the other studies that made by Getachew (2017) from Ethiopia (0.12%) and Tanzania (0.02%) by Mwanziva *et al*. (2011). The highest sporozoite rate in our study was due to the presence of large proportion of the *Anopheles* mosquitoes across the year. These populations had better access to human blood meals. The expansion of maize farming in the district is also increased the chance of infection rate (Ye-ebiyo *et al*., 2000 &

2003; Kebede *et al*., 2005). On the contrary, the overall sporozoite rate of all *Anopheles* species in our study was very lower as compared with the other reports that conducted in various parts of Ethiopia (Tirados *et al*., 2006; Animut *et al*., 2013a; Kibret *et al*., 2017; Abraham *et al*., 2017) and in Africa (Mboera *et al*., 2010; Mzilahowa *et al*., 2012; Lwetoijera *et al*., 2014). This very low overall sporozoite rate may be due to cumulative effects of intensively usage of LLINs and IRS, resulted in a decline in human-mosquito contact, a decrease the number of mosquitoes and a reduction sporozoite inoculation rates into people (Norris *et al*., 2010; Bekele *et al*., 2012 & 2013).

The present result showed that the intensity of malaria transmission in the study area was very low. In line with low sporozoite rate, recently made epidemiological survey from health facilities indicated the reduction of malaria cases in most Amhara region including Bure-surrounding districts (Toyama *et al*., 2016). Many works also documented that all ELISA tested *Anopheles* species (e.g., *An. arabiensis*, *An. pharoensis*, and *An. funestus*) were without *Plasmodium* species in various parts of Ethiopia (Woyessa, 2001; Aklilu, 2008; Tesfaye *et al*., 2011; Bekele *et al*., 2012 & 2013; Gari *et al*., 2016; Kenea, 2016; Taye *et al*., 2016).

The overall annual *P.f*-sporozoite rate of *An. arabiensis* was 0.2 %, which was similar to (0.2%) Shililu *et al*. (2003) report in Eritrea. On the other hands, it was higher than Waka *et al*. (2005) (0.01%) and Getachew (2017) (0.06%) reports that conducted in Eritrea and Ethiopia, respectively. The closeness of breeding habitats and presence of large numbers of *An. arabiensis* in this study may be favoring such difference. Massebo *et al*. (2013a), Animut *et al*. (2013a), Yewhalaw *et al*. (2014) and Daygena *et al*. (2017) obtained relatively higher average annual sporozoite rates in *An. arabiensis* in various

parts of Ethiopia. Similarly, higher annual overall *P. f*-sporozoite rates of *An. arabiensis* have documented in different parts of Africa (Shililu *et al*., 2004; Cohuet *et al*., 2004; Mboera *et al*., 2010; Mzilahowa *et al*., 2012).

In this study, sporozoite infected *An. arabiensis* identified during main rainy season (July), as reported by Kibret *et al*. (2017); however, Daygena *et al*. (2017) detected from both in small rainy (October) and dry season (January). Generally, the reduction of *An. arabiensis* sporozoite rate in the study villages was due to the widespread adoption of LLINs that are known to be able to delay biting rate and reduce the mean survival of vector populations (Smith & McKenzie, 2004; Killeen & Smith, 2007) and the presence of very little or the absence of malaria parasites in the study district.

The overall annual sporozoite rate of *An. funestus* was 0.57%, is higher as compared to 0.03%, which reported from Senegal (Dia *et al*., 2008). However, it was very low as compared with the other reports in African (Dery *et al*., 2010; Mzilahowa *et al*., 2012; McCann *et al*., 2014; Stevenson *et al*., 2016) and in Ethiopia (Kibret *et al*., 2017). The low sporozoite rate could be due to the impact of LLINs and IRS (Smith & McKenzie, 2004; Killeena & Smith, 2007), and the presence of very little or the absence of malaria parasites in the study district.

The current study revealed the presence of *P.vivax*-247 infected *An. funestus* in the district. However no one showing *P.vivax* infected *An. funestus* in Ethiopia (Kibret *et al*., 2009 & 2017) and Africa countries (Dia *et al*., 2003; Mboera *et al.*, 2007; McCann *et al*., 2014; Stevenson *et al*., 2016). This could be an indication of shift of carrying the parasite from *P. falciparum* to *P. vivax.* *P.v*-247 infected *An. funestus* was collected only from outdoor during the small rainy (9.1%, December) and dry season (4.8%, January). Occurrence of an infection in December was consistent with the major

malaria transmission season of Amhara region and Ethiopia (Midekisa *et al*., 2012; Ayele *et al*., 2012). Similarly, Atangana *et al.* (2009) reported sporozoite infected *An. funestus* in dry season in Western Cameroona highlands.

In this study, *An. funestus* had high sporozoite rate than *An. arabiensis* and *An. coustani*. Previously, Fontaine *et al.* (1961) and MoH (2004) reported that *An. gambiae* s.l (*An. arabiensis*) was the only responsible vector for most of the transmission of malaria in the highland parts of Amhara region. However, the presence of dominantly infectious *An. funestus* in the current study suggesting that the populations of *An. funestus* experienced an adaptation to the new ecology of the highland parts of Bure district. Moreover, it indicated a replacement of the main malaria vector (*An. arabiensis*) due to climate change.

Anopheles coustani was third species obtained infected by *P.vivax*-210. This infectious *An. coustani* was collected from outdoor in December. Similar to the present finding, Kelel (2010) and Yewhalaw *et al.* (2014) reported the same *P.vivax*-210 infected *An. coustani* from Gilgel-Gibe hydroelectric dam area, southwestern Ethiopia. The overall annual sporozoite rate for *An. coustani* was 0.34%, which was very lower than the other reports that conducted in Ethiopia (0.68%) (Degefa *et al*., 2015) and in Kenya (1.3%) (Mwangangi *et al*., 2013). Likewise, higher allover sporozoite rate of *An. coustani* reported from Ethiopia (Yewhalaw *et al*., 2014) and from Kenya (Antonio-Nkondjio *et al*., 2006; Ogola *et al*., 2017) than the present study. In Kenya, Taveta district, *An. coustani* was well known outdoor malaria transmitter (Mwangangi *et al.* 2013). Moreover, the occurrence of infected *An. coustani* in December corresponds with the major malaria transmission season of Ethiopia (Ayele *et al*., 2012). Generally, until now *An. coustani* was not incriminated as vector of malaria in Ethiopia, but stand from

the aforementioned facts, their density (as described in chapter four) and human blood meal sources (as indicated in chapter five), this species might be a potential malaria vector in Ethiopia. Therefore, it needs a regular entomological surveillance and monitoring and appropriate incrimination of this species to determine its role in malaria transmission in Ethiopia.

The current study revealed the presence difference in sporozoite rate between villages. All infected mosquitoes were found in non-irrigated villages, in Shnebekuma and Workmidr. The average annual sporozoite rates of *An. arabiensis*, *An. funestus* and *An. coustani* were higher in non-irrigated than irrigated villages. Similar to our results, various study indicated the presence of significantly higher annual sporozoite rate in non-irrigated than irrigated villages in different parts of Africa (Appawu *et al*., 2004; Muturi *et al.*, 2008; Diakite *et al*., 2015). Ijumba *et al*. (2002) and Sogoba *et al.* (2007) also reported highest annual sporozoite rates for *An. arabiensis* and *An. funestus* in non-irrigated sites than irrigated (e.g., rice irrigated area) in Africa. However, contradicts to the current result, Kibret *et al*. (2014) obtained higher overall annual sporozoite rate of *An. arabiensis* (1.67%) from the irrigated village than non-irrigated villages (0.43%) in Zeway area, central Ethiopia. Similarly, Jaleta *et al*. (2013) from Ethiopia, Dolo *et al.* (2004), Muturi *et al*. (2008) and Mboera *et al*. (2010) in Africa found highest annual sporozoite rates for *An. gambiae* s.l and *An. funestus* in irrigated than non-irrigated sites.

The presence of infected mosquitoes in non-irrigated villages might be connected with the occurrence very large numbers (over 80%) of the adults of these three sporozoite infected vectors in the two non-irrigated villages throughout the year. The chance of being infected is very high for those mosquitoes with higher proportion. Non-irrigated

villages had more breeding habitats throughout the year, which helped to increase the vector survival rate by releasing enough moisture to the surrounding areas (Sogoba *et al*., 2007). Therefore, population dynamics and adult age structure are important determining factors for the ability of the vector to transmit malaria (Beck-Johnson *et al*., 2013). The presence of altitudinal difference could be the other source of variation. Kibret *et al*. (2017) didn't get any infected mosquitoes (e.g., *An. arabiensis*, *An. funestus* and *An. pharoensis*) in higher altitude (Koga Dam, 1950 m.a.s.l) than the lower altitude (Kessem Dam, 912 m.a.s.l; Koka Dam, 1,551m.a.s.l) in Ethiopia. In Tanzanian, Maxwell *et al.* (2003) documented 2.5 times higher sporozoite rate in low land than highlands. Moreover, the absence or the presence of little malaria in irrigated community could be the possible reason for the absence infectious mosquitoes in irrigated village (Ijmuba & Lindsay, 2001; Dolo *et al.*, 2004).

Generally, this study revealed that only *P. falciparum* and *P. vivax* were the common *Plasmodium* species in Bure district. This result is in agreement to the epidemiological study conducted in Bure surrounding districts and in the other Amhara zones that both *P. falciparum* and *P. vivax* were the prevalent parasites (Emerson *et al*., 2008; Alemu *et al*., 2012; Ayale *et al*. 2012; Toyama *et al*., 2016). In the current study, the proportion of *P. vivax* (75%, ¾) was over *P. falciparum* (25%, ¼) in mosquito's body, this shows the presence shift of parasite load. Consistent with the present finding, epidemiological studies made in various parts of Ethiopia indicated the reduction of the proportion of *P. falciparum* and the rise of the of *P. vivax* (onward 2011) (Deressa *et al*. 2003b; Alemu *et al*., 2011 & 2012, Tesfaye *et al*., 2012, Bekele *et al*., 2013; Tefera, 2014; Molla & Ayele, 2015; Abeku *et al.*, 2015). Similarly, Yeshiwondim *et al*. (2009) indicated the occurrence of high prevalence of *P. vivax* over *P. falciparum* in the highlands parts of Ethiopia. Totally, WHO (2016b) reported that *P.vivax* is only a major health problem in

Ethiopia than any African country. In the present study, the presence of high proportion of *P. vivax* over *P. falciparum* might be the result of very less attention (negligence) which given by the Ethiopia's ministry of health for 'less fatal' *P. vivax* parasite than *P. falciparum* (more prevalent and fatal malaria). Other possible reasons might be climate variability and *P. vivax* might have developed resistance for the currently used drug to control *P. falciparum* parasite (Alemu *et al*., 2012).

This study indicated that the combined sporozoite inoculation rate for both *Anopheles* species was 5.7 ib/p/y (overall annual EIR). It was extremely lower than those records from similar altitude and altitudes below this study district. In Chad, Kerah-Hinzoumbe *et al*. (2009) found 311 ib/p/y; in Ghana Appawu *et al*. (2004) reported 418 ib/p/y and in Malawi Mzilahowa *et al*. (2012) reported 183 ib/p/y. In Ethiopia (Jaleta *et al*., 2013; Kibret *et al*., 2017) and in Africa (Ijumba *et al*., 2002; Diakite *et al*., 2015), high infectivity bites per person per year were reported. Generally, the overall annual EIRs of *Anopheles* mosquitoes were very low, this mainly due to the influence of LLINs and IRS which reduces human-mosquito contact and sporozoite inoculation rates into people (Magesa *et al*., 1991; Bekele *et al*., 2012 & 2013). Moreover, the presence very minimal numbers of sporozoite infected mosquitoes in the district and the occurrence little or total absence of malaria parasite in the community (Ijmuba & Lindsay, 2001; Dolo *et al.*, 2004) could be the possible reason for such minimal annual EIRs in the three villages.

The overall estimated EIRs of the three individual species were varied across villages and seasons in Bure district. The estimated overall annual *P.f*EIR of *An. arabiensis* was 1.4 ib/p/y. It is very low as compared with those reported by Antonio-Nkondjio *et al*. (2006) (16.5 ib/p/y), Mboera *et al.* (2010) (728 ib/p/y) and Massebo *et al*. (2013a) (17.4

ib/p/y) in Cameroon, Tanzania and southwestern Ethiopia, respectively. In this study, the overall annual *P.v*EIR of *An. funestus* was 3.3 ib/p/y. It is very low as compared with Antonio-Nkondjio *et al*. (2006), Dery *et al*. (2010) and Mboera *et al*. (2010) reports, who reported between 6.5 - 151.4 ib/p/y (in various sites), 141ib/p/y and 12ib/p/y in Cameroon, Ghana and Tanzania, respectively. Similarly, *An. coustani* had lower overall annual *P.v*EIR which was 1.3 ib/p/y. It is not comparable from Antonio-Nkondjio *et al*. (2006) finding, who documented 3.4 ib/p/y for *An. coustani* in Cameroon.

In our study, from twelve months, infectious mosquitoes were detected only in three months (July, December and January), mainly during the main transmission season though the main malaria vectors were collected in all survey months. This is highly linked with large scale distribution and acceptance of LLINs and the application of IRS; resulted in a decline in human-mosquito contact and a reduction sporozoite inoculation rates into people (Magesa *et al*., 1991; Bekele *et al*., 2012 & 2013). The absence or the presence of little malaria cases in the community, the existence very low sporozoite or absence of infectious of these mosquitoes could be the possible reasons. In this situation, the probability of the mosquitoes to be infected by malaria parasite get reduce because mosquitoes bite more likely uninfected people (Churcher *et al*., 2015).

The overall annual EIR of all *Anopheles* mosquitoes in non-irrigated villages was higher than irrigated village. The annual *P.f*EIR of *An. arabiensis*, *An. funestus* and *An. coustani* showed similar trend. In agreement with these, Ijumba *et al*. (2002), Muturi *et al.* (2008) and Sogoba *et al*. (2007) found higher overall annual *P.f* EIR of *An. arabiensis* and *An. funestus* in non-irrigated areas than rice irrigated area/ villages in Tanzania, Kenya and Mail, respectively. However, inconsistent with the current

findings, Jaleta *et al*. (2013) documented higher estimated annual EIR (102 ib/p/y) of *An. arabiensis* in irrigated sugarcane agro-ecosystem, than in the traditional irrigated (22 ib/p/y) and non-irrigated agro-ecosystem (18 ib/p/y) in western Ethiopia. Recently, Kibret *et al*. (2017) reported extremely very large annual *P.f* EIR of *An. arabiensis* (129.8 ib/p/y) and *An. funestus* (47.8 ib/p/y) in dam-villages than non-dam villages, 15.6 ib/p/y for *An. arabiensis* and nil for *An. funestus* in lowland (Kesem dam) altitude in Ethiopia. Similarly, Mboera *et al.* (2010) found extremely high annual EIR of *An. gambiae* s.l (1350 ib/p/y) and *An. funestus* (270ib/p/y) in flooding rice irrigation ecosystem than non-irrigated ecosystem (sugarcane) (727ib/p/y, 55.7ib/p/y) or wet savannah (nil for both) in Tanzania, respectively.

The presence of sporozoite infected mosquitoes in non-irrigated villages might be due to the absence or the presence of little malaria in the community and low survival rates of the mosquitoes in irrigated village (Ijmuba & Lindsay, 2001; Dolo *et al.*, 2004). Within this situation, the probability of the mosquitoes to be infected by malaria parasite get reduce because mosquitoes bite more likely uninfected people (Churcher *et al*., 2015). In Gambia and Ethiopia, many reports have indicated the absence of sporozoite infected mosquitoes (e.g., *An. arabiensis, An. funestus* and *An. pharoensis*) (Jawara *et al*., 2008; Aklilu, 2008; Tesfaye *et al*., 2011; Bekele *et al*., 2012 & 2013; Gari *et al*., 2016; Kenea, 2016). Recently made survey (using health facilities) indicated the reduction of malaria cases in most Amhara region including Bure surrounding districts (Toyama *et al*., 2016). The presence of small proportion of *Anopheles* mosquitoes in irrigated village could be the other possible reason for the absence of annual EIRs in irrigated village. Moreover, the presence of altitudinal difference has played a great role for such variation. In Tanzanian, Maxwell *et al.* (2003) found 17 times greater average annual EIR in lowland than highland area. In the same country,

Kulkarni *et al*. (2006) obtained 47ib/p/y in low altitude and 2ib/p/y in higher altitude area. Kibret *et al*. (2017) didn't detect any infected mosquitoes (*An. arabiensis, An. funestus* and *An. pharoensis*) in higher altitude (Koga Dam, 1950 m.a.s.l) than the lower altitude (Kessem Dam, Koka Dam) in Ethiopia. The community may have better information on malaria (Audibert *et al*., 1990; Sissoko *et al*., 2004) because information sources such as the health center and primary school were established at the hub of irrigated village than non-irrigated villages.

6.6 Conclusions

The average parous rates and longevity of *An. arabiensis, An. funestus An. pharoensis* and *An. coustani* were higher in non-irrigated than irrigated village, except for *An. cinereus*. Generally, the average annual longevity of these vectors was not sufficient to complete the life cycle of malaria parasite for malaria transmission in the study period.

Following irrigation activity, the numbers of breeding habitats increase; result in an increased number of mosquitoes and a raised of malaria prevalence. However, this trend was not worked in this study area, because sporozoite infected mosquitoes found only in non-irrigated villages, Shnebekuma and Workmidr. *An. arabiensis, An. funestus* and *An. coustani* were involved in malaria parasites transmission. Proportion of *P.vivax* (75%) was over *P. falciparum* (25%) in *Anopheles* mosquitoes in Bure district; this indicated the dominance of *P. vivax* in the study area. However, the absence of epidemiological study was one of the limitations of this study.

Infectious mosquitoes were detected only in three months (July, December and January); it indicated the presence of continues malaria transmission in the area.

Annual *P.f*-EIR of *An. arabiensis*, *P.v*-EIR of *An. funestus* and *An. coustani* were higher in non-irrigated villages (Shnebekuma and Workmidr) than irrigated village (Bukta). The infectivity rate of the three mosquitoes in the study area was very low. However, the contribution of *An. funestus* s.l in the transmission of malaria at Bure area was higher than those of the other two vector species. Generally, the obtained infective bites per person per year was lowest (annual EIRs) and found to be less than ten (EIR < 10); therefore, malaria transmission status of the study area is very low and said to be unstable (Takken & Lindsay, 2003). However, it is not less than one infective bite per person per year to reach elimination level; therefore, much work is remained and it has to be done (Beier *et al*. (1999). Generally, the CSP-ELISA results indicated the availability of epidemiological studies in Bure district to know the exact proportion of *P. falciparum* and *P. vivax* to take other better action. Breeding habitats found in non-irrigated villages should be managed throughout the year. LLINs and IRS should be used continuous through the whole months. Malaria education must be given strictly.

Chapter 7: Composition, Distribution, Abundance and Habitat Characterization of Anophelines Mosquitoes Larvae in Bure District, Northwestern Ethiopia

7.1 Introduction

Mosquito larval habitats are the places where eggs are laid, larvae hatch and change into instars, then become pupate and adults (Overgaard *et al*., 2002, Eckhoff, 2011). *Anopheles* mosquitoes larval habitats are species-specific (Ramirez *et al*., 2009; Forstinus *et al*., 2015) and survive in large varieties of habitats (Washburn, 1995; Silver, 2008). Breeding habitats vary from place to place (Rozendaal, 1997; Dotson, 2010) include both natural (in more) and anthropogenic sites (Gimnig *et al*., 2002; Silver, 2008; Manguin & Boete, 2011), permanent or temporary, freshwater (Rueda, 2008) or saltwater (Varnado *et al*., 2012), and shaded or sunny (Machault *et al*., 2009).

The productivities of mosquito breeding habitats are determined by the interaction and the combination of both biotic and abiotic factors (Service 1977). The combined effects of these factors determine densities and rate of mosquito larvae development (Chase & Knight, 2003; Paaijmans, 2008; Stresman, 2010). Therefore, the presence and quality of mosquito larval habitats are said to be the most important inputs to adult distribution, abundance and fitness (Chen *et al*., 2006; Mwangangi *et al*., 2007b; Forstinus *et al*., 2015).

Previously, in Ethiopia malaria was commonly occurred less than 2,000 m.a.s.l (Fontaine *et al*., 1961; Lindsay & Martens, 1998; Woyessa *et al*., 2002); however, this trend has changed and the major malaria epidemics were seen in highland parts of the country, up to 3,000 meters (Graves *et al*., 2009; Tchuinkam *et al*., 2010) and still this problem is continuing in Amhara region (Tamiru *et al*, 2014; Lake *et al*., 2016) due to

climate (Senay & Verdin, 2005; Alemu *et al*., 2011; Mbogo, 2012) and land-use changes (Zhou *et al*., 2004), population movements, prevalence of drug and pesticide resistance (Fisiha, 2002; Heggenhougen *et al*., 2003), failure of malaria control programs, degradation of public health infrastructure and socioeconomic factors (Eskindir & Markussen, 2010).

In Ethiopia, four vector species are known by transmit malaria, but *An. arabiensis* Patton is the main malaria vector (Woyessa *et al.*, 2004); whereas *An. funestus* Giles, *An. pharoensis* Theobald and *An. nili* Theobald are secondary vectors (Krafsur & Armstrong, 1978; Balkew *et al*., 2006). Currently, the country is applying the three primary interventions to stop the impacts of malaria: mass distribution of LLINs, IRS, RDTs coupled with prompt and effective case management with artemisinin-based combination therapy (ACT) (Otten *et al*., 2009; Jima *et al*., 2010; Shargie *et al*., 2010).

However, in general, SSA Africa countries are not controlling malaria through larval habitats management due the lack of information and the presence of complicated vector biology of the three main vector species (*An. gambiae* s.s., *An. arabiensis* and *An. funestus*) and the shift in focus to the adult vectors (Walker & Lynch, 2007). As compared to adults control using LLINs and IRS, controlling malaria by controlling larval populations (via the breeding sites) is relatively simple and has many advantages because the larvae are usually found in masses, relatively immobile, often readily accessible, cannot change their habitat to avoid control activities and occupy minimal habitat area (Killeen *et al*., 2002; Pfaehler *et al.*, 2006; Soleimani-Ahmadi *et al*., 2013). Besides, the community is the primary player in larval management activity, this makes very effective and simple. Previously, through environmental modification of breeding sites, it was possible to reduced malaria transmission throughout the tropics (Fillinger

& Lindsay, 2011; Killeen *et al*., 2002) by eliminated African primary vectors species from larger areas of Brazil, Egypt, and Zambia (Soper, 1966; Utzinger *et al*., 2001; Killeen, 2003). In Tigray, Ethiopia, it was also possible to reduce the adult populations of *An. arabiensis* by 49% via source reduction (Yohannes *et al*., 2005).

Therefore, proper understanding and management of the larval habitat ecology, characterization of the larval habitats, identifying the relationships between biotic and abiotic factors are so important in determining larval densities and species assemblage, in controlling the adult vectors, and in designing effective malaria control programmes (Service, 1989; Rozendaal, 1997; Minakawa *et al*., 1999; Mwangangi *et al*., 2007a; Rueda, 2008; Li *et al*., 2009; Asmare *et al*., 2017). However, these are possible by having accurate knowledge and identification of the different types of vector breeding sites (Carter *et al*., 2000; Rueda, 2008), proper understanding of the aquatic stages of vectors (Minakawa *et al*., 1999) and the environmental factors that affect mosquito abundance (Overgaard *et al*., 2002; Rueda, 2008).

Totally, our understanding of *Anopheles* larval ecology is limited, and the knowledge is insufficient to achieve effective vector control through larval control means (Oaks *et al*., 1991). In the current study area, there was no any kind baseline data (study) in relation with the identification and dynamics of *Anophenes* larvae and characterization of their breeding habitats. Hence, this study was majorly focus on the assessments of composition, spatio-temporal distribution and abundance of *Anopheles* larvae, identification and characterization of the larval habitats in the highland fringe of Bure district, northwestern Ethiopia. Such base-line data is very essential to realize effective control mechanism in Bure in particular and other similar area across the country, in general.

7.2 Materials and Methods

7.2.1 Descriptions of the Study Area

The study was conducted in Bukta, Workmidr and Shnebekuma villages in Bure district, Northwestern Ethiopia. The larva of *Anopheles* mosquito was collected from different breeding habitats once a month in each village from July 2015 to June 2016. The detail descriptions of the three study villages are described in chapter three (section 3.1).

7.2.2 Sampling and Characterization of Mosquito Larva Breeding Habitats

7.2.2.1 Mosquito Larvae Sampling, Processing and Identification

Mosquito larvae collection, processing, identification and habitats characterization were carried-out longitudinally from July 2015 - June 2016 in Bukta, Workmidr and Shnebekuma villages.

Sampling techniques: Collection was carried out using WHO (2003 & 2013b) procedures. Larvae were collected using a standard 350 ml white dipper (Clarke Mosquito Control Products, Roselle, IL) from different breeding habitats such as rain pools, stream-pockets, stream-edges, marshlands, ditches, dam-edges and pits. From a large breeding habitat types, 10-dips were taken. Samples from very small breeding habitats such as hoof-prints and cart/car-track were taken using 5ml graduated pipettes and finally pooled into 10-dip.

After collection, the larvae and/ or pupae in the dipper (pipettes) were poured into white enamel sorting tray. And then, larvae were sorted and counted into genus *Anopheles* and *Culex*. *Culex*, 1st and 2nd *Anopheles* larvae and pupae stages were discarded. Finally, only 3rd and 4th instars of genus *Anopheles* were transferred in

separated labeled vial using a plastic pipette, and then immediately 75% ethanol alcohol was added to preserve. Soon after mounting each larva with EUPARAL-ESSENCE (Eucalyptol and aldehyde, # 6372D) on microscope slide, it was dried at room temperature and morphological identification at species level was carried out based on morphological criteria (Verrone, 1962b; Gillies & Coetzee, 1987) using compound microscope. Samplings were always done by the same individual in the morning (04:00 - 12:00AM) for about 30 min at each larval habitat. Dipper, funnel, sieve, large white tray, pipette, larval vials, 75% alcohol/, cotton wool, a pencil, and labeling paper were used for collection, sorting and packing purpose (WHO, 2003).

7.2.2.2 Environmental Characterization of Larval Habitats

Concurrently with larval sampling, the environmental characteristics of each larval habitat were measured and recorded for one consecutive year, from July 2015 - June 2016 in each month in three villages. The recorded environmental characteristics included water depth, water surface area, water current, permanent, light coverage, distance to the nearest house, surface debris coverage, algal coverage, vegetation status emergent plant coverage, turbidity, habitat type, and substrate type. The presence of larvae at high or low densities was determined by dipping. If *Anopheles* species could be seen without dipping or nearly every dip, the site was defined as having a high *Anopheles* species density. On the other hands, if sites without *Anopheles* larvae from ten dips, they were recorded as empty. Pupae were not recorded as they cannot be differentiated from non-*Anopheles* species in the field (Sattler *et al*., 2005). Water depth was measured using a plastic ruler at different points of each habitat and average depth was taken. Then, it was classified into less than 0.5m, between 0.6m and 1.0m and greater than 1.1m depth. The perimeter (area) of each breeding site was categorized by the estimation as shorter than 10 m, 11 - 100 m, and longer than 101 m. The distance

to the nearest house was measured by measuring tape, if less than 100m, if not it was estimated by visually. Finally, the distance to the nearest house was classified into four-classes: between 100m ranges (0-100, 101-200, 201-300, >301). Turbidity was measured by placing a water sample in a clean glass test tube and holding it against a white background; it was classified into clean and turbid level only. Intensity of light of the habitat was visually categorized as full sunlight, partial sunlight and shade visually (Minakawa *et al*., 1999; Mwangangi *et al*., 2007a; Majambere *et al*., 2008).

Permanent habitats were sampled at exactly the same location throughout the year, while sampling location of temporary habitats was changed depending on the availability of water. Temporary habitats are those containing water for a short period of time, approximately two weeks after the end of rainy season. Semi-permanent habitats are those containing water for over two weeks and less than 3 months after the rainy season ends. Permanent habitats are those containing water over 3-months (fed by surface or ground water) and are more stable systems. Breeding habitats categorized into temporary were rain-pools, cart/ track-prints and animal foot-prints; semi-permanent was stream-fringes, and permanent habitat types were marshlands, stream-edges, dam-edge, irrigation-canals, ditch and pits (for nursery purpose). Again, all these habitats were categorized into natural (marshlands, rain-pools, stream-edges and pockets) and artificial (pits, irrigation canal and dam-edge, car-tracks and animal prints). Substrate types were classified into muddy, sandy, gravel with soil. Surface debris coverage was classified visually as present and absent. Plants status was grouped into absent, emergent, submerged of a habitat using visually estimate (Liu *et al*., 2012).

Vegetation status (grass along the edges present) of the breeding areas was categorized into absent, fully-covered or partially-covered. Algal coverage was assigned as fully-

covered, periphery/ offshore or totally absent (Minakawa *et al*., 1999; Mwangangi *et al*., 2007a). Generally, all visual classifications were done by the researcher to maintain consistency.

7.3 Data Analysis

Data were subjected into SPSS Version-20 and analyzed. Densities of *Anopheles* with *Culex* larvae were compared by Independent-Samples T-Test ($p < 0.05$). Descriptive analysis (percentages, tables and graphs) was used to show the trend of the frequency of each *Anopheles* immature in each habitat types, villages and months. Variations in mean densities of the larvae among villages and environmental factors (characteristics) of the larval habitats were analyzed using one way analysis of variance (ANOVA) ($p < 0.05$). When significant differences were observed in ANOVA, Tukey test (HSD-Test) was used to separate the means ($p < 0.05$). Pearson correlation analysis was used to determine the association between abundance of mosquito's with environmental variables ($p < 0.05$). Before using of multiple step-wise linear regression, simple correlation analysis was applied; then only significant associations were further examined by multiple step-wise linear regression to determine the best predictor variables associated with relative abundance of each *Anopheles* larval species. All data were first transformed [log10 (x + 1)] to normalize the distribution before any analysis was carried out. The existence of larva was characterized as present when at least one larva was present or absent when the absence of any larva in each supervised breeding habitats.

7.4 Results

7.4. 1 Diversity, Distribution and Productivity of Larval Habitats

In the present study, all larval breeding habitats were found within one kill meter radius from each settlement. Of 323 potential breeding habitats surveyed, mosquito larva was

collected from only 212 habitats in ten different habitats in the three villages throughout the year. Of 212 habitats positive for mosquito larvae, 22% were productive for only *Anopheles* larvae while 11.1% were productive for only *Culex* larvae. However, the largest proportions, 32.5% were productive for both *Anopheles* and *Culex* larvae. Generally, from 323 supervised habitats, 54.5% had *Anopheles* larvae while 45.5% had no any *Anopheles* mosquito at all (Table 7.1).

Table 7.1: Breeding sites of *Anopheles* and *Culex* larvae in Bure area, from July 2015 - June 2016

Types of Surveyed Habitats	No of Visited breeding Sites	Habitats Held		
		Only *Anopheles spp.*	Only *Culex*	Mosquitoes
		No (%)	No (%)	No(%)
Marshes	60	2(3.3)	22(36.7)	24(40)
Rain-pools	36	7(19.4)	3(8.3)	26(72.2)
Stream -edges	30	15(50)	3(10)	6(20)
Stream-pockets	34	14(41.2)	0	20(58.8)
Pits for nursery	32	24(75)	0	8(25)
Irrigation-canals	27	9(33.3)	5(18.5)	4(14.8)
Drainage-ditches	12	0	0	9(75)
Dam-margins	8	0	0	8(100)
Hoof-prints	60	0	0	0
Tyre-tracks	24	0	3(12.5)	0
Total Numbers	323	71(22)	36(11.1)	105(32.5)

Overall ten potential breeding habitat types (marshes, rain-pool, stream-pockets and edges, ditch, dam-edge, irrigation-canals, pits, hoof-prints and tyre-tracks) were identified throughout the year in the three villages (Figure 7.1). However, these breeding sites were not uniformly distributed in the three villages. Marshes, rain-pools,

and hoof-prints were common in the three sites; whereas tyre-tracks, stream-pockets and edges found only in Bukta and Shnebekuma villages. Besides, pits in Workmidr, ditch in Shnebekuma, and dam-edge and irrigation canals in Bukta were restricted. Of 10 habitats, only marshes, stream pockets and edges, pits, irrigation-canals and ditch were present during the dry months. From all surveyed habitats, hoof-prints and tyre-tracks were devoid of any single *Anopheles* larva; however, tyre-tracks had few numbers of *Culex* larvae (Table 7.1 and 7.3). Productivity of eight habitats were measured, only marshland (1.37 ± 0.05) and ditch (1.33 ± 0.05) were significant productive than others (ANOVA, $F_{7, 88} = 2.806$; $p = 0.011$) and dam-edge was the least productive (0.45 ± 0.19) (Table 7.2).

Table 7.2: Mean (larvae/10dips) of *Anopheles* mosquitoes larvae in various breeding habitats in Bure area (M= mean; SE = standard error of mean)

Habitat Types	**M ± SE**	**P- value**
Marshes	1.37 ± 0.05^{a}	0.011
Rain-pools	0.77 ± 0.23^{ab}	
Stream –edges	1.11 ± 0.16^{ab}	
Stream-pockets	0.95 ± 0.21^{ab}	
Pits for nursery	1.01 ± 0.19^{ab}	
Irrigation-canals	1.19 ± 0.26^{ab}	
Drainage-ditches	1.33 ± 0.05^{a}	
Dam-edge	0.45 ± 0.19^{b}	

Note: Means of the same letter (s) are not significantly different from each other. Tukey HSD Test ($p < 0.05$)

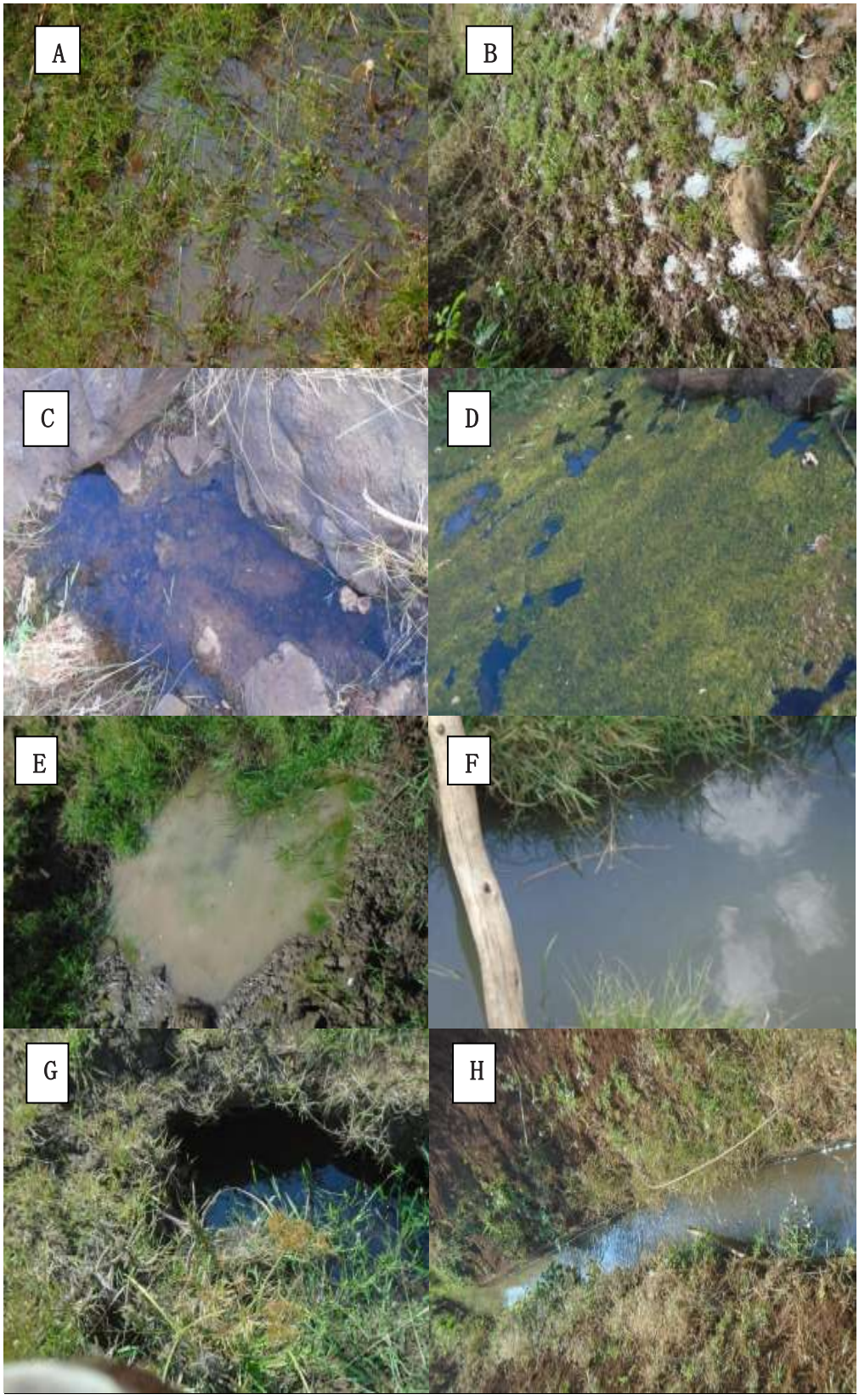

Figure 7.1: Types of breeding habitats [A) marshes, B) cow prints, C & D) stream-pockets with and without algae E) rain-pools, F) stream-edges, G) pit, and H) irrigation canal] found in the three sites

Table 7.3: Distribution and abundance of mosquito's larvae by breeding habitats and by villages, from July 2015- June 2016 (Values in parentheses indicate percentage)

Surveyed Habitats	***An. gambiae***	***An. funestus***	***An. pharoensis***	***An. coustani***	***An. squamosus***	***An. cinereus***	***An. demeilloni***	**Total**
Marsh lands	104 (13.1)	110 (21)	22 (91.7)	129 (28.2)	94 (34.7)	57 (28.1)	351 (28.9)	867 (24.8)
Rain pools	265 (33.3)	151 (28.8)	2 (8.3)	29 (6.3)	14 (5.2)	0	171 (14.1)	632 (18.1)
Stream edges	107 (13.4)	80 (15.3)	0	32 (7)	7 (2.6)	40 (19.7)	179 (14.7)	445 (12.8)
Stream pockets	146 (18.3)	84 (16)	0	89 (19.4)	8 (3)	16 (7.9)	128 (10.5)	471 (13.5)
Pits	98 (12.3)	22 (4.2)	0	0	0	0	101 (8.3)	221 (6.3)
Irrigation canals	16 (2)	40 (7.6)	0	135 (29.5)	101 (37.3)	63 (31)	144 (11.9)	499 (14.3)
Dam-edges	5 (0.6)	0	0	15 (3.3)	7 (2.6)	19 (9.4)	40 (3.3)	86 (2.5)
Ditch	55 (6.9)	37 (7.1)	0	29 (6.3)	40 (14.8)	8 (3.9)	100 (8.2)	269 (7.7)
Hoof prints	0	0	0	0	0	0	0	0
Tyre tracks	0	0	0	0	0	0	0	0
All Total %	796 (100)	524 (100)	24 (100)	458 (100)	271 (100)	203 (100)	1214(100)	3490 (100)
Villages								
Bukta	261 (32.8)	161 (30.7)	11(45.8)	243 (53.1)	73 (26.9)	140 (69)	264 (21.7)	1153 (33.0)
Workmidr	261 (32.8)	95 (18.1)	5 (20.8)	53 (11.6)	28 (10.3)	6 (3)	401 (33)	849 (24.3)
Shnebekuma	274 (34.4)	268 (51.1)	8 (33.3)	162 (35.4)	170 (62.7)	57 (28)	549 (45.2)	1488 (42.6)
All Total %	796 (100)	524 (100)	24 (100)	458 (100)	271 (100)	203(100)	1214(100)	3490 (100)

7.4. 2 Composition and Mean Densities of *Anopheles* larvae in Various Breeding Habitats

The composition of each *Anopheles* larval in different breeding habitats is described in Table 7.3. Totally, 9, 243 III and IV stages of mosquito larvae were collected from July 2015 to June 2016 from the three surveyed villages. Of these, 37.8% were *Anopheles* while 62.2% were *Culex.*

Of 3,490 *Anopheles* larvae, morphologically seven species of *Anopheles* larvae were identified, composed of *An. demeilloni* (34.8%), *An. gambiae* s.l (22.8%), *An. funestus* s.l (15.0%), *An. coustani* s.l (13.1%), *An. squamosus* (7.8%), *An. cinereus* (5.8%), and *An. pharoensis* (0.7%) (Table 7.3 and Figure 7.2). All the seven species were collected in each village. There was significant difference in mean density among different species of *Anopheles* mosquito larvae ($p < 0.001$) (Table 7.4).

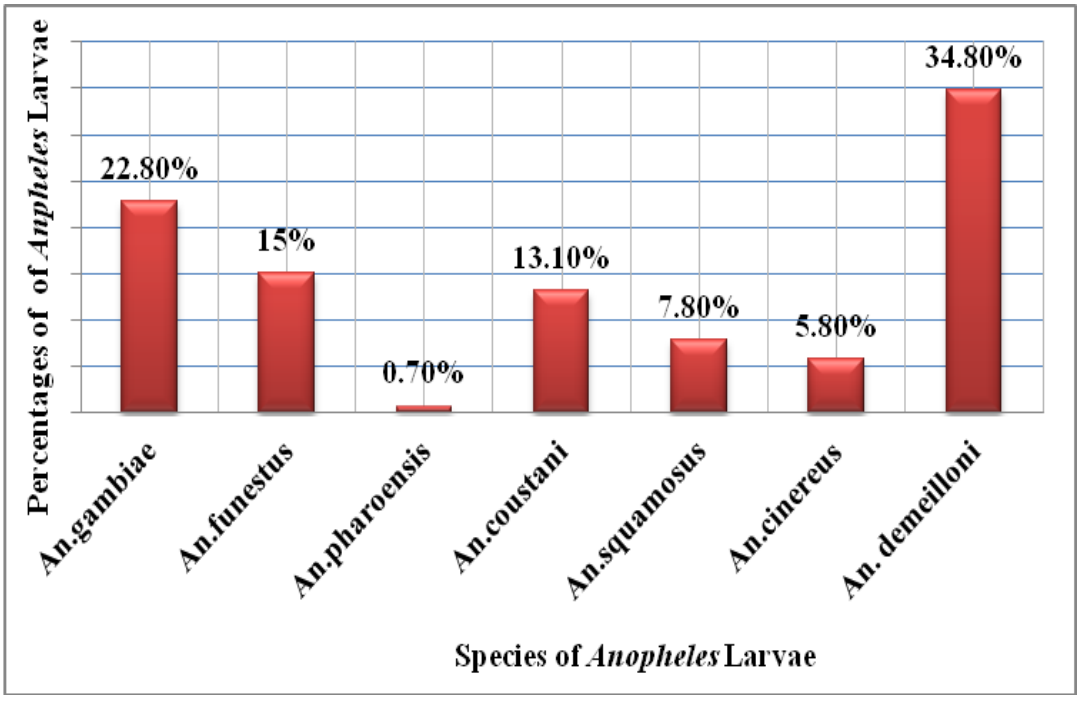

Figure 7.2: Species percentages of *Anopheles* larva in Bure district

Table 7.4: Mean densities of *Anopheles* larvae by sites and by species (larvae/ 10dips)

Sites	**M ± SE**	**P-value**
Bukta	23.68 ± 4.79	0.212
Workmidr	18.88 ± 3.32	
Shnebekuma	27.86 ± 1. 79	
Anopheles Species	**M ± SE**	0.001
An. gambiae s.l	1.79 ± 0.06^{ab}	
An. pharoensis	0.18 ± 0.04^{e}	
An. funestus s.l	$1.63 \pm 0.12a^{bc}$	
An. coustani s.l	$1.42 \pm 0.10b^{cd}$	
An. squamosus	1.26 ± 0.18^{cd}	
An. cinereus	$0.98 \pm 0 .06^{d}$	
An. demeilloni	1.97 ± 0.03^{a}	

Note: Means of the same letter (s) are not significantly different from each other. Tukey HSD Test ($p < 0.05$)

Generally, most identified species were observed over six different types of habitats. *Anopheles gambiae* s.l and *An. demeilloni* were found in all habitats except in hoof-prints and tyre-tracks; whereas *An. funestus* s.l was collected in all habitats except in hoof-prints, tyre-tracks and dam-edges. *An. coustani* s.l, *An. squamosus* and *An. cinereus* were collected in the same seven habitats, except pits, hoof-prints and tyre-tracks. However, different from all *Anopheles* larvae, the larva of *An. pharoensis* was found only in marshlands and rain pools during the rainy season only (Table 7.3).

7.4.3 Spatio-Temporal Occurrence of *Anopheles* Larvae from Three Villages

Of three thousand four hundred ninety *Anopheles* larvae collected, 42.6% were from non-irrigated village (Shnebekuma) while 33% and 24.3% were from Bukta and

Workmidr villages, respectively (Figure 7.3). However, mean densities of the larvae of *Anopheles* mosquitoes indicated the absence significant difference between the three different villages (ANOVA: $F_{2, 33} = 1.628$; p= 0.212) (Table 7.4).

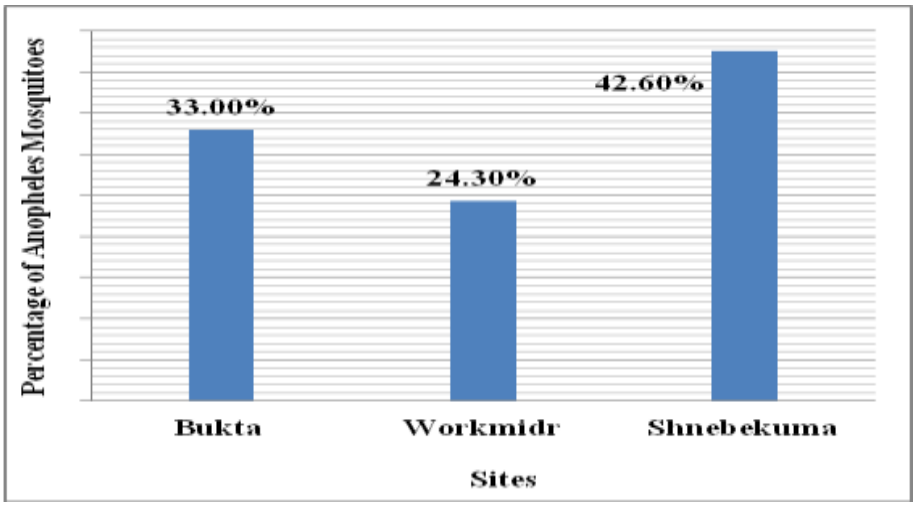

Figure 7.3: Percentages of the larva of *Anopheles* mosquitoes in three sites

In Shnebekuma, the highest *Anopheles* larvae were collected during main rainy season (September) with mean monthly density 35.2 larvae/ 10dips; whereas the lowest was recorded during the end of dry season (April) with mean density 16.2 larvae/ 10dips. Contrast to Shnebekuma, in Bukta the peak mean densities of *Anopheles* mosquitoes were observed during dry season, February (46.8 larvae/ 10dips). Similarly, in Workmidr the highest mean densities of *Anopheles* larvae were recorded during the dry season, January (57.5 larvae/ dip). In general, most of the larvae were collected during dry season, from January to March than in main rainy and small rainy season (Figure 7.4).

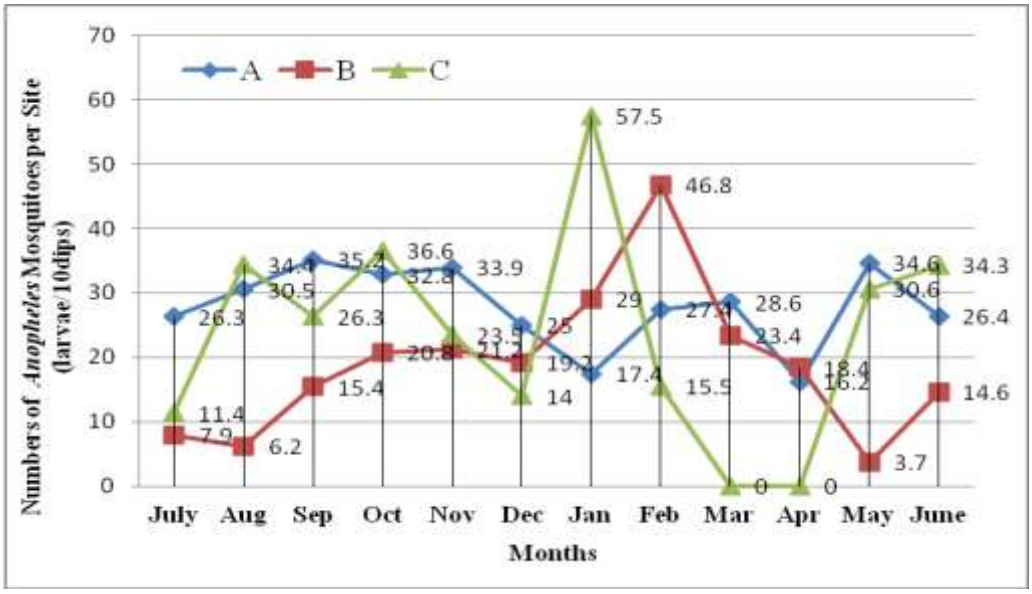

Figure 7.4: Overall temporal abundance of *Anopheles* larvae in three villages, from July 2015- June 2016. A = Shnebekuma, B = Bukta and C = Workmidr. Main rainy season = July – Sep; Small rainy = Oct – Dec and May – June; and Dry = Jan - April

7.4.4 Characterization of Larval Habitats and Environmental Factors Associated with Larval Occurrence and Abundance

Thirteen environmental characteristic of the larval habitats and the mean densities of six *Anopheles* larvae are described in Appendix 7.1 and 7.2. Significantly higher mean densities of *An. gambiae* s.l and *An. funestus* s.l larvae were collected from natural, permanent and temporary aquatic habitats that had clean and standing water, muddy, without mats of algae and plants. Fully sun-exposed breeding habitats with less than 0.5m depth and 10m diameters were the most favorable environmental characteristics for these two species. Additionally, habitat with debris was other supportive characteristics of the occurrence of *An. funestus* s.l larvae ($p < 0.05$).

Similarly, significantly higher mean densities of *An. coustani* s.l, *An. squamosus* and *An. demeilloni* larvae were also sampled from permanent and natural habitats that had

clean and still water current. The majority of these habitats hadn't algae and plants, and were fully sun-exposed and closer to the inhabitants (<100m), and had less than 0.5m depth and 10m diameter. Muddy habitats with peripheral vegetation or without vegetations were the most favorable breeding habitats for these three larvae. Moreover, still water habitat was the other conducive parameter for both *An. coustani* s.l and *An. demeilloni* larvae ($p < 0.05$). The larvae of *An. cinereus* also were found in permanent habitats. Stagnant water, fully sun-exposed habitats with peripheral vegetations, less than 1m depth and 10m diameters were the most favorable characteristics for the occurrence of highly significant densities of *An. cinereus* larvae. Moreover, muddy, debris habitats without plants and algae were the others fovrable parameters for the occurrence and the development of *An. cinereus* ($p < 0.05$).

Correlation analysis of each environmental variable and the densities of *Anopheles* larvae are described in Table 7.5. Mean density of *An. gambiae* s.l larvae were significantly and positively correlated with still water current, shallow habitat depth, small diameters and habitats closer to the house. *An. coustani* s.l larval density was negatively correlated with habitats situated close to houses. The occurrence and the abundance of *An. squamosus* larvae were significantly and positively correlated with natural habitat, matted algae, shallow habitat depth and habitats closer to the house.

Table 7.5: Correlation coefficients between environmental variables and densities of *Anopheles* larvae

Environmental variables	*An. gambiae*	*An. funestus*	*An. coustani*	*An. squamosus*	*An. cinereus*	*An. demeilloni*
Permanence habitat	0.085	0.07	0.485	-0.024	0.03	0.052
Sun light exposed	0.547	0.204	0.224	0.312	-0.0132	-0.074
Clean water	0.181	0.135	0.55	0.101	-0.081	0.122
Still water current	0.676*	0.281	0.021	0.419	-0.14	-0.137
No surface debris	0.306	0.506	0.42	0.266	-0.224	0.047
Presence of algae	0.442	-0.09	-0.05	0.744**	0.071	-0.193
Presence of plants	0.559	0.237	0.257	0.281	-0.19	-0.021
Presence of grasses	0.512	0.146	0.167	0.367	-0.226	-0.049
Natural habitats	0.541	0.281	0.045	0.619*	-0.257	-0.032
Without substrate	0.392	0.257	0.518	0.312	-0.132	-0.074
Habitat depth < 0.5m	0.600*	-0.073	-0.038	0.611*	-0.287	-0.185
Diameter < 10m	0.630*	0.197	0.060	0.394	-0.110	-0.195
Nearest distance to house (< 100m)	0.578*	0.155	-0.593*	0.624*	-0.373	-0.294

Note: ** and * Correlation is significant at the 0.01 and 0.05 level, respectively

Further multiple step-wise linear regression analysis was run to assess key environmental variables associated with abundance of *Anopheles* larvae (Table 7.6). Based on multiple step-wise linear regression analysis, from still water current, habitat depth, diameter and habitat distance closer to the house, only still water current was statistically significantly predicted the density of *An. gambiae* s.l [$F_{1,10} = 8.417$; $p < 0.016$, $R^2 = 0.457$] ($p < 0.05$). Distance to house was the main predictor for *An.*

coustani s.l larvae [$F_{1,10} = 5.426$; $p < .042$; $R^2 = 0.352$] ($p < 0$ .05). A multiple regression was run to predict density of *An. squamosus* from natural habitat, algae presence, shallow habitat depth and distance to house. Only presence of algae statistically significantly predicted *An. squamosus* larvae [$F_{1,10} = 12.377$, $p < .006$, $R^2 = 0.553$] ($p < 0.05$). Generally, based on this analysis, the relative abundance of *An. gambiae* s.l and *An. squamosus* were positively associated with still water current and presence of matted algae, respectively; whereas the larvae abundance of *An. coustani* s.l was negatively correlated to distance to the nearest house.

Table 7.6: Multiple step-wise regressions for three common species of *Anopheles* larvae in relation to habitat characteristics

Species	**Environmental parameter**	**t**	**P-value**
An. gambiae s.l	Still water current	2.901	0.016*
An. coustani s.1	Habitats near to house	-2.329	0.042*
An. squamosus	Presence of algae	3.518	0.006*

*Significant at the 0.05 level. C = Coefficient and SE = Standard Error

7.5 Discussion

This study was identified the types of *Anopheles* larvae, potential breeding habitats and characterized all potential breeding habitats in the three villages in Bure district. Seven species of *Anopheles* larvae (*An. gambiae* s.l, *An. funestus* s.l, *An. pharoensis*, *An. coustani* s.l, *An. squamosus*, *An. cinereus* and *An. demeilloni*) were identified in Bukta, Workmidr and Shnebekuma. Of these, *An. demeilloni* (34.8%) was the most predominant larvae and found up to 2157 m.a.s.l. The occurrence of *An. demeilloni*

larva at higher altitude is similar to the other findings, Gone *et al*. (2014) reported the presence of *An. demeilloni* at 2,650 m.a.s.l in highland parts of Derashe district, southern Ethiopia and Animut *et al*. (2012) reported at 2,196 m.a.s.l in Wurib, south-central Ethiopia. Previous works have showed the presence of *An. demeilloni* in various highland parts of Ethiopia (Ovazza & Neri, 1955; O'Connor, 1967), in highlands of Western Kenya (Mulambalah *et al*., 2011) and in highlands of Eretria (Shililu *et al*., 2003). However, in this study the proportions of *An. demeilloni* were very high; this is connected with incidence of climatic changes (global warming) due to ecological changes (Afrone *et al*., 2006; Checchi *et al*., 2006; Tesi, 2011; Siraj *et al*., 2014) and the presence extensive maize cultivation in Bure district. Due to maize cultivation, tremendous quantities of pollens are produced, which favored for the positive growth of these larvae (Ye-ebiyo *et al*., 2000 & 2003; Kebede *et al*., 2005; Minakawa *et al*., 2005). Moreover, the presence of breeding habitats during the dry months (January to March) have played great role for such abundance.

In our study, *Anopheles gambiae* s.l (22.8%) (Presumably *An. arabiensis*) was the second dominant larvae at altitude of 2,157 m.a.s.l. In line with this finding, Tesfaye *et al*. (2011) found *An. gambiae* s.l in Butajira highland (2,280 m.a.s.l); Wayessa *et al.* (2004) reported from outskirts of Addis Ababa (2,110 m.a.s.l); Animut *et al*. (2012) collected from Wurib town (2,196 m.a.s.l), and Kibret *et al*. (2017) found from Koga villages (1,950 m.a.s.l) in Ethiopia. Similarly, Minakawa *et al*. (1999), Chen *et al*. (2006), and Kweka *et al*. (2015) reported the presence of *An. gambiae* s.l larvae in the highland parts of Kenya. However, the proportion of *An. gambiae* s.l in our study was higher than the above reports; this is due to the above described reasons.

In Ethiopia, *An. arabiensis* is the leading malaria vector (Krafsur & Armstrong, 1978; White *et al*., 1980). Previously, the occurrence of malaria epidemics in the highland

parts of Amhara region (Kiszewski & Teklehaimanot, 2004; Negash *et al*., 2005; Graves *et al*., 2009) were due to *An. gambiae* s.l, the solely responsible vector for *Plasmodium* parasites (Fontaine *et al*., 1961). Moreover, this study detected sporozoite infected *An. arabiensis* (as described in chapter six). Therefore, the occurrence of *An. gambiae* s.l larvae in a large proportions have increased disease transmission role by increasing adult densities; this indicate the necessity of managing the larval habitats to minimize the adult density and to stop their transmission role.

Anopheles funestus s.l was the third largest larval species found in a higher altitude (up to 2,157 m.a.s.l). The incidence of this larva was reported previously by Verrone (1962b) and O'Connor (1967) in Amhara region, Ethiopia. Recently, Kibret *et al*. (2017) collected very few numbers (n = 6) of *An. funestus* in Koga villages at 1,950 m.a.s.l. Totally, different from our finding, study conducted in Ethiopia (Animut *et al*., 2012; Gone *et al*., 2014; Kibret *et al*., 2017) and in Africa (Minakawa *et al*., 2005; Imbahale *et al*., 2011; Kweka *et al*., 2015) indicated the presence of *An. funestus* below 1,950m.a.s.l. In Bure district, *An. funestus* was collected in each month throughout the year with large proportions; this indicate Bure district is very suitable for this species due to the presence of increased temperature (global warming), extensive maize cultivation and larval breeding habitats during dry months. Furthermore, this study detected sporozoite infected *An. funestus* (as described in chapter six). Therefore, the results of this study suggests that there should be surveillance and monitoring of this species by the national program for targeted control as historically it was one the secondary vectors of malaria in some parts of Ethiopia (MoH, 2010).

Anopheles pharoensis was the least available larval species (n = 24) in higher altitude of Bure area. This observation was consistent with Animut *et al*. (2012) and Kibret *et*

al. (2017) report in Ethiopia. The rarity could be attributed by the influence of predators; predation rate is high in large and permanent than temporary habitats (Service, 1977; Washburn, 1995; Sunahara *et al*., 2002). Naturally, this species needs permanent and fully swamps habitats. Besides, the presences of human and cattle interfere were the other possible reason for rarity of the larvae of *An. pharoensis* because the larvae of mosquitoes need very calm environment. Furthermore, this study discovered the larva of *An. coustani* s.l, *An. squamosus* and *An. cinereus* at 2,157 m.a.s.l. Consistent to these results, Woyessa (2001), Woyessa *et al*. (2004), Animut *et al*. (2012) and Gone *et al*. (2014) reported the larva of *An. cinereus* between 2,110 m.a.s.l to 2, 650 m.a.s.l in various parts (Akaki town and its environs, Wurib and Derashe district) of Ethiopia. Moreover, the occurrence of *An. coustani*, *An. squamosus* and *An. cinereus* have documented by previous studies conducted in Addis Ababa around Filwoha (2,457 m.a.s.l), in Entoto (3,000 m.a.s.l) and in Gojam (Amhara region), Ethiopia (Ovazza & Neri, 1955; Verrone, 1962b; O'Connor, 1967).

The result of current study revealed the presence of relatively noticeable monthly and seasonal variation in *Anopheles* larvae. The overall higher density of *Anopheles* larvae were observed during dry season (January – March). This was contrary with various studies (Shililu *et al*., 2003; Kibret *et al*., 2009; Kenea *et al*., 2011). Kibret *et al*. (2009 & 2012) demonstrated very peak larval densities during and immediately after the main rainy season; whereas the least larval density was recorded between January and March. Kenea *et al*. (2011) also reported low and high mean density of *Anopheles* larvae in December and in March, respectively. The presence of breeding habitats in dry months together with appropriate temperature could be attributed factors of variation in densities across month and season in this study. In workmidr, mosquitoes were not collected during dry season (March and April); this is probably due to the

reduction water level and the presence of disturbance of the pits water during fetching time for seedlings purpose. On the other hand, in Bukta, the proportion of collected mosquitoes during May was very low, which might be linked with flooding as a result of rain.

The numbers and productivity of larval habitats ultimately determines the distribution and the abundance of the adults (Gimnig *et al*., 2001 & 2002). In parallel, habitat types influence the development and the survivorship of *Anopheles* larvae (Munga *et al*., 2007). This study discovered ten important breeding habitats; both *Culex* and *Anopheles* were coexisted in all habitats except in vehicle ruts and hoof-prints. In western Kenya (Minakawa *et al*., 1999; Fillinger *et al*., 2004) and in Ghana (Kudom, 2015), both the subfamilies Culicinae and Anophelinae shared the majority of the habitats. Similarly, Dejenie *et al*. (2011) observed the same situational between 2,000 and 2,070 m.a.s.l in Tigray region, Ethiopia, both genera (*Anopheles* and *Culex*) shared the same habitats. In western Ethiopia (1775 m.a.s.l), Kiszewski *et al.* (2014) collected both *Anopheles* and *Culex* from the same habitats. It indicates the presence of equal physiological need of the two different families, where habitats could provide/ fulfill their interest/. Entirely, the existence of both sub-families in various habitats throughout the year implies that larval control of selected *Anopheles* habitats will not be possible.

Though every species has its own habitat preference (Forstinus *et al*., 2015), this study found that six (*An. gambiae* s.l, *An. funestus* s.l, *An. coustani* s.l, *An. squamosus*, *An. cinereus* and *An. demeilloni*) larval species shared the same habitats: in marshlands, rain-pools, stream-edges, stream-pockets, pits, irrigation-canals, dam-edge and ditch. There was no any single habitat with a single species of *Anopheles* mosquitoes. Similar

to this result, Mwangangi *et al*. (2007a) found both *An. arabiensis*, *An. funestus*, and *An. coustani* in stream pools, puddles, ponds and swamps. Correspondingly, Animut *et al*. (2012) and Kibret *et al*. (2017) collected *An. arabiensis*, *An. pharoensis*, *An. funestus*, *An. coustani*, *An. cinereus* and *An. demeilloni* in streams-edges, swamps, rain-pools, irrigation canals and rain pool in various parts (Hobe, Dirama, Wurib, Kessema, Koka, koga district) of Ethiopia. Moreover, Kenea *et al*. (2011) obtained *An. arabiensis*, *An. pharoensis*, *An. squamosus*, *An. coustani* and *An. cinereus* in swamps, irrigation canals and sand pools in the Rift Valley, central Ethiopia. Kiszewski *et al.* (2014) found both *An. gambiae* s. l and *An. coustani* in borrow pits in western Ethiopia.

In our study, marshlands and ditches were the most productive breeding sites as compared with the other habitat types. The productivity partly associated with presence non-fluctuated water level throughout the year and partly the presence of sufficient nutrients for the growing larvae. In Bure area, maize is the leading crop. Being this, maize is considered the main engine to speed-up for vector density, mosquito longevity, and malaria infection (Kebede *et al*., 2005). Thus, not only higher numbers of adults but also infected *Anopheles* mosquitoes were detected in the district. Furthermore, Ye-ebiyo *et al*. (2000 & 2003) and Minakawa *et al*. (2005) have confirmed the presence of positive role of pollens produced by maize to the development of the mosquito larvae (e.g., *An. gambiae* s.l) to develop more quickly into larger and longer-lived adult vectors.

The result of this study discovered that some breeding habitats including hoof-prints and tyre-tracks were devoid of *Anopheles* larvae. These results were in line with Sattler *et al*. (2005), Mala *et al*. (2011a) and Animut *et al*. (2012) reports. However, conflicting to the current result, Mwangangi *et al*. (2007a) and Imbahale *et al*. (2011),

Tiku *et al*. (2013) and Getachew (2017) found *Anopheles* larvae from hoof-prints and tyre-tracks from western Kenya coast and Ethiopia, respectively. The unproductive natures of hoof-prints and vehicle ruts could be linked to the rapid percolation of the rain water into the soil or the high evaporation nature of the water or the heavy rain may flush out larvae and eggs during the rainy season (Munga *et al*., 2007). Usually, mosquito's larvae do require only calm (undisturbed) habitats; however, the presence of day to day interference to these habitats by cattle, humans and carts was common and practically confirmed during sampling time, and this could be the other reason for the absence of *Anopheles* mosquitoes in such habitats (Personal Obser.).

In general, marshes, stream-edge and pockets, dam-edges, irrigation canals, ditch and pits were the most important habitats for *Anopheles* larvae growth during the dry season in this area. Equivalent to this, Mouchet *et al*. (1998) and Imbahale *et al*. (2011) found large numbers of *Anopheles* larvae in river fringes, dams and irrigation canals during the dry season. Among the productive habitats, dam-edge was the least supporting habitat due to the presence of regular fluctuation of water level. On the other hands, in the present study rain pools were found among the most effective breeding habitat during rainy season. This contrary to other similar studies (Martens *et al*., 1995; Paaijmans *et al*., 2007); where most of rain pools were not productive during the rainy time, because too much rainfall either sweeping away mosquito larvae from breeding sites (both eggs and larvae) or directly kill them, which lead to reduction in emerging adults. Thus, controlling these habitats during dry season is not only important to reduce adult density but also to minimize the cost of intervention and the coming consequence during the rainy season (Fillinger *et al*., 2004; Sogoba *et al*., 2007).

This study characterized larval habitats and studied spatial heterogeneity of *Anopheles* mosquito' larvae in various environmental characteristics. Out of thirteen parameters, *Anopheles gambiae* s.l was primarily dominant in natural, permanent and temporary aquatic habitats that had clean and standing water, without matted algae, vegetations and plants. Besides, fully sun-exposed breeding habitats had less than 0.5m depth with less than 10m diameter was the most productive habitats. These findings corroborates to previous reports (Gillies & Coetzee, 1987; Sattler *et al*., 2005), *An. gambiae* s.l bred in clean and temporary sites. Minakawa *et al*. (1999) reported the occurrence of *An. gambiae* s.l larvae mainly in small temporary and sunlit pools. Others (Gimnig *et al*., 2001; Ye-ebiyo *et al*., 2003; Chen *et al*., 2006; Sinka *et al*., 2010) also reported that the larvae of *An. arabiensis* more preferred to natural habitats, small, temporary, sunlit, clean and shallow fresh water pools without vegetations or surface films. However, Gimnig *et al*. (2001) obtained positive association between *An. arabiensis* and matted algae and increased turbidity. Environmental characteristics such as presence of shade from plants, vegetations, algae, turbidity and excessive debris coverage were not liked by *An. gambiae* s.l in this study due to prevention of sun ray to the habitats that lead to incapable of the larvae to reach for food resources (Ye-ebiyo *et al*., 2003). Moreover, plants and vegetations that surround the breeding habitats may act as a refuge for predators, which are not liked by *Anopheles* larvae.

On the other hand, correlation analysis revealed that *An. gambiae* s.l larvae abundance were significantly and positively correlated with four (still water current, shallow habitat, < 10m diameter, habitats nearer to house) out of thirteen parameters; but multiple step-wise linear regression analysis was only found still water current was the main predictor of *An. gambiae* s.l abundance. This was inconsistent with the findings of Shililu *et al*. (2003) and Kenea *et al*. (2011). Because *An. gambiae* s.l is preferred

stagnant water more to stay closer to the surface to get air for breathing through spiracles and to feed (Rozendaal, 1997; Dotson, 2010; CDC, 2015). Also, standing water is so important for *An. gambiae* s.l larvae for capable of terrestrial displacement (Miller *et al*., 2007). *An. squamosus* abundance was significantly and positively correlated with four (matted algae, natural habitats, shallow habitat and habitats nearer to house) from thirteen parameters. However, multiple step-wise linear regression analysis distinguished that only matted algae were significantly positively predicted to the relative abundance and the occurrence of *An. squamosus.* It was in agreement with Shililu *et al.* (2003) and Kenea *et al*. (2011) reports. The presence of algae is an important food items and as refuge for *Anopheles* larvae (Manguin *et al*., 1996; Gimnig *et al*., 2001 & 2002; Kaufam *et al*., 2006).

Anopheles coustani was the other larval species this study discovered; the larvae were significantly and negatively correlated with breeding habitats nearest to house. This is disagreement with the work of Minakawa *et al*. (1999), suggesting that *Anopheles* preferred laying eggs in habitats near houses. This species is medically very important and was reported as vector in various Africa countries (Gillies, 1964; Bekele *et al*., 2012; Kawada *et al*., 2012; Mwangangi *et al*., 2013). In Ethiopia (in Jima zone), Kelel (2010), Yewhalaw *et al.* (2014) and Degefa *et al.* (2015) found sporozoite infected *An. coustani*. Moreover, this study identified sporozoite infected *An. coustani* (as described in chapter six). Hence, all these evidences implied the availability of larvae habitat management and the reconsideration *An. coustani* as a vector.

Generally, our study also collected the larvae of *An. cinereus* and *An. demeilloni*; however, correlation analysis didn't indicate presence of any relation between abundance of these larvae and environmental characteristics while some biological and most physical factors were included in the analysis. These results similar with Animut

et al. (2012) report, *An. cinereus* and *An. demeilloni* had not any significant association with turbidity, surface debris and surface algae though shallow and stagnant habitats support to grow. Therefore, the absence of full chemical (pH, sulphate, phosphate, total dissolved nitrate, chloride, total alkalinity, calcium and magnesium), few physical (e.g., dissolved oxygen, temperature) and biological parameters (predators) in the analysis may be considered as a limitation of this study. These parameters alone or in together have an effect on their abundances. In this regard, Ishak *et al*. (2014) indicated that *Anopheles* larvae densities were closely associated with physicochemical variables such as temperature, pH, and salinity**.** Additionally, Tiku *et al*. (2013) reported presence of direct relation between abundance of *Anopheles* larvae (increase) with physico-chemical parameters (increase) (water temperature, total dissolved solids, alkalinity and nitrate).

7.6 Conclusions

In conclusion, morphologically seven types of *Anopheles* larvae were documented. *Anopheles demeilloni* and *An. gambiae* s.l were the most abundant. Larvae of secondary malaria vectors of Ethiopia such as *An. funestus and An. pharoensis* were sampled. In this survey, except hoof-prints and vehicle ruts, all habitats had *Anopheles* larvae with various proportions. The largest overall abundance of *Anopheles* larvae were recorded during dry season than the others. This indicated the absence of larval habitat management during the dry season, and an indication of the high probably of malaria prevalence in the wet season. Marshes, ditches, irrigation canals, pits, stream-edge and fringes were the most important breeding sites during dry season and responsible for the continuous production of the adult vectors throughout the year and need to be considered vector control operations. Anthrophogic effects have their own contribution on the abundance of *Anopheles* mosquitoes. Generally, this study fully-furnished groundwork information on composition, distribution and abundance, and habitat characterization of *Anopheles* mosquitoes and indicated the availability of larval management across all months, particularly during dry months to control the adult vectors in combination with LLINs, IRS and case management.

Chapter 8: General Discussion, Conclusions and Recommendations

8.1 Discussion

This study assessed adult species composition, distribution and abundance, blood meal sources, parous rates, longevity, sporozoite infection rates; and larval species composition, distribution and abundance, and characterization of the larval habitats of *Anopheles* mosquitoes in Bure district, northwestern Ethiopia.

A total of nine adult *Anopheles* species were identified from the highland fringe of Bure district, composed of *An. arabiensis*, *An. funestus*, *An. pharoensis*, *An. coustani*, *An. squamosus*, *An. cinereus*, *An. demeilloni*, *An. rupicolus*, and *An. natalensis.* These species have documented previously in different parts of Ethiopia (Ovazza & Neri, 1955; Verrone, 1962a; Rishikesh, 1966; O'Connor, 1967; Woyessa *et al*., 2004; Dejenie *et al*., 2012; Animut *et al*., 2013a).

In this study, the adult of *An. demeilloni* was the most dominant mosquito (over 50.0%) followed by *An. arabiensis* and *An. funestus*. Higher adults *Anopheles* were collected in non-irrigated villages (Shnebekuma and Workmidr) than irrigated (Bukta). The occurrence of this difference was linked with the presence of diversified breeding habitats (including marshlands and ditches) throughout the years more in non-irrigated villages, that allowed the mosquitoes to have a gonotrophic cycle in each month (Mwangangi *et al*., 2012). Economic difference was also another reason, community near to irrigation scheme and dam areas have a greater wealth which was created by irrigation cultivation (Bure District Administration office, Unpub., 2016), this might be helped them to buy and use mosquito nets and other protective equipment, to get urgent treatment and better life too (Konradsen *et al.*, 2003; Dolo *et al.*, 2004; Sissoko *et al*.,

2004; Diuk-Wasser *et al*., 2005; Atieli *et al*., 2009; Njie *et al*., 2009; Animut *et al*., 2013b). Altitudinal difference could play a great role for such variation. In Tanzanian, Maxwell *et al.* (2003) and Kulkarni *et al*. (2006) found very high densities of *Anopheles* mosquitoes in the lowland than highland areas. In Ethiopia, Animut *et al*. (2013a) and Kibret *et al*. (2017) collected large numbers of mosquitoes in low land than highland areas. Therefore, the low temperature reduces the growth rate by slow down the metabolic rate of mosquitoes in the highland area. Moreover, presence of enough information about malaria (Sissoko *et al*., 2004) might be possible source of variation, because information sources such as health center and primary school are found at center of irrigated village.

Highest parous rates of *An. arabiensis* and *An. funestus* were recorded in non-irrigated village than irrigated villages. It is in agreement with Mboera *et al*. (2010) finding, who reported higher proportion of parous rate of *An. funestus* in non-irrigation villages than both large-scale and traditional irrigation villages. This could be connected with the presence of high exposure to the hosts when the mosquitoes need to bite. However, the average parous rates and the longevity of the six species (*An. arabiensis*, *An. funestus*, *An. coustani*, *An. squamosus* and *An. cinereus*) were very low as compared with other reports (Massebo *et al*., 2013a; Kenea *et al*., 2016; Gari *et al*., 2016; Taye *et al*., 2016 & 2017), this might be the result of mass emergence of nulliparous adults from the nearest breeding sites (Massebo *et al*., 2013a). Generally, the average longevity of these five species was not enough to complete the life cycle of the malaria parasite in mosquitoes (WHO, 1975).

The blood-ELISA result indicated that *An. arabiensis*, *An. funestus*, *An. coustani*, *An. squamosus* and *An. cinereus* had extremely high mixed blood (human and cattle) than

single blood meals sources. The presence of highest proportion of mixed feeding is the charter of mixed dwellings (Bruce-Chwatt *et al*., 1966; Hadis *et al*., 1997) and properly characterized present study villages. The proportion of indoor mixed blood meal was higher than outdoor, this incidence could be connected with feeding interruption; the vectors were interrupted while feeding outdoors on cattle and moved into houses to complete their feeding in a single night or on consecutive nights (Boreham & Garrett-Jones, 1973; Norris *et al*., 2010; Bashar *et al*., 2012) or climatic factors (Hadis *et al*., 1997; Bashar *et al*., 2012). In this study sites, villagers house were traditional (made of mud) and served as cooking, sleeping and tethering livestock, which increases indoor temperature. Being this, outdoor mosquitoes enter to the house and get more access to blood meal sources (Afrane *et al*., 2006; Paaijmans & Thomas, 2011; Bashar *et al*., 2012).

The presence of higher proportions of mixed blood meals (humans and others) are very important than single human meals because mixed feeds tend to diminish the density of gametocytes in the mosquito stomach, thereby reducing the chance of fertilization of the female gametes and diminish the chances of malaria vector becoming infected. Thus, mixed dwelling activate should be reinforced as a means of vector control (Muriu *et al*., 2008; Bashar *et al*., 2012; Ngom *et al*., 2013) in this area.

This study discovered the presence of sporozoite infected *An. arabiensis*, *An. funestus*, and *An. coustani* in non-irrigated villages only. These three species involved in malaria parasites transmission in the district. Therefore, the current study has documented for the first time the roles of *An. funestus* in malaria transmission at higher altitude (an altitude 2,025 meters); this is might be due to the climate change (global warming) (Checchi *et al*., 2006; Tesi, 2011). Previously, Kelel (2010), Yewhalaw *et al.* (2014) and Degefa *et al*. (2015) have reported sporozoite infected *An. coustani* in Jima zone

(below 2,000 m.a.s.l), Ethiopia. However, the presence of sporozoite infected *An. coustani* at higher altitude in this area was the first report and indicated that, this species could be an active malaria vector both in low land and highland parts of Ethiopia.

The overall annual EIR for *Anopheles* mosquitoes in non-irrigated villages was 0.31 ib/p/y. No single mosquito species were found infected from irrigated village. Similar to this study, Ijumba *et al.* (2002) reported higher allover annual EIR in non-irrigated than irrigated villages. The absence of sporozoite infected *Anopheles* mosquitoes in irrigated village could be associated with the presence of small numbers of mosquitoes, altitudinal difference (temperature variation) and the absence or the presence of little malaria in the community.

Seven species of *Anopheles* larvae (*An. gambiae* s.l, *An. funestus* s.l, *An. pharoensis, An. coustani* s.l, *An. squamosus, An. cinereus* and *An. demeilloni*) were identified in the three villages. The occurrences these species have been reported in various highland parts of Ethiopia (Ovazza & Neri, 1955; Woyessa, 2001; Woyessa *et al*., 2004; Animut *et al*., 2013b; Gone *et al*., 2014). This could be largely connected with incidence of climatic changes (global warming) (Checchi *et al*., 2006; Tesi, 2011; Siraj *et al*., 2014) and the expansion of maize farms in the district (Ye-ebiyo *et al*., 2000 & 2003; Kebede *et al*., 2005). Ten breeding habitats were discovered which including marshes, rain-pools, stream-edges and pockets, pits, irrigation-canals, dam-edges, ditch, tyre-tracks and hoof-prints. Of these, vehicle ruts and hoof-prints were devoid of *Anopheles* larvae due to the presence of regular disturbance, high rate of percolation or evaporation.

Most habitats were productive during the dry season, which have been previously reported (Mouchet *et al*., 1998; Imbahale *et al*., 2011; Mala *et al*., 2011a). These

habitats are not only used as breeding grounds, but also release moisture to the surrounding area in which the adults are found. Due to these reasons, better longevity of adult mosquitoes was observed in nonirrigated than irrigated village. On the other hand, in irrigated village, not only the number and the diversity of breeding habitats too low but also marshlands habitats were used for grazing purpose in all months except during the main rainy season. As a result, there densities, parous rate and longevity were very low.

8.2 Conclusions

This study assessed adult species composition, distribution and abundance, blood meal sources, parous rates, longevity, sporozoite infection rates; and larval species composition, distribution and abundance, and characterization of the larval habitats of *Anopheles* mosquitoes in Bure district, northwestern Ethiopia. Based on the obtained results, the following conclusions were drawn. Nine adults of *Anopheles* species were identified; *An. demeilloni* was the most dominant followed by *An. arabiensis* and *An. funestus.* These three adults were found throughout the year than other species. Mean densities of indoor and outdoor host seeking (*An. demeilloni*, *An. arabiensis*, *An. funestus*, *An. coustani*, *An. squamosus*, *An. cinereus* and *An. pharoensis*) of adult *Anopheles* mosquitoes collected by CDC-LTs were equal, implying that each species was not showed behavioral change. The largest proportions of adult *Anopheles* mosquitoes were collected in non-irrigated villages than the irrigated village. Thus, irrigation activity hasn't any positive correlation with the densities and the distributions of adult *Anopheles* mosquitoes in the three villages.

Blood meal analysis using ELISA indicated that *An. arabiensis*, *An. funestus*, *An. squamosus*, *An. coustani* and *An. cinereus* had very large HBIs and BBIs. These species

had higher proportions of mixed blood (human and cattle) than single blood meals. Generally, both the five species showed opportunistic feeding behavior.

Better average parous rates and longevity were recorded in non-irrigated than irrigated village. The observed average parous rates and longevity of *An. arabiensis*, *An. pharoensis*, *An. funestus*, *An. coustani*, *An. squamosus* and *An. cinereus* were very low and insufficient to complete the life cycle of malaria parasite for malaria transmission.

Detection of *Plasmodium* circumsporozoite protein (CSP) by ELISA indicated that only sporozoite infected *An. arabiensis*, *An. funestus* and *An. coustani* were found in only non-irrigated villages. Overall infective bites per year (5.7 ib/p/y) were very low (annual EIRs) and found to be less than ten (EIR < 10). This was similar to each species. However, it is not less than one infective bite per person per year; therefore, it indicates the presence of much work for interruption of malaria transmission (Beier *et al*. (1999). However, the contribution of *An. funestus* in the transmission of malaria at Bure area is higher than those of the other two vectors species. Totally, irrigation activity hasn't any positive linkage with EIRs of *Anopheles* mosquitoes.

Morphologically, seven types of *Anopheles* larvae were identified, *An. gambiae* s.l and *An. demeilloni* were the most dominant. Ten different breeding habitats were identified, marshes, stream-edges and pockets, irrigation canals and ditches were found in each month. Therefore, gonotrophic cycle was taken place in all months. Totally, this study revealed that distribution and abundance, blood meal sources, parous rate, longevity, sporozoite infection rates and larval distribution and abundance were not associated with various land use land cover changes areas in Bure district, northwestern Ethiopia.

8.3 Recommendations

The numbers of identified adult species (n = 9) were varied as compared with larvae species (n = 7); this suggest the availability of surveying both the adult and larval stages not to miss obtainable species in specific area during malaria vectors surveillance.

The abundance of human blood origins from *An. coustani*, *An. squamosus* and *An. cinereus* indicate the potential of these species to transmit malaria; therefore, proper investigation is required to know their exact role in malaria transmission, though reports indicated that these species are vectors of malaria elsewhere in Africa. The mixed blood feeding habits of the five *Anopheles* mosquitoes indicated the necessity of combination zooprophylaxis; this mosquito diversion system should be appreciated because the sites are mixed dwelling.

CSP-ELISA indicated the predominance of *P. vivax* (75%) proportions over *P. falciparum* (25%) in *Anopheles* mosquitoes. The disproportion of parasite load in mosquitoes indicated the distribution of the parasites in the community and the absence of equal attention by the ministry of health to eliminate the parasite *P. vivax*; hence, equal attention should be given and further epidemiological study is available to know their exact proportions in the community to take an appropriate action.

Infected mosquitoes were only found in non-irrigated villages during the wet (from July to December) and dry seasons (January); this indicated the availability of continuous use of LLINs. Usually, the Ethiopia ministry of health has a custom of using IRS once in a year before malaria epidemic happens, but because of the aforementioned findings IRS should be applied during the dry season too, especially in the nonirrigated villages.

The status of resistance should be measured and other alternative control mechanism should be set because reduction of indoor host seeking density was not observed after using new LLINs and application of IRSs onward September. Malaria control strategies should be planned based on land use patterns.

Potential breeding sites must be overseen by community health extension works throughout the year, especially in non-irrigated villages. Moreover, strict malaria education should be prepared and given to the community (more for non-irrigated villages).

Generally, the obtained infective bite per year was low (annual EIRs) and found to be less than ten (EIR < 10). Malaria transmission status is very low and unstable; therefore, it is possible to manage by the current control and prevention strategies (larval management, LLINs, IRS, and case management).

Generally, the result of this study indicated the presence of the following research gaps. In particular:-

- ➔ Resting behaviors (indoor & outdoor) were unknown and further study is so important.
- ➔ The presence of resistance to insecticides was not confirmed & warrant to further study.
- ➔ Other blood meal sources (except humans and cattle) were not known and needs further study.
- ➔ Prevalence of *Plasmodium* parasites in human blood at different groups was not identified; thus it must be studied.
- ➔ The influence full chemical, few physical (e.g., DO, T°) and biological parameters (predators) on the composition, abundance and distribution of mosquito larvae were not determined and requires further study.
- ➔ Morphologically, the family of Culiclinae (larva & adult) was not identified to species level; hence it needs additional study.

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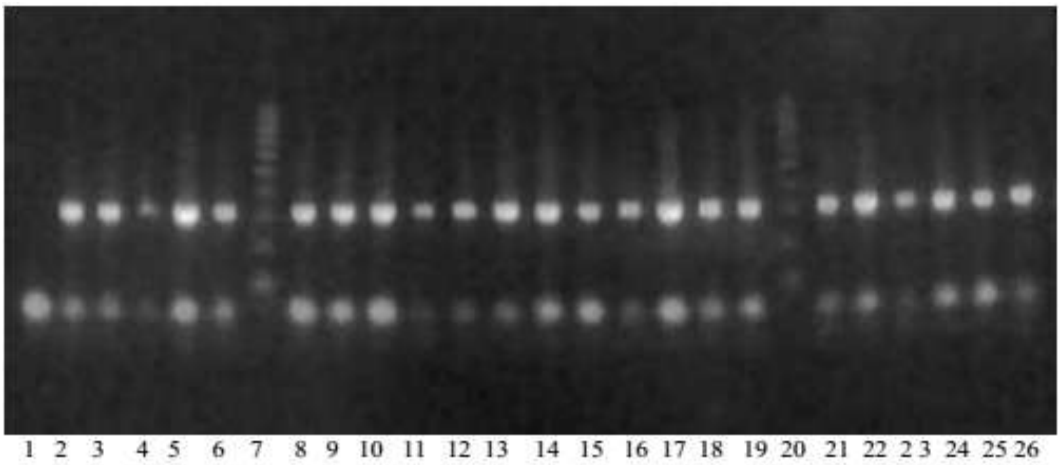
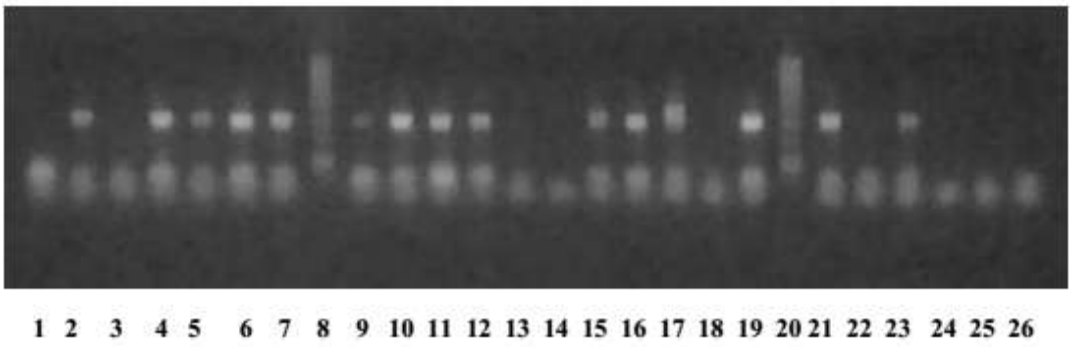
Appendices

Appendix 4a: Photos taken from gel electrophoresis of the PCR products for the identification of *An. gambiae* complex, Lane/L/ (7 & 20) = ladder (1000bp), the band of each ladder = 100bp; L1 = negative control; L2 = positive control (lab reared *An. arabiensis*); L3 - 6, 8-19 and 21- 26 = *An. arabiensis.* Gel condition = 2% agarosel gel with Etbr



Appendix 4b: Photos taken from gel electrophoresis of the PCR products for the identification of *An. gambiae* complex, Lane/L/ (8 & 20) = ladder (1000bp), the band of each ladder = 100bp; L1 = negative control; L2 = positive control (lab reared *An. arabiensis*); L3, 13, 14, 18, 22, 24 -26 = Unamplified; L4-7, 9-12, 15-17, 19, 21, 23 = *An. arabiensis.* Gel condition = 2% agarosel gel with Etb



Appendix 7.1a: Environmental characteristic of larval habitats and mean densities of *An. gambiae* s.l, *An. funestus* s.l and *An. coustani* s.l larvae in Bure area, from July 2015- June 2016

Characteristics of breeding sites	Variables	*An. gambiae* s.l		*An. funestus* s.l		*An. coustani* s.l	
		M ± SE	P-value	M ± SE	P- value	M ± SE	P- value
Permanence	Permanent	0.95 ± 0.06a	.001	0.82 ± 0.04a	.001	0.78 ± 0.10a	0.001
	Semi-permanents	0.37 ± 0.09b		0.26 ± 0.06c		0.28 ± 0.08b	
	Temporary	0.80 ± 0.05a		0.62 ± 0.03b		0.18 ± 0.03b	
Intensity of Light	Full sunlight	16 ± 2.39a	.001	0.99 ± 0.39a	.001	0.81 ± 0.10a	0.001
	Partial sunlight	0.45 ± 0.07b		0.29 ± 0.02b		0.30 ± 0.05b	
	Shaded	0.30 ± 0.05b		0.05 ± 0.01c		0.00 ± 0.00c	
Water turbidity	Clean	1.03 ± 0.06	.007	7.23 ± 0.69	.001	0.78 ± 0.09	.002
	Turbid	0.796 ± 0.05		3.03 ± 0.28		0.39 ± 0.06	
Algal coverage	Absent	1.15 ± 0.06a	.001	0.96 ± 0.04a	.001	0.78 ± 0.09a	.001
	Periphery	0.42 ± 0.07b		0.32 ± 0.06b		0.23 ± 0.04b	
	Fully covered	0.12 ± 0.01c		0.11 ± 0.01c		0.27 ± 0.05b	
Plants status	Absent	1.20 ± 0.06a	.001	0.99 ± 0.04a	.001	0.82 ± 0.10a	.001
	Emergent	0.00± 0.00b		0.00 ± 0.00c		0.00 ± 0.00c	
	Sub-emergent	0.08 ± 0.01b		0.25 ± 0.02b		0.25 ± 0.04b	
Substrate Status	Sand	0.00 ± 0.0b	.001	0.00 ± 0.00b	.001	0.00 ± 0.00b	.001
	Mud	1.20 ± 0.06a		1.03 ± 0.04a		0.86 ± 0.11a	
	Gravel	0.0 0.0b		0.00 ± 0.00b		0.00 ± 0.00b	
Surface debris coverage	Absent	0.99 ± 0.6	.114	4.09 ± 0.39	.007	3.53 ± 0.75	.307
	Present	0.86 ± 0.06		6.17 ± 0.59		4.87 ± 1.03	
Vegetations/	Absent	0.97 ± 0.06a	.001	0.73 ± 0.04a	.001	0.48 ± 0.07ab	.001

grasses coverage	Periphery	0.87 ± 0.6a		0.75 ± 0.04a		0.69 ± 0.09a	
	Fully	0.08 ± 0 .01b		0.25 ± 0.02b		0.25 ± 0.04b	
Water current	Still	1.20 ± 0.06	.001	1.03 ± 0.04	.001	0.77 ± 0.10	.008
	Slow moving	0.08 ± 0.02		0.03 ± 0 .01		0.39 ± 0.09	
Origin (Types) of habitats	Natural	1.14 ± 0.06	.001	0.99 ± 0.04	.001	0.79 ± 0.10	.007
	Artificial	0.52 ± 0.04		0.17 ± 0.02		0.41 ± 0.08	
Distance to the nearest inhabited houses	< 100m	0.78 ± 0.05a	.001	3.15 ± 0.30a	.004	0.65 ± 0.09a	.001
	101-200m	0.55 ± 0.05b		2.06 ± 0.19b		0.42 ± 0.06ab	
	201-300m	0.48 ± 0.04b		2.11 ± 0.19b		0.20 ± 0.04b	
	>301m	0.83 ± 0 .06a		2.93 ± 0.28ba		0.33 ± 0.05b	
Maximum depth	<0.5m	1.12 ± 0.06a	.001	0.98 ± 0.04a	.001	0.84 ± 0.10a	.001
	0.6 -1m	0.27 ± 0.06c		0.19 ± 0.04b		0.17 ± 0.04b	
	>1.1m	0.46 ± 0.04b		0.21 ± 0.02b		0.00 ± 0.00b	
Diameter of breeding area	<10m	1.18 ± 0.06a	.001	1.01 ± 0.04a	.001	0.84 ± 0.10a	.001
	11-100m	0.23 ± 0.06b		0.18 ± 0.02b		0.14 ± 0.04b	
	>101m	0.00 ± 0.00c		0.00±0.00c		0.00 ± 0.00b	

Note: The table included the mean differences of (Tukey Test) “each environmental variable”. Means of each characterized breeding habitats of a particular parameter followed by the same letter (s) are not significantly different from each other ($p < 0.05$, Tukey HSD)

Appendix 7.1b: Environmental characteristic of larval habitats and mean densities of *An. squamosus*, *An. cinereus* and *An. demilloni* larvae in Bure district, from July 2015- June 2016

Characteristics of breeding sites	Variables	*An. squamosus*		*An. cinereus*		*An. demilloni*	
		M ± SE	P-value	M ± SE	P-value	M ± S E	P-value
Stability	Permanent	0.70 ± 0.07a	.001	0.54 ± 0.10a	.001	1.28 ± 0.07a	.001
	Semi-permanents	0.00 ± 0.00b		0.07 ± 0.02b		0.36 ± 0.08c	
	Temporary	0.07 ± 0.01b		0.00 ± 0.00b		0.63 ± 0.05b	
Intensity of Light	Fully exposed	0.63 ± 0.07a	.001	0.52 ± 0.10a	.001	1.34 ± 0.07a	.001
	Partially sunlight	0.29 ± 0.04b		0.14 ± 0.03b		0.39 ± 0.04b	
	Shaded	0.00 ± 0.00c		0.00 ± 0.00b		0.22 ± 0.09c	
Water turbidity	Clean	3.02 ± 0.57	.120	2.22 ± 0.53	.251	1.22 ± 0.07	.002
	Turbid	1.93 ± 0.36		1.47 ± 0.35		0.91 ± 0.06	
Algal coverage	Absent	0.68 ± 0.07a	.001	0.52 ± 0.10a	.001	1.3 ± 0.07a	.001
	Periphery	0.13 ± 0.03b		0.14 ± 0.04b		0.62 ± 0.1b	
	Fully	0.00 ± 0.00b		0.00 ± 0.00b		0.05 ± 0.01c	
Plants status	Absent	0.67 ± 0.07a	.001	0.53 ± 0.01a	0.001	1.36 ± 0.07a	.001
	Emergent	0.00 ± 0.00c		0.00±.00b		0.00 ± 0.00c	
	Sub-emergent	0.17 ± 0.03b		0.13 ± 0.03b		0.33 ± 0.03b	
Substrate Status	Sand	0.00 ± 0.00b	.001	0.00 ± 0.00b	0.001	0.00 ± 0.00b	.001
	Mud	0.71 ± 0.07a		0.55 ± 0.11a		1.37 ± 0.07a	
	Gravel	0.00 ± 0.00b		0.00 ± 0.00b		0.00 ± 0.00b	
Surface debris coverage	Absent	2.58 ± 0.49	.727	0.18 ± 0.04	.005	14.9 ± 2.13	.156
	Present	2.35 ± 0.44		0.51 ± 0.09		11.0 ± 1.58	
Vegetation/ grass coverage	Absent	0.39 ± 0.05a	.001	0.31 ± 0.06ab	.005	1.05 ± 0.06a	.001
	Periphery	0.53 ± 0.06a		0.42 ± 0.08a		1.11± 0.07a	

	Fully	0.17 ± 0.03b		0.11 ± 0.03b		0.33 ± 0.03b	
Water current	Still	0.68 ± 0.07	.001	0.51 ± 0.10	.003	1.4 ± 0.07	.001
	Slow moving	0.12 ± 0.03		0.15 ± 0.04		0.31 ± 0.08	
Origin (Types) of habitats	Natural	0.59 ± 0.07	.015	0.46 ± 0.09	.121	1.27 ± 0.07	.001
	Artificial	0.32 ± 0.07		0.27 ± 0.08		0.81 ± 0.06	
Distance to the nearest inhabited houses	< 100m	0.34 ± 0.05ab	.002	0.26 ± 0.05	.058	0.78 ± .06ab	.028
	101-200m	0.45 ± 0.06a		0.35 ± 0.07		0.8 ± 0.06ab	
	201-300m	0.21 ± 0.03b		0.16 ± 0.03		0.73 ± 0.06b	
	>301m	0.25 ± 0.04b		0.19 ± 0.04		0.98 ± 0.06a	
Maximum depth	<0.5m	0.69 ± 0.08a	.001	0.49 ± 0.10a	.001	1.32 ± 0.07a	.001
	0.6 -1m	0.12 ± 0.05b		0.21 ± 0.06b		0.50 ± 0.1b	
	>1.1m	0.00 ± 0.00b		0.00 ± 0.00b		0.00 ± 0.00c	
Diameter of breeding area	<10m	0.67 ± 0.08a	.001	0.5 ± 0.10a	.001	1.4 ± 0.07a	.001
	11-100m	0.13 ± 0.04b		0.18 ± 0.06b		0.3 ± 0.07b	
	>101m	0.00 ± 0.00c		0.00 ± 0.00b		0.00 ± 0.00c	

Note: The table included the mean differences of (Tukey Test) “each environmental variable”. Means of each characterized breeding habitats of a particular parameter followed by the same letter (s) are not significantly different from each other ($P < 0.05$, Tukey HSD)