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Assessment of Entomological Parameters in Anophelines and Insecticide Susceptibility Status of *Anopheles gambiae* sensu lato in Selected Zones of Gambella Region, Southwestern Ethiopia

BY

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A thesis submitted to Department of Environmental Health Sciences and Technology, College of Public Health and Medical Sciences, Jimma University; in partial fulfillment for the degree of Master of Science in Environmental Health

Assessment of Entomological Parameters in anophelines Mosquitoes and Insecticide Susceptibility Status of *Anopheles gambiae* sensu lato in Selected Zones of Gambella Region, Southwestern Ethiopia

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Abstract

Gambella region is characterized by high precipitation, high humidity and warm temperature. These climatological conditions are favorable for survival and distribution of malaria vectors. The region was using DDT for over four decades alternatively with malathion until it was terminated from use in 2009 and replaced by deltamethrin for IRS. Currently, deltamethrin and propoxur are in use for malaria vector control. The study was conducted in Gambella Town, Lare and Abobo districts, southwestern Ethiopia from June to October 2013. Longitudinal and cross-sectional study design was employed to assess species composition, density, parous rates, fed/gravid, gonotrophic cycle, peak hourly activity and insecticide susceptibility status in three selected sites southwestern Ethiopia. *Anopheles* mosquitoes were collected using CDC light trap from indoor and outdoor to assess of entomological parameters. Anopheline mosquito larvae were collected and reared to adult for insecticide susceptibility test. Data were analyzed using SPSS software package version 16.0. Mean indoor and outdoor density of adult anopheline mosquito species were compared using ANOVA and mean mosquito density pre and post spray operation was compared using T-test. $P < 0.05$ was considered statistically significant during the analysis. The fed/gravid ratio was determined. Overall 3,177 anopheline mosquitoes belonging to five species were collected over the 5 months study period. Of these 1,417 (44.6%) belong to *An. gambiae* s.l. presumably *An. arabiensis* followed by *An. pharoensis 676* (21.3%), *An. funestus group, 538* (16.9%), *An. wellcomei* 309 (9.7%) and *An. nili* 237 (7.5%). Mean probability of daily survival pre spray operation was significantly higher than mean probability of daily survival post spray operation ($P < 0.05$). In deltamethrin sprayed areas, mean parous rate did not show significant variation (Gambella town and Lare district). However, significantly higher reduction (more than 50%) in parous rates of anopheline mosquito species was recorded in a propoxur sprayed area (Abobo district). Mean mosquito densities did not differ significantly between pre and post-IRS operation in deltamethrin sprayed areas. In contrast, there was significant difference in mosquito density pre and post spray operation in propoxur sprayed area. The duration of gonotrophic cycle was approximately two days for all anopheline mosquito species. *An. gambiae* s.l., *An. pharoensis* and *An. wellcomei* showed early activity (before 22:00 hours). Moreover, *An. gambiae* s.l. showed resistance to DDT and deltamethrin but susceptible to bendiocarb, propoxur and primiphos-methyl in all sampling sites. The development resistance by anopheline mosquitoes against deltamethrin has been determined by assessment of entomological parameters. Resistance development of *An. gambiae s.l.* in susceptibility test against DDT and deltamethrin could be from the long term use of the insecticides for IRS.

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ABBREVIATIONS AND ACRONYMS

CDC	Centre for Disease Control and Prevention
DDT	Dichloro-diphenyl-trichloroethane
FMOH	Federal Ministry of Health
IRM	Insecticide Resistance Management
IRS	Indoor Residual Spraying
ITNs	Insecticide Treated Nets
IVM	Integrated Vector Management
Kdr	knockdown resistance
KDT_{50}	The exposure time to obtain 50% knockdown
KDT_{95}	The exposure time to obtain 95% knockdown
LLINs	Long Lasting Insecticide Nets
PMI	President Malaria Initiative
VILR	Vlaamse Interuniversitaire Road
WHO	World Health Organization

1 Introduction

1.1 Background

In tropical Africa, the share of malaria cases was more than 90%. Except in some highland zones where it is too cold for the vector mosquitoes and in some urban areas in which malarial infections are rare, most people in tropical Africa get several *falciparum*-infected mosquito bites every year. In some areas, the inoculation rate is far elevated up to several hundred infected bites per annum on average (Laumann, 2010). According to WHO (2013a) world malaria report, there was high malaria transmission (Greater than 1 cases per 1,000 population) in 1%, low transmission (0 to 1 case per 1,000 population) in 66% and malaria-free (0 case per 1,000 population) in 33% of Ethiopian population. The report of Federal Ministry of Health Ethiopia (FMoH, 2012) shows the reduction of malaria cases since 2006.

Malaria transmission in most parts of Ethiopia is seasonal with bimodal rainfall pattern usually occurring in March-April and June/July-September. The prevalence patterns are also affected by differences in altitude, precipitation and population movement (FMOH, 2012). Thus the country is prone to focal and multifocal malaria epidemics due to instability of the disease transmission (Gebre & Negash, 2004). The disease is caused by *Plasmodium* parasites such as *P. falciparum, P. vivax, P. malariae P. ovale curtisi and P. o. wallikeri* (Alemu *et al.*, 2013). The parasites are transmitted by the bite of *An. arabiensis*, the primary malaria vector in Ethiopia and *An. funestus* group, *An. pharoensis* and *An. nili* secondary vectors (Krafsur & Armstrong, 1978). Selected vector control using IRS and LLINs are the main malaria vector control approach in Ethiopia (FMoH, 2008) that led to remarkable decreases in malaria transmission in some regions and these interventions are the basis of malaria control programmes in Ethiopia.

Residual insecticides such as DDT, malathion for Indoor Residual Spray (IRS) resulted in rapid reduction and complete interruption of malaria transmission from different areas (Laumann, 2010). Thus, the most widely used insecticide for malaria vector control in Ethiopia for more than four decades was DDT (Biscoe *et al*., 2004). However, because of the development of resistance by *An. arabiensis* against the insecticide, its use for IRS has been terminated in 2009 (Djènontin *et al.*, 2009).

Gambella is one of the national regional states in Ethiopia located in the southwestern part of the country adjacent to South Sudan. It is characterized by high precipitation, high humidity and warm temperature. These climatological conditions are favorable for survival and distribution of malaria vectors (Abebe and Singburaudom, 2006). Similar to other parts of the country, the region was using DDT for over four decades alternatively with malathion until it was terminated from use in 2009 and replaced by deltamethrin for IRS. However, entomological monitoring and surveillance is lacking and the resistance status of the principal malaria vector in the region is not yet updated. Therefore, the current study, which was conducted in the selected zones (Agnuwa and Nuwer) of the region, aimed to assess entomological parameters in malaria vectors and determine susceptibility status of *An. gambiae* s.l. to four classes of insecticides used in public health.

1.2 Statement of the problem

The worldwide risk of malaria is estimated to 3.4 billion people in 2012. The highest risk of acquiring malaria, approximately 207 million cases and 627,000 deaths in 2012 alone were estimated to occur with populations living in Sub-Saharan Africa. Children under five years of age and pregnant women were of the most affected population groups of Sub-Saharan African region. About 1%, 66% and 33% of the Ethiopian populations were estimated to live in high risk, low risk and malaria free areas of the country respectively (WHO, 2013a). The transmission patterns are seasonal in highland fringe and relatively longer in duration in lowland areas.

A study conducted around Gilgel-Gibe hydroelectric dam, southwestern Ethiopia, indicated knockdown resistance (*kdr)* to DDT and pyrethroid. The study also highlighted the need to evaluate the status of knockdown resistance *(kdr)* throughout the country to implement vector control strategies designed to manage insecticide resistance (Yewhalaw *et al.,* 2010). Another comparable study conducted in four districts (Omo Nada, Kerssa, Tiro Afeta and Sekoru) also showed multiple-resistance in populations of *An. arabiensis* to conduct study and to come up with alternative vector control options (Yewhalaw *et al.,* 2011). Previous study done in Sri Lanka also suggested that resistance surveillance should be conducted first in areas where malaria transmission and intensive agricultural pest control coincide, because these areas are most likely to develop insecticide resistance in mosquito vectors (Overgaard, 2006).

In Gambella Region, there is high malaria transmission throughout the year although case build up differs from season to season. Privately owned large agricultural activities are ongoing using Alwero Dam. Hence there are abundant temporary mosquito breeding sites. There are also large state farms that utilize pesticides which may result in selection pressure. In spite of the calls from FMOH and others to carry out insecticide susceptibility test in different parts of the country and to hamper malaria transmission, no study has been currently conducted on some entomological parameters and on resistance status of *An. gambiae* s.l. in the region.

1.3 Significance of the study

The major cause of poverty and underdevelopment worldwide and especially with the greatest share in Sub-Saharan Africa is malaria. Malaria is the major health problem in Ethiopia in general and in Gambella region in particular. The disease significantly contributes to the major adverse effects such as maternal mortality, malaria-induced anemia, spontaneous abortion, neonatal death and low birth weight during pregnancy (Steketee, Wirima & Campbell, 1996).

To alleviate the problems associated with the disease, important elements that are available are vector control by means of LLINs and IRS which are mainly dependent on synthetic insecticides (WHO, 2006). However, the emergence of widespread insecticide resistance posed the greatest difficulty in the prevention and control of the disease. The development of resistance has also been reported from different regions of Ethiopia (Balkew *et al.*, 2012; Yewhalaw, 2011; 2010).

In Gambella region, in which intensive agriculture and stable malaria transmission coexist, studies conducted on different insecticide resistance status and entomological assessment of malaria vectors were done before a decade. Therefore, by undertaking this study, the extent of resistance or susceptibility of *An. gambiae* s.l. to insecticides in use and potential insecticide for future use was determined. Furthermore, some entomological parameters of malaria vectors were assessed and updated by this study. Hence, the present study will be helpful for policy makers and stakeholders for the best alternatives of malaria vector control strategy. Additionally, the present study is important to recommend on the appropriate time for application of residual insecticide.

2 Literature review

2.1 Global and sub Saharan African region malaria situation

Malaria is one of the most important public health concerns at global level. It causes morbidity, mortality, economic loss and social disruption (Shiff *et al*., 2011). It is an infectious parasitic disease which has been a deadly human companion for many years. As populations migrated from tropical Africa into Eurasia and later across the ocean to the Americas, malaria parasites moved with their human hosts. Hence, malaria became a worldwide disease. For instance, at the mid of the twentieth century 77 percent of the global population was at risk of the disease (Laumann, 2010).

Despite its long association with human evolution, the burden of malaria in sub-Saharan Africa and in distant rural areas of Asia and Latin America was unbearably high. According to WHO (2006), malaria remained as a major cause of poverty and underdevelopment, and it was estimated that 3.2 billion people live at continuous risk of this disease. Each year, there were more than 350 million cases of malaria and more than one million deaths from the disease. The disease still remains as one of the most important disease of public health importance. According to WHO (2012a), 99 out of 104 countries have ongoing malaria transmission, and the disease killed an estimated 655,000 people in 2010, most of them were children under five years of age.

The previous decade has authenticated a remarkable rise in commitment to control malaria in Africa (Gething *et al*., 2014). However, malaria rank is high among the major killer diseases which are endemic to the tropics. The disease takes the life of one African child every minute (WHO, 2013a; WHO, 2012b).

2.2 The public health problem of malaria in Ethiopia

Although there was relatively low malaria prevalence compared to most other malaria endemic countries in Africa, the disease remained as a major public health problem in Ethiopia. Malaria is seasonal in most parts of Ethiopia, with variable transmission and prevalence patterns affected by the large diversity in altitude, rainfall, and population movement (FMOH, 2012). Thus, unstable malaria transmission patterns make Ethiopia prone to focal and multifocal epidemics that have every now and then caused little immunity in the community; hence malaria epidemics are common and lead to high mortality and morbidity (Gebre & Negash, 2004). In these unstable malaria transmission areas, the disease significantly contributes to the major adverse effects such as maternal mortality, malaria-induced anemia, spontaneous abortion, neonatal death and low birth weight during pregnancy (Steketee *et al.*, 1996). Particularly, the densely populated highland fringes and the semi-arid lowlands of the Afar and Somali regions are liable to seasonal and unstable malaria transmission. As the study conducted by Kiszewski and Teklehaimanot (2004) shows, epidemic malaria in the country is very strongly linked with topography.

Gambella is one of the national regional states of Ethiopia with high malaria endemicity. Most of malaria morbidity and mortality occur in children and pregnant women. Children predominantly under-five years of age are at risk of acquiring sever malaria due to their comparatively less developed immunity to malaria and the decline of passively acquired perishable immunity (Alameraw, 1998).

2.3 Anopheline mosquito vectors and their distribution in Ethiopia

Anopheles species of Afrotropical region includes *An. gambiae* s.l. *(An. gambiae* s.s., *An. arabiensis, An. quadriannulatus species A, An. quadriannulatus species B, An. bwambae, An. melas* and *An. merus), An. pharoensis*, *An. funestus* group consists *An. funestus* sub-group (*An. funestus* Giles, *An. aruni*, *An. parensis, An. confusus* and *An. vaneedeni) and* Rivulorum sub-group (*An. brucei*, *An. fuscivenosus*, *An. rivulorum leeson,* and *An. rivulorum) An. nili* and *An. wellcomei* (Gillies & Coetzee 1987).

An. funestus group has been found more widely scattered areas in Ethiopia than *An. gambiae s.l.*. Furthermore, *An. gambiae s.l.* appears to be less widely distributed than *An. pharoensis* that is dispersed from the lowlands to the high plateau and is most common in irrigated areas (O'connor, 1967). O'connor (1967) also reported that the swamps along the Baro River in western Ethiopia offer an environment that favors *An. funestus* to exceed *An. gambiae* in numbers throughout most of the year. *An. nili* and *An. wellcomei* were found in Gambella regional state, along the swamps of Baro River (Krafsur, 1977) and *An. wellcomei* in Adami Tulu District, South Central Ethiopia (Bekele, *et al.*, 2012).

An. arabiensis, *An. funestus*, *An. pharoensis* and *An. nili* are responsible for the transmitting *Plasmodium* parasite such as *P. falciparum, P. vivax, P. malariae P. knowlesi (*mainly a monkey parasite, recently identified in humans in South East Asia), *P. ovale curtis and P. o. wallikeri (* found in most of sub-Saharan Africa, Southeast Asia, and the Indian subcontinent) (WHO, 2012b).

These two sub-species, *P. o. curtis and P. o. wallikeri were* recently reported from north Gonder, Northwestern Ethiopia (Alemu *et al*., 2013).

A member of the *An. gambiae* complex, *Anopheles arabiensis,* is the primary malaria vector in Ethiopia, with *An. funestus* group, *An. pharoensis* and *An. nili* secondary vectors (Balkew et al., 2006; Krafsur & Armstrong, 1978). For example, the study conducted by Krafsur (1977), shows that *An. nili* was responsible for nearly 20% of the total inoculations in Gambella, south western Ethiopian lowlands during the wet season of 1968.

One of the essential factors of the epidemiological status of the malaria vectors is physiological age of female *Anopheles* mosquitoes. It is an indirect indication of the possible infectivity of *Plasmodium* in the salivary glands of a female, in the case in which it has taken in *Plasmodium* gametocytes during one of its previous blood feedings. *Plasmodium* develops in a female mosquito to an invasive stage within 2 – 3 or more gonotrophic cycles (the period in a female life which begins with the search for a host, continues with the process of blood-feeding, and finishes with oviposition into a water reservoir, depending on the ambient temperature) (WHO, 2001). Assuming mortality rate to be constant throughout the ovarian cycle, the gonotrophic cycle can be estimated by fed/gravid ratio. Hence, 1:1 and 1:2, is approximately 2-days and 3-days respectively (Davidson, 1954). Then the probability of daily survival (***P)*** can be estimated for species based on the following formula:

$$P = \sqrt[x]{\frac{Np}{Np + Nn}}$$

Where ***x*** is the duration of the gonotrophic cycle in days, ***Np*** and ***Nn*** the numbers of parous and nulliparous females respectively in the population (Donald, 1957).

Understanding of vector behavior will allow more wise use of control interventions and may further influence the choice of control method used (Kulkarni *et al.,* 2006). For instance, mosquito species differ in their breeding habitats, biting behavior, flight range and in many other ways. Therefore, different strategies are needed to control different species of mosquitoes (Goddard, 2003). Accordingly, behavioral monitoring is conducted to assess if vector behaviors change, especially early outdoor biting, in response to the changes in the insecticide used for IRS. Insecticide residual life monitoring to obtain evidence for the selection of best alternative insecticide is also a priority activity (PMI, 2012).

The aforementioned major malaria vectors in sub-Saharan Africa, such as *An. gambiae* s.s. and *An. funestus* group are primarily anthropophilic, endophagic, and endophilic. *An. arabiensis* is more variable in its foraging behavior. Although the vector can be found feeding and resting indoors in some localities, in other areas it is mainly exophagic and exophilic. Additionally, *An. arabiensis* has been reported to be highly anthropophilic in some regions, including southern Zambia, whereas elsewhere it predominantly displays zoophagic foraging tendencies (Fornadel, *et al.,* 2010).

Many of the important malaria vectors enter human dwelling or animal barn then they rest on the walls, ceilings and other interior surface before or after feeding on the inhabitants for searching blood meals. Thus endophilic and endophagic characteristics of malaria vectors is an important for the disease control intervention using IRS. As the vectors comes in contact with insecticide sprayed on walls or ceilings and other surfaces, they absorb lethal dose of the insecticide by their tarsi, and by so doing reduce their lifetime. This results in gradual decrease in vector density and longevity and favors to a decline in malaria transmission (WHO, 2013a).

2.4 Malaria vector control and insecticide resistance profile in Ethiopia

Huge scale implementation of IRS and LLINs that incorporate synthetic insecticide for malaria vector control have led to remarkable decreases in malaria transmission in some regions and these interventions are the basis of malaria control programmes in Ethiopia and in most African countries (Balkew *et al.,* 2012; Beier *et al*., 2008;Collins *et al.*, 2000). Abate and Hadis (2011) also reported that IRS is a major component of malaria control in Ethiopia. IRS, conducted according to the national guideline with effective insecticide, is a powerful intervention to quickly reduce endophilic adult mosquito vector density and longevity and, therefore, to reduce malaria transmission (WHO, 2013b).

DDT was one the most widely used insecticide for malaria vector control in Ethiopia for more than four decades (Biscoe, Mutero & Kramer, 2004). Abose *et al.* (1998) and Nigatu *et al.* (1994) reported DDT resistance in Gambella, Southwestern Ethiopia. Different scholars have also reported the development of resistance by malaria vectors in different areas of Ethiopia; for example, DDT and deltamethrin resistance in *An. arabiensis* and multiple insecticide resistance in the same species have been reported by Yewhalaw *et al.* (2011, 2010); Cross-resistance between DDT and permethrin *and* propoxur resistance in *An. arabiensis* have been reported by Balkew *et al.* (2010, 2003); Furthermore, DDT resistance has been reported (Balkew *et al*., 2006). Hence its use for IRS has been terminated in 2009 (Djènontin *et al.*, 2009).

Entomological assessment provides vital information concerning the presence, distribution, behavior and susceptibility status of malaria vectors to different classes of insecticides in a given area (WHO, 2013b). Accordingly, WHO (2003) identified entomological indicators to be monitored for adult mosquito control. These includes: adult vector density, parous rates, and adult insecticide susceptibility.

Reduction in vector density in areas where effective IRS conducted can be indicative of the effect of the intervention implemented (PMI, 2013; WHO, 2003). Therefore, mosquito density can be used for early warning of malaria epidemics FMOH (2012). Fed/gravid ratio is used to estimate the duration of gonotrophic cycle (Tchuinkam *et al.*, 2010). Again the length of gonotrophic cycle is used to compute estimate of the probability of daily survival of mosquitoes (Davidson, 1954). As the number of gonotrophic cycles increases, the greater is its epidemiological importance (WHO, 2002). IRS functions by reducing the female mosquito daily survival rate and human biting frequency (Massebo, 2013).

By measuring the proportion of parous mosquitoes in a vector population one can monitor changes in vector populations and evaluate the impact of an intervention. Changes in vector populations and the impact of an intervention can be evaluated by the proportion of parous mosquitoes in a vector population. The aim of killing mosquitoes that rest on sprayed surface area of a given unit structure is to reduce their longevity and ability to transmit malaria. If residual spraying is effective there will be less parous mosquitoes compared to nulliparous mosquitoes following spraying than before spraying (WHO, 2003).

Insecticide resistance monitoring is crucial to support programmers to implement more efficient and sustainable malaria control strategies in endemic countries (Djègbè *et al*., 2011). Nonetheless, low priority is given in most sub-Saharan African countries (Betson, Jawara, & Awolola, 2009). Insecticide resistance monitoring studies were carried in Oromia, Ethiopia by President Malaria Initiative from 2008 to 2012. Hence, widespread DDT resistance along with a reduced level of deltamethrin efficacy was confirmed. Based on the findings, the FMOH decided to discontinue the use of DDT in 2009 and made an interim decision to use deltamethrin for IRS (PMI, 2013; 2012). The simplest form of resistance management is likely to be insecticide based, and this could take the forms of rotation, mixture, fine scale mosaic and Insecticide Resistance Management (IRM) in an Integrated Vector Management context (IRAC, 2011).

WHO susceptibility bioassay for insecticide resistance monitoring is a direct response-to-exposure test; it measures mosquito mortality to a known standard dose of a given insecticide (i.e. the diagnostic or discriminating concentration). Accordingly, the mortality of test sample and control is calculated by the following formula.

$$\text{Observed mortality (\%)} = \left(\frac{\text{Total No. of dead mosquito}}{\text{Total sample size}}\right) \text{X } 100$$

$$\text{Control mortality(\%)} = \left(\frac{\text{No. of mortality of control mosquito}}{\text{Total No. of control mosquito}}\right) \text{X } 100$$

When control mortality is between 5% and 20%, then the observed mortality has to be corrected using Abbots formula, as follows:

$$\text{Observed mortality (\%)} = \left(\frac{\text{\% observed mortality} - \text{\% control mortality}}{100 - \text{\% control mortality}}\right) \text{X } 100$$

3 Objectives of the study

3.1 General objective

To undertake assessment of entomological parameters in anopheline mosquitoes and insecticide susceptibility status of *Anopheles gambiae* s.l. in selected zones of Gambella Region, Southwestern Ethiopia

3.2 Specific objectives

- To determine the species composition of anopheline mosquitoes
- To compare anopheline mosquito species density and parous rates of pre and post-IRS
- To determine the fed to gravid ratio of anopheline mosquitoes
- To determine the peak hourly activity of anopheline mosquito in the study area
- To assess the susceptibility status of *Anopheles gambiae* s.l. against the 4 classes of insecticides

4 Hypotheses

- Anopheline mosquito species composition, parous rate and density are similar throughout the study period.
- *An. gambiae* s.l. is susceptible to all four classes of insecticides.

5 Materials and Methods

5.1 Study area and period

Gambella People's Regional State (GPNRS) is located southwestern Ethiopia between the geographical coordinates $6^0$28'38" to $8^0$34' North Latitude and 33^0 to $35^0$11’11" East Longitude, which covers an area of about 34,063 km^2. It is situated at 766 kms South West of Addis Ababa. The total population of the region is 406,606. The region is a lowland Savannah and the topography is relatively flat. The people live in "tukuls", which are the common type of rural dwellings. Most of the residential areas are near the rivers and streams. Mean annual rainfall of the region was 1031mm. The main rainy season lengthen from June to the end of October. Annual mean minimum and maximum temperature was 21.1^{0}C and 35.9^{0}C respectively. IRS and LLINs are the main strategy for malaria vector control in the area (FMOH, 2008). Deltamethrin was sprayed in Gambella town and Lare district and propoxur was sprayed in Abobo district in 2013. The study areas were selected based on insecticide use for agricultural purpose, sentinel site for malaria survey and prevalence of malaria in the districts.

Lare is one of the five districts in the Nuer Zone. It is 80 kms away from the capital city of the region, Gambella Town. Average altitude is 430 meters above sea level. The people engage in agriculture and stock herding. Cattle are herded in open enclosure close to human habitations. It has been nationally used as sentinel site for Malaria survey. Flood during heavy rainfall is the common annual phenomena. There is low agricultural activity. Accordingly, insecticide usage for agricultural purposes around the study area did not exist.

Abobo is one of the six districts in Agnuwa Zone. It is 45 kms away from the capital city of the region. Average altitude is 461 meters above sea level. The people engage in agriculture and fishery. Large estate farm (3,000 hectares of land) has been occupied by Abobo Agricultural Development Enterprise in the district. The state farm has been utilizing deltamethrin and Ethio-sulfane for pest control since 2011 (Ato Habtamu Deresse- Head of Agricultural Development Enterprise, personal communication). Cotton and maize were the main crops harvested from the farm. There were also privately owned farm lands around the study area in the district.

Peripheral villages around Gambella town were included in this study. Average altitude is 453 meters above sea level. Baro River, a permanent stream of greatly variable volume, arises in the highlands and flows nearly to the east of the region. Jabjabe stream that originate from the northern highlands is one of the temporary tributary of Baro River. Both join in the capital city of the region. The Baro River flows westerly crossing the Lare district. Numerous seasonal streams also exist throughout the study areas and their flow corresponds with periods of heavy rainfall, usually from June through October. This study was conducted from June to October 2013.

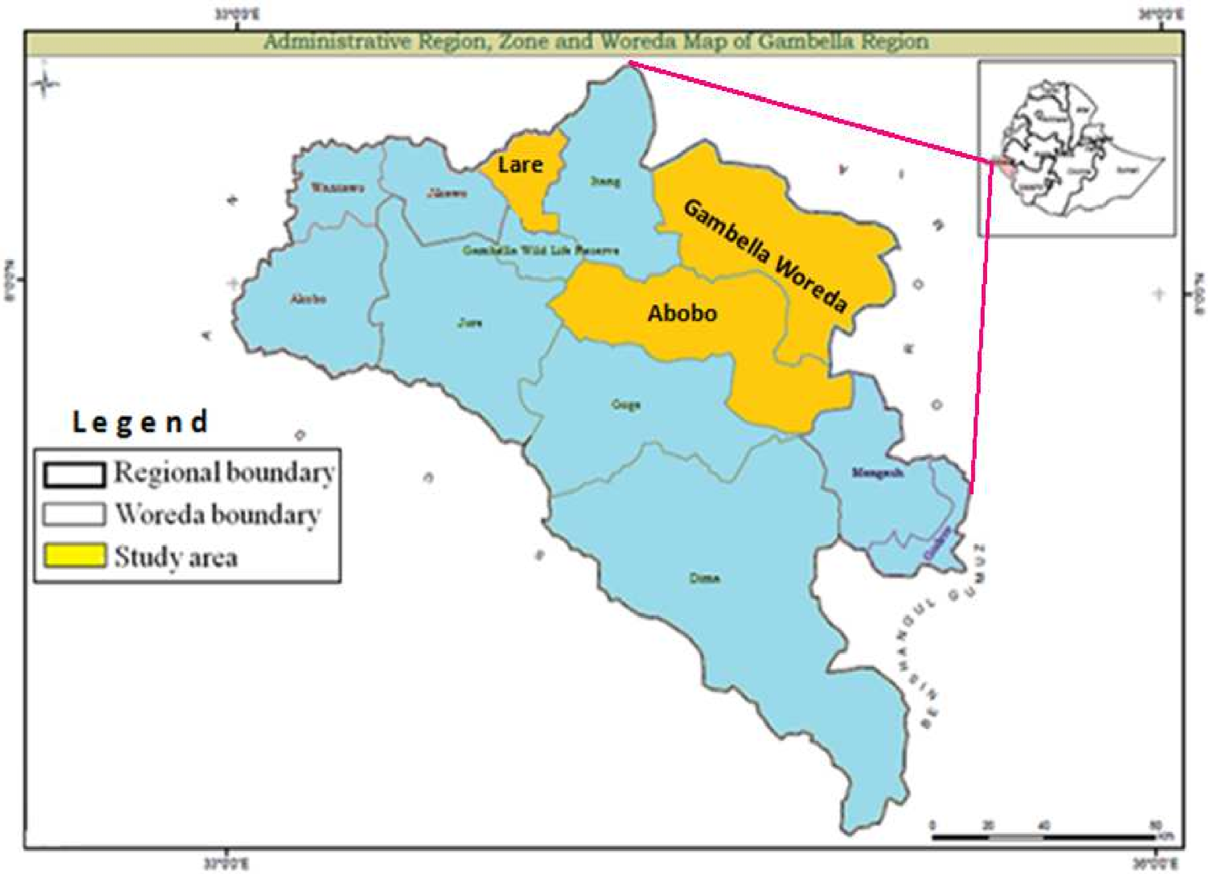

Figure 1: Map of the study area

5.2 Study design

Both longitudinal and cross-sectional study designs were employed for entomological assessment and insecticide susceptibility test respectively.

5.3 Mosquito sampling

After obtaining verbal consent from the heads of households, mosquito collection was conducted at fortnight interval in each village of the selected districts, from June to October 2013, using two motorcycle battery operated Centers for Disease Control and Prevention (CDC) light traps (BioQuip Products, Inc, CA, USA).

Mosquito collection

One light trap was installed in the sleeping room beside a bed. Another trap was installed outdoor at five to eight meters distance from residential houses in the same household. Each selected household was provided with untreated mosquito net. The trap was hanged at about 1.5 m above the floor next to the foot of the bed in the indoor and at the same height in the outdoor. Human volunteer slept under the net during the night, while the light traps attracting mosquitoes. Trapped mosquitoes were removed at an hour interval starting from 18:00 to 06:00 (WHO, 2003). The collected mosquitoes were transferred to paper cups and then transported to temporary examination post in the field.

Mosquito identification

Species identification was made using morphological keys (Gillies, 1987). Female *Anopheles* mosquitoes were counted and determination of physiological status was undertaken as unfed, freshly fed, half gravid and gravid (WHO, 2003).

Mosquito dissection for parity

Legs and wings of unfed female *Anopheles* mosquitoes from the light trap collections were removed after anesthetized by chloroform. A drop of Phosphate Buffer Saline (PBS) on clean microscope slide was placed. Then the mosquitoes were dissected under the dissecting microscope by gently grabbing the thorax with forceps, and placing ventral side up with their abdomen in the PBS. While viewing the specimen under the dissecting microscope, a fine tip forceps was taken and the 7th and 8th abdominal segments of the female *Anopheles* were gently removed by grasping and pulling away slowly. Then other tissues were dissected away and ovaries were isolated. Under a compound microscope (x10 objective), ovaries in which the terminal skiens of the tracheoles become uncoiled were considered to be parous (Detinova, 1962).

5.4 Insecticide susceptibility test

Larval collection and adult rearing

Anopheles larvae/pupae were collected by dipping from different breeding sites available in the area. The collected larvae were feed yeast until they reared to adults. The reared mosquitoes were identified using standard key (Gillies, 1987). Quadruplet and duplicate of 25 female *An. gambiae* s.l. were obtained for each insecticide test (WHO, 2013c).

Insecticides for susceptibility test

The mosquitoes obtained from larval collection were exposed to discriminating dose of five insecticides from all four classes i.e., DDT 4%, deltamethrin 0.05%, Primiphos-methyl 0.25%, Bendiocarb 0.1% and Propoxur 0.1% using WHO standard susceptibility test kit. The test was conducted at field in the building free from insecticidal contamination and at a temperature of $25^oC \pm 2^oC$. The relative humidity during the test was also maintained between $80\% \pm 10$ by using wet towel (WHO, 2013c).

Test methods

Six sheets of clean white papers were inserted into six holding tubes and fastened into position with a steel spring-wire clip. One hundred and fifty female mosquitoes were aspirated from a mosquito cage in to the six holding tubes to give four replicate samples and two replicate of 25 mosquitoes per tube. Then, the slide unit was closed and the holding tubes set in an upright position for one hour. At the end of this time, the damaged insects were removed. Six exposure tubes were prepared (4 red-dotted for insecticide-impregnated papers and 2 green-dotted controls for oil-impregnated papers). Then mosquitoes were blown gently into the empty exposure tubes. Mosquitoes were kept in the exposure tubes for1 hour and knockdown was recorded at 0, 10 15, 20, 30, 40, 50 and 60 minutes. At the end of 1-hour exposure period, the mosquitoes were transferred back to the holding tubes. A pad of a cotton-wool soaked in 10% sugar solution was placed on the mesh-screen of each holding tube. Mosquitoes were maintained in the holding tubes for 24 hours in a shady, sheltered place. Temperature and humidity was recorded. After recovery period, the number of dead mosquitoes was counted and recorded (WHO, 2013c). Identified mosquitoes were cross checked after mortality count. Finally, mosquitoes were individually preserved in 1.5 ml labeled Eppendorf tubes (dead and survive kept in different tubes) over silica-gel for further analysis.

Data interpretation

Mortality of test result is interpreted as susceptible if mortality ranges between 98-100%, suggest the existence of resistance if mortality is less than 98% and further investigation is required if observed mortality is between 90% and 97%, presence of resistant genes in the vector population must be confirmed. If the control mortality is less than 5%, no correction is necessary but above 20%, the tests must be discarded (WHO, 2013c).

6 Study variables

6.1 Dependent variables

Susceptibility of *An. gambiae* s.l. to an insecticide, parous rate, vector density, fed/gravid ratio, length of gonotrophic cycle, biting activity, Mosquito species composition

6.2 Independent variables

Discriminating dose of insecticides, temperature, relative humidity, time, study site, vector control intervention

7 Data analysis

Data was analyzed using SPSS version 16.0 software package. The parous rate was determined as the proportion of parous mosquitoes to the sum total of parous and nulliparous mosquitoes caught by CDC light traps that had ovaries with uncoiled tracheoles. Monthly vector densities in each village were calculated as the means of the monthly averages of the traps/day for each sampling sites (Bigoga *et al*., 2007). Students' t-test was applied to compare the indoor and outdoor mean density of adult anopheline mosquitoes. The monthly fed/gravid ratio was determined for different sites and used to estimate the duration of gonotrophic cycle (Donald, 1957). The resistance status of *An. gambiae* s.l. against 5 insecticides was determined based on the WHO protocol (WHO, 2013c). $P < 0.05$ was considered significant.

8 Data quality assurance

Standard protocol was used, support from experienced entomology technician and further morphological identification of preserved mosquitoes was made by VLIR insectory workers. Furthermore, quality of insecticide susceptibility test was controlled through replication and using control.

9 Ethical consideration

Ethical clearance was obtained from Jimma University Research and Ethics Committee. Written consent was also obtained from Gambella Regional Health Bureau, Abobo, Lare and Gambella Town health offices and households selected for this study.

10 Result

Mosquito species abundance

A total of 3,177 female anopheline mosquitoes belonging to at least 5 species were collected from the three sampling sites. The majority of the mosquitoes species were collected in August (26.8% of 853) followed by September (22.9% of 726), July (22.4% of 712), October (15.7% of 499) and June (12.2% of 387). From the total mosquitoes collected, *An. gambiae* s.l. (44.6% of 1417) was the predominant from the overall samples collected during the study period followed by *An. pharoensis* (21.3% of 676), *An. funestus* group (16.9% of 538), *An. wellcomei* (9.7% of 309) and *An. nili* (7.5% of 237). There was significant difference in abundance among the 5 anopheline mosquito species ($P < 0.05$). *An. gambiae* s.l. was also the predominant species collected from all sampling sites (Abobo 49.0%, Lare 44.8% and Gambella Town 41.0%) (Table 1).

Table 1: Abundance of anopheline mosquitoes collected from the study sites (June to October 2013)

Study sites	***An. gambiae n(%)***	***An. pharoensis n(%)***	***An. nili n(%)***	***An. funestus n(%)***	***An. wellcomei n(%)***	***Total n(%)***
Gambella Town	451(41.0)	237(21.6)	98(8.9)	193(17.6)	120(10.9)	1099(34.6)
Lare	557(44.8)	253(20.4)	117(9.4)	188(15.1)	128(10.3)	1243(39.1)
Abobo	409(49.0)	186(22.3)	22(2.6)	157(18.8)	61(7.3)	835(26.3)
Total	1417(44.6)	676(21.3)	237(7.5)	538(16.9)	309(9.7)	3177(100)

Density of anopheline mosquito species pre and post IRS operation

Mean *Anopheles* mosquito species density per trap increased from June to beginning of September (the time of IRS operation started in the district). However, mean anopheline mosquito species density per trap decreased post IRS operation (September to October) using deltamethrin in Gambella Town and Lare district by 23.4% and 40.3% respectively when compared to the density per trap recorded in August in the same site (Table 2a and Table 2b). Mean anopheline mosquito density per trap sharply decreased by 62% following propoxur spray (Abobo district) as compared to the density per trap documented in August (Table 2c). However, the difference in mean anopheline mosquito species density per trap among deltamethrin sprayed area (Gambella Town and Lare district) was not significant ($P > 0.05$). But significantly reduced ($P < 0.05$) in propoxur sprayed area (Abobo district). Overall, density per trap of *An. gambiae* s.l. was the highest (23.6

mosquitoes per trap) followed by *An. pharoensis* (11.4 mosquitoes per trap) and the lowest density per trap was recorded for *An. nili* (5.2 mosquitoes/trap /night) (Table 2a, Table 2b and Table 2c).

During pre and post-IRS, density per trap for *An. gambiae* s.l. collected from Gambella Town, Lare and Abobo districts in all the months of collection is greater as compared to other anopheline mosquitoes density of the study site in respective months of collection. Except in Gambella Town, *An. gambiae* s.l. density Post-IRS was reduced in other study sites (Table 2a, Table 2b and Table 2c). Significant reduction of density per trap of *An. gambiae* s.l. (32.0, 25.3 and 12.3 *An. gambiae* s.l. per trap in August, September and October respectively) following propoxur spray was obtained from Abobo district (Table 2c). In other study sites, density per trap of *An. gambiae* s.l. was not significantly reduced after deltamethrin spray (Table 2b, Table 2c).

Table 2: Density of anopheline pre and post spray as determined by CDC light trap collection

a) Gambella Town

Anopheline species	Pre-IRS			Post-IRS	
	June	July	August	September	October
An. gambiae	10.3	21.3	22.5	29.5	29.3
An. pharoensis	6.3	12.8	16.8	12.3	11.3
An. funestus	5.5	11.0	15.0	10.5	6.3
An. wellcomei	1.0	5.5	10.0	9.3	4.3
An. nili	3.0	6.5	5.8	6.8	2.5

b) Lare

Anopheline species	Pre-IRS			Post-IRS	
	June	July	August	September	October
An. gambiae	14.0	30.5	33.5	34.8	26.5
An. pharoensis	8.8	14.0	17.0	13.5	10.0
An. funestus	7.3	10.8	14.5	9.0	5.5
An. wellcomei	5.5	8.8	8.3	6.3	3.3
An. nili	5.0	6.5	9.0	5.0	3.8

c) Abobo

Anopheline species	Pre-IRS			Post-IRS	
	June	July	August	September	October
An. gambiae	11.3	21.5	32.0	25.3	12.3
An. pharoensis	8.3	12.0	14.5	7.8	4.8
An. funestus	7.0	9.0	10.3	8.3	4.8
An. wellcomei	3.8	2.5	4.3	3.5	1.3
An. nili	0.0	5.0	0.0	0.0	0.0

Parous rates of anopheline mosquitoes

Of 1,606 unfed mosquito collected and dissected, 40.4% (n = 649) were parous. The overall parous rate pre-IRS was 45.8%. However, during post-IRS operation, there was an overall reduction of parous rate by 11.6%. Parous rate was increased during pre-IRS (June through August) and decreased post-IRS operation (September and October) in all study sites. In Gambella Town, the highest parous rate reduction (from 52.0 in August to 25.0 in October) was recorded for *An. funestus* followed by *An.wellcomei* (from 47.1 in August to 30.8 in October) and the least parous rate reduction (from 45.9 in August to 38.3 in October) was recorded for *An. gambiae* s.l. (Table 3a). In Lare district, the highest parous rate reduction after spray was observed for *An. wellcomi* (from 46.2 In August to 10.0 in October) followed by *An. nili* (from 46.7 in August to 16.7 in October) and the least reduction was recorded for *An. funestus* (from 47.8 in August to 33.3 in October) (Table 3b). No parous *An. nili* was recorded in Abobo district from August through October. The highest reduction in parous rate (from 55.6 in August to 0.0 in October) was recorded for *An. wellcomei* followed by *An.pharoensis* (from 46.2 in August to 7.7) in the district (Table 3c).

Table 3: Parous rate of anopheline mosquitoes collected from the study sites (June to October 2013)

a) Gambella Town

Anopheline species	Pre-IRS			Post-IRS	
	June	July	August	September	October
An. gambiae	43.8	45.7	45.9	45.6	38.3
An.pharoensis	45.5	42.9	48.1	48.0	35.5
An.nili	33.3	45.5	40.0	42.9	25.0
An. funestus	40.0	45.0	52.0	50.0	25.0
An.wellcomei	33.3	40.0	47.1	45.0	30.8

b) Lare

Anopheline species	Pre-IRS			Post-IRS	
	June	July	August	September	October
An. gambiae	43.5	42.6	49.1	40.8	30.8
An.pharoensis	46.7	52.0	50.0	44.4	29.6
An.nili	50.0	46.2	46.7	36.4	16.7
An. funestus	46.2	47.4	47.8	35.0	33.3
An.wellcomei	33.3	53.3	46.2	35.7	10.0

c) Abobo

Anopheline species	Pre-IRS			Post-IRS	
	June	July	August	September	October
An. gambiae	42.9	24	47.1	33.3	15.8
An.pharoensis	44.4	14	46.2	28.6	7.7
An.nili	0.0	5	0.0	0.0	0.0
An. funestus	43.8	17	44.4	33.3	13.3
An.wellcomei	25.0	4	55.6	22.2	0.0

In deltamethrin sprayed areas, (Gambella town and Lare district), mean percentage of parous rate was reduced from 46.1% to 37.2%. Nearly 50% reductions in mean percentage of parous rate of anopheline mosquito species was recorded in propoxur sprayed area (Abobo district) following IRS operation (from 44.9% to 24.0%). There was significant mean percentage of parous rate reduction following IRS operation in propoxur sprayed area ($P < 0.05$) (Figure 2).

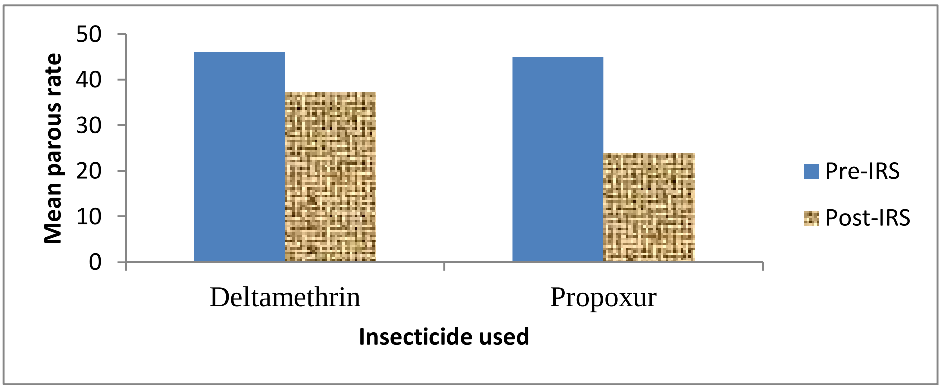

Figure 2: Mean parous rates of anopheline mosquito species pre and post-IRS in deltamethrin and propoxur sprayed districts (June to October 2013)

Physiological status of anopheline mosquitoes

The highest percentage of unfed mosquitoes collected from Gambella Town was *An. gambiae* s.l (41.3% of 227) followed by *An. pharoensis* (21.1% of 116) and *An. funestus* (16.9% of 93) and the smallest percentage was recorded for *An. nili* (9.1% of 50). The percentage of half gravid and gravid was also highest for *gambiae* s.l (43.4% of 112) followed by *An. pharoensis* (22.9% of 59) and *An. funestus* (17.8% of 46) and the least percentage of half gravid and gravid was recorded for *An. nili* (7.8% of 20). The highest percentage of unfed mosquitoes collected from Lare district was *An. gambiae* s.l (45.2% of 274) followed by *An. pharoensis* (19.8% of 120) and *An. funestus* (14.9% of 90) and the smallest percentage was recorded for *An. nili* and An.wellcomi (10.1% of 61 each). The percentage of half gravid and gravid was also highest for *gambiae* s.l (45.5% of 131) followed by *An. pharoensis* (20.8% of 60) and *An. funestus* (15.6% of 45) and the least percentage of half gravid and gravid was recorded for *An. nili* (7.6% of 22) in Lare district. The highest percentage of unfed mosquitoes collected from Abobo district was *An. gambiae* s.l (48.3% of 218) followed by *An. pharoensis* (21.1% of 95) and *An. funestus* (19.7% of 89) and the smallest percentage was recorded for *An. nili* (2.4% of 11). The percentage of half gravid and gravid was also highest for *gambiae* s.l (53.6% of 81) followed by *An. pharoensis* (24.5% of 37) and *An.*

funestus (15. 2% of 23) and the least percentage of half gravid and gravid was recorded for *An. nili* (2.7% of 4) in Abobo district (Table 4).

Table 4: Physiological status of anopheline mosquitoes collected from the study sites (June to October 2013)

Study site	*An. gambiae*				*An.pharoensis*				*An.nili*				*An. funestus*				*An.wellcomei*			
	Unfed	Freshly fed	Half gravid	Gravid	Unfed	Freshly fed	Half gravid	Gravid	Unfed	Freshly fed	Half gravid	Gravid	Unfed	Freshly fed	Half gravid	Gravid	Unfed	Freshly fed	Half gravid	Gravid
Gambella Town	227	112	75	37	116	62	40	19	50	28	18	2	93	54	34	12	63	36	18	3
Lare	274	152	86	45	120	73	44	16	61	34	16	6	90	53	34	11	61	37	26	4
Abobo	218	110	55	26	95	54	24	13	11	7	3	1	89	45	19	4	38	17	5	1

Probability of daily survival

The difference was significant in mean probability of daily survival between pre and post-IRS (P < 0.05) being lower during post-IRS. Mean monthly probability of daily survival of the mosquito species was highest during pre-IRS; in August (0.69) followed by July and June (0.68 and 0.64 respectively). However, mean probability of daily survival was not significant among anopheline mosquito species (*P* > 0.05) in deltamethrin sprayed districts. The highest mean probability of daily survival was recorded for *An. gambiae* (0.39) and *An. pharoensis* ((0.39) in deltamethrin sprayed districts and the lowest was for *An. nili* (0.30) and An. wellcomi (0.30). In propoxur sprayed area, *An. gambiae* (0.25) and *An. pharoensis* (0.23) yet again showed the highest mean probability of daily survival (Figure 3).

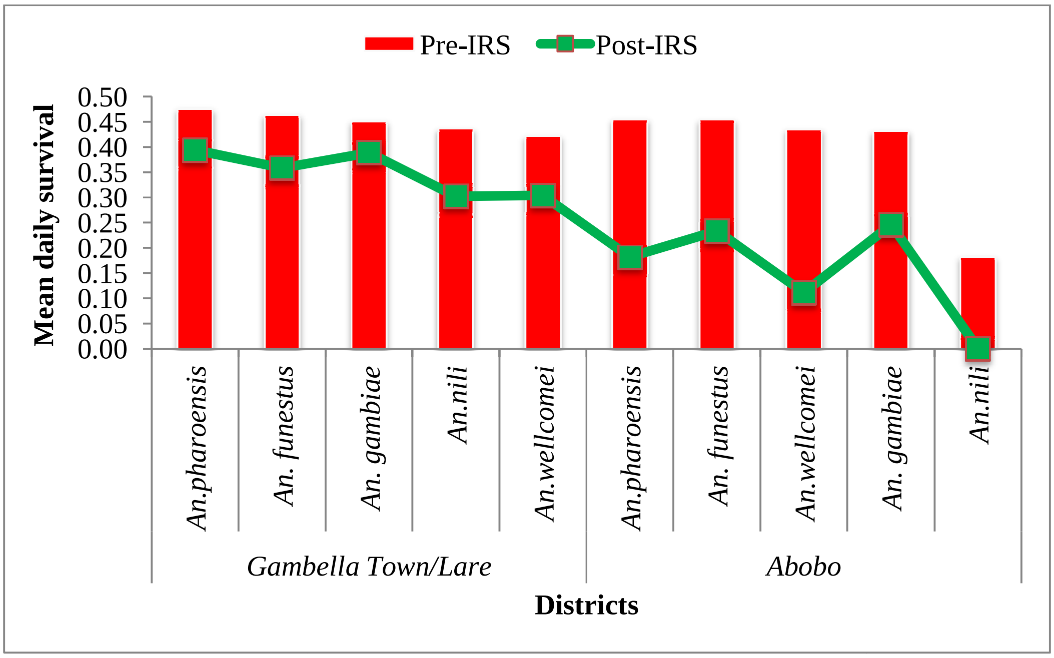

Figure 3: Mean probability of daily survival of anopheline mosquito species in deltamethrin and propoxur sprayed districts (June to October 2013)

Hourly activity of anopheline mosquito species

Of 3,177 mosquitoes collected, indoor and outdoor catches accounted 1,921 (60.5%) and 1,256 (39.5%), respectively and indoor mosquito catches was higher than outdoor catches ($P < 0.05$) with indoor to outdoor ratio of 1.53:1.0. The overall indoor caches were decreased from 61.3% to 59.1% following IRS operation. This trend was reversed and the percentage of outdoor resting anopheline mosquito species increased from 38.7% to 40.9% following IRS operation. Outdoor density of *An. gambiae* s.l. increased following post IRS operation from 34.4% to 41.4%. Similarly, the outdoor density of *An. wellcomei* increased from 31.3% to 34.2% (Table 5).

Table 5: Mean indoor and outdoor anopheline mosquito pre and post spray (June to October 2013)

Mosquito species	**Pre-IRS**		**Post-IRS**	
	In	Out	In	Out
	n (%)	**n(%)**	**n(%)**	**n(%)**
An. gambiae	516(65.6)	270(34.4)	368(58.3)	263(41.7)
An. pharoensis	223(50.6)	218(49.4)	125(53.2)	110(46.8)
An. nili	105(64.0)	59(36.0)	51(69.9)	22(30.1)
An. funestus	220(60.9)	141(39.1)	107(60.5)	70(39.5)
An. wellcomei	123(61.5)	77(38.5)	68(62.4)	41(37.6)

Peak outdoor and indoor density of *An*. *gambiae* s.l. was observed from 21:00-22:00 hours. Peak outdoor and indoor activities of *An*. *pharoensis* occurred between 21:00-22:00 and 20:00-21:00 hours, respectively. For *An*. *wellcomi* its peak outdoor and indoor activities were recorded between 20:00-21:00 and 21:00-22:00 hours, respectively, which sharply went down afterwards but with a considerable peak between 01:00-02:00 hours outdoors. Peak outdoor and indoor activities of *An*. *nili* was recorded between 02:00-03:00 and 01:00-02:00 hours, respectively, and then sharply decreased until 04:00-05:00. Peak outdoor and indoor activities of *An*. *funestus* group *was recorded* between 01:00-02:00 hours, and then sharply decreased until 04:00-05:00hours. In general, about 44%, 57%, and 69% of *An. gambiae, An. pharoensis* and *An. wellcomi* density occurred before 22:00 hours. Except for *An. nili* ($P < 0.05$), there was no significant difference in peak mosquito density among the study sites ($P > 0.05$) (Figure **4**).

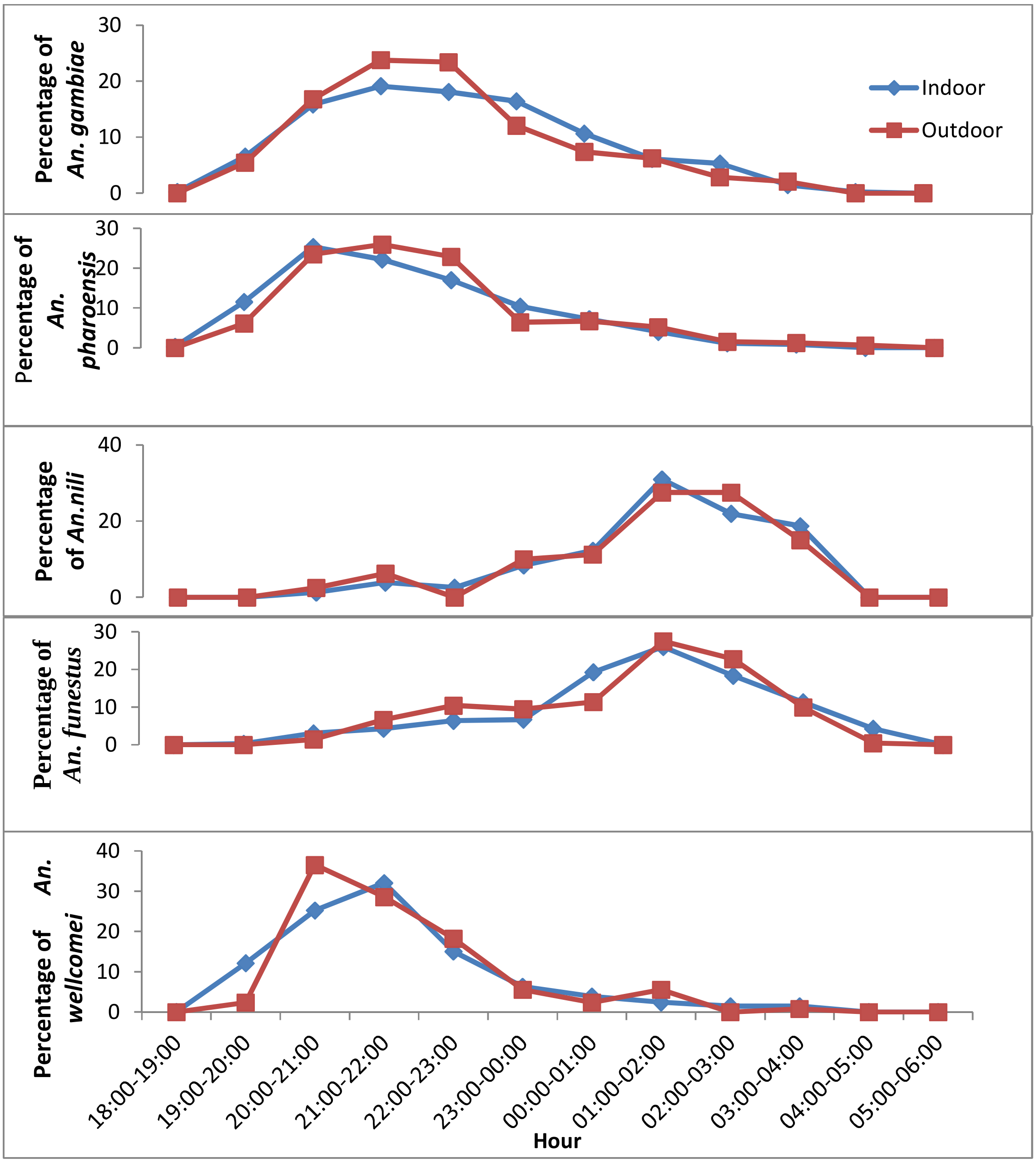

Figure 4: Hourly indoor and outdoor hourly activity of anopheline mosquito species, Gambella, southwestern Ethiopia (June to October 2013).

Susceptibility status of *An. gambiae* s.l.

A total of 2250 adult mosquitoes reared from larval/pupa collections in all sampling sites were identified morphologically as *An. gambiae* s.l. Of these, 1500 *An. gambiae s.l* (100 mosquitoes per insecticide) were exposed to the discriminating doses of insecticides (DDT 4%, deltamethrin 0.05%, primiphos-methyl 0.25%, bendiocarb 0.1% and propoxur 0.1%). The remaining 750 (50 mosquitoes per insecticide) were used as control. Observed mortality of field populations of *An. gambiae* s.l. exposed to deltamethrin was 16%, 2% and 16% in Gambella Town, Lare and Abobo districts respectively. Except to bendiocarb which had 99% mortality, field populations of *An. gambiae* s.l. were 100% susceptible to both primiphos-methyl and propoxur 24 hours post exposure. Difference in observed mortality rates between the three sampling sites was not significant ($P > 0.05$). However, there was significant difference in mortality rate among the insecticides in all sampling sites ($P < 0.05$) (Figure 5). Two percent and four percent control mortality was recorded at the end of 24 hours holding time in Gambella Town and Abobo district as determined by susceptibility test of *An. gambiae s.l.* against DDT and propoxur respectively.

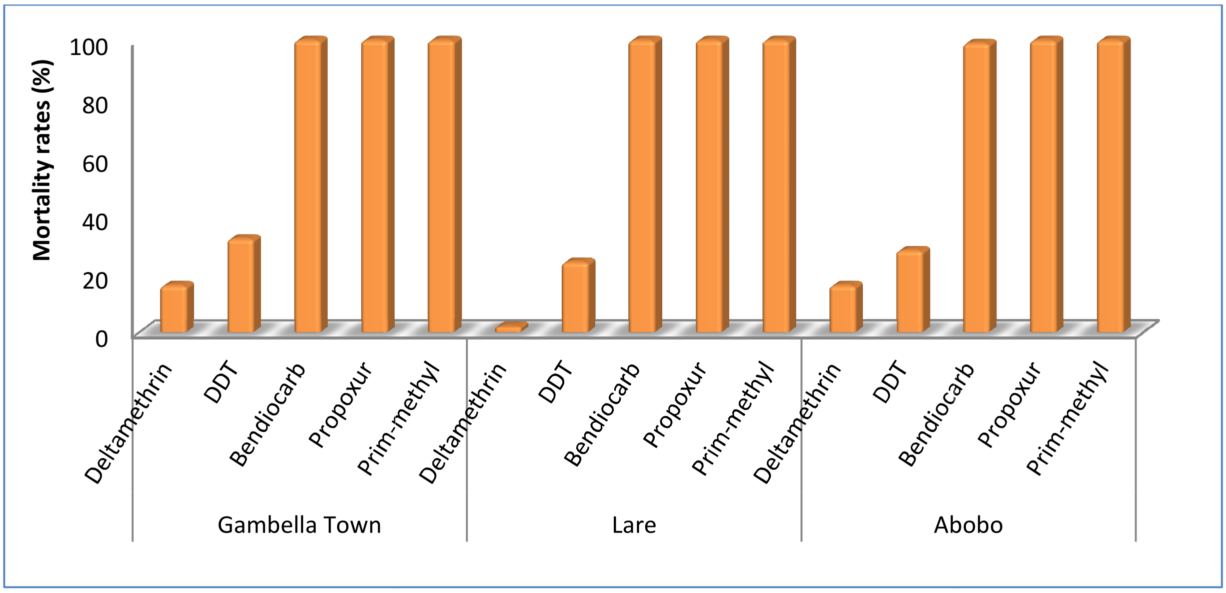

Figure 5: Susceptibility of *An. gambiae* s.l. to discriminating doses insecticides, southwestern Ethiopia

For both DDT and deltamethrin 50% and 95% knockdown times were not observed within 60 minutes of the exposure period. Therefore, it would not be possible to determine KDT_{50} and KDT_{95} values for both insecticides in all the study areas (Table 6).

Table 6: Knockdown of An. gambiae s.l. exposed to discriminating concentrations of DDT and deltamethrin, southwestern Ethiopia

Study site	Insecticide	KDT (minute)						
		10	**15**	**20**	**30**	**40**	**50**	**60**
Gambella Town	DDT	0	1	2	2	4	11	18
	Deltamethrin	0	0	0	0	1	7	12
Lare	DDT	1	9	19	23	24	27	29
	Deltamethrin	1	1	1	1	5	9	13
Abobo	DDT	0	3	10	14	17	21	24
	Deltamethrin	0	0	2	4	8	12	14

11 Discussion

In the current study anopheline mosquito species composition were similar in all the study areas. The result shows that five anopheline mosquito species were collected, these include: *An. gambiae* s.l., *An. pharoensis, An. funestus* group*, An. wellcomi* and *An. nili*. This finding is similar to the findings reported from Gambella Town and villages around Baro River (Krafsur, 1977). *An. gambiae s.l* was the predominant species in the study areas. Similarly, the report of Krafsur and Armstrong (1978) indicated that a member of *An. gambiae* complex, *An. arabiensis* was the predominant species.

Analysis of the results from the entomological assessment shows that mosquito densities per trap did not differ significantly between pre and post-IRS operation in areas where deltamethrin was sprayed (Gambella Town and Lare district). This could be from resistance development of anopheline against deltamethrin sprayed in the areas. The finding is also consistent with the present susceptibility test conducted in which *An. gambiae* s.l. is highly resistant to deltamethrin. However, there was considerable reduction in anopheline mosquito species density per trap in Abobo district, which was sprayed by propoxur. Reduction of vector density in this district could be indicative of the effective action of propoxur sprayed (PMI, 2013; WHO, 2003).

Mean percentage of parous rate of all anopheline mosquitoes pre-IRS was less than 50%. This observation could indicate that a small proportion of anopheline mosquitoes in the study areas had blood fed (Olayemi & Ande, 2008) or it could be from the time of sampling the mosquitoes as high parous rate mosquitoes are more in drier areas or in dry season than wet areas or wet season (Warrell & Gilles, 2002). Mean percentage of parous rate of all anopheline mosquito was further declined following IRS. The reduction in parous rate of the species could be from the application of IRS (Olayemi & Ande, 2008). Furthermore, less parous rate is directly proportional to less probability of daily survival. The relatively high mean percentage of parous rate of *An. gambiae* s.l. during post-IRS may possibly be from more resistance development of the species than other anopheline mosquitoes. Evaluation of the areas according to the insecticides used shows that the mean percentage of parous rate in propoxur sprayed area is almost reduced by 50% as compared to deltamethrin sprayed areas where percentage of parous rate is reduced only by 20%.

There was no significant difference in fed to gravid ratio among anopheline mosquito species. The ratio of all *Anopheles* species in the present study was approximately 1.0:1.0. Thus the duration of gonotrophic cycle was just about two days for all anopheline mosquito species in the study areas. Gonotrophic cycle of three days has been reported in the highland areas of east Africa and in Cameroon. In agreement with the present study, Gillies & Meillon (1968), two day cycle has been reported to be common throughout Africa. Therefore, infective anopheline mosquitoes in the study areas require at least four blood meals, with possibility for malaria parasite transmission. As the number of gonotrophic cycles of malaria vectors increase, the greater is its epidemiological importance (WHO, 2002). The likelihood of the parasite transmission within two days of gonotrophic cycle has also been reported by previous study from Nigeria (Olayemi & Ande, 2008).

The overall probability of daily survival of anopheline mosquito species in this study was 0.62 which was lower than that of the study conducted on some malaria vectors in a lowland region of Ethiopia (0.89 in wet season and 0.79) in dry season (Krafsur, 1970). Mean probability of daily survival of mosquito species decreased post-IRS operation. These reductions in survival rate could be from intensive use of IRS and LLINs to fight malaria vectors in the region because both IRS and LLINs function by reducing female mosquito probability of daily survival (Massebo, 2013). Related studies conducted in Benin and Malawi showed that daily survival of *An. gambiae* s.l. was reduced in IRS operated areas (Ossè *et al.* & Wondji *et al.*; 2012). The probability of daily survival of *An. gambiae* s.l. and *An. pharoensis* was relatively higher in deltamethrin sprayed areas but significantly reduced in propoxur sprayed area. This finding might suggest propoxur is more effective in reducing the daily survival of these species. On the other hand *An. gambiae* s.l. had the highest daily survival probability in both deltamethrin and propoxur sprayed areas. This also shows that *An. gambiae* s.l. could be well adapted to the environmental conditions of the study areas. Comparable finding was also reported from Nigeria (Olayemi & Ande, 2008).

This study revealed that more number of *An. wellcomei* and *An. gambiae* s.l. were caught indoors than outdoors. The recent baseline survey conducted in Ethiopia is also in consistent with our findings (PMI, 2013). Reduction in indoor density of anopheline mosquito species was observed post-IRS operation using deltamethrin and propoxur. This could be due to behavioral resistance when the resistant insects stay away from the insecticide treated surfaces by shifting their usual behavior (Ménard *et al*., 1997). Indoor density of *An. gambiae* s.l., which is the primary malaria vector of Ethiopia, decreased post-IRS. In contrary, the outdoor density increased. The change in resting habit of the species could be challenge in malaria vector control program in the region where LLINs and IRS which target indoor resting mosquitoes. In agreement with the present finding, studies conducted in different African countries on distributions of malaria mosquitoes revealed the effect of IRS and insecticide treated nets in reducing indoor resting habit and increasing the rate of exophily (Coetzee, Craig & Ie Seur, 2000). Furthermore, the previous study conducted in Gambella, Ethiopia showed that mosquitoes escape from living houses during the spraying and immediately post spray period (Krafsur, 1977).

The majority of *An. gambiae* s.l., *An. pharoensis* and *An. wellcomi* were trapped earlier than 22:00 hours, before people go to bed. In-line with the present, study conducted in Tigray and Zwai Ethiopia showed that the peak biting time of *An. arabiensis* was from 18:00 to 20:00 (Yohannes & Boelee, 2012). Moreover, the highest peak biting activity of the same species in Tanzania was detected at 20:00. However, peak activity for *An. arabiensis* varies in different countries of Africa. Accordingly, in Kenya, Chad and Senegal wee small hours was the highest peak activity time recorded for the species. On the other hand, in Mozambique and Tanzania, high activity of the species were recorded at 20:00 (Fornadel *et al*., 2010). A peak outdoor and indoor activity of *An. nili* was recorded between 02:00-03:00 and 01:00-02:00 hours respectively. Comparable hourly activity of *An. nili* was recorded in earlier study conducted in villages nearby Baro River banks around Gambella Town, southwestern Ethiopia (Krafsur, 1977).

The level of mortality of *An. gambiae* s.l. recorded in the resistance tests of the present study using DDT and deltamethrin was low and didn't exceeds 32% in all the study areas. This suggests that *An. gambiae* s.l. from all the 3 study sites developed resistance against DDT and deltamethrin. Similarly, DDT resistance has been reported from Gambella (Abose *et al*. 1998; Nigatu *et al*., 1992). Furthermore, the previous longitudinal study conducted in Omo Nada, Kerssa, Tiro Afeta and Sekoru districts, Ethiopia, showed that a member of *An. gambiae* complex, *An. arabiensis*, resistant to DDT and deltamethrin (Yewhalaw *et al.,* 2011; 2010). The development of resistance of *An. gambiae* s.l. to deltamethrin could be from the prolonged use for IRS and community-wide use of deltamethrin impregnated LLINs.

Populations of *An. gambiae* s.l. were 100% susceptible to propoxur (Carbamate) and primiphos-methyl (Organophosphate) 24 hours post exposure. Furthermore, 99% mortality against bendiocarb (Carbamate) was found only in Abobo district. Likewise, complete susceptibility of *An. arabiensis* to propoxur was reported from Omo Nada, Kerssa, Tiro Afeta and Sekoru districts, southwestern Ethiopia (Yewhalaw *et al*., 2011). In addition to this, the recently conducted study in central, northern and southwestern Ethiopia indicated the susceptibility of *An. arabiensis* to primiphos-methyl, propoxur, and fenithrotion (Balkew *et al*., 2012). A previous study conducted in Sudan also showed susceptibility of *An. arabiensis* to bendiocarb and propoxur (Abdalla *et al.*, 2008). These results indicated that the three insecticides can be potential alternatives in IRS use in the study sites.

Fifty and ninety five percent knockdown was not observed in populations of *An. gambiae* s.l. for DDT and deltamethrin within 60 minutes of exposure period indicating the high resistance of this mosquito species to DDT and deltamethrin. Therefore, determining KDT_{50} and KDT_{95} is not possible for both insecticides. Similarly, the result from previous study in villages around Gilgel-Gibe dam, southwestern Ethiopia shows unfeasibility of determining KDT_{50} and KDT_{95} for DDT and deltamethrin (Yewhalaw *et al*., 2011). In line with this study, the study conducted in Senegal where mosquitoes were relatively resistant to DDT, KDT_{50} was long (Ndiath *et al*., 2012).

12 Conclusion

The development resistance by anopheline mosquitoes against deltamethrin and peak hourly activity of *An. gambiae* s.l., *An. pharoensis* and *An. wellcomi* before the people go to bed has been determined by assessment of entomological parameters. Resistance development of *An. gambiae s.l.* in susceptibility test against DDT and deltamethrin could be from the long term use of the insecticides for IRS.

13 **Recommendations**

Community awareness on personal protection against the bite malaria vectors before they go to bed need to be raised in the study areas by coordination of Gambella Region Health Bureau. Gambella Region Health Bureau need to replace deltamethrin with potential alternative insecticide such as Bendiocarb and primiphos-methyl. Entomological surveillance and insecticide resistance monitoring need to be conducted by Federal ministry of Health and Gambella Region Health Bureau for effective malaria vector control and to slow down the development and spread of insecticide resistance.

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15 List of Annex

Annex I: Form for recording susceptibility test data in the field (WHO, 2013c)

Village Code ☐ ☐ ☐ Test Number ☐ ☐ ☐ ☐ Date (dd-mm-yy) ☐ ☐ / ☐ ☐ / ☐ ☐

Investigator name: .... Code investigator ☐ ☐ ☐

Area information

Country: .... Province: ....

District: .... Commune: .... Village: ....

GPS position **UTM_X** ☐ ☐ ☐.☐ ☐ ☐ **UTM_Y** ☐ ☐ ☐.☐ ☐ ☐

Sample information

Species tested: .... Species control: ....

Sex: .... Age (days): .... (only if known: colony & F1)

Collection method

Human Landing Indoor ☐ Resting night Indoor ☐ Resting morning Indoor ☐

Cattle Collect ☐ Human Landing Outdoor ☐ Resting night Outdoor ☐

Other: specify .... Larval collection ☐ Progeny F1 ☐

Colony ☐ Name of colony strain: ....

Physiological stage

Non-blood fed ☐ Blood fed ☐ Semi-gravid ☐ Gravid ☐

Test insecticide information

Insecticide tested: .... Date of expiry: ☐ ☐ / ☐ ☐ / ☐ ☐

Impregnated papers prepared by: .... Date box first open: ☐ ☐ / ☐ ☐ / ☐ ☐

Concentration: .... Number of times this paper is used: ☐ ☐

Storage conditions: Room temperature ☐ Refrigerated ☐

Test conditions

	Exposure period: Start	**End test**
Temperature °C	☐ ☐.☐	☐ ☐.☐
Relative humidity (%)	☐ ☐	☐ ☐

Annex II: Form for recording results of susceptibility testing in the field (WHO, 2013c**)**

	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Control 1	Control 2
No. exposed						

Number of knocked down (KD) mosquitoes after exposure for minutes

	Replicate 1		Replicate 2		Replicate 3		Replicate 4		Control 1		Control 2	
	Time	No.	Time	No.	Time	No.	Time	No.	Time	No.	Time	No.
START												
10'												
15'												
20'												
30'												
40'												
50'												
60'												

Number of dead/alive mosquitoes at the end of holding period (24 hours)

	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Control 1	Control 2
No. dead						
No. alive						

To be completed by a supervisor at the end of the test

Code of supervisor ☐ ☐ ☐

Comments ....

....

I confirm that the form is complete.

Date: ☐ ☐ / ☐ ☐ / ☐ ☐ ☐ ☐

Name ....

Signature ....

To be completed by data entry clerks during data entry

Data entry clerk 1	**Data entry clerk 2**
Date ☐ ☐ / ☐ ☐ / ☐ ☐ ☐ ☐	Date ☐ ☐ / ☐ ☐ / ☐ ☐ ☐ ☐
Signature	Signature ....

Annex IV: Formats for hourly mosquito collection by CDC light trap

Region: ___________________, District: ___________________, Kebele: ____________________

Locality name: _______________, House code: __________, Altitude_______________ Date of collection: __________, Round: ________

Collection time	Types of anopheline mosquito												Total anopheline mosquito		Total *Culicine*	
	An. gambiae s.l		*An. pharoensis*		*An. nili*		*An. funestus* group		*An. wellcomei*		Others					
	In	Out	In	Out	In	Out	In	Out	In	Out	In	Out	In	Out	In	Out
18:00-19:00																
19:00-20:00																
20:00-21:00																
21:00-22:00																
22:00-23:00																
23:00-00:00																
00:00-01:00																
01:00-02:00																
02:00-03:00																
03:00-04:00																
04:00-05:00																
05:00-06:00																
Total																

Collector's name: -----------------------Sign. ------------------------- Checked by----------------------------------Sign. -------------------

Annex V: Individual mosquito collection registration format

District: ________________, Kebele: ______, Average rain fall & temperature------- & -------- respectively

Altitude of the localities---------------------Months of collections: _______

Date	Round of collection	Locality	House code	No. of An. mosquito	Vector species identified	Indoor/outdoor		Abdominal status				No. dissected	Parity	
						Indoor	Outdoor	UF	FF	HG	G		NP	P

UF = Unfed, FF = Freshly Fed, HG = Half gravid, G = Gravid, NP = Nulliparous and P = Parous

Collector's name --------------------, Signature------------- Checked by-----------------------Signature------------ Date -----------

Annex VI: List of plates

Plate 1: Preparation for mosquito collection by CDC light trap (Photo credit: Hailu Turura)

Plate 2: Mosquito collection by CDC light trap (Photo credit: Hailu Turura)

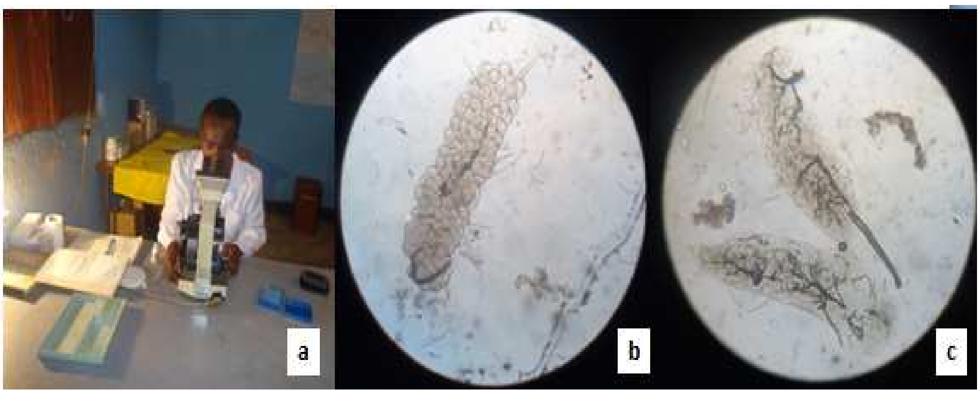

Plate 3: Identification of nulliparous and parous mosquitoes (Photo credit: Teshome Gobena)

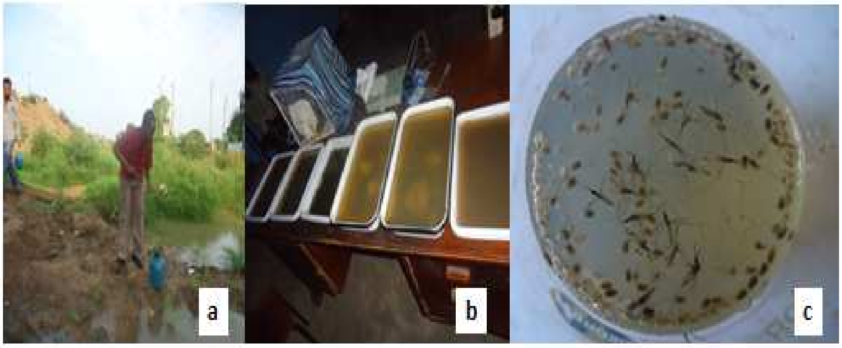

Plate 4: Mosquito larval collection and rearing for susceptibility test (Photo credit: Teshome Gobena)

Plate 5: Running Insecticide susceptibility test (Photo credit: Teshome Gobena)

Plate 6: Mosquito Mortality 24 hrs post exposure and preservation (Photo credit: Hailu Turura)