

# **Anopheles species and malaria transmission risk in a highland area, south-central Ethiopia**

**Abebe Animut Ayele**



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## **Dedication**

*To my late father Animut Ayele Adgeh,  
And my late brother Delelew Animut and sister Saysh Animut,  
who are always in my memory*

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## Summary

Malaria is a growing public health problem in Butajira area, a highland in south-central Ethiopia. However, the occurrence of vectors and the entomological aspects of the disease remain poorly described. This thesis describes abundance, host feeding preference, resting behaviour and entomological inoculation rates (EIRs) of *Anopheles* mosquitoes in low- (Hobe), mid- (Dirama) and high- (Wurib) altitude villages of the area. Housing conditions and the exposure of households to the bite of *Anopheles arabiensis* are also described.

A larval survey and collection were undertaken in the villages following standard entomological methods to describe breeding habitats and their dynamics. Habitats were characterized and late larval instars were identified to species. Adult mosquitoes were sampled from indoors and outdoors and identified to species, and their host preferences and sporozoite infection rates were determined.

From larval and adult collections 10 and nine *Anopheles* species, respectively, were identified. During the dry seasons, the streams serve as the main breeding habitats of *Anopheles* mosquitoes, including *An. arabiensis*. The occurrence of immature *An. arabiensis* was correlated positively with habitat temperature ( $r = 0.33$ ,  $p < 0.05$ ) and negatively with habitat depth ( $r = -0.56$ ;  $p < 0.05$ ). Adult *An. arabiensis* fed on human and cattle with a similar preference. From CDC light trap catches, the annual *P. falciparum* EIR for *An. arabiensis* was 3.7 in the first year (July 2008 - June 2009) in the low-altitude village, while in the same village, the annual *P. falciparum* EIR was zero in the second year (July 2009 - June 2010). The annual *P. vivax* EIR for *An. arabiensis* was 33 in the first year and 14.5 in the second. Sporozoite-positive *An. arabiensis* and *An. pharoensis* were caught inside houses closer to streams. Moreover, houses located in the low-altitude village, and in mid-altitude houses with open eaves, were associated with a high density of indoor-resting *An. arabiensis*.

The density of *An. arabiensis* larvae and the densities of adult *An. arabiensis* and *An. pharoensis*, including sporozoite-positive ones, decreased with an increasing altitude starting from the low-altitude village, whereas densities of the other anophelines increased with an increase in altitude.

**Scientific environment**

Aklilu Lemma Institute of Pathobiology, Addis Ababa University, P.O. BOX 1176,  
Addis Ababa, Ethiopia

Hobe, Dirama and Wurib villages, south-central Ethiopia, Southern Nations  
Nationalities Peoples Regional State (SNNPR)

Centre for International Health, Faculty of Medicine and Dentistry, University of  
Bergen, P. O. Box 7804, Norway

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## List of original articles

**This thesis is primarily based on the following articles, which will be referred to in the text as Paper I, Paper II or Paper III.**

Paper I: Abebe Animut, Teshome Gebre-Michael, Meshesha Balkew and Bernt Lindtjørn. Abundance and dynamics of anopheline larvae in a highland malarious area of south-central Ethiopia. *Parasites and Vectors* 2012, 5: 117.

Paper II: Abebe Animut, Meshesha Balkew, Teshome Gebre-Michael and Bernt Lindtjørn. Blood meal sources and entomological inoculation rates of anophelines along a highland altitudinal transect in south-central Ethiopia. *Malaria Journal* 2013, 12: 76.

Paper III: Abebe Animut, Meshesha Balkew and Bernt Lindtjørn. Impact of housing condition on indoor-biting and indoor-resting *Anopheles arabiensis* density in a highland area, central Ethiopia. *Malaria Journal* 2013, 12: 393.

## List of abbreviations

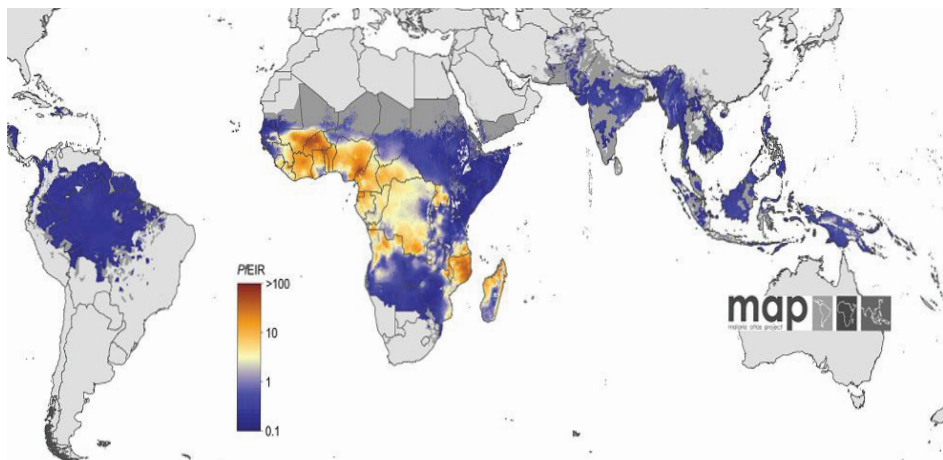
AAU	Addis Ababa University
ALIPB	Aklilu Lemma Institute of Pathobiology
An	<i>Anopheles</i>
APS	Artificial Pit Shelter
CDC	Centers for Disease Control
CSP	Circumsporozoite proteins
DDT	Dichlorodiphenyltrichloroethane
EIR	Entomological Inoculation Rate
ELISA	Enzyme-Linked Immunosorbent Assay
ENSO	<i>El Nino</i> Southern Oscillation
FF	Fresh Fed
GPIRM	Global Plan for Insecticide Resistance Management
GR	Gravid
HBR	Human Biting Rate
HG	Half Gravid
HLC	Human Landing Catch
Ib/p/year	Infective bites per person per year
IRS	Indoor Residual Spraying
ITN	Insecticide Treated Net
IVM	Integrated Vector Management
LHM	Larval Habitat Management
LLIN	Long-Lasting Insecticidal Net
m	Meters
m.a.s.l.	Meters above sea level
NERC	National Health Research Ethics Committee
P	<i>Plasmodium</i>
PSC	Pyrethrum Spray Collection
s.l.	sensu lato
s.s.	sensu stricto

SNNPR Southern Nations, Nationalities and People's Region  
spp. species  
SR Sporozoite Rate  
UF Unfed  
WHO World Health Organization

## 1. Introduction

### 1.1. Global malaria situation

Malaria is a disease caused by protozoan parasites of the genus *Plasmodium*. *Plasmodium falciparum*, *P. vivax*, *P. ovale* and *P. malariae* cause human malaria, of which *P. falciparum* is responsible for severe morbidity and mortality followed by *P. vivax*. During the period from 1900 – 2002, the global population has grown from 1 to 6 billion, with the malaria risk population increasing from 0.9 to 3 billion [1]. An estimated 2.6 billion people in the tropical countries were at risk of *P. falciparum* malaria infection in 2010 (Figure 1). Among these, 1.13 and 1.44 billion people were at risk of unstable and stable infection, respectively [2]. The highest level of *P. falciparum* transmission occurs in Africa, which contributes to 99% of the global- and 95% of the African falciparum malaria cases.

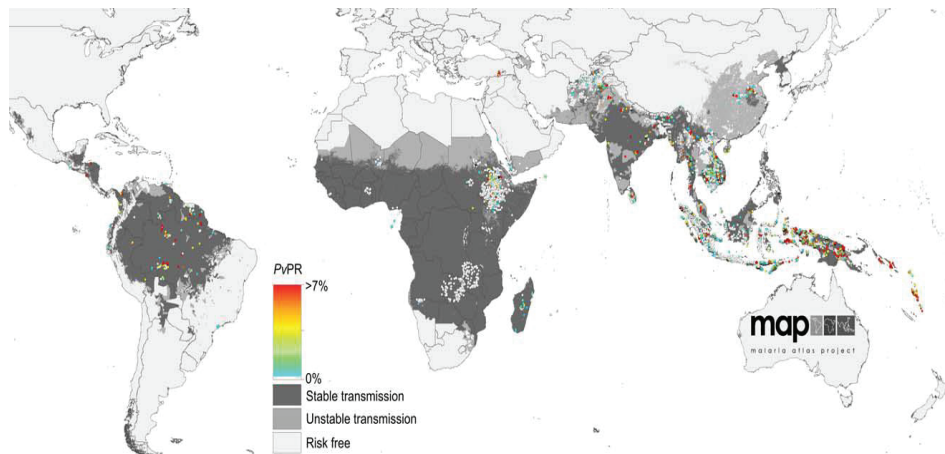


Source: Gething *et al. Malaria Journal*, 2011, 10: 378

**Figure 1:** Distribution of *Plasmodium falciparum* based on entomological inoculation rate (PfeIR) in 2010. The medium grey areas had an annual parasite incidence (PfAPI) < 0.1 per 1,000 per annum (pa) and the light grey had a PfAPI = 0 per 1,000 pa which is with no risk.

In the same year, approximately 2.5 billion people were at risk of *P. vivax* infection (Figure 2). Among these, 1.5 and 1 billion were at risk of unstable and stable *P. vivax* malaria infection, respectively [3]. The highest population at risk of *P. vivax* infection lived in Central Asia (2.05 billion), which was followed by Southeast Asia (215

million) and South and Central America (137.4 million). Africa has a population of 74.4 million at risk of *P. vivax*.



Source: Gething *et al.* PLOS Neglected Tropical Diseases, 2012, Volume 6 | Issue 9 | e1814

**Figure 2:** Distribution of *Plasmodium vivax* malaria endemicity in 2010. The dark grey areas had a PvAPI  $\geq 0.1$  per 1,000 pa, medium grey areas had a PvAPI  $< 0.1$  per 1,000 pa and the light grey areas had a PvAPI = 0 per 1,000 pa. *P. vivax* prevalence between January 1985 and June 2010 are presented as a continuum of light green to red, with zero-valued surveys shown in white.

A substantial reduction in malaria transmission has been achieved globally, particularly in endemic countries between 2000 and 2012 [4]. Over this period, the malaria mortality rate was reduced by 42% in all age groups and by 48% in children under five years of age. Approximately 3.3 million deaths were prevented between 2001 and 2012, of which 91% were children under five years of age in Africa. The reduction was mainly associated with a scaled-up support by international donors, socioeconomic developments, the deployment of artemisinin-based combination treatment, a wider coverage of long-lasting insecticidal nets (LLINs) and indoor residual spraying in malarious areas [5].

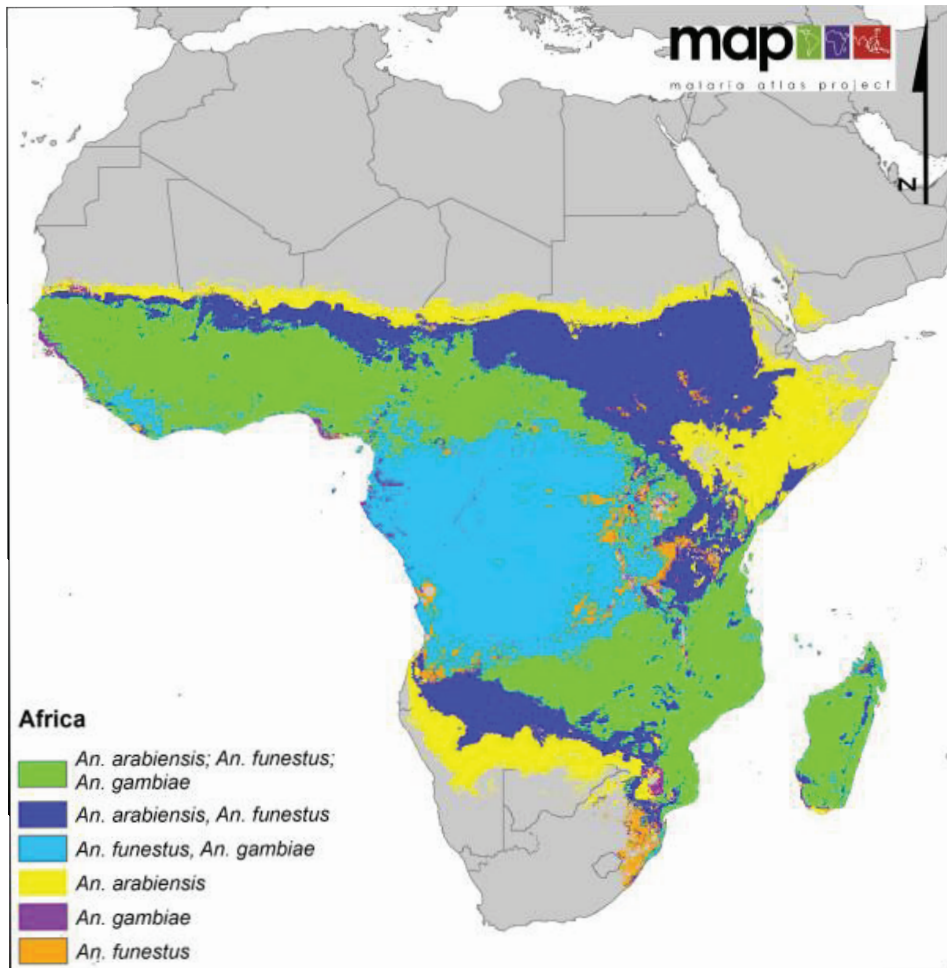
Despite the scaled-up intervention efforts and positive gains, malaria continues to be a major public health problem [4]. In 2013, there were still 104 malaria-endemic countries and territories, where an estimated 3.4 billion people lived. Approximately 207 million cases and 627,000 deaths were documented in 2012. Most cases (80%)

and deaths (90%) occurred in Africa, where children under the age of five years contributed to 77% of the total deaths [5].

Malaria is transmitted by the bite of female mosquitoes of the genus *Anopheles*. Globally, there are over 537 species of *Anopheles*, most (87%) of which have been formally named [6]. Among these, 70 species can transmit human malaria parasites, and 41 are dominant vectors globally. In Africa, there are over 140 *Anopheles* species, of which at least eight are effective vectors of malaria. The three most dominant vectors in the continent (Figure 3) are *An. gambiae* s.s. and *An. arabiensis* (in the *Anopheles gambiae* complex) and *An. funestus* [6, 7].

*Anopheles gambiae* complex contains the most widespread species in sub-Saharan Africa. These are *An. gambiae* s.s. Giles, *An. coluzzii* Coetzee and Wilkerson, *An. arabiensis* Patton, *An. bwambae* White, *An. melas* Theobald, *An. merus* Dönitz, *An. quadriannulatus* Theobald, *An. amharicus* Hunt, Wilkerson, Coetzee and Fettene and *An. comorensis* Brunhes, le Goff and Geoffroy, among which the first three are the major malaria vectors [8, 9].

Most female *Anopheles* mosquitoes require a blood meal on a regular basis to support the development of their eggs. Male and female gametocytes are taken up by female mosquitoes during blood feeding, and passed to their gut. Within the gut of the mosquito, both the male and female gametocytes escape from their erythrocytes, mature and fuse to form zygote. The zygote then develops into sporozoites after passing through successive developmental stages and invades the mosquito's salivary gland. This mosquito is infective and injects the sporozoites into susceptible human hosts during its successive bites [10].



Source: Sinka *et al.* Parasites and Vectors, 2012, 5:69

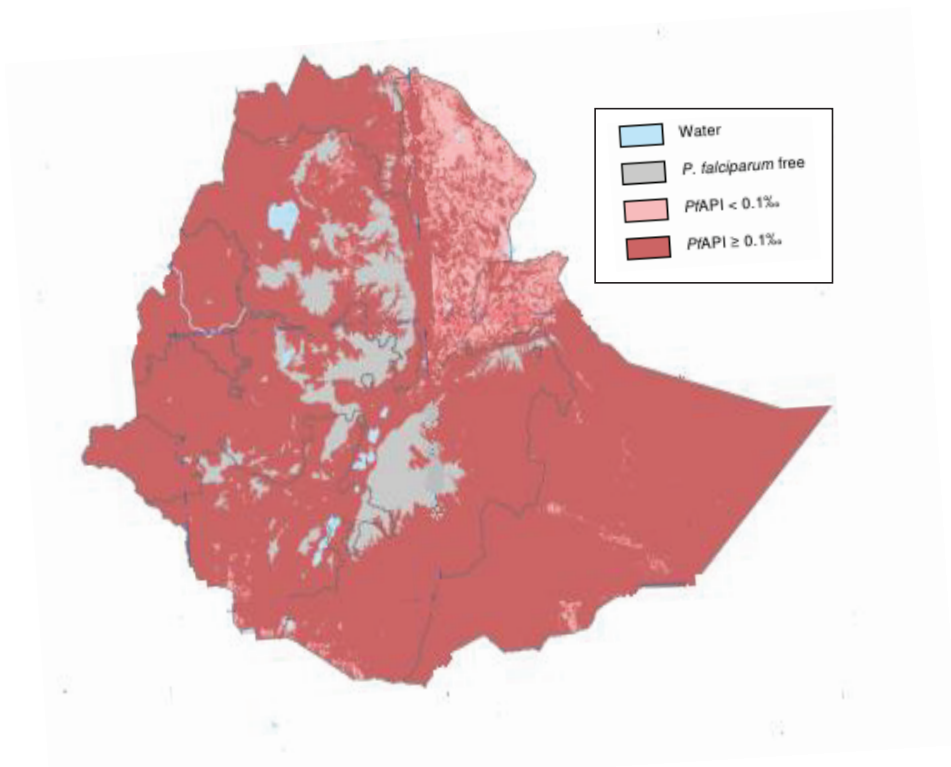
**Figure 3:** A regional map showing the distribution of the three most dominant malaria vectors in Africa.

## 1.2. Malaria in Ethiopia

Malaria is the leading public health problem in Ethiopia, and is a risk for the life of approximately 70% of the population [11], with roughly 75% the land surface being malarious. Areas below an elevation of 1,500 m are considered to be lowlands, and are affected by seasonal malaria transmission. Those located from 1,500 m to less than 1,750 m are considered as highland fringes, and are characterized by a high transmission and an epidemic of malaria. Areas located at approximately 1,750 m or



higher elevations are highlands, which are affected by occasional epidemics. *Plasmodium falciparum* and *P. vivax* are the most dominant parasites responsible for the majority of malaria cases, while *P. ovale* and *P. malariae* contribute to less than 1% of the cases [12]. Malaria transmission exhibits a seasonal and unstable pattern [12, 13], and differs with altitude, rainfall and regional climate variability (Figure 4). The current global malaria mapping studies show that the majority of the Ethiopian land surface is characterized by a stable type of *P. falciparum* and *P. vivax* transmission [2, 3].



Source: Gething *et al. Malaria Journal*, 2011, 10: 378

**Figure 4:** Spatial distribution of *Plasmodium falciparum* transmissions in Ethiopia, 2010

The duration of disease transmission also varies greatly with locality and altitude. Most endemic areas of central and western Ethiopia experience malaria transmission for a period of less than three months per year, while the lowlands in the eastern part of the country maintain the transmission near water bodies. Malaria case distribution is therefore characterized by a spatial and temporal heterogeneity in the majority of the areas. The disease is both endemic and seasonal in most areas, and has been presented in the form of epidemics at intervals of five-eight years in highlands and highland fringe areas [12, 14]. The heterogeneity of malaria transmission is primarily associated with the pattern of regional rainfall, temperature, humidity and land use [11, 13].

### **1.3. Highland malaria**

Highland areas of Africa have been experiencing unstable and spatially focal malaria transmission [15]. There has been documentation of epidemic malaria since the 1920s in highland areas of East Africa, including western Kenya, Uganda, Ethiopia, Tanzania, Rwanda and Madagascar. Compared to the 1920s and 1950s, the current pattern of malaria is characterized by increased frequencies and expanded geographic areas [16].

Factors such as land use, climate change, socio-economic, insecticide-resistant vectors and drug resistant parasites have been cited as the contributors of increasing malaria transmission in the highlands. Riparian forest and swamp clearing has increased the number of man-made mosquito breeding habitats and increased indoor temperatures, thereby maintaining the reproductive fitness of vectors in the highlands [17, 18]. Climate change has been considered as a driving factor for the increasing trend of malaria transmission in the East African highlands, as temperature affects the development and survivorship of the parasites and vectors. Rainfall increases the availability of the mosquito larval habitat, and thus the vector population [16]. However, no agreement has been reached on the level of the effect of climate change on malaria due to a lack of good quality data, variations in impact assessment methods and variations in local climates. The 1997 – 1998 *El Nino* southern oscillation

(ENSO) was one of the largest climatic changes ever, which resulted in exceptionally heavy rainfall and raised temperatures. The ENSO event caused 2.4 times more rainfall than normal, and was expected to precipitate malaria epidemics in Tanzania. Nonetheless, it was found to cause a significantly lower prevalence of malaria parasitaemia and splenomegaly [19].

Socio-economic and housing conditions such as living within 450 m of a vector breeding site, regular or recent travel to malarious areas, low or no vegetation cover in the living compound, houses without ceilings, houses with a separate kitchen building, living within 200 m of a maize field and houses where female household heads had no education are at an increased risk of malaria infection in the highlands [15, 20].

In Ethiopia, malaria transmission has been documented in high-altitude areas of the country, starting from the widespread epidemic that took place in 1958 [18]. Several studies [11, 21, 22] have reported an occurrence of malaria cases in areas located between 1,750 m and 3,000 m.a.s.l. This indicates the expansion of the disease into higher altitude areas of the country, showing that malaria is no longer only a lowland disease. Even so, it remains vital to describe the occurrence of the vectors and the entomological aspects of malaria (human blood index, sporozoite rate, entomological inoculation rate and the indoor-biting and indoor-resting densities of anophelines) that drive the disease in the highlands, in order to draw a complete picture for the risk of malaria transmission in such settings.

#### **1.4. *Anopheles* species and vectors of malaria in Ethiopia**

About 45 species of *Anopheles* mosquitoes are believed to occur in Ethiopia [23, 24], among which *An. gambiae* s. l. is the most prevalent. *An. gambiae* s. l. occurs in most parts of the country and breeds in different types of water collections from small sunlit natural pools and temporary breeding habitats created by man on the shores of lakes. *An. arabiensis*, a member of the *An. gambiae* complex, is the major malaria vector in Ethiopia, and is responsible for malaria epidemics in most parts of the country [24].

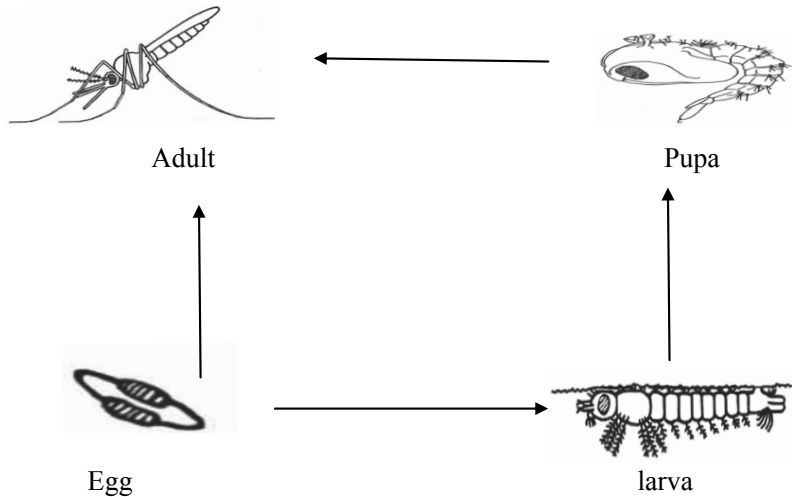
*An. amharicus* (previously known as *An. quadriannulatus* sp. B) is the other member of the complex reported to occur in the country and is mainly zoophilic, having no role in the transmission of malaria. *Anopheles arabiensis* transmits malaria in most endemic areas and *An. pharoensis*, *An. funestus* and *An. nili* may also transmit the disease in some areas [12, 24-26], but at present the role of the latter two species is not known. *Anopheles cinereus*, *An. coustani*, *An. rhodesiensis*, *An. d'thali*, *An. maculipalpis* and *An. paludis* are indicated to be susceptible to malaria parasites [24, 27], but their importance in the transmission of malaria is not yet known because of little or no entomological studies that have targeted these species.

Although malaria cases have been reported consistently in the highlands of Ethiopia, including in the south-central highlands [11, 12, 21, 28, 29], the species of *Anopheles* that occur in the area and their role in transmitting the disease are not clearly described. This study was undertaken to document the species of *Anopheles*, their distribution and the entomological aspects of malaria transmission risk in a highland area of south-central Ethiopia.

## **1.5. Bionomics of *Anopheles* mosquitoes**

### **1.5.1. Life cycle**

After mating and blood feeding, a female *Anopheles* mosquito lays 50-200 small brown or blackish boat-shaped eggs per oviposition, singly onto a water surface (Figure 5). In tropical countries, eggs hatch into larvae within two-three days, but may take two-three weeks depending on the local temperature. Larvae develop through four instars (first, second, third and fourth) before they metamorphose into pupae after five-ten days and finally into adults [10, 30].



Source: World Health Organization (2013). Larval Source Management: A supplementary Measure for Malaria Vector Control, an Operational Manual.

**Figure 5:** *Anopheles* mosquito life cycle

The body of a larva is divided into three regions: the head, the thorax and the abdomen. The head bears mouth brushes, a pair of antennae and a pair of compound eyes, while mouth brushes serve to sweep water containing minute food particles into the mouth. The thorax is roundish and has hairs, which are usually long and conspicuous. Its segmented abdomen has hairs (either unbranched or branched). The last abdominal segment has two paired groups of long hairs that form the caudal setae, and a larger fan-like group comprising the ventral brush. It ends in two pairs of transparent, sausage-shaped anal papillae, which undertake osmoregulation. Most of the abdominal segments have a pair of palmate hairs, which aid larva in keeping parallel to the water surface. *Anopheles* larva must come to the surface to breathe, and take in atmospheric air through a pair of dorsally situated spiracles. It feeds on yeast, bacteria, protozoa and numerous other micro-organisms, as well as on decaying plant and animal materials found on the water's surface [10, 30].

The fourth instar larva moults into comma-shaped pupa. The head and thorax of the pupa are combined to form cephalothorax, which dorsally has a pair of respiratory trumpets. It comes to the surface frequently to breathe through its trumpets, but does

not feed. After a few days, depending on the local temperature, the dorsal surface of the cephalothorax splits and the adult mosquito emerges [10, 30].

After emerging from pupa, the female mates with a male and finds a blood meal for its eggs to mature. An adult *Anopheles* has a slender body divided into a head, thorax and abdomen. The head contains the eyes and a pair of long many-segmented antennae, which help to detect the host odour and breeding site. The head also has an elongated forward-projecting proboscis used for feeding and two sensory palps. The thorax is specialized for locomotion, and is the place where three pairs of legs and a pair of wings are attached. The abdomen is specialized for food digestion and egg development, and expands considerably during the blood meal. The blood is digested over time, and serves as a source of protein for egg production [10, 30].

### **1.5.2. Habitats of larvae**

Naturally, rainfall is the primary source for the formation of larval habitats, which include the landward edges of floodplains, footprints, ponds, rain pools, puddles, tire tracks and hoof prints [31]. In some cases, however, rainfall can negatively affect mosquitoes by flushing their eggs and larvae, and also by killing them directly [32]. Not all water collections serve for mosquito breeding, as a great majority of them could be transient and live a short amount of time before the maturation of larvae into pupae and adults. It is the stable and relatively bigger habitats that contribute to pupae and adult mosquito production. In western Kenya, pupal occurrence was found to be positively correlated with habitat stability, and also with habitat size [33].

*Anopheles* mosquitoes inhabit diverse larval habitats, including water overflows, irrigation ditches, borrow pits, wheel ruts, hoof prints, foot prints, rice field puddles, small streams, dams, riverbed pools, seepage springs, shallow wells, ponds, irrigation channels, the edges of lakes, lake lagoons, slow flowing rivers, natural depressions in the ground, swamps, pools in drying stream beds, disused goldmines, plant hollows and cavities, epiphytic arboreal and terrestrial bromeliads, rock holes in stream beds, tree holes, water-filled bamboo stump, pitcher plants, leaf axils in a banana tree,

pineapples and other plants, water-filled split coconut husks and snail shells. Larvae also occur in “man-made container-habitats”, such as wells, clay pots, water-storage jars, tin cans, discarded kitchen utensils and motor vehicle tires. A few mosquitoes breed almost exclusively in brackish or salt water, while some species are less specific in their requirements and can inhabit a wide range of breeding habitats including lakes [30, 34, 35].

In Eritrea, the larvae of *An. arabiensis* were predominant in stream edges and stream bed pools [36], in addition to rain pools, ponds, dams, swamps and drainage channels at communal water supply points. In the dry seasons of western Kenya, burrow pits and pools in stream beds have contributed to a significant increase in *An. arabiensis* pupal productivity [37]. In Eritrea, mosquito breeding persists year round in stream bed pools, but significantly decreases with an increase in rainfall [36].

The major malaria vectors of Africa, *An. arabiensis* and *An. gambiae* s.s. often inhabit small and sunlit temporary water pools [33]. However, they adapt to existing local conditions. *Anopheles funestus*, *An. arabiensis* and *An. gambiae* s.s. were found to breed on the shores [35] and in the large backwater pools (lagoons) of Lake Victoria [38]. In the study, *An. arabiensis* was significantly higher in lakeshore habitats with short grass compared to habitats containing tall vegetation. The density of *An. arabiensis* in habitats surrounded by non-woody tall plants was significantly higher than the density in habitats exposed to waves. *Anopheles arabiensis* was the most dominant mosquito in Lake Victoria, thereby suggesting that it may proliferate in big and permanent habitats, in addition to small and sunlit temporary pools [33]. Lagoons supported greater densities of *An. funestus* s.s. and *An. Rivulorum*, as well as several patches of open habitats that maintained *An. arabiensis* and *An. gambiae* s.s. [35].

Members of the *An. gambiae* s.l. breed more abundantly in the aquatic habitats of pasture land than in farmland, indicating their preference for sunlight and higher temperatures. Larval habitats in pasture lands are exposed to sunlight for a long time, which helps to provide suitable habitats for larval growth and oviposition by gravid

female *Anopheles* mosquitoes [37, 39]. They occur in small, open disused goldmines, hoof prints and in cultivated swamps. Grass covered habitats cause a decrease in the abundance of *An. gambiae* s.l. and an increase in *An. funestus* and other *Anopheles* larvae [40]. In the shores and lagoons of Lake Victoria, the occurrence of *An. arabiensis* was significantly greater in habitats with short grass and uncovered areas compared to habitats containing tall vegetation [35].

*Anopheles* larval management can play a significant role in the control of malaria, especially in areas where vectors are resistant to chemicals used for indoor residual spraying and mosquito net impregnation, exophilic species and also where antimalarial drug resistance is a problem. However, the control of *Anopheles* mosquito larvae requires an adequate knowledge of the local breeding habitat types and dynamics of the immature stages [30].

### **1.5.3. Survival strategies in dry seasons**

In order to maintain a continuation of life, *Anopheles* mosquitoes must survive dry seasons during which little or no larval development occurs. Exhibiting an aridity tolerance is essential for *Anopheles* mosquitoes distributed over wide areas that have different rainfall patterns and longer dry periods [41]. Members of the *An. gambiae* and *An. funestus* occur in a variety of environments, including dry savannas, semi-deserts and dry seasons, where the surface water required for larval development disappears for four to eight months each year [42]. They breed in a wide array of habitats ranging from smaller intermittent pools of water, including hoof prints, to big stagnant water bodies and slow-moving rivers [31, 35].

Aquatic stages of some *Anopheles* mosquitoes may survive on wet soil in transient breeding habitats and contribute to upcoming adult populations. A study by Koenraadt *et al.* [43] indicated that the eggs of *An. gambiae* s.s. hatched on damp soil, and that the emergent larvae were capable of moving up to 10 cm to reach the nearest surface water, thus enabling further development. In the study, larvae reaching nearby surface water decreased with an increasing distance. Moreover, first-, second- and third-instar



larvae survived on damp soil for a period of 64, 65 and 69 hours, respectively, while fourth-instar larvae survived for 113 hours [43]. The eggs of *Anopheles* mosquitoes survived on moist soil for up to two weeks [44]. Under identical breeding conditions, female *An. arabiensis* showed significantly higher desiccation resistance than female *An. gambiae* s.s. at emergence and post emergence from the egg. Water content was found to be higher in *An. arabiensis* than in *An. gambiae* s.s. at emergence, which might be one possible reason for the physiological variation in desiccation resistance between the species [45].

In a semi-arid part of the Sudan, during a period of 11 dry months between November 1966 and December 1967, *An. gambiae* having a fresh or older blood meal, but not fully distended abdomen, was found. This indicates that the adult stage is adapted to survive through severe drought seasons in hot arid zones of the country. Its feeding activity continued while the ovarian development was retarded, and only one batch of eggs matured during a nine-month dry period. But in the Nile Valley of the area, *An. gambiae* were subjected to a continuous year-round breeding [46]. A mark release-recapture experiment undertaken in Mali provided evidence that *An. gambiae* s.s. undergo aestivation up to a period of seven dry months [42].

The members of the *An. gambiae* complex differ in their abundance according to season, local rainfall and latitude, which reflects their differences in physiology and behaviour [47]. In the Sahel region of Mali, *An. arabiensis* and *An. gambiae* s.s. were found together, though the M form was predominant during the long dry seasons, while *An. arabiensis* and the S form were more common during the wet seasons [42, 48]. When the M and S forms were compared, the M form was more drought tolerant than the S form [8]. In East Africa, *An. arabiensis* was found to be more arid tolerant than the S form of *An. gambiae* [49, 50]. These comparisons suggest variations in drought adaptation within a single *Anopheles* species. The less dry resistant populations might therefore be re-established or enriched via migration during each rainy season [51].

Flight increases metabolic rates approximately 18- to 22-fold in *An. gambiae* s.l. Hence, aestivation may reduce the energy demand of a female mosquito and increase its age. A resting female mosquito may have a 40% reduction in its energy requirement for active flight and consequently have a longer lifespan. Resting in a cooler shelter and avoiding flight activity may favour a longer survival for mosquitoes in dry seasons. Since aestivation reduces the energy requirements and activities associated with sugar and blood source searches, as well as oviposition sites, the survival of the mosquitoes will be longer during the dry seasons [47].

The utilization of blood meals to fulfil energy requirements, rather than reproductive demands, can be a costly strategy for aestivating females. Freshly blood fed and gravid females need mean metabolic rates of approximately 2.6- and 1.6-fold higher than unfed females, respectively [47]. For an active female destined to oviposit, a blood meal sustains her for an interval of 3.8 days compared to a sugar meal, which sustains her for 3.1 days. On the other hand, a sugar meal provides a greater survival (7.8 days) than a blood meal (6.0 days) for aestivating female mosquitoes. For this reason, feeding on sugar provides a greater benefit to aestivating *Anopheles* mosquitoes than feeding on blood [42, 48]. Thus, behavioural changes, such as a reduced flight activity and seeking a cooler resting location, may lower the metabolic rate of the malaria transmitting mosquitoes [47].

Changes in the *Anopheles* genetic materials can also help them adapt local ecological conditions. For example, in *An. funestus* a chromosomal inversion (inversion 3Ra comprising roughly 30% of the right arm of its chromosome 3) was found to be correlated with humidity [52, 53], resting behaviour, host preference and wing shape [53]. It is therefore suggested to contribute to genetic isolation between populations in Burkina Faso [54]. In *Anopheles* mosquitoes, chromosomal inversions have also been observed to affect habitat preference, feeding behaviour [55], aridity tolerance, temperature tolerance and susceptibility to parasites [56-59].

#### 1.5.4. Dispersal

Dispersal refers to a goal-oriented flight of *Anopheles* mosquitoes from one place to another. In normal atmospheric circumstances, most individuals of the tropical *Anopheles* mosquitoes apparently fly within a range of 1-3 km, although there are records of a few species or occasional individual mosquitoes flying much further [60].

Knowledge regarding the dispersal of adult vectors from their breeding sites helps to identify areas where control methods such as LLINs and IRS are better applicable. The movement of mosquitoes is governed by a number of factors, including temperature, humidity, host attractiveness and the attractiveness of breeding sites depending on their physiological conditions. The flight of gravid female *Anopheles* mosquitoes to breeding places is stimulated by fully developed ovaries and the characteristics of the breeding site, as they disperse in the direction of post emergence or oviposition, resting, feeding, daytime resting and breeding sites [61].

The typical active flight of most *Anopheles* mosquitoes is short and under their control, but some such as *An. pharoensis* can actively fly long distances [62]. The population size of *Anopheles* mosquitoes decreases with an increasing distance from their source of breeding places or release points [61] and is non-random, but related primarily to their distribution, number of resting sites and blood meal sources. In Sri Lanka, Curtis and Rawlings (1980) caught a marked number of *An. culicifacies* 498 m from a release point within a day after marking [63]. Another study showed that the proportion of dispersing *An. gambiae* s.l. declined exponentially with an increasing distance starting from a larval habitat along The River Gambia, and that 90% of their movements were within 1.7 km [64].

Flight distance differs among the *Anopheles* species. In Senegal, Trape *et al.* [65] observed a significant decrease in the density of indoor-occurring *An. arabiensis* with an increase in distance up to 910 meters from a permanent marshy area. Inhabitants

close to the marsh experienced a maximum risk of malaria infection than those further away. In Burkina Faso, the mean distance moved by individual *An. arabiensis* and *An. gambiae* s.s. mosquitoes ranged from 350-650 m per day [66] under a condition where the daily survival of the mosquitoes was estimated to be 80–88%. In Korea, 85% of the released *An. sinensis* were recaptured (where the number of livestock such as cows and pigs was higher) within 6 km from the release point [67]. Highly localized dispersion activities provide *Anopheles* mosquitoes with a better opportunity for breeding [66].

When a female *Anopheles* mosquito feeds on humans to nourish its eggs, it may acquire *Plasmodium* gametocytes from a carrier. After several feeding cycles, the mosquito becomes infectious, and on biting a second human host it transmits the parasites. During a single rainy season, proximity to mosquito breeding sites predicted human malaria infection when homesteads were upwind of larval sites, but not when they were downwind of larval sites. This indicates that following oviposition, female *Anopheles* mosquitoes fly upwind searching for human hosts, and hence increasing the risk of malaria transmission. Because of this, malaria transmission could be disrupted by targeting vector larval sites in close proximity to human dwellings and downwind of malaria hotspots [68]. In addition, the adult *Anopheles* mosquito prevention tools, such as LLINs and IRS, can better be applied in the upwind direction in order to minimize the risk of malaria transmission.

Host availability, proximity to breeding site, sugar source, resting site, preferred flight direction and season could affect dispersal of vectors [68]. In Mali, large population size and migration was observed during the wet season, but with very low numbers and no sign of migration during the dry season. The study suggested that vector control measures could be more efficient in the region and other seasonal riparian habitats by targeting the disruption of mosquito populations by the river during the dry season. This would decrease the size of an already small population, and would likely delay an explosive growth in vector abundance in inland villages as rainfall increases [51].

After emerging from pupa, the female *Anopheles* mosquito rests for some hours in the vicinity of the breeding site and undertakes mating. It then flies to areas where hosts are available, orientated by the hosts' stimuli. Males generally tend to be more concentrated in the area of their breeding site and to remain in outdoor shelters, although a good number of males of endophilic species accompany females to their resting places [60, 61].

Passive dispersion occurs when a mosquito is transported by external factors, including cattle, air currents, ships, airplanes, trains and vehicles. The movement of cattle from a breeding place in the evening to remote villages led mosquitoes to longer distances by accompanying cattle [67]. Wind also causes dispersion over a wide range. For example, *An. pharoensis* in Egypt was found at distances of 56 km and 29 km from the nearest possible breeding places. In contrast, under a condition with a very low wind speed, mosquitoes can detect air-carried, host-specific odours from a distance and orient themselves to the host by flying upwind [60].

#### **1.5.5. Feeding and resting behaviour**

Flight, host seeking and the feeding activities of *Anopheles* mosquitoes can take place if the relative humidity and temperature are not limiting. Many female *Anopheles* mosquitoes bite humans to obtain a blood meal, and a few feed on humans in preference to animals. Mosquitoes are attracted to hosts by various stimuli emanating from their breath or sweat, such as carbon dioxide, lactic acid, octenol, body odours and warmth. Some species feed more or less indiscriminately at any time of the day or night [69].

After having their blood meal, mosquitoes seek resting places in which to shelter until their meal is digested and their ovaries are matured. Adults of *An. gambiae* s.l. are primarily indoor-feeding (endophagic) and indoor-resting (endophilic), as opposed to outdoor-feeding (exophagic) and outdoor-resting (exophilic) mosquitoes. Few mosquitoes entirely feed on humans (anthropophagic) or animals (zoophagic), or

possibly zoo-anthropophagic feeding on both depending on availability. Feeding occurs between dusk and dawn in species associated with open terrain or sunlit habitat [30]. Even so, the feeding behaviour of a species may change over time [10, 60].

The biting behaviour of female *Anopheles* mosquitoes is important in the epidemiology of malaria. Mosquitoes feeding on people predominantly outdoors and late at night may not bite many young children, because children will be indoors and asleep at this time. Consequently, young children will be less likely to be infected with any disease that these mosquitoes transmit. During the hot and dry seasons, a substantial number of people may sleep outdoors and as a result, be bitten more frequently by exophagic mosquitoes. Some mosquitoes bite predominantly in forests or wooded areas, so people will only get bitten when they visit these places. Thus, the behaviour of both people and mosquitoes is relevant in malaria transmission [10].

The resting and biting behaviour of vectors is important in planning control measures. In malaria control campaigns, interior surfaces of houses such as walls and ceilings are sprayed with residual insecticides to kill resting adult mosquitoes, and LLINs are also used to prevent indoor-biting mosquitoes at night [12]. These approaches remain effective in controlling malaria if the vectors are endophilic, endophagic and susceptible to IRS and LLIN insecticides.

The human blood feeding activity of female *Anopheles* mosquitoes is responsible for malaria transmission. This activity is part of their intrinsic behaviour, as blood proteins are essential nutrients for egg production, metabolic energy and reproductive fitness. Blood quality, and hence host type, affects reproductive output, which suggests the host preference is likely to be more common given the evolutionary association between insect vector and pathogen. According to Takken and Verhulst [70], host preference is defined as the trait to preferentially select certain host species above others. This selective behaviour has a great influence on disease transmission. Host preference resulting from selective behaviour exists not only between different

species, but also between populations of the same species, and even within a given population due to several extrinsic and intrinsic factors [70].

External factors, such as an absence of the preferred host and a reduced response threshold for host selection owing to low metabolic energy or adverse weather, prevent mosquitoes from venturing far from their local habitat. This may force them to change their feeding and resting preference [71]. The extrinsic determinants of host preference include odorants (and their production by skin bacteria), carbon dioxide, blood quality/host species, colour, body heat, relative humidity, body mass, gender, age, defensive behaviour, parasites and climate [70, 72] and the potential suitability of a host. Skin emanations contain host-specific cues that play a role in host preference. For example, (s)-lactic acid is an excretory product of humans and an important cue in the host selection process of *An. gambiae* s.s. [73].

The body mass of a host may affect preference, presumably because a larger host would exude a higher quantity of olfactory cues. A well-known example of this is the production of metabolic carbon dioxide, which is positively associated with body size [74]. Young children are bitten less often by mosquitoes than their parents are, with mosquitoes expressing different degrees of preferences for humans. These preferences are supposed to be associated with differences in odour profiles, which differ between men and women, as well as between people of the same sex [75]. Lindsay *et al.* [76] demonstrated that *An. gambiae* s.s. were more attracted to pregnant women than to women who were not pregnant.

The intrinsic factors that determine the host preference of mosquitoes include physiology, genetics and plasticity (learning, divergence after the implementation of insecticide-treated bed nets and indoor residual spraying and host abundance) [70]. Soon after emergence from the pupal stage, male and female mosquitoes express a strong behavioural response to nectar that serves them as a source of the metabolic energy needed for flight and anemotactic behaviours [77]. Following mating, female mosquitoes search for blood. Choice experiments showed a preference of *An.*

*quadriannulatus* and *An. arabiensis* for a cow's odour, while *An. gambiae* s.s. preferred a human's volatiles [78]. Nonetheless, the nutritional state of the insects may overrule the inherent host preference, because the principal strategy of the insect is to safeguard reproduction, for which animal blood is required. Under such circumstances, the mosquitoes lower their threshold for host preference, and may feed on a non-preferred host. The age of the mosquito does not affect host preference, though adaptive learning through a memorized host encounter was shown to affect the choice for a specific host species [79].

Host choice depends not only on the innate host preference of the mosquito species, but also on the tendency of the mosquito to feed indoors or outdoors and the time of feeding. These behavioural characteristics may be driven by selection, and therefore have a genetic background. Studies have confirmed the existence of genetic control for the behavioural differences between the strains, although none of the behavioural preferences was strongly fixed in the population. The anthropophilic behaviour of *An. gambiae* s.s. is found to be strongly fixed in a population, but not complete [70].

Intervention strategies should not only consider the feeding preferences of vectors but also their peak biting time, which varies between species, populations of the same species and the age of individual mosquitoes. Nulliparous female *An. gambiae* s.l. in Sierra Leone and *An. punctulatus* in Papua New Guinea showed a tendency to bite earlier than the parous ones [80]. Additionally, *Anopheles* mosquitoes infected with *P. vivax* were observed to bite earlier than those infected with *P. falciparum*. On average, mosquitoes containing *P. vivax* sporozoites are expected to be younger than those infected with *P. falciparum* sporozoites. This is because the duration of *P. vivax* sporogony (seven days) is shorter than that of *P. falciparum* (nine days) at 30<sup>0</sup>C. The early biting tendency of younger parous females than older ones may help explain the early biting habit of mosquitoes infected with *P. vivax* in comparison to mosquitoes with *P. falciparum* [80].



IRS is mostly targeted against the indoor-resting malaria vectors. However, these mosquitoes may avoid the impact of IRS by changing their behaviour to outdoor feeding and outdoor resting [81]. *Anopheles sundiacus* and *An. albimanus* [82] modified their indoor-biting and indoor-resting behaviours in response to residual house spraying with DDT. On Bioko Island in Equatorial Guinea, *An. gambiae* s.s., which was primarily an indoor-feeding and indoor-resting vector, was observed to seek hosts outdoors at least as much as it did indoors [83]. In the Temotu Province of the Solomon Islands, *An. farauti* showed the tendency of early and outdoor biting following intensive IRS (DDT and lambda-cyhalothrin spray) and LLIN use [84]. In southern Zambia, a doubling in the amount of rainfall in the 2005 – 2006 rainy season resulted in a 10-fold increase in the number of *An. arabiensis* resting inside human sleeping quarters each night [85].

The introduction of insecticide-treated nets brought behavioural changes such as shifts toward outdoor and/or earlier biting. Like other aspects of its behaviour, the nightly biting activity of *An. arabiensis* varies dramatically across Africa, as peak biting after midnight has been observed in Senegal, Chad and Kenya [86, 87]. However, in Mozambique, Tanzania and Ethiopia, biting was observed as early as 9 pm [88, 89]. In southern Zambia, *An. arabiensis* biting was observed throughout the night, with peak activity starting before midnight at approximately 10 pm. Although most persons have gone to bed by this hour, roughly 14% of the *An. arabiensis* biting occurred prior to this time when residents were finishing dinner and preparing for bed and were not protected by ITNs. In the area, *An. arabiensis* remained highly antropophilic despite ITN use, and also appeared to be relatively exophagic, biting outdoors immediately after sunset and before sunrise, thereby circumventing the protective effect of ITNs [90].

## **1.6. Sampling methods of *Anopheles* mosquitoes**

### **1.6.1. Larval sampling methods**

Larval sampling is carried out for different purposes, including the identification and characterization of vector breeding habitats, identifying preferred habitats and

monitoring and evaluating the impact of vector control interventions. *Anopheles* larvae are found in a variety of water collection ranging from lakes, swamps, marshes and rice fields to tree holes, hoof- and footprints. The starting point in larval surveys is identifying topographical features that support mosquito breeding. Humans settle close to water and serve as blood meals and gametocyte sources for the vectors, so locating water sources and undertaking exhaustive surveys remain important in identifying pockets of breeding habitats. The breeding habitats can also be identified through remote sensing with the help of high flying aircraft and earth-orbiting satellites [61].

Several methods have been employed in larval sampling, among which dipping, netting and pipetting are most common [34, 61]. Dipping using a ladle is the most commonly and widely used sampling method. For example, a soup ladle 9–10 cm in diameter with a capacity of 100 – 150 ml or a diameter of 15 cm and a capacity of 350 ml or more can be used depending on the condition. For relatively inaccessible habitats, a long handle can be attached to dippers. Pipettes and/or spoons may be used for collecting larvae from the surface of smaller breeding habitats, including hoof prints, tree holes and leaf axils. A pond net, which is constructed using a ring of iron wire to which a nylon bag is attached, is also used to collect larvae from bigger habitats, including the edge of streams, lake shores and dams [34, 61].

Although several larval sampling techniques have been used, each has its own limitations. Dipping catches larvae at the surface of breeding habitats, though this causes a sampling bias since those which remain at the surface are caught. The unequal dispersal of larvae and changes in vegetation cover in the habitats affect sampling by dipping. A fluctuation of habitat size with changes in rainfall, temperature and human activity, plus the escape of larvae by swimming away, makes use of dipping difficult. Dipping is not convenient for sampling *Anopheles* larvae when the population density is low and when they remain submerged after disturbance at the water surface, either by shadow or the movement of the water. Because larvae usually have a very patchy distribution and most aggregate along the edges of water

collections or around clumps of emergent or floating vegetation, they may not be sampled adequately using the dipping technique [61].

After collection, larvae may be handled or processed depending on the purpose of collection (e.g. the initiation of laboratory colonies, insecticide susceptibility studies, the monitoring of intervention, species identification).

### **1.6.2. Adult sampling methods**

Adult mosquitoes are sampled to identify the species that prefer humans and animals, know their distribution, determine the human biting rate and density, the peak biting time, longevity, sporozoite rate, EIR, human and bovine blood meal index, preferred resting habit, status of insecticide susceptibility/resistance and the mechanisms that confer resistance. The major methods for adult female *Anopheles* mosquito sampling are aspirator, PSC, human or animal baits, traps, experimental huts and pit shelters [34, 61]. With the aid of a torch, *Anopheles* mosquitoes can be collected from indoor-resting sites by aspirator or test tube, and transferred to paper cups. Mosquitoes can also be collected from outdoor-resting sites such as animal burrows, tree holes, cracks and crevices in the ground and rock fissures using aspirators. This method avails itself of live mosquitoes for insecticide resistance or other studies. But it only catches a small proportion of the resting mosquitoes, and hence loses those that leave the house after feeding or are irritated by insecticides and those escaping disturbance. The method is useful but labour intensive, and requires a skilled technician.

Pyrethrum spray sheet collection is used to catch endophilic mosquitoes. Early in the morning, the inside of the house is prepared and a white sheet is spread over the floor and openings, including windows, and the doors are closed. A person sprays the room with the insecticide of choice (e.g. pyrethrum) using a hand sprayer. At the same time, another person sprays openings on the outside to deter mosquitoes from flying out. After 10 minutes, the knocked-down mosquitoes are collected from the sheets. The method is used to quantify density, human blood index, longevity, infection and the

infectivity of indoor-resting mosquitoes [34, 61, 91]. On the down side, it is expensive and may expose humans to chemical hazards [92].

Human bait is used to collect female mosquitoes as they attempt to feed on exposed body parts [34, 93]. This is the gold standard method to sample antropophagic mosquitoes and determine man-mosquito contact and preferred biting location and time [93, 94]. The procedure involves one or two persons sitting indoors or outdoors and exposing their legs to mosquitoes in the period from dusk to dawn. When mosquitoes land on the body, and before they start biting, they are captured by aspirator or test tube using a torch light. The number caught each hour is recorded to determine the peak biting hours and density. In addition, catches from human baits serve as the determination of the human biting rate, parity rate, infection and infectivity, host preference, duration of gonotrophic cycle and epidemiological studies. However, it exposes collectors to infection, requires high level of organization and expense, and might be conducted inefficiently due to fatigue, flat batteries, various disturbances and human error [34, 91, 93].

Methods such as exit- and entry traps, bed net traps, PSC, pit shelters and light traps are alternatives to human bait collections, as they minimize the risk of exposure to infective bites and sampling errors [93]. Exit traps fitted to the windows, doors, eaves, walls or verandas of a house or hut indicate daily movements, exophily/endophily, responses to insecticides and the physiological stages of mosquitoes [34]. Still, these are not productive for poorly constructed houses, as the mosquitoes fly through other openings there by escaping the traps [91].

A bed net trap hung around an animal or human, leaving a free space of approximately 15 – 20 cm between the floor and the bottom of the net, can be used to collect host-seeking *Anopheles* mosquitoes, although a net having a door-like opening can be used instead. In the case of humans, the person is enclosed within an inner net to protect the bite of infectious vectors. The catches can be used to study host preference, density,

availability and the insecticide susceptibility status of *Anopheles* mosquitoes in a given area [34, 91].

The CDC light trap remains a preferred alternative to human bait for collecting host-seeking mosquitoes [94]. The light trap is suspended by a string from the ceilings of bedrooms, near a person sleeping under an insecticide-free mosquito net or from a tree branch in a cattle enclosure. Light traps collect endophilic mosquitoes and species that leave houses after feeding, and which consequently would not be caught in a pyrethrum spray sheet, indoor human bait or other indoor collection techniques [91].

Artificial resting sites constructed outdoors can attract exophilic mosquitoes, among which the best is the pit shelter. A pit with a depth of 1.75–2 m, a width of 1 m and a 20 cm deep horizontal cavity is dug underneath a tree or bush shed, and outdoor-resting mosquitoes are then collected from the small horizontal cavity and from the sides of the pit [91].

### **1.7. Age determination methods in female *Anopheles* mosquitoes**

One of the entomological determinants in the transmission of malaria in an area is the age status of individual females in the population of the vector. Older females are responsible for much of malaria transmission, as age also indicates the efficacy level of vector control interventions in an area [91]. It is also useful for the qualitative assessment of vector density in general, and of man-mosquito contact in particular. Most current control programmes aim at shifting the age structure of the vector population towards a younger age since the young are incapable of transmitting sporozoites. Residual insecticides and insecticide-treated nets reduce the longevity of malaria vectors [91, 95].

Mosquito age grading methods include morphological changes in the skeletal apodemes, ovarian dissection, cuticular hydrocarbon, transcriptional profiling and pteridine fluorescence [95-97]. The growth of the layers of the skeletal apodemes of the *Anopheles* can be observed on a daily basis. This provides the actual calendar age,

and is a better estimate than the physiological age determination. The length of the thoracic apodemes is directly related to the size of the thoracic muscles. Therefore, the length of the apodemes reflects the amount of growth of the thoracic muscles and hence the calendar age of anophelines up to the age of 13 calendar days [34, 98]. However, this method is not adequate, as it may not address older mosquitoes.

The physiological age of female mosquitoes can be determined by counting the number of dialations or follicular relics in the ovary as either 0-parous (nulliparous) or 1-parous, 2-parous, 3-parous, etc... based on the number of ovipositions. Yet, it is technically difficult and labourious, and may be of limited value as the proportion of diagnostic ovarioles decline with age [99, 100]. The tracheation method distinguishes between nulliparous (tightly coiled tracheols) and parous (stretched tracheols) [101], which is relatively faster and easier to use in the field. The Pteridine fluorescence method is unreliable due to the difference in the concentration of the pteridine with respect to the mosquito's physiological condition [96, 97].

Transcriptional profiling, a method of age-grading based on genes that display an age-dependent expression in mosquitoes, was found to determine the chronological age of mosquitoes under field conditions. It can determine the age of adult mosquitoes to a much higher degree of accuracy and precision than the previous methods used [96]. This method was found to be consistent with the ovarian dissection method and also valuable for the determination of the age of *An. gambiae* mosquitoes in two malaria-endemic areas in western Kenya. It may therefore be useful for the determination of the age, vectorial capacity and survivorship of a population of a vector where vector control interventions are ongoing [95].

### **1.8. Methods of host preference studies in *Anopheles* mosquitoes**

The identification of the blood meal source of freshly fed female mosquitoes remains important to understand their host preference and vectorial role. Techniques such as the precipitin test, the enzyme-linked immunosorbent assay (ELISA) and the

polymerase chain reaction (PCR) have been used in mosquito blood meal identifications [34, 102, 103].

The precipitin test has been the most commonly used serological technique to identify the blood meal source of mosquitoes. However, the test is neither sensitive nor specific, which results in the underreporting of feeding habits of arthropods that take small blood meals. The test also demonstrates multiple blood meals, indicating a lack of specificity. As a result, other serological methods have been adapted for mosquito blood meal source identification, among which the ELISA is most preferred [104, 105].

The advantages of the ELISA technique over the precipitin test are that blood meals can be rapidly identified in microtiter plates, the test results are more objective and the sensitivity is very high. The ELISA can be quantified and automated, and the automated equipment is relatively cheap, compact and easy to operate [103, 105]. A single mosquito can be tested, both by the blood meal ELISA and the malaria sporozoite ELISA, for host preference and infection, respectively. An experienced technician can more easily diagnose the blood meal sources of larger number of mosquitoes with accuracy when using ELISA than precipitin [103].

Although the ELISA method has been used to determine the blood meal sources of *Anopheles* mosquitoes, it still has its own limitations, including the difficulties of obtaining specific antisera against a broad diversity of host species [106]. The PCR-based technique overcomes the limitations of the serological tests in identifying mosquito blood meal sources, especially for laboratories using DNA-based techniques. In this technique, individual DNA extracts serve multiple purposes, such as species confirmation, the determination of blood meal sources, the infection status for various pathogens, insecticide resistance mechanisms and vector population genetic studies. Fed mosquitoes can also be preserved dry, stored for long periods of time and tested at facilities physically distant from the point of collection [102]. Human-specific genetic markers within fresh fed anophelines may allow for

identification of the individual human host [107]. A multiplexed PCR targeting cytochrome b was found to identify the mammalian blood host in engorged vector mosquitoes two–seven months after collection in Zambia. The host DNA was detectable in frozen mosquito abdomens 24–30 hours post-feeding. This test is advantageous, as multiple blood hosts can be directly identified by size-specific fragments [102].

The proportion of *Anopheles* giving a positive reaction for human blood is the result of the human blood index (HBI), which is a valuable guide to the potential importance of an *Anopheles* mosquito species as a malaria vector. Sampling bias must be taken into consideration in interpreting the results as, e.g. it is to be expected that a high proportion of adults caught from houses will have fed on humans, and most of those caught from cattle sheds will have fed on cattle [91].

### **1.9. The sporozoite rate and its detection methods**

The sporozoite rate is the proportion of vectors that carry *Plasmodium* sporozoites in their salivary glands. The sporozoite infection status of anophelines can be detected using methods including dissecting salivary glands, ELISA and PCR. The sporozoite infection status of *Anopheles* mosquitoes has been detected by dissecting and observing salivary glands using a microscope [34, 108]. Although the microscopic evaluation of dissected salivary glands is the gold standard for the determination of mosquito infection, it is labour intensive, requires a trained and experienced technician and may not differentiate among the *Plasmodium* species [108, 109]. Microscopical examination often fails to differentiate oocysts and sporozoites of human *Plasmodium* parasites because they are morphologically similar. Thus, it is primarily replaced by the circumsporozoite protein enzyme-linked immunosorbent assay (CSP ELISA)[110, 111]. This test is species-specific and can detect *P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae* or mixed species [110, 112-114], and also serves in testing single and pooled mosquito specimens. Testing pooled specimens is a highly efficient and economically cheap method for determining sporozoite rates, particularly when vector infection rates are low [115].



A CSP ELISA may overestimate the true salivary gland infection rate as a result of the spread of the circumsporozoite protein throughout the mosquito after being shed [109, 116] or detecting the CSP from the oocysts bursting, which occurs two to three days before the sporozoites actually reach the salivary glands [117]. The CSP ELISA may be less sensitive than the microscopic examination of dissected salivary glands for detecting low-level sporozoite infections in the *Anopheles* mosquitoes [114]. The long-term storage of mosquitoes in chemicals, such as in ethanol or isopropanol for later analysis, makes the specimens unsuitable for ELISA testing. A mosquito must be separately subjected to four assays each for one *Plasmodium* species that causes human malaria, but in practice many studies only test for the presence of one or two of the species. Additionally, neither microscopy nor ELISA assays allow for the detection of genetic diversity in the *Plasmodium* sporozoites. Furthermore, CSP ELISA overestimates the mosquito infectivity rate by detecting the circulating sporozoites, even in non-infective mosquitoes [109, 114, 116, 118].

The limitations of the microscopy and the ELISA-based detection of *Plasmodium* parasites in the thorax and salivary glands of vector mosquitoes led to the adoption of molecular tools, including the multiplex PCR, real-time PCR and duplex real-time PCR. These methods helped to improve the sporozoite detection rate of all four *Plasmodium* species much better than the ELISA and microscopy, but still have their own limitations [109, 118]. The real-time quantitative PCR assay is probably highly sensitive and more specific than multiplex and duplex PCRs in detecting *P. falciparum* in the salivary glands of vectors [118].

The proportion of *Anopheles* mosquitoes positive for *Plasmodium* sporozoites is the sporozoite rate, which is a valuable guide to incriminate mosquitoes as malaria vectors and to describe the epidemiology of the disease. Sporozoite detection is also useful for defining the season of transmission and in evaluating the effect of mass drug administration [34]. The presence of oocysts in a mosquito indicates that the mosquito

is a potential vector, but not that it is infective. When sporozoites are found in the salivary glands, the mosquito is assumed to be capable of transmitting malaria. There may be considerable seasonal variations in sporozoite rates, reflecting, in part, changes in adult survival rates of the mosquitoes [91].

### **1.10. Entomological inoculation rate and its implications**

Malaria transmission intensity is determined by the number of infective *Anopheles* mosquito bites received per person per unit of time, and is described as the entomological inoculation rate (EIR) [119, 120]. The EIR is a favoured measure for assessing malaria endemicity and risk of epidemic development [121], and is a product of the human biting rate (HBR) and the sporozoite rate (SR) [122, 123]. Nevertheless, the methods used to determine the HBR are not standardized [124].

The human bait catch has been considered as the gold standard method to determine HBR [34]. However, it is technically difficult to replicate and unethical in areas where malaria parasites are resistant to drugs [123], and where other mosquito-borne diseases are common. Indoor spray collection and the exit trap have been used in some cases but are less sensitive, as the anophelines could be less directly associated with feeding on humans [85, 123]. A CDC light trap suspended close to sleeping people at night can be used to estimate HBR, as it catches mosquitoes that attempt to feed on humans [94, 125]. Even so, the relationship between the CDC light trap and HLC, as well as the variations with respect to the level of endemicity and the behaviour of vectors is not determined. Hence, it is important to consider *Anopheles* characteristics and their ecological niches into account [124], as their vectorial role varies greatly depending upon land use, population density, elevation and climatic variables [1, 126]. As a result, the choice of the method depends on the local condition and behaviour of the vectors.

The EIR is a measure of the level of exposure to infectious *Anopheles* bites. The mean annual EIR value of 159 spatially distinct sites of Africa was 121 infected *Anopheles*

mosquito bites per annum (range: 0 – 884) [123]. The “rural” class had the highest value (mean=146; range 0 – 884), followed by areas surrounded by irrigated rice (mean= 99; range= 0 – 601) and urban areas (mean =14; range = 0 – 43). The result depicts the diverse transmission pattern of malaria on the continent resulting from variations in the environmental factors, such as sampling method, the relative effects of breeding habitats on species abundance and sporozoite rate, the number and type of breeding habitats, the surrounding human population and level of breeding site contamination, the number of non-human blood meal sources in the locality, as well as the degree of local immunity.

Over a two-year study period (2005 – 2006) in southern Zambia in two villages, estimates of EIR depended greatly on the sampling method used. In Chidakwa village, the yield of mosquitoes from HLC was few, so the EIR was none during the two years. However, in the other village of Lupata, it was 3.75 during November-May. Alternatively, a huge number of mosquitoes was obtained from PSC; therefore, the EIR was 1.6 infective bites per person per season in Chidakwa, and 18.3 infective bites per person in Lupata [85].

In Zambia, the transmission intensity by *An. arabiensis* increased gradually throughout the 2005 – 2006 rainy season peaking in April 2006. The seasonal pattern of transmission intensity by *An. arabiensis* directly corresponded with malaria cases admitted to the Macha Mission Hospital during this period [85]. Although the Chidakwa and Lupata communities were separated by approximately 5 km, the indoor-resting density, human biting rate and malaria transmission by *An. arabiensis* were all much higher in Lupata than in Chidakwa, thereby suggesting a localized and spatial heterogeneity of malaria transmission intensity.

In Uganda [127], a one-year entomological study in seven ecologically different sites showed that *An. gambiae* s.s. was the main vector in five of the sites, and *An. funestus* was the most prevalent vector in the remaining two sites. In a peri-urban village, *An. arabiensis* contributed substantially to malaria transmission. Moreover, clear

differences in annual EIRs were observed between the study sites, ranging from four infective bites per person per year by *An. gambiae* s.s. in the southwestern part of the country to > 1,500 infective bites per person per year by *An. funestus* in a swampy area near the Nile River.

In a high-altitude and large-scale sugarcane growing zone in the Kakamega District of western Kenya, there were 29.2 infective bites per person per year (ib/p/year) for *An. gambiae* s.l. and 17.5 ib/p/year for *An. funestus*. The *P. falciparum* parasite rate among asymptomatic children was 55.4% and 44% in the wet and dry seasons, respectively. This indicates that a low level of malaria transmission by vectors may contribute to a significantly higher malaria prevalence within a given population [128]. It is possible in some cases to have a 0 ib/p/year while there is malaria in the population, possibly due to the low number of vectors, which are below a detectable level, but which are efficient in transmitting the disease [123]. Still, the magnitude of entomological inoculation rate is influenced by the species type, the rate at which vectors feed on humans, biting locations, biting hours, mosquito density, feeding habits and sampling technique used.

The EIR values are useful to assess the impact of vector control interventions on malaria parasite transmission and elimination. Shaukat *et al.* [122] analysed the impact of eight malaria vector control interventions and found a 90% reduction in EIR in the ITN-using community and a 93% reduction in EIR in the IRS-using community in Tanzania relative to a community without the intervention. In the Solomon Islands, the group found EIR values 94% lower in the ITN community and EIR values 56% lower in the IRS community.

The annual values of the EIR within Africa vary from as low as 0.1 to > 1,000 depending on the eco-epidemiological conditions of the locality [123, 129]. Generally speaking, when the EIR is < 10 on the continent, an area is considered to have unstable malaria, and when the EIR >100, the area is considered to have stable malaria transmission [120, 127]. Areas with EIR values in between these extremes

vary in their level of endemicity depending on environmental and demographic conditions, such as rainfall, vegetation cover, human population density and land use patterns [130].

### **1.11. Living conditions and exposure to infectious *Anopheles* bites**

In Africa, the primary malaria vectors are nocturnal, endophilic or exophilic and endophagic or exophagic. They mainly transmit the disease at home while interacting with humans to imbibe blood. However, all homes are not equally accessible for the mosquitoes [131]. Malaria transmission is heterogeneous in that it varies among villages, households and individuals due to factors, including altitude, vector distribution and abundance, household distance from nearby mosquito breeding site, house construction, household crowding and personal protection methods employed against mosquito bites [65, 132-134].

During an epidemic season in a highland area of western Kenya, malaria transmission was found to be clustered in low-altitude areas due to a relatively high temperature, which affects the development and survival of the vector and also the development of *Plasmodium* parasites within the vector [135].

An increase in the number of households in an area increases the number of human-made breeding habitats, consisting mainly of broken pipes, roadside ditches and potholes, and temporary pools of water along unpaved roads and paths within and around family compounds, drainage or abandoned swimming pools, tire tracks and shallow garden wells, which expose inhabitants to an increased risk of malaria infection [136].

In a dry area of Kenya (Baringo), the odds of *An. arabiensis* occurrence increased with a decreasing distance to the animal shelter and the nearest larval habitat and an increasing number of houses, sleepers and the size of eaves. *An. arabiensis* was also more likely to be encountered in grass-thatched- than in metal-roofed houses and in

the absence rather than the presence of animals [137]. Human activities also increased human-vector contact. In the study, lightly-dressed residents who stayed out late in the evening to irrigate their farms exposed themselves to mosquito bites.

In Sri Lanka, the risk of getting malaria was greater for inhabitants of poorly constructed houses compared to complete brick, plaster walled and tiled-roof houses. Such houses might be a preferred resting place as they offer dark and cool micro-environments, such as crevices in mud walls and thatched roofs [132]. In Burundi, a lower rainfall, an absence of vector control measure and houses near breeding sites were all associated with a higher indoor *Anopheles* density [138].

In a north-central dry zone of Sri Lanka, Konradsen *et al.* [139] found that houses closer than 750 meters to a breeding stream had a 4.7-fold higher risk of harbouring *An. culicifacies* and a 1.5-fold higher risk of harbouring *An. subpictus* than houses at least 750 meters away. Rooms where more than two people slept the night before PSC had increased risk of having *An. culicifacies*. Using selected traditional fumigants appeared to attract these vectors [139].

On the south bank of the River Gambia, some 180 km inland, Lindsay *et al.* [140] found that children's exposure to a *An. gambiae* s.l. bite increased in houses adjacent to mosquito breeding sites, with open eaves and sleeping in a room without a ceiling in the wet season. In the dry season, the group found an increased number of *An. gambiae* s.l. related to living next to a mosquito breeding site, living in an unfenced compound, sleeping in a room without a ceiling and using no insecticide aerosol [141].

In southern Tanzania, *An. gambiae* s.s. and *An. arabiensis* were observed to prefer eaves as an entry point [142]. In Guinea Bissau, significantly greater numbers of indoor-resting *Anopheles* mosquitoes were present in rooms with open eaves, in houses with a well on the compound and in houses where pigs were present. In

addition, an abundance of female *Anopheles* mosquitoes increased with increasing human biomass per square meter of bedroom area [143].

In Adama, a city located 100 km southeast of Addis Ababa, malaria prevalence was highly clustered, and 65% of the cases occurred in only 5% of households [20]. The incidence of malaria was significantly higher in children (127.5 per 1,000 population) than in adults (64.9 per 1,000 population). Household level factors significantly associated with malaria were age, distance from vector breeding site and the number of adults with indoor jobs. The mean malaria incidence in children below the age of 18 in houses at a distance 150 m from a nearby breeding site was 1,374 per 1,000 population, compared with 373 per 1,000 population residing at 350 m [20].

Ayele *et al.* [144] observed region, socioeconomic status, age and gender to be associated with the risk of malaria transmission in Ethiopia. The Amhara region was at a higher risk of malaria than the SNNP and Oromiya regions. Houses sprayed with insecticides were less likely to be affected by malaria, whereas the risk of malaria infection was observed to increase with a per unit increase in family size. Furthermore, malaria parasite prevalence was highest in children and females.

In northern Ethiopia, Ghebreyesus *et al.* [134] found that the use of irrigated land, an earth roof, animals sleeping inside houses, windows, open eaves, no separate kitchen and one sleeping room were all significantly associated with malaria infection. In this area, children living closer to a dam had a seven-fold increase in the risk of malaria infection compared to those living farther away from dams. The malaria prevalence rate was significantly higher for all age groups in wet lowland ecological zones [133]. In Eritrea, houses having a mud wall were positively associated with malaria infection [145].

In a holoendemic area of North West Burkina Faso, the prevalence of *P. falciparum* infection among inhabitants of iron sheet-roofed houses (12.7%) was two times less than the prevalence among residents of mud-roofed houses (25.6%) [146]. In a

highland area of western Kenya, living close to swamp and forest and at a lower elevation were associated with greater risk of malaria infection [147]. An increase in malaria risk was also associated with a low education level of female household heads, overnight travel, living near a channelled swamp and keeping livestock near a residential house at night. Living in a house with a metal roof, no ceilings or a separate kitchen was also related to higher risk of infection [15].

In addition to the overall variation in vector abundance, biting density is influenced by factors such as local climate, topology, house design, house proximity to mosquito breeding site, host availability, personal protection methods and mosquito avoidance behaviour [140]. Human subjects vary in their attractiveness towards malaria vectors [148]. Some individuals have a greater susceptibility to be either inoculated with sporozoites, (i.e. a non-homogeneous contact between an individual and the mosquito vector) or to be inoculated to develop the disease (e.g. due to innate or some degree of acquired immunity to malaria) [132].

Improved housing generally protects the entry of indoor-feeding and indoor-resting mosquitoes that transmit malaria, filariasis and arboviruses [142]. Vector control campaigns involve environmental management, the implementation of educational programmes and the use of insecticides either to impregnate fabrics (i.e. mosquito nets and curtains) or through the use of sprays (indoors and outdoors) [149].

Scant consideration has been given to house design and construction as an environmental strategy in controlling malaria. The addition of a simple ceiling to traditionally designed houses reduced the exposure to vectors of malaria and other diseases in rural Gambia. In the area, all nettings and insect screen ceilings reduced the house entering of *An. gambiae* by approximately 80% and *Mansonia* spp. by approximately 70% [150].

In The Gambia, net ceilings and screened eaves installed into typical houses resulted in a significant reduction in the density of mosquitoes that occurs indoors [151].



House screening reduced the indoor densities of *An. gambiae* s.l. as well as *Mansonia* spp., both of which are vectors of several tropical diseases in rural areas of Africa and some parts of Asia. As a control tool against house-entering mosquitoes, blocking eaves and screening houses may help reduce nuisance mosquitoes and thus encourage the uptake of control interventions that which rely on acceptance, participation and even investment by the community [142].

In Burkina Faso, a substantial reduction in malaria transmission was achieved in houses where permethrin-treated curtains were hung on doors, windows and eaves [152]. The protection of all members within a household, beyond merely young children and pregnant women who are at a higher risk, is essential to achieve maximum control and even the elimination of the disease [153]. The mosquito proofing of a house therefore offers the advantage of equitably protecting all the members of the household, including those who are not sleeping under a bed net [154].

The type of plant used to smoke or as mosquito repellent may also affect the indoor density of malaria vectors. In Tanzania, a significant reduction in *An. gambiae* s.s. (56%) and *An. funestus* s.s. (83%) was observed in houses where a tall and densely foliated repellent plant *Lantana camara* L. was planted in the compound [155].

In western Kenya, Atieli *et al.* [149] found that a papyrus mat ceiling modification reduced the density of house-entering *An. gambiae* s.l. and *An. funestus* by 78–80% and 86%, respectively, compared to unmodified houses. Houses with screens had a higher average humidity (62.9%) than those with no screens (57.8%). In addition, simple insecticide-impregnated ceilings fixed above sleeping rooms of traditionally designed houses reduced house-entering *An. gambiae* s.l. by approximately 76–82%.

In Tanzania, house proofing with ceilings, window screens and closed eaves significantly prevented the entry of *Anopheles* mosquitoes [154]. Many residents installed ceilings to protect themselves from mosquito bites, malaria infection, for

fashionability and to lower the indoor temperature. Ceilings can therefore be promoted for having multiple benefits. Screens and ceilings reduced the densities of anophelines and culicines that occur indoors in west and central Africa [154, 156].

Consequently, house proofing may be used for protecting households against mosquito bites, and hence for a community-level suppression of malaria transmission. In Tanzania, an increased coverage of screens and ceilings was associated with a decline in malaria prevalence between April 2004 and March 2007 [142].

## **1.12. Malaria vector control**

Mosquito control activities are mostly employed at the local level depending on the season, environmental conditions, biology and behaviour of both mosquitoes and humans. It is therefore important to have a basic knowledge of the bionomics of the mosquitoes. The basic knowledge of their bionomics includes the development of immature stages (egg, larva, and pupa) and the life of the adults under the influence of the local environment, since vector control is directed against the larval and adult stages [60].

### **1.12.1. Larval habitat management**

Since the discovery of *Anopheles* mosquitoes as vectors of malaria, larval habitat management (LHM) has been used in reducing and eliminating malaria transmission [157]. LHM refers to the planning, organizing, carrying out and monitoring of activities for the modification and/or manipulation of mosquito breeding habitats or their interaction with man, with a view towards preventing or minimizing vector propagation, thereby reducing man-vector-pathogen contact. LHM primarily includes habitat modification, habitat manipulation, larviciding and biological control [30].

#### **1.12.1.1. Habitat modification**

Habitat modification is a form of environmental management consisting of a physical transformation that is permanent or long-lasting of land, water and vegetation, which

is aimed at preventing, eliminating or reducing the habitats of vectors without causing unduly adverse effects on the quality of the human environment' [30]. It was also the major vector control method before the advent of pesticides [30], causing a physical change to mosquito breeding areas and help in preventing, eliminating or reducing vector density. It includes drainage, filling, land levelling and transformation and impoundment margins. The work of habitat modification is usually permanent in nature; however, proper design and adequate maintenance are essential for their effectiveness. Draining includes creating ditches or drains to keep water moving and to carry water used as mosquito breeding sites in a managed way. Drains may be lined or unlined and located at the surface or on the subsoil level [157]. It involves techniques for drainage and surface water management that depend on the local topography [30].

Habitat modification also involves the elimination of wetlands, thereby creating channels to increase water flow in areas of standing water, filling small ponds or water collecting depressions, or changing banks of water impoundments to help reduce mosquito populations. As slow-moving rivers and streams create larval breeding habitats for certain vector species, straightening their banks reduces vector populations. Modification can also involve human-made vector breeding habitats associated with water-holding structures in mini-dams and small-scale irrigation projects. The creation of a larval habitat can be avoided through careful design and collaborations with other sectors such as agriculture and construction [30].

#### **1.12.1.2. Habitat manipulation**

Habitat manipulation refers to producing temporary conditions that are unfavourable to the breeding of vectors in their habitats [30, 158]. It refers to activities that reduce vector abundance through a temporary change of aquatic environments. Habitat manipulation must be repeated to remain efficacious and is primarily directed at a specific vector species [30]. Water salinity change, stream flushing, the regulation of water levels in reservoirs, the dewatering or flooding of swamps or boggy areas, vegetation removal and management, shading and exposure to sunlight, and

intermittent irrigation to agricultural fields, are all examples of the activities. This is appropriate where permanent habitat modification is not feasible or in areas of irrigated agriculture [157, 158].

With proper planning, design and maintenance, LHM can reduce or eliminate mosquitoes. LHM offers a number of advantages over other vector control methods, including long-term effects, low cost, mutual benefit for agriculture and health, only a slight environmental impact, a low level of exposure to chemicals, prevention and the control of other vector-borne and water associated diseases. Nonetheless, habitat management should be preceded by in-depth ecological studies to help avoid undesired environmental change [158] .

In recent years, there has been a renewed interest in vector control using LHM alone or by employing LHM as a supplement to existing strategies [30, 159]. Larval control played a major role in the eradication of *An. gambiae* from northeast Brazil in the 1930s and early 1940s [160], and suppressed malaria transmission significantly in Zambia and Tanzania [161]. Larval habitat modification was important for malaria eradication in the United States, Israel, and Italy. Effective larval control measures depend on locally derived ecologic concepts that can be adapted to each vector species and applied continuously without any time limit. *An. gambiae* and *An. arabiensis* occur in diverse types of habitats [31, 35], thus creating difficulty for environmental management.

The lack of basic sanitary installations, proper use and maintenance can produce several breeding habitats that may go undetected and escape environmental management. In developing countries, high population growth is associated with unreliable services, frequent breakdowns and leakages of water supply. As a result, people store water in their houses, underground cisterns, roof tanks, water jars and other vessels, most of which are usually left uncovered and then become suitable habitats for vectors, such as *An. dthali*, *An. stephensi*, *An. claviger* and *An. varuna*. Such habitats can be managed by covers such as a plastic floating mesh screen [158].

### 1.12.1.3. Larviciding

Larviciding can be achieved through treating breeding habitats with chemical or biological agents, which is feasible and effective when habitats are relatively few in number and are easily accessible [157]. Chemical and biological larvicides were important to malaria control programmes in the early 20th century, and played the primary role in the eradication of *An. gambiae* s.l. from rural Brazil in the 1930s [157, 160].

Chemicals used as larvicides include petroleum oils, Paris Green (copper acetoarsenite), monolayer surface films, DDT, organophosphate-based larvicidal formulations (for example temephos), synthetic pyrethroids and insect growth regulators. Their efficacy depends on factors including formulation, water quality, habitat size and speed, and susceptibility of the target larvae [157]. Although chemicals are effective in reducing mosquitoes [157, 160, 162], they exhibit mammalian toxicity, a high persistence and non-target effects, a lower effectiveness as an adult side through a selective pressure for resistance, and a high toxicity to aquatic non-target organisms [162]. Larvicides are used on breeding sites that cannot be drained or filled, and where the use of larvivorous fish is expensive or impossible [163].

Larvicidal oils kill larvae when they rise to the surface to breathe, either by suffocation or by poisoning with toxic vapour. Different grades of oil may be suitable for larval mosquito control, depending on local conditions. At higher temperatures a thicker oil is required, e.g. crude or fuel oil, while in the presence of vegetation a lighter oil with a greater spreading power, e.g. kerosene or diesel oil, is necessary. Oils kill larvae quickly, but only last between a few hours and several days. Because of their relatively high cost and limited persistence, their use of mosquito control has decreased. They are important in situations where mosquitoes are resistant to insecticides and in small-scale applications by individual households or communities [163].

In the 1940s, the organochlorine insecticides were adopted for the spraying of breeding sites, but were resisted in the 1950s. They persist in soil and in the tissues of plants and animals. The organophosphorus compounds, the carbamates and the pyrethroids are less persistent, breaking down quickly in the environment, and are recommended as larvicides. However, the pyrethroids are very toxic to fish and crustaceans. Temephos (Abate) is the current insecticide of choice for larviciding because of its reduced persistence and relative safety for non-target organisms [163].

#### **1.12.1.4. Biological control**

Biological control is the introduction of the natural enemies of larvae, including predatory fish, predatory invertebrates, bacteria, fungi and viruses, into their habitats [30]. Such a method can be considered as an alternative in areas where mosquito larvae develop a resistance to insecticides, and adults are exophilic and exophagic [163]. However, an effective use of the biological methods requires a good knowledge of the bionomics of the vectors and the local ecological conditions. The method can be most effective when used in combination with the others [157, 163].

Several viruses have been studied in mosquitoes, but have shown little practical applicability. The bacteria *Bacillus thuringiensis* H-14 and *B. sphaericus* form spores that produce toxins, which poison the gut of mosquito larvae when ingested. They tend to be more specific in terms of which mosquito species they can control and what habitats, and their short persistence of activity often requires repeated applications, which increases costs and logistical complications [157, 162]. Field trials of *B. thuringiensis* var. *israelensis* and *B. sphaericus* to control *An. gambiae* s.l. larvae exhibited a good control, but a short duration of efficacy [159, 164]. In addition, several genera of fungi, nematodes of the family Mermithidae, predatory mosquitoes of the genus *Toxorhynchites*, dragonflies, small crustaceans and *Azolla*, a free-floating fern that can completely cover water surfaces and prevent breeding by mosquitos, showed a strong biological activity against *Anopheles* larvae [162, 163]. In comparison to chemical controls, biological agents can be effective at relatively low doses, are safe to humans and non-target wildlife, have a low toxicity and a simple

application procedures, are low-cost and have a lower risk of resistant development [157, 162]. However, of all the biological control interventions, the use of larvivorous fish has been most successful in different areas of the world [91, 163].

The most successfully used fish species are *Gambusia affinis* and *Poecilia reticulata*, of which the first is efficient in clean water, while the second can be used in organically polluted water [165]. The annual killifishes, *Cynolebias*, *Nothobranchius* and *Aphyosemion*, have dry-resistant eggs that make them useful in mosquito breeding habitats that temporarily dry out [163].

Exotic fish species should be evaluated for their suitability to the local vector species and ecology. Imported fish species may cause unwanted side-effects in the natural habitats by replacing local species or affecting other aquatic animals. The practice of importing *G. affinis* (Baird & Girard, 1853), a freshwater species native to the southeastern US, has been discouraged as the efficacy is highly variable and negative impacts of this voracious and aggressive fish on native fauna have been quite significant [158]. The introduction of *Gambusia* has actually led to the elimination of native fish from certain habitats [166]. However, it can be freely used in man-made breeding habitats such as water tanks and cisterns for the storage of drinking water, swimming pools, garden ponds and water reservoirs in desert locations without a risk of escaping into the natural environment [91, 163].

## **1.12.2. Adult control**

### **1.12.2.1 Indoor residual spraying (IRS)**

IRS is the application of chemical insecticides on the walls and roofs of houses and domestic animal shelters, with the purpose of killing the adult vector mosquitoes resting on these surfaces. It reduces the lifespan and density of the vector. In some cases, the insecticides repel mosquitoes, thereby reducing human-vector contact in sprayed rooms [167].

IRS saved hundreds of millions of lives between the 1940s and the 1980s in Europe, Asia and the Americas [168], as well as contributing to malaria eradication from Europe, the former USSR and several countries in Asia and the Caribbean [167]. It is effective when properly applied, but requires capacity, structures and systems [168]. In Africa, malaria eradication pilot projects, initiated from the 1950s to the 1970s, demonstrated a significant reduction of malaria and the vectors following the application of IRS. Subsequent evidence over several decades has confirmed the effectiveness of IRS in reducing the level of infection and incidence of malaria, but was not fully implemented in large parts of sub-Saharan Africa [167]. The scaled-up implementation of IRS, together with LLINs and case treatment, brought a remarkable decline in the malaria burden during the last decade in Africa [5].

The consistent application of IRS has altered the vectors and epidemiological pattern of malaria in Botswana, Namibia, South Africa, Swaziland and Zimbabwe, as *An. funestus* has been eliminated or reduced to negligible levels. *An. gambiae* s.s. and *An. arabiensis* are also well-controlled in some areas of Africa.

The IRS requires detailed and rigorous planning, management and supervision, and the strategy in IRS management and its implementation has been changed in recent years. The changes are associated with universal LLIN coverage, insecticide resistance management and the reorientation of many national malaria control programmes towards an integrated vector management (IVM) approach. Effective IRS operations require an adequate socio-political commitment, a health system capable of delivering good-quality implementation, information on local vectors, indoor versus outdoor feeding and resting behaviours and sustainable financial, logistical and human resources. Twelve insecticides that belong to the four chemical classes (organochlorines, organophosphates, carbamates and pyrethroids) have been used in IRS. Nonetheless, insecticide resistance has been reported to most of these chemicals in the African malaria vectors [168].



### 1.12.2.2 Insecticide treated nets (ITNs)

The use of mosquito nets as physical barriers against mosquitoes, flies and other arthropods has been practiced from early times. During the past 30 years, the protective effect of the nets against mosquitoes has been enhanced by treating them with insecticides. The insecticides have killing and excito-repellent effects against mosquitoes that add a chemical barrier to the physical one, thus further reducing human-vector contact. Sleeping under ITNs protects humans from the night-biting malaria vectors, hence reducing transmission of the disease [169]. When properly made and used, they prevent disease-transmitting and annoying mosquitoes. In areas where most people sleep under insecticide treated nets, large numbers of mosquitoes are killed and do not survive long enough to transmit the disease [162]. High ITN coverage provides community-level malaria protection through prevention for both users and non-users [170]. ITN use by the majority of entire populations could protect all children, even those not actually covered by achieving existing personal protection targets [153]. Even so, the day-biting habits of some mosquito species, inadequate maintenance/use of the nets, a simple lack of care, resistance of the vector to insecticides in the net fabrics, ecology and the population genetics of the vectors can all help reduce their value [158].

ITNs are considered to be effective in all types of malarious areas where mosquito biting patterns coincide with the time when most people are likely to be sleeping under a net [162]. The use of the LLIN, a factory-treated mosquito net that is expected to retain its biological activity for at least 20 WHO standard washes under laboratory conditions and three years of recommended use under field conditions, has been distributed over the last 10 years. It avoids the need for visits by a re-treatment team and re-treatment by the owners [162]. In Africa, this tool is effective against *An. gambiae* s.l. and *An. Funestus*, which prefer to bite at night when people are in bed. The species are efficient malaria vectors, because of their anthropophilic, endophilic and endophilic characteristics, their longevity and their abundance. LLINs reduce the vector populations by mass killing, leading to a significant reduction in the lifespan, reducing human contact and the malaria sporozoite rate of *Anopheles* mosquitoes, and

the use of excito-repellent insecticides that cause mosquitoes to leave rooms for outdoors [171].

The insecticides commonly used for net impregnation are the pyrethroids, in particular permethrin, deltamethrin and lamdacyhalothrin, because of their low toxicity hazard and good residual effect. However, pyrethroid resistance is reported in most malaria vectors [172]. Moreover, a low level of ITN usage [173, 174], the increased outdoor-feeding tendency of vectors following ITN use [175], the feeding behaviour of mosquitoes as a result of ITN use immediately after sunset and before sunrise [90] and a low level of ownership and the misuse of ITNs [176] are among the major problems in using ITNs as a malaria vector control strategy.

### **1.12.2.3 Improved housing**

Houses located away from nearby breeding habitats experience a low density of indoor-biting malaria vectors to its occupants. The maximum active flight range of most *Anopheles* species from their breeding places does not exceed 2 km [64], with the great majority occurring within a radius of 1 km from their breeding site [177]. The few species that may fly four-five km or more, supported by environmental factors such as wind and vehicles, can be controlled by other measures or may be too small to establish disease transmission [158].

Houses on high ground and exposed to wind currents harbour a lower mosquito density and experience a facilitated draining of rain water to lower lands, thereby reducing potential breeding habitats. Houses located on the leeward side of breeding places, and at the foot of hills or in enclosed valleys, experience a high density of *Anopheles* mosquitoes due to calmer air and more abundant water. Sandy and porous soils that do not easily become waterlogged are preferred village sites compared to clay and impermeable soils which form water pools [158].

Well-designed and mosquito proof houses contribute to a significant reduction in malaria transmission, as wire mesh cloth screens designed to give adequate protection,

maximum ventilation and long life are preferable and sustainable mosquito screening strategies. In humid areas, wire screens could be exposed to the corrosive action of air and to vibrations induced by strong winds; for such situations, a plastic mesh is less liable to deteriorate, although it may require backing with a welded mesh of thick wire to prevent sagging under the wind pressure.

In the early 20th century, house improvement and screening were the methods given a priority for the control of malaria [150]. People living in poor houses are more exposed to malaria than those occupying complete brick and plastered houses. House screening was found to reduce human biting rates of mosquitoes and malaria infections in the United States, Greece and Italy, with clinical trials showing that house screens and ceilings alone provide protection against anaemia and exposure to malaria infection in rural parts of The Gambia. Window screening, closed eaves and ceilings prevent the entry of mosquitoes into houses in Africa [154, 178]. In southwest Ethiopia, screening windows and doors with a metal mesh, in addition to closing all openings with mud, reduced the overall indoor densities of *An. arabiensis* by 40% [179].

In the tropics, people may remain outdoors until late at night and become exposed to infectious mosquito bites, consequently leading to a minimal effect of house screening on malaria transmission [158]. Mosquito proofing involves not only the closure of windows and doors with screens, but also the repair of cracks and holes and the blockage of all other openings through which mosquitoes might gain entrance.

#### **1.12.2.4. Repellents**

Repellents are substances applied to the skin, clothing or mosquito nets to repel mosquitoes and other biting insects and prevent them from biting. They can be used in the outdoors, in the early evenings and in the mornings in places where IRS and LLINs cannot be used [180]. Spatial repellents are, “Chemicals that change the behaviours of mosquitoes resulting in driving away mosquitoes from a potential

human host” and reduce human–vector contact and therefore offer personal protection. Depending on efficacy and application modality, it creates a vector-free area both in- and outdoors. The protected space range depends on the active ingredient, application platform and environmental conditions such as air flow, temperature and humidity. Spatial repellents discourage mosquitoes from entering a space occupied by a potential human host, thus reducing encounters between humans and vectors [162, 181].

Repellents delay the emergence of insecticide resistance and reduce the toxic effects of chemicals to human and non-target organisms. They can reduce malaria transmission by forcing mosquitoes to either feed upon non-human hosts or to search for an alternative blood source. A longer exposure period of a vector species to outdoor conditions such as predation, stressful environments and excessive energy expenditure during host-seeking, or identifying a resting or oviposition site, reduces the longevity and size of the mosquito population. The reduction in human-vector contact could ultimately lead to reduced numbers and the survival of older mosquitoes that transmit mature infectious stage parasites [181, 182].

Repellents can be either synthetic or plant-based products. In the US alone, 7,000 synthetic organic chemicals were being tested at one point. The mixture of compounds has produced repellents several times more effective than single-molecule chemicals. At present, dimethyl phthalate (DMP), dibutyl phthalate (DBP) and diethyltoluamide (DEET) are those most commonly used [158]. Metofluthrin, a newly synthesized pyrethroid, has offered a strong knockdown and lethal activity against mosquitoes. It has high vapour pressure that enables itself to vaporize at normal temperatures without heating compared to the other pyrethroids, which require heating for evaporation. A multilayer paper strip impregnated with metofluthrin caused a significant spatial repellency effect against mosquitoes in laboratory- and open-field conditions for a month at a 200 mg concentration in Indonesia [183]. Metofluthrin-impregnated polyethylene latticework plastic strips (approximately 600 mg per 2.6–5.52 m<sup>2</sup>) reduced *Ae. aegypti* resting inside houses for at least eight weeks in Vietnam [184].

Plants contain natural chemicals to prevent themselves from the attacks of predator insects. These chemicals may serve as repellents, feeding deterrents, toxins or growth regulators. Mankind has been using plant repellents since time immemorial by hanging, fumigating or as oil formulations applied to the skin or clothes to drive away nuisance mosquitoes, which is still practiced in most poor rural communities of the tropics. There are a diverse amount of plant species containing repellent chemicals. Although plant-based repellents are better for the environment than synthetic ones, they may contain compounds that need adequate formulation and monitoring [185]. Some of the plant-based repellents include PMD (para-methane 3-8, diol), citronella and artemesia oils.

A repellent might also be used in combination with other intervention tools in view of seeking a radical reduction in malaria transmission. In a community-based study in the Bolivian Amazon, a significant reduction in the episodes of *P. vivax*, *P. falciparum* and reported fever was observed in the group that used treated nets and a repellent (PMD) [186]. The use of repellents with LLINs or IRS may contribute to a significant reduction of the disease in areas where vectors feed in the early evening and outdoors [89].

### **1.12.3. Problem of insecticide resistance and management**

Insecticides play a central role in controlling major disease vectors such as mosquitoes, sandflies, fleas, lice, tsetse flies, bedbugs, ticks and triatomid bugs. However, insecticide resistance has been documented in insect vectors from every genus. Insecticide resistance refers to the situation in which disease vectors are no longer killed by the standard dose of insecticide or manage to avoid coming into contact with the insecticide. The resistance of anopheline mosquitoes has been documented in almost all countries with ongoing malaria transmission to most of the available insecticides [172].

Insecticide resistance is a growing concern in many countries, which requires immediate attention because of the limited chemical resources available for vector control. Countries in west and central Africa (particularly Benin, Burkina Faso, Cameroon, Côte d'Ivoire, Mozambique, South Africa and Ghana) have long been reporting high frequencies of resistance to the four classes of insecticides, such as organochlorines, pyrethroids, carbamates and organophosphates. In Ethiopia, resistance has been reported to all four classes of insecticides, including DDT and pyrethroids, with a similar condition documented in Uganda, Kenya, Tanzania, Malawi and Zambia. Insecticide resistance leads to a reduced efficacy of chemical-based interventions or the possibility of control failure [172]. However, the pattern of resistance is very heterogeneous, even over relatively small distances [187], which is possibly due to a misuse of insecticides and a cross resistance with the locally used pesticides, as well as herbicides.

The widespread use of LLINs resulted in the development of vectors resistant to insecticides impregnated within the net fabrics. This problem could be exacerbated in households owning damaged nets. In addition, the benefit of a community-wide reduction in the number of infectious vectors could be reversed. However, the LLINs can maintain their physical protective effect, against malaria vectors, as long as they are not damaged and used properly [188].

In order to minimize the increasing trend of insecticide resistance in malaria vectors, the WHO and its partners have developed a Global Plan for Insecticide Resistance Management (GPIRM) [172]. The plan is developed to serve as the basis for a national vector control strategy, including the use of IRS. The basis of the plan is the building of capacity and systems for basic epidemiological and entomological monitoring, including bioassays for the insecticide susceptibility of vectors to insecticides in order to delay the further development of resistance. This remains important for the pyrethroids, the only class of insecticide that can be used on nets. Because pyrethroids are safe for close contact and have a rapid, persistent effect on mosquitoes at low doses, they are recommended for the treatment of nets.

Nevertheless, effective non-pyrethroid alternatives are being sought because of the consequence that the emergence of a strong resistance to pyrethroids would have on the effect of insecticide-treated mosquito nets [162]. The GPIRM recommends that pyrethroids need to be “protected” through judicious use and through a rotation among the four classes of insecticides that can be used for IRS [168].

The four main strategies for managing insecticide resistance are: 1) rotating insecticides, with different modes of action, from one year to the next, 2) using two or more insecticide-based vector control interventions in a house (e.g. pyrethroids on nets and an insecticide of a different class on the walls), 3) using one compound in one geographic area and a different compound in neighbouring areas, the two being in different insecticide classes, and 4) using a mix of two or more compounds of different insecticide classes in a single product or formulation, so that the mosquito is guaranteed to come into contact with the two classes at the same time [172].

#### **1.12.4. Integrated vector management**

The reliance on a single method of vector control may be challenging in several respects (e.g. insecticide resistance or the outdoor- and early biting behaviour of the vectors), hence making single-intervention method such as IRS or ITNs is useless. For this reason, a combination of two or more methods of vector control may have to be worked out for effective control depending on the local condition of the area. Such an approach is the concept of integrated vector management (IVM), which is a rational decision-making process designed to optimize the use of resources for vector control based on evidence and integrated management, promoting the use of a range of interventions – whether alone or in combination – selected on the basis of local knowledge about the vectors, diseases and disease determinants [189]. In Zambia, an IVM that integrated chemical and non-chemical approaches resulted in a marked reduction in malaria-related morbidity and mortality, while ensuring a better protection of the environment [190].

## **2. Statement of the problem and rationale of the study**

Malaria is an important public health problem in the Butajira area, a highland in south-central Ethiopia [21, 28, 29, 191]. In this area, the occurrence of the *Anopheles* species and their role in malaria transmission remain poorly described. This necessitated undertaking the study on the species' composition, the distribution of the *Anopheles* species and the entomological risk factors in relation to the risk of malaria transmission. As a result, the occurrence and dynamics of *Anopheles* larvae and their breeding habitats were described (Paper I) to inform different health sectors on the type, location and seasonality of breeding habitats that drive adult vector populations in the area. The feeding and resting behaviours of adult *Anopheles* mosquitoes and their *Plasmodium* sporozoite vectorial role was studied (Paper II) in order that concerned public health authorities practice evidence-based vector control and disease management strategies. This was also done to document the *Anopheles* species, including the malaria vectors in the area. The study assessed the impact of the local housing conditions on indoor-biting and indoor-resting *Anopheles* mosquitoes (Paper III) in order to identify the house designs that minimize the risk of indoor exposure to infectious *Anopheles* mosquito bites, and which inform the community as well as policymakers on the type of house construction that best prevents malaria infection.

In general, this study was envisaged to document the occurrence of the *Anopheles* species and the entomological aspects of malaria transmission risk in a highland area of south-central Ethiopia, so that the concerned health authorities and policymakers undertake evidence-based and appropriate disease intervention strategies, and make preparations for future malaria epidemics in the area.

## **3. Inception of the study**

The study was part of a bigger project entitled, “*The Ethiopian Malaria Prediction System (EMaPS)*” [<http://www.malariajournal.com/content/13/S1/P56>]. EMaPS was a



collaborative project, undertaken from 2007 to 2012, between Ethiopian universities (Addis Ababa University, Hawassa University and Arba Minch University) and the University of Bergen (in Norway) to develop a malaria prediction model for Ethiopia. The study shows a new classification of climate zone, weather variability as the main driver of malaria and indigenous malaria transmission during a non-epidemic year above 2,000 m attitude in the country. A computer model, an open malaria warning, was also developed and validated, although long-term data (clinical and entomological) was recommended by the research team to validate such models.

#### **4. Objectives of the study**

##### **4.1. General objective**

The overall objective of the study was to determine the *Anopheles* species and entomological aspects of malaria transmission risk in an altitudinal transect of the Butajira area, a highland in south-central Ethiopia.

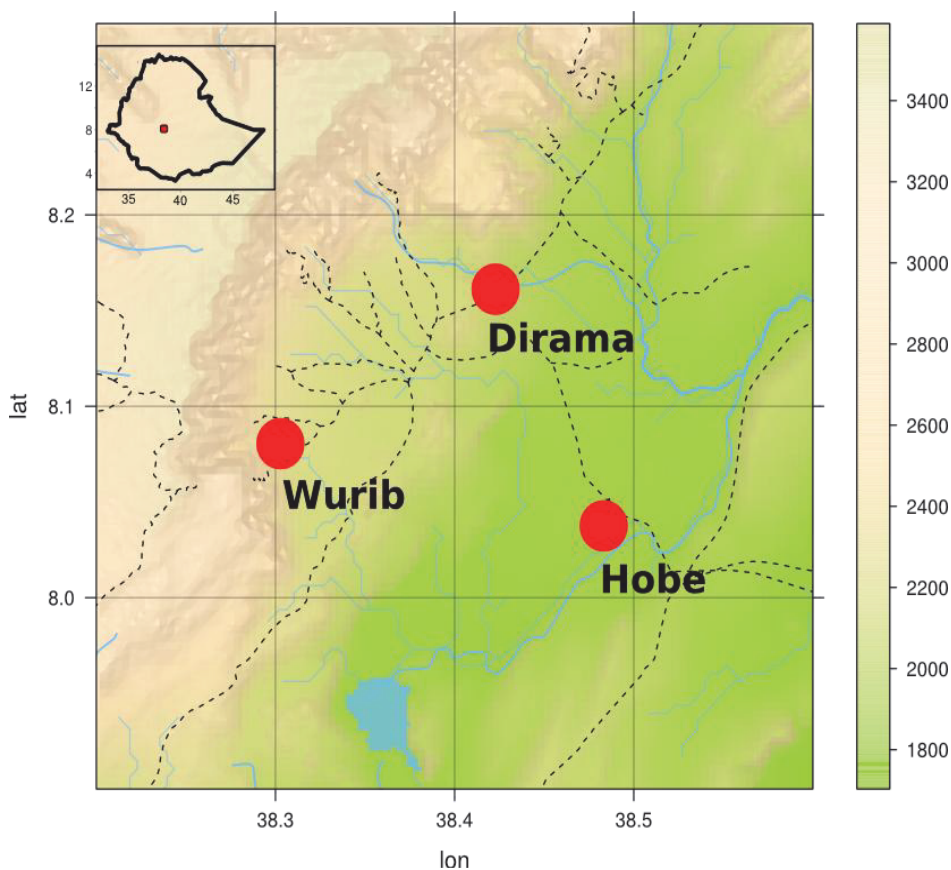
##### **4.2. Specific objectives**

1. To determine the occurrence and dynamics of *Anopheles* larvae;
2. To study the feeding preferences of the adult *Anopheles* species;
3. To estimate the entomological inoculation rates (EIRs) of the *Anopheles* species; and
4. To assess the impact of housing conditions on the indoor-biting and indoor-resting density of the major malaria transmitting *Anopheles* species.

## 5. Study area and methods

### 5.1. Study area and population

The study was conducted in a district, formerly called the Meskan and Mareko, in the Gurage Zone of the Southern Nations, Nationalities and People's Region (SNNPR) in south-central Ethiopia (Figure 6).



Source: Anmut *et al.* Parasites and Vectors, 2012, 5:117

**Figure 6:** Location of Hobe, Dirama and Wurib villages in Butajira area, Southern Nations Nationalities and People's Region (SNNPR), southern Ethiopia

The Butajira Town, the district's capital, is located approximately 135 km south of Addis Ababa, and the district had 86 villages (*Kebeles*). In the district, there is a

Demographic Surveillance Area (DSA) constituting 10 villages. The area is divided into three ecological zones, namely the lowland (Hobe, Jaredemeka and Bati villages), the midland (Bido, Dirama, Butajira Town *Kebele* 04 and Dobena villages) and the highland (Wurib and Yeteker villages) [192]. Among these, the Hobe (N=08<sup>0</sup>01'.912; E=038<sup>0</sup>29'.179), Dirama (N=08<sup>0</sup>10'.061; E=038<sup>0</sup>25'.142) and Wurib (N=08<sup>0</sup>04'.877; E=038<sup>0</sup>17'.991) villages were randomly selected and used in the study. In each village, a study site was selected along a permanent stream and used for immature- and adult mosquito study. During the selection, the sites were noted for having a higher number of malaria cases by the community health workers. The villages are located at elevations of 1,800 to 2,300 m above sea level and form an altitudinal transect. These were among the six villages where seasonal prevalence and the risk factors of malaria, within the Ethiopian Malaria Prediction System (*EMaPS*) project, were being studied [22]. The villages had a total population of 14,475 during July 2009, of which 6,140 were in Wurib, 5,278 in Hobe and 3,057 in Dirama (Table 1). The overall female population (7,306; 50.5%) was almost equal to the male population (7,169; 49.5%) in the area.

The main rainy season of the area is from June to September, as the area remains dry and does not support surface water collection during the majority of the year. This study was part of the *EMaPS* project, a multi-disciplinary research project designed to develop a model to be used for early malaria transmission prediction in Ethiopia [<http://www.malariajournal.com/content/13/S1/P56>].

Table 1: Population size of Wurib, Dirama and Hobe villages, Butajira area, south-central Ethiopia, July 2009

Age	Population		Wurib		Dirama		Hobe	
	Male	Female	Male	Female	Male	Female	Male	Female
<1	127	127	59	59	26	28	42	40
1-4	861	799	358	334	148	152	355	313
5-14	1,937	1,950	879	857	409	402	649	691
15-49	3,551	3,777	1,347	1,633	771	812	1,433	1,332
50 <sup>+</sup>	693	653	319	295	150	159	224	199
Total	7,169	7,306	2,962	3,178	1,504	1,553	2,703	2,575

Source: Butajira Rural Health Programme, Addis Ababa University; n = number of individuals

## **5.2. Methods**

### **5.2.1. *Anopheles* larvae survey and sampling (Paper I)**

A monthly mosquito larvae survey was undertaken alongside- and nearby streams in Hobe, Dirama and Wurib villages for two years (July 2008 – 2010). All available aquatic habitats, including streams, water wells, small rain pools, pools in hoof- or footprints and *Enset* (a false banana) (*Ensete ventricosum*) leaf axils, were surveyed. Habitats were first inspected for the presence of mosquito larvae visually. Positive habitats were sampled with a soup ladle (a 350 ml capacity). Habitat characteristics, including water speed, length, width, depth, pH, turbidity, vegetation cover and distance from the nearest inhabited house, were also recorded. Culicines were discarded after counting, whereas *Anopheles* were identified into early (first and second) and late (third and fourth) instars in the field. Late instars were preserved in 70% alcohol after being killed in hot water (ca. 60<sup>0</sup>C) [193] and transported to The Aklilu Lemma Institute of Pathobiology, Addis Ababa University, mounted in a gum-chloral mountant on microscope slides, dried at room temperature and identified to a species on the basis of a morphology under a microscope using appropriate keys [23, 34, 193].

### **5.2.2. Indoor-biting mosquito collection (Paper II)**

Twenty houses were selected from each village for indoor-biting anopheline mosquito collection. The day before the actual CDC light trap-based mosquito collection, a member of the researchers visited each target house and informed the householders of the purpose of the trap, the CDC light trap schedule and what the households were expected to do. A miniature CDC light trap (John W. Hock Ltd, Gainesville, FL., USA), fitted with a 150mA incandescent bulb and operated by a 6 V power source, was hung in each selected bedroom, about 1 m to 1.5 m from the floor and about 50 cm from one of the occupied untreated bed nets. Each CDC light trap was set to run from sunset (6:00 pm) to dawn (6:00 am) for two consecutive nights, resulting in a total of 40 CDC trap nights per village per month [34]. The traps were collected later

in the morning, and enquiries made as to whether the trap fan and light had both worked well and all night. Most CDC light traps worked well and the reported failures were rare. On the few occasions when the light traps failed, the light trap catches were multiplied by 3/2 [194]. Mosquitoes trapped in the cages (fitted to the CDC light trap) were collected in the morning by mouth aspirator. Culicines and male *Anopheles* mosquitoes were discarded after counting, and the females were processed for later use.

### **5.2.3. Indoor-resting mosquito collection (Paper II)**

Indoor-resting mosquito collection was undertaken in 10 selected houses in each village using the PSC method. The day before the PSC was conducted, a member of the research team travelled to the target houses and informed the householders of the purpose and details of the PSC, and what the residents were expected to do in preparation. The PSC was carried out in the mornings (7:00 am to 8:30 am) in each village once every month. Before spraying, the occupants and their domestic animals left the house. In addition, the utensils used for food, drinking water and clothes were taken out of the houses, the house apertures were carefully covered with clothes and the available floor space was entirely covered by two–three white plastic sheets (each with an area of 4 m × 5 m). Spraying was made using KILIT™ insecticide aerosol (Miswa Chemicals LTD, Caswell Road, Brackmills, Northampton, NN4 7PW England) according to the manufacturer's instructions and the collectors waited outside for approximately 15 min. The principal active ingredients of the insecticide are Dichlorvos, permethrin and tetramethrin, which belong to the class of insecticides called pyrethroids. The sheet was then carefully taken out of the house and knocked down mosquitoes were collected using forceps [34, 193]. Culicine and the male *Anopheles* mosquitoes were discarded after counting and the females were processed for later tests.

### **5.2.4. Outdoor-resting mosquito collection (Paper II)**

Five pit traps constructed in shaded areas were used for outdoor-resting mosquito collection in each village. Pits 1.5–2 m deep, 1.2–1.5 m long and 1 m wide were dug into the ground under a tree shed and within a distance of 20–50 m from the nearest

inhabited house. Four small cavities were then hollowed out to a depth of roughly 0.3 m in the sides of the pit from 0.5 to 0.6 m above the bottom. Mosquitoes were collected from inside the cavities or on the sides of the pit by a mouth-held aspirator using a torch as a light source [34]. During the time of collection, each pit was covered with a transparent net to prevent mosquitoes from escaping. Culicine and male *Anopheles* mosquitoes were discarded after counting and the females were preserved.

#### **5.2.5. *Anopheles* mosquito processing (Paper II)**

Female *Anopheles* mosquitoes from all catches were counted, categorized according to their abdominal status and identified to species on the basis of their external morphological characteristics under a dissecting microscope [23]. Their abdomen was categorized as fresh fed (FF), half gravid (HG), gravid (GR) or unfed (UF) and identified to species on the basis of their external morphology under a stereoscopic dissecting microscope. Unfed *Anopheles* were dissected and their parity determined microscopically as either parous or nulliparous based on changes in their ovarian tracheal system. A mosquito was kept in a labelled 1.5 ml Eppendorf tube containing a silica gel desiccant and cotton for the identification of blood meal sources (when FF), sporozoite infection status (when HG, GR or parous) or sibling species (when *An. gambiae* s.l.). Samples were stored at room temperature while in the field and in a 20°C refrigerator at the Akililu Lemma Institute of Pathobiology, Addis Ababa University until tested [34].

#### **5.2.6. Blood meal source identification (Paper II)**

The abdomen of FF *Anopheles* mosquitoes from all catches was simultaneously assayed for human and bovine blood antigens by ELISA [195, 196]. The abdomen of each FF mosquito was ground in a 50 µL phosphate-buffered saline (PBS) and the volume was brought to 200 µL with a PBS buffer. 50 µL of the triturate was coated in duplicate wells on two separate U-bottomed, 96-well microtitre plates simultaneously: one plate for human blood meal identification and the other for bovine. Plates were incubated overnight at room temperature and washed twice with PBS-Tween 20. 50

µL of peroxidase-conjugated anti-human IgG was added in the first plate and the same volume of peroxidase-conjugated anti-bovine IgG in the second plate, which was incubated for one hour at room temperature and washed thrice with PBS-Tween 20. Finally, 100 µL of ABTS peroxidase substrate was added, incubated at room temperature for 30 min and visually observed for green colour reaction and read for absorbance at 405 nm (by MRX Microplate Reader, Dynex Technologies, 14340 Sullyfield Circle, Chantilly, VA. 20151–1683, USA). Positive controls (either human or bovine blood meal) and negative controls (the unfed abdomen of laboratory-bred *An. arabiensis*) were included in each plate. The human blood index (HBI) and bovine blood index (BBI) of each *Anopheles* species was determined by dividing the number of mosquitoes with human and cattle, respectively, to the total tested.

#### **5.2.7. Sporozoite rate (SR) and entomological inoculation rate (EIR) determination (Paper II)**

The dried head and thorax of half gravid, gravid and parous *Anopheles* mosquitoes from all catches were simultaneously tested for *P. falciparum* and *P. vivax* circumsporozoite proteins (CSPs) [113]. Entomological inoculation rates were determined from CDC light trap collections [94] and PSC [193]. The dried head and thorax of the GR or parous mosquito from all catches were carefully separated from the abdomen and simultaneously tested for *P. falciparum* and *P. vivax* CSPs [34, 35]. Three U-bottomed 96-well micro plates were coated separately with a 50 µL solution of *P. falciparum*, *P. vivax*-210 and *P. vivax*-247 monoclonal antibodies (MAB), respectively, and incubated at room temperature overnight. The contents of the plates were drained, washed three times with PBS-Tween 20, filled with 200 µL of blocking buffer (BB) and incubated for one hour at room temperature. During the incubation period, the mosquitoes were ground individually in 50 µL of boiled casein containing Igepal CA-630, and the final volume was brought to 250 µL with BB. The BB was removed from plates and 50 µL of mosquito triturate was added to each of the three test wells. The CSP-positive sample and laboratory-bred *An. arabiensis* were used as positive and negative controls, respectively. Plates were incubated for two hours and washed with PBS-Tween 20 twice. 50 µL aliquots of homologous peroxidase-



conjugated MAB (0.05 µg/50 µL BB) were added to each triplicate well in the plates and incubated for one hour.

The plates were washed thrice with PBS-Tween 20, 100 µL ABTS (2,2'-azinobis[3-ethylbenzthiazoline-6-sulfonic acid] diamonium salt) peroxidase substrate added per well and incubated for 30 and/or 60 min. Plates were visually observed for green colour and their optical density was read at 405 nm in the micro plate reader. Samples with green colour and with optical density values of greater than two times the average optical density of the negative controls were considered sporozoite-positive. Positive samples were also retested for confirmation. The *P. falciparum* and *P. vivax* SRs of each *Anopheles* species was determined by dividing *P. falciparum* and *P. vivax*-positive *Anopheles*, respectively, to the total tested. The sporozoite rate (SR) was separately determined for CDC light trap catches and also for PSCs.

Since no human landing catch (HLC) was performed due to ethical concerns, the daily EIR was estimated based on a CDC light trap and PSC. For a CDC-based EIR, the factor determined for *An. arabiensis* in Zambia [94], where a CDC light trap represents 1.91 of indoor HLCs, was used. Thus, the EIR was determined by the formula:  $1.91 \times (\text{no. sporozoite-positive ELISAs/no. mosquitoes tested}) \times (\text{no. mosquitoes collected by CDC light traps/no. CDC catches})$ .

Similarly, the daily EIR based on PSC was calculated according to the WHO [193] as  $(\text{no. FF mosquitoes caught by PSC/no. human occupants who spent the night in the sprayed house}) \times (\text{no. human fed mosquitoes/no. mosquitoes tested for human blood meal}) \times (\text{no. sporozoite-positive ELISAs/no. mosquitoes tested})$ .

#### **5.2.8. *Anopheles gambiae* sibling species identification (part of Paper II)**

Approximately 12.5% (n = 305) of the collected adult *Anopheles gambiae* s.l. were randomly selected and identified to species using a species-specific PCR [197]. Briefly, a leg was removed from each mosquito and mixed with 12.5 µl of PCR master mix (containing 10x dNTPs, MgCl<sub>2</sub> Solution, QD primer, UN Primer, GA

primer, ME primer, AR primer, deionized water and RTag) in a 0.2 ml PCR tube, centrifuged for 20s-20min at 16 K r.p.m. and amplified in a PCR apparatus (PTC-100™ Programmable Thermo cycler, MJ Research, Inc., USA) with a PCR cycle condition (95°C/5 min × 1 cycle; [95°C/30s, 50°C/30s, 72°C/30s] × 30 cycles; 72°C/5 min × 1 cycle; 4°C hold). 5 µl of PCR product mixed with 2 µl of loading dye and 4 µl of DNA ladder was electrophoresed through a 2% agarose-tris-borate-EDTA containing ethidium bromide gel (with a 100 V and 150 mA power source) and visualized under a UV light box (Alpha Innotech, MultiImage™, Light Cabinet, Pacific Image Electronics Co. Ltd, Taiwan). All the 305 *An. gambiae* s.l. mosquitoes were found to be *An. arabiensis*; hence, all other *An. gambiae* s.l. samples were regarded as *An. arabiensis* [in Paper II].

### **5.2.9. Assessing housing condition and exposure to *Anopheles arabiensis* bite (Paper III)**

A two-year repeated cross-sectional study on the relationship between housing conditions and the abundance of indoor-biting and indoor-resting *Anopheles arabiensis* was undertaken in the villages once a month for two years (from July 2008 to June 2010). During the time of the CDC light trap and PSC, the condition of each house was recorded. The data on housing conditions included the presence of apertures (holes in the roof, holes in the wall, open eaves, window fitness, door fitness), the number of occupants who slept there the previous night, the number and type of domestic animals tethered indoors the previous night, the altitudinal location and distance from the closest breeding site. The density of indoor-resting *Anopheles* mosquitoes was determined as the number of female anophelines caught by PSC per house per day, while the density of the indoor-biting *Anopheles* was estimated based on the CDC light trap catches per house per night [94, 193].

### **5.3. Data quality and management**

Standard entomological tools and procedures were employed in sampling the immature and adult stages of *Anopheles* mosquitoes. Standard protocols [23, 197]

were also used in identifying *Anopheles* mosquitoes morphologically into species and sample preservation. Blood meal source identification was made using blood meal ELISA, and the *Plasmodium* sporozoite infection status was tested using species-specific sporozoite ELISA as described elsewhere in the text. Appropriate onsite training and advice on entomological data collection was obtained from the leaders of the project. Consent was obtained from the district health bureaus, and the data was collected as per the schedule, and then computerized, cleaned and analysed.

Data collectors (the principal investigator and technicians) had experience in mosquito sampling, handling and *Anopheles* mosquito colony maintenance at the Vector Biology and Control Unit of the Akililu Lemma Institute of Pathobiology (ALIPB), Addis Ababa University (AAU). The principal investigator developed the data collection instruments and undertook the data collection, assisted by the technicians and field assistants. The reliability of the data was checked in the field, and the data was then entered in a computer data base using SPSS versions 16.0, and later exported to PASW Statistics Version 18.0 (SPSS Inc, Chicago, IL, USA).

#### **5.4. Data analysis**

A descriptive analysis was made and tables were used to present the frequency of each *Anopheles* species (immature and adult) by collection method, village, altitude, abdominal status, blood meal source and *Plasmodium* sporozoite infection status. Mean numbers and confidence intervals of *Anopheles* larvae species, human/cattle fed and *Plasmodium*-infected *Anopheles* mosquitoes and *P. vivax*-infected *An. arabiensis* were determined and presented in tables. *Anopheles* mosquito counts were log transformed after adding one, from which one was subtracted and then means and confidence intervals were calculated. A Spearman's correlation coefficient analysis, the Mann-Whitney *U* test, the extended Mantel – Haenszel chi-square test for linear trend and the intra rater agreement test (Kappa) were used where appropriate. Graphs were used to show trends and associations, and univariate (binary regression) and multivariate (generalized estimating equations for repeated measures) analyses were also made where necessary.

## **5.5. Ethical considerations**

The EMaPS study was approved by the Ethical Committee of the Faculty of Medicine, Addis Ababa University and The National Health Research Ethics Review Committee (NERC) of Ethiopia, with reference number RDHE/48 – 85/2009, and by the Regional Ethical Committee in Norway. Permission to undertake the study was obtained from district and regional health bureaus, residents and authorities of the study villages were informed about the study and permission was obtained prior to its start. All immature and adult *Anopheles* mosquito collections were made following verbal consent from the head of the household. Pit shelters were prepared after obtaining the consent of the land owners. Every PSC and CDC light trap mosquito collection activity was undertaken following the informed verbal consent of the household heads. Participation in the study was strictly on a voluntary basis, and all records, data forms and computer files were kept in a secure place to maintain confidentiality.

## 6. Results

The main findings of the study are published in three articles (Paper I, Paper II and Paper III) and are referred to in the text.

Some of the results that are not depicted in the published articles are presented in tables (Table 2, Table 3 and Table 4) and texts in the thesis in order to make a better documentation and discussion of the findings on the occurrence and entomological risk factors of malaria in south-central Ethiopia. Paper I, which addresses the abundance and dynamics of anopheline larvae, does not show density along the three altitudes. The abundance and relative frequency of each species of the late instars (third instar or fourth instar larvae) of the *Anopheles* mosquitoes at a particular sampling point was determined as the number of mosquitoes that belong to the species in the sampling point per the number of dips employed in the sampling point. This was then expressed per 100 dips to determine its density in the sampling point. The density of each *Anopheles* species in the swamp or a stream was then the total of its densities at several sampling points along the specified habitat. The densities of each *Anopheles* species larvae were then compared among the three altitudes. Accordingly, the mean and confidence interval of the density of the five abundant *Anopheles* species is shown in Table 2.

Table 3 shows the mean numbers of the fresh fed (FF), human fed (HF), bovine fed (BF), human and bovine fed (HBF), *P. vivax*-positive and *P. falciparum*-positive mosquitoes among each of the five major *Anopheles* species collected during the study period. This information is missing from Paper II, which deals with the blood meal sources and entomological inoculation rates of *Anopheles* in the area during the study period. Similarly, the relationship between the average numbers of *P. vivax*-infected *An. arabiensis* and housing condition in the study villages that is not shown in Paper III (the impact of housing condition on indoor-biting and indoor-resting *An. arabiensis* density in a highland area of central Ethiopia) is presented in Table 4.

We made 2,544 visits to collect adult mosquitoes in the low- (Hobe), mid- (Dirama) and high- (Wurib) altitude villages of the Butajira area. Among the 2,544 visits, 2,160 were to residential houses using CDC light traps and PSC, 360 to artificial pit shelter traps and 24 to the potential breeding habitats.

### **6.1. Occurrence and dynamics of *Anopheles* larvae (Paper I and Table 2)**

A total of 24 surveys of immature *Anopheles* mosquitoes were carried out from July 2008 to June 2010 in the low- (Hobe), mid- (Dirama) and high- (Wurib) altitude villages of the Butajira area. Among the potential mosquito breeding habitats surveyed, Odamo Stream in the low-, Akamuja Stream in the mid- and the Assas Stream and Beko Swamp in the high-altitude village were found to harbour culicines (not included in the paper) and *Anopheles* larvae [Paper I]. In addition, two small temporary rain pools (one in the low- and the other in the mid-altitude village) were found to support early instars (first instar or second instar larvae) of *Anopheles* mosquitoes.

We collected 9,532 immature *Anopheles* mosquitoes of which 3,171 (33.3%) were first instars, 2,414 (25.5%) were second instars, 2,266 (23.8%) were third instars and 1,681 (17.6%) were fourth instars. Among 3,100 late (third and fourth) instars, the most dominant was *An. cinereus* (32.5%), followed by *An. gambiae* s.l. (= *An. arabiensis* during the study period) (31.4%), *An. chrysti* (23%) and *An. demeilloni* (12.2%). *Anopheles arabiensis*, *An. cinereus*, *An. christyi* and *An. demeilloni* occurred in the low-altitude village. *Anopheles arabiensis*, *An. cinereus*, *An. christyi*, *An. demeilloni*, *An. garnhami*, *An. azaniae* and *An. pharoensis* occurred in the mid-altitude village. Similarly, *An. arabiensis*, *An. cinereus*, *An. christyi*, *An. demeilloni*, *An. pretoriensis*, *An. azaniae*, *An. rufipes*, *An. sergenti*, and *An. garnhami* were observed in the high-altitude village.

The density of *An. arabiensis* larvae was highest in the lowest elevation village and lowest in the highest elevation village. Generally speaking, the density of *Anopheles*

larvae was highest during the dry months along the beds and shallow surfaces of the streams. The larval density increased with an increase in habitat temperature and a decrease in habitat depth.

The mean number of catches of the larval stages of the five abundant *Anopheles* species and their differences in the low-, mid- and high-altitude villages is shown in Table 2. The mean numbers of *An. arabiensis* larvae sampled from the Odamo Stream (mean = 2.5; 95%CI = 0.5-7.2) and the Akamuja Stream (mean = 2.7; 95% CI =1.06-6.02) were higher than the mean numbers collected from the Assas Stream (mean = 0.1; 95% CI = 0 – 0.2) and the Beko Swamp (mean = 0.3; 95% CI = 0 – 0.7) located in the high-altitude village. On the other hand, the mean counts of *An. chrysti* and *An. demeilloni* increased from the low- to the high-altitude village.

Table 2: Mean numbers of *Anopheles* larvae sampled from the Hobe, Dirama and Wurib villages, south-central Ethiopia, July 2008 – June 2010

Immature anopheline species	Hobe	Dirama	Wurib	
	Odamo Stream	Akamuja Stream	Assas Stream	Beko Swamp
	M (95% CI)	M (95% CI)	M (95% CI)	M (95% CI)
<i>An. arabiensis</i>	2.5 (0.5-7.2)	2.7 (1.0-6.2)	0.1 (0-0.2)	0.3 (0-0.7)
<i>An. pharoensis</i>	0	0.03 (0-0.1)	0	0
<i>An. chrysti</i>	0.3 (0.1-0.6)	1.1 (0.3-2.4)	1.0 (0.1-2.0)	5.2 (2.1-11.3)
<i>An. cinereus</i>	0.2 (0-0.5)	4.5 (1.5-11.4)	1.0 (0-2.3)	1.4 (0.4-3.3)
<i>An. demeilloni</i>	0.2 (0-0.5)	1.0 (0.3-2.0)	1.0 (0.1-2.4)	2.2 (1.0-4.4)

Note: *An* = *Anopheles*; M (95% CI) = mean (95% confidence interval)

During the non-rainy months, no surface water was observed in the three villages, with the exception of the natural permanent streams and some wells built close to residential houses. Additionally, some false banana (*Ensete ventricosum*) trees in the high-altitude village were found to have small water collections in their leaf axils.

The water collections in the wells and leaf axils were found to hold the larvae of culicine mosquitoes, but not *Anopheles*.

## 6.2. Feeding preferences of adult *Anopheles* species (Papers II, III and Table 3)

The adults of *An. arabiensis*, *An. pharoensis*, *An. christyi*, *An. cinereus*, *An. demeilloni* and *An. coustani* were collected from the low- and mid-altitude villages. Similarly, *An. arabiensis*, *An. pharoensis*, *An. christyi*, *An. cinereus*, *An. demeilloni*, *An. coustani*, *An. culicifacies*, *An. garnhami* and *An. rhodesiensis* were found in the high-altitude village. *An. arabiensis*, *An. pharoensis* and *An. coustani* were the most frequently collected mosquitoes in the low-altitude village and decreased with an increase in altitude, whereas the densities of the remaining *Anopheles* species increased with an increase in altitude [Paper III].

A total of 2,433 fresh fed *Anopheles* mosquitoes composed of *An. arabiensis* (1,336; 54.9%), *An. demeilloni* (605; 24.9%), *An. pharoensis* (243; 10%), *An. christyi* (175; 7.2%) and *An. cinereus* (74; 3%) were tested for their blood meal source.

Approximately 40% of the fresh fed *An. arabiensis* had their blood meal from cattle, 32.2% from humans and 12.2% from both cattle and humans (mixed), while the blood meal source of the remaining 15.2% was neither cattle nor human. Similarly, 55.9% of *An. pharoensis* were cattle fed, 18.6% were human fed and 15% mixed (human and cattle) fed [Paper II].

Table 3 shows the mean number of fresh fed (FF), human fed (HF), bovine fed (BF), human and bovine fed (HBF), *P. vivax*-positive and *P. falciparum*-positive mosquitoes among each of the five major *Anopheles* species. The mean catches of FF, HF, BF and HBF were reduced along the altitude transect, from the lowest-altitude village to the high-altitude village.



### 6.3. Sporozoite rates and entomological inoculation rates of *Anopheles* species (Paper II and Table 3)

We tested 1,117 *Anopheles* mosquitoes representing five species for *Plasmodium* sporozoite infection. From among 819 *An. arabiensis*, 14 (1.7%) were positive for *P. vivax* CSP (*P. vivax*-210, *P. vivax*-247 or both) and 2 (0.2%) for *P. falciparum* CSP. From the total of 79 *An. pharoensis* tested, only 2 (2.5%) were positive for *P. vivax* CSP. The sporozoite-positive *Anopheles* mosquitoes were collected from the low-altitude village (14= *An. arabiensis*; 2=*An. pharoensis*) and the mid-altitude village (2= *An. arabiensis*) [Paper II].

Based on the CDC light trap catches, there were 3.7 annual *P. falciparum* infective bites per person for *An. arabiensis* in the year from July 2008 to June 2009 in the low-altitude village. In this village, the annual *P. falciparum* sporozoite infective bite from July 2009 to June 2010 was zero. The annual *P. vivax* EIR for *An. arabiensis* was 33 from July 2008 to June 2009 and 14.5 from July 2009 to June 2010. In the mid-altitude village, there was zero annual *P. vivax* EIR for *An. arabiensis* in the first year and 2.3 annual *P. vivax* EIR for *An. arabiensis* in the second year [Paper II]. The average catches of *P. vivax* sporozoite-positive *An. arabiensis* were highest in the low-altitude village (mean = 0.05; 95% CI = 0.02–0.08) and decreased with an increase in altitude along the transect [Table 3]. The number of *An. pharoensis* caught was also observed to decrease with a decrease in altitude (Paper II).

Table 3: Mean number of fresh fed, human/cattle fed and *Plasmodium* sporozoite-infected *Anopheles* mosquitoes in the Hobe, Dirama and Wurib villages of south-central Ethiopia, July 2008 – June 2010

Species	Status (n)	Hobe	Dirama	Wurib
<i>An. arabiensis</i>	FF (1,336)	2.0 (1.7-2.4)	1.0 (0.7-1.1)	0.6 (0.3-1.0)
	HF	1.1 (1.0-1.3)	0.5 (0.4-0.7)	0.4 (0.1-0.8)
	BF	1.2 (1.0-1.5)	0.6 (0.5-0.8)	0.1 (0-0.2)
	HBF	0.4 (0.3-0.5)	0.1 (0.1-0.2)	0.1 (0-0.2)
	Pv-positive	0.05 (0.02-0.08)	0.02 (0-0.05)	0
	Pf-positive	0.01 (0-0.02)	0	0
<i>An. pharoensis</i>	FF	1.5 (1.1-2.0)	1.3 (1.0-1.7)	1 (0-3.4)
	HF	0.5 (0.3-0.7)	0.2 (0-0.4)	0
	BF	1.5 (1.1-1.9)	1.0 (0.5-1.4)	0.4 (0-1.8)
	HBF	0.4 (0.2-0.6)	0.2 (0-0.4)	0
	Pv-positive	0.04 (0-0.1)	0	0
	Pf-positive	0	0	0
<i>An. cinereus</i>	FF	0.5 (0.1-1.1)	1.0 (0.4-1.2)	0.7 (0.5-1.0)
	HF	0	0.3 (0-0.6)	0.3 (0.2-0.5)
	BF	0.4 (0-1.8)	0.6 (0.1-1.2)	0.8 (0.5-1.2)
	HBF	0	0.1 (0-0.4)	0.2 (0-0.3)
<i>An. chrysti</i>	FF	0.3 (0-0.8)	0.6 (0.3-1.0)	0.9 (0.7-1.1)
	HF	0	0.1 (0-0.4)	0.5 (0.3-0.7)
	BF	0	0.6 (0.2-1.0)	1.0 (0.7-1.3)
	HBF	0	0.1 (0-0.2)	0.1 (0.1-0.2)
<i>An. demeilloni</i>	FF	0	1.0 (0.5-1.1)	1.2 (1.0-1.4)
	HF	0	0.1 (0-0.2)	0.3 (0.2-0.4)
	BF	0	1.0 (0.5-1.3)	1.5 (1.2-1.9)
	HBF	0	0.03 (0-1.0)	0.1 (0.1-0.2)

Note: FF = fresh fed; HF = human fed; BF = bovine fed; HBF= human and bovine fed; Pv = *Plasmodium vivax*; Pf = *Plasmodium falciparum*

#### 6.4. Housing condition and exposure to bite of *Anopheles arabiensis* (Table 4 and Paper III)

A total of 16,894 mosquitoes were collected, of which 71.7% (12,106/16,894) were culicines and 28.3% (4,788/16,894) were *Anopheles*. Among the 4,788 *Anopheles* mosquitoes, 96% (4,597) was collected from inside residential houses. *An. arabiensis* was the most common in the area (2,489; 52%) followed by *An. demeilloni* (1,261; 26.3%), *An. chrysti* (432; 9.0%), *An. pharoensis* (408; 8.5%), *An. cinereus* (166; 3.5%) and *An. coustani* (16; 0.3%).

Among the 2,489 *An. arabiensis*, 41.3% (1,027) were caught by the CDC light trap, 58% (1,443) by PSC and the remaining 0.8% (19) by artificial pit shelter (APS). Furthermore, from the total of 408 *An. pharoensis* catches, most (93%; 381) were collected using CDC light trap and the remaining (6.6%; 27) with PSC.

In the univariate analysis, the number of indoor-biting *An. arabiensis* (mean = 1.1; 95% CI = 0.70-1.42) in houses with two or more goats tethered the previous night was significantly ( $p = 0.035$ ) higher than the number (mean = 0.6; 95% CI = 0.38-0.82) in houses with less than- or equal to one goat. Houses with no window had significantly more mosquitoes (mean = 1.0; 95% CI = 0.78-1.27) than those with at least one window (mean = 0.3; 95% CI = -0.04-0.60). Houses with a hole in their roof had a significantly higher number of mosquitoes than houses with no such hole ( $p = 0.023$ ). The number of indoor-biting *An. arabiensis* also varied significantly with respect to altitudinal location, and was highest in houses located in the low- altitude village (mean = 1.8; 95% CI = 1.53-2.12).

Similarly, the mean number of *An. arabiensis* (mean = 3.0; 95% CI = 1.84-4.27) resting inside houses with greater than- or equal to five occupants who slept the previous night was significantly ( $p = 0.042$ ) higher than the number (mean = 1.5; 95% CI = 0.58-2.38) in houses with less than- or equal to four occupants. The mean number of *An. arabiensis* in houses with less than- or equal to two cattle tethered the previous night was also significantly higher than the corresponding mean number in houses with greater than- or equal to three cattle tethered ( $p = 0.004$ ). The density of *An. arabiensis* in houses with a hole in the roof (mean = 4.8; 95% CI = 3.31-6.31), with a hole in the wall (mean = 3.3; 95% CI = 2.22-4.32) and with an open eave (mean = 5.7; 95% CI = 4.22-7.12) was significantly higher than the density inside those with no hole in the roof (mean = 1.1; 95% CI = 0.18-2.05), no hole in the wall (mean = 0.7; 95% CI = -0.48-1.94) and no open eave (mean = 0.7; 95% CI = -0.16-1.69), respectively. The average number of indoor-resting *An. arabiensis* either at the low-altitude village (mean = 5.3; 95% CI = 4.14-6.57) or at the mid-altitude village (mean = 0.8; 95% CI = -0.42-2.08) was significantly higher than the number at the high-altitude village (mean = 0.02; 95% CI = -1.17-1.21). The number (mean = 3.2;

95% CI = 2.08-4.37) of indoor-resting *An. arabiensis* during the dry seasons was significantly ( $p = 0.023$ ) higher than the number (mean = 1.2; 95% CI = -0.06-2.51) during the wet seasons.

In the multivariate analysis, the number of *An. arabiensis* that bite inside houses located at the low-altitude village was 4.5 (95% CI = 3.47-5.48;  $p < 0.001$ ) times higher than the number at the high-altitude village. Similarly, the number at the mid-altitude village was 2.8 (95% CI = 1.97-3.72;  $p < 0.001$ ) times higher than the number at the high altitude. Houses with a window had a 57% lower number of indoor-biting *An. arabiensis* ( $\beta = -0.6$ ; 95% CI = -1.05-0.094;  $p = 0.02$ ) compared to those with no windows. Similarly, the location of the house at the low- or mid-altitude village and the presence of open eaves were strong predictors of indoor-resting *An. arabiensis*.

The impact of the housing condition on the average number of indoor-occurring (indoor-resting and indoor-biting) *P. vivax* sporozoite-infected *An. arabiensis* is shown in Table 4. Among the CDC light trap catches, the mean number of *P. vivax* sporozoite-positive *An. arabiensis* inside houses having one or no chickens the previous night was significantly higher than the number inside the houses with two or more chickens ( $p = 0.015$ ). Although the difference was marginally significant ( $p = 0.043$ ), the mean number of *P. vivax* sporozoite-positive *An. arabiensis* collected from inside the houses with an open eave was higher than the corresponding number caught from houses having no open eave. From the mosquitoes collected by PSC, no significant difference was observed in the mean number of *P. vivax* sporozoite-positive *An. arabiensis* between- or among housing conditions compared.

Table 4: Average number of *P. vivax*-positive *An. arabiensis* caught per housing condition in Hobe, Dirama and Wurib villages of south-central Ethiopia, July 2008 - June 2010

Housing condition	Indoor-biting <i>An. arabiensis</i>		Indoor-resting <i>An. arabiensis</i>	
	Mean (95% CI)	P value	Mean (95% CI)	P value
<b>Occupants</b>				
≤ 4	0.04 (0 – 0.1)	0.647	0.05 (0 – 0.1)	0.053
≥ 5	0.06 (0 – 0.1)		0	
<b>No. cattle</b>				
≤ 2	0.05 (0 – 0.1)	0.917	0.05 (0 – 0.1)	0.268
≥ 3	0.05 (0 – 0.1)		0.01 (0 – 0.04)	
<b>Sheep</b>				
≤ 1	0.03 (0 – 0.1)	0.070	0.03 (0 – 0.1)	0.271
≥ 2	0.09 (0 – 0.2)		0	
<b>Goat</b>				
≤ 1	0.05 (0 – 0.1)	0.772	0.02 (0 – 0.05)	0.990
≥ 2	0.05 (0 – 0.1)		0.02 (0 – 0.1)	
<b>Horse</b>				
0	0.06 (0 – 0.1)	0.197	0.02 (0 – 0.05)	0.758
≥ 1	0		0	
<b>Donkey</b>				
0	0.05 (0 – 0.1)	0.469	0.02 (0 – 0.05)	0.720
≥ 1	0.08 (0 – 0.2)		0	
<b>Chicken</b>				
≤ 1	0.01 (0 – 0.03)	0.015	0.01 (0 – 0.04)	0.562
≥ 2	0.09 (0 – 0.1)		0.03 (0 – 0.1)	
<b>Window</b>				
Absent	0.06 (0 – 0.1)	0.299	0.01 (0 – 0.04)	0.335
Present	0.02 (0 – 0.1)		0.04 (0 – 0.1)	
<b>Hole in the roof</b>				
Absent	0.04 (0 – 0.1)	0.455	0.01 (0 – 0.05)	0.575
Present	0.07 (0 – 0.1)		0.03 (0 – 0.1)	
<b>Holes in the wall</b>				
Absent	0.06 (0 – 0.1)	0.726	0.02 (0 – 0.1)	0.938
Present	0.05 (0 – 0.1)		0.02 (0 – 0.05)	
<b>Open eaves</b>				
Absent	0.03 (0 – 0.1)	0.043	0.01 (0 – 0.04)	0.538
Present	0.10 (0 – 0.2)		0.03 (0 – 0.1)	
<b>Altitude</b>				
High	0	0.512	0	0.864
Mid	0.02 (0 – 0.1)		0.02 (0 – 0.1)	
Low	0.06 (0 – 0.1)		0.03 (0 – 0.1)	
<b>Season</b>				
Wet	0.07 (0 – 0.1)	0.216	0.02 (0 – 0.1)	0.902
Dry	0.03 (0 – 0.1)		0.03 (0 – 0.1)	

## 7. Discussion

### 7.1. Methodological discussion

#### 7.1.1. Study design

A two-year repeated cross-sectional study design [Paper I, Paper II, Paper III] was undertaken in three ecological villages of the Butajira Demographic Surveillance Area in the former Meskan and Mareko District [192]. Monthly visits were made to low- (Hobe), mid- (Dirama) and high- (Wurib) altitude villages that were randomly selected.

The main strengths of the design are that by collecting mosquitoes over a two-year period, it would provide information about both seasonal and spatial variations in mosquitoes. This includes information on the occurrence and density of the *Anopheles* mosquito populations, sporozoite rates and entomological inoculation rates. The aggregation of the samples over the study period enabled the capture of rare *Anopheles* species such as *An. coustani* and *An. culicifacies*. This design captured *Plasmodium* sporozoite-infected *An. arabiensis* and *An. Pharoensis*, which are highly rare in highland areas. It also enabled us to document months with a high and low mosquito population density. As a repeated cross-sectional survey undertaken once a month for 24 consecutive months, it enabled us to generate the key findings.

In this repeated cross-sectional study design, the same houses were not followed over the study period, and the sequence of the intended outcome and exposure variables were not documented. The measurement of the variables was made at the same time; as a result, it was possible to draw associations between exposure and outcome variables but not causal relations. Strong and weak statistical associations were observed between the variables. For example, there was a strong positive association between open eaves and the density of indoor-resting *An. arabiensis*. The association seemed plausible, and biologically the presence of an open eave could allow easy entry for mosquitoes. Hence, it could be one component of several causes for the indoor occurrence of the mosquito, although the effect of the open eave regarding the indoor density of the mosquito could be neither strong nor weak. The strength of the

association might not be biologically stable, and could change from village to village and over time with changes in the distribution of the exposure variables. On top of these, it was not possible to demonstrate evidence that the presence of an open eave was predisposed towards an increased density of the mosquito inside houses or vice versa. Consequently, the association generated could be partly true from a biological perspective.

### **7.1.2. Sample size**

The sample size depends on the objective, nature and expected outcome of the research, but in entomological studies it has not been customary to estimate the sample size. Therefore, we based our sample size estimation on similar studies done in East Africa [198] and selected 90 houses and 15 artificial pit shelters that were equally allocated among the three ecological zones. These sample sizes were considered adequate for the study considering the required regular house-to-house visit, installing CDC traps, recording household conditions, collecting trapped mosquitoes, undertaking pyrethrum spray-based mosquito collections, collecting mosquitoes from pit shelters, surveying the aquatic stages of mosquitoes and processing the collections.

The 2,544 visits made to collect adult mosquitoes, among which 2,160 were made to houses, 360 to artificial pit shelters and the remaining 24 to the available potential breeding habitats along the streams over the total 24 study months, contributed to reasonable mosquito catches in this particular highland area.

Our study that looked into the effect of household members in relation to the indoor-biting *An. Arabiensis*, which had a total size of 1,025. This sample was grouped into household members of  $\leq 4$  (mean = 0.57, n = 453, variance = 9.89) and household members of  $\geq 5$  (mean = 0.91, n = 572, variance = 13.45). The power of the study to detect the observed mean difference of 0.34 was 35.8%, which was low. This indicates that the sample size was low. This also suggests that the intended outcome could likely be overlooked or missed to detect the significance difference that would have been evident if the statistical power had been greater. The study that looked into

the effect of household size on indoor-resting *An. arabiensis* had a population of 1,417. This size was further grouped into households having  $\leq 4$  members (mean = 1.48, n = 666, variance = 48.1) and households having  $\geq 5$  members (mean = 3.05, n = 751, variance = 179.5). The power of the study to detect the mean difference of 1.57 was 80.4%, which was adequate.

### **7.1.3. Internal validity**

#### **7.1.3.1. Selection bias**

Selection bias is a systematic error in a study that stems from the procedures used to select subjects, as well as from factors that influence the study participation [199]. In the study, selection bias could arise in the process of selecting larval and adult sampling habitats.

Mosquito catches were undertaken along permanent streams in each study village based on previous studies that malaria vectors in Africa often occur and maintain a high disease transmission closer to breeding habitats [198, 200-202]. Although this could have increased the catch size of mosquitoes, it might also have led to a non-representative selection of *Anopheles* mosquito populations that occurred in the villages, which is probably the cause of the difference in results found by Woyessa and colleagues [191]. They found malaria transmission to occur after the main rains, while our finding is that larvae and adult mosquito densities were highest during the dry seasons [Papers I, II]. We believe this selection bias occurred at all our study sites. However, from an entomological perspective, surveying the potential breeding sites and residential houses along the streams increased the size of the mosquito catches and the entomological aspects of malaria transmission risk in the area. In addition, considering three villages at different altitudes, adult mosquito collections from indoors (using CDC light trap and PSC) and outdoors (using APS), and as observed in other studies, considering monthly mosquito catches for two consecutive years enhanced the representativeness of the mosquito catches [200, 201]. However, undertaking weekly or fortnightly data collection could have helped improve this [201, 203][Papers I, II].



The number of the artificial pit shelters (n = 15) used for outdoor adult mosquito collection was not comparable to the number of houses (n = 90) used for indoor collection, which could potentially underestimate the outdoor-resting mosquito population in the area. Pit shelters may not be the ideal method to collect mosquitoes to estimate transmission outside houses. Because the main livelihood in the area is subsistence farming on small plots of land, it was not feasible to construct an equivalent number of artificial pit shelters to that of the houses.

### **7.1.3.2. Information bias**

Information bias can arise when the information collected about- or from study subjects is erroneous [199]. In this study, information bias could have arisen while collecting, documenting and reporting on the *Anopheles* species. More specifically, bias could arise in the process of identifying the species, their blood meal sources, sporozoite infection status and age of *Anopheles*. Bias could also result in the process of larval sampling [Papers I, II and III]. In order to minimize these possible sources of bias, adequate training was given to the various field staff by the principal investigator, and standard procedures were employed as documented in the methods section. Nevertheless, mosquitoes that may have developed resistance to the spray chemical could have been missed from spray catches. Unfortunately, the insecticide susceptibility status of the *Anopheles* was not determined during the investigation due to the low number of larvae during most of the months.

The identification of *Anopheles* mosquitoes into species could be less precise, especially at the beginning of the study, which could have led to some bias. However, this potential bias was lessened through experience and re-identifying the species that were identified at the beginning of the study. The CSP detection by ELISA [Paper II] may have overestimated the true *Plasmodium* sporozoite rate as a result of the spread of the CSP throughout the mosquito after being shed [109, 116]. The method could also detect the CSP from the oocysts bursting, two to three days before the sporozoites actually reach the salivary glands [117]. In turn, these could overestimate the EIRs in the area. There could also be an inter-observer difference in identifying the different

larval stages of anopheline mosquitoes. In this regard, 10% of the identified *Anopheles* larvae were re-identified by another more experienced researcher, which resulted in a very good agreement ( $\kappa = 0.89$ ,  $p < 0.01$ ) between the identified species [Paper I].

### **7.1.3.3. Confounding**

Confounding is the confusion or mixing of effects, in which case the effect of the exposure is mixed together with the effect of another variable, thereby leading to a bias. It is a systematic error that investigators aim to either prevent or remove from a study [199]. Several factors could affect the density of the larval and adult stages of *Anopheles* mosquitoes. For example, the temperature and depth of a breeding habitat affect the density of the larvae of *An. arabiensis*. An increase in habitat temperature is strongly correlated with an increased density of *An. arabiensis* larvae, while a decrease in habitat depth was strongly associated with an increase in the density of the larvae of the mosquito [Paper I]. These variables were therefore among the potential confounding factors. Several other factors could also affect the density of indoor-occurring adult *Anopheles* mosquitoes, including *An. arabiensis*. Hence, the confounding role of some of the exposure variables on the indoor-biting and indoor-resting densities of *An. arabiensis* was controlled by stratifying (grouping) the data and employing a multiple regression analysis. The variables that were thought to be predictors of the outcome were listed in the design stage, and the study area was stratified into low-, mid- and high-altitude villages. Besides, since the density of mosquitoes varies with habitat type and collection method, the catches were stratified into indoor and outdoor, and also CDC light trap and PSC [Paper III].

### **7.1.3.4. Chance**

In estimating the mean number of each species of *Anopheles* larvae [Table 2], a 95% CI was used for the mean numbers of adult female *Anopheles* species with respect to their collection method, abdominal status, blood meal source, *Plasmodium* sporozoite infection status and entomological inoculation rates [Table 3]. The 95% CI was used to indicate that if the data collection and analysis could be repeated many times, the

correct value of the estimate would lie within the set CI 95% of the time in terms of the variability (random error) of the estimates. Thus, the difference from the specified CI would be attributed to a statistical or chance factor in the data. To assess the probability that the data obtained in the study would demonstrate an association with the outcome variable of interest, a  $p$  value cut-off point of 0.05 and a 95% CI were used [Paper I].

#### **7.1.4. External validity**

External validity is the extent to which the results of a particular study in a given population apply to other populations. This study was undertaken for 24 consecutive months, since it is evident that the density of mosquitoes is not similar across different seasons and years due to changes in weather and environmental conditions. The study was conducted along the streams of the Hobe, Dirama and Wurib villages in order to have reasonable mosquito catches across different climatic and altitudinal settings. Previous studies showed that *Anopheles* density is high along breeding sites such as streams and houses near to streams. The advantage of undertaking the study along the streams was that it was possible to have immature mosquito catches during both the dry months (on the beds and shallow surfaces of the streams) and the wet seasons from temporary breeding sites created in marshy areas created adjacent to streams, and also with adult mosquitoes from nearby areas. For this reason, the results of the entomological study can be generalized to the Butajira area, particularly to areas that lay along the streams and at the altitudinal range of 1,800 to 2,300 m.a.s.l. These can also be further generalized to similar altitude areas in Ethiopia and elsewhere with similar ecologic and socioeconomic contexts.

#### **7.2. Discussion of main findings**

With the aims of determining the occurrence and dynamics of *Anopheles* larvae, describe the feeding preferences of adult *Anopheles* species, estimate the entomological inoculation rates of *Anopheles* species and assess the impact of housing conditions on the indoor-biting and indoor-resting density of the major malaria transmitting *Anopheles* species, we found *An. arabiensis*, *An. pharoensis*, *An.*

*cinereus*, *An. chrysti* and *An. demeilloni* to be the most frequently occurring mosquitoes. Their larvae were collected from streams, and most adults from houses close to streams. *Anopheles cinereus* larvae were observed most frequently, followed by *An. arabiensis* and *An. chrysti*. Larvae of the *Anopheles* species on the edges/beds of streams occurred more often during the dry- than the wet seasons. The density of *An. arabiensis* larvae increased with a rise in habitat temperature, and also with a decrease in habitat depth. The density of *An. arabiensis* in sandy habitats was higher than in the muddy habitats, whereas the densities of the larval and adult stages of *An. arabiensis* and the adults of *An. pharoensis* decreased with an increase in altitude, but those of others increased with an increase in altitude. The adults of the five *Anopheles* species fed on human blood, but *An. arabiensis* and *An. pharoensis* were observed to have human *Plasmodium* parasites in addition to human blood, thus indicating their role as malaria vectors in the area. Houses having open eaves, no windows and located either in the low- or mid-altitude villages had a high density of *An. Arabiensis*, which put households at a greater risk of infectious malaria mosquito bites.

*Anopheles arabiensis* and other *Anopheles* species breed along the pools, beds and shallow surfaces of natural streams in the low- (Hobe), mid- (Dirama) and high- (Wurib) altitude villages of the Butajira area [Paper I]. This finding is in agreement with previous studies in the central Rift Valley of Ethiopia [204], northern Ethiopia [205], Eritrea [36] and Kenya [37, 203, 206]. It shows that the permanent streams of the villages serve in maintaining the local *Anopheles* mosquito populations, including the main malaria vectors. The density of *Anopheles* larvae along the streams was generally low during the rainy seasons, which could result from the increased volume and speed of streams, following rains that carry away the eggs, larvae and pupae of mosquitoes. It could also result from the direct lethal effect of heavy rain showers on the larvae [32]. *Anopheles arabiensis*, the predominant and widespread malaria vector in Ethiopia [12], is adapted to dry environments [41, 42, 207] and breeds abundantly along pools and the edges of streams, with an increased density during the dry seasons [33, 36].

*Anopheles arabiensis* was the most common malaria vector in the low-altitude village, followed by the mid- and high-altitude villages [Papers I and II]. *Anopheles pharoensis* was also the second most common vector in the low-altitude village, though very low or scarce in the mid- and high-altitude villages [Paper II]. *Anopheles arabiensis* was observed to feed on human and cattle with a similar preference, which is also in line with the reports from southern Ethiopia [208, 209]. This puts the inhabitants of south-central Ethiopia at a greater risk of malaria infection since both households and their cattle (including all other domestic animals) stay inside the same living quarters at night.

Although a reasonably high density of adult *An. pharoensis* (including two *An. pharoensis* mosquitoes that were infected with *P. vivax* sporozoites) was collected towards the end of the main rainy season, only one larval stage of the mosquito was identified from the stream located in the mid-altitude village. This indicates that the available natural streams may not support the breeding of *An. pharoensis* during the dry seasons. As a result, the majority of adult *An. pharoensis* collected in the study sites might be those which came from adjacent or nearby villages having potential breeding habitats. It is also possible that there could be undetected *An. pharoensis* breeding habitats in the villages, as we were not able to undertake fortnightly or weekly larval surveys in the study sites, and also because all the villages were not considered for the larval survey.

Based on the CDC light trap collection, the annual *P. falciparum* infective *An. arabiensis* bites per person in the low-altitude village for the year from July 2008 to June 2009 was greater than in the year from July 2009 to June 2010 (Paper II). The annual *P. vivax* EIR for *An. arabiensis* from July 2008 to June 2009 was also higher than from July 2009 to June 2010. This finding, which is the first from an EIR study in the area, and based on two years of a repeated cross-sectional study design, revealed that *An. arabiensis* is a major vector of *P. falciparum* and *P. vivax* malaria in the Hobe and Dirama villages of Butajira area, and that the entomological risk for malaria transmission varied from year to year. This also indirectly strengthens the

reports on human malaria prevalence by some studies undertaken in the area [21, 28, 29, 191].

*Plasmodium vivax* and *P. falciparum* sporozoite-infected *An. arabiensis* and *An. pharoensis* mosquitoes were collected from houses that were located closer to streams in the low- and mid-altitude villages of the Butajira area. As reported in previous studies, the density of indoor-occurring *An. arabiensis* decreased significantly with an increase in distance from a nearby permanent breeding habitat [65, 66]. Individuals living near the streams could therefore be bitten more frequently by infectious vectors, and are more likely to be infected by malaria. These households could also serve as carriers of *P. vivax* and *P. falciparum* gametocytes, thereby maintaining and amplifying the transmission of the disease in the villages [210]. From these areas, the disease might be carried over a long distance by gametocyte-carrying travellers, who will carry the parasite to the available vectors at their destination. It can also be carried longer distances by *An. pharoensis* and *An. arabiensis* with the support of external factors, such as wind, cattle and vehicles [67]. As a consequence, the inhabitants of the low- and mid-elevation villages, especially those living closer to the streams, could serve as malaria hotspots. These households therefore need a scaled-up malaria intervention strategy, as they are at risk of infection and serve as potential hotspot sites.

This study revealed that *An. arabiensis* is the primary malaria vector, followed by *An. pharoensis* in south-central Ethiopia [Paper II]. Other *Anopheles* mosquitoes, including *An. cinereus*, *An. demeilloni* and *An. Chrysti*, were also common, particularly in the mid- and high-altitude villages. These mosquitoes fed substantially on human blood, but were negative for *Plasmodium* sporozoites. *Anopheles coustani*, which was less frequent and not tested for its blood meal source and sporozoite infection status, was also observed. Although negative for *Plasmodium* sporozoites in this study, their occurrence could be an entomological risk factor for malaria transmission. This is because most of them have been documented as malaria vectors in other parts of Africa. In Kenya, *An. coustani* was observed to transmit *P. falciparum*, which was the same rate as *An. arabiensis* but higher than *An. funestus*

[211]. *Anopheles cinereus* is reported as a potential malaria vector in Eritrea [212], and many of these mosquitoes harboured human blood, therefore indicating their importance as biting nuisances [Paper II]. The low annual *P. falciparum* EIR (lower than 10) indicates an unstable falciparum malaria transmission intensity in the area, which could result in unexpected epidemics [127, 213].

In the study area, a single house serves for living, tethering domestic animals (cattle, sheep, goats, donkeys, horses and chickens), catering and keeping household belongings [Paper III]. Most of the *Anopheles* mosquitoes were from inside the residential houses [Paper III]. In such a house, night-biting *Anopheles* mosquitoes have a chance to acquire their blood meal from alternative sources with minimal physiological energy expenditure, which increases their age and malaria transmission role. In this particular highland area, the living conditions could provide an appropriate microclimate for the mosquitoes [18, 214], and hence a faster and higher risk of malaria transmission compared to outside the house [214]. Houses with open eaves, built either in the low- or mid-altitude village, and with no window, were associated with higher densities of *An. Arabiensis*, putting their inhabitants at a greater risk of malaria infection.

Although densities of both the aquatic and adult stages of malaria vectors (*An. arabiensis* and *An. pharoensis*) decreased with an increase in altitude, the occurrence of *An. arabiensis* in the high-altitude village (Wurib) indicates that malaria vectors are adapting and occurring in the highlands of Ethiopia, especially in the south-central highlands [Papers I, II and III]. This also suggests an expansion of the vector into the highlands of south-central Ethiopia, which could partly be explained by the effect of global warming [215, 216] and changes in the local ecology as a result of the increased population pressure.

## 8. Conclusions and recommendations

### 8.1. Conclusions

- Larval stages of *An. arabiensis*, *An. cinereus*, *An. christyi* and *An. demeilloni* occurred in the low-altitude village, whereas *Anopheles arabiensis*, *An. cinereus*, *An. christyi*, *An. demeilloni*, *An. garnhami*, *An. azaniae* and *An. pharoensis* occurred in the mid-altitude village. Similarly, *An. arabiensis*, *An. cinereus*, *An. christyi*, *An. demeilloni*, *An. pretoriensis*, *An. azaniae*, *An. rufipes*, *An. sergenti*, and *An. garnhami* were observed in the high-altitude village.
- Adults of *An. arabiensis*, *An. pharoensis*, *An. christyi*, *An. cinereus*, *An. demeilloni* and *An. coustani* were collected from the low- and mid-altitude villages. Similarly, *An. arabiensis*, *An. pharoensis*, *An. christyi*, *An. cinereus*, *An. demeilloni*, *An. coustani*, *An. culicifacies*, *An. garnhami* and *An. rhodesiensis* were found in the high-altitude village.
- Densities of *An. arabiensis*, *An. pharoensis* and *An. coustani* decreased with an increase in altitude, while densities of the other species increased with an increase in altitude.
- Freshly fed *An. arabiensis*, *An. pharoensis*, *An. christyi*, *An. cinereus* and *An. demeilloni* were observed to feed on both human- and cattle blood.
- Some of the adult *An. arabiensis* and *An. pharoensis* from the low-altitude village were found carrying *P. falciparum* and *P. vivax* sporozoites, while only two *An. arabiensis* mosquitoes were *P. vivax* sporozoite-positive in the mid-altitude village. This may suggest that malaria transmission occurs in the area.
- During the dry season, natural streams serve as the major *Anopheles* mosquito breeding habitats in the area.
- Most of the adult *Anopheles* mosquito catches were made inside residential houses, and most of them were *An. arabiensis*.
- Houses having open eaves, located either in the low- or mid-altitude villages, which had no windows or were located close to streams, had a high density of



indoor-occurring *An. arabiensis* (including *Plasmodium* sporozoite-infected ones).

## **8.2. Recommendations**

### **8.2.1. For practice**

- Because the streams serve as the major *Anopheles* mosquito breeding habitats during the dry seasons, larval source management alongside them might want to be considered in vector control strategies.
- An improved house construction may be important in minimizing indoor densities and the bites of *An. arabiensis*, *An. pharoensis* and other nuisance biting mosquitoes.

### **8.2.2. For research**

- A study that involves weekly larval surveys could be important in helping to describe the role of temporary water collections in *Anopheles* mosquito breeding in the villages.
- The vectorial role of *An. cinereus*, *An. demeilloni* and *An. chrysti* should be described as they are prevalent in the villages and could possibly play a role in malaria transmission in the highlands of Ethiopia.
- Most of the *Anopheles* catches were made from inside residential houses, whereas the corresponding outdoor catches were made from fewer artificial pit shelters. Because of this, exophilic mosquitoes could be overlooked. Thus, similar studies should consider the same number of houses and artificial pit shelters.

### **8.2.3. For policy measures**

- Studies on EIRs, as well as the resting- and biting behaviours of vectors might want to be considered in control strategies, since they are directly associated with the risk of malaria transmission.

- Larval source management along the streams, particularly during the dry seasons, might also be worth considering in the existing vector control strategy.
- Lastly, improved and screened houses should be considered in order to prevent indoor-occurring and indoor-biting mosquitoes.

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## RESEARCH

## Open Access

# Abundance and dynamics of anopheline larvae in a highland malarious area of south-central Ethiopia

Abebe Animut<sup>1,2\*</sup>, Teshome Gebre-Michael<sup>2</sup>, Meshesha Balkew<sup>2</sup> and Bernt Lindtjørn<sup>1</sup>

## Abstract

**Background:** Malaria is a public health problem in Ethiopia, and increasingly so in highland areas, possibly because of global warming. This study describes the distribution, breeding habitat and monthly dynamics of anopheline larvae in Butajira, a highland area in south-central Ethiopia.

**Methods:** A study of the abundance and dynamics of *Anopheles* larvae was undertaken at different sites and altitudes in Butajira from July 2008 to June 2010. The sites included Hobe (1817 m.a.s.l.), Dirama (1995m.a.s.l.) and Wurib (2196m.a.s.l.). Potential anopheline larval habitats were surveyed once per month in each village. The recorded characteristics of the habitats included habitat type, pH, surface debris, emergent plants, algae, substrate, turbidity, temperature, length, width, depth, distance to the nearest house and anophelines. The Spearman correlation coefficient and Mann-Whitney *U* test were used to calculate the degree of association between the density of anopheline species and key environmental factors.

**Results:** Among the different types of habitat surveyed, the Odamo, Akamuja and Assas streams and Beko swamp were positive for anopheline larvae. A total of 3,957 third and fourth instar larvae were collected from the three localities, and they represented ten species of anophelines. These were: *Anopheles cinereus* (32.5%), *An. arabiensis* (31.4%), *An. chrysi* (23%), *An. demeilloni* (12.2%), *An. pretoriensis* (0.6%), *An. azaniae* (0.1%), *An. rufipes* (0.1%), *An. sergentii* (0.06%), *An. garnhami* (0.06%) and *An. pharoensis* (0.03%). The density of anopheline larvae was highest during the dry months. *An. arabiensis* was widely distributed, and its density decreased from the lowest elevation in Hobe to the highest in Wurib. The density of *An. arabiensis* larvae was correlated positively with larval habitat temperature ( $r = 0.33$ ,  $p < 0.05$ ) and negatively with depth of larval habitat ( $r = -0.56$ ,  $p < 0.05$ ).

**Conclusion:** Ten species of anophelines were identified, including two known vectors of malaria (*An. arabiensis* and *An. pharoensis*), along streams in Butajira. Larvae of *An. arabiensis* were found in streams at 2200m.a.s.l. This possible expansion of the malaria vector to highland areas indicates an increasing risk of malaria because a large proportion of the Ethiopian population live above this altitude.

## Background

Malaria is the leading cause of mortality and morbidity in Ethiopia in areas up to 2500 metres above sea level (m.a.s.l.) [1], although cases have been reported up to 3000m.a.s.l. in some areas [2]. About 70% of the population is estimated to be at risk of infection every year [3]. Transmission of the disease is unstable in many highland

areas of the country, where the population has low immunity, and these regions experience malaria epidemics [4]. *Anopheles arabiensis*, a member of the *An. gambiae* complex, is the main vector of malaria in the country [5], while *An. pharoensis*, *An. funestus* and *An. nili* represent secondary vectors [6,7].

The transmission of malaria in high altitude areas of Ethiopia [1,2,8] might possibly be due to global warming [9], land use practices [10,11] and ecological changes [12,13] that could favour the breeding and survival of vectors. Warmer weather and increased water temperature

\* Correspondence: animut2004@yahoo.com

<sup>1</sup>Center for International Health, University of Bergen, Bergen, Norway

<sup>2</sup>Akilu Lemma Institute of Pathobiology, Addis Ababa University, Addis Ababa, Ethiopia

enhance malaria transmission in the highlands by shortening the development time from eggs to adult mosquitoes [14], increasing the number of human blood meals taken by adults, increasing the frequency of egg laying and increasing the survival rate of adult mosquitoes [14,15]. Increased warmth also shortens the sporogonic cycle of the parasite in the vector, which results in increased intensity of malaria transmission [16,17]. The continuation of global climate change could therefore allow malaria to expand into the highlands of east Africa [18], threatening the lives of millions of people.

The existing malaria intervention strategy, which includes indoor residual insecticide spraying, nets treated with long-lasting insecticide, and case management, has been reducing the impact of the disease in Ethiopia. Nevertheless, spread of insecticide-resistant vectors [19,20] and drug-resistant malaria parasites [21,22] may result in disease outbreaks. Therefore, control of larvae, which has so far been given little attention, should be reintroduced and implemented together with the existing strategy. Larval control will result in the reduction of the adult mosquito population, subsequently limiting malaria transmission [23]. However, current knowledge of the distribution and dynamics of the aquatic stages of mosquitoes is not adequate. *Anopheles* mosquitoes breed at the edges of rivers and streams, in temporary rain pools, ponds, dams, drainage ditches, burrow pits, rice fields, swamp margins, roadside puddles and in tree holes close to human dwellings [23-25]. However, mosquitoes differ in their preference for the type, size, turbidity, algal cover and stability of the habitat [26-28]; these factors determine the density, size and disease transmission competence of vectors [25,29]. Although malaria has become an important health problem in the south-central highland area of Butajira [30,31], information on the dynamics of the immature stages of the vectors is scarce. The aim of this study was to describe the species distribution and seasonal dynamics of anopheline larvae in the south-central highland area of Butajira. Such information is important in order to implement effective interventions and establish an early warning system for the disease in this country.

## Methods

### Study area

The study was undertaken in the Butajira area in the south-central highlands of the Southern Nations and Nationalities Regional State of Ethiopia, which is located 135 km south of Addis Ababa (Figure 1).

For larval sampling, three study sites were selected. These included Hobe (1817 metres above sea level), Dirama (1995m.a.s.l.) and Wurib (2196 m.a.s.l.). The sites are villages close to the Odamo, Akamuja and Assas streams, respectively. They were selected by Health Extension

Workers on the basis of habitat availability, accessibility and malaria case reports. Rainfall data for the area (July 2008 to March 2010) was obtained from the National Meteorological Agency of Ethiopia from the only station in Butajira town, which is located 5–20 km from the study areas. On the basis of the previous thirty years of meteorological data from the area (National Meteorological Agency of Ethiopia), the average monthly rainfall is 94.6 mm and the relative humidity is 60.8%, while the average maximum and minimum temperatures are 25.5°C and 11.5°C, respectively. Peak rainfall occurs between July and August, while the lowest level occurs in November and December, with little rain between March and May.

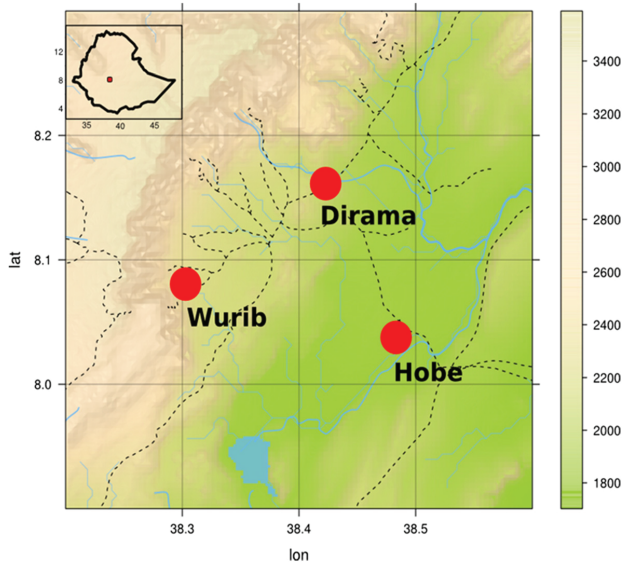
### Larval sampling and processing

Sampling for anopheline larvae was undertaken once a month from July 2008 to June 2010. Streams, water wells, small rain pools, pools in hoof- or foot-prints and false banana (*Ensete ventricular*) leaf axils were surveyed for the presence of larvae, and collections were made by applying a standard sampling procedure [27,32-34]. Three to ten samples were taken with a soup ladle (350 ml capacity) from each breeding habitat, depending on the size of the habitat and the availability of larvae. In streams, dipping was performed at the edges and stream beds for a distance of 600 to 1600 m, depending on presence of larvae. Along the streams the average distance between two consecutive larval sampling points was 100 m.

Larvae were sorted into culicines and anophelines. All anopheline larvae sampled from each sampling point were identified as 1<sup>st</sup>, 2<sup>nd</sup>, 3<sup>rd</sup> or 4<sup>th</sup> larval instars, and the corresponding counts were recorded after transferring the larvae from the sampling dipper to white enamel trays. All culicine larvae and the 1<sup>st</sup> and 2<sup>nd</sup> anopheline larval instars were discarded. All late anopheline instars (3<sup>rd</sup> and 4<sup>th</sup>) were preserved in 70% alcohol after being killed in hot water (ca. 60°C) [35]. In the laboratory, the larvae were mounted in gum-chloral mountant on slides and the species identified on the basis of morphology under a microscope [36]. Furthermore, about 10% of the larval species identified morphologically by the first author (AA) were selected randomly and subjected to reidentification and confirmation by one of the senior and more experienced co-authors (MB). Larvae that were members of the *An. gambiae* complex were inferred from the results of species-specific PCR conducted on the adults collected from the same study sites (manuscript under preparation). After identification of the late instars, the density of the most common species was expressed as the number of larvae per 100 dips.

### Characterization of larval habitat

The types of larval habitats and their characteristics, such as speed, length, width, depth, pH, turbidity, trees



**Figure 1** Location of the Butajira study area in the south-central Ethiopian Highlands.

nearly (shade), distance to the nearest inhabited house, availability of emergent plants and substrate types were described by technicians and the first author (AA). The flow speed of aquatic habitat was described visually as fast flowing, slowly flowing or stagnant (not flowing). Habitat length, width, depth and distance to the nearest house was measured using measuring tape; shade was recorded as present or absent by observing terrestrial vegetation and/or trees and their branches near the breeding habitat. Emergent plants included both aquatic and immersed terrestrial vegetation [27]. 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 four levels: clear, low, medium and high [27]. Substrate type was classified as muddy or sandy. The pH of the water was measured using a portable pH meter, and the water temperature was measured using a minitherm HI 8753 (Romania) digital thermometer.

#### Statistical analysis

The data were entered and analysed using SPSS version 16.0 statistical software (SPSS Inc., Chicago, IL). Monthly dynamics of the density of the major anopheline species and the corresponding monthly rainfall data are presented in line charts. The association of the density of the major species with habitat characteristics such as temperature, depth and pH was analysed using the Spearman correlation coefficient, while associations with substrate type (muddy

or sandy), turbidity (low or medium), surface debris (present or absent), and surface algae (present or absent) were analysed using the Mann Whitney *U* test. The extended Mantel-Haenszel chi-square test for linear trend was used to investigate the trends in major anopheline density at the Hope, Dirama and Wurib sites. The Kappa value was calculated to study the agreement between the researchers in the identification of larval species.

#### Results

Potential anopheline breeding habitats surveyed from July 2008 to June 2010 in Hobe, Dirama and Wurib villages are presented in Table 1. Among the different types of habitat surveyed, three streams (Odamo, Akamuja and Assas) and one swamp (Beko) were found to harbour anopheline larvae. No anopheline larvae were found in water wells, false banana axils, hoof-prints and most temporary rain pools.

During the study period, 9532 immature anopheline larvae were collected, of which 3171 (33.3%) were 1<sup>st</sup> instars, 2414 (25.3%) were 2<sup>nd</sup> instars, 2266 (23.8%) were 3<sup>rd</sup> instars and 1681 (17.6%) were 4<sup>th</sup> instars. Among the total sampled, 2302 were from Odamo stream, 37 from a rain pool in Hobe, 1961 from Assas stream, 2294 from Beko swamp, 2925 from Akamuja Stream and 13 from a foot-print in Dirama village.

Of 3947 late (3<sup>rd</sup> and 4<sup>th</sup>) instar *Anopheles* larvae, 3100 (78.5%) were identified to species level (Table 2). The



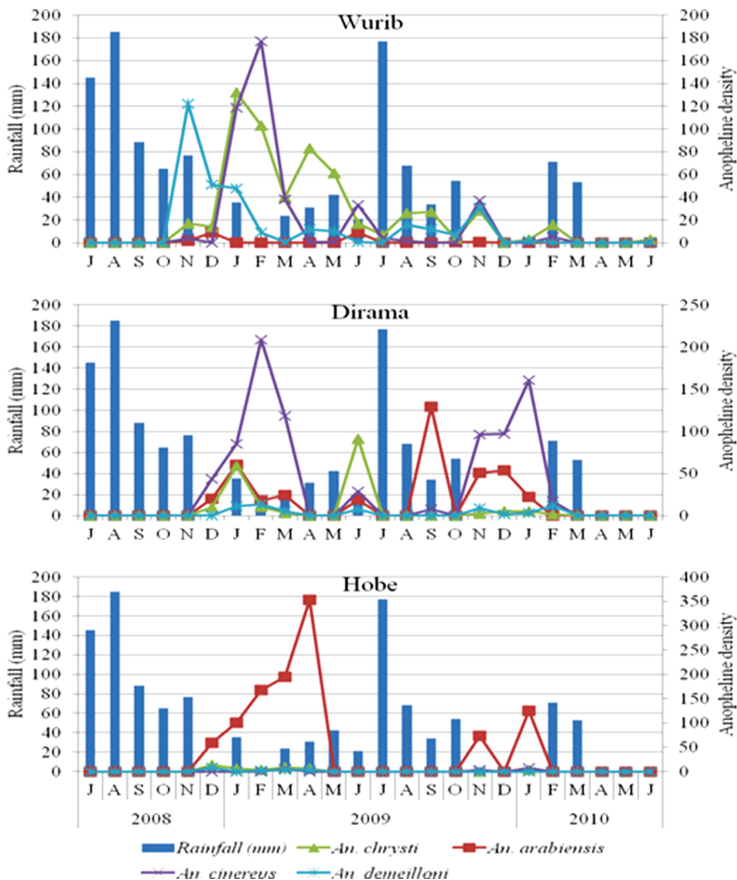


the lowland to the highland areas (chi-square for linear trend = 1794,  $p < 0.01$ ). On the other hand, the density of *An. cinereus*, *An. chrysti* and *An. demeilloni* increased from Hobe to Wurib. The six other species, *An. pretoriensis*, *An. rufipes*, *An. sergentii*, *An. azaniae*, *An. garnhami* and *An. Pharoensis*, were rare; the first five were sampled from the high altitude village while the last species was obtained from the intermediate altitude.

Figure 2 shows the seasonal density of the four common *Anopheles* species, expressed as the number of larvae per 100 ladle dips, and the corresponding monthly rainfall of the area. *An. arabiensis* larvae were predominant in Hobe, with high density from December 2008 to April 2009. This was the dry season, when the monthly rainfall was below 40 mm. The density of *Anopheles* larvae was generally

lowest during July and August, corresponding to the highest amount of monthly rainfall. The density of *An. demeilloni*, *An. chrysti* and *An. cinereus* larvae showed similar trends. Among the three villages, Wurib had diverse species of anopheline larvae.

Beko swamp showed the presence of anopheline larvae most frequently (during 16 surveys), followed by Akamuja stream (11 surveys), among the 24 larval surveys (Table 3). The highest average water temperature was recorded along Odamo stream (26°C) and the lowest along Assas stream and in Beko Swamp (23°C). On average, a 1600 m stretch of the Akamuja stream was surveyed once each month for the presence of anophelines, and the shortest habitat distance surveyed, 600 m, was along the Beko swamp. Beko was the deepest permanent breeding habitat



**Figure 2** Monthly rainfall and *Anopheles* larva density in Hobe, Dirama and Wurib villages of Butajira area, south-central Ethiopia, July 2008- June 2010.

**Table 3 Characteristics of streams during anopheline larvae occurrence, south-central Ethiopia, July 2008-June 2010**

Local name of Stream	Frequency of occurrence	Habitat characteristic (M ± SD)					
		Temperature (°C)	pH	Length (m)	Width (m)	Depth (cm)	Nearest domicile (m)
Odamo	7	26.1 ± 2.5	7.2 ± 0.3	1597 ± 7.6	4.3 ± 0.5	3.4 ± 0.5	350
Akamuja	11	24.5 ± 1.8	7.5 ± 0.7	1600	5.4 ± 0.5	4.4 ± 0.8	200
Assas	6	22.7 ± 4.1	7.1 ± 0.1	1033 ± 81.7	3.2 ± 0.3	5.0 ± 0.6	120 ± 42.2
Beko Swamp	16	23.0 ± 2.3	7.2 ± 0.3	600	4.4 ± 0.5	5.3 ± 1.5	20

M ± SD = mean ± standard deviation.

(5.3 ± 1.5 cm) and had the closest human inhabitants (20 m).

The density of *An. arabiensis* late instars increased significantly with increasing habitat temperature ( $r = 0.33$ ,  $p < 0.01$ ) and also with decreasing depth of habitat ( $r = -0.56$ ,  $p < 0.05$ ) (Table 4). Analysis using the Mann-Whitney *U* test revealed significantly higher larval density in sandy habitats ( $z = -3.648$ ,  $p < 0.01$ ) when compared with habitats with muddy substrate. The density of *An. demeilloni* was negatively associated with habitat temperature ( $r = -0.387$ ,  $p < 0.05$ ). *An. arabiensis*, *An. chrysti*, *An. cinereus* and *An. demeilloni* were not significantly associated with habitat characteristics such as pH, turbidity, surface debris and surface algae in any of the streams. These habitats supported larval development at their shallow edges, where the speed of flow was low, and on their beds in small and stagnant pools. No emergent vegetation was available along the three streams, but was

present in Beko swamp. There was no canopy cover along the anopheline-positive habitats, except for some scattered trees, with no measurable shade on the breeding habitats. All the land close to the breeding habitats was cultivated by farmers.

### Discussion

Ten anopheline species were identified in Butajira. The predominant species was *An. Arabiensis*, which is the main vector of malaria in the country [5,6]. Its density decreased from Hobe at the lowest elevation (about 1800 m.a.s.l.) to Wurib at 2200 m.a.s.l. Two of these species (*An. gambiae* s.l, presumably *An. arabiensis* and *An. chrysti*) have been reported from neighbouring villages at about the same altitude [37]. This shows that malaria transmission in the area [30,31] decreases with increasing altitude. Malaria-related mortality in the area was reported previously to follow a similar altitudinal trend [31].

**Table 4 Association between habitat characteristics and anopheline larval density, south-central Ethiopia, July 2008 to June 2010**

Habitat characteristics	Species			
	<i>An. arabiensis</i>	<i>An. chrysti</i>	<i>An. cinereus</i>	<i>An. demeilloni</i>
<b>Correlation</b>				
pH	-0.2	0.0	0.1	-0.2
Temperature (°C)	0.3*	-0.1	0.2	-0.3*
Depth (cm)	-0.6**	0.2	0.0	0.2
<b>Differences of means (medians)</b>				
Substrate				
Muddy	1.9 (0.0)**	52.5(25.8)*	19.2 (2.3)	15.8 (14.3)
Sandy	61.5 (23.3)	17.7 (4.1)	54.1 (12.5)	16.7 (4.2)
Turbidity				
Low	39.5 (2.5)	31.1 (7.7)	42.2 (7.5)	15.9 (5.0)
Medium	1.1(1.1)	40.9(40.9)	0.0	23.9 (24.0)
Surface debris				
Present	38.0 (2.2)	32.4 (8.7)	42.2 (7.5)	15.0 (5.1)
Absent	30.3 (30.3)	17.7 (17.7)	0.0	42.1 (42.1)
Surface Algae				
Present	38.6 (2.2)	32.2 (8.9)	41.1 (7.4)	16.7 (7.6)
Absent	0.0	8.6 (8.6)	2.9 (2.9)	0.0

\*  $P < 0.05$ ; \*\*  $P < 0.01$ .

We found that *An. arabiensis* breeds at 2196 m.a.s.l., which is above the altitude reported previously from Kenyan highlands [38,39]. This suggests that malaria vectors are breeding in highland areas, and global warming [9] could be one explanation for the expansion of *An. arabiensis* and *An. pharoensis* in the Butajira highlands. The 1958 malaria epidemic that affected most highland areas, including at 2600 m.a.s.l. [8], and a recent report of malaria prevalence of 3.2% at an altitudinal range of 2500 to 3000 m.a.s.l. [2] could be attributed partly to the expansion of the vectors into areas of higher elevation. Expansion of these vectors to highland areas is a serious threat because most of the Ethiopian population lives in the highlands.

The study revealed that the edges and beds of streams serve as anopheline breeding habitats in the Butajira area during months with low precipitation, as reported previously in the central Rift Valley of Ethiopia [40] and Western Kenya [41]. Streambed pools were also productive breeding habitats of *An. arabiensis* during low rainfall seasons in Eritrea [42]. Similar findings have also been documented in other areas of East Africa [32,42]. Streams can produce large vector populations during dry seasons, and hence larval management that targets streambed pools and stream edges may bring substantial reduction in vector density, and subsequently the incidence of malaria [23,43], in the south-central highlands of Ethiopia. The absence of larvae along the streams during the rainy months could result from increased stream flow, which carries away immature stages of mosquitoes from their breeding points. Heavy rainfall could also kill larvae directly [44].

Larvae could not be investigated in temporary water collections formed during rain, in water wells, or in *Ensete* leaf axils. The absence of larvae from most of the temporary collections of surface water could be due to rapid infiltration of the rain water into the soil and high evaporation. Many permanent water wells did not support anopheline larvae, except culicines. This could be due to their depth, which ranges from 15 to 20 m from the surface, and their water volume, which prevents the entry of direct sunlight and could in turn lower habitat temperature and reduce the availability of the food necessary for larval development. Although temporary habitats may dry out or be flushed out before immature anophelines complete their development [28], they are unpredictable in occurrence and may make a small contribution to overall adult productivity [43]. In addition, their contribution to vector breeding should not be ignored [13] because some may support anopheline breeding. Given that we were not able to perform weekly or daily sampling of larvae, we might have missed some potential and temporary breeding habitats between the monthly surveys, and this could have biased our results.

We recommend that future studies should be carried out at frequent intervals to produce more detailed information on the dynamics of anopheline larvae.

The anopheline breeding points were shallow edges and beds of streams that were sunlit, slow flowing or stagnant, with or without debris and surface algae. Similar habitat types were reported from the Ethiopian Rift Valley [40] and Eritrea [25,42]. The larval density of *An. arabiensis* increased with increasing habitat temperature and decreasing habitat depth. The occurrence of *An. arabiensis* larvae in Beko Swamp is an indication of its adaptation to habitats with emergent grass and its expansion to higher elevations, which results in an increased risk of highland malaria. Variability in the pH, turbidity, surface debris and surface algae of the streams did not affect the density of *An. arabiensis*, *An. chrysti*, *An. cinereus* and *An. demeilloni* larvae significantly. *An. arabiensis* is adapted to diverse habitats [25,27]. The density of *An. chrysti*, *An. demeilloni* and *An. cinereus* was not significantly correlated with habitat temperature and depth, which indicates that these anophelines can breed at a greater range of depths and temperatures than *An. arabiensis*. The lower density of the vector (*An. arabiensis*) in the Beko and Assas habitats of Wurib village may have been due to the relatively low temperature in the area, which may affect its breeding negatively. However, this area supported more of other anopheline species for much of the study period, when compared with the other three permanent breeding habitats. This may be because the grass present in this habitat might have prevented the loss of immature forms in running water or by the direct splashing of rainfall, and the grass might have served as a resting site for newly emerging and gravid anopheline mosquitoes [41].

## Conclusion

This study has revealed that *An. arabiensis* breeds on the edges and beds of streams in south-central Ethiopia at elevations up to 2200 m.a.s.l. during the dry months. This observation underlines the importance of streams as breeding habitats of *An. arabiensis* during dry periods. The edges and pools of streams may be important for maintenance of the *Anopheles* population and for small-scale transmission of malaria during dry seasons. Hence, policy makers and organizations involved in malaria control activities need to consider options for the management of larvae that target streams during dry seasons. This strategy may reduce *An. arabiensis* density, and thus reduce the risk of malaria transmission [23,32,42]. However, streams might not be the only breeding habitats for anophelines in the area, and hence weekly surveys of all the available habitats and habitat chemistry need to be performed to design a comprehensive and effective larval control strategy.

### Competing interests

The authors declare that they have no competing interests.

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### Authors' contributions

AA designed the study, collected data in the field, carried out the data analysis and wrote the first draft of the manuscript. TGM participated in the study design, interpretation of the results and editing of the manuscript. MB participated in the conception of the study, in the study design and editing of the manuscript. BL conceived the idea for the study and took part in the study design, data entry and analysis, data interpretation and editing the manuscript. All authors have read and approved the final manuscript.

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## RESEARCH

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# Blood meal sources and entomological inoculation rates of anophelines along a highland altitudinal transect in south-central Ethiopia

Abebe Animut<sup>1,2\*</sup>, Meshesha Balkew<sup>2</sup>, Teshome Gebre-Michael<sup>2</sup> and Bernt Lindtjørn<sup>1</sup>**Abstract**

**Background:** The role of anophelines in transmitting malaria depends on their distribution, preference to feed on humans and also their susceptibility to *Plasmodium* gametocytes, all of which are affected by local environmental conditions. Blood meal source and entomological inoculation rate of anophelines was assessed along a highland altitudinal transect in south-central Ethiopia.

**Methods:** Monthly adult anopheline sampling was undertaken from July 2008 to June 2010 in Hobe (low altitude), Dirama (mid altitude) and Wurib (high altitude) villages located at average elevations of 1800 m, 2000 m and 2200 m, respectively. Anophelines were collected using CDC light trap, pyrethrum space spray catches (PSC) and artificial pit shelter methods. Upon collection, females were categorized according to their abdominal status and identified to species. Their human blood index, sporozoite rate and entomological inoculation rate was determined.

**Results:** A total of 4,558 female anophelines of which *Anopheles arabiensis* was the most prevalent (53.3%) followed by *Anopheles demeilloni* (26.3%), *Anopheles christyi* (8.9%), *Anopheles pharoensis* (7.9%) and *Anopheles cinereus* (3.6%) were caught and tested for blood meal source or sporozoite infection depending on their abdominal status. The proportions of human fed and bovine fed *An. arabiensis* were generally similar. In the low altitude village, there were 0.3% (1/300) and 0.2% (1/416) *Plasmodium falciparum* infected *An. arabiensis* among the CDC trap catches and PSC respectively. The percentage of *Plasmodium vivax* infected *An. arabiensis* were 3% (9/300) and 0.7(3/416) among the CDC and PSCs respectively in the village. In addition, there were 1.4% (1/71) and 50% (1/2) *P. vivax* infected *An. pharoensis* from the CDC light trap and PSCs, respectively. In the mid altitude village, 2.5% (1/40) and 1.7% (1/58) from among the CDC and PSCs of *An. arabiensis* respectively carried *P. vivax* sporozoites. Among the CDC light trap catches; there were 3.7 and 0 *P. falciparum* infective bites per year per household for *An. arabiensis* in the years July 2008 to June 2009 and July 2009 to June 2010 respectively in the low altitude village. The corresponding numbers for *P. vivax* infective bites for *An. arabiensis* were 33 and 14.5 in the same village. Space spray catches revealed 0.32 *P. vivax* infective bites per household for *An. pharoensis* during the first year in the low altitude village.

**Conclusion:** *Anopheles arabiensis* was the most prevalent vector of *P. vivax* and *P. falciparum* malaria in the low and mid altitude villages followed by *An. pharoensis*. Annual entomological inoculation rates showed that vivax malaria transmission was higher than that of the falciparum and both decreased with increase in altitude.

\* Correspondence: animut2004@yahoo.com

<sup>1</sup>Center for International Health, University of Bergen, Bergen, Norway<sup>2</sup>Akilu Lemma Institute of Pathobiology, Addis Ababa University, P. O. Box 1176, Piazza, Ethiopia

## Background

*Plasmodium falciparum* and *Plasmodium vivax* are the most prevalent malaria parasites in Ethiopia [1] of which the first is the most notable cause of sickness and death. Transmission of the disease is unstable and occurs mainly from September to December following the June-September rains while the minor transmission occurs in April to May following the February-March small rains. Areas between 1,500 m and 2,500 m altitude have been affected by epidemics at intervals of 5–8 years while those below 1,500 m are affected by seasonal transmission. Moreover, the increasing magnitude of the global temperature and ecological changes [2–5] might have contributed in the expansion of the disease to areas higher than 2,500 m altitude [6–8].

*Anopheles arabiensis* is the principal malaria vector in Ethiopia [9] while *Anopheles pharoensis*, *Anopheles funestus* and *Anopheles nili* are secondary vectors [1,10,11]. *Anopheles arabiensis* is adapted to diverse ecology, feeding preference, seasonal occurrence and vectorial capacity resulting in diverse spatial and temporal malaria transmission patterns [12–14]. The role of anophelines in transmitting the disease depends on their occurrence and preference to feed on humans [15], which in turn is affected by local socio-economic as well as environmental factors [14,16]. Thus, preventing humans from the bite of vectors can reduce malaria transmission. However, implementation of prevention tools requires knowledge on occurrence, feeding behaviour and entomological inoculation rate of the vector in the local setting [17,18].

Preference of anophelines to feed on humans can be estimated using human blood index (HBI). HBI is the proportion of human fed among a total of fresh fed anophelines. However, as a vector may feed on alternative hosts depending on availability and accessibility, it remains imperative to assess its blood meal source in local settings. Tests such as enzyme-linked immunosorbent assay (ELISA) [19,20], precipitin test [10] and polymerase chain reaction (PCR) [16] can be employed to identify the blood meal source of a vector of which the first is preferable.

Risk of malaria infection can be measured using entomological inoculation rate (EIR) [20,21]. EIR of a vector depends on its human biting frequency and susceptibility to *Plasmodium* gametocytes [15,22]. It is the product of the human biting rate (HBR) and the sporozoite rate (SR) [17]. The human bait catch is considered as the gold standard method to determine HBR or human landing collection (HLC) [23]. However, it is technically difficult to replicate and unethical in areas where malaria parasites are resistant to drugs [24] and where other mosquito-borne diseases are common. Indoor spray collection and exit trap have been used in some cases but are less sensitive as the anophelines could be less

directly associated with feeding on humans [16,24]. The Centers for Disease Control (CDC) light trap hang nearby sleeping people, at night, can also be used to estimate HBR as it catches mosquitoes that attempt to feed on humans [22,25,26]. However, the relationship between a CDC light trap catch and a HLC varies by locality based on the behaviour of the local vectors. SR is the proportion of vectors that carry *Plasmodium* sporozoites in their salivary glands. Anophelines can be diagnosed for sporozoite infection by dissecting their salivary glands [23], by polymerase chain reaction (PCR) [27] or by ELISA [19] of their thorax and head. In the present study, human blood indices and entomological inoculation rates of anophelines was assessed in a highland malarious area of south-central Ethiopia [28,29].

## Methods

### Study area

Adult anopheline sampling was undertaken along a highland transect of south-central Ethiopia consisting of Hobe (low altitude), Dirama (mid altitude) and Wurib (high altitude) villages once per month for 24 months (July 2008 to June 2010). The villages are located at average elevations of 1,800 m, 2,000 m and 2,200 m, respectively. The low altitude village (N=08°01'.912; E=038°29'.179) is adjacent to Odamo stream, the mid (N=08°10'.061; E=038°25'.142) to Akamuja stream and the high (N=08°04'.877; E=038°17'.991) to Assas stream and Beko Swamp. The streams serve as permanent anopheline breeding habitats during dry seasons [30]. The average annual rainfall of the area is 1,135 mm while the annual average minimum and maximum temperature is 11.5°C and 25°C respectively. The average number of occupants per house in the study villages is 4.3 and the inhabitants keep their small number of livestock in their residential houses during the night. Most of the houses were constructed of mud plastered wood and thatched roof. Like the rest of the country, malaria vector control is one of the strategies for the prevention and control of the disease and activities include implementation of LLINs (PermaNet®) in the low and mid altitude villages and a once per year indoor residual spraying in the low altitude village (personal communication with district health officers). During the study period, LLINs ownership was 28.5% (Woyessa, personal communication).

### Collection, identification and processing of anopheline mosquitoes

Anophelines were sampled using CDC light traps (John W. Hock Ltd, Gainesville, FL., USA) and pyrethroid space spray collections (PSCs) from indoors and artificial pit traps from outdoors [23]. In each village, CDC light traps were set running from 6:00 pm to

6:00 am for two consecutive nights in 10 houses (one trap/ house) and the same was repeated in another 10 houses resulting in 40 CDC trap-nights per village per month. A trap was hung next to occupants' foot sleeping under untreated mosquito net about one metre above the ground [23,31] and the trapped female anophelines were collected in the morning by mouth aspirator. PSC was made in the morning (7:00 am to 8:30 am) in ten randomly selected houses in each village once every month. Before spraying, occupants and their domestic animals left the house. In addition, utensils used for food, food, drinking water and clothes were taken out of houses, house apertures carefully covered with clothes, and the available floor was entirely covered by 2–3 white plastic sheets (each having area of 4 m × 5 m). Spraying was made by KILIT™ insecticide aerosol (Miswa Chemicals LTD, Caswell Road, Brackmills, Northampton, NN4 7PW England) according to the manufacturer's instruction and collectors waited outside for about 15 min. The sheet was then carefully taken out of the house and knocked down mosquitoes were collected using forceps. Five pit traps, constructed in shaded areas, were used for outdoor resting mosquito collection in each village. Each pit shelter was 1.5 m deep, 1.2 m long and 1 m wide. In each pit, four small horizontal cavities of 0.3 m deep were dug out from 0.5 m above the bottom on the walls. Anophelines resting in pit shelters were collected by mouth held aspirator using torch as light source.

Female anophelines from all catches were counted, their abdominal status determined [fresh fed (FF), gravid (GR) or unfed (UF)] and identified morphologically to species under stereoscopic dissecting microscope [23,32]. Unfed anophelines were dissected and their parity determined microscopically as either parous or nulliparous based on changes in their ovarian tracheal system [23]. Each mosquito was kept in a labelled 1.5 ml Eppendorf tube containing silica gel desiccant and cotton. Samples were stored at room temperature while in the field and in -20°C refrigerator at the main laboratory in Addis Ababa until used. FF anophelines were used for blood meal source identification while those of GRs and parous females were used for sporozoite rate determination.

#### Identification of *Anopheles gambiae* sibling species by polymerase chain reaction (PCR)

About 12.5% of the *Anopheles gambiae* s.l were selected randomly and identified to their sibling species using species specific polymerase chain reaction (PCR) [33]. A leg was removed from each mosquito and mixed with 12.5 µl PCR master mix (containing 10x dNTPs, MgCl<sub>2</sub> Solution, QD primer, UN Primer, GA primer, ME

primer, AR primer, deionized water and RTag) in 0.2 ml PCR tube, centrifuged for 20s–20min at 16 K r.p.m. and amplified in a PCR apparatus (PTC-100™ Programmable Thermo cycler, MJ Research, Inc., USA) with PCR cycle condition (95°C/5 min × 1 cycle; [95°C/30s, 50°C/30s, 72°C/30s] × 30 cycles; 72°C/5 min × 1 cycle; 4°C hold). 5 µl PCR product loaded with 2 µl loading dye and 4 µl DNA ladder were electrophoresed through a 2% agarose-tris-borate-EDTA containing ethidium bromide gel (with 100 V and 150 mA power source) and visualized under UV light box (Alpha Innotech, MultiImage™, Light Cabinet, Pacific Image Electronics Co. Ltd, Taiwan).

#### Blood meal source identification and human blood index determination

FF anophelines, from all catches, were assayed for human and bovine blood antigens simultaneously by ELISA [19]. Abdomen of each FF mosquito was ground in 50 µl phosphate-buffered saline (PBS) and final volume brought to 200 µl with PBS buffer. 50 µl of the triturate was coated in duplicate wells on two separate U-bottomed 96-well microtitre plates simultaneously: one plate for human blood meal identification and the other for bovine. Plates were incubated overnight at room temperature and washed twice with PBS-Tween 20. 50 µl peroxidase-conjugated anti-human IgG was added in the first plate and the same volume of peroxidase-conjugated anti-bovine IgG in the second plate incubated for one hour at room temperature and washed thrice with PBS-Tween 20. Finally 100 µl ABTS peroxidase substrate was added, incubated at room temperature for 30 min and observed for green colour reaction visually and absorbance read at 405 nm (by MRX Microplate Reader, Dynex Technologies, 14340 Sullyfield Circle, Chantilly, VA. 20151–1683, USA). Positive control (either human or bovine blood meal) and negative controls (abdomen of laboratory-bred UF *An. arabiensis*) were included in each plate. Human blood index (HBI) and bovine blood index (BBI) of each anopheline species was determined by dividing human fed and cattle fed anophelines respectively to the total tested [13].

#### Sporozoite rate (SR) and entomological inoculation rate (EIR) determination

Dried head and thorax of GR or parous mosquito, from all catches, were carefully separated from the abdomen and tested for *P. falciparum* and *P. vivax* circumsporozoite proteins (CSPs) simultaneously [34,35]. Three U-bottomed 96-well micro titre plates were coated separately with 50 µl solution of *P. falciparum*, *P. vivax*-210 and *P. vivax*-247 monoclonal antibodies (MAB) respectively and incubated at room temperature overnight. Contents of plates were drained, washed three times with PBS-Tween 20, filled with

200  $\mu$ L blocking buffer (BB) and incubated for one hour at room temperature. During the incubation period, mosquitoes were grounded individually in 50  $\mu$ L boiled casein containing Igepal CA-630 and the final volume brought to 250  $\mu$ L with BB. BB was removed from plates and 50  $\mu$ L of each mosquito triturate was added to each of the three test wells. CSP positive sample and laboratory-bred *An. arabiensis* were used as positive and negative controls, respectively. Plates were incubated for two hours and washed with PBS-Tween 20 twice. 50  $\mu$ L aliquots of homologous peroxidase-conjugated MAB (0.05  $\mu$ g/50  $\mu$ L BB) were added to each triplicate well in the plates and incubated for one hour. Plates were washed thrice with PBS-Tween 20, 100  $\mu$ L ABTS peroxidase substrate added per well and incubated for 30 and or 60 min. Plates were observed visually for green colour and also their optical density determined at 405 nm in the micro plate reader. Samples with green colour and with optical density values of greater than two times the average optical density of the negative controls were considered sporozoite positive. Positive samples were retested for confirmation. The *P. falciparum* and *P. vivax* SRs of each *Anopheles* species was determined by dividing *P. falciparum* and *P. vivax* positive anophelines respectively to the total tested. SR was determined for CDC light trap catches and also for PSCs separately.

Since no human landing catch (HLC) was performed, the daily EIR was estimated based on CDC light trap and PSC. For CDC based EIR, the factor determined for *An. arabiensis* in Zambia [22], where a CDC represents 1.91 of an HLC indoors was used. Thus,  $1.91 \times$  (no. sporozoite positive ELISAs/ no. mosquitoes tested)  $\times$  (no. mosquitoes collected by CDC/no. CDC catches).

Similarly, the daily EIR based on PSC was calculated according to WHO [36] as (no. FF mosquitoes caught by PSC/no. human occupants who spent the night in the sprayed house)  $\times$  (no. human fed mosquitoes/no. mosquitoes tested for human blood meal)  $\times$  (no. sporozoite positive ELISAs/no. mosquitoes tested).

#### Statistical analysis

Data entry and analysis was made using SPSS version 16.0 soft ware (SPSS Inc., Chicago, IL). The significance of differences between proportions of human fed and bovine fed anophelines was analysed using Chi-square test. The daily EIR was multiplied by the number of days of the corresponding month to get estimated monthly EIR in each village. Then, the monthly EIRs in each village were summed up to obtain the annual EIR [16].

#### Ethical issues

The investigation was ethically approved by the Ethical Committee of the Faculty of Medicine of Addis Ababa University and The National Health Research Ethics

Review Committee (NERC) of Ethiopia with reference number RDHE/48-85/2009.

## Results

### Composition and blood meal source of *Anopheles* species

A total of 4558 adult female *Anopheles* mosquitoes were caught of which *Anopheles gambiae* s.l (= *An. arabiensis*) was the most prevalent (53.3%) followed by *Anopheles demeilloni* (26.3%), *Anopheles christyi* (8.9%), *Anopheles pharoensis* (7.9%) and *Anopheles cinereus* (3.6%) (Table 1). PCR identification of the sample of *An. gambiae* s.l (n=305) showed all to be *An. arabiensis*; hence all other *An. gambiae* s.l samples were regarded to be *An. arabiensis*. *Anopheles arabiensis* was highest in the low altitude village (86.0%) and lowest in the high altitude village (1.2%). Similarly, *An. pharoensis* was highest in the low altitude village (13.4%) and lowest in the high altitude village (0.3%). On the other hand, catches of *An. christyi*, *An. demeilloni* and *An. cinereus* were highest in the high altitude village and very low or scarce in the low altitude village.

In almost all species and villages (Table 1), FF anophelines were predominant indoors (in CDC and PSC collections) despite the use of nets by the occupants, whereas these were very low outdoors (in pit shelter collections). Furthermore, UF females of *An. arabiensis* and *An. pharoensis* were surprisingly the lowest indoors in CDC collections. Likewise, significant number of GR females was collected indoors.

Table 2 reveals the blood meal sources of different anopheline species in south-central Ethiopia. In CDC traps, *An. arabiensis* had human blood index (HBI) ranging from 32% in the low altitude village to 57% in the high altitude village (average HBI=34%). In PSC, the same species had HBI of 25% in the high altitude village to 31.5% in the low altitude village (average HBI=31%). In outdoors, very small number of FF *An. arabiensis* were caught and tested from the low altitude village only, which had 66.7% HBI. Thus, the overall HBI of *An. arabiensis* in the study area was 32.2%. Regarding *An. pharoensis*, the HBI in CDC traps ranged from 19% in the low altitude village to 21.4% in the mid altitude village (average HBI=18.8), whereas its values in the PSC ranged from 25% in the low to 0% in the mid village (average HBI=17.4%); no specimen was analysed from pit shelters. Thus, the overall HBI of *An. pharoensis* was 18.6% in the study area.

Regarding the zoophilic feeding behaviour of the two species, *An. arabiensis* had bovine blood feeds of 14.3% in the high elevation village to 39.1% in the low (average=38%) in CDC catches, while the values ranged from 0% in the high altitude village to 49% in the mid-altitude village (average=40.5%) in PSCs. In outdoor catches, only 33.3% were bovine fed. Thus,

**Table 1 Anopheline species and their abdominal status by village and collection method in south-central Ethiopia, July 2008- June 2010**

Village	Species	Total	CDC			PSC			Pit shelter		
			UF	FF	GR	UF	FF	GR	UF	FF	GR
Hobe (n=2442)	<i>An. arabiensis</i>	2101	127	436	272	49	771	427	10	3	6
	<i>An. pharoensis</i>	328	34	212	64	0	16	2	0	0	0
	<i>An. christyi</i>	7	0	2	5	0	0	0	0	0	0
	<i>An. cinereus</i>	5	0	2	1	0	1	1	0	0	0
	<i>An. demeilloni</i>	1	0	0	1	0	0	0	0	0	0
Dirama (n=481)	<i>An. arabiensis</i>	311	22	65	41	6	118	59	0	0	0
	<i>An. pharoensis</i>	26	0	14	3	1	7	1	0	0	0
	<i>An. christyi</i>	26	5	12	5	0	0	3	0	1	0
	<i>An. cinereus</i>	23	4	12	1	1	3	2	0	0	0
	<i>An. demeilloni</i>	95	22	53	14	1	2	1	0	0	2
Wurib (n=1635)	<i>An. arabiensis</i>	19	3	6	5	0	4	1	0	0	0
	<i>An. pharoensis</i>	5	0	4	1	0	0	0	0	0	0
	<i>An. christyi</i>	373	91	149	54	2	45	20	3	2	7
	<i>An. cinereus</i>	135	14	56	25	3	13	8	2	2	12
	<i>An. demeilloni</i>	1103	128	588	117	9	90	33	22	30	86
<b>Total</b>		<b>4558</b>	<b>450</b>	<b>1611</b>	<b>609</b>	<b>72</b>	<b>1070</b>	<b>558</b>	<b>37</b>	<b>38</b>	<b>113</b>

Note: n= total anophelines collected per village; CDC=Centers for Disease Control light trap; PSCs= pyrethroid spray catches; UF=Unfed; FF=Fresh Fed; GR=Gravid.

the overall zoophilic feeding pattern (index) was about 39.6% in the study area. For *An. arabiensis*, its overall BBI was not statistically different from the HBI.

Similarly, *An. pharoensis* which was absent in the high altitude village had 55.8% and 64.3% of similar bovine

feeding rates in the low and mid villages in CDC catches, respectively (average=51.2%). It also had BBIs of 43.8% in the low and 85.7% in the mid village (average=56.5%). In the absence of bovine feeds outdoors, its overall bovine feeding rate was 55.9% showing to have a

**Table 2 Blood meal sources of indoor and outdoor resting anophelines of three highland villages (Hobe, Dirama and Wurib) of south-central Ethiopia, July 2008- June 2010**

Village and anopheline	CDC					PSC					Pit shelter				
	n	HBI	BBI	Mix	Un	n	HBI	BBI	Mix	Un	n	HBI	BBI	Mix	Un
<b>Hobe</b>															
<i>An. arabiensis</i>	422	32	39.1	13.7	15.2	723	31.5	39.4	12.2	16.9	3	66.7	33.3	0	0
<i>An. pharoensis</i>	206	18.9	55.8	14.6	11.6	16	25	43.8	25	6.2	0	0	0	0	0
<b>Dirama</b>															
<i>An. arabiensis</i>	64	43.7	34.4	12.5	9.4	114	28.1	49.1	7	15.8	0	0	0	0	0
<i>An. pharoensis</i>	14	21.4	64.3	14.3	0	7	0	85.7	14.3	0	0	0	0	0	0
<i>An. christyi</i>	9	11.1	66.7	11.1	11.1	1	0	100	0	0	1	100	0	0	0
<i>An. cinereus</i>	10	20	60	20	0	2	50	50	0	0	0	0	0	0	0
<i>An. demeilloni</i>	41	9.8	70.7	2.4	17.1	1	0	0	0	0	0	0	0	0	0
<b>Wurib</b>															
<i>An. arabiensis</i>	6	57.1	14.3	0	28.6	4	25	0	25	50	0	0	0	0	0
<i>An. christyi</i>	125	26.4	55.2	8	10.4	37	27	48.6	18.8	5.6	2	50	50	0	0
<i>An. cinereus</i>	49	20.4	51	14.3	14.3	12	16.7	66.7	8.3	8.3	1	100	0	0	0
<i>An. demeilloni</i>	471	11.5	69	5.7	13.8	70	5.7	72.9	1.4	20	22	9.1	68.2	0	22.7

Note: n= number tested; HBI=human blood index in%; BBI=bovine blood index in%; Mix= human and bovine mixed blood index (%); Un=unidentified blood meal in%.

more zoophilic behaviour than that of *An. arabiensis*. However, the overall BBI of *An. arabiensis* was not significantly different from that of the BBI of *An. pharoensis*.

Apart from either of the two main blood meal sources (human and bovine), a small proportions of the two species also had mixed feeding patterns ranging from 0 to 14% in CDC traps and from 7 to 25% in PSCs with averages of 12.2% for *An. arabiensis* and 15% for *An. pharoensis*. Furthermore, 15.2% *An. arabiensis* and 11.6% of *An. pharoensis* from Hobe had blood meals of undetermined origin; no such blood meals were detected in outdoor pit shelters since specimens were generally low. Other non-vector anophelines (*An. christyi*, *An. cinereus* and *An. demeilloni*) caught indoors or outdoors in all villages exhibited far more zoophilic behaviour (48.6% to 100%) than anthropophilic behaviours.

#### Sporozoite rates

A total of 1117 indoor caught anophelines, representing five species, were tested for *Plasmodium* circumsporozoite proteins (CSPs) (Table 3). Sporozoites were only detected in 18 mosquitoes belonging to two species (*An. arabiensis* and *An. pharoensis*) collected from the low and mid-

altitude villages. A total of 819 *An. arabiensis* tested from both CDC and PSC had overall *P. vivax* and *P. falciparum* sporozoite rates of 1.7% and 0.2%, respectively. In the low altitude village, the *P. vivax* sporozoite rate in the same species was 3% and 0.7% from CDC and PSC, respectively, where highest number of *An. arabiensis* was caught and analysed. The *P. falciparum* sporozoite rate for the same mosquito in the village was 0.3% and 0.2% in the CDC and PSC, respectively. In the mid altitude village, where small number of *An. arabiensis* were analysed, the *P. vivax* rates were 2.5% (1/40) (CDC) and 1.7% (1/58) (PSC).

Similarly, analysis of only 79 *An. pharoensis* from all the three villages resulted in an overall *P. vivax* rate of 2.5% (2/79) with no *P. falciparum* infection. Most of the *An. pharoensis* caught and analysed was from the low altitude village where *P. vivax* sporozoite rate was 1.4% (1/71) in CDC and 50% (1/2) in PSC. None of the very few mosquitoes tested in the two other villages were positive for either of the two *Plasmodium* sporozoites.

Although sporozoite infections were generally low, they were higher among CDC light trap catches (Table 4) than PSC catches (Table 5). Among the *An. arabiensis* caught by the CDC trap, the daily *P. vivax* sporozoite

**Table 3 Sporozoite infection rates of anophelines in three highland villages of south-central Ethiopia, July 2008- June 2010**

Villages and parameters	<i>An. arabiensis</i>		<i>An. pharoensis</i>		<i>An. demeilloni</i>		<i>An. christyi</i>		<i>An. cinereus</i>		Total
	CDC	PSC	CDC	PSC	CDC	PSC	CDC	PSC	CDC	PSC	
<b>Hobe</b>											
No. tested	300	416	71	2	0	0	1	0	1	0	791
No. PvS+ (%)	9 (3)	3 (0.7)	1 (1.4)	1 (50)	0	0	0	0	0	0	14 (1.8)
No. PFS+ (%)	1 (0.3)	1 (0.2)	0	0	0	0	0	0	0	0	2 (0.3)
<b>Dirama</b>											
No. tested	40	58	4	1	12	0	3	2	1	3	124
No. PvS+ (%)	1 (2.5)	1 (1.7)	0	0	0	0	0	0	0	0	2 (1.6)
No. PFS+ (%)	0	0	0	0	0	0	0	0	0	0	0
<b>Wurib</b>											
No. tested	4	1	1	0	85	22	45	10	28	6	202
No. PvS+ (%)	0	0	0	0	0	0	0	0	0	0	0
No. PFS+ (%)	0	0	0	0	0	0	0	0	0	0	0
<b>Total</b>											
No. tested	344	475	76	3	97	22	49	12	30	9	1117
No. PvS+ (%)	10 (2.9)	4 (0.8)	1 (1.3)	1 (33)	0	0	0	0	0	0	16 (1.4)
No. PFS+ (%)	1 (0.3)	1 (0.2)	0	0	0	0	0	0	0	0	2 (0.3)
<b>Overall</b>											
No. tested	819		79		119		61		39		1117
No. PvS+ (%)	14 (1.7)		2 (2.5)		0		0		0		16 (1.4)
No. PFS+ (%)	2 (0.2)		0		0		0		0		2 (0.3)

PvS+ (%) = number *P. vivax* sporozoite positive (rate in percent) ; PFSR (%) = number *P. falciparum* sporozoite positive (rate in percent).

**Table 4 CDC light trap based assessment of sporozoite and entomological inoculation rates in two highland villages of south-central Ethiopia, July 2008- June 2010**

Study period	Hobe <i>An. arabiensis</i>				Hobe <i>An. pharoensis</i>		Dirama <i>An. arabiensis</i>	
	Daily PvSR	Monthly PvEIR	Daily PFSR	Monthly PFIR	Daily PvSR	Monthly PvEIR	Daily PvSR	Monthly PvEIR
Jul 2008	0	0	0	0	0	0	0	0
Aug 2008	0.17	6.23	0	0	0	0	0	0
Sep 2008	0	0	0	0	0	0	0	0
Oct 2008	0.04	4.00	0	0	0	0	0	0
Nov 2008	0	0	0	0	0	0	0	0
Dec 2008	0	0	0	0	0	0	0	0
Jan 2009	0	0	0	0	0	0	0	0
Feb 2009	0	0	0	0	0	0	0	0
Mar 2009	0.05	3.85	0	0	0	0	0	0
Apr 2009	0.03	11.56	0	0	0	0	0	0
May 2009	0.03	7.31	0.02	3.66	0	0	0	0
Jun 2009	0	0	0	0	0	0	0	0
<b>Year I Total</b>	<b>0.32</b>	<b>32.95*</b>	<b>0.02</b>	<b>3.66*</b>	<b>0</b>	<b>0*</b>	<b>0</b>	<b>0*</b>
Jul 2009	0	0	0	0	1	2.3	0	0
Aug 2009	0	0	0	0	0	0	0.5	2.58
Sep 2009	0	0	0	0	0	0	0	0
Oct 2009	0	0	0	0	0	0	0	0
Nov 2009	0	0	0	0	0	0	0	0
Dec 2009	0	0	0	0	0	0	0	0
Jan 2010	0	0	0	0	0	0	0	0
Feb 2010	0	0	0	0	0	0	0	0
Mar 2010	0	0	0	0	0	0	0	0
Apr 2010	0	0	0	0	0	0	0	0
May 2010	0.2	14.5	0	0	0	0	0	0
Jun 2010	0	0	0	0	0	0	0	0
<b>Year II Total</b>	<b>0.2</b>	<b>14.5*</b>	<b>0</b>	<b>0*</b>	<b>1</b>	<b>2.3*</b>	<b>0.5</b>	<b>2.58*</b>

PvEIR=*Plasmodium vivax* entomological inoculation rate; PFIR=*P. falciparum* entomological inoculation rate; \* = annual EIR.

rate was highest in May 2010 (which was 0.2) and was lower or zero during most of the months in the low altitude village (Table 4) where most of the sporozoites were observed. No distinct seasonal pattern was apparent for *An. pharoensis* since only two mosquitoes were found positive for *P. vivax* during the whole study period. Generally, very low *P. falciparum* sporozoite rate were observed in all catches and study villages.

#### Entomological inoculation rates (EIR)

In the absence of direct human landing catches, EIR for each village was estimated based on the sampling methods employed (CDC and PSCs). However, a small number of mosquitoes were found sporozoite positive on both catches and in all villages. This resulted in low EIR estimates varying from 0 (in most months) to

14.5 (May 2010) monthly *P. vivax* infectious bites of *An. arabiensis* in the low altitude village (Hobe) based on the CDC trap catches (Table 4), while it had only 2.58 in the mid-altitude village (Dirama) in August 2009. Based on CDC based EIR estimates, there was evidence of *P. vivax* transmission in August and October of 2008, in March, April and May of 2009, and in May 2010 coinciding with small rainy seasons of the year in the area.

Although the number of *An. arabiensis* caught by PSC (n=1,247) was much higher than the number caught by CDC traps (n=835), in the low altitude village, the total number of sporozoite infected mosquitoes was very low in the PSC (Table 5). Monthly *P. vivax* EIRs of 0.13 and 0.73 were observed in October 2008 and in June 2009 in the village. In addition, there was *P. falciparum* EIR of

**Table 5 PSC based assessment of sporozoite and entomological inoculation rates in two highland villages of south-central Ethiopia, July 2008- June 2010**

Study period	Hobe <i>An. arabiensis</i>				Hobe <i>An. pharoensis</i>		Dirama <i>An. arabiensis</i>	
	Daily PvSR	Monthly PvEIR	Daily PFSR	Monthly PFEIR	Daily PvSR	Monthly PvEIR	Daily PvSR	Monthly EIR
Jul 2008	0	0	0	0	0	0	0	0
Aug 2008	0	0	0	0	0	0	0	0
Sep 2008	0	0	0	0	0.5	0.32	0	0
Oct 2008	0.08	0.13	0	0	0	0	0	0
Nov 2008	0	0	0	0	0	0	0	0
Dec 2008	0	0	0	0	0	0	0	0
Jan 2009	0	0	0	0	0	0	0	0
Feb 2009	0	0	0	0	0	0	0	0
Mar 2009	0	0	0	0	0	0	0	0
Apr 2009	0	0	0	0	0	0	0	0
May 2009	0	0	0	0	0	0	0	0
Jun 2009	0.03	0.73	0	0	0	0	0	0
<b>year I Total</b>	<b>0.11</b>	<b>0.86*</b>	<b>0</b>	<b>0*</b>	<b>0.5</b>	<b>0.32*</b>	<b>0</b>	<b>0*</b>
Jul 2009	0	0	0	0	0	0	0	0
Aug 2009	0	0	0	0	0	0	0	0
Sep 2009	0	0	0.04	0.93	0	0	0	0
Oct 2009	0	0	0	0	0	0	0	0
Nov 2009	0	0	0	0	0	0	0	0
Dec 2009	0	0	0	0	0	0	0	0
Jan 2010	0	0	0	0	0	0	0	0
Feb 2010	0	0	0	0	0	0	0	0
Mar 2010	0	0	0	0	0	0	0	0
Apr 2010	0	0	0	0	0	0	0	0
May 2010	0	0	0	0	0	0	0.09	0.2
Jun 2010	0	0	0	0	0	0	0	0
<b>Year II Total</b>	<b>0</b>	<b>0*</b>	<b>0.04</b>	<b>0.93*</b>	<b>0</b>	<b>0*</b>	<b>0.09</b>	<b>0.2*</b>

0.93 in September 2009 in the village. While the only *P. vivax* infection in the mid-altitude village in May 2010, resulted in the monthly EIR of 0.2.

Annual EIRs varied between the first and the second - years and also between the low and mid-altitude villages (Table 4). From the CDC light trap collections; there were 3.66 and 0 *P. falciparum* infective bites per year per person for *An. arabiensis* in the years July 2008 to June 2009 and July 2009 to June 2010 respectively in the low altitude village. The corresponding values for *P. vivax* infective bites by *An. arabiensis* were 33 and 14.5 in the village. In addition, there were 0 and 2.3 *P. vivax* infective bites for *An. pharoensis* in the village during the first year and the second year, respectively. The space spray catch revealed 0.32 *P. vivax* infective bites per person for *An. pharoensis* during the first year with zero value in the second year.

## Discussion

*Anopheles arabiensis* was the predominant malaria vector followed by *An. pharoensis* along the altitudinal transect consisting of Hobe (low altitude; 1,800 m), Dirama (mid altitude; 2,000 m) and Wurib (high altitude; 2,200 m) villages in south-central Ethiopia. Although sampling was not made for anophelines that could escape through eaves and windows, the highest number of *An. arabiensis* was caught by pyrethroid spray revealing its indoor resting behaviour [37,38]. The majority of the anophelines were collected from inside houses which could be associated with the indoor occurrence of blood meal sources, higher indoor temperature and with limited outdoor-resting places [38,39]. Most *An. arabiensis* and other anopheline species caught indoors (*An. pharoensis*, *An. christyi*, *An. demeilloni*, and *An. cinereus*) were fresh fed and



gravid indicating their indoor or outdoor feeding with indoor resting behaviour. The higher number of fresh fed and gravid mosquitoes in the CDC light trap catches might be due to their attraction to CDC light traps and their possible repeated feeding behaviour [13,22,27]. The human fed catches by the CDC light traps, despite the presence of nets, might be due to the early biting behaviour of *An. arabiensis* [40] before bed time and blood feeding on exposed occupants who sleep traditionally on floor mats in which case nets do not provide adequate protection.

The HBI of *An. arabiensis* was similar to that of its BBI indicating its opportunistic feeding behaviour in the area. Similar feeding preferences are reported from southern Ethiopia where people and livestock either share the same houses or where cattle are kept separate but close to houses during the night [41]. Our result can also be strengthened by the study from Fuchucha village in the Konso District of Ethiopia where cattle- and human-fed *An. arabiensis* mosquitoes were found to have similar rates of *Plasmodium* infection [42]. However, the HBI observed in this study is very low compared to the value from human dwellings alone (91.5%) and higher compared to that from human and bovine mixed dwellings (20.2%) reported in the country [43]. The variations in the HBI of the vector could result from differences in the relative distance and accessibility of hosts.

The HBI of *An. pharoensis* observed in this study (18.6%) is lower than that of *An. arabiensis*, but is higher compared to that of the Kenya (8.2%) [13]. In addition, it had the highest mixed human and bovine blood index among the five anopheline species. An experimental study in southern Ethiopia [44] documented similar number of *An. pharoensis* catches both in human- and cattle-baited traps. Thus, it can be suggested that *An. pharoensis* of south-central Ethiopia may have a moderately opportunistic feeding behaviour probably due to its similar responsiveness to cattle and human host cues [44]. This tendency of the mosquito to feed on humans increases its vectorial capacity. *Anopheles christyi*, *An. cinereus* and *An. demeilloni* also had considerably high human blood indices depicting their importance as biting nuisances. *Anopheles cinereus* has previously been reported as a potential vector of malaria in Eritrea [45]. Significant number of blood meals of *An. arabiensis*, *An. pharoensis* and other anophelines could not be identified by ELISA, which most could have been identified by PCR [33]. Limitations of primers and reagents hindered the use of such a technique in the study. The quality of some of the blood samples might have also been degraded during storage before analysis. However, the unidentified blood meal sources could be of sheep, goat, donkey, horse, chicken and dogs which are available in the area.

*Anopheles arabiensis* was the most abundant, most anthropophilic and the most sporozoite laden species proving its role as the primary malaria vector in the area [1]. Very few *An. pharoensis* (n=2) were found carrying *P. vivax* sporozoites which might be attributed to its occurrence mainly following the main rainy season. *P. vivax* sporozoite carriage was higher than that of *P. falciparum* which is also similar to previous reports from southern Ethiopia [41,42,46]. This describes dominance of vivax malaria transmission over falciparum in the region. It is, therefore, imperative to undertake epidemiological studies on *P. vivax* in view of the current reports that revealed severe clinical manifestation resulting from the infection [47,48].

Annual *Plasmodium falciparum* infectious bite was lower than 10 in the study villages indicating its unstable transmission intensity [21,49] and risk of epidemics [50]. Apart from this, the study area is a highland fringe where vector density is lower resulting in low transmission intensity compared to typical lowland malarious areas such as in southern Ethiopia [42], Tanzania [51], Eritrea [14], Zambia [16] and Uganda [49]. For example, in southern Ethiopia, more than 45, 000 *An. arabiensis* were collected in 12 months at a locality with an average altitude ranging from 800 m to 1,300 m a.s.l. [42] compared to the present 2,431 *An. arabiensis* in the two years study time. However, since adult anopheline sampling was undertaken only once per month, this value may underestimate the risk of malaria transmission. *Plasmodium falciparum* and *P. vivax* infective *An. arabiensis* bites and *P. vivax* infective *An. pharoensis* bites decreased starting from the low altitude village to the higher. An increase in altitude is related to a decrease in temperature that limits vector occurrence and development of the parasites in the vector thereby reducing the number of infectious anopheline bites [50,52,53]. Mortality due to malaria was also reported to have a decreasing magnitude with increasing altitude in the area [28].

Recent studies, in the study area [7,54], reported *P. vivax* and *P. falciparum* malaria transmissions at elevations ranging from 2,100 m to 2,280 m. Although the relationship between EIR and malaria prevalence rate is not direct [20,55], EIR may vary between 0 and 1,500 infective bites per person per year in endemic countries of Africa and is a useful index in assessing malaria endemicity and transmission intensity [20,49]. The number of infective bites by both *An. arabiensis* and *An. pharoensis* were higher during dry months compared to the rainy months as was observed in western Kenya [56] and eastern Sudan [57]. However, this trend is different from the report in Eritrea [14] where EIR in wet season was nine times higher than in the dry season and also from that of southern Zambia [16], Tanzania [51] and Kenya [58]. This seasonal difference could result from diverse ecological adaptation and

behavioural changes of *An. arabiensis*. Malaria infectious bites were observed during the months of August to October and March to June which generally corresponds to the major and minor malaria transmission seasons respectively in the country [59]. This finding suggests that malaria transmission is seasonal and unstable in Hobe, Dirama and Wurib villages of south-central Ethiopia. As in most parts of Ethiopia, the unstable malaria transmission in the study area could result from variations in the meteorological factors, movement of inhabitants from non malarious to malarious areas and vice versa, and human population growth increasing activities that create increased and suitable vector breeding habitats along natural wetlands and foothills [60]. In addition, *Plasmodium* infectious bites were more frequent in the first study year (July 2008 to June 2009) and decreased from the low elevation village to the suggesting temporal and spatial variation of malaria transmission intensity.

## Conclusion

*Anopheles arabiensis* was the most prevalent vector of *P. vivax* and *P. falciparum* malaria along a south-central highland transect of Ethiopia consisting of Hobe (low altitude), Dirama (mid altitude) and Wurib (high altitude) villages followed by *An. pharoensis*. Both anopheline species fed on human and bovine of which the first was opportunistic feeder while the second being moderately anthropophilic. The annual EIRs were generally lower compared to typical endemic areas and showed a decreasing trend from the low altitude village to the high altitude village.

## Competing interests

The authors declare that they have no competing interests.

## Authors' contributions

AA designed the study, collected data in the field, carried out the data analysis and wrote the first draft of the manuscript. TGM participated in the study design, interpretation of the results and editing the manuscript. MB participated in the conception of the study, in the study design and editing the manuscript. BL conceived the idea for the study and took part in the study design, data entry and analysis, data interpretation and editing the manuscript. All authors have read and approved the final manuscript.

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## RESEARCH

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# Impact of housing condition on indoor-biting and indoor-resting *Anopheles arabiensis* density in a highland area, central Ethiopia

Abebe Animut<sup>1,2\*</sup>, Meshesha Balkew<sup>2</sup> and Bernt Lindtjørn<sup>1</sup>

## Abstract

**Background:** Exposure of individuals to malaria infection may depend on their housing conditions as houses serve as biting and resting places of vectors. This study describes the association of housing conditions with densities of indoor-biting and indoor-resting *Anopheles arabiensis* in Hobe, Dirama and Wurib villages of a highland area in central Ethiopia.

**Methods:** Data on housing conditions, including presence of house apertures, number of occupants and number and the type of domestic animal tethered inside, were collected. Indoor-biting mosquitoes were sampled using Centers for Disease Control (CDC) light traps and indoor-resting mosquitoes sampled with pyrethrum spray catches (PSCs) monthly for two years (July 2008 to June 2010). Female anophelines were identified to species and processed. Univariate and general linear estimating equation allowing for repeated measures were used to assess the contribution of housing conditions for indoor-biting and indoor-resting *An. arabiensis*.

**Results:** About 96% (4,597/4,788) of anophelines were caught inside residential houses. Nine anopheline species were identified, among which *An. arabiensis* was most prevalent (2,489; 52%). Vectors entering houses were higher in those situated at low ( $\beta = 4.475$ ; 95% CI = 3.475-5.476;  $p < 0.001$ ;  $\beta$  = strength of the association) and medium ( $\beta = 2.850$ ; 95% CI = 1.975-3.724;  $p < 0.001$ ) altitudes compared to high altitude, and where houses have no windows ( $\beta = -0.570$ ; 95% CI = -1.047-0.094;  $p = 0.019$ ) compared with those that have. Numbers of indoor-resting vectors were higher in those situated at low ( $\beta = 6.100$ ; 95% CI = 4.571-7.629;  $p < 0.001$ ) and medium ( $\beta = 4.411$ ; 95% CI = 2.284-6.537;  $p < 0.001$ ) altitudes compared to high altitudes, and where houses had open eaves ( $\beta = 1.201$ ; 95% CI = 0.704-1.698;  $p < 0.001$ ) compared with those that had closed eaves.

**Conclusion:** Housing conditions such as presence of open eaves, absence of window, location at low and mid altitudes, were strong predictors of indoor exposure to *An. arabiensis* bite in a highland area of south-central Ethiopia.

## Background

Malaria affects 68% of the Ethiopian population [1]. Although control efforts brought reduction in malaria-related mortality compared to the previous years [1,2], the disease is still among the top causes of morbidity in the country [3,4]. It is seasonal in most areas below 1,500 m altitude and unstable in areas above 1,500 m [1].

Transmission of the disease depends largely on local topography, climate and land use. It may also be influenced by housing conditions [5-7]. Conditions, such as nearby irrigated land, earth roof, tethering livestock inside, window presence, open eaves, absence of separate kitchen and presence of a single sleeping room, were associated with high incidence of child malaria in northern Ethiopia [8]. In Burkina Faso, children living in mud-roofed houses were at a higher risk of *Plasmodium falciparum* infection compared to those in iron sheet-roofed houses [9]. In The Gambia, eaves were the main routes of *Anopheles gambiae* and *Mansonia* spp. entry

\* Correspondence: animut2004@yahoo.com

<sup>1</sup>Center for International Health, University of Bergen, Bergen, Norway

<sup>2</sup>Akilu Lemma Institute of Pathobiology, Addis Ababa University, PO Box 1176, Addis Ababa, Ethiopia

[10,11]. Houses with a grass roof were associated with increased malaria risk in Mozambique [12].

The association of poorly constructed houses with high malaria infection risk may result from their suitability to indoor abundance of vectors [9,10,13]. Houses are the principal site where malaria vectors bite and rest [10,11,14], hence improved housing may reduce indoor occurrence and the risk of malaria transmission in Ethiopia. However, housing conditions and their impact on indoor abundance of vectors may vary with respect to geography, socio-economy and individual household factors. This study was undertaken to assess the contribution housing conditions make to indoor-biting and indoor-resting *Anopheles arabiensis* in a highland area of central Ethiopia.

## Methods

### Study area and housing conditions

A longitudinal study on the relationship between housing conditions and number of indoor-biting as well as indoor-resting *An. arabiensis* was undertaken in Hobe, Dirama and Wurib villages of south-central Ethiopia once a month for two years (July 2008 to June 2010). The same villages and houses were used for related studies [15,16].

Most of the houses were constructed with mud-plastered wooden walls and grass roofs. They did not have ceilings or separate kitchen. A single living house is used for sleeping, keeping all household belongings, cooking and dining, keeping warm by burning wood and also for tethering domestic animals at night (Figure 1). Data on housing conditions, including presence of house apertures, number of occupants that slept the previous

night and number and type of domestic animals tethered indoor the previous night were recorded, while undertaking mosquito sampling, once per month. In addition, the location of each house where mosquitoes were sampled was categorized into either low altitude (Hobe), mid altitude (Dirama) or high altitude (Wurib). The study period was categorized into either dry or wet. Wet were months with average rainfall of greater than 1 mm. They include May, June, July, August, September and October. The number of occupants and domestic animals (cattle, sheep, goat, horse, donkey, and chicken) was recorded by interviewing the head of household or the next elder occupant. House apertures, such as door (unfit or fit), window (absent or present), open eaves (absent or present), hole on wall (absent or present), and hole on roof (absent or present) were recorded by direct observation. All the houses (except one) had unfit doors; therefore the variable door fitness was excluded from the analysis.

### Mosquito sampling

Mosquito sampling was undertaken using Centers for Disease Control (CDC) light trap, pyrethrum spray collection (PSC) and artificial pit shelter (APS) [17] from Hobe, Dirama and Wurib villages. CDC light trap-based collection was made for two consecutive nights inside 20 houses resulting in 40 trap nights per month per village. PSC was made in ten randomly selected houses where no CDC light trap catches was undertaken. Five APSs constructed in shaded areas were used for outdoor-resting mosquito collection in each village. CDC light trap catches were used to collect mosquitoes that attempted to bite humans inside houses during night



**Figure 1** Typical housing in south-central Ethiopia. **A** = door of the house from the outside; **B** = inside the house.



hours. PSC was used to collect mosquitoes that rest indoors during daylight hours. All female anopheline catches were identified to species, counted and processed, while culicines were discarded after counting. The detailed method is described elsewhere [16].

### Statistics

Indoor- and outdoor-sampled mosquitoes were depicted in a frequency table. Association of each housing condition with the number of either indoor-biting or indoor-resting *An. arabiensis* catches was assessed independently using univariate analysis from which the mean number of *An. arabiensis* catches, including 95% confidence interval (CI) for the mean and significant level was calculated. In the univariate analysis, an independent variable with p value less than 0.1 was considered as a potential predictor and was re-analysed using generalized estimating equation (GEE) multivariate analyses for repeated measures. The dependent variable, number of *An. arabiensis*, fitted to a negative binomial distribution with a log link function [18]. Variables with p values <0.05 in the GEE were considered as strong predictors. Data were analysed using PASW Statistics version 18 (SPSS Inc, Chicago, IL, USA).

### Ethics

The study was ethically cleared by the Ethical Committee of the Faculty of Medicine, Addis Ababa University and The National Health Research Ethics Review Committee (NERC) of Ethiopia with reference number RDHE/48-85/2009. All anopheline collections were undertaken following verbal consent of households.

### Results

A total of 16,894 mosquitoes were sampled of which 71.7% (12, 106/16,894) were culicines and the remaining 28.3% (4,788/16,894) were anophelines (Table 1). Among the total 4,788 female *Anopheles* catches, 96% (4,597) was from inside residential houses. The highest number of anophelines was collected from Hobe (low altitude village) and the lowest from Dirama (mid altitude). *Anopheles arabiensis* was the most common vector in the area (2,489; 52%) followed by *Anopheles demeilloni* (1,261; 26.3%), *Anopheles christyi* (432; 9.02%), *Anopheles pharoensis* (408; 8.52%), *Anopheles cinereus* (166; 3.5%), *Anopheles coustani* (16; 0.33%), *Anopheles culicifacies* (12; 0.25%), *Anopheles garnhami* (3; 0.06%) and *Anopheles rhodesiensis* (1; 0.02).

Wurib had nine anopheline species while Hobe and Dirama had six species each. *Anopheles arabiensis* was highest in Hobe (2,146) followed by Dirama (323) and Wurib (20). Similar distribution pattern was observed for *An. pharoensis* and *An. coustani*. Catches of *An. christyi*, *An. demeilloni* and *An. cinereus* were highest in Wurib followed by Dirama and very low or scarce in Hobe. From the total 191 outdoor catches, the highest number of anopheline species (n = 169; comprising *An. demeilloni* = 141, *An. cinereus* = 16 and *An. christyi* = 12) was from Wurib while the lowest (n = 3; composed of *An. demeilloni* = 2 and *An. christyi* = 1) being from Dirama. Only one species (*An. arabiensis*; n = 19) was collected from the APS in Hobe.

Table 2 presents housing conditions and associated mean number of *An. arabiensis* catches. Mean number of indoor-biting *An. arabiensis* was significantly higher (p = 0.035) in houses with two or more goats tethered the previous night (mean = 1.06; 95% CI = 0.70-1.42)

**Table 1 Diversity and abundance of anopheline mosquitoes in three villages of central Ethiopia, July 2008-June 2010**

Mosquito	Hobe			Dirama			Wurib			Total (%)
	CDC	PSC	APS	CDC	PSC	APS	CDC	PSC	APS	
<i>An. arabiensis</i>	874	1253	19	138	185	0	15	5	0	2,489 (52)
<i>An. pharoensis</i>	359	18	0	17	9	0	5	0	0	408 (8.52)
<i>An. christyi</i>	7	0	0	23	4	1	312	73	12	432 (9.02)
<i>An. cinereus</i>	3	2	0	19	7	0	95	24	16	166 (3.5)
<i>An. demeilloni</i>	1	0	0	91	4	2	882	140	141	1,261 (26.3)
<i>An. coustani</i>	11	0	0	2	1	0	2	0	0	16 (0.33)
<i>An. culicifacies</i>	0	0	0	0	0	0	7	5	0	12 (0.25)
<i>An. garnhami</i>	0	0	0	0	0	0	3	0	0	3 (0.06)
<i>An. rhodesiensis</i>	0	0	0	0	0	0	1	0	0	1 (0.02)
Total anopheline	1,255	1,273	19	290	210	3	1,322	247	169	4,788 (100)
Total culicine	4,557	578	1,024	988	113	490	1,483	324	2,549	12,106
Total mosquitoes	5,812	1,851	1,043	1,278	323	493	2,805	571	2,718	16,894

CDC = Centers for Disease Control light trap catches; PSC = pyrethrum spray catches; APS = Artificial pit shelter catches.

**Table 2 Estimation of average number of indoor *Anopheles arabiensis* catches per housing conditions using univariate analysis in three villages of central Ethiopia, July 2008-June 2010**

Housing condition	Indoor-biting <i>An. arabiensis</i>		Indoor-resting <i>An. arabiensis</i>	
	Mean (95% CI)	P value	Mean (95% CI)	P value
<b>Occupants</b>				
≤4	0.57 (0.33–0.80)	0.055	1.48 (0.58–2.38)	0.042
≥5	0.91 (0.65–1.18)		3.05 (1.84–4.27)	
<b>Number of cattle</b>				
≤2	0.61 (0.34–0.89)	0.429	5.62 (3.56–7.68)	0.004
≥3	0.75 (0.54–0.96)		1.81 (0.29–3.32)	
<b>Sheep</b>				
≤1	0.73 (0.49–0.97)	0.921	2.36 (1.44–3.28)	0.402
≥2	0.71 (0.40–1.02)		1.61 (0.12–3.10)	
<b>Goat</b>				
≤1	0.60 (0.38–0.82)	0.035	1.97 (1.11–2.83)	0.333
≥2	1.06 (0.70–1.42)		2.99 (1.12–4.87)	
<b>Horse</b>				
0	0.70 (0.50–0.90)	0.441	2.21 (1.41–3.01)	0.501
≥1	0.96 (0.33–1.58)		0.73 (–3.51–4.96)	
<b>Donkey</b>				
0	0.75 (0.55–0.95)	0.493	2.32 (1.50–3.14)	0.155
≥1	0.56 (0.07–1.06)		0.28 (–2.42–2.97)	
<b>Chicken</b>				
≤1	0.62 (0.36–0.88)	0.263	1.91 (0.92–2.91)	0.459
≥2	0.84 (0.57–1.12)		2.52 (1.26–3.79)	
<b>Window</b>				
Absent (n = 157)	1.02 (0.78–1.27)	<0.001	2.35 (1.30–3.39)	0.628
Present (n = 120)	0.27 (–0.04–0.60)		1.95 (0.72–3.18)	
<b>Hole on roof</b>				
Absent (n = 210)	0.61 (0.38–0.83)	0.023	1.17 (0.24–2.10)	<0.001
Present (n = 97)	1.12 (0.75–1.50)		4.81 (3.31–6.31)	
<b>Holes on wall</b>				
Absent (n = 138)	0.67 (0.32–1.02)	0.628	0.73 (–0.48–1.94)	0.002
Present (n = 171)	0.77 (0.54–1.01)		3.27 (2.22–4.32)	
<b>Open eaves</b>				
Absent (n = 198)	0.66 (0.43–0.88)	0.160	0.77 (–0.15–1.69)	<0.001
Present (n = 98)	0.97 (0.60–1.34)		5.67 (4.22–7.12)	
<b>Village</b>				
Low	1.82 (1.53–2.12)	<0.001	5.35 (4.14–6.57)	<0.001
Mid	0.30 (–0.002–0.598)	<0.001	0.83 (–0.42–2.08)	<0.001
High	0.03 (–0.263–0.315)		0.02 (–1.17–1.21)	
<b>Season</b>				
Wet	0.65 (0.34–0.96)	0.271	1.23 (–0.06–2.51)	0.023
Dry	0.89 (0.61–1.17)		3.22 (2.08–4.37)	

compared to the houses with less than or equal to one goat (mean = 0.60; 95% CI = 0.38-0.82). Houses with no window had significantly more mosquitoes (mean = 1.02; 95% CI = 0.78-1.27) compared to those with a window (mean = 0.28; 95% CI = -0.04-0.60). Houses with holes on their roof had significantly higher mosquitoes (mean = 1.12; 95% CI = 0.75-1.50) compared to the houses with no holes (mean = 0.61; 95% CI = 0.38-0.83). Density of indoor-biting *An. arabiensis* also varied significantly with respect to altitudinal location and was highest in the houses located at the low altitude village (mean = 1.82; 95% CI = 1.53-2.12).

Mean number of *An. arabiensis* resting in houses where greater than or equal to five occupants slept the previous night (mean = 3.05; 95% CI = 1.84-4.27) was significantly higher ( $p = 0.042$ ) than in those with less than or equal to four occupants (mean = 1.48; 95% CI = 0.58-2.38). The mean number of mosquitoes in houses where less than or equal to two cattle tethered the previous night (mean = 5.62; 95% CI = 3.56-7.68) was also significantly higher ( $p = 0.004$ ) than the number in houses where greater than or equal to three cattle tethered (mean = 1.81; 95% CI = 0.29-3.32). Density of mosquitoes in houses with hole on their roof (mean = 4.81; 95% CI = 3.31-6.31), with hole on wall (mean = 3.27; 95% CI = 2.22-4.32) and with open eaves (mean = 5.67; 95% CI = 4.22-7.12) was significantly higher than in those with no hole on roof (mean = 1.11; 95% CI = 0.18-2.05), with no hole on wall (mean = 0.73; 95% CI = -0.48-1.94) and with no open eaves (mean = 0.76; 95% CI = -0.16-1.69), respectively. Density of indoor-resting *An. arabiensis* either at the low altitude village (mean = 5.35; 95% CI = 4.14-6.57) or the mid (mean = 0.83; 95% CI = -0.42-2.08) was significantly higher than at the high altitude village (mean = 0.02; 95% CI = -1.17-1.21). The number of indoor-resting mosquitoes during the dry season (mean = 3.22; 95% CI = 2.08-4.37) was significantly higher ( $p = 0.023$ ) than the number during the wet season (mean = 1.23; 95% CI = -0.06-2.51) in the area.

Housing conditions that predict indoor-biting and indoor-resting *An. arabiensis* are presented in Table 3. The number of *An. arabiensis* that bite inside houses located at the low altitude village (Hobe) was 4.475 (95% CI = 3.475-5.476;  $p < 0.001$ ) times relative to the number in the high altitude village. Similarly, the number in the mid altitude village was 2.850 (95% CI = 1.975-3.724;  $p < 0.001$ ) times relative to the high altitude. Houses with window had 57% lower number of indoor-biting *An. arabiensis* ( $\beta = -0.570$ ; 95% CI = -1.047-0.094;  $p = 0.019$ ) relative to those with no window. Similarly, house location at the low or mid altitude village relative to the high altitude and presence of open eaves relative to no open eaves were strong predictors of indoor-resting *An. arabiensis*.

The mean number of indoor-biting *An. arabiensis* characterized by feeding status, blood meal source and *Plasmodium* sporozoite infection status with respect to housing condition is presented in Table 4. Houses located in the low altitude village were observed to have significantly highest mean number of fresh fed (2.58), half gravid (0.89), gravid (0.72), unfed (0.75) and bovine fed (1.31) *An. arabiensis* caught by CDC light trap. Houses with no window had higher mean number of fresh fed, unfed, bovine fed, human fed and human and cattle mixed blood fed *An. arabiensis* and the differences were significant.

The mean numbers of indoor-resting (caught by PSC) fresh fed, half gravid, gravid, bovine fed, human fed, and human and bovine mixed blood fed *An. arabiensis* were significantly higher in houses having open eaves than in those with no open eaves and also in houses located at either the low or mid altitude village than in the high altitude village (Table 5).

## Discussion

Most *Anopheles* mosquito species in Hobe, Dirama and Wurib villages of central Ethiopia occur inside residential houses. Houses having open eaves, no window, and located at either low or mid altitude village were associated with higher risk of malaria. The indoor occurrence of anophelines in these highland villages could be attributed to several factors among which appropriate indoor microclimate is one [19,20]. The tradition of cooking, sleeping and tethering livestock inside residential houses could contribute to the indoor occurrence of mosquitoes by increasing indoor temperature and providing access to blood meal sources. This in turn contributes to the survival and increased malaria transmission potential of the vectors in the area. Indoor-resting mosquitoes of East Africa are estimated to transmit malaria between 0.3 and 22.5 days earlier than those of outdoor-resting mosquitoes [19]. This study reveals that *An. arabiensis* and *An. pharoensis*, which are malaria vectors in the area [16] and the remaining seven anopheline species, exhibit endophilic behaviour indicating the need to construct mosquito proof houses.

Densities of both indoor-biting and indoor-resting *An. arabiensis* were highest in the low altitude village and decreased with increasing altitude. Similarly, densities of both immature and adult stages of the vector were observed to decrease significantly with increasing altitude in the area during the period [15,16] and so was the risk of acquiring *P. falciparum* and *Plasmodium vivax* malaria [16,21,22]. Density of vectors generally decreases with increasing altitude in highland areas [23].

In this study, houses with open eaves were strongly associated with indoor-resting *An. arabiensis* relative to the houses with no such opening. Eaves could enhance

**Table 3 Housing condition and indoor abundance of *Anopheles arabiensis* based on generalized estimating equation model, south-central Ethiopia, July 2008-June 2010**

Housing condition	Indoor-biting <i>An. arabiensis</i>		Indoor-resting <i>An. arabiensis</i>	
	$\beta$ (95% CI)	p	$\beta$ (95% CI)	p
<b>Number of occupants</b>				
≥5	0.010 (-0.559-0.580)	0.278	0.135 (-0.364-0.634)	0.596
≤4	0*		0	
<b>Number of cattle</b>				
≥3	NA	NA	0.007 (-0.444-0.459)	0.975
≤2	NA			
<b>Number of goats</b>				
≥2	-0.027 (-0.498-0.444)	0.530	NA	NA
≤1	0		NA	
<b>Window</b>				
Present (n = 120)	-0.570 (-1.047-0.094)	0.019	NA	NA
Absent (n = 157)	0		NA	
<b>Holes on roof</b>				
Present (n = 97)	0.289 (-0.368-0.947)	0.388	0.258 (-0.156-0.671)	0.222
Absent (n = 210)	0		0	
<b>Holes on wall</b>				
Present (n = 171)	NA	NA	0.243 (-0.241-0.727)	0.325
Absent (n = 138)	NA		0	
<b>Open eaves</b>				
Present (n = 98)	NA	NA	1.201 (0.704-1.698)	<0.001
Absent (n = 198)	NA		0	
<b>Village</b>				
Low	4.475 (3.475-5.476)	<0.001	6.100 (4.571-7.629)	<0.001
Mid	2.850 (1.975-3.724)	<0.001	4.411 (2.284-6.537)	<0.001
High	0		0	
<b>Season</b>				
Dry	NA	NA	0.479 (-0.435-1.393)	0.304
Wet	NA		0	

NA = housing condition not applicable.

*An. arabiensis* entry to houses and its blood meal sources (human and cattle) which stay indoor during night hours [16] and then rest in the house until oviposition. Houses with open eaves and no ceilings were observed with higher number of *An. gambiae* than those with closed eaves and ceilings [10]. Open eaves were associated with increased risk of *An. gambiae s.l.* and *Culex pipiens s.l.* entry in The Gambia [11,24]. *Anopheles gambiae s.s.*, *An. arabiensis*, *Mansonia africana* and *Ma. uniformis* were noted to prefer eaves as the main entry points in Tanzania [25]. The high density of *An. arabiensis* inside houses with open eaves could result from the upward-flying behaviour of the mosquito when encountering wall surfaces and entering houses through these holes having been attracted by microclimatic

conditions and odours of humans and cattle coming from the houses [10,11,19,20,26].

This study indicates the need to construct houses with closed eaves, roof and ceilings in Hobe, Dirama and Wurib villages of central Ethiopia in order to minimize indoor-resting *An. arabiensis*, which is the most prevalent and major malaria vector in the area [15,16]. House ceilings made of plywood, synthetic-netting, insecticide-treated synthetic-netting, and plastic insect screen, all installed below open eaves and mud-closed eaves, reduced entry of *An. gambiae* into experimental huts in Gambia [10]. Closing eaves resulted in a three-fold reduction in *An. gambiae s.l.* caught indoors [11]. Eaves screening reduced density of indoor *An. gambiae s.l.*, *Ma. africana* and *Ma. uniformis* significantly in southern

**Table 4 Differences in the mean number of indoor biting *Anopheles arabiensis* status (feeding, blood meal source and *Plasmodium* infection) with respect to selected housing conditions in three villages of central Ethiopia, July 2008-June 2010**

Anopheline status	Window		Village		
	Absent	Present	Low	Mid	High
<b>Fresh fed</b>					
Mean	2.49	0.89	2.58	0.84	0.55
p	0.005		0.001		
<b>Half gravid</b>					
Mean	0.87	0.40	0.89	0.29	0.27
p	0.053		0.013		
<b>Gravid</b>					
Mean	0.68	0.38	0.72	0.25	0.18
p	0.196		0.051		
<b>Unfed</b>					
Mean	0.74	0.29	0.75	0.29	0.27
p	0.032		0.034		
<b>Bovine fed</b>					
Mean	1.34	0.44	1.31	0.56	0.14
p	0.018		0.039		
<b>Human fed</b>					
Mean	1.15	0.61	1.07	0.72	0.57
p	0.036		0.234		
<b>Human and bovine fed</b>					
Mean	0.46	0.08	0.46	0.21	0
p	0.008		0.065		
<b><i>P. vivax</i> positive</b>					
Mean	0.09	0.03	0.09	0.03	0
p	0.299		0.512		
<b><i>P. falciparum</i> positive</b>					
Mean	0.01	0	0.01	0	0
p	0.346		0.847		

**Table 5 Differences in the mean number of indoor-resting *Anopheles arabiensis* status (feeding, blood meal source and *Plasmodium* infection) with respect to three housing conditions in three villages of central Ethiopia, July 2008-June 2010**

Anopheline status	Open eaves		Village		
	Absent	Present	Low	Mid	High
<b>Fresh fed</b>					
Mean	3.03	9.00	8.76	1.87	1.00
p	0.001		<0.001		
<b>Half gravid</b>					
Mean	0.73	2.40	2.22	0.62	0
p	0.013		0.031		
<b>Gravid</b>					
Mean	0.83	2.71	2.64	0.32	0.25
p	0.017		0.006		
<b>Unfed</b>					
Mean	0.20	0.59	0.56	0.10	0
p	0.324		0.418		
<b>Bovine fed</b>					
Mean	1.39	4.14	3.85	1.12	0
p	0.001		0.001		
<b>Human fed</b>					
Mean	1.32	2.90	3.08	0.64	0.33
p	0.007		<0.001		
<b>Human and bovine fed</b>					
Mean	0.27	1.32	1.19	0.16	0.33
p	0.002		0.004		
<b><i>P. vivax</i> positive</b>					
Mean	0.02	0.04	0.05	0.03	0
p	0.538		0.864		
<b><i>P. falciparum</i> positive</b>					
Mean	0	0.02	0.02	0	0
p	0.310		0.743		

Tanzania [25]. Screening houses fully and also equipping them with screened ceilings can reduce indoor exposure to *An. arabiensis* bites as noticed in The Gambia [27] and Kenya [28]. In addition, constructing houses with iron-sheet roof instead of thatched roof may reduce malaria infection risk in south-central Ethiopia as reported from Burkina Faso [9].

The number of *An. arabiensis* that attempted to bite indoors at night was 57% lower in houses with windows than in those with no window. The presence of windows might have increased aeration inside houses, which could reduce indoor temperature. Low indoor temperature in these highland villages could deter the indoor-biting mosquitoes at night. In The Gambia [11],

windows and doors were found less important for *An. gambiae* s.l. entry into houses but were the main entry routes of culicines.

*Anopheles arabiensis*, which is the principal malaria vector in Hobe, Dirama and Wurib villages in particular [16] and in Ethiopia in general, was prevalent inside houses located in the low altitude village and in the mid altitude village. Houses with open eaves were also observed to have high density of indoor-resting *An. arabiensis*. Better designed houses and house screens, together with existing malaria control programmes, may help to reduce indoor-biting as well as indoor-resting *An. arabiensis* and hence transmission of the disease significantly.

## Conclusion

Nine species of anopheline mosquitoes, including *An. arabiensis*, which is the primary malaria vector in Ethiopia, were more abundant inside residential houses than outdoors (in pit shelters) in Hobe, Dirama and Wurib village of south-central Ethiopia. Housing conditions such as the presence of open eaves, location at either low or mid altitude village, and absence of windows, were found to be strong predictors of indoor-occurring *An. arabiensis*.

## Competing interests

The authors declare that they have no competing interests.

## Authors' contributions

AA designed the study, collected data in the field, carried out the data analysis and wrote the first draft of the manuscript. MB participated in the conception of the study, in the study design and editing the manuscript. BL conceived the idea for the study and took part in the study design, data analysis, data interpretation and editing the manuscript. All authors have read and approved the final manuscript.

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## 11. Appendices

### MOSQUITO LARVAE COLLECTION FORM

#### 1. Identification of collection site

1.1 Region \_\_\_\_\_

1.2 district \_\_\_\_\_

1.3 Locality \_\_\_\_\_

1.4. Geographic coordinates:

1.4.1 Latitude \_\_\_\_\_

1.4.2 Longitude \_\_\_\_\_

1.4.3 Elevation \_\_\_\_\_

#### 2. Characterization of the breeding site

2.1. Type (e.g. Permanent, Semi-permanent, Temporary) \_\_\_\_\_

2.2. Origin of the water (e.g. rain, river, lagoon, man-made) \_\_\_\_\_

2.3. Nature of the water collection (e.g. puddle, rice field, ditch) \_\_\_\_\_

2.4. Characteristics of the water (e.g. clear, turbid, polluted, dark) \_\_\_\_\_

2.5. Temperature \_\_\_\_\_

2.6. pH \_\_\_\_\_

2.7. Exposure to sunlight (Shaded, Partially Shaded, Sunlit) \_\_\_\_\_

2.8. Presence of vegetation (emergent, submerse, floating, shed) \_\_\_\_\_

2.9. Habitat/water speed (stagnant, slow, fast) \_\_\_\_\_

2.10. Nature of the adjacent land surface (e.g. cultivated, grazing) \_\_\_\_\_

2.11. Distance from nearby inhabited house \_\_\_\_\_

#### 3. Sampling description

3.1. Sampling time and minute \_\_\_\_\_

3.2. Number of dips \_\_\_\_\_

3.3. Presence of larvae (Anopheline, Culicine, Negative) \_\_\_\_\_





## ADULT MOSQUITO COLLECTION FORM

### 1 Identification of collection site

1.1 Region \_\_\_\_\_

1.2 District \_\_\_\_\_

1.3 Locality \_\_\_\_\_

### 1.4 Geographic coordinates

1.4.1. Latitude \_\_\_\_\_

1.4.2. Longitude \_\_\_\_\_

1.4.3. Elevation \_\_\_\_\_

### 2 Type of collection

2.1 Human landing catches: Indoor \_\_\_\_\_ Outdoor \_\_\_\_\_

2.2 Resting collections: Indoor \_\_\_\_\_ Outdoor \_\_\_\_\_

2.3 Pyrethrum Spray Sheet collection \_\_\_\_\_

2.4 Exit trap \_\_\_\_\_

2.5 Other \_\_\_\_\_

### 3 Characteristics of the collection site

#### 3.1 Indoor collection

3.1.1. Type of house \_\_\_\_\_

3.1.2. Construction materials \_\_\_\_\_

3.1.3. Presence of (yes/no):

3.1.3.1 Eve \_\_\_\_\_

3.1.3.2 Hole on wall \_\_\_\_\_

3.1.3.3 Hole on roof \_\_\_\_\_

3.1.3.4 Window \_\_\_\_\_

3.1.3.5 Fit door \_\_\_\_\_

3.1.4. Number of bedrooms \_\_\_\_\_

3.1.5. Number of rooms \_\_\_\_\_

3.1.6. Number of people that slept in the house the previous night \_\_\_\_\_

3.1.7. Number of people slept under bed net \_\_\_\_\_

3.1.8. Number slept without bed net \_\_\_\_\_

3.1.9. Type of bed net used (Non-impregnated, Impregnated, LLIN) \_\_\_\_\_

3.1.10. Do occupants and cattle share same room (yes/no)? \_\_\_\_\_

3.1.11. If yes in 3.1.10 above:

3.1.11.1. No of cattle tethered the previous night \_\_\_\_\_

3.1.11.2. No sheep tethered the previous night \_\_\_\_\_

3.1.11.3. No of goat tethered the previous night \_\_\_\_\_

3.1.11.4. No of horse tethered the previous night \_\_\_\_\_

3.1.11.5. No of donkey tethered the previous night \_\_\_\_\_

3.1.11.6. No of chicken tethered the previous night \_\_\_\_\_

3.1.12. Is kitchen separated from bed room (yes/no)? \_\_\_\_\_

3.1.13. House distance from nearby breeding site (in metres) \_\_\_\_\_

**4. Date of the last time the house was sprayed by residual insecticide** \_\_\_\_\_

**5. Type and characteristics of outdoor mosquito sampling habitats**

**5.1.** Animal shelter \_\_\_\_\_

**5.2.** Vegetation \_\_\_\_\_


**5.3.** Artificial Pit helter \_\_\_\_\_

**5.4.** Tree bark \_\_\_\_\_

**5.5.** If any other type, please specify \_\_\_\_\_

**6. Sampling description**



	<b>Addis Ababa University, Medical Faculty, Institutional Review Board</b>	SOP# AAUMF 007  <b>Version 1.0</b> <b>Effective date:</b> <b>1 Jan. 2007</b> <b>Page 13 of 13</b>
	<b>Title:</b> <b>3.1. Use of Study Assessment Form</b>	

**ANNEX 3**  
Form AAUMF 03-008

**IRB's Decision**

Meeting No: 007/08

Date (D/M/Y): July 24, 2008

Protocol number: 018/08/SPH

Assigned No.....

Protocol Title : Ethiopian Malaria Prediction System EMAPS	
Principal Investigators:	Dr. Wakgari Deressa
Institute:	School of Public Health, AAU
Elements Reviewed (AAUMF 01-008) :	<input checked="" type="checkbox"/> Attached <input type="checkbox"/> Not attached
Review of Revised Application <input checked="" type="checkbox"/> Yes <input type="checkbox"/> No	Date of Previous review:
Decision of the meeting:	<input checked="" type="checkbox"/> Approved <input type="checkbox"/> Approved with Recommendation <input type="checkbox"/> Resubmission <input type="checkbox"/> Disapproved

IRB Approval Period **From 14/08/08 to 13/08/2010**

\_\_\_\_\_

**Dr. Yeweyenhareg Feleke**  
**Chairperson, IRB**

Date: 14/08/2008



.....  
**Associate Dean for Post  
Graduate and Research  
Faculty of Medicine**

Date:.....