Comparative studies on bionomics and behavior of phlebotomine sandflies in highland and lowland areas of kala-azar transmission, northwest Ethiopia

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This is to certify that the thesis prepared by Essayas Aklilu, entitled: ***Comparative studies on bionomics and behavior of phlebotomine sandflies in highland and lowland areas of kala-azar transmission, northwest Ethiopia*** and submitted in fulfillment of the requirements for the Degree of Doctor of Philosophy in Biology (Insect Science) complies with regulations of the University and meets the accepted standards with respect to originality and quality.

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Abstract

Comparative studies on bionomics and behavior of phlebotomine sandflies in highland and lowland areas of kala-azar transmission, northwest Ethiopia

Essayas Aklilu, Addis Ababa University, 2015

The aim of this study was to investigate and compare the bionomics, ecology and behaviors of the vector (s) of visceral leishmaniasis (VL) in two endemic and ecologically distinct areas of northwestern Ethiopia. This included species composition, population dynamics, habitat preference, host preference, vectorial potential and nocturnal activity studies in the field. Phlebotomine sandflies were collected using CDC light traps, sticky traps and pyrethrum space spray catches from Libo-Kemkem district (May 2011-April 2012) and Metema district (October 2012-September 2013). A total of 51,411 sandfly specimens were collected: 10,776 from highland and 40, 635 from the lowland areas. Seven species were found in the highland area: two *Phlebotomus* sandflies (*P. orientalis* and *P. rodhaini*) and five *Sergentomyia* species, whereas 19 species were found in the lowland area: six *Phlebotomus* (*P. orientalis*, *P. rodhaini*, *P. bergeroti, P. duboscqi, P. papatasi* and *P. martini*) and 13 *Sergentomyia* species. Of the *Phlebotomus* spp., *P. orientalis* was the predominant species in both the highland (99.9%) and lowland (93.7%) areas. In both areas, this species showed seasonal a occurrence and mainly abundant during the dry months (March-May/June) of the year and increasing in numbers till the rains began, when numbers dropped dramatically. In both areas, *P. orientalis* exhibits both strong exophilic and exophagic behaviors and its parous rates of the species were 45.6% and 66.2% in highland and lowland, respectively. Identification of blood meals in wild caught blood fed *P. orientalis* using cytochrome *b* polymerase chain reaction (Cyt *b* –PCR) and reverse line blotting (RLB) and enzyme linked immunosorbent assay (ELISA) showed that this species has strong predilection for bovine in both the highland (67.8%) and the lowland (49.8%) areas. A total of 1087 *P. orientalis* from the highland area and 344 *Phlebotomus* spp. from lowland area were dissected for detection of natural infection, but none of them was infected. A similar result was obtained in 347 *P. orientalis* females processed using PCR for detection of *L. donovani* from the highland area. Hourly activity period of the two populations of *P. orientalis* showed variation. The nocturnal activity of *P. orientalis* in the highland peaked between 22:00 and 23:00hours for both sexes; whereas in the lowland area, this peak was between 3:00 and 4:00hours. Although natural infection was not detected in the current study, *P. orientalis* is strongly implicated as vector of VL in both study areas due to its abundance and other circumstantial evidences. Therefore, control of the disease in these particular areas should involve tools that mainly target the vector of the disease by considering its seasonal abundance, ecology and behaviors in the respective areas.

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Chapter One. General Introduction

1.1. Taxonomy of phlebotomine sandflies

Phlebotomine sandflies are minute bloodsucking insects in the suborder Nematocera of the Order Diptera (two-winged flies). Although the classification below the suborder is a matter of controversy and no universally accepted system exists, they are generally placed in the Family Psychodidae, subfamily Phlebotominae (Killick-Kendrick, 1999). This subfamily is distinguished from other subfamilies of Psychodidae (Trichomyiinae, Sycoracinae, Psychodinae and Bruchomyiinae) by the following traits; presence of five segmented palps, biting mouth parts, antennal segments almost cylindrical, the wing has five-branched radial vein and lack of an eye-bridge (Lane, 1993). Furthermore, they tend to have an elongate and more fragile structure, in contrast to a squatter and more robust appearance of the other (Munstermann, 2004).

Phlebotomine taxonomists have segregated the subfamily into Old World (Africa, Asia and Europe) and New World (North, Central and South America). Throughout the world, six genera of the subfamily phlebotominae sandflies are recognized, viz., *Phlebotomus*, *Chinius*, *Sergentomyia*, *Brumptomyia*, *Lutzomyia*, and *Warileya*. The former three genera are found in the Old World and the rest are in the New World. Under these genera, approximately 800 species and subspecies are so far found (Bates, 2008; Ready, 2013). Species and subspecies in the three genera, *Phlebotomus, Lutzomyia* and *Sergentomyia*, suck blood from vertebrate hosts, but the former two are the more medically important groups since they contain disease vectors (Lane, 1993; Killick-Kendrick, 1999; Ready, 2013).

1.2. Distribution

Phlebotomine sandflies are found mainly in warm parts of the world (tropics and subtropics), and also a few species penetrating into the temperate regions of the northern (50^0N) and the southern (40^0S) hemispheres. These insects have not been reported either in New Zealand or on Pacific islands (Lane, 1993). Sandflies have a wide altitudinal distribution from below sea level in areas adjoining the Dead Sea in Israel and Jordan to 3300meters above sea level (masl) in Afghanistan (Killick-Kendrick, 1999).

1.3. Biology, Ecology and Behavior of phlebotomine sandflies

1.3.1. Biology

The biology of every species of phlebotomine sandflies is peculiar and complex and it covers many aspects, such as life cycle and other activities that have a significant role in the transmission of sandfly borne diseases and vector control (WHO, 2010).

Like all true flies and many other insects, sandflies develop through four distinct life stages: egg, larva (four stages), pupa and adult (Fig 1). Unlike mosquitoes and other subfamilies of Psychodidae, the preimaginal stages of sandflies do not require an aquatic environment to complete development, though they do require relatively warm and moist environment (Claborn, 2010). As the different stages of sandflies are highly affected by the ambient temperature, it is hard to give veracity periods for the development of the stages. After mating and feeding blood or vice-versa, the adult females develop eggs and oviposit average number of 50 eggs individually or in small batches in a suitable habitat such as forest floor, rodent burrows, soils in animal pens and dens, caves, termite hills and cracks under rocks (Magnarelli *et al*., 1984; Lane, 1993; Killick-Kendrick, 1999).

The eggs of sandflies are initially white or light gray in color but often turn to dark brown or black within a few hours of ovipostion, depending on the species. They are banana-shaped, and nearly microscopic in size (0.3-0.5mm). Time to hatch is highly temperature dependent. In 4-10 days the eggs hatch into the next stage (larvae).

Sandfly larvae are caterpillar-shaped with head capsules and small leaf-like antennae. They are mainly scavengers, feeding on organic matter such as fungi, decaying forest leaves, semi-rotting vegetation, and animal feces and decomposing bodies of arthropods. Distinctive caudal setae can help to identify the larvae as sandflies, but larvae are rarely used in taxonomy because very few are ever located in nature (Lawyer and Perkins, 2004). There are four larval instars ranging in size from 0.55mm long in the 1st to about 3.2mm long in the 4th. The 1st instar larvae usually have two long caudal setae, but the rest larval instars have four caudal setae. Diapause occurs in the fourth instar of several species during unfavorable conditions, for instance, the 4th instar of two populations (Addis Zemen and Melka Worer) of laboratory colonies of *P. orientalis* stopped feeding and went into a dormant phase owing to the poor quality of food provided (Seblova *et al.*, 2013). The duration of the larval instars varies greatly, both between and within species, and is regulated mainly by temperature (Lane, 1993). At low temperature, the period of larval development can be as short as a month, whereas at high temperature the duration of the development can be as long as three months (Tesh and Guzman, 1996). Before pupation, sandfly larvae cease feeding and some species may travel a short distance upward to a drier location.

Pupae resemble a small butterfly chrysalis except that the 4th stage larval exuvium (cases of the exoskeleton) is attached at one end. The exuvium acts as glue which is attached to a solid substrate and holds the pupa upright. After pupation, adults emerge in 4-6 days during the hours of darkness, often just before dawn.

Most adult sandflies are within a size range of 2.5-3.5mms. Depending on the species, color of adult sandflies varies from silvery gray to nearly black. The overall dimensions of male and female are similar, but the two are readily distinguished. The abdomen of the female is round and robust; whereas the male is slender and the terminalia claspettes in side view form a „C" shape. In males, the terminalia rotate through 180^0 in 24 hours immediately after emergence and then they become sexually mature (Lane, 1993; Munstermann, 2004; Lawyer and Perkins, 2004).

Killick-Kendrick (1999) pointed out three basic features which can help to distinguish adult sandflies from other flies. These are, while at rest they characteristically hold their wings at an angle above the abdomen by forming "V" shape; their body and wings are entirely covered by hair; and, when coming to engorge, they typically hop around on the host before settling down to bite.

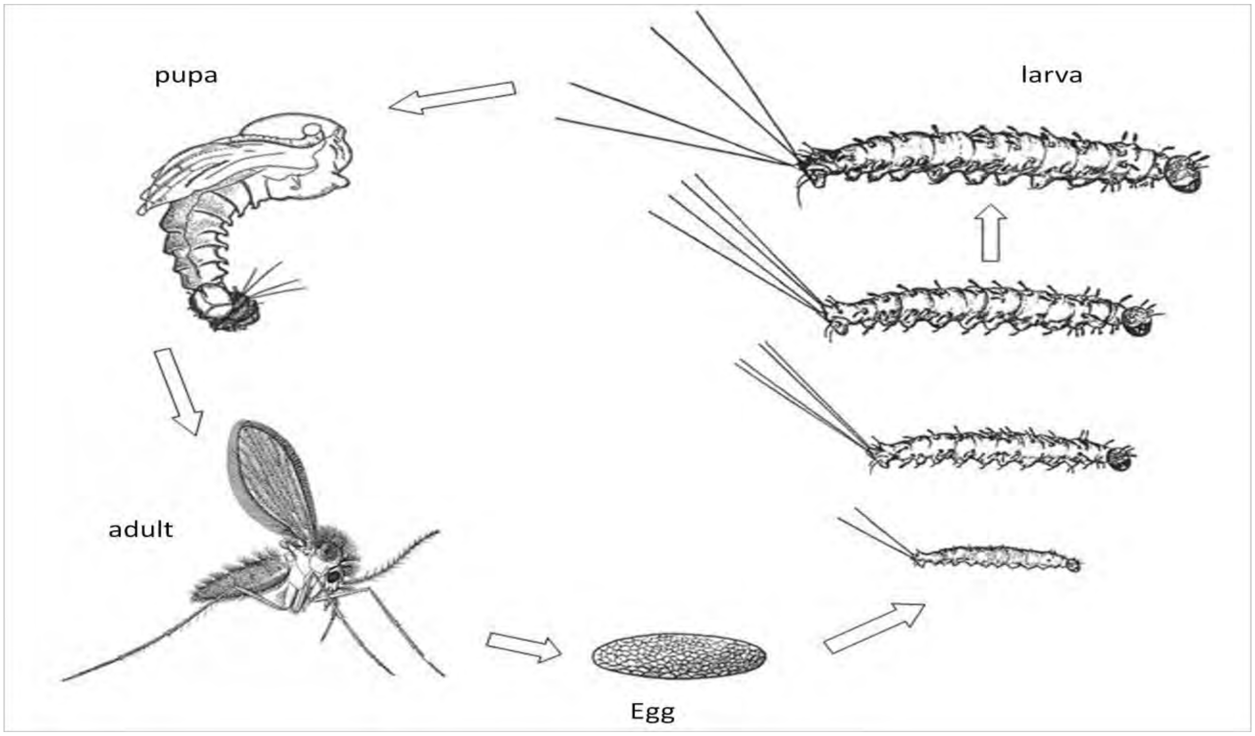

Figure 1. Sandfly life cycle, showing egg, larva, pupa and adult (Lawyer and Perkins, 2004)

1.3.2. Ecology

Breeding and resting sites

Most sandflies use the same sites for breeding and resting (Lane, 1993). However natural breeding sites for most species of sandflies are poorly identified and looking for preimaginal stages in their natural biotopes is difficult, tiresome and proven to be unproductive. As locating the breeding sites of sandflies in nature is hard, control of the immature stages is difficult, if not impossible (Feliciangeli, 2004). Therefore, most control efforts should be entirely dependent on combating full-grown insects (Alexander and Maroli, 2003).

A number of attempts have been made to elucidate the breeding habitats of certain species of sandflies both in the Old and New Worlds although a few numbers of immature stages have been recovered (Feliciangeli, 2004). Among these efforts, some of the works that have been done in the Old World are mentioned below. In Kenya, Mutinga *et al.* (1986) reported animal burrows as the main breeding habitat of *P. duboscqi* (the proven vector of *L. major*) using the soil incubation technique. Killick-Kendrick (1987a) in France found six larvae of *P. ariasi* from rotted manure in a cellar housing goats using soil desiccation. Similarly in India, Singh *et al*. (2008) reported 70 adult sandflies emerging from soil samples (255.3kg) using the soil incubation method. Of these, 59 of them were *P. argentipes* and 11 were *P. papatasi*. The most productive habitat for *P. argentipes* was cattle shed, whereas for *P. papatasi* was human dwelling.

In general, sandflies breed and/or rest in habitats with stable cool temperature and high humidity such as caves, latrines, cellars, stables, tree holes, tree trunks, cracks in the rocks and cavities between boulders, fissures in the ground/tree bark, leaf litter, buildings, termite hills, animal burrow, cattle shed, inside a human dwelling and bird nest (Killick-Kendrick, 1999; Feliciangeli, 2004).

Population dynamics

In order to develop appropriate control strategy against vector species in a particular area, knowledge on the population dynamics of the species over extended periods is essential. The number of generations and population peaks determine the most effective time to mount an attack on the target species (Killick-Kendrick, 1978). Different abiotic factors, including wind, temperature, rain, relative humidity, atmospheric pressure and lunar light affect the population dynamics of vector species (WHO, 2010).

A number of studies have been carried out to determine the seasonal variation of sandflies and the influence of various non-biotic factors on the dynamics of the flies both in the New and Old Worlds. For instance in the New World (Brazil), Fatima *et al.* (2006) reported that females of *Lu. longipalpis* have one peak in May whereas two peaks for males, one in May and a second in October-November. Correlation analysis with various abiotic variables indicated that density of the species positively and negatively correlated with humidity and wind velocity, respectively. Similarly, Oliveira *et al*. (2013) noticed populations‟ peaks of this species during the rainy season whose density showed a reduction as relative humidity decreased and wind velocity increased in the same country.

Different attempts have also been made to elucidate the seasonality of vector species and other sandflies and, their correlation with various environmental variables in different countries of the Old World. In India, Srinivasan *et al*. (1993) investigated the population dynamics of *P. papatasi* from the indoor resting collection. The density of adult decreased during the drier months of January to May and increased following the rainfall in June and July. This indicated that density had positive correlation with rainfall in the area. In contrast, Tarallo *et al*. (2010) reported the peak density of *P. perniciosus*, *P. neglectus*, *P. perfiliewi*, *P. papatasi* and *S. minuta* in the dry months (July and August) in southern Italy. Monthly abundance of flies was positively and negatively associated with monthly mean temperature and relative humidity, respectively. Likewise, Boussaa *et al*. (2005) in Morocco determined the seasonality of *P. sergenti*, *P. papatasi*, *S. fallax* and *S. minuta*. The former species has a single density peak (November) while the rest have two density peaks (November and May). Temperature was the only environmental variables correlated with the density of *P. papatasi* but no significant association was reported. The seasonality of *P. papatasi* was also studied in Egypt where two population peaks (May, July) were observed (Hanafi *et al*., 2007).

Studies have also been conducted on seasonal abundance of East Africa sandfly species. In Kenya, Minter (1964) compared the seasonality of *P. martini* in two ecologically distinct areas (Baringo and Kitui districts). In Baringo, a population peak in density of *P. martini* was observed in June whereas in Kitui in April. This discrepancy was mainly the two areas differ in the pattern of rainfall. Similarly, Basimke *et al*. (1992) observed seasonal variations of *P. martini* and *P. duboscqi* in Baringo. *Phlebotomus martini* had two peaks, the first in December and the second in April, whereas *P. duboscqi* had only one in April. Such variations in the density of sandflies were also attributed by rainfall. Likewise, seasonality of *P. guggisbergi*, vector of CL due to *L. tropica*, was determined in another district of Kenya (Johnson *et al*., 1999). The species was found throughout the year with one peak in December.

In the former southern Sudan, Quate (1964) studied the seasonal dynamics of sandflies of the Paloich area. He categorized sandfly fauna of the area into “seasonal” and “non-seasonal” species. The seasonal species of the area were *P. orientalis*, *P. papatasi*, *S. heischi*, *S. clydei* and *S. schwetzi* and they were prevalent during the dry months of the year and disappeared as the rains started, whereas *S. africana*, *S. antennata* and *S. squamipleuris* were included in the second group and occurred throughout the year. In the same area, Hoogstraal and Heyneman (1969) collected few *P. orientalis* in the first part of the dry season (January to April) and a plethora of flies in the second part of the season (April-June). Elnaiem *et al.* (1997) compared the seasonal distribution of *P. orientalis* between a small village of Umsalala and Dinder National Park in the eastern Sudan. In the village area, the number of flies collected was small and did not show clear monthly fluctuation throughout the year. In the park area, however, *P. orientalis* showed monthly variation with two peaks in density: the short one in January and the larger peak in June.

Studies on the seasonality of sandflies in Ethiopia are quite few. Foster (1972a) investigated the seasonality of *P. longipes* in Addis Ababa and Meta Abo area and he found this species to exhibit bimodal peaks (September-October and May). The density of flies increased immediately following the rains. Gebre-Michael and Lane (1996) assessed the seasonality of *P. martini* and *P. celiae* in Aba Roba area in southern Ethiopia. *Phlebotomus martini* was found in much of the year without distinct seasonal pattern; however, *P. celiae* showed clear seasonality with two annual peaks associated with the wet months of the year (February-May and September-October).

1.3.3. Sandfly Behaviors

Feeding behavior

Both sexes of adult sandflies consume sugar as an essential part of their diet since it is a main source of energy. Sugar is also essential for the development of *Leishmania* parasites in the gut of female sandflies. Although most sources of natural sugars consumed by sandflies are unknown, some species may obtain sugar of plant origin directly by piercing stem, leaves or other parts

(Schlein and Warburg, 1986) or obtain it from aphid or coccid honeydew from the surface of different plants (Killick-Kendrick and Killick-Kendrick, 1987b).

In addition to sugar, almost all female sandflies need a blood meal from vertebrate hosts for the maturation and production of eggs. There are also a few species of sandflies that do not require a blood meal to develop and lay their first batch of eggs (e.g. *P. papatasi*) albeit blood is crucial for subsequent production of eggs. Such behavior is known as autogeny (El Kammah, 1973; Killick-Kendrick, 1999). For non-autogenous sandflies, the frequency of taking blood meal during the gonotrophic cycle varies from species to species. Some species of sandflies take one bloodmeal for each batch of eggs and they are known as gonotrophically concordant (e.g. *P. ariasi*, *P. orientalis*, *P. perniciosus*), whereas females of others species are gonotrophically disconcordant (e.g. *Lu. longipalpis*, *P. argentipes*), which take more than one blood meal for each batch of eggs. In terms of epidemiology, gonotrophically disconcordant sandflies are more efficient vectors as contact of vector and hosts increase due to this behavior of sandfly (Ghosh and Bhattacharya, 1992; Killick-Kendrick, 1999).

Host preferences

Female sandflies feed on a range of vertebrate hosts, including humans, cattle, equines, dogs, rodents, birds and reptiles (Service, 2008). Each species of sandflies has its own specific host preferences, although availability of host is an important determining factor for such preference (Foster *et al.*, 1972b; WHO, 2010). An unequivocal understanding of this behavior of sandflies in general and vector species in particular is fundamental in identifying the natural transmission cycles of sandfly-borne diseases and developing efficacious control strategies in vector control programs (Garlapati *et al.*, 2012).

Various studies on host preference indicated that both Old and New World species of sandflies exhibit varying degrees of preferences, however in general all are opportunistic feeders (Montoya-Lerma and Lane, 1996). For instance, Morrison *et al*. (1993) identified 579 blood meals of *Lu. longipalpis* using precipitin test in rural village of Colombia. The authors reported strong preferences of the species for cow and pig over chicken, equian, human, dog and opossum. In Brazil, varying host preferences of *Lu. longipalpis* was reported by Sant"Anna *et al*. (2008). They analyzed 58 blood-engorged females by polymerase chain reaction (PCR) using FTA databasing and found strong predilection of the species to chicken (>70%) than dogs and humans. Such opportunistic feeding habits of sandflies have also been revealed in the Old World species. In West Bengal in India, Palit *et al*. (2005) analyzed blood meals of 304 *P. argentipes* and 206 *P. papatasi* using enzyme linked immunosorbent assay (ELISA). The authors reported that blood meals sources of 57. 2% of fed *P. argentipes* and 22.8% of fed *P. papatasi* were that of bovine while the rest were from humans and mixed feedings. This indicates that *P. argentipes* is relatively zoophilic whereas *P. papatasi* is anthropophilic. Although *P. argentipes* in West Bengal showed its propensity to feed more on bovine, in Bihar state this species is more anthropophilic as revealed by Cytochrome *b* (cyt *b*) PCR- reverse line blotting (RLB) analysis (Garlapati *et al*., 2012). In Turkey, Svobodova *et al*. (2003) conducted blood meal analysis on 54 fed *P. seregnti* using ELISA and they found 65% of flies fed on chicken and the rest fed on mouse, rat, cattle, sheep/goat, and human in descending order. Recently, in Israel, Valinsky *et al*. (2014) identified twenty-one different hosts from 221 engorged females *P. seregnti* using molecular method. Of these hosts, hyrax was the main (42%) source of blood meal for the flies.

In Africa, a few host preference studies using different serological techniques and molecular methods have been reported. In Ethiopia, Ashford (1970) collected 12 blood fed *P. longipes* from caves colonized by hyrax and porcupine near Kutaber. All of the blood meals originated from hyrax. Foster *et al*. (1972b) analyzed the blood meal of 465 fed *P. longipes* from Meta Abo and Addis Ababa using precipitin test. A number of hosts were reported; viz. bovide, sheep/goat, canidae, equidae, bird and human. Recently, Gebre-Michael *et al*. (2010) analyzed the blood meal sources of 273 freshly fed *P. orientalis* collected from Metema using ELISA. They reported a high zoophilic nature (92%) of this species in the area. Furthermore, Jaouadi *et al*. (2013) in Tunisia determined blood meal origin for 146 blood-fed four *Phlebotomus* spp. using molecular method. *Phlebotomus sergenti* fed on sheep and horse only, whereas *P. perniciosus, P. longicuspis* and *P. papatasi* had diverse sources of blood.

Dispersal behavior

As compared to other nematocerous biting flies, sandflies appear to be weak fliers. They are travelling at short hops rather than sustained flight in search of a host, sugar meal, a mate or resting and ovipostion sites. Such hopping flight behavior of sandflies has been the basis for the assumption that these flies are incapable of flying far from their breeding sites. However, Killick-Kendrick *et al*. (1984) pointed out that a single female *P. ariasi* was able to move further than 2km in a field experiment using mark-release-recapture techniques in south France.

Dispersal ability of sandflies is influenced by a number of factors. The first factor is the type of habitat in which sandflies inhabit. The impact of such factor is clearly observed in the species of Old and New Worlds. The Old World sandflies, most of the times, inhabit in open habitats and can disperse greater distance. For example, *P. orientalis* in flat open *Acacia* woodlands in Sudan flew as far as 730m (Quate, 1964). In contrast, sandflies of the New World inhabit rainforest habitats. In this habitat, sandflies exhibit regular vertical movement in addition to horizontal movement patterns. Owing to such kinds of movements, sandflies of this region can only disperse a short distance from their breeding sites. For instance, studies in Panama (Chaniotis *et al.*, 1974) and Colombia (Alexander, 1987) revealed that sandflies were recaptured no farther than 200m from their release point as majority of the flies were recollected at distances less than 100m. The other factor which determines the dispersal of sandflies is the wind speed. Although slight air movement is important for detection of host odour, wind speeds of greater than 1.5m/s preclude flight and totally halt activities even in light winds of 4-5m/s (Lane, 1993). In addition to these factors, sex differences might be also an important factor for dispersal as female sandflies move a longer distance from the breeding and resting sites in search of either sugar meal, blood or both (Yuval *et al.*, 1988).

Nocturnal activity patterns

Sandflies are crepuscular or nocturnal in their activity (Killick-Kendrick, 1999). Nocturnal activity consists of many distinct components, principally searches for blood meals, sugar meals, mates and breeding sites (Yuval and Schlein, 1986) though such activity patterns vary greatly from species to species. Identifying species-specific patterns provide important epidemiological information for vector incrimination and development of appropriate control tools (El Said *et al.*, 1986). Like other insects, this activity of sandflies is probably controlled by a number of factors such as an internal biological clock, daily changes in light intensity and other abiotic factors (temperature, relative humidity, rain, and wind speed).

The nocturnal activity patterns of sandflies have been recorded for New World species in many studies. For instance, Morrison *et al.* (1995) compared nocturnal activity patterns of *Lu. longipalpis* in the cattle corral and pigpen in Colombia. The activity of the flies increased shortly after sunset and then remained elevated throughout the night in the cattle corral whereas in pigpen the peak activity occurred between 18:30-23:30hours and declined steadily. They also noted differences in activity patterns between sexes as males usually arrive before females at both sites. Similarly, Souza *et al.* (2005) assessed the activity patterns of *Lu. whitmani* and *Lu. intermedia* in a forest and peridomestic habitats in Brazil. *Lutzomyia whitmani* caught in the first habitat was most active in the middle of the night, on the other hand *Lu. intermedia* in the second habitat showed a peak activity between 19:00 and 22:00 hours.

Besides New World species, nocturnal activity patterns of Old World species also have been extensively studied. Roberts (1996) determined such activity of *P. alexandri*, *S. clydei*, *S. fallax* and *S. tiberiadis* in Oman. All of them were nocturnal and most of the flies were collected between 20:00 and 5:00 hours, although the activity patterns among them differ during this period. In India, Dinesh *et al.* (2001) studied the nocturnal activity of *P. argentipes* and the hourly catches of the study revealed that the activity of flies concentrated between 21:00 and 01:00 hours, with a peak just before midnight (23:00-24:00). Females became more prevalent as the night progressed. Unlike *P. argentipes*, *P. tobbi* in Turkey showed no significant difference in activity pattern (Kasap *et al.*, 2009). The study revealed the importance of relative humidity on the activity of the species. In Iraq, Coleman *et al.* (2007) found that more than 80% of the total sandflies collected were active between 20:00 and 02:00 hours. They also observed changes in nocturnal activity of sandflies from season to season. From April to October (cold season), sandflies were active early in the evening, whereas from May to June (hot season) they were active throughout the night.

Like other parts of the Old World, studies on nocturnal activity of African sandfly species have been carried out. Hoogstraal *et al.* (1962) in former southern Sudan noted that the biting activity of *P. orientalis* commenced immediately after sunset (19:00 hour) and increased throughout the night. Similarly, in Paloich area of former southern Sudan, Quate (1964) found a large number of human biting sandflies (including *P. orientalis*) between 21:30 and 22:00 hours. He also reported the influence of wind velocity on the activity of flies since it was ceased when the wind velocity was greater than 1.5m/sec. In Egypt, such behavior of *P. papatasi* and *P. langeroni* was studied by El Said *et al.* (1986). Males and females of the same species showed similar activity patterns. *Phlebotomus papatasi* showed a single peak between 24:00 and 2:00 hours, but *P. longeroni* had two peaks with the highest activity between 2:00 and 4:00 hours. More recent nocturnal activity of sandflies was also investigated in Morocco (Guernaoui *et al.*, 2006a) and observed seasonal variation. For instance, in August (summer), *P. sergenti* ocurred throughout the night with a highly marked crepuscular activity, whereas in October (autumn) its activity was strong until midnight and reached peak between 20:00 and 22:00 hours.

In Ethiopia, a few attempts have been made to elucidate the nocturnal activity of sandflies. Ashford *et al.* (1973a) indicated the biting activity of *P. orientalis* to peak shortly after sunset in Arbaya, northern Ethiopia. According to the authors the activity of this species considerably reduced when the temperature became less than 16^{0}C. Similarly, in southern Ethiopia, Gebre-Michael and Lane (1996) determined the biting cycles of *P. martini* and *P. celiae* in Aba Roba. These species showed peak activity pattern between 20:00 and 22:00 hours.

Resting behavior

After engorgement or feeding, sandflies rest either inside (endophilic) or outside (exophilic) human dwellings. Endophily is a tendency to rest in a shelter (indoor) until the time of oviposition (Lane, 1993). For example, *P. papatasi* exhibits strong endophilic behavior throughout its range (Srinivasan *et al.*, 1993; Orshan *et al.*, 2010; Arroub *et al.*, 2012). In contrast to this species, certain species of sandflies such as *P. sergenti* and *P. orientalis* prefer to rest outdoors in a variety of natural habitats (Hoogstraal and Heyneman, 1969; Elnaiem *et al*., 1997; Orshan *et al*., 2010). Attempts to control flies which exhibit such behavior using conventional control methods (e.g. indoor residual spraying) is not effective. So, knowledge of the resting behavior of sandflies in a particular area is quite important for recommending or suggesting appropriate control methods against a particular vector species.

1.4. Age grading of adult sandflies

Determining the reproductive age of adult insects is a fundamental component of any entomological study, including population dynamics and vectorial role (Ready *et al*., 1984). Age of flies can be studied using different methods. For instance, the age of mosquitoes and other Diptera, parous (those that have taken a blood meal and oviposited at least once) and nulliparous (are young ones that have not taken blood meal and have not laid eggs) can be distinguished by observing the ovarian tracheoles as either coiled or uncoiled (Detinova‟s method) (Detinova, 1962) and by counting the number of follicular relics in the ovarioles (Polovodova‟s method) (Polovodova, 1949). However, such techniques are not fruitful for detecting the age of sandflies as their ovaries do not show obvious changes in tracheolar coiling or follicular dilatations to associate with parity (Anez and Tang, 1997).

Various methods have been employed to discern parous female sandflies from nullipars. One method of aging sandflies involves dissecting and extracting out the ovaries and detecting the presence or absence of granules in the accessory glands (Lewis *et al*., 1970). Although such method has limited success in New World and few Old World species (Foster *et al*., 1970), it is a reliable method of distinguishing the physiological age of female sandflies for the majority of Old World species, particularly in African species (Lewis and Minter, 1960; Gebre-Michael, Pers.

Comm). The other method depends by observing on state of ovaries (Gebre-Michael *et al.*, 1993; Anez and Tang, 1997). In this case, a parous female has large, opaque, pale and stretchable ovaries; whereas nulliparous female has small, clear, refractile, transparent, shiny and fragile ovaries. Additionally, parous flies tend to sink in 0.7% saline solution whereas nulliparous flies remain to float (Ashford, 1974). The presence or absence of crease in the genital atrium (Anez and Tang, 1997) has also been used. Further investigations are needed on the utility of the later two methods. The most reliable method for all species of sandflies is the presence or absence of follicular dilatations/relics in the ovaries (Lewis and Minter, 1960), although multiple dilatations are rarely seen in some sandflies.

1.5. Sandflies and disease transmission

More than 90 species and subspecies of phlebotomine sandflies are a proven or suspected vector of a number of human pathogens (Maroli *et al*., 2013). These species of sandflies belong to either the genus *Lutzomyia* or genus *Phlebotomus*. They are vectors of the bacterium *Bartonella bacilliformis*, several viruses and, most importantly, nearly 20 species of protozoan parasites in the genus *Leishmania*.

1.5.1. Leishmaniasis

Leishmaniasis is an important vector-borne disease caused by obligate intramacrophage protozoa belonging to the genus *Leishmania* of the family Trypanosomatidae (Order Kinetoplastida). These parasitic unicellular protozoans are usually transmitted between vertebrate hosts by the bite of female sandflies. Of the known 30 species and subspecies of *Leishmania*, about 20 species are pathogenic to humans. The disease is endemic in many areas of the tropics, subtropics and Mediterranean basin. Currently, it threatens 310 million people in 88 countries around the world and the majority of these countries are developing countries (WHO, 2014). It affects an estimated

12 million people with approximately 2 million new cases each year (WHO, 2014). Wide ranges of clinical symptoms have been subsumed under leishmaniasis, most notably cutaneous and visceral leishmaniasis (Desjeux, 2004; Chappuis *et al*., 2007).

A. Cutaneous leishmaniasis

Cutaneous leishmaniasis (CL) is the most common type of leishmaniasis. Nearly 95% of CL burden occur in the Americas, Mediterranean basin, Middle East and Central Asia. Throughout the world, between 0.7 and 1.3 million new cases of CL occur annually (WHO, 2014). Two-thirds of these cases come from six countries: Afghanistan, Algeria, Brazil, Colombia, Iran and Syria (WHO, 2014). Cutaneous leishmaniasis can be categorized as either localized cutaneous leishmaniasis (LCL) or diffuse cutaneous leishmaniasis (DCL). LCL is characterized by one or more ulcer (s) or lesion (s) on the exposed parts of the body to the bite of female sandflies. These ulcers usually develop within several weeks or months after the first exposure. DCL occurs in individuals with defective cell-mediated immune response (Desjeux, 2004). Unlike LCL, DCL has no ulcerative nodules but it is characterized by disseminated cutaneous nodules from the initial site of infection and may cover a patient‟s entire body. As compared to LCL, treatment of DCL is quite difficult and patients do not self-cure (Reithinger *et al*., 2007a).

B. Visceral leishmaniasis

Visceral leishmaniasis (VL), also known as kala-azar, is a human systemic disease and it is typically fatal if left untreated, with a mortality rate of 75-95% (Ready, 2014). The disease is characterized by a multitude of clinical manifestations, such as a prolonged undulant fever, weight loss, weakness, poor appetite, anemia, pancytopenia, and abdominal distention with splenomegaly and hepatomegaly (Hailu *et al*., 2005).

Kala-azar is endemic in 70 countries, with a total of 200 million people at risk and an estimated 0.2 to 0.4 million new cases of VL are contracting each year and the disease claims approximately one-tenth of these new cases (WHO, 2014).. More than 90% of new cases occur in six countries: Bangladesh, Brazil, Ethiopia, India, South Sudan, and Sudan (Alvar *et al*., 2012; WHO, 2014).

The etiological agents of VL are two closely related members of *Leishmania donovani* complex: *L. donovani* in East Africa and the Indian subcontinent and *L. infantum* in Europe, North Africa and Latin America. *Leishmania donovani* infects all age groups and its transmission is anthroponotic (human-to-human transmission), whereas *L. infantum* infects mostly children (1-4years) and those weakened by different diseases (e.g. HIV and tuberculosis) and the transmission is zoonotic (canine reservoir host to human transmission) (Chappuis *et al*., 2007).

Next to the Indian subcontinent, the largest kala-azar burden occurs in East Africa. The disease is prevalent in numerous foci in Eritrea, Ethiopia, Kenya, Somalia, Sudan, South Sudan and Uganda. Of these countries, Sudan, South Sudan and Ethiopia are the most affected by VL. In the region, the annual number of cases of VL is estimated between 29,400 and 56,700 (Alvar *et al*., 2012).

Various factors have contributed for such high incidence of the disease in East Africa, such as armed conflicts which have induced massive movements of susceptible or infected populations into VL-endemic or non-endemic areas triggering major epidemics like the one that happened between 1984 and 1994 in the western upper Nile region in South Sudan which claimed an estimated 100,000 people (Seaman *et al*., 1996; Reithinger *et al*., 2007b). Additionally, large scale agricultural development schemes such as in northwest Ethiopia, which usually attracts massive migratory laborers from the surrounding highland areas are also known for the spread of the disease (Mengesha and Abuhoy, 1978; Hailu *et al*., 2006a).

In areas where *L. donovani* transmission prevail, considerable number of kala-azar patients develop macular, maculo-papular or nodular skin rash after successful treatment for VL. Such complication of VL is known as post kala-azar dermal leishmaniasis (PKDL) or dermal leishmanoid. PKDL usually appears 0-6 months in 50% of kala-azar cases in Sudan and six months to three years in India (WHO, 2010). Cases of PKDL other than these countries have low and limited foci. For instance, in Ethiopia almost all cases with such conditions have been reported from northwestern VL endemic areas of the country (such as Libo-Kemkem and the Metema-Humera plains) and the disease is more frequent among HIV patients (Desjeux *et al*., 2013; Diro *et al*., 2014). PKDL patients have been considered as a putative reservoir for anthroponotic VL between epidemic cycles as the nodular lesions of the patients contain many parasites, which can be taken by sandflies (Chappuis *et al*., 2007).

There are a number of sandfly species involved in the transmission of kala-azar in different parts of the world. For instance, in the New World, *Lu. longipalpis* is the principal vector of zoonotic VL and *Lu. cruzi* and *Lu. evansi* are suspected vector of the disease in the region (Ready, 2013). In the Old World a range of species are incriminated as vector of VL in different countries such as *P. argentipes* in Indian subcontinents (Bern *et al*., 2010), *P. perniciosus* in Italy (Rossi *et al*., 2008), *P. ariasi* in France (Killick-Kendrick, 1990), *P. martini* in Kenya and Ethiopia (Perkins *et al*., 1988; Gebre-Michael and Lane, 1996) and *P. orientalis* in Sudan and Ethiopia (Ashford *et al*., 1992; Hailu *et al*., 1995; Elnaiem *et al*., 1998).

1.6. Vector incrimination

Of known species of sandflies, a few sandfly species play a role in the transmission of human leishmaniasis. This might be due to the fact that the majority of the flies may never have anthropophilic behavior; their distribution may not coincide with a reservoir host (for zoonotic type of transmission); their feeding preferences may not include a reservoir host; or they may be unable to support the development of the parasites (Killick-Kendrick, 1999; WHO, 2010).

Incrimination of a species as a vector of *Leishmania* proceeds in five widely accepted criteria, more of analogous to Koch"s postulates (Killick-Kendrick, 1999; WHO, 2010). These criteria were first proposed by Killick-Kendrick and Ward (1981) and then Killick-Kendrick (1988) cited in Killick-Kendrick (1990) presented them in simplified forms and ways of studying the criteria. The basic criteria for incriminating a certain species of sandfly as *Leishmania* vectors are the following; 1) the vector must feed on humans. Exclusive anthropophily is never the case as most sandflies feed on a range of hosts. Such anthropophilic behavior of flies can be determined either by human-bait catch (to collect flies at night as they come to bite humans) or by analysis of blood-meals in female flies using serological methods such as ELISA. 2) In zoonotic type of leishmaniasis, the vector must also bite the reservoir host (s) of the parasite. 3) The promastigotes isolated from the wild caught sandflies will be indistinguishable from isolates from patients, and this must be ascertained by comparison of isolates from dissected wild caught sandflies with that of the patient"s parasite using isoenzymes or molecular methods. 4) The vector must support the complete development of the parasite after an infected blood-meal has been digested and voided. 5) The vector must be able to transmit the parasite by the bite of a susceptible host while taking a blood-meal (Killick-Kendrick, 1999; WHO, 2010; Maroli *et al*., 2013).

Besides the above mentioned basic vector incrimination criteria, recently Ready (2013) proposed two more new criteria. According to the author, these new criteria reflect the desirability of demonstrating that a species is actually an important vector in a specific focus, by modeling its role and showing the direct effect of control on transmission. The first criterion is using retrospective data; mathematical modeling demonstrates that the vector is essential for maintaining transmission with or without the involvement of other vectors, and the other criterion is mathematical modeling based on a planned control program demonstrating that the disease incidence significantly decreases following a significant decrease in the biting density of the specific vector. However, none of the vectors described so far fulfills these requirements.

1.7. Control of phlebotomine sandflies

The rationale behind the control of sandflies is to alleviate or interrupt transmission of sandfly-borne diseases. As the breeding habitats of the immature stages of sandflies are hard to locate, control methods of sandflies are entirely directed to the adult stage (Alexander and Maroli, 2003). Prior to developing control mechanisms against the adult sandflies, adequate knowledge on the local epidemiology of the disease (anthroponotic or zoonotic), and also the vector species involved, its habitats (peridomestic or sylvatic), host feeding preferences (anthropophilic or zoophilic), resting habits (endophilic or exophilic), nocturnal activity and seasonality should be taken into account (WHO, 2010). Currently available control methods of sandflies are chemical controls, environmental management and prophylactic.

1.8. Leishmaniasis in Ethiopia

Leishmaniasis is one of vector-borne diseases of major public health problem in Ethiopia. Both CL and VL are known in the country with endemic areas of the diseases are continually spreading (Hailu *et al*., 2006a).

Cutaneous leishmaniasis

In Ethiopia, CL was first described in 1913 by Martoglio (Ashford *et al*., 1973b). Since then, the disease has affected thousands of people in the highland parts (1400masl-2900masl) of the country such as Aleku, Kutaber, Ochollo, Sebeta (Lemma *et al*., 1969; Ashford *et al*., 1973b; Gemetchu *et al*., 1990), lower Omo valley (Fuller *et al*., 1979), Sidamo (Lindtjorn, 1981), Silti (Negera *et al*., 2008), Addis Ababa (Lemma *et al*., 2009), Libo-Kemkem and around Debre-Tabor area (Personal Observation).

Annually, about 20,000-50,000 new cases of CL are estimated to occur in the country and the majority of the cases are children between 10 and 15 years of age (Alvar *et al*., 2012; Deribe *et al*., 2012). The most important cause of CL in Ethiopia is *L. aethiopica* in the highlands (Hailu *et al*., 2006a) while in the lowlands *L. tropica* and *L. major* rarely cause CL (Hailu *et al*., 2006b). The disease manifests in three clinical forms: localized, mucosal and diffuse cutaneous. It has zoonotic type of transmission with two hyrax species (*Procavia habessinica* and *Heterohyrax brucei*) as the main reservoir hosts for *L. aethiopica* (Bray *et al*., 1973; Ashford *et al*., 1973b).

Visceral leishmaniasis

In Ethiopia, VL was first discovered before seven decades ago by Cole *et al*. (1942) in the lower Omo valley. Like other East African countries, the disease is caused by *L. donovani* (Hailu *et al*., 2006a). Unlike CL, the transmission cycle of VL in the country is believed to be anthroponotic although some studies suggested zoonotic transmission (Ayele and Ali, 1984; Malaria consortium, 2010). Estimated 3700-7400 new cases of kala-azar are reported every year (Alvar *et al*., 2012). Kala-azar is largely restricted in the lowlands of the country, albeit the disease shows variation in magnitude in the endemicity from place to place (Hailu *et al*., 1995). The disease is prevalent in northwest, south and southwest, southeast and northeast of Ethiopia (Malaria consortium, 2010).

The most important foci of VL in north western Ethiopia bordering with Sudan are the Metema and Humera lowlands where the disease accounts approximately 60% of the total cases in the country (Hailu *et al*., 2006a). The first report of VL cases in the northwest was by Tekle *et al*. (1970) who diagnosed agricultural migrant workers at Metema and Humera. Following this, Fuller *et al*. (1976a) tested 1057 farmers and non-farmers using leishmanin skin test (LST) in the Setit-Humera and they found 45-60% of farmers and 8-30% of the non-farmers were skin test positive. Two years later, Mengesha and Abuhoy reported a high mortality (55.6%) rate in 27 kala-azar patients from the Metema-Humera lowlands, most of whom were labour migrants. Also, Maru (1979) described clinical and laboratory features of 18 VL patients who had travel history to the lowlands from surrounding highland areas as seasonal workers. Hailu *et al*. (2006a) reviewed the most recent outbreak of kala-azar, which started in 1995 and continued until 2004 in Humera. The disease claimed the lives of about 100-200 temporary farm laborers mainly from Maykadra village. Similarly, in Metema from 2008-2013, 1189 primary cases (1148 males and 41 females) were admitted and treated in Metema hospital. Of these, 69 died due to the disease (Metema Hospital unpublished data).

While VL is highly endemic in the lowland parts of the northwest Ethiopia, there are also reports of the disease from highland areas of this region. For instance, Ashford *et al.* (1973a) reported six cases of the disease from Belessa, which lies at 1800 masl, in Amhara regional state. However, only two of them were autochthonous cases. The rest were believed to have travelled either Metema or Humera as seasonal migrant laborers and contracted the disease while they were working. More recently, an epidemic of the disease occurred in other highland districts of the Amhara regional state, in Libo-Kemkem and Fogera (average altitude 2,000 masl). The outbreak started in 2003 in Bura (one of the villages in Libo-Kemkem) and expanded to the neighboring villages and subsequent cases of the disease peaked in 2005. During the epidemic the lives of more than 200 people were claimed. By 2007, more than 2500 primary cases and 200 deaths were reported by Addis Zemen health center since the start of the epidemic. Still now VL is a major public health problem for the inhabitants (Alvar *et al*., 2007; Herrero *et al*., 2009; Gebre-Michael *et al*., 2007; Bashaye *et al*., 2009).

Other important foci of VL are found in the south and southwest of Ethiopia. Coles *et al.* (1942) reported 31 cases of VL in military battalion, who had travel history to the lower Omo. Similarly, 136 cases of VL were reported in a brigade stationed in northern Kenya (Anderson, 1943 cited in Ali and Ashford, 1994). Of these cases, 11 of them had acquired the disease inside Ethiopia on the Moyale-Addis Ababa road, although the place of infection was not revealed. In the same area (lower Omo), Fuller *et al.* (1979) found higher positive skin tests (>64%) in three (Dassanetch, Nyangatom, and Kerre) of the seven tribes, who had been examined by LST. In addition, two autochthonous cases were also found. Such high LST rates in the lower Omo were also corroborated by Hailu *et al*. (1996). In the same way, Ali and Ashford (1993) tested the people of Aba Roba (another focus area of VL in southwest) and found 34.6% positive skin test. In the same area, Ali and Ashford (1994) recorded 142 new cases of VL. Besides to the above mentioned foci of VL in south and southwest, sporadic cases have been reported from Woyto, Gelana and Dawa valleys in the same region (Lindtjorn and Olafsson, 1983; Ayele and Ali, 1984; Lindtjorn, 1984; Lindtjorn, 1987).

In addition to the northwest and southwest foci of VL, endemic areas of kala-azar are found in southeast and northeast parts of Ethiopia. In the former area, Marlet *et al*. (2003) reported seven active cases of VL in Afder and Liben zones of the Somali region. All of the patients were children, which might indicate prior endemicity of the disease in the areas. In northeast Ethiopia, VL occurs sporadically and in association with HIV-co-infection (Hailu *et al*., 2006a). Active case findings by several investigators (Fuller *et al*., 1976b; Ali, 1997; Ali *et al*., 2002:2004) did not document VL cases, although LST rates were very high (average rate of 40-59.5%) in the indigenous Afars. It is difficult to explain why the disease has remained sporadic despite the high rates of leishmanin reaction and presence of abundant population of the potential vector (*P. orientalis*) (Gebre-Michael *et al*., 2004a).

1.9. Vectors of leishmaniasis in Ethiopia

There are six subgenera of *Phlebotomus* sandflies (*Adlerius*, *Anaphlebotomus*, *Larroussius*, *Paraphlebotomus*, *Phlebotomus* and *Synphlebotomus*) found in Ethiopia. These subgenera consist of twenty species. The largest of these is the subgenus *Larroussius* comprising at least seven species. Of the twenty species, at least eight *Phlebotomus* spp. are known or implicated to transmit either of the two forms of leishmaniasis in the country. These are *P. orientalis*, *P. martini*, *P. celiae*, *P. pedifer*, *P. longipes*, *P. duboscqi*, *P. sergenti* and *P. saevus* (Ashford *et al*., 1973b;

Gebre-Michael *et al*., 1993; Hailu *et al*., 1995; Gebre-Michael and Lane, 1996; Gebre-Michael *et al*., 2004a).

Vectors of visceral leishmaniasis

Phlebotomus orientalis, *P. martini* and *P. celiae* are vectors of kala-azar in Ethiopia. The former one was incriminated as vector of VL in the lower Omo plains by Hailu *et al*. (1995) following detection of *L. donovani* from a single specimen of *P. orientalis* by molecular means, though further studies are needed to fulfill essential criteria for vector incrimination (WHO, 2010). The species has long been suspected as a vector of the disease in the region based on its man biting behavior and previous evidence from Sudan (Gemetchu *et al*., 1976). Furthermore, based on circumstantial evidence, *P. orientalis* has strongly been suspected as a vector of VL in many other endemic foci of the country, including the Metema-Humera plains, Awash valley, Belessa valley and Libo-Kemkem, although this has never yet been confirmed, despite several investigations (Gemetchu *et al*., 1975; Fuller *et al*., 1976a; Ashford *et al*., 1973a; Gebre-Michael *et al*., 2007: 2010).

Detailed entomological investigations conducted in a highly endemic VL focus in the Segen valley (Aba Roba) of southwest Ethiopia, *P. martini* and it close relative *P. celiae* were confirmed as the vectors of VL based on dissection/isolation and subsequent characterization of the parasites by isoenzyme analysis or DNA probes (Gebre-Michael and Lane, 1996). The infection rate was 0.7 % (16/2326) in *P. martini* and 0.3% (3/1044) in *P. celiae*. Based on this and other observations, the authors concluded *P. martini* is the primary vector where as *P. celiae* is the secondary vector of the disease in the area. A closely related species, *P. vansomerenae*, has recently been recorded (together with *P. martini* and *P. celiae*) from Liben district, southeastern Ethiopia (Gebre-Michael *et al*., 2013). One or more of these species are regarded as important vectors of VL in much of the south and southwest Ethiopia except the Omo valley (Balkew *et al*., 1999; Yimer, 2010; Mohammed, 2010; Gebre-Michael, unpub data). These three species are also associated with VL transmission in Kenya, *P. martini* playing the major role (Perkins *et al*., 1988; WHO, 2010).

Ecologically, these vector species are found in two distinct settings (Gebre-Michael *et al*., 2004b). The first is in the semi-arid regions in the north, northwest and also in southwest where *P. orientalis* has strong association with *Acacia seyal-Balanites aegyptiaca* forests. These forests in turn are linked with deeply cracking „black cotton soil (vertisols)". Such type of soil is rich in montmorillonite; this is 2:1 lattice clay mineral with an exceptionally high cation exchange capacity. When the mineral gets excess water during the rainy season it expands and then contracts when it loses its water during the dry season resulting in very deep crack, creating essential micro-habitats for *P. orientalis* (Hoogstraal and Heyneman, 1969; Fuller *et al*., 1979; Thomson *et al*., 1999; Gebre-Michael *et al*., 2004b). The second type of ecology is found in southern Ethiopia where *P. martini, P. celiae* and *P. vansomerenae* have strong association with termite mounds of different types, constructed by *Macrotermes subhyalinus* or *M. bellicosus* (Minter, 1964; Gebre-Michael *et al*., 2013). The ventilation shafts of these termite mounds are believed to provide ideal breeding and resting habitats for the species.

Vectors of kala-azar do not only show variation in terms of ecological preferences, but also show contrasting seasonal difference. Studies on the seasonality of *P. orientalis* in Ethiopia and in neighboring Sudan (Quate, 1964; Hoogstral and Heyneman, 1969; Gemetchu *et al*., 1975; Elnaiem *et al*., 1997) revealed that *P. orientalis* is a seasonal species and mainly prevalent in the low precipitation period of the year (November-May). Unlike *P. orientalis*, its counterparts (*P. martini,* *P. celiae* and *P. vansomerenae*) are more prevalent during the rainy months of the year (September-October; March-May) (Minter, 1964; Wijers and Ngoka, 1974; Gebre-Michael and Lane, 1996).

Vectors of Cutaneous leishmaniasis

Phlebotomus pedifer, *P. longipes*, *P. duboscqi*, *P. sergenti* and *P. saevus* are responsible for transmitting different *Leishmania* spp., which are causing various forms of cutaneous leishmaniasis in Ethiopia. *Phlebotomus pedifer* and *P. longipes* are known to transmit *L. aethiopica* in the highland of the country (Ashford *et al*., 1973b; Gemetchu *et al*., 1990). Another vector of CL due to *L. major* in the country is *P. duboscqi*, from which the parasites were isolated and characterized in southern Ethiopia (Gebre-Michael *et al*., 1993), although the parasite appears to be rare in Ethiopia. In addition, *P. sergenti* and *P. saevus* are implicated as vector of CL due to *L. tropica* in the Awash valley (Gebre-Michael *et al*., 2004b). The authors also isolated *L. aethiopica* from *P. sergenti* in the same locality. Cutaneous leishmaniasis caused by *L. tropica* is also rare in Ethiopia.

1.10. Statement of the problem

As indicated above VL has been known in the Metema lowland, northwestern Ethiopia, for a long time and remains as a major health problem in the area (Hailu *et al*., 2006a). Kala-azar, in this area, is mainly linked with the influx of non-immune laboures from surrounding highland regions to the extensive agricultural development schemes in the area (Mengesha and Abuhoy, 1978). In addition to this lowland area of the region, VL recently has spread to the highland area of the region mainly in Libo-Kemkem district (Alvar *et al*., 2007). Different entomological studies have been conducted in both lowland (Metema) and highland (Libo-Kemkem) areas of the region

(Gemetchu *et al.*, 1976; Gemetchu *et al.*, 1983; Gebre-Michael *et al.,* 2007:2010). However, these studies were based on short duration and lacked detailed investigations regarding species composition, feeding behavior, population dynamics, habitat preferences, indoor resting habits, nocturnal activity and role of *Phlebotomus* species in the transmission of the disease in these endemic areas. Based on these knowledge gaps the following objectives were set.

1.11. Research Objectives

1.11.1. General Objective

The overall objective of the studies outlined herewith was to identify the phlebotomine vector (s) of kala-azar, study the ecology, behaviors and dynamics of the vector (s) in relation to VL transmission in the endemic areas of Metema and Libo-Kemkem districts for the management of the disease in both areas.

1.11.2. Specific Objectives

- To determine and to compare species composition, relative abundance and indoor resting habits of phlebotomine sandflies in the study areas
- To determine and to compare population dynamics and habitat preferences of *Phlebotomus orientalis* in the study areas
- To determine and to compare physiological age, host preference and vector potential of sandflies in the study areas
- To determine and to compare the nocturnal activity pattern of *P. orientalis* in the study areas

Chapter Two. General Materials and Methods

2.1. Description of the study areas

The study was conducted in two ecologically distinct areas of Amhara region in northwestern Ethiopia, namely Libo-Kemkem and Metema districts. The former is situated in a highland and the latter in a lowland area. The distance between the two areas is approximately 255kms. In both localities, kala-azar is a major public health problem.

2.1.1. Libo-Kemkem district

In Libo-Kemkem, entomological investigation was carried out for one year from May 2011 to April 2012. The district is found about 645km north of Addis Ababa on Addis-Gondar main road and 65km north of Bahir Dar. It is located at $12^0$04'N of latitude, 37^0 45'E of longitude and average altitude of 2000 masl. Addis Zemen is the main town of the district. The mean annual temperature of the area is 20.3^0C. The mean maximum and mean minimum monthly temperatures of the district is 24.4 ^{0}C in April and 19.4^0C in August, respectively. The area receives annual rainfall of 1350mm. It has unimodal type of rain. The rainy season often starts at the mid of May and lasts until September. During this period heavy and continuous rain is common. The heavy rain, especially from surrounding mountainous area of the district causes over flooding leading to water-logging in the majority of the flat-plain as much of these flat-plains contain vertisol (black cotton soil), which cracks during the dry season and expands when the rain commences.

The natural vegetation coverage of the area (mostly *Acacia seyal*) has been immensely reduced mainly for agricultural purpose, construction of houses and fire wood. This natural vegetation has been replaced by *Eucalyptus* trees. Nowadays, there are a few scattered clumps of *Acacia* spp. including *A. seyal* in the area.

Based on the 2007 national census conducted by the Central Statistical Agency of Ethiopia (CSA), this district had a total population of 176, 381, which reside in 32 sub districts (*Kebeles*) (CSA, 2007). The inhabitants of the district belong to Amhara ethnic group. Most of the people live in huts constructed of mud walls and thatched roofs with some living in iron-roofing. Agriculture and allied activities are the most important source of subsistence for the majority of the population. They principally produce *teff*, maize, millet, bean, sunflower, rice and cotton during the main rainy season. During the dry season, they also produce onion, tomato and pea in irrigated fields, though in limited scale. They also raise a large number of livestock, including cattle, sheep, goats and poultry. Furthermore, sand mining from *Sheni* River, which bisects the district, is an important source of income for a few people.

For the entomological study, three villages of the district were selected mainly based on previous reports of VL cases in the district. These villages were Angot (N 12^{0}04.912‟/ E 037^0 45.088‟), Bura (N 12^{0}02.606‟/ E 037^0 44.882‟) and Yifag (N 12^{0}05.126‟/ E037^{0}43.349‟) (Fig 2). The largest population is found in Angot (n=7922) followed by Bura (n=5962) and Yifag (n=4071). The villages are located approximately 5, 12 and 10km south of Addis Zemen, respectively. The distance between these villages varies from 5 to 7kms. Angot and Bura are typical type of rural villages and they are situated in flat-plains which comprised of mainly vertisol whereas Yifag is more of semi-rural village found in rocky hills with much of the soil being alluvial clay soil. During the dry season (October-April), adult men usually sleep outdoor for guarding their cattle from theft.

In addition to domestic animals, wild rodents (presumably *Arvicanthus niloticus*) were occasionally observed around small animal burrow and soil cracks.

2.1.2. Metema district

Field studies were also conducted in Metema district for one year (October 2012 - September 2013), following the study in Libo-Kemkem. The district is located about 860km northwest of Addis Ababa and 180km southwest of Gondar city. It has international boundary of more than 60km distance between Ethiopia and Sudan. It lies at latitude of 12^0 46‟ N, longitude of 36^0 24‛E and altitudes between 700 and 750 masl. The district has unimodal rainfall, with annual rainfall ranging between 850 and 1000mm. The rain starts in June and extends until the end of September. Unlike Libo, rainfall in Metema can be erratic. The dry season of the area starts in October and extends until the end of May. During this period, the temperature usually ranges between 27^0C and 32^0C and the daily maximum temperature reaches 41^0C in April. During this time, the majority of the people sleep outdoors because of the hot weather as well as to look after cattle, which are usually kept in open enclosures. The soil type in the area is predominately black-cotton soil.

Like Libo-Kemkem, the previous natural and dominant vegetations (*Acacia seyal-Balanites aegyptiaca* forest) of the area have been largely cleared for large scale mechanized agricultural production of cash crops such sesame (*Sesamum indicum*) and cotton (*Gossypium* sp.), and staple crops (*teff,* maize and sorghum) and also for other purposes such as fire wood, charcoal production and construction of houses and fences. At present time, the characteristic vegetation of the area is wooded savannah which is alternating with grasslands. The main trees are *Acacia syeal*, *Balanites aegyptiaca*, *Zyzyphus spina-christa* and other trees and bushes. In addition to these, the introduced neem (*Azadiracta indica*) plants are abundantly available as roadside plantations in the towns, and as shade plants in the rural areas.

According to 2007 CSA census, the total population of the district was 110,252, which reside in 19 *kebeles* (CSA, 2007). The administrative center of the district is *Gende Wuha* (Shehdi) town. The human population of Metema belongs to many ethnic groups, most of whom have come from various droughts affected areas of the country mainly from highlands (Wello, Gondar, and others). The five largest ethnic groups of the area are Amhara (78.9%), Qemant (10.3%), Tigrayan (7.0%), Gumuz (2.1%) and Agaw Awi (1.3%). The majority of the people live in huts constructed of mud walls and thatched roof with some living in houses covered with iron-corrugated sheets. Besides production of cash and staple crops, farmers in Metema produce fruit tree (Mango, Papaya and other) and vegetables on a small scale. Charcoal production is also an important source of income for Gumuz people.

The domestic animals of the area are mainly cattle, goats, sheep, donkeys, dogs and chicken. Wild animals known to occur in the area are warthog, jackal, hyena, monkey, mongoose, squirrel, wild rodents, and porcupine.

For entomological study, three villages were randomly selected. These villages were Aftit (N 12^{0}48.367‟/ E036^{0}21.307‟), Kokit (N12^{0}51.938‟/ E 036^{0}16.264‟) and Mender-6 (N12^{0}55.206‟/ E 036^{0}15.204‟) (Fig 2). A total of 3542, 6864 and 3771 people were living in Aftit, Kokit and Mender-6, respectively. The villages are situated approximately 8, 20, and 35km north of Gende Wuha. The distance between these villages varies approximately from 12km to 20km.

2.2. Entomological studies

2.2.1. Sandfly sampling and preservation in the field work

2.2.1.1. CDC light traps

CDC light traps (John W. Hock, Gainesville, Florida, USA) were used to collect nocturnally active phlebotomine sandflies. The trap was connected to 6 volts rechargeable batteries and sandflies cages. During the sampling night, it was hanged 30-50cm above the ground from dusk to dawn

2.2.1.2. Sticky trap

Sandflies were also sampled using sticky traps (STs) of white A4 sized polypropylene (21cm x29.7cm) smeared on both sides with sesame oil. During the sampling night, the traps were placed horizontally from dusk to dawn. Captured sandflies were separated from other insects and preserved in absolute alcohol for identification.

2.2.2. Mounting and identification of sandflies

For species identification, the head, tip of the abdomen and the rest parts of dissected sandflies (see below), and male and undissected female *Phlebotomus* and *Sergentomyia* spp. (both male and female) were individually mounted on a slide with the head ventral-side uppermost in Hoyer mountant and kept aside. Identification of the species was made by examining male genitalia, and the female spermathecae and pharynx according to the morphological keys of Quate (1964) and Abonnenc and Minter (1965). Additional morphological keys of Lane and Fritz (1986) and Gebre-Michael and Medhin (1997) were also used to separate sympatric species of the subgenus *Phlebotomus* in Metema.

2.2.3. Meteorological Data

In order to observe the relation of weather variables with the monthly abundance of sandflies, total rainfall, minimum and maximum temperatures for each month for the years 1997-2012 (Appendices 1, 2 and 3) for Libo and 1999-2013 (Appendices 4, 5 and 6) for Metema, and relative humidity for each month from 2004-2013 for Metema (Appendix 7) were obtained from the National Meteorological Services Agency of Ethiopia Bahir Dar branch**.**

2.4. Ethical Approval

The project was approved by National Research Ethics Review Committee. Ethical clearance was obtained from Addis Ababa University, Medical Faculty, Department of Microbiology, Immunology and Parasitology. The study was part of a larger study on VL transmission dynamics supported by the Bill and Melinda Gates Foundation: “Studies on the ecology and transmission dynamics of visceral leishmaniasis in Ethiopia”. In addition, formal consent was obtained from owner of the house to collect sandflies using sticky traps and pyrethrum space spray catches inside their homes.

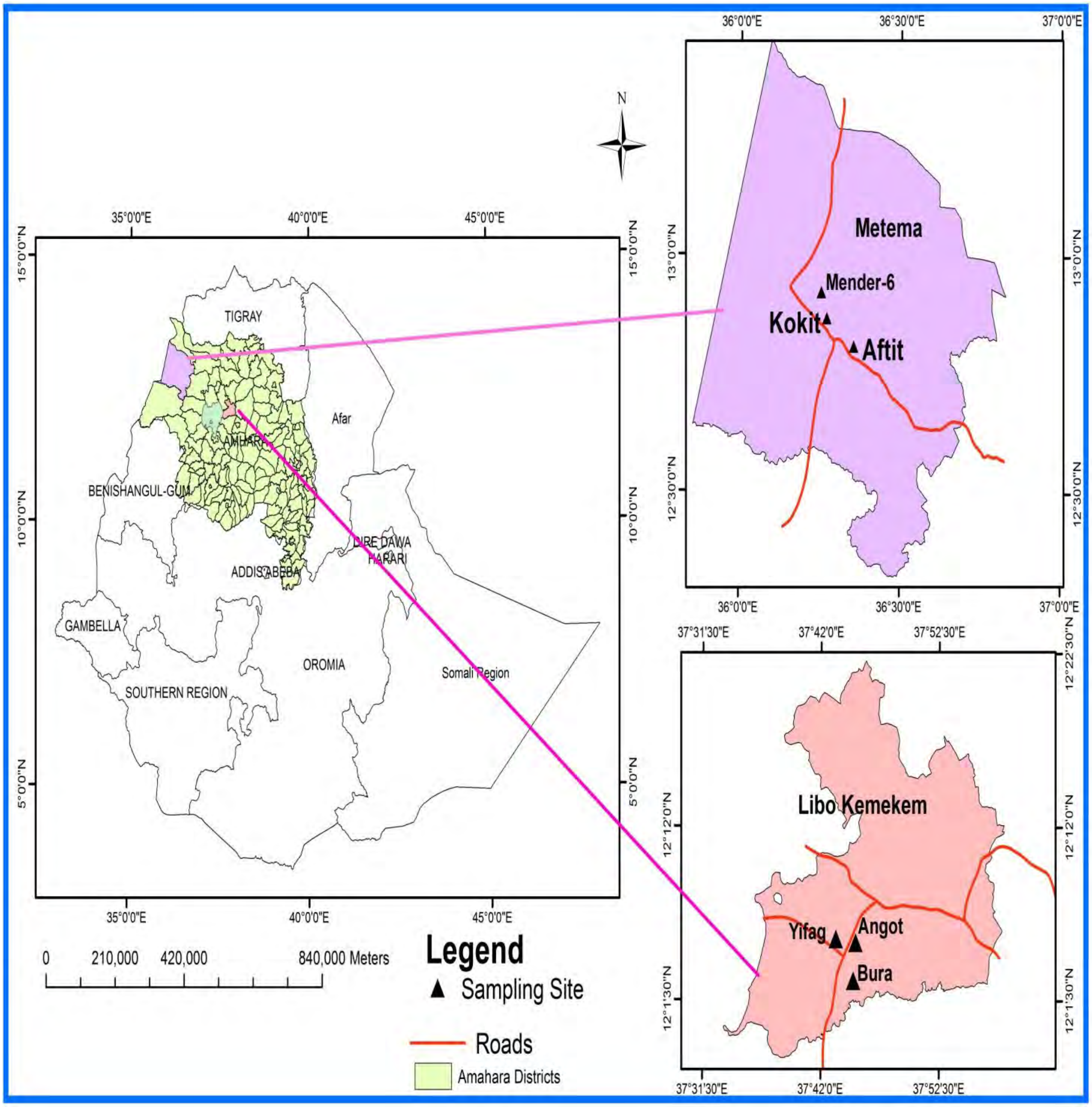

Figure 2. Map of the study villages in Libo-Kemkem and Metema districts northwestern Ethiopia

Chapter Three. Studies on species composition, relative abundance and indoor resting habits of phlebotomine sandflies in the highland and lowland foci of visceral leishmaniasis, northwest Ethiopia

3.1. Introduction

Visceral leishmaniasis (VL), also known as kala-azar, is a human systemic disease caused by *Leishmania donovani* (Chappuis *et al*., 2007), and typically left untreated with a mortality rate of 75-95% (Ready, 2014). The pathogen is transmitted from human to human or from animal to human by the bite of female sandflies. A total of 200 million people are at risk of the disease in 70 countries, and annually between 0.2 and 0.4 million new cases of VL are reported (Alvar *et al*., 2012). More than 90% of these cases occur in six countries: Bangladesh, Brazil, Ethiopia, India, South Sudan, and Sudan (WHO, 2014).

In Ethiopia, the foci of VL are highly confined in the lowlands although the endemicity of the disease shows variation from place to place (Hailu *et al*., 1995). The disease is prevalent in northwest, south and southwest, southeast and northeast of the country (Malaria consortium, 2010).

The most important foci of VL in Ethiopia are the Metema and Humera lowlands (northwest) bordering with Sudan where the disease accounts for approximately 60% of the total cases (Hailu *et al*., 2006a). In these plains, the disease is particularly associated with influx of non-immune seasonal laborers from the surrounding highland regions to the extensive agricultural development areas here in the lowlands. The first report of VL cases in the northwest was that of Tekle *et al*. (1970). Since then several cases have been reported from both areas (Mengesha and Abuhoy, 1978; Maru, 1979; Hailu *et al*., 2006a). During the last five years alone (2008-2013), 1189 primary cases (1148 males and 41 females) were admitted and treated in Metema hospital. Of these, 69 died due to the disease (Metema Hospital, unpublished data). Obviously, these numbers would have been much more if it were to include those treated in nearby hospital (Gondar Hospital) or elsewhere.

Besides, VL has also spread to highland areas where it was previously rarely known. In Libo-Kemkem district VL was not known prior to 2005. Since the start of the epidemic in 2003 up to 2007, more than 2500 primary cases had been admitted and treated in Addis Zemen Health Center. Of these cases, more than 200 deaths were reported. Since then VL has remained as a major public health problem for the inhabitants of the district and neighboring districts (Alvar *et al*., 2007; Herrero *et al*., 2009; Gebre-Michael *et al*., 2007; Bashaye *et al*., 2009).

Following the first report of VL in northwest Ethiopia, several entomological studies have been carried out in both the lowland and highland foci (Gemetchu *et al*., 1975; Gemetchu *et al*., 1983; Gebre-Michael *et al*., 2007; Gebre-Michael *et al*., 2010). Studies in the lowlands pointed out the presence of six *Phlebotomus* spp. (*P. orientalis*, *P. papatasi, P. bergeroti, P. duboscqi, P. rodhaini* and *P. alexandri*) and various *Sergentomyia* spp.. Whereas the study in the highland focus revealed the presence of two *Phlebotomus* spp. (*P. orientalis* and *P. longipes*) and a few *Sergentomyia* spp. However, these studies were based on short duration; hence the present study was designed to determine and to compare the species composition, relative abundance and resting habits of phlebotomine sandflies in these two ecologically distinct kala-azar endemic areas of northwestern Ethiopia.

3.2. Materials and Methods

3.2.1. Collection of sandflies

Adult phlebotomine sandflies were collected using three sampling techniques. These were CDC light traps, sticky traps and pyrethrum space spray methods (Alexander, 2000; Service, 1993).

A. CDC light traps

In both districts, three permanent sampling habitats were selected for collection of sandflies using CDC miniature light traps (LTs). These habitats were household compound, farm field and mixed forest. The distance between the habitats ranged between 150 to 200m. In each habitat, two LTs were deployed. In the morning, the trapped sandflies were collected with aspirator from cage and killed using chloroform. *Phlebotomus* females were sorted out from the rest (male *Phlebotomus* and male and female *Sergentomyia*) for dissection to detect natural infection and, freshly fed *Phlebotomus* females were also preserved for later blood meal analysis (see Chapter five). The rest of sandflies were preserved in 70% alcohol for later species identification. Sampling using LTs was carried out weekly in Libo (May 2011-April 2012) and bimonthly in Metema (October 2012-September 2013).

B. Sticky traps

Three sampling habitats in each village were selected to collect sandflies using sticky traps. These sampling habitats were indoor, farm-field and mixed forest. For indoor collection, five STs per hut were tied on nylon string in a line about 30cm apart and placed on the wall above sleeping location. In sampling night four huts were used (i.e. a total of 20 STs per night). In each of the other habitats (farm field and mixed forest) similar number of STs with that of indoor collection were deployed horizontally (i.e. 20 STs in farm field and 20 STs in mixed forest). Deployment and retrieval of traps was done at the same time with the LTs. Sandflies collected by this method were removed from STs and stored in absolute alcohol in labeled vials for later species identification.

C. *Pyrethrum space spray catches*

Collection of sandflies using pyrethrum space spray catches (PSCs) was carried out in one village of Libo (Bura) and another village in Metema (Kokit) to determine any endophilic sandflies. In each village five huts were used. PSCs were conducted early in the morning from 6:00-8:00 hrs once a month for six months (November 2011-April 2012) in Bura and seven months (December 2012-June 2013) in Kokit. For this purpose, all occupants, removable objects, exposed food and water were removed from each hut. Doors, windows and any openings were properly closed. The entire floor was covered with white plastic sheets (4×3m), after which an aerosol insecticide (Roach-Killer-M/s Kafr Elzayat, Maybanz Plc, Egypt) containing Fenitrothion, Cypermethrin, and Bioallethrin locally purchased was sprayed for about 5min inside the hut. After spraying, the operator waited outside, hut remained closed for 15min to produce a knockdown effect. Then, the white plastic sheet was taken outside and all knocked down sandflies were collected using forceps and placed in 70% alcohol for species identification (Service, 1993).

3.2.2. Data Analysis

Prior to data analysis, sandfly numbers were log-transformed [log (n+1)] to fit normal distribution and checked for normality by Shapiro-Wilk test. When trapping data did not conform to the normal distribution, the non-parametric equivalent test of Mann Whitney U-test was made to compare the sex ratio in sampling methods. Ecological comparisons between the study districts and between the villages in the district were performed using the following indices:

Shannon-Wiener index: $H' = -\sum_{i=1}^{s}(Pi) \ln Pi$; where S is the number of species and Pi is the proportion of the total samples belonging to i-th species. H'' expresses the differences in the diversity of the sandfly fauna between villages and districts

Evenness: $E = H'/\ln(S)$, Where H'' is the value of Shannon-Wiener, E= expresses how evenly the individuals in the community are distributed over the different species (Heip *et al.*, 1998)

Species richness: S= the number of species in each district (Spellerberg and Fedor, 2003)

Similarity in sandfly fauna between the districts was estimated by Jaccard Coefficient: Sij = a/(a + b + c), where a is the number of species present in both areas, b is the number of species present in district i only, and c is the number of species present in district j only.

3.3. Results

3.3.1. Species composition

A total of 51,411 phlebotomine sandflies (26, 584 males: 24,827 females) were collected during the course of the study period from six villages of the two districts using LTs, STs and PSCs. Of these, 21% (10, 776, i.e. 6980 males: 3796 females) were caught from three villages of Libo-Kemkem, whereas the rest 79% (40, 635, i.e. 19, 604 males: 21, 031 females) were from the three villages of Metema.

In Libo-Kemkem, seven species of sandflies were caught which comprised two species of two subgenera of the genus *Phlebotomus* (86.6%): *Phlebotomus* (*Larroussius*) *orientalis* and *P* (*Anaphlebotomus*). *rodhaini* and five *Sergentomyia* spp. (13.4%) of four subgenera: *Sergentomyia* (*Sergentomyia*) *bedfordi* group, *S.* (*S*). *schewtzi, S* (*Grassomyia*). *squamiplueris, S* (*Parrotomyia*). *africana* and *S* (*Sintonius*). *clydei* (Table 1, Fig 3).

On the other hand, in Metema, 19 species of sandflies comprising of six *Phlebotomus* spp. (11.7%) belonging to four subgenera: *P* (*Larroussiust*). *orientalis*, *P (Phlebotomus). bergeroti, P* (*Phlebotomus*). *papatasi, P* (*Phlebotomus*). *duboscqi, P* (*Anaphlebotomus). rodhaini* and *P* (*Synphlebotomus*). *martini* and 13 *Sergentomyia* spp. of four subgenera (*Sergentomyia*, *Parrotomyia*, *Grassomyia* and *Sintonius*), which accounts for 88.3% of the total collection (Table 2, Fig 3).

Seven species (*P. orientalis*, *P. rodhaini*, *S. bedfordi*, *S. squamiplueris, S. schewtzi*, *S. africana* and *S. clydei*) occurred at both districts and the Jaccard similarity index was 0.37.

Table 1. Species composition and relative abundance of phlebotomine sandflies collected using CDC light traps and sticky traps from three villages of Libo-Kemkem district in northwest Ethiopia

Species	**Sampling villages**									**Overall Total (%)**
	Angot			**Bura**			**Yifag**			
	M	F	Total (%)	M	F	Total (%)	M	F	Total (%)	
P. orientalis	937	337	1274(13.7)	5113	2879	7992(85.7)	29	34	63(0.6)	**9329(86.6)**
P. rodhaini	0	0	0	0	1	1	0	0	0	**1(0.001)**
S. africana	27	20	47(64.4)	4	19	23(31.5)	3	0	3(4.1)	**73 (0.68)**
S. clydei	1	0	1(14.3)	1	5	6(85.7)	0	0	0	**7(0.001)**
S. bedfordi	414	173	587(84.5)	40	63	103(14.8)	0	5	5(0.7)	**695(6.5)**
S. schewtzi	190	2	192 (88.1)	21	5	26(11.9)	0	0	0	**218(2.02)**
S. squamiplueris	109	121	230(50.7)	81	129	210 (46.4)	0	3	3(0.6)	**453(4.2)**
Total (%)	1678 (72.0)	653(28.0)	**2331(21.7)**	5260(63)	3101(37.1)	**8361(77.6)**	32(43.2)	42(57)	**74(0.7)**	**10,766**

M= male, F= female

Table 2. Species composition and relative abundance of phlebotomine sandflies collected using three collection methods from three villages of Metema district, northwest Ethiopia

Species	**Sampling villages**									**Overall Total (%)**
	Aftit			**Kokit**			**Mender-6**			
	M	F	Total (%)	M	F	Total (%)	M	F	Total (%)	
P. orientalis	305	69	374 (8.4)	2245	1131	3376 (76.1)	554	131	685(15.4)	**4435(10.9)**
P. duboscqi	0	0	0	3	0	3 (37.5)	2	3	5 (62.5)	**8(0.02)**
P. papatasi	0	0	0	2	0	2 (66.3)	1	0	1 (33.3)	**3(0.007)**
P. bergeroti	0	0	0	20	3	23 (76.7)	6	1	7(23.3)	**30(0.74)**
P. rodhaini	8	22	30(11.9)	55	134	189 (75.3)	11	21	32(12.7)	**251(0.6)**
P. martini	0	0	0	1	0	1 (50)	0	1	1 (50)	**2(0.005)**
S. schewtzi	202	328	530 (18.1)	648	1079	1727 (58.9)	251	421	672 (22.9)	**2929(7.3)**
S. africana	887	452	1339(31.5)	602	549	1151(27.0)	1262	502	1764(41.5)	**4254(10.5)**
S. bedfordi	171	202	373 (5.0)	2318	3398	5716(78.3)	606	609	1215 (16.6)	**7302(18.0)**
S. squamipleuris	1297	1403	2700(45.8)	878	1082	1960(33.3)	437	792	1229(20.9)	**5889(14.6)**
S. clydei	964	2391	3355(29.9)	1432	2691	4123(36.8)	1502	2236	3738(33.3)	**11216(27.6)**
S. adleri	27	5	32(35.2)	23	7	30(32.9)	16	13	29(31.9)	**91(0.22)**
S. antennata	136	133	269(6.4)	441	586	1027(24.6)	2280	602	2882(68.9)	**4178(10.3)**
S. yusafi	1	0	1(14.3)	3	0	3 (42.8)	3	0	3(42.8)	**7(0.017)**
S. affinis	0	0	0	0	0	0	2	0	2(100)	**2(0.005)**
S. buxtoni	0	0	0	2	1	3(100)	0	0	0	**3(0.074)**
S. dubita	0	3	3(9.4)	1	20	21(65.6)	0	8	8(25)	**32(0.08)**
S. suberecta	1	0	1(50)	0	0	0	0	1	1(50)	**2(0.005)**
S. christophersi	0	0	0	1	0	1(100)	0	0	0	**1(0.005)**
Total (%)	3999(44.4)	5008(55.6)	9007(**22.2**)	8675(44.8)	10681(55.2)	19356(**47.6**)	6933(56.5)	5341(43.5)	12274(**30.2**)	**40635**

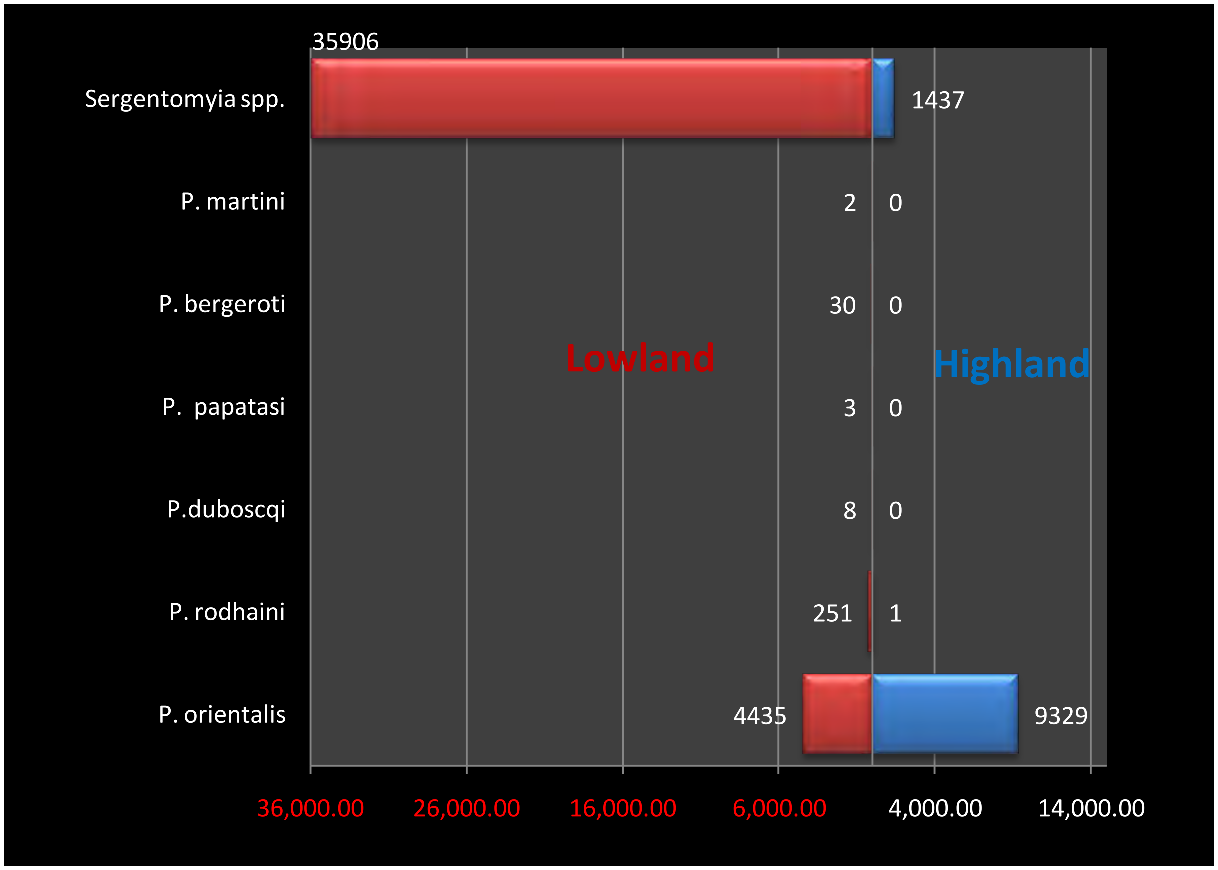

Figure 3. Total number of *Sergentomyia* spp. and different *Phlebotomus* spp. collected using light trap, sticky trap and pyrethrum space spray catches from highland and lowland areas in northwest Ethiopia

3.3.2. Sandfly diversity

There was a difference in the diversity of the sandfly fauna between the two districts as indicated by the values of Shannon-Weiner index (H"). The H" for Metema (1.95) was higher than that of Libo (0.548). Equally, the richness (S) and evenness (E) of sandfly fauna were maximal at the low altitude district (Table 3).

Table 3. The Shannon-Weiner diversity index (H‘), evenness (E) and richness (S) of the sand fly species from the study areas

District and villages	H′	E	S
Libo-Kemkem	**0.548**	**0.2816**	**7**
Angot	1.19	0.664	6
Bura	0.23	0.118	7
Yifag	0.58	0.42	4
Metema	**1.95**	**0.66**	**19**
Aftit	1.59	0.64	12
Kokit	1.82	0.66	17
Meneder-6	1.81	0.63	17

3.3.3. Abundance of phlebotomine sandflies

In the highland area, *P. orientalis* was significantly the most prevalent sandfly than all other species and accounted 86.6% of the total collection and 99.9% of the total *Phlebotomus* species. The remaining species in descending order were *S. bedfordi* (6.5%), *S. squamiplueris* (4.2%), *S. schewtzi* (2.02%), *S. africana* (0.68%), *S. clydei* (0.001%) and *P. rodhaini* (0.0001 %) (Table 1), whereas in the lowland, *S. clydei* was the most abundant species and accounted 27.6% of the total collection and followed by *S. bedfordi* (18.0%), *S. squamipleuris* (14.6%), *P. orientalis* (10.9%), *S. africana* (10.5%), *S. antennata* (10.3%) and *S. schewtzi* (7.3%). These seven species of sandflies together accounted for 99% of the total collection in the area. The remaining species were less abundant and are depicted in Table 2. Among the *Phlebotomus* spp. in

Metema, *P. orientalis* was the most predominate species (93.8%) followed by *P. rodhaini* (5.3%), *P. bergeroti* (0.6%), *P. duboscqi* (0.2%), *P. papatasi* (0.06%) and *P. martini* (0.04%).

3.3.4. Species diversity and abundance based on trapping methods

Light traps. In Libo, 77.2% of the total collection and all the seven species in the area were collected by this method. The four most common species were the same as the total collection (*P. orientalis*, *S. bedfordi*, *S. squamiplueris*, and *S. schewtzi*) (Table 4). In Metema, 77.5% of the total collection and 18 out of 19 species of sandflies captured were collected by this method. The four most abundant species in ascending order were *S. antennata*, *S. squamipleuris*, *S. bedfordi* and *S. clydei* (Table 5).

Sticky traps. In Libo, 22.8% of the total collection was collected using this method and only five species of sandflies were caught. The four most common species are similar to that of LTs (Table 4). In Metema, STs collected 21.7% of the total collection and 12 out of the total species caught. The four most frequent species were *S. clydei*, *S. squamipleuris*, *P. orientalis* and *S. schwetzi* (Table 5).

Pyrethrum space spray. In Libo, attempt to collect sandflies by this method was not productive, whereas, in Metema, it collected 0.8% of the total catch. The four most abundant species collected indoors by this method were all *Sergentomyia* species: *S. antennata, S. africana*, *S. bedfordi* and *S. clydei.* Also, two female *P. orientalis* were also collected by the method (Table 5).

3.3.5. Sex ratio

In Libo, sex ratios (males: females) for different sandfly species demonstrated that males caught by all methods were higher than that of females (6980 male: 3796 female), with an overall sex ratio of 1.84:1 (Table 3). For *P. orientalis*, the sex ratio in LTs was 1.3:1, which did not show any significant difference between sexes (Mann Whitney U-test, P>0.05). Although there was no statistical difference, the sex ratio of male to female (21.2:1) in the STs was clearly higher than that of LTs collection. Unlike Libo, the sex ratios (males: females) in Metema for different sandfly species demonstrated that females caught by all methods were higher than that of males (19604 male: 21031 female), with an overall sex ratio of 0.93:1 (Table 4). For *P. orientalis*, the sex ratio in LTs was 1.4:1, which did not show any significant difference between sexes (Mann Whitney U-test, P>0.05) as opposed to a very high ratio of male to female (15.3:1) in the STs that was clearly significant (P=0.014).

Table 4. Species composition and sex ratio of phlebotomine sandflies collected by various collection methods in Libo-Kemkem district in northwest Ethiopia

Species	Collection methods									
	Light trap			Sticky trap			Pyrethrum space spray		Total	
	M/F	Total (%)	M:F	M/F	Total (%)	M:F	M/F	Total (%)	M/F	M:F
P. orientalis	4154/3159	7313(87.8)	**1.3**	1925/91	2016(82.2)	**21.2**	0/0	0	6079/3250	**1.87**
P. rodhaini	0/1	1(0.01)	-	0	0 (0)	0	0/0	0	0/1	-
S. africana	14/31	45(0.5)	0.45	20/8	28 (1.1)	2.5	0/0	0	34/39	0.87
S. clydei	2/5	7(0.08)	0.4	-	-	-	0/0	0	2/5	0.4
S. bedfordi	316/176	492(5.9)	1.8	138/65	203(8.2)	2.1	0/0	0	454/241	1.9
S. schewtzi	161/4	165(1.98)	40.3	50/3	53 (2.2)	16.7	0/0	0	211/7	30.1
S. squamiplueris	144/154	298 (3.73)	0.94	56/99	155 (6.3)	0.57	0/0	0	200/253	0.79
Total	4791/3530	8321(**77.2**)	1.4	2189/266	2455(**22.8**)	8.2	0/0	0	6980/3796	**1.84**

M= male, F=female

Table 5. Species composition and sex ratio of phlebotomine sandflies collected by various collection methods in Metema district, northwest Ethiopia

Species	Collection methods										
	Light trap			Sticky trap			Pyrethrum space spray			Total	
	M /F	Total (%)	M:F	M/F	Total (%)	M:F	M/F	Total (%)	M:F	M/F	M:F
P. orientalis	1729/1239	2967 (**9.4**)	1.4	1375/90	1466(**16.6**)	15.3	0/2	2(0.6)	-	3104/1331	**2.3**
P. duboscqi	5/3	8(0.025)	1.7	0/0	0	-	0/0	0	-	5/3	1.7
P. papatasi	2/0	2(0.006)	-	1/0	1(0.01)	-	0/0	0	-	3/0	-
P. bergeroti	26/4	30(0.095)	6.5	0/0	0	-	0/0	0	-	26/4	6.5
P. rodhaini	52/147	199(0.63)	0.4	22/30	52(0.6)	0.7	0/0	0	-	74/177	0.41
P. martini	1/1	2(0.006)	1	0/0	0	-	0/0	0	-	1/1	1
S. schewtzi	564/1148	1712(5.4)	0.5	537/680	1217(**13.8**)	0.8	0/0	0		1101/1828	0.6
S. africana	2267/1092	3359(**10.7**)	2.1	462/357	819(**9.3**)	1.3	22/54	76(**23.9**)	0.4	2751/1503	1.8
S. bedfordi	2731/3840	6571(**20.87**)	0.7	360/355	715(**8.1**)	1.01	2/14	16(**5.04**)	0.1	3093/4209	0.73
S. squamipleuris	1702/2179	3881(**12.3**)	0.8	909/1097	2006(**22.7**)	0.8	1/1	2(0.6)	1	2612/3277	0.79
S. clydei	2914/6164	9078(**28.8**)	0.5	982/1152	2134(**24.2**)	0.9	2/2	4(1.2)	1	3898/7318	0.53
S. adleri	40/24	64(0.2)	1.7	26/1	27(0.3)	26	0/0	0	-	66/25	2.64
S. antennata	2666/911	3577(**11.4**)	2.9	178/207	385(4.4)	0.9	13/203	216(**68.1**)	0.06	2857/1321	2.1
S. yusafi	4/0	4(0.012)	-	3/0	3(0.03)	-	0/0	0	-	7/0	-
S. affinis	2/0	2(0.006)	-	0/0	0	-	0/0	0	-	2/0	-
S. buxtoni	2/1	3(0.009)	2	0/0	0	-	0/0	0	-	2/1	2
S. dubita	1/27	28(0.08)	0.04	0/4	4 (0.05)	-	0/0	0	-	1/31	0.03
S. suberecta	1/1	2(0.006)	1	0/0	0	-	0/0	0	-	1/1	1
S. christophersi	0/0	0	-	0/0	0	-	0/1	1(0.3)	-	0/0	-
Total	14709/16781	31490(**77.5**)	**0.9**	4855/3973	8828(**21.7**)	**1.2**	40/277	317(**0.8**)	0.14	19605/21030	**0.93**

3.3.5. Indoor resting habits (Endophilic behavior) of phlebotomine sandflies

Sampling of endophilic phlebotomine sandflies in Libo-Kemkem using PSCs was not productive in collecting single sandflies (Table 3), whereas in Metema, 317 phlebotomine sandflies were collected. The most endophilic sandfly was *S. antennata* (68.1%) followed by *S. africana* (23.9%) and *S. bedfordi* (5.0%). Only two (0.06%) *P. orientalis* were found resting indoor (Table 4).

3.4. Discussion

In the present study, more than fifty thousand of phlebotomine sandflies of two genera (*Sergentomyia* and *Phlebotomus*) were collected from the high altitude site Libo-Kemkem and the low altitude site Metema in northwest Ethiopia. Between these sites there was a remarkable difference in the diversity, evenness and richness of sandfly fauna. Such variation in the diversity of sandfly fauna indicated that sandfly populations are distributed patchily in nature. This may have resulted in the difference in elevation between the two sites, which, in turn, creates variation in biotic and abiotic factors (Özbel *et al.*, 2011). Similarly, Guernaoui *et al.* (2006b) in Morocco noted high species richness and diversity at low altitude as compared to high altitude.

Abundance of sandflies significantly differed between the three sampling villages of Libo-Kemkem, with Bura and Yifag being the most and the least productive sites, respectively. The difference in productivity of sandflies among the villages might be due to differences in the soil type as Bura and Angot are found in a flat-plain with much black cotton soil (vertisol) in which *P. orientalis* is suspected to breed and rest (Gebre-Michael *et al.*, 2004b; Moncaz *et al.*, 2014) whereas in Yifag clay soil is the most prevalent. Although Bura was the most productive site in the total sandfly collection, the results of H“ and E showed that it was the least diverse and also had the least evenness than the rest of sampling villages. Such low diversity and evenness of sandfly in this study village are caused by a high abundance of *P. orientalis* (95.5%). In contrast to the highland study villages, sandfly abundance in the lowland villages was not different.

Seven species of sandfly were encountered in the highland district; *P. orientalis, P. rodhaini, S. bedfordi*, *S. squamiplueris, S. schewtzi*, *S. africana* and *S. clydei*. The former one was the predominant species and is the suspected vector of VL in the area and in northwestern Ethiopia (Gebre-Michael *et al.,* 2007: 2010) and the proven vector of the disease in neighboring Sudan (Ashford *et al*., 1992; Elnaiem *et al*., 1998). Although there are some variations in sandfly fauna, this result is in agreement with Gebre-Michael *et al*. (2007). One of the variations was the absence of *P. longipes* in the present study since no collection was made in the nearby monastery located at a higher altitude (2300 masl) from where it was collected by Gebre-Michael *et al*. (2007) and such higher altitude is a typical ecology of the species. On the other hand, *P. rodhaini* and *S. clydei* are new records for the area and this might be due to the short duration of the investigation by Gebre-Michael *et al*. (2007).

In addition to the above seven species of sandflies, additional 13 species of sandfly species were recorded in the lowland district. Unlike Libo-Kemkem, the majority of these species in Metema belonged to the genus *Sergentomyia* (88.9%). The predominance of *Sergentomyia* spp. over *Phlebotomus* spp. were also previously reported in neighboring Sudan, which have similar ecology and climatic condition with the present study area (Metema) (Elnaiem *et al*., 1997; Lambert *et al*., 2002). The most abundant species in the district was *S. clydei* followed by *S. bedfordi, S. squamipleuris*, *P. orientalis*, *S. africana*, *S. antennata* and *S. schewtzi*. The predominance of *S. clydei* was previously reported by Elnaiem *et al.* (1997) from eastern Sudan, which is adjoining and sharing similar ecology to the present study area. In contrast to these reports, in southern Ethiopia *S. schewtzi* was found to be the most prevalent species (Gebre-Michael and Lane, 1996). Both species are known to occasionally bite humans, but all *Sergentomyia* spp. are not known to transmit mammalian leishmaniasis (Lewis, 1982; Lane, 1993).

The genus *Phlebotomus* in the Metema district consists of six species, namely *P. orientalis*, *P. bergeroti, P. papatasi, P. duboscqi, P. rodhaini* and *P. martini*. Like Libo-Kemkem, *P. orientalis* is the prevalent species and the first five species are previously recorded in the area (Gebre-Michael *et al*., 2010). However, *P. martini*, the proven vector of VL in southern Ethiopia (Gebre-Michael and Lane, 1996) and Kenya (Perkins *et al*., 1988), is the first record for the area even though only two specimens were recorded.

The absence of the three species of the subgenus *Phlebotomus* (*P. bergeroti*, *P. papatasi,* and *P. duboscqi*) in the highland site (Libo) might be due to the members of the subgenus preferring areas with high ambient temperature (Lewis, 1982; Lane and Fritz, 1986) although a few specimens of *P. duboscqi* were previously collected in the high altitude of Belessa valley (1800 masl), north of Libo-Kemkem (Ashford *et al*., 1973a). The role of these sympatric species in the epidemiology of leishmaniasis in Metema is unclear, although recently a man had been admitted and treated for CL (the causative agent was not revealed) at Metema hospital (unpublished data). Elsewhere these species are either suspected or proven vectors of zoonotic cutaneous leishmaniasis. For instance, *P. papatasi* is a proven vector for CL due to *L. major* throughout North Africa, Middle East, Central and Eastern Asia (Lane, 1993; WHO, 2010), whereas *P. duboscqi* has also a similar role in the Sahel region of Africa (Dedet *et al*., 1980;

WHO, 2010). This species was also reported to be a vector of *L. major* in the southern VL foci of Ethiopia (Gebre-Michael *et al*., 1993).

Among the species of the genus *Phlebotomus*, *P. orientalis* was the most abundant species in both the highland (99.9%) and lowland (93.7%) areas. Such high numbers and dominance of the species in the northwestern Ethiopia has also been reported from previous studies (Gemetchu *et al*., 1975; Gemetchu *et al*., 1983; Gebre-Michael *et al*., 2010). Due to its abundance, these previous studies hypothesized that the species is the likely vector of VL in the region.

Following *P. orientalis*, *P. rodhaini* was the second most abundant species in the genus *Phlebotomus* in the lowland area. This observation is in contrast to the earlier report in the area (Gebre-Michael *et al*., 2010). The second most abundant species was *P. bergeroti* in the earlier study. The difference might be due to the seasonality of the species as this earlier study was done on a brief period of time. The epidemiological role of *P. rodhaini* in the area and also in other parts of the country is a subject of further investigation. A few attempts have been made to detect parasite by dissection in the country. Balkew *et al*. (2002) detected natural infections with flagellates in nine *P. rodhaini* from Rift valley areas (Ziway-Langano); however these parasites differ from mammalian *Leishmania* species by their position in the insect gut, morphology and behavior in cultures. Gebre-Michael *et al*. (2004a) also dissected a few females of this species in the Awash valley and they also found similar result as above. Unlike the above reports, recently, Elnaiem *et al*. (2011) found three females *P. rodhaini* infected with *Leishmania* parasites in Dinder National Park in eastern Sudan (which is found in the same ecology and climatic condition to Metema). Of these, two were typed by molecular method as

L. donovani. This finding might indicate that this species in eastern Sudan and also in Metema area play a secondary role next to *P. orientalis* in the transmission of VL.

Sampling of phlebotomine sandflies for determining indoor resting habits of phlebotomine sandflies in general and *P. orientalis* in particular using pyrethrum space spray catches failed to collect sandfly in the highland while few specimens of *P. orientalis* in the lowland area were collected. This observation indicated that *P. orientalis* in both areas has a propensity of exophilic (resting outdoor) behavior and it is in agreement with those of earlier studies in Sudan (Hoogstraal and Heyneman, 1969; Elnaiem *et al*., 1997) and neighboring districts in northern Ethiopia (Gebresilassie *et al*., in press). Furthermore, from epidemiological point of view, this result will have a significant role in the control of VL based on vector control. Conventional vector control methods, such as indoor residual spraying (IRS), which is currently used for the control of the vector of kala-azar in India (Gupta *et al*., 2013) and also for malaria vectors in Africa, including Ethiopia (WHO, 2006) may not have a significant impact on the density of *P. orientalis* in these districts.

In the present study, more numbers of sandfly specimens were collected in CDC light traps compared to the other collection methods. More than 75% of sandflies were caught using LTs which is in accord with the findings of Toprak and Ozer (2007) who found highest capture efficiency for LTs over aspirators and STs in Turkey. A similar result was also reported by Dinesh *et al*. (2008). Besides collecting a bulk of sandflies, this trap also collected a number of species than the other two traps which result in a different description of the species composition among the trapping methods. This difference in species composition might be due to LTs could collect additional phototropic species of sandfly together with non-phototropic flies.

The overall sex ratio for *P. orientalis* was largely male-biased in both study sites (1.87 in Libo and 2.3 in Metema). Male-biased sex ratio for different sandfly species also noted by other workers (Yuval, 1991; Wasserberg *et al.,* 2003; Signorini *et al*., 2013), and such male-biased ratio might be as result of either male of *P. orientalis* exhibiting „lekking" behavior for awaiting host-seeking females (Yuval, 1994) or females of this species move farther than males as that of *P. ariasi* (Killick-Kendrick *et al*., 1984). On the other hand, sex ratio for *Sergentomyia* species in the highland and lowland areas showed contrasting results, the ratio in highland area was more of male-biased, but it was female biased in the lowland area.

In conclusion, the present study is the first to compare the species composition, relative abundance and indoor resting habits of phlebotomine sandflies in two ecologically distinct foci of kala-azar in northwestern Ethiopia. The study shows the variation in sandfly fauna between the highland and lowland. It also revealed the occurrence of seven and 19 species of sandfly in the highland (Libo-Kemkem) and lowland (Metema) areas, respectively. Furthermore, the study pointed out the presence of one of the major kala-azar vectors in East Africa, *P. orientalis*, which was the predominant *Phlebotomus* species and hence, the most likely vector of VL in both of study areas.

Chapter four. Population dynamics and habitat preferences of *Phlebotomus orientalis* in two ecologically distinct foci of kala-azar in northwestern Ethiopia

4.1. Introduction

Phlebotomine sandflies (Diptera: Psychodidae) are vectors of a number of human diseases such as arboviral, bartonellosis and leishmaniasis (Maroli *et al.*, 2013). Leishmaniasis is caused by obligate intra-macrophage protozoa with wide clinical symptoms: cutaneous, muco-cutaneous and visceral leishmaniasis (VL). Of the different forms of leishmaniasis, VL or also known as kala-azar is the most serious and result in loss of life if left untreated (Chappuis *et al.*, 2007).

In Ethiopia, kala-azar due to *Leishmania donovani* (Hailu *et al.*, 2006a), is a growing health problem with an estimated annual VL incidence between 3700 and 7400 (Alvar *et al.*, 2012). The well known endemic areas of the disease are found in the Metema-Humera lowlands in the northwest bordering with Sudan, where it accounts for about 60% of the total cases and in arid areas of south and southwest of the country (Hailu *et al.*, 2006a). Besides, recently the disease has spread to the highlands of Libo-Kemkem and Fogera districts, where it claimed the lives of more than 200 people. Nowadays the disease is a serious health concern in the districts and also neighboring areas (Alvar *et al.*, 2007; Herrero *et al.*, 2009; Gebre-Michael *et al.*, 2007; Bashaye *et al.*, 2009).

Phlebotomus orientalis is a proven vector of VL in eastern Sudan (Elnaiem *et al.*, 1998) and the suspected vector in the northwestern Ethiopia (Gemetchu *et al.*, 1983; Gebre-Michael *et al.*, 2010). In these areas, it is strongly associated with *Acacia-Balanites* forest and deeply cracking „black cotton soil‟, which the species may use as breeding and resting sites (Gebre-Michael *et* *al*., 2004b; Moncaz *et al.,* 2014). Previous studies on population dynamics and distribution of this species in Sudan revealed that *P. orientalis* is a seasonal species and abundant mainly in the drier period of the year (February-June) although results of population peak vary from place to place (Quate, 1964; Hoogstraal and Heyneman, 1969; Elnaiem *et al*., 1997). For instance, Quate (1964) observed seasonal abundance of *P. orientalis* in Paloich area of southern Sudan with one population peak in April. Similarly, Elnaiem *et al*. (1997) also noted seasonality of this species in Dinder National Park in eastern Sudan but the peak density of the flies was at the end of dry season (June). They also reported positive relationship between the density of *P. orientalis* and mean temperature and relative humidity. In contrast, in Ethiopia attempts to elucidate population dynamics, distribution and behaviors of the suspected vector of VL are few. Ashford *et al*. (1973a) collected *P. orientalis* in the highland area of Belessa valley for seven months (September-March), but the authors did not observe difference in abundance of the species throughout the seven months of collection.

Information on the population dynamics, distribution and behaviors of vector species are very important to understand when, where and how humans are infected with *Leishmania* parasites. Furthermore, this knowledge is the basis towards developing appropriate vector control methods (Janini *et al*., 1995). Hence, the aim of this study was to determine and compare the population dynamics and habitat preferences of *P. orientalis* and other sandfly species in two ecologically distinct kala-azar foci of northwestern Ethiopia.

4.2. Materials and Methods

4.2.1. Collection of sandflies

Adult phlebotomine sandflies were collected using two sampling techniques. These are CDC light traps (LTs) and sticky traps (STs) (Alexander, 2000).

A. CDC Light traps (*LTs*)

In both districts, three permanent sampling sites in each village of the two districts were selected for collection of sandflies using LTs. These sites were household compounds, farm fields and mixed forests. The distance between the habitats ranged between 150 to 200m. In each sampling habitat, two LTs were deployed from dusk to dawn (18:00-6:00). The Light trap was placed 30-50cm above the ground. In the morning, the trapped sandflies were collected using aspirators from cages and killed by chloroform. *Phlebotomus* females were sorted out from the rest (male *Phlebotomus* and male and female *Sergentomyia*) for dissection to detect natural infection (Chapter five). Fresh fed *Phlebotomus* females were preserved for late blood meal analysis (Chapter five). The rest were preserved in 70% alcohol for later species identification.

B. *Sticky traps* (*STs)*

Three sampling sites in each village were selected to collect sandflies using sticky traps (STs) of white A4 sized polypropylene (21cm x29.7cm) smeared on both sides with sesame oil. These sampling sites were indoor, farm-field and mixed forest. For indoor collection, five STs per hut were tied on nylon string in a line about 30cm apart and placed on the wall above sleeping location. In sampling night four huts were used (i.e. a total of 20 STs per night). In each of the other habitats (farm field and mixed forest) similar number of STs with that of indoor collection were deployed horizontally (i.e. 20 STs in farm field and 20 STs in mixed forest). Deployment and retrieval of traps was done at the same time with the LTs. Sandflies collected by this method were removed from STs and preserved in absolute alcohol in labeled vials for later species identification (see Chapter three).

4.2.2. Data Analysis

The mean densities of sandflies were computed as numbers of flies per trap per night for both trapping methods. Before analysis of the data, the normality of the data was assessed using Shapiro-Wilk test in SPSS version 20. As the data were not normally distributed non-parametric tests such as Mann-Whitney U-test, Spearman and Pearson correlation coefficients were used. Mann-Whitney U-test was used to compare efficiency in collection of *P. orientalis* between LTs and STs. Correlation between meteorological variables and density of *P. orientalis* was made using either Spearman or Pearson correlation coefficient.

4.3. Results

4.3.1. Population dynamics of *P. orientalis* in the highland and lowland areas

Population dynamics of *P. orientalis* in Libo-Kemkem

The seasonal fluctuation of *P. orientalis* collected by LTs and STs pooled from the three villages is depicted in Fig 4. There was a distinct seasonal fluctuation in the abundance of *P. orientalis* over 12 months of collection periods, showing an overall increased in density between January and May, with its highest peak of abundance in April (19.7± 17.1 flies/trap/night) for LTs and May (0.54±0.49 flies/trap/night) for STs. The mean temperature during these months in Libo-Kemkem was 24.0^{0}C. However, *P. orientalis* population density drastically declined during the peak rainy season (July-September) being completely absent in August and September.

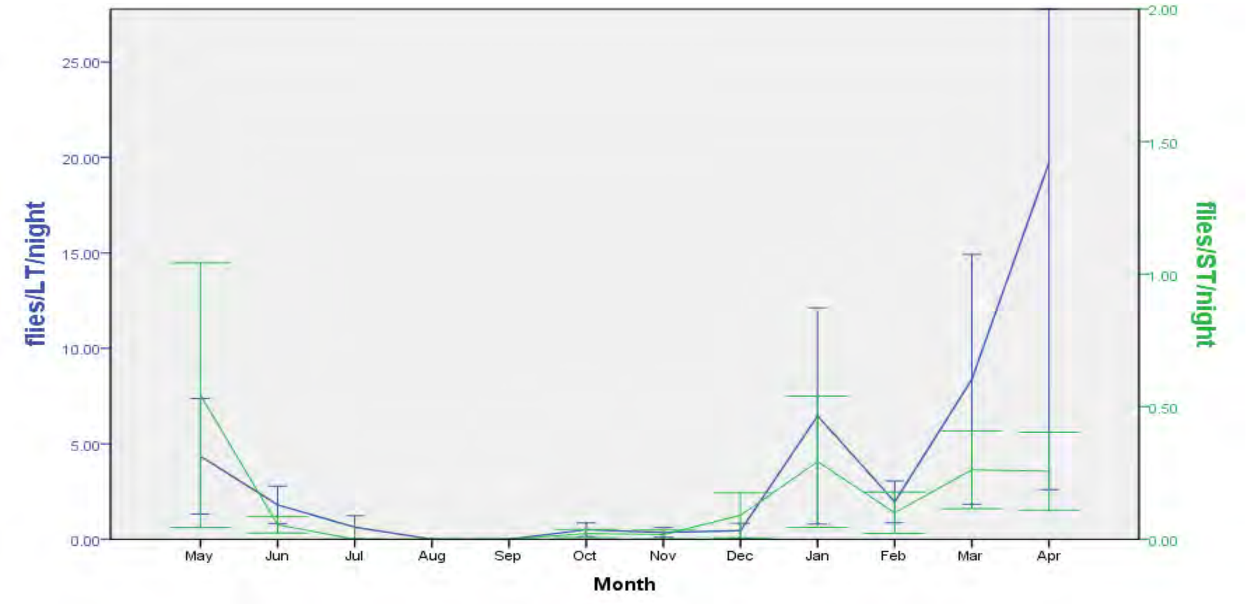

Figure 4. Seasonal pattern of *P. orientalis* in Libo-Kemkem district collected by light and sticky traps (May 2011-April 2012) in northwest Ethiopia

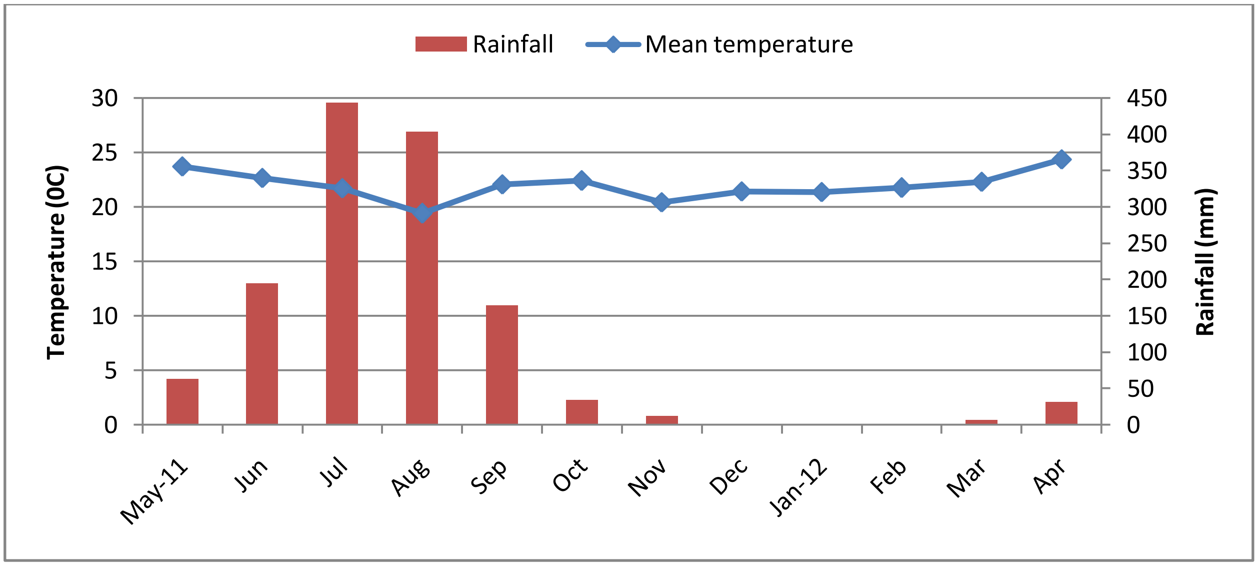

Figure 5. Monthly total rainfall and mean temperature of Libo-Kemkem district (May 2011-April 2012) in northwest Ethiopia

During the dry season (October-May), monthly maximum temperature of the study area ranged between 31.6 and 35^{0}C while monthly minimum temperature varied from 9.3 to 14^{0}C. The total monthly rainfall in this period ranged between 0 and 33.9mm. In the wet season (June-September), mean monthly maximum temperature ranged between 25.5 to 29.6^{0}C and mean monthly minimum temperature varied between 13.3^{0}C to 15.7^{0}C. The total monthly rainfall during this time ranged from 165 to 443.3mm (Fig 5).

Correlation between weather variables and density of **P. orientalis**

The density of *P. orientalis* was correlated with monthly mean temperature and total rainfall using Pearson (for LTs collection) and Spearman (for STs collection) correlation coefficients (Table 6). In LT collections, density of *P. orientalis* had significant positive correlation with mean temperature (r= 0.671, P=0.017) and a non-significant negative association with the rainfall (r= -0.452, P>0.05). In ST collections, density of *P. orientalis* positively correlated with the mean temperature but not significant (P>0.05) and negatively correlated with the rainfall and also statistically significant (r=-0.627, P<0.05).

Table 6. Correlation analysis of mean temperature and rainfall with the density of *P. orientalis* in Libo-Kemkem district

	Collection methods			
	Light trap		Sticky trap	
Weather variables	r	p-value	ρ	p-value
Mean Temperature	0.671*	0.017	0.416	0.179
Rainfall	-0.452	0.079	-0.627*	0.029

r- Pearson correlation coefficient, ρ - Spearman correlation coefficient; * Correlation is significant at the 0.05

Population dynamics of *P. orientalis* in Metema

The seasonality of *P. orientalis* collected using LTs and STs is shown in Fig 6. Like in Libo, the activity of *P. orientalis* was in sharp decline or completely absent during the rainy season (July-September) and only present during the dry season (November-June) with increase in abundance towards the end of the dry season. During the dry season, the peak density of the species was observed in June for both trapping methods (16.5±10.7 flies/LT/night and 0.58±0.27 flies/ST/night). The mean temperature during this month in Metema was 27.7^{0}C.

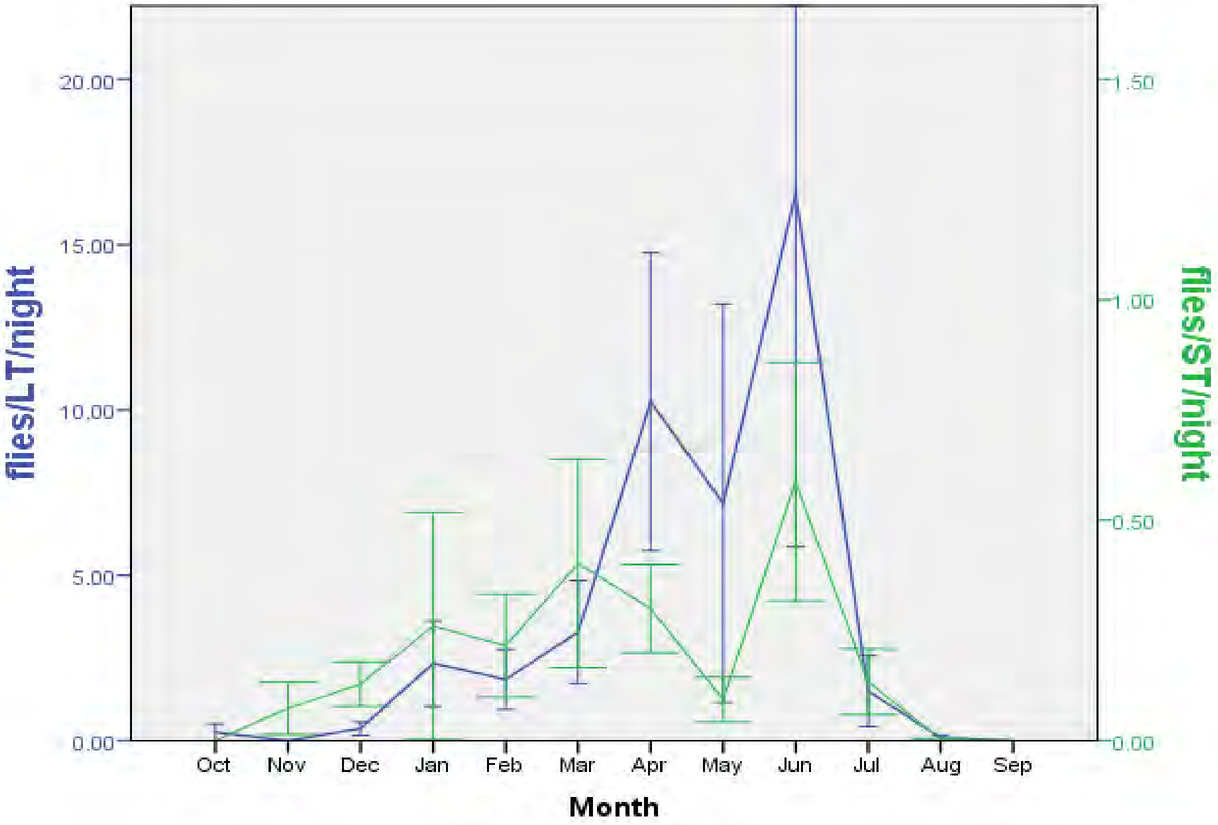

Figure 6. Seasonality of *P. orientalis* that were collected using light trap and sticky trap in Metema (October 2012 - September 2013) in northwest Ethiopia

During the dry season (October-May) of the study period, monthly maximum temperature of the area ranged from 36.3^{0}C to 41.3 ^{0}C while monthly minimum temperature ranged from 16.3^{0}C to 23.4^{0}C. The total monthly rainfall and relative humidity in this period ranged between 0 and 47.8mm and 29 to 56.2%, respectively. In wet season (June-September), monthly maximum temperature ranged between 29.4^{0}C to 34.7^{0}C and monthly minimum temperature varied between 19.3^{0}C to 20.6^{0}C. The monthly rainfall and relative humidity during this time ranged from 150.4mm to 352.7mm and 76.5 to 80.3%, respectively (Fig 7).

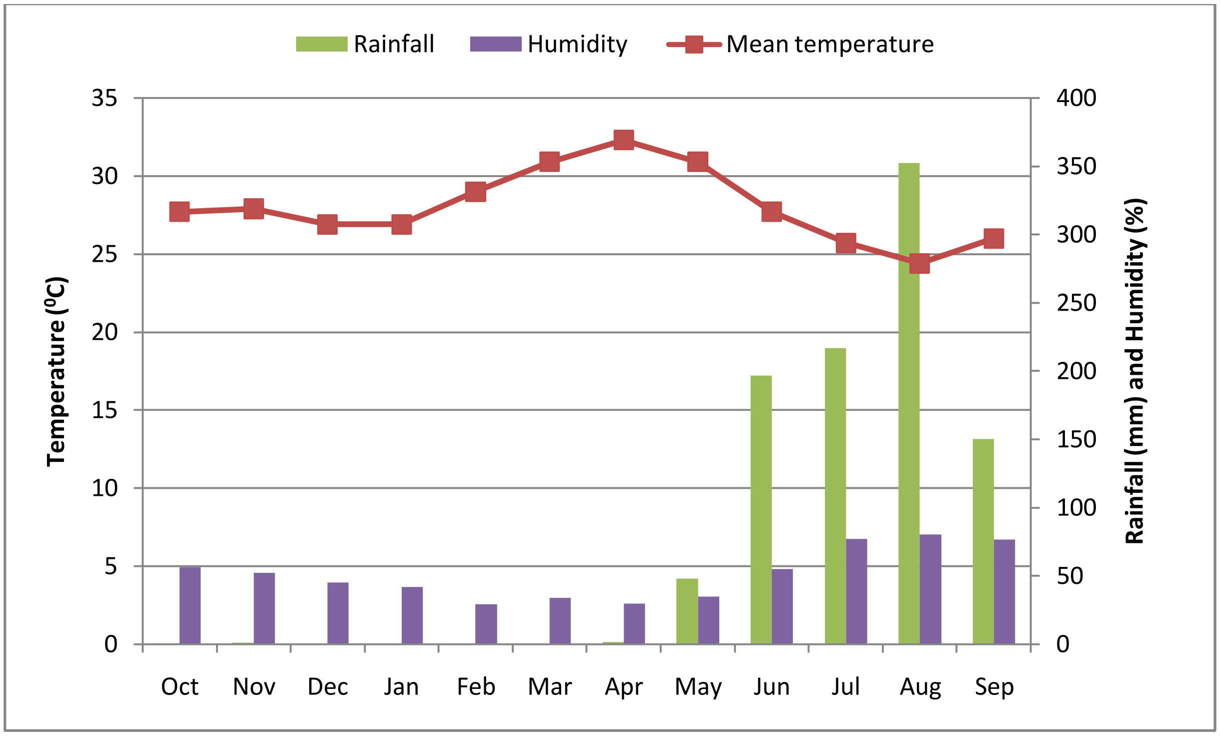

Figure 7. Monthly total rainfall, relative humidity and mean temperature of Metema (October 2012- September 2013)

Correlation between weather variables and density of **P. orientalis**

Correlation between the density of *P. orientalis* and weather variables such as mean temperature, relative humidity and rainfall of the area was performed using Spearman and Pearson correlation coefficients. In LTs collection, density of *P. orientalis* had statistically significant positive correlation with the mean temperature ($r= 0.639$, $P<0.05$), and negatively correlated with rainfall and humidity but not significant ($P>0.05$). Furthermore, in STs collection, the correlation analysis showed that the density of *P. orientalis* was positively associated with the mean temperature and, negatively associated with rainfall and relative humidity but not significant ($P>0.05$) (Table 7).

Table 7. Correlation between weather variables and the density of phlebotomine sandflies collected by light trap and sticky trap in Metema district

Weather variables	CM	Species													
		P. orientalis		*S. clydei*		*S. bedfordi*		*S.squamiplueris*		*S. schewtzi*		*S. africana*		*S. antennata*	
		r/ ρ	p-value	ρ	p-value	r/ ρ	p-value	r/ ρ	p-value	ρ	p-value	r/ ρ	p-value	r	p-Value
MT	LT	**0.639***	0.025	0. 62*	0.031	**0.73****	0.008	**0.45**	0.13	0.13	0.66	**0.547**	0.06	**0.57**	0.051
	ST	**0.395**	**0.204**	0.58*	0.045	0.61*	0.03	-0.36	0.251	0.54	0.07	0.28	0.36	**0.611***	0.035
Rainfall	LT	-0.068	0.834	-0.12	0.69	**-0.54**	0.07	**0.17**	0.57	-0.27	0.39	**-0.67***	0.017	**-0.64***	0.026
	ST	**-0.148**	**0.645**	-0.25	0.43	-0.31	0.33	-0.15	0.64	0.01	0.98	-0.60*	0.03	**-0.473**	0.12
Humidity	LT	-0.536	0.073	-0.65*	0.02	**-0.85****	0.00	-0.49	0.102	-0.39	0.19	**-0.89****	0.001	**-0.88****	0.00
	ST	**-0.463**	**0.129**	-0.63*	0.027	-0.69*	0.01	**0.06**	0.84	-0.54	0.06	-0.63*	0.027	**-0.75****	0.005

*Correlation is significant at the 0.05 level; **Correlation is significant at the 0.01 level ; MT- Mean Temperature; CM- collection method; LT-light trap; ST-sticky trap; ρ = Spearman correlation; r-Pearson correlation. N.B. figures in bold are analyzed using Pearson correlation if not Spearman correlation

4.3.2. Habitat-specific seasonal dynamics of *P. orientalis*

Libo-Kemkem

Seasonal dynamics of *P. orientalis* collected by LTs and STs in different habitats are shown in Fig 8 and Fig 9, respectively. In LTs, seasonality of *P. orientalis* in the three habitats appears to have similar pattern but peak in density showed variation among the habitats. Peak in density of *P. orientalis* first observed at mixed forest in January, and followed by compound habitat and farm field habitat simultaneously in April. However, in STs clear seasonality of the species was observed only in farm field habitat with three peaks in density. The first peak was in May and the other peaks were in January and April.

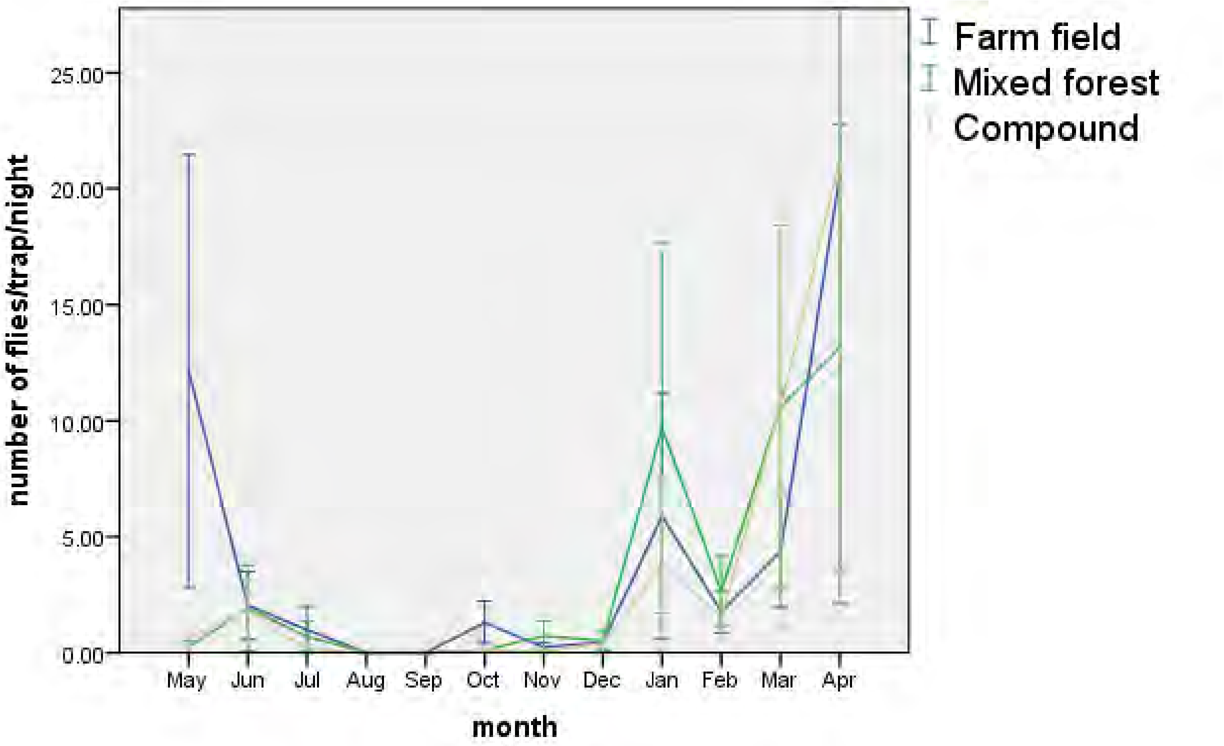

Figure 8. Seasonal abundance of *P. orientalis* in different sampled habitats using light trap in Libo-Kemkem (May 2011 - April 2012) in northwest Ethiopia

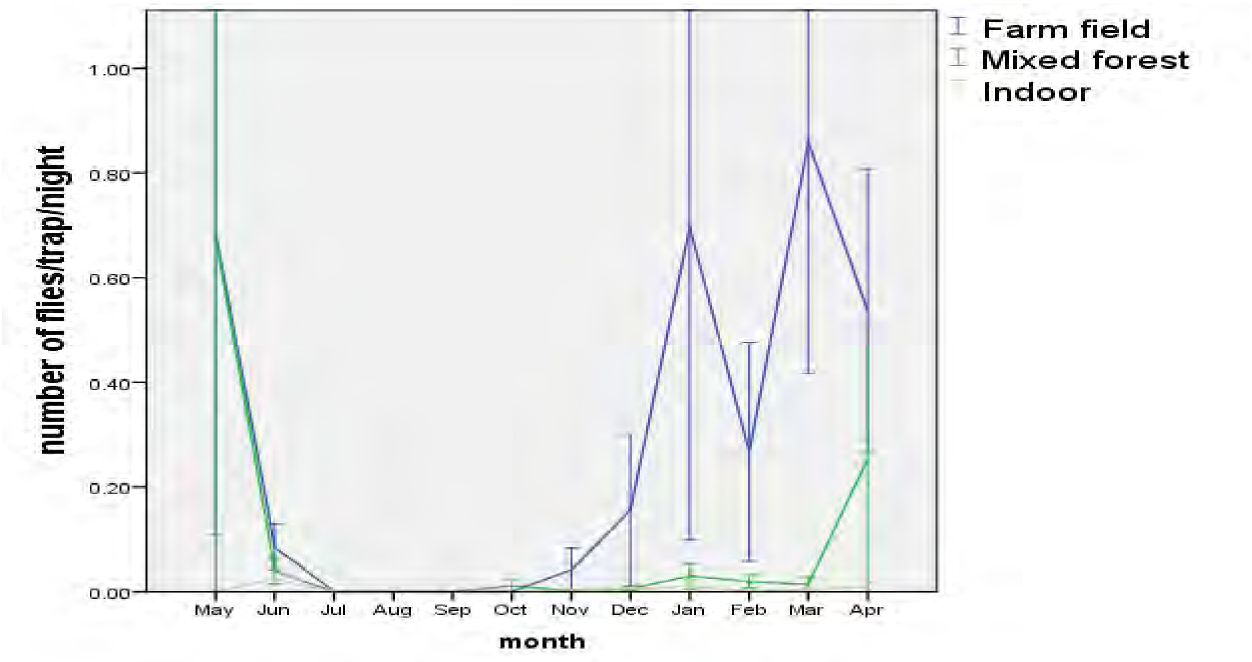

Figure 9. Seasonal abundance of *P. orientalis* in different sampled habitats using sticky trap in Libo-Kemkem (May 2011 - April 2012) in northwest Ethiopia

Metema

Seasonal dynamics of *P. orientalis* collected by LTs and STs in different habitats are depicted in Fig 10 and Fig 11, respectively. In LTs, differing trends in seasonal dynamics of *P. orientalis* were notable among the three habitats. For the species, activity peaked first at farm field habitat and followed by household compound habitat and mixed forest habitat. In farm field habitat, the flies were active between October and July. In this period, two peaks in density were observed. The short peak was in January whereas the larger peak was in April. In mixed forest habitat, the activity of the species concentrated between January and July with two peaks in density. The major one was in June whereas the minor one was in April. In household compound habitat, *P. orientalis* was active between February and July with a single peak density in April. Similarly, different patterns in seasonal dynamics of *P. orientalis* were noted among the three sampled habitats in STs collections. The activity of the species peaked first at farm field habitat in January and followed by mixed forest habitat in March. Indoor collections of *P. orientalis* did not show clear seasonality as the density was too few.

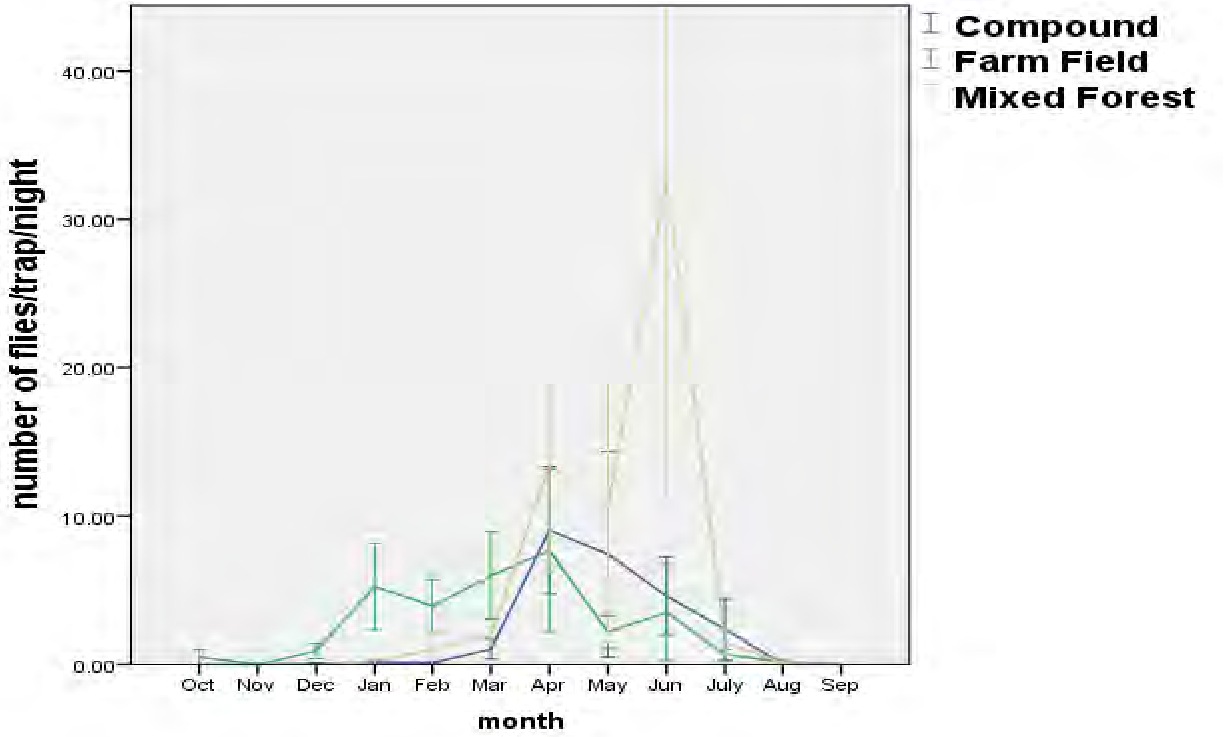

Figure 10. Seasonal abundance of *P. orientalis* in different sampled habitats using light trap in Metema (October 2012 - September 2013) in northwest Ethiopia

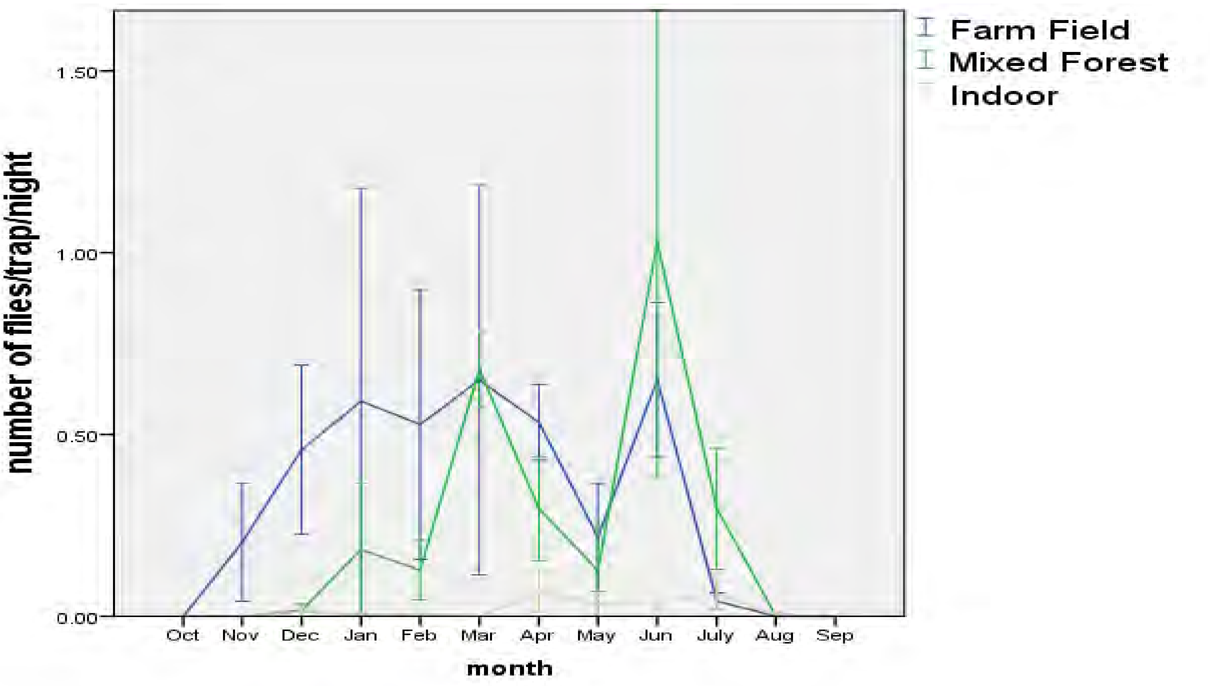

Figure 11. Seasonal abundance of *P. orientalis* in different sampled habitats using sticky trap in Metema (October 2012 - September 2013) in northwest Ethiopia

4.3.3. Population dynamics of *Sergentomyia* spp.

The monthly incidence of the six common *Sergentomyia* spp. (*S. clydei, S. bedfordi, S. squamipleuris, S. africana, S. antennata* and *S. schewtzi*) in Metema collected using LTs and STs are depicted in Fig. 12 and Fig. 13, respectively. All appear to follow the same pattern to that of *P. orientalis* being absent or greatly reduced during the wet season (July-September) and common during the dry season (November-June). Some species like *S. clydei* were exceptionally high in the month of June in both LTs (65.4±36.6/LT/night) and STs (1.67±0.369/ST/night). Others like *S. squamipleuris* were suddenly high in early dry season in December in STs (1.52±0.72/ST/night) (Fig. 12) but with no comparable increase in the same month by LTs (14.7±2.5/LT/night) (Fig. 13).

Correlation between weather variables and Sergentomyia *spp.*

Correlation between the densities of the six most abundant *Sergentomyia* spp. and the aforementioned weather variables were performed using the above mentioned correlation coefficients. Results of both correlation analyses in general indicated that mean temperature has positive, but rainfall and relative humidity had negative correlations with the densities of *Sergentomyia* species. For both trapping techniques, *S. clydei* and *S. bedfordi*, and *S. antennata* (only for STs) have statistically significant positive and negative associations with mean temperature and relative humidity, respectively. Densities of *S. africana* (for both methods) and *S. antennata* (for only LTs) have a significant negative correlation with rainfall (Table 7).

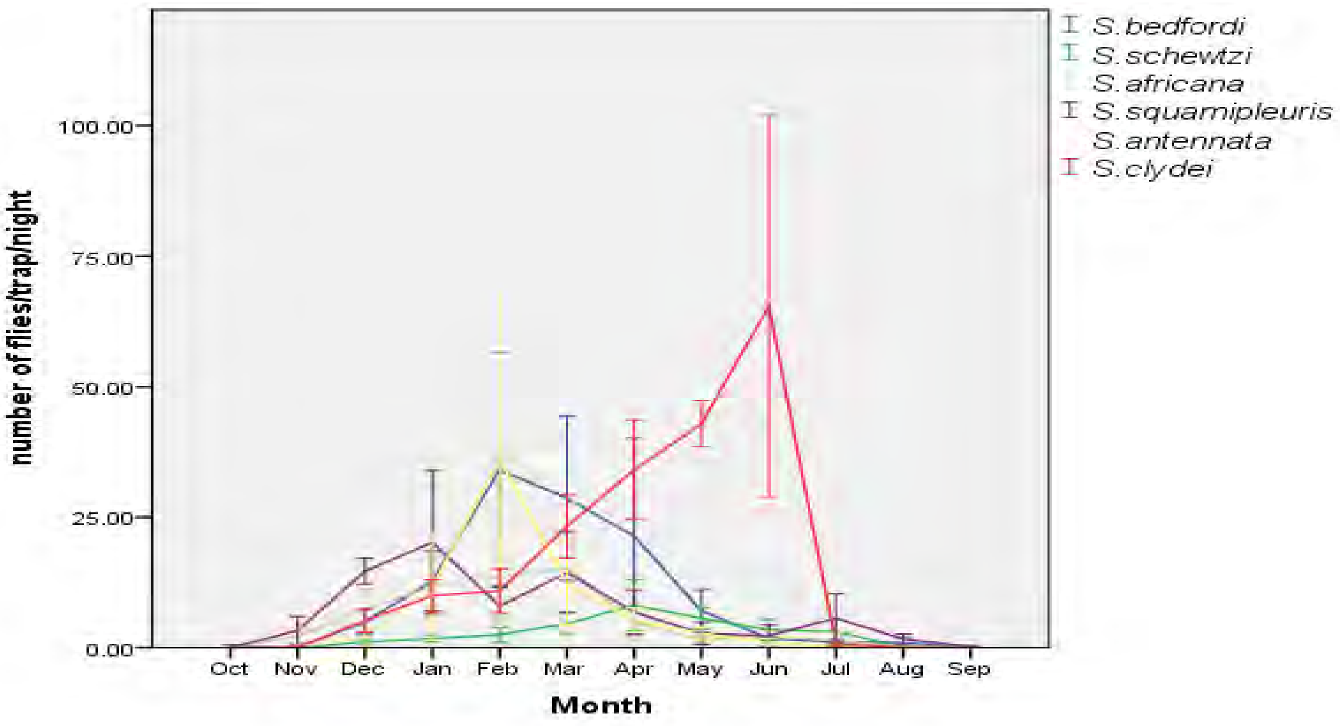

Figure 12. Seasonality of *Sergentomyia* spp. that were collected using light trap from Metema district (October 2012-September 2013)

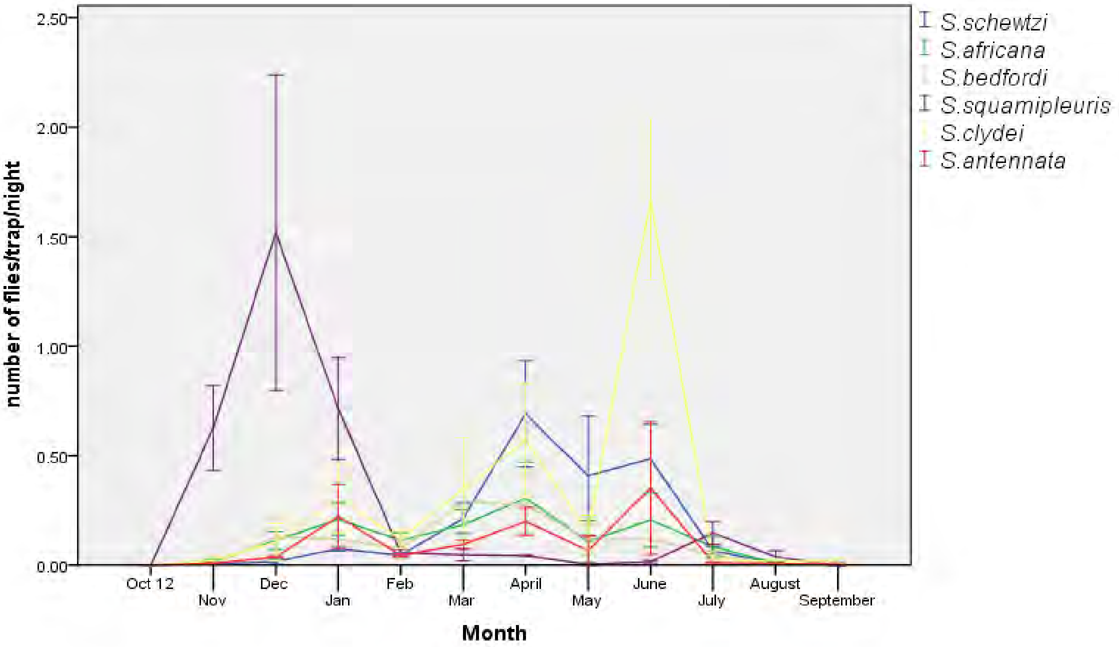

Figure 13. Seasonality of *Sergentomyia* spp. that were collected by sticky trap from Metema district (October 2012- September 2013)

4.3.4. Habitat preferences of *P. orientalis* in Libo-Kemkem and Metema

In Libo-Kemkem, significant difference was recorded in density of *P. orientalis* among the three sampled habitats using STs (χ^2 =6.174, df=2, P=0.046). *Phlebotomus orientalis* was significantly more abundant in farm field (0.093±.03201flies/ST/ night) than in mixed forest (0.026±0.015flies/ST/night) or indoor (0.005±0.02flies/ST/night). Similar significant difference was also observed in density of males of *P. orientalis* among three habitats with similar trend with total population of the species (χ^2 =6.05, df=2, P<0.05). In the case of females non significant but similar trend in habitat preferences was noted (P>0.05). In contrast, there was no significant difference among the three sampled habitats (household compound, farm field and mixed forest) in density of *P. orientalis* observed using LTs (P>0.05) (Fig 14)

In Metema, similar significant difference in habitat preferences of *P. orientalis* collected by STs was obtained as in Libo (χ^2 =5.9, df=2, P=0.05). Mean density of *P. orientalis* was more abundant in farm field (0.177±0.03 flies/ST/ night), followed by mixed forest (0.0862±0.03 flies/ST/ night) and indoors (0.07±0.02 flies/ST/ night). When habitat preferences of *P. orientalis* analyzed based on sexes, males of *P. orientalis* significantly abundant (P=0.04) in farm field (0.112±0.03) than mixed forest (0.08±0.034) and indoor (0.006±0.002) whereas for females non significant but similar trend in habitat preferences was obtained (P>0.05). In contrast to the STs, mean density of *P. orientalis* collected by LTs was more abundant in mixed forest (2.88±1.6 flies/LT/ night) and least abundant in compound (1.01±0.49 flies/LT/ night) but not significant (P>0.05) (Fig 15).

4.3.5. Comparison between trapping methods for collecting *P. orientalis*

The mean ± SE of *P. orientalis* was significantly higher in LT collections than in ST collections in Libo-Kemkem: 2.82±1.2 flies/LT/night and 0.0425±0.014flies/ST/night, respectively (Mann Whitney U-test, χ^2 =20, df=1, P<0.05). Similarly, LTs (6.7±3.23) caught higher number of *P. orientalis* than STs (0.24± 0.08) in the lowland area but the difference was not significant (P>0.05).

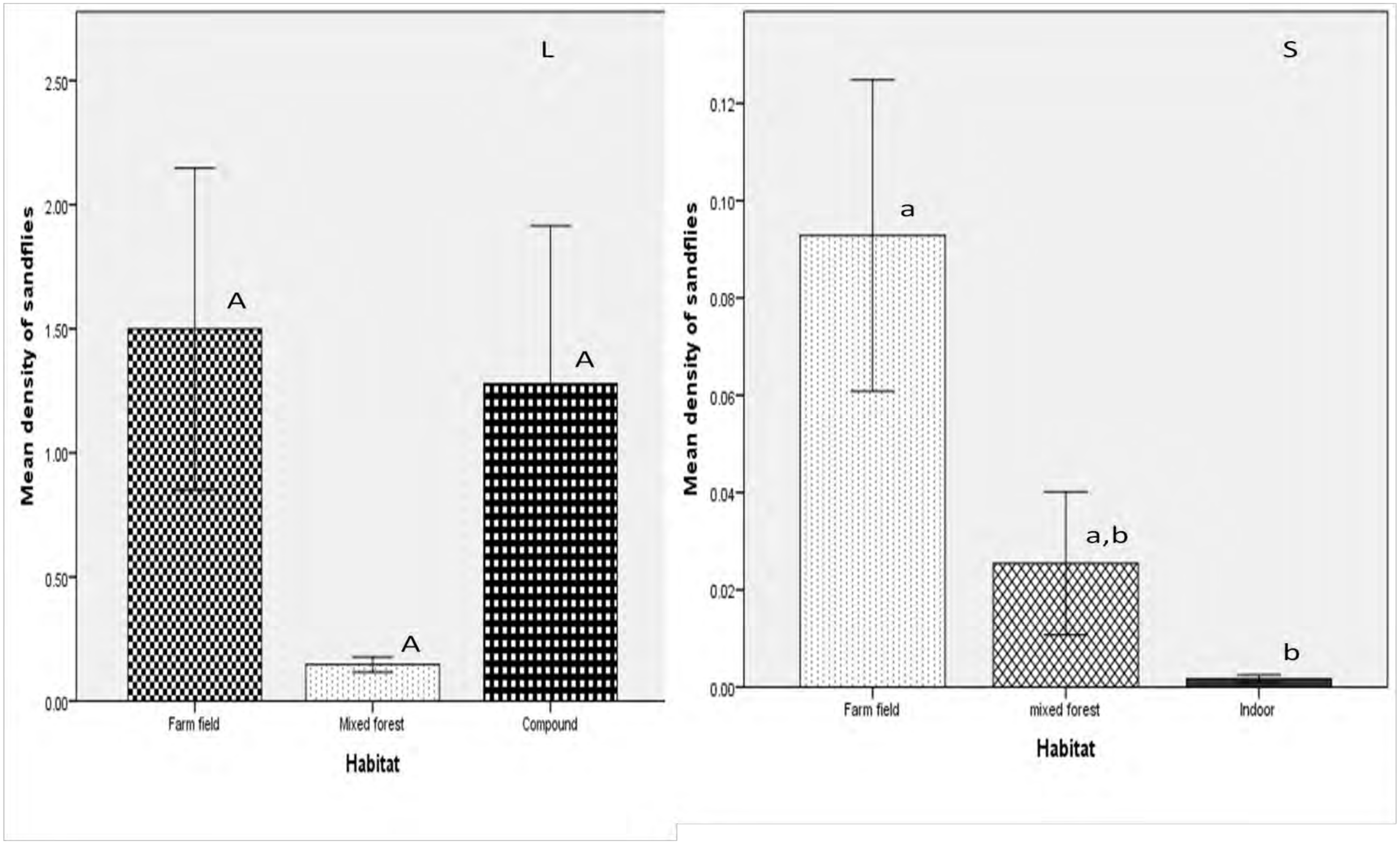

Figure 14. Habitat preferences of *P. orientalis* collected using light trap and sticky trap in Libo-Kemkem district. N. B. Significant differences between habitats are indicated by different letters above the bars ($P<0.05$); L= light trap, S= sticky trap

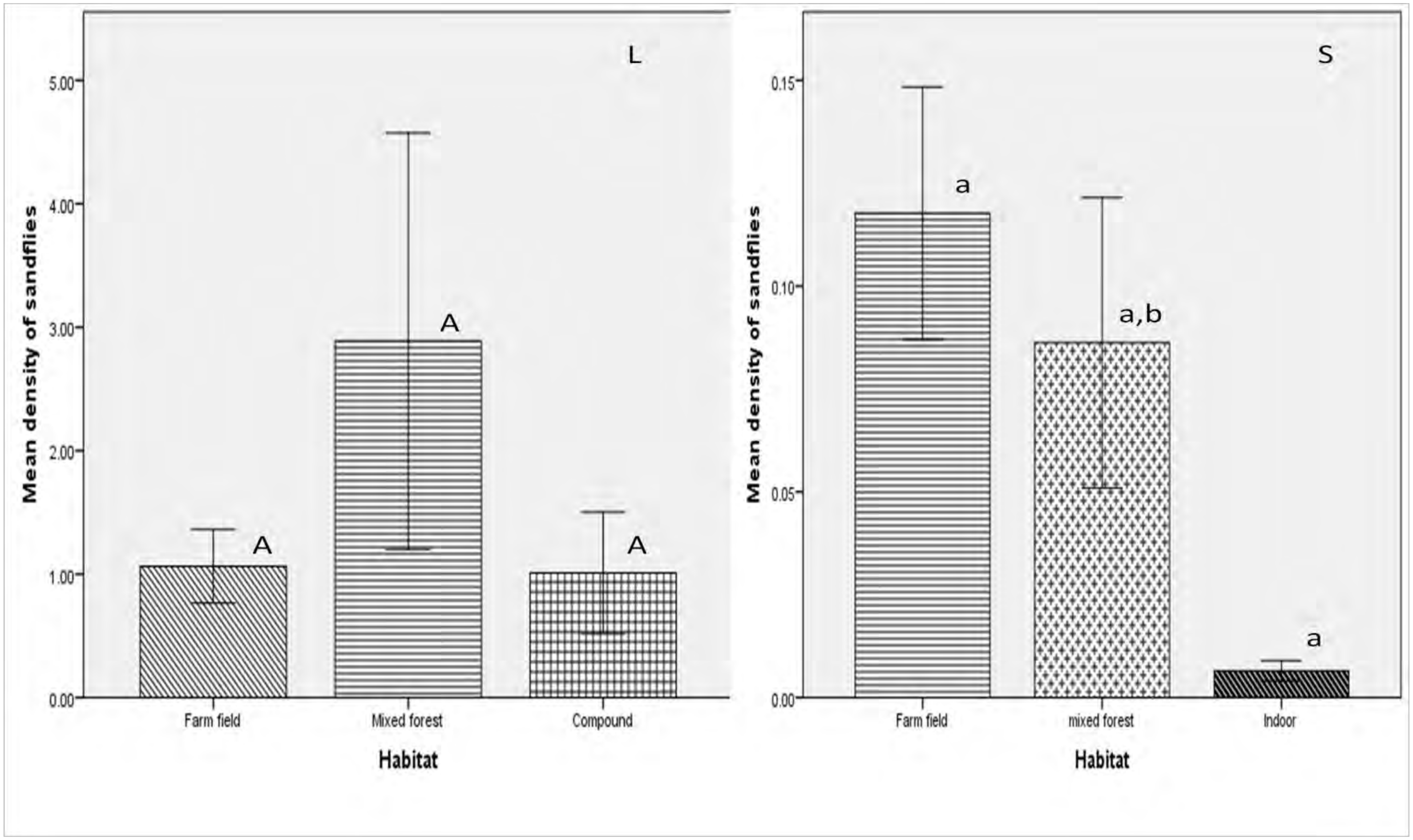

Figure 15. Habitat preferences of *P. orientalis* collected using light trap and sticky trap in Metema district. N.B. Significant differences between habitats are indicated by different letters above the bars ($P<0.05$); L= light trap, S= sticky trap

4.4. Discussion

From the results obtained in this study, it has clearly shown that most sandflies, including *P. orientalis*, the probable vector of VL in two districts, was virtually absent during the rainy season and abundant in the dry season (November-May/June). Dry season occurrence and abundance of *P. orientalis* has previously also noted by several investigators in Sudan (Hoogstraal and Heyneman, 1969; Elnaiem *et al*., 1997) and in Ethiopia (Gemetchu *et al*., 1975; Lemma *et al*., 2014a), although peak months of abundance may vary with locality and method of collection. Thus, in the highland area, *P. orientalis* was mainly active during the dry season (October-April) of the year and showed two distinct population peaks between the trapping methods, the highest density being in April (LTs) and May (STs). Like the highland population, in the lowland area the species activity concentrated in the low precipitation period of the year with similar peak in the density albeit the months of the peaks were different. The major peak was in June (on the onset of the rainy season) for both trapping methods. Such difference in population peaks between the two areas may be due to climatic difference as the highland area is more cool and humid during the dry season than the lowland area. Similarly, seasonal patterns of *P. orientalis* were also noted by earlier studies in different parts of Sudan (Quate, 1964; Hoogstraal and Heyneman, 1969; Elnaiem *et al*., 1997). In contrast to the present observations, previous study by Ashford *et al*. (1973a) did not observe any seasonal variation in the density of *P. orientalis* throughout the seven months of collection from Belessa valley, which is located about 100-130 km north of the highland study area.

Habitat-specific seasonal dynamics of *P. orientalis* also appeared in both study areas especially in LT collections. In Libo, the number of flies collected by LTs started activity in October and gradually increased and peaked in different habitats between January and April. The activity of

P. orientalis peaked first at mixed forest habitat in January and lasted in household compound and farm-field habitats in April. This discrepancy in population dynamics in different habitats may be associated with the variation in the time of formation of the deep cracks of black cotton soil between the habitats as this soil usually forms such deep cracking (~1m) in the late dry season (February–April). Similar seasonal differences between different habitats also were observed in the lowland area (Metema). However, in this area the activity of *P. orientalis* was in contrast to that of the highland area. The general seasonal pattern of *P. orientalis* showed a first peak in the farm field habitat in January followed by household compound habitat in April and mixed forest habitat in June.

In the lowland area, migrant workers from the surrounding highland regions (Gondar, Gojam and Tigray) make their first arrivals usually at the end of May. This migration is seasonal, and coincides with the different agricultural activities such as clearing of the natural habitat/forest for preparing a land and subsequent weeding and harvesting. This period also coincides with the peak density of *P. orientalis* in May and June (just before the rainy season) in the area leading the migrant workers to be exposed to biting challenges of *P. orientalis* and increased chances of being infected with *L. donovani*. The situation is more exacerbated in Metema where most of the migrant labourers sleep outdoors nearby the natural habitats around the farmlands without using any protection such as bed net (Pers. observation). Argaw *et al*. (2013) pointed out those migrant workers who slept under *Acacia* tree in Kafta-Humera area were associated with high risk of the disease.

The correlation analysis performed between the densities of *P. orientalis* and mean temperature in both districts showed positive correlation and it is in agreement with Elnaiem *et al*. (1997). In both areas, density of *P. orientalis* reached peak before the onset of the main rainy season when the mean temperature was <30^0C (24.4^0C for highland area and 27.7^0C for lowland area). However, rainfall (for both districts) and humidity (for Metema) had negative correlations with the densities of the species. The negative association between the density of the species and rainfall in the present study corroborated previous works by Quate (1964) and Hoogstraal and Heyneman (1969) as all of them could not find single adult *P. orientalis* during the rainy period in Sudan. Such indirect association was also pointed out by Gebre-Michael *et al.* (2004b). A similar negative association between rainfall patterns and abundance of *P. orientalis* was recorded in the district of Tahtay Adiyabo, northern Ethiopia (Gebresilassie *et al.*, in press). However, the association between the relative humidity and density of *P. orientalis* in the present study contradicts with the work of Elnaiem *et al.* (1997) who found direct correlation between the two in eastern Sudan.

Like *P. orientalis,* the six most abundant *Sergentomyia* species in the Metema district (*S. clydei*, *S. schwetzi*, *S. bedfordi*, *S. africana, S. antennata* and *S. squamipleuris*) exhibited seasonality. Their activity began in the first part of the dry season and continued until the beginning of the rainy season. During the rainy season the activity of these species became limited or completely absent. Similarly, Quate (1964) in Sudan also noted seasonal behavior in some *Sergentomyia* spp. such as *S. clydei* and *S. schwetzi*. In contrast to these seasonal species, he also reported other *Sergentomyia* species, which were non-seasonal species, including *S. africana, S. antennata* and *S. squamipleuris*. Of the *Sergentomyia* species in the present study, *S. clydei* and *S. schwetzi* are known to bite human in other parts of Ethiopia (Hailu *et al.,* 1995; Gebre-Michael and Lane, 1996), however they are not implicated as vector of kala-azar as these *Sergentomyia* spp. are unable to support the development of human *Leishmania* parasite in their gut (Kaddu *et al*., 1986; Sadlova *et al*., 2013).

Habitat preference study of *P. orientalis* collected by STs in both districts showed that farm field was the most productive habitat than that of mixed forest and indoor habitats. Similar findings have been observed by Lemma *et al.* (2014a) in Kafta-Humera district, north of Metema district. In both areas, majority of the farm field consists of black cotton soil (vertisol). This soil type is previously associated with the distribution of *P. orientalis* in East Africa (Thomson *et al*., 1999; Gebre-Michael *et al*., 2004b). Such type of soil is characterized by swelling when it gets water during the high precipitation period of the year leading the apparent absence of *P. orientalis* during that period and expanding when it loses water during the dry season resulting in very deep cracks where *P. orientalis* may use as breeding and resting habitats, leading to increased adult population. Furthermore, a study conducted in Humera area by Shabtai *et al.* (2014) indicated that vertisols from farm fields have high swelling capacity upon wetting than vertisol in a nearby forest. Accordingly, cracks in farm fields were markedly wider and deeper than cracks in forest habitat. Such wider and deeper cracks have high humidity and stable temperatures which could provide suitable breeding and resting sites for *P. orientalis* (Moncaz *et al*., 2014). However, very little is known about how *P. orientalis* survives the rainy season, but it may be by diapausing as late instar larvae as observed in laboratory colonies (Seblova *et al*., 2013) or Palaearctic species or as eggs as in warmer and wetter habitats (Killick-Kendrick, 1999).

Besides abundance of *P. orientalis* in the farm field, it is noteworthy to mention the low abundance of this sandfly species inside human dwelling (indoor) collected by non attractive STs in both study areas. The result indicated that this species is principally an outdoor species wherever it occurs as has previously been shown by several investigators (Hoogstraal and Heyneman, 1969; Elnaiem *et al*., 1997; Gebre-Michael *et al*., 2010), but in disagreement with

Lambert *et al*. (2002) where 79% of the total collected *P. orientalis* by LTs were from inside human dwellings in eastern Sudan. This difference may be due to variation in the type of trapping methods used as LTs lure phototropic exophagic flies from outside human dwelling to enter inside (Wheeler *et al.,* 1996). This observation in both districts has significance in planning control measures against VL.

In conclusion, the present data showed that *P. orientalis* is a seasonal species which is mainly active during the dry period of the year although activity peak showed variation between the highland and lowland populations. It also pointed out that the seasonal activity of the species varies depending on different types of habitats in the study areas. In addition, *P. orientalis* has strong association with the black cotton soil which may serve as breeding and resting habitats. Furthermore, in both areas this species of sandfly showed exophagic behavior. Therefore, control of kala-azar in both areas should involve designing of tools that mainly target the vector of the disease by considering its seasonal abundance, distribution and behavior.

Chapter five. Physiological age, host preference and vectorial potential of sandflies in Libo-Kemkem and Metema districts, northwestern Ethiopia

5.1. Introduction

Visceral leishmaniasis (VL) like other forms of leishmaniasis in the Old World is transmitted by bloodsucking female *Phlebotomus* species (Maroli *et al*., 2013). Females of this group use blood of vertebrate hosts to supply protein for the maturation of their eggs, while both the females and males are dependent on plant sugar sources (direct from plants/flowers or honeydew of aphids) for their energy requirements (Lane, 1993). During blood feeding, a sandfly may take the etiological agent of VL along with the blood meal from infected host and transmits the parasites during a subsequent feeding on another host (Garlapati *et al.,* 2012).

Based on the circumstantial evidences, *P. orientalis* has been implicated as a vector of VL in northwest and southwest Ethiopia. Some of these foci are the lower Omo in the southwest (Gemetchu *et al*., 1976; Fuller, 1979) and the Metema-Humera plains in the northwest (Gebre-Michael *et al*., 2010). So far, demonstration of natural infection in *P. orientalis*, which is one of the criteria of vector incrimination (WHO, 2010), has not been an easy task. Hailu *et al*. (1995) found *L. donovani* promastigotes in one of the *P. orientalis* dissected in the lower Omo plains. Gebre-Michael *et al*. (2004a) dissected 1219 females of the species in the Awash valley but none was infected with *Leishmania* spp. Similar unsuccessful attempts have been made with dissection of 607 and 618 *P. orientalis* dissected from Libo-Kemkem and Metema-Humera lowlands, respectively (Gebre-Michael *et al*., 2007: 2010).

Blood meal identification of haematophagous insects provides information on host-feeding preferences (whether anthropophagic or zoophagic) since nowadays, man-biting or man-landing catches are rarely carried out in endemic areas due to ethical concerns. In addition, such information also helps to implicate the potential reservoir host (s) (Jaouadi *et al*., 2013). A good reservoir host is a major blood meal source for the sandfly vector (Bray, 1982; WHO, 2010). In malaria epidemiology, it is always common to study the proportion of mosquitoes that have fed on humans (anthropophagic) and animals (zoophagic) in order to determine the human blood index (HBI) (Garrett-Johns, 1964). However, this is rarely used in phlebotomine sandflies.

Identification of the blood meal source of haematophagous insects has been performed using both serological and molecular techniques. The earliest and well known serological methods are precipitin test; hemagglutination inhibition assays, counter-current immune-electrophoresis (CCIE) and enzyme linked immunosorbent assay (ELISA) (Boreham, 1975; Dhanda and Gill, 1982; Beier *et al*., 1988; Ogusuku *et al*., 1994; Svobodova *et al*., 2003). Although these methods have yielded valuable information on the identity of the hosts of many blood feeding arthropods, they have some limitations, including the need to produce species-specific antibodies against each potential animal host and the requirement for relatively fresh blood (Blackwell *et al*., 1994).

Following the introduction of polymerase chain reaction (PCR), the above mentioned limitations have been resolved or minimized (Mukabana *et al*., 2002). Molecular method involves the amplification of either mitochondrial or nuclear DNA by PCR followed by species identification using restriction digestion (PCR-RFLP), terminal restriction length polymorphisms, heteroduplex mobility assays, and sequencing. All of these approaches require relatively large amounts of PCR product and do not detect multiple blood sources in a single insect. In order to overcome these setbacks Cytochrome *b* (cyt *b*) PCR- reverse line blotting

(RLB) assay was developed and used for identifying the blood meal sources (Abbasi *et al*., 2008).

Various attempts have been made to delineate the blood meal source of sandfly vectors of VL in different parts of the Old World. Guy *et al*. (1984) in France examined the blood meal sources of 530 fed *P. ariasi* using both precipitin ring test and CCIE and revealed the majority of the flies had fed on canids (44%). Likewise in India, Mukhopadhyay and Chakravarty (1987) determined blood meals of 725 *P. argentipes* using gel diffusion technique and pointed out the species has strong preference to cattle (68%). Similarly, Ngumbi *et al*. (1992) conducted blood meal analysis on 224 fed *P. martini* using ELISA in Kenya. The majority of the flies had fed on goat (28.5%) followed by rabbit (22.7%) and human (8.9%). In Italy, Rossi *et al*. (2008) also analyzed the blood of 80 engorged *P. perniciosus* using direct dot-ELISA and found avian blood 60.3% of the sandfly specimens.

Studies on the host preferences of the probable vector of kala-azar in Ethiopia are a few. Mamo (1999) analyzed some blood meal specimens of *P. orientalis* collected from the Awash Valley using CCIE and he showed the species to be predominantly zoophilic, feeding on a number of domestic animals. Similarly, Gebre-Michael *et al*. (2010) analyzed the blood meal sources of freshly fed *P. orientalis* females collected from Metema northwest Ethiopia using ELISA and found a similar result showing its predominant zoophilic behavior (92%) compared to only 2.2% that showed its anthropoghagic behavior.

The study of the physiological age (parous or nulliparous) of vector populations is of great importance in the epidemiology of vector-borne diseases, as parous females will have fed on vertebrate blood and may have been infected with *Leishmania* parasites (Lewis *et al*., 1970).

High parous rates in sandflies or other haematophagous insects implies high proportion of longer lived insects in the population, thus high number of potentially infected insects capable of transmitting the organism during the next feeding. There are various methods used to determine parous rates of sandflies such as observing for the presence or absence of granules in the accessory glands or follicular dilatations/relics in the ovaries or observing the general state of ovaries (Lewis and Minter, 1960; Gebre-Michael *et al.*, 1993; Anez and Tang, 1997).

In order to control sandflies and thereby to reduce leishmaniasis, the first important step is to identify the sandfly species, which has the ability to support full development of *Leishmania* parasites and transmit the parasites to human (vector species) (Killick-Kendrick, 1978). Identification or incrimination of vector species in a *Leishmania* endemic focus should fulfill at least one of the five basic vector incrimination criteria set by WHO (2010).

The aim of this study was to determine and compare physiological age, blood feeding habits and to detect natural infection of *P. orientalis* in two ecologically distinct foci of VL in northwestern Ethiopia (Libo-Kemkem and Metema districts).

5.2. Materials and Methods

5.2.1. Sandfly collection, dissection and blood meal sample collection

Sandflies were collected using CDC LTs (Chapters 3, 4 and 6) between May 2011-April 2012 in Libo and October 2012-September 2013 in Metema. During these activities, *Phlebotomus* females were categorized into different abdominal status (unfed, half-gravid, fully gravid, and freshly fed) under the dissecting microscope.

All freshly engorged *Phlebotomus* females collected by LTs and STs from both of the study areas were preserved for blood meal analysis. For this purpose, the head and tip of each fed female were severed from the rest of the body and slide-mounted separately for species identification (see Chapter three). The rest of the body corresponding to the parts that were slide mounted was individually placed either in silica gel or absolute alcohol. The specimens preserved in silica gel stored at room temperature whereas those in absolute alcohol were stored at -20^0C (for Libo samples) until blood meal analysis by either cyt *b* PCR-RLB or ELISA (see below).

5.2.2. Blood meal analysis using cyt *b* PCR-RLB

Blood meal analysis using cyt *b* PCR-RLB was conducted according to the procedure of Abbasi *et al*. (2008) in August 2012 in the Department of Microbiology and Molecular Genetics, Hadassah Medical School, Hebrew University, Jerusalem, Israel. Analysis of blood meal by this technique was carried out for some of the specimens collected from Libo-Kemkem (May 2011-April 2012). The rest of blood meal samples from both Libo-Kemkem and Metema were analyzed by ELISA at Aklilu Lemma Institute of Pathobiology, Ethiopia (see below).

DNA extraction

For DNA extraction, each specimen (thorax and abdomen of the flies) was individually placed in eppendorf tube which contained a mixture of 200µl lysis buffer (50mM NaCl, 10mM ethylene diamine teracacetic [EDTA], 50mM Tris-HCL 1% trition X-100) and 10µl proteniase K. The specimen was grinded with pestle. This was followed by extraction of DNA with phenol and precipitation using ethanol. Phenol extraction of DNA was carried out by adding of 180µl phenol and followed by addition of 8µl NaCl solution and centrifugated at maximum speed

(1400 rpm) for about two minutes. Then, precipitation of DNA was done by transferring 150µl the aqueous part (which contained the DNA) into another eppendorf tube, then adding 400µl ethanol onto it. The mixture with ethanol was preserved at -20^{0}C overnight, after which cold centrifugation at maximum speed (1400rpm) for 10 min was done to precipitate the DNA. Following the precipitation, the supernatant was discarded and the precipitated DNA was left to dry in oven for about 10min. After this, the DNA pellet was resuspended in 50 µl double distilled water (ddH_2O).

DNA amplification by polymerase chain reaction. 5 µl of DNA sample was mixed with 20 µl master mix, which contained forward and backward primers, *Taq* DNA polymerase, dNTPs, $MgCl_2$, reaction buffers and ddH_2O, to have 25µl of solution for PCR reaction. The target DNA for amplification was 344 base pairs of the conserved region of the cyt *b* gene. Amplification of this region was made using the following primer pairs: forward Cyto1: 5“-CCA TCA AAC ATA TCA GCA TGA TGA AA-3“ and reverse, Cyto2: 5“-CCC CTC AGA ATG ATA TTT GTC CTC -3“. The thermo-cycling conditions consisted of 35 cycles at 94°C for 30 sec, 55°C for 30 sec, and an elongation step at 72°C for 1 min.

Electrophoresis. The PCR products were loaded on agarose gel (1.5%) and electrophoresed at 120 V in 1x Tris-Acetate (TAE) buffer containing 10µl ethidium. The gels were visualized under UV light for determination of the sizes of the amplicon. The PCR amplified products were used as probes in RLB hybridization reaction.

RLB hybridization reaction. This procedure was done in two steps. These are

1. *Immobilization of Oligonucleotide to the membranes*

Biodyne C nylon membrane (5.5 × 15 cm, Gelman USA) was activated by washing three times using 0.1M HCL for 10min. Afterward, the membrane was rinsed in ddH$_2$O three times for 6 min and soaked in 10% solution of 1-ethyl-3-[3"-dimethyl amino propyl] carbodiimide (EDC) for half an hour. Then, the membrane was rinsed in ddH$_2$O and left to dry. Species specific 5"-end amino linked oligonucleotide probes for human, cow, sheep, goat, camel, donkey, dog, mouse, rat, chicken, and bird (avian) developed by Abbasi *et al*. (2008) were diluted to 5pmol/μL and added to the membrane. The above probes were linked to nylon membrane through the formation of amide bonds between the carboxyl groups on the nylon membrane and the amino groups linked to the oligonucleotides using a manifold blotter apparatus (Immunetics, Cambridge, MA).

2. Hybridization and Detection

The nylon membrane sheet with above mentioned oligonucleotide probes was cut into strips at 90^0 to the direction of the blot; hence each strip contained all the eleven probes. Strips were placed in the incubation tray, which had eight lanes. Strips were incubated in pre-hybridization solution (2 x sodium chloride and sodium citrate [SSC] with 0.1 Sodium dodecyl sulfate [SDS]) for 30min at 46^{0}C with gentle shaking. Biotinylated PCR products were denatured in water bath at 95^{0}C for 10min. Hybridization of the denatured PCR products took place at the same temperature of incubation of strips for an hour. Hybridized biotinylated DNA was detected by incubating the strips in streptavidin horseradish peroxidase (HRP) for 30min at room temperature. After washing the strips three times using 2 xSSC, 0.1%SDS, freshly prepared

TMB solution (0.1mg/ml of 3, 3“, 5, 5“ tetramethylbezidine, 0.003% H_2O_2 in 0.1M sodium citrate [pH 5.0]), was added for chromogenic detection. After a few minutes bands were observed.

5.2.3. Blood meal analysis using ELISA

Blood meal analysis using ELISA was performed on remaining specimens from Libo-Kemkem and all of the specimens from Metema following the procedure of Beier *et al*. (1988) with some modifications. Briefly, each fed fly was triturated in 1.5ml eppendrof tube with micro tissue grinders to which 50µl of 0.01 M phosphate buffered saline (PBS), pH 7.4, was added. The triturated sample was kept at -20^0C until analysis. Then, 50µl of the triturate sample was diluted in carbonate/bicarbonate coating buffer (CBB) (1:50) and 50µl of the mixture was added to wells of polyvinyl chloride, U-shaped, 96-well microtiter plates (Dynatech Laboratories, Inc., Alexandria, Va), which were covered and incubated at 4^0C overnight. Each well was washed three times with washing solution (200µl PBS containing Tween-20). Plates were subsequently blocked by adding bovine serum albumin (BSA) and incubated for one hour at 37^0C. Each well was washed three times with washing solution (PBS-Tw-20). This was followed by the addition of 50µl host specific conjugate (antihost IgG, Human, bovine, donkey, goat, sheep and dog) diluted 1:2000 for human, 1:250 for bovine, 1:5000 for the rest of animals) in 0.5% boiled casein containing 0.025% Tween-20. The boiled casein was prepared by boiling 5g casein in 100ml 0.5 N NaOH and adding 900ml PBS (pH 7.4), 0.1g Thimerosal (sodium ethyl mercuri thio salicylate) and 0.02 gm phenol red. After 1h, wells were washed three times with PBS-Tw-20, and 100 µl of ABTS peroxidase substrate were added to each well. Absorbance at 405 nm was determined with an ELISA reader 30 min after the addition of substrate. Each blood meal sample was considered positive if the absorbance value exceeded the mean plus three standard deviations of the mean of three negative controls and also by observing color change (green color). Negative controls were prepared using unfed laboratory-reared female *P. orientalis* from ALIPB‟s colony. Positive controls were blood specimens of the six hosts.

The anti-immunoglobulin antisera were pre-screened to verify their antigentic specificity by reactions with blood meal samples of the six hosts. Cross- reaction was noted only between anti-goat and anti-sheep antisera and the result of these (goat and sheep) reported as one, i.e. goat/sheep.

5.2.4. Dissection of sandflies for determination of physiological age and detection of natural infection

Unfed (including those without the remains of previous blood meal) females of *Phlebotomus* spp. were dissected for determination of parous state. Flies were first rinsed twice in 2% savlon in saline and once in sterile physiological saline. Then, each female fly was dissected in a drop of physiological saline on a glass slide under a dissecting microscope. The ovaries were pulled out along with the gut of the fly, and then covered with a small cover slip for examination under the microscope for determination of parous states. Parous females were distinguished from nulliparous by the presence of granules in the accessory glands (Lewis *et al*., 1970) as well as ovarian features described by Gebre-Michael *et al*. (1993). The gut of parous female was instantaneously examined with 10 x and 40 x objectives of the microscope for the presence of flagellated parasites. The guts of half and fully gravid females *Phlebotomus* sandflies were also drawn out and examined for *Leishmania* promastigotes under the microscope. After microscopic examination, the guts of parous, half-gravid and gravid females in the saline were transferred to 70% alcohol for further detection of parasites using molecular method in case infection might have been overlooked (described below). In addition, leftover undissected females *Phlebotomus* were also preserved in alcohol to detect the parasites by the same technique.

5.2.5. Detection of *Leishmania* parasites using molecular method

Four procedures were followed to detect *Leishmania* spp. parasites in the preserved guts of dissected sandflies, undissected wild caught sandflies. These were DNA extraction, DNA amplification, gel electrophoresis and sequencing of DNA samples to identify the species of *Leishmania* (Abbasi *et al*., 2013). DNA extraction and gel electrophoresis steps were similar to that of the steps (described above) used in blood meal analysis using molecular method. However, DNA amplification step was quite different as it targeted different DNA base pairs and used different primers to that of the blood meal analysis. The target DNA for amplification was 346bp of the whole internal transcribed spacer (ITS) in the ribosomal operon. The DNA was amplified using the primers LITSV (5‟-ACACTCAGGTCTGTAAC-3‟) and LITSR (5‟-CTGGATCATTT-TCCGATG-3‟). The positive controls were *L. donovani*, *L. major* and *L. aethiopica* whereas the negative control was distile water.

5.3. Results

5.3.1. Abdominal status of *Phlebotomus* spp. in both areas

Table 8 shows the abdominal status of female *Phlebotomus* species during dissection in the two study areas. Of the total 1314 female *P. orientalis* examined under the microscope in Libo-Kemkem, the great majority, 958 (72.9%) were unfed, followed by 205 (15.6%) that were freshly fed and 151 (11.5%) were gravid or half-gravid females. In Metema, of the total 484 *P.*

orientalis females examined for their abdominal status, 213 (44.0%) were unfed, 192 (39.7%) were freshly fed and the rest (16.3%) were gravid or half-gravid females.

Table 8**.** Abdominal status of female *Phlebotomus* species determined during dissection from both study areas

Species	No. collected		No. Unfed (%)		No. gravid (%)		No. Freshly fed (%)	
	Libo	Metema	Libo	Metema	Libo	Metema	Libo	Metema
P. orientalis	1314	484	958(72.9)	213(44)	151(11.5)	79(16.3)	205(15.6)	192(39.7)
P. rodhaini	1	46	1 (100)	39(84.8)	0	7(15.2)	0	0
P. papatasi	0	3	0	1(33.3)	0	2(66.7)	0	0
P. duboscqi	0	2	0	1(50)	0	1(50)	0	0
P. bergeroti	0	1	0	0	0	1(100)	0	0

5.3.2. Parous rates

Results of dissection of *Phlebotomus* females for determination of physiological age and monthly parous rates in both districts are depicted in Table 9 and Fig 16, respectively. In Libo, a total of 936 unfed *P. orientalis* were dissected. Of these, average parous rate was 45.6% (n=427), ranging from 37.0 in October to 61.6% in the middle of the dry season (February) with obvious seasonal trend. In Metema, out of the total of 213 unfed *P. orientalis* dissected the mean parous rate was 66.2% (n=141), ranging from 0 during early dry season (October-December) to 100% at the beginning of rainy season (July), thus showing clear seasonal pattern. From the small number of *P. rodhaini* dissected (n=39) in Metema, 46.1% (n=18) were parous.

Table 9. Parous rates of *Phlebotomus* females dissected in Libo-Kemkem and Metema districts

Species	Libo-Kemkem		Metema	
	No. dissected	No (%) parous	No. dissected	No. (%) parous
P. orientalis	936	427 (45.6)	213	141 (66.2)
P. rodhaini	1	1 (100)	39	18 (46.2)
P. papatasi	0	0	1	1 (100)
P. duboscqi	0	0	1	1 (100)
P. bergeroti	0	0	1	0
Total	937	428 (**45.7**)	252	161 (**63.8**)

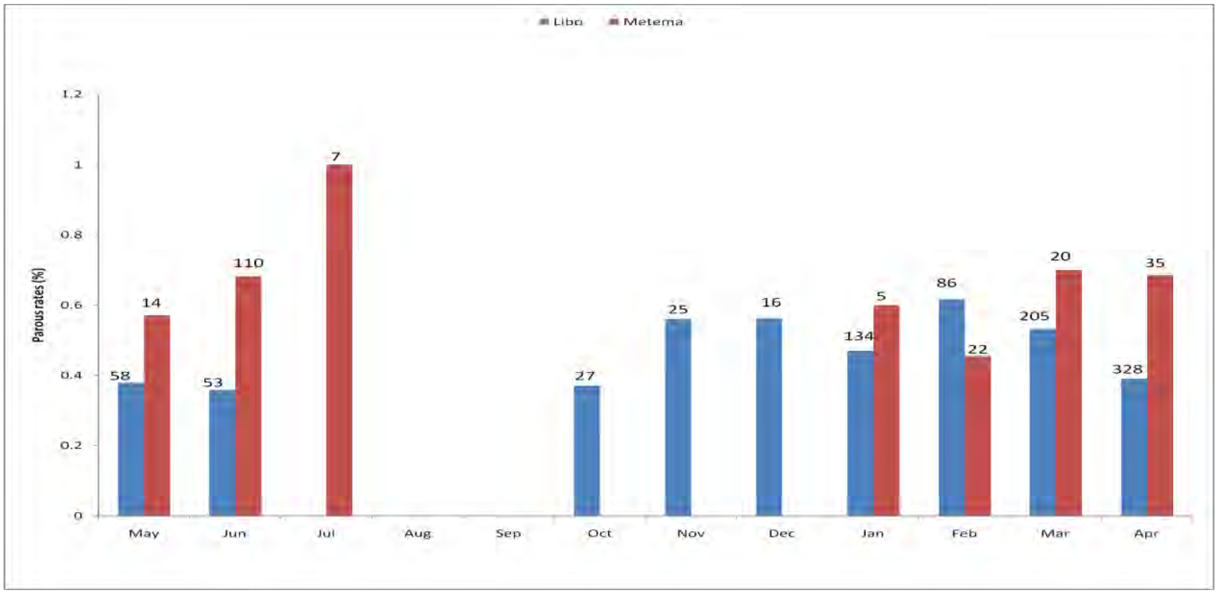

Figure 16. Monthly parous rate of *P. orientalis* dissected from Libo-Kemkem (May 2011- April 2012) and Metema (October 2012- September 2013) districts. N. B. number above the bar indicates the total number of *P. orientalis* dissected per month

5.3.3. Blood meal identification based on Cyt *b* PCR-RLB

A total of 216 blood fed females *P. orientalis* were collected from May 2011 to April 2012 in Libo-Kemkem mainly from two villages (Angot and Bura). Out of these fed sandflies, 115 flies were analyzed using cyt *b* PCR-RLB to identify the blood meal source for the flies. The remaining blood samples were analyzed by ELISA. Of 115 blood fed *P. orientalis*, 113 (98.3%) were positive to cyt *b* PCR (Fig 17) and were used for blood meal identification using RLB. Out of 113 blood fed sandflies positive to PCR, the results after immobilization, hybridization and chromogenic detection (RLB) revealed the following blood meals sources: 75 (66.4%) were of cow origin and 6 (5.3%) were from human origin. Mixed blood meal for human-cow detected in 18 (15.9%). The rest 14 (12.4%) were unidentified (Table 10 and Fig 18).

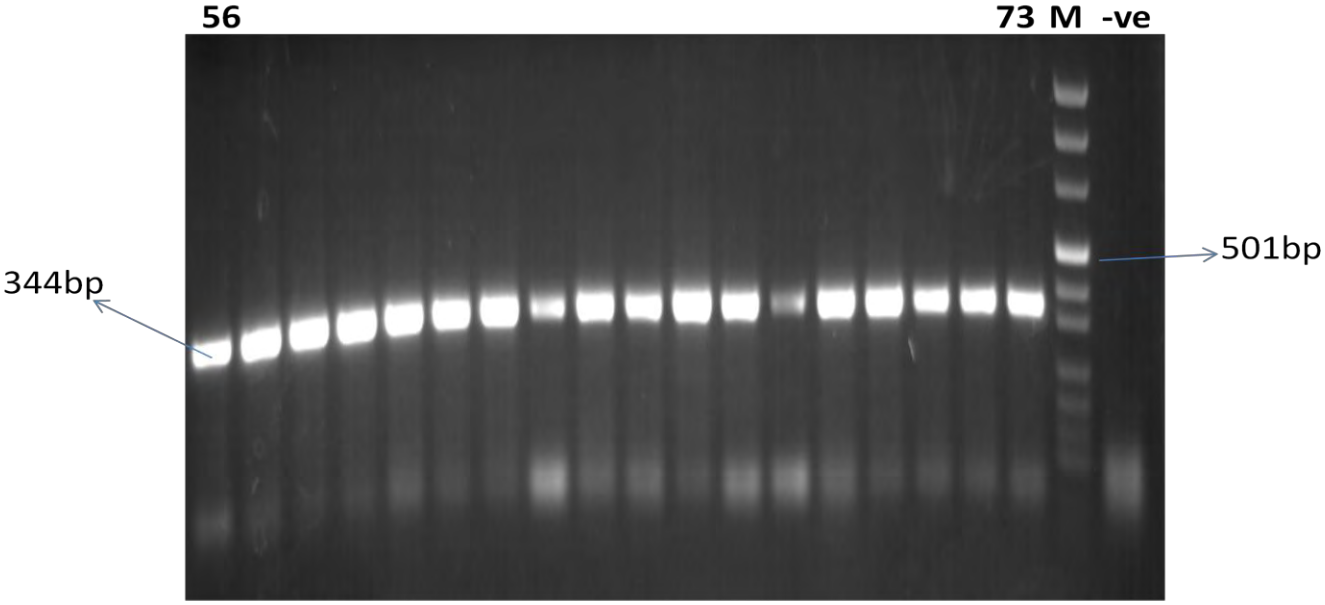

Figure 17. Gel image of cyt *b* PCR targeting DNA extracted from wild caught blood fed *P. orientalis*. Lanes 56 to 73 are PCR products of blood fed sandfly amplified for cyt *b* region. M is DNA marker. –ve, negative control

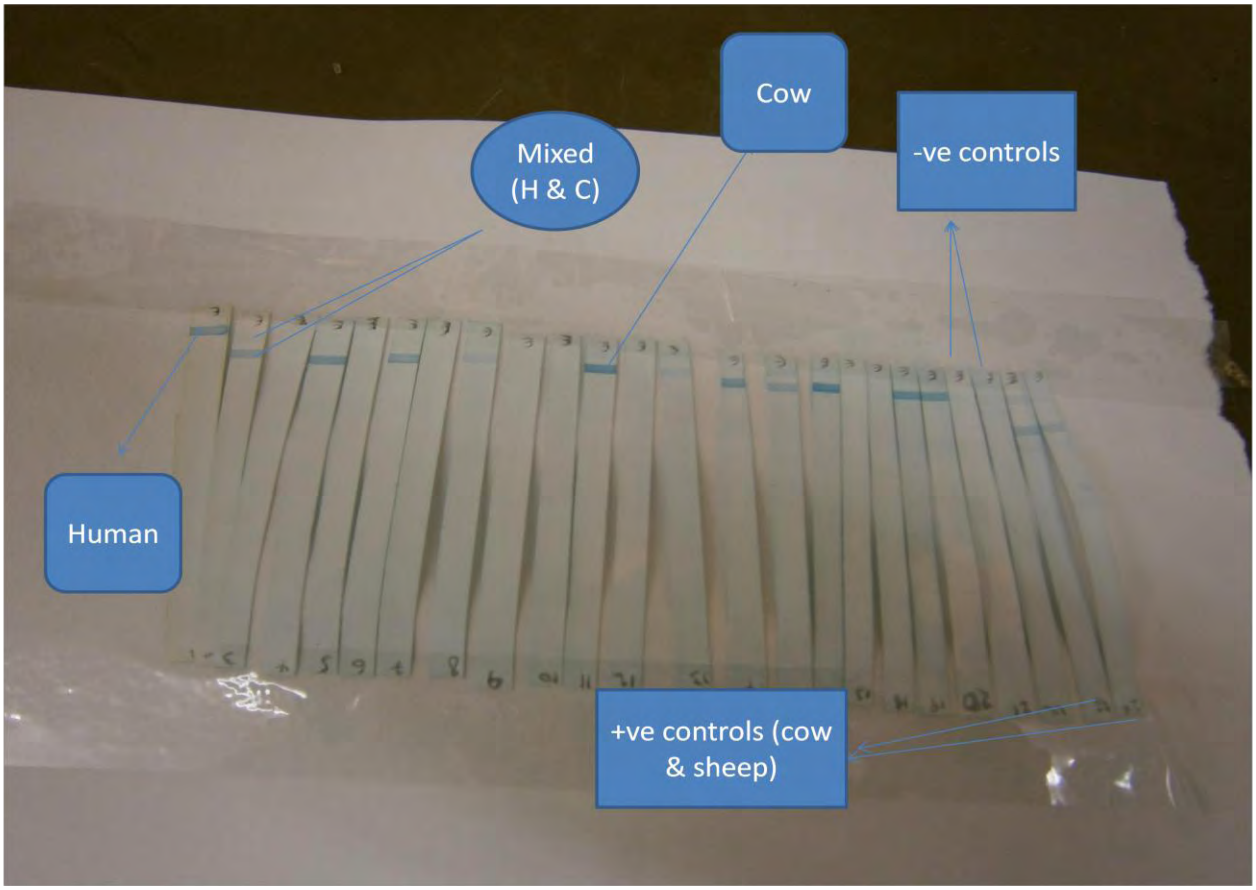

Figure 18. Reverse line blotting results of cyt *b* PCR products from wild caught blood fed *P. orientalis*. H=human, C= cow

5.3.4. Blood meal identification based on ELISA

Libo-Kemkem

A total of 101 blood fed females *P. orientalis* were analyzed for blood meal source using ELISA. Results are summarized in Table 10, which shows that five animals served as blood meal source for *P. orientalis* with 69.3% (n=70) positive for bovine blood alone, 1.9% (n=2) positive for human alone, and 0.99% (n=1) positive for goat/sheep alone. Mixed blood meals were also identified that included; 10.9% (n=11) for human-bovine blood, 0.99% (n=1) for human-donkey blood, 0.99% (n=1) for bovine-donkey blood and 0.99% (n=1) for bovine-dog blood. The remaining 13.9% (n=14) blood meal samples were unidentified.

Table 11 shows blood feeding habits of *P. orientalis* in different sampling habitats of the area. The majority of the blood feds were from the compound habitat (163/214) and seven of the eight human fed *P. orientalis* were also caught in the compound. The other hosts, which were identified in this habitat, were bovine, goat/sheep, bovine-human, human-donkey, bovine-donkey, and bovine-dog. Only a single fed *P. orientalis* collected from indoor and it had fed on both cow and human.

Blood meal Indices based on combined cyt *b* PCR-RLB and ELISA

The HBI (including the mixed feeding) of *P. orientalis* in Libo-Kemkem based on both methods of analysis (cyt *b* PCR-RLB and ELISA) was 17.3%, whereas the bovine blood index (BBI) (including the mixed feedings) was 82.2%, showing its higher predilection for cattle.

Table 10. Sources of blood meals of *P. orientalis* sampled from Libo-Kemkem and Metema districts identified by cyt *b* PCR-RLB and ELISA

Blood meal sources	District				Total (%)
	Libo-Kemkem			Metema	
	RLB= 113	ELISA = 101	Combined = 214	ELISA=277	491
Bovine	75 (66.4)	70 (69.3)	145 (67.8)	138 (49.8)	283 (57.6)
Human	6 (5.3)	2 (1.9)	8 (3.7)	16 (5.7)	24 (4.9)
Donkey	0	0	0	15 (5.4)	15 (3.1)
Dog	0	0	0	4 (1.4)	4 (0.8)
Sheep/Goat*	0	1 (0.99)	1 (0.5)	4 (1.4)	5 (1.0)
Human-Bovine	18 (15.9)	11 (10.9)	29 (13.6)	5 (1.8)	34 (6.9)
Human-Donkey	0	1 (0.99)	1 (0.5)	9 (3.2)	10 (2.0)
Human-Dog	0	0	0	3 (1.1)	3 (0.6)
Bovine-Donkey	0	1 (0.99)	1 (0.5)	6 (2.2)	7 (1.4)
Bovine-Dog	0	1 (0.99)	1 (0.5)	9 (3.2)	10 (2.0)
Bovine-Sheep/Goat	0	0	0	2 (0.7)	2 (0.4)
Bovine-Donkey-Dog	0	0	0	1 (0.4)	1 (0.2)
Unidentified	14(12.4)	14 (13.9)	28 (14.0)	65 (23.5)	93 (18.9)

***** Since there was cross-reaction between the two antisera the results are presented as goat/sheep

Table 11. Blood meal identification for *P. orientalis* from different sampling habitats of Libo-Kemkem using cyt *b* PCR-RLB and ELISA

Blood meal sources	**Sampling habitat**				
	Indoor (%)	Compound (%)	Farm field (%)	Mixed forest (%)	**Total** (%)
Bovine	0	114 (69.1)	27(75.1)	4(28.6)	145(67.7)
Human	0	7(4.2)	1(2.7)	0	8(3.7)
Sheep/Goat*	0	1(0.6)	0	0	1(0.5)
Human-Bovine	1 (100)	18(10.9)	5(13.9)	5(35.7)	29(13.5)
Human-Donkey	0	1(0.6)	0	0	1(0.5)
Bovine-Donkey	0	1(0.6)	0	0	1(0.5)
Bovine-Dog	0	1(0.6)	0	0	1(0.5)
Unidentified	0	22(13.3)	3(8.3)	5(35.7)	28(13.1)
Total (%)	1(0.5)	165(76.3)	36(16.7)	14 (6.5)	214

In Metema, a total of 277 blood fed females *P. orientalis* were collected during the course of the study period in Metema (October 2012-September 2013) and were all analyzed by ELISA. The majority of these flies (94%, n=259) were collected from household compounds. Results of the analysis are shown in Table 10 above. Thus, the pattern for single hosts was: 138 (49.8%) for bovine blood, 16 (5.7%) for human blood, 15 (5.4%) for donkey blood, 4 (1.4) for dog blood, 4 (1.4%) for sheep/goat blood. Mixed blood meals from either two hosts (six) or three hosts (one) were also identified that included: 9 (3.24%) of bovine-dog, 9 (3.24%) of human-donkey, 6 (2.2%) of bovine-donkey, 5 (1.8%) of bovine-human, 3 (1.1%) of human-dog, 2 (0.7%) of bovine-sheep/goat and including one tri-host (0.36%) from bovine-donkey-dog. Specimens for 65 (23.5%) blood meals remained unidentified.

Blood meal Indices

The HBI for *P. orientalis* (including the mixed blood feedings) in Metema was 11.9% while the bovine blood index (including the mixed feedings) was 58.1%, showing again, a predilection for cattle, though much lower than in Libo-Kemkem.

5.3.5. *Leishmania* detection based on dissection and molecular method

Results of dissection for determination of leishmanial infection rates in female *Phlebotomus* spp. from both highland and lowland study areas are depicted in Table 12. In the highland area (Libo), the guts of 578 females *P. orientalis* (427 parous and 151 gravid) and 1 parous *P. rodhaini* were observed for detection of natural infection during dissection. None of these flies was found infected with *Leishmania* parasites microscopically. Similar results were also obtained from 251 *Phlebotomus* spp. (141 parous and 79 gravid *P. orientalis*, 18 parous and 7 gravid *P. rodhaini*, 1 parous and 2 gravid *P. papatasi* and 1 parous and 1 gravid *P. duboscqi*) dissected in Metema.

In addition to microscopic dissection to determine natural infection, attempt was made to detect natural infection using molecular method. A total of 347 *P. orientalis* specimens collected from Libo were processed using PCR. Of these, 10 (2.8%) specimens were found to be positive to *Leishmania* spp. (Fig 19). However, sequencing of these *Leishmania* positive specimens for identification of the *Leishmania* species did not give positive result due to low concentration of DNA in the specimens.

Furthermore, a total of 2230 females of *P. orientalis* (parous, gravid and undissected) from Libo-Kemkem and 1131 *P. orientalis,* 111 *P. rodhaini,* 3 *P. papatasi,* 4 *P. duboscqi* and 4 *P. bergeroti* from Metema were preserved in 70% alcohol and stored in refrigerator at ALIPB to be processed by molecular method.

Table 12. Dissection results for leishmanial infection rates in Libo-Kemkem and Metema districts

Species	Libo-Kemkem			Metema			Total dissected (% infec)
	No. parous diss.	No. gravid diss.	No. inf. (%)	No.parous diss.	No. gravid diss.	No. inf. (%)	
P. orientalis	427	151	0	141	79	0	798 (0)
P. rodhaini	1	0	0	18	7	0	26 (0)
P. papatasi	0	0	0	1	2	0	3 (0)
P. duboscqi	0	0	0	1	1	0	2 (0)
P. bergeroti	0	0	0	0	1	0	1 (0)
Total	428	151	0	161	90	0	830 (0)

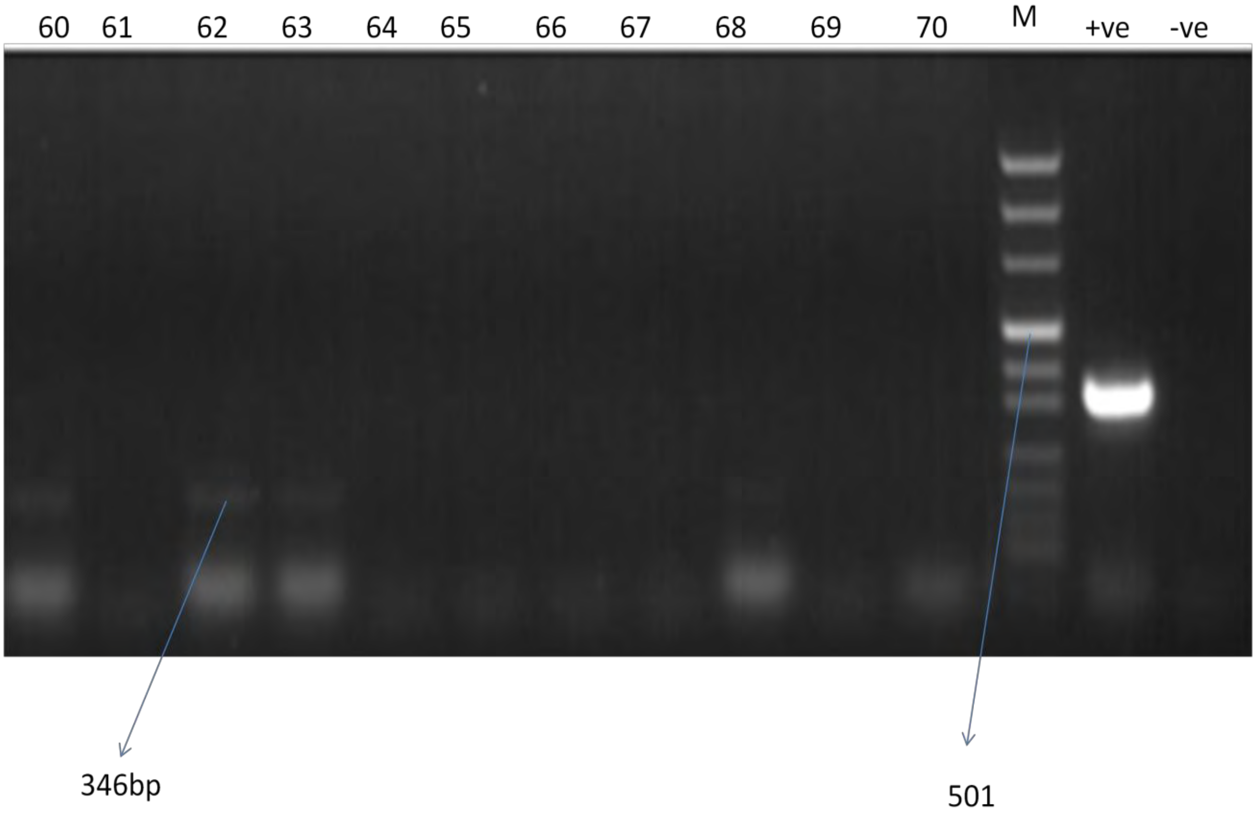

Figure 19. Agarose gel electrophoresis of PCR amplification of extracted from wild caught *P. orientalis*; Lanes 60 to 70 PCR product sandfly amplified for ITS region. M is DNA marker. +ve- positive control, -ve- negative control

5.4. Discussion

Recognition of parous females of sandflies has epidemiological significance as these flies must survive at least one oviposition cycle to be potentially infective with *Leishmania* spp. parasites (Lewis, 1971). The overall parous rates of the dissected *P. orientalis* females were 45.6% and 66.2%, in Libo-Kemkem and Metema, respectively. This perhaps indicates that highland population of *P. orientalis* are short-lived, with most failing to survive long enough to take a second blood meal, presumably dying during or immediately after oviposition in comparison with the lowland population of the species. Similar observation was also noted by Anez *et al*. (1994) in Venezuela. However, parous rates of the species in both populations were higher than that of previous observations reported from these areas (Gebre-Michael *et al*., 2007:2010). Such discrepancy might be due to seasonal differences as earlier investigations were done during a brief period of time in both study areas and relatively smaller numbers of females were dissected in Metema in the present study. However, low parous rates have been reported in *P. ariasi* in southern France: 33% (Dye *et al*., 1987); 14-26% (Gibb *et al*., 1988) and in *P. papatasi* in Jordan Valley: 39% (Yuval *et al*., 1988). In the current study, parous rates of *P. orientalis* showed seasonal trend being higher in February (61.6%) in Libo (dry season) and July (100%) in Metema (rainy season) and lower during the rainy season in both areas. Likewise, parous rates of *P. martini* and *P. celiae* showed seasonal variation in southern Ethiopia, being lower during the rainy season and higher during the dry season (Gebre-Michael and Lane, 1996).

This is the first attempt to describe the natural blood meal sources of *P. orientalis* in the highland kala-azar endemic area, whereas some information is already available for *P. orientalis* from the lowland kala-azar endemic area (Gebre-Michael *et al*., 2010). Of the analyzed flies using the molecular technique in Libo, 98.3% of them were positive for cyt *b* PCR reaction. The flies that were not positive for the PCR may have had dried out in which the DNA could not be extracted or <0.1pg DNA was present in the ingested blood, which could not be detected (Abbasi *et al*., 2008). Five hosts (single and mixed) were identified as sources of blood meals for *P. orientalis* by both techniques of analysis, of which bovine was the most preferred host with about the same magnitude (66% RLB and 69% ELISA, the overall being

68%). The results also indicate that humans were comparatively less attractive to *P. orientalis* with overall feeding rate of only 3.7% by both methods. Thus, the human blood index (17.3%) was much lower than the bovine blood index (82.2%), showing the zoophilic feeding habits of *P. orientalis* in particular cattle. On the other hand, blood meal analysis of *P. orientalis* from the lowland area using ELISA showed the presence of at least five hosts (single and mixed) for *P. orientalis*. However, the mixed combinations of hosts were wider in scope (at least five) than in the highland, with bovine and human being the commonest combinations. As in the highland, *P. orientalis* showed a higher preference of blood feeding on bovine (49.8%) than on human (5.7%) showing its predominant zoophilic behavior, but this behavior was lower than it was observed in the highland. Such difference might be due to the lowland population of *P. orientalis* having more access to other hosts such as goat, sheep, and donkey as compared to the highland population, where most of goats and sheep were kept inside the hut during the night.

These data from both areas agree with a previous observation in Metema by Gebre-Michael *et al*. (2010) who showed *P. orientalis* was a predominantly zoophilic species, cattle being the major host (92%) and much higher than the present study. Studies on host choice conducted recently in Humera and Sheraro, northwestern and north Ethiopia, respectively, have corroborated the present observation where cattle were found to attract the highest number of *P. orientalis* (Gebresilassie *et al*., in press; Yared unpub. data). In contrast, a study on host attractiveness in a limited number of animal hosts (dog, mongoose, Nile rat and genet) to *P. orientalis* in eastern Sudan, showed that dog baited trap significantly attracted the highest number of *P. orientalis,* though no other domestic animals were used in the experiment (Hassan *et al*., 2009); there is yet no results available on blood meal analysis of *P. orientalis* from Sudan. Ngumbi *et al.* (1992) analyzed the blood meal sources of 224 *P. martini* using ELISA in

Kenya. The species fed mainly on goat, rabbit and human. In India, Palit *et al*. (2005) analyzed blood meals of 304 *P. argentipes* and 206 *P. papatasi* using ELISA and showed *P. argentipes* to be more zoophilic (57. 2%) than *P. papatasi* (6.3%). In contrast to zoophilic behavior of *P. argentipes* in West Bengal, this species showed anthropohilic behavior as revealed by cyt *b* PCR-RLB in Bihar state (Garlapati *et al*., 2012). Recently, in Kafta-Humera (northwest Ethiopia) Lemma *et al*. (2014b) analyzed 25 blood-fed *P. orientalis* using cyt *b* PCR-RLB and found 7 (28%) for human blood, 9 (26%) for mixed human and cattle blood and 2 (8%) for cattle blood.

The low HBI both in the highland (17.3%) and lowland (12%) is comparable, but were much higher than the previous finding (4.7%) in Metema (Gebre-Michael *et al*., 2010). Elsewhere in Sheraro and Humera in northern and northwestern Ethiopia, respectively, *P. orientalis* also exhibited similar bovine feeding behavior (Gebresilassie and Yared, pers.com) as was also the case in the Awash Valley, northeast Ethiopia (Mamo, 1999).

The driving force behind preference of *P. orientalis* for cattle to other hosts in the present study is not known; however from previous knowledge on host preferences of other haematophagous insects, different explanations can be given. One of these reasons could be the relative abundance of the hosts which might determine the host preference of vector species (Takken and Verhulst, 2013). In both study areas large numbers of cattle with small number of goats, sheep, donkeys and dogs were present outside of human dwellings and these animals were guarded by two to three adult men during the night. The other reason could be difference in body size of the animals. This difference in size in turn results in variation in the amount of CO_2 released by the animals (Quinnell *et al*., 1992). Thus, larger animals like cattle could be more attractive than the rest, although some authors suggested that animal size had no any effect on host selection by sandflies in case of *Lu. olmeca, Lu. panamensis* and *Lu. vespertilionis* (Christensen and Herrer, 1980).

The role of cattle in the epidemiology of visceral leishmaniasis is controversial. In India, Barnett *et al*. (2005) concluded that owning a large number of cattle is a risk factor for VL transmission. A similar result was also reported by Bucheton *et al*. (2002). These studies hypothesize the presence of plethora cattle in the areas might serve as blood meal source for the vector species and also the byproduct of these animals could be an important source of food for sandfly larvae and as a result of which the density of the vector species increase. This increase in abundance of vector species increases vector-host contact and accordingly the chance for transmission of the disease. In contrast, Bern *et al*. (2005) reported that increasing cattle density around house decreased kala-azar transmission by sandflies in Bangladesh. It has been suggested that the proximity of cattle to human dwellings diminish kala-azar transmission by enabling vector species to feed preferentially on animals, not susceptible to leishmaniasis (dead end host), thereby decreasing vector-human contact (zooprophylaxis effect) (Mahande *et al*., 2007). However, the role of cattle in both of our study areas other than serving as main blood meal sources is not clear.

The detection of other blood meal hosts, though at lower proportion, might indicate the opportunistic behavior of *P. orientalis,* enabling it to feed on those readily available hosts. Such behavior is common in a number of species observed elsewhere (Foster *et al*., 1972b; Guy *et al*., 1984; Ngumbi *et al*., 1992; Mamo, 1999; Bongiorno *et al*., 2003).

In both areas, particularly in the lowland region, a considerable proportion of *P. orientalis* analyzed had contact with more than one host (mixed feeding) during a single gonotrophic cycle. Of these, 13.7% had double meals and 0.4% contained triple meals, presumably on the same night. Such mixed blood meals are commonly observed in other sandflies (Guy *et al.,* 1984; Ngumbi *et al*., 1992; Rossi *et al*., 2008). The exact cause of mixed feeding for this and other species of sandflies in nature is not clear, however, it is believed to be a consequence of interrupted feedings due to difficulties faced while they engorging on a single host-due to host defensive behavior (Coleman and Edman, 1987; Bongiorno *et al*., 2003), or when the fly encounters an unnatural host it is diverted to another nearby host before repletion from the first host, or the fly might be infected by *Leishmania* parasite and this infection by the parasite promotes multiple feeding (Rogers and Bates, 2007).

The numbers of unidentified blood meals in both areas were considerable: 28 (14.0%) in Libo-Kemkem and 65 (23.5%) in Metema. The failure to determine the blood meal sources of *P. orientalis* may have occurred as a result of either not using probes/antisera of both large (e.g. warthog) and small (e.g. murines and canines) wild mammals which were seen frequently in the study areas especially in Metema. It could also be due to the poor quality of the blood meal resulting from enzymatic degradation of the blood.

Owing to the populations of *P. orientalis* in both localities having a strong predilection for cattle, treating of these animals by topical application of insecticides (Courtenay *et al*., 2009) or systematic insecticides such as ivermectin (Kassem *et al*., 2001; Mascari *et al*., 2008) may help in alleviating the burden of the disease in the study areas. However, proper evaluations on these potentialities are required.

Identification of vectors and determination of natural infection rates with *Leishmania* spp. in wild flies are important for definition of risk factors and epidemiologic control of leishmaniasis

(Oshaghi *et al*., 2009). In the current study, no natural infection was detected in 1379 *P. orientalis* females dissected (1087 from Libo and 292 from Metema), nor in other *Phlebotomus* females dissected (47 *P. rodhaini*, 3 *P. papatasi*, 2 *P. duboscqi* and 1 *P. bergeroti*) from both study areas. Similarly, attempt to detect natural infection using molecular methods was also made in 347 specimens of *P. orientalis* from Libo. Of these, 2.8% of the specimens were found positive for *Leishmania*; however, sequencing of the DNA of the *Leishmania* positive samples to identify the *Leishmania* spp. was not successful. This might indicate low prevalence of the kala-azar in the population during the study period. However, absence of promastigotes in the gut of dissected *P. orientalis* is not uncommon in Ethiopia. Ashford *et al*. (1973a) dissected a total of 1006 females of this species in Belessa and found no infection. Similarly, no infection was detected in small number of *P. orientalis* (n=607) dissected in Libo (Gebre-Michael *et al*., 2007) nor in the 618 *P. orientalis* dissected in Humera and Metema districts (Gebre-Michael *et al*., 2010). Natural infection rates in phlebotomine sandflies are usually low, and it is sometimes necessary to dissect thousands of sandflies to find an infected specimen (Killick-Kendrick, 1990). This might be due to deficiencies in sampling, intensity of the disease or due to longevity of the vector. In previous studies by Gebre-Michael *et al*. (2007:2010), the majority of dissected flies were nulliparous, while the parous rates were only 30-34% in both the highland (Libo) and lowlands (Humera and Metema). On the other hand, previously Hailu *et al*. (1995) found natural *L. donovani* infection in one of 70 (1.4%) dissected female *P. orientalis* in southwest Ethiopia, while Ashford *et al*. (1992) detected 4 infected among 48 *P. orientalis* (8.3%) females dissected during an epidemic of the disease in former southern Sudan.

Although natural infection was not detected in either methods in the present observations, *P. orientalis* appears to be a principal vector of VL in northwestern Ethiopia mainly by the following reasons; first, *P. orientalis* is the only species in the genus *Phlebotomus* which is found in large numbers in both areas; second, in a recent study on susceptibility of *P. orientalis* (colony of Libo-Kemkem population) to *L. donovani* showed that this species supports full development of *L. donovani* promastigotes in the gut and the parasite colonized anterior parts of the midgut and the stomodeal value of the flies (Seblova *et al*., 2013) and lastly, *P. orientalis* is a proven vector in neighboring Sudan (Hoogstraal and Heyneman, 1969; Ashford *et al*., 1992; Elnaiem *et al*., 1998).

In conclusion, the present study shows that the difference in the parous rates between the highland and lowland populations of *P. orientalis* being higher in the lowland population. It also indicates that both populations of *P. orientalis* have strong zoophilic behavior. Furthermore, in both areas the species is strongly implicated as a vector of kala-azar. Therefore, in the future management of the disease based on the vector control should target on the vector of the disease and also its feeding behavior.

Chapter six. Nocturnal activity pattern of *Phlebotomus orientalis* in two ecologically distinct foci of kala-azar in northwestern Ethiopia

6.1. Introduction

Phlebotomine sandflies (Diptera: Psychodidae) are small biting insects of considerable public health importance in many parts of the world, where they are the vectors of human pathogens. Most importantly, they transmit etiological agents of leishmaniasis (Killick-Kendrick, 1999). Leishmaniases are a group of diseases caused by protozoan parasites of the genus *Leishmania*. The diseases manifest from self-healing cutaneous leishmaniasis to life threatening visceral leishmaniasis (Desjeux, 2004). In the Old World, the implicated vectors of the disease belong to the genus *Phlebotomus* (WHO, 2010).

Leishmania donovani, the causative agent of visceral leishmaniasis, is transmitted mainly by two sandfly species in east Africa: *P. martini* and *P. orientalis*. *Phlebotomus martini* is frequently associated with termite mounds in southern Ethiopia and in Kenya, whereas *P. orientalis* is associated with *Acacia-Balanites* forests and black cotton soil in Sudan, south Sudan and northwestern Ethiopia (Hoogstraal and Heyneman, 1969; Gebre-Michael and Lane, 1996; Elnaiem *et al*., 1998; Gebre-Michael *et al*., 2004b).

Most sandfly species are either crepuscular, with peaks of activity soon after sunset and before dawn, or nocturnal (Lewis, 1971; Kravchenko *et al*., 2004). Nocturnal activity of sandflies consists of a number of discrete components, predominantly searches for blood meals, sugar meals, mates and breeding sites (Yuval and Schlein, 1986). Such behavior of sandflies is governed by a range of factors such as internal biological clock, daily changes in light intensity and other abiotic factors, including temperature, relative humidity, rain, and wind speed (Souza *et al*., 2005). Adequate knowledge of the activity pattern of sandflies in general and vector species in particular would contribute to the understanding of the epidemiology of sandfly-borne diseases as it indicates the time when a person is most likely to be bitten and get the diseases (Feliciangeli, 1997; Guernaoui *et al*., 2006a).

Previous investigations on nocturnal activity pattern of vectors of kala-azar in Ethiopia are limited. Ashford *et al*. (1973a) reported that a peak activity of *P. orientalis* reached shortly after sunset in Arbaya after which the activity of the sandflies subsided concomitantly as the temperature dropped from 16^0C. Similarly, Gebre-Michael and Lane (1996) studied the biting activity of the vectors of kala-azar (*P. martini* and *P. celiae*) in southern Ethiopia and the two species showed similar peak in activity pattern between 20:00 and 22:00 hours. The authors also observed the same pattern in nocturnal activity of the more epidemiologically dangerous parous females of the species. Elsewhere, Quate (1964) in Sudan reported a peak activity period of *P. orientalis* between 21:30 and 22:00 hours. In contrast, Schorscher and Goris (1992) pointed out that biting activity of *P. orientalis* occurred throughout the night till sunrise. In India, Dinesh *et al*. (2001) studied the nocturnal activity of *P. argentipes* and hourly catches of the study revealed that the flies activity concentrated between 21:00 and 01:00 hours, with a peak just before midnight. In Israel, Kravchenko *et al*. (2004) noticed different activity pattern of males and females of *P. tobbi*. Activity of males of the species was nocturnal whereas females exhibit crepuscular behaviour.

As studies on the nocturnal activity pattern of the probable vector of kala-azar in northwestern Ethiopia are scarce particularly in the current study areas, the present study was designed to determine and compare such behavior of both sexes of the species in two ecologically distinct foci of VL in the region. In addition, attempt was also made to elucidate the effect of local weather such as temperature and relative humidity on the nocturnal activity of the sandflies in both foci.

6.2. Materials and Methods

The study was carried out in Bura, Libo-Kemkem district, from January to May 2012, and in Kokit, Metema district from March to June 2013. These months were selected because of the relatively high abundance of *P. orientalis* in study areas.

6.2.1. Sandfly sampling

The nocturnal activity of sandflies was determined using CDC LTs placed in compounds occupied by both cattle and humans, so as to adequately sample the host seeking sandflies. In both areas, collection of sandflies was conducted bimonthly which started at 18:00hrs and ended at 6:00hrs. Every hour, a cage was replaced by another cage labeled with date and time of collection.

6.2.2. Recording weather variables

Hourly weather variables (temperature and relative humidity) were recorded from dusk to dawn using data logger (HOBO Microstaion) during the collection nights.

6.2.3. Dissection of sandflies for age determination

In the mornings, the hourly catches of females *Phlebotomus* were sorted out according to their abdominal status (unfed, blood fed and gravid/semi-gravid) under the dissecting microscope. Dissection was performed on unfed females to separate into parous and nulliparous as described elsewhere (Chapter five). Then, the parous females and gravid *Phlebotomus* spp. were further processed for the detection of *Leishmania* parasites (see chapter five). The rest (male

Phlebotomus spp. and both sexes of *Sergentomyia* spp.) were preserved in 70% alcohol for mounting and species identification as described previously (Chapter three).

6.2.4. Data Analysis

The mean densities of sandflies were computed as numbers of flies per LTs per hour. Prior to analysis of the data, normality of the data was checked using Shapiro-Wilk test. T-test was used to compare the activity of females and males of *P. orientalis* before and after midnight. Kruskal-Wallis was used to compare the hourly activity of males, females and total *P. orientalis* populations and proportion of parous rates. Correlation between hourly recorded weather variables (temperature and relative humidity) and density of *P. orientalis* was made using Spearman Correlation Coefficient.

6.3. Results

6.3.1. Species composition of sandflies

Table 13 shows species of phlebotomine sandflies collected during the study period in both study areas. In Libo, a total of 3438 (1943 males and 1457 females) sandflies were collected from January to May 2012. Of these, 3319 (96.5%) (1943males and 1376 females) were *P. orientalis* and the rest were *Sergentomyia* spp. (3.5%). In Metema, a total of 6041 (2193 males and 3848 females) sandflies were caught from March to June 2013 which included six species of *Phlebotomus* (*P. orientalis, P. duboscqi, P. papatasi, P. bergeroti, P. rodhaini* and *P. martini*) and several species of *Sergentomyia*. *Phlebotomus orientalis* accounted 13.5% (n=813) of the total collection in the area, *Sergentomyia* spp. being the most prevalent (86%).

Table 13. Number and species of sandflies collected using light trap in Libo–Kemkem and Metema

Species	Libo-Kemkem			Metema		
	Male	Female	Total (%)	Male	Female	Total (%)
P. orientalis	1943	1376	3319(96.5)	442	371	813 (13.5)
P. duboscqi	-	-	-	2	2	4 (0.07)
P. papatasi	-	-	-	1	5	6 (0.09)
P. bergeroti	-	-	-	7	8	15(0.25)
P. rodhaini	-	-	-	4	9	13(0.22)
P. martini	-	-	-	1	0	1 (0.02)
Sergentomyia spp.	38	81	119 (3.5)	1736	3453	5189 (85.8)
Total	1981	1457	3438	2193	3848	6041

6.3.2. Nocturnal activity pattern of *P. orientalis* in Libo-Kemkem

During each collection night, the male:female ratio varied with the hour of collection. The overall ratio and range was 1.4 and 0.9-3.4. The hourly activity pattern of *P. orientalis* was significantly different for female, male and both sexes of the species: females (Kruskal-Wallis χ^2 =31.9, df=11, P<0.05), males ($\chi2$ =21.7, df=11, P<0.05) and both sexes ($\chi2$ =28.1, df=11, P<0.05). Higher numbers of flies for both sexes were observed between 21:00 and 24:00hours with a peak density between 22:00 and 23:00hours (Fig 20). When the activity of females grouped into before and after midnight, there was a significant difference in the activity of females before and after midnight (F=0.974, df=10, P= 0.026) where large numbers of females caught before midnight, but such difference was not observed on the activity of males and total population of *P. orientalis* (P>0.05) for both. After midnight, the densities of both sexes subsided steadily throughout the night. During the peak activity hours of the species, the mean temperature and relative humidity were, 17.9±2.43^{0}C and 43.1± 6.2 (%), respectively (Fig 20).

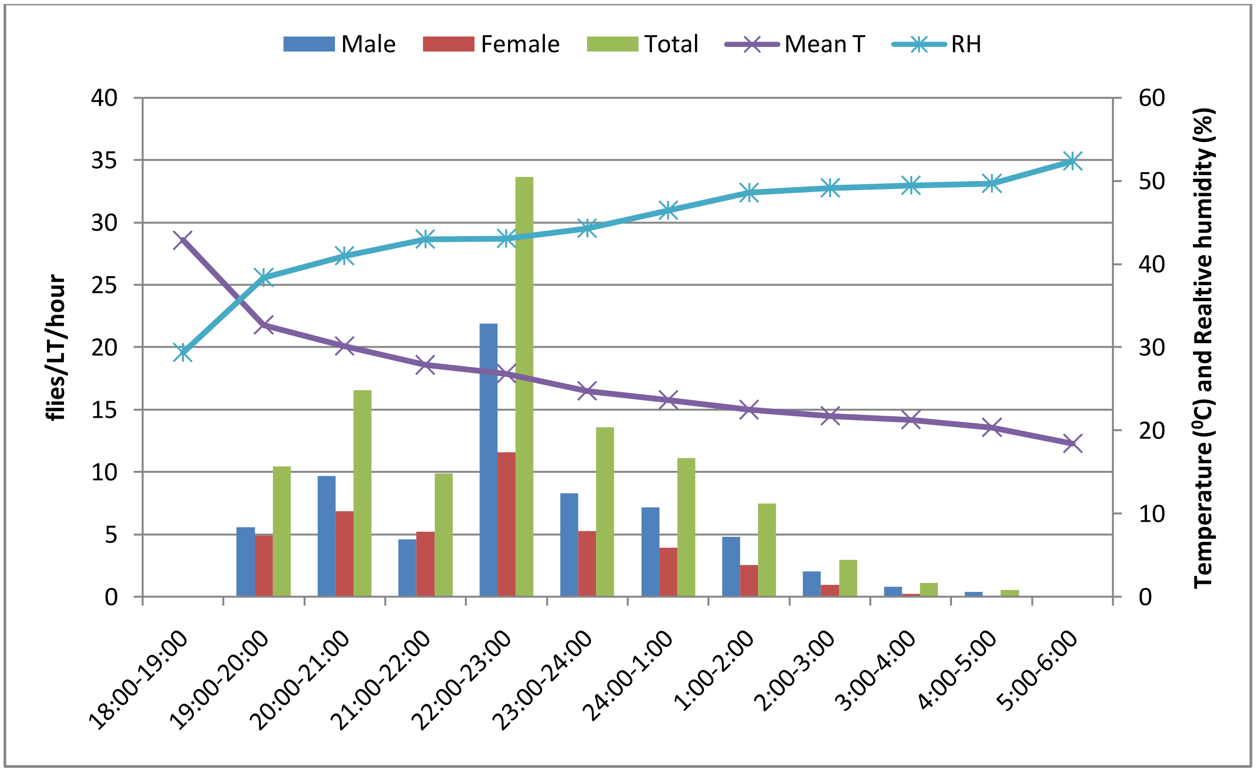

Figure 20. Nocturnal activity of *P. orientalis* and mean hourly recorded temperature and relative humidity in Libo-Kemkem district (January-May 2012)

Of the total 1376 females *P. orientalis* collected, only 285 unfed were dissected for parous rates of which 48.8% (n=139) were parous. These parous females and 44 gravid/semi-gravid females (total 329) were checked for *Leishmania* detection under the microscope. None was positive for *Leishmania* parasites.

Fig. 21 shows hourly activity of parous and nulliparous females of *P. orientalis* in Libo-Kemkem. Significant difference in the hourly proportion of parous females was observed (Kruskal-Wallis χ^2 =21.4, df=11, P=0.029), where the majority of parous population came before midnight (105/139, 75.5%). In general, parous females have two major peaks of activity during the night, one before mid night (19:00-22:00hours) and another after midnight (1:00-2:00hours). Similar bimodal peaks in activity for nulliparous females were also observed. The first peak in the activity was between 22:00 and 23:00hours and the second peak activity was between 3:00 and 4:00hours.

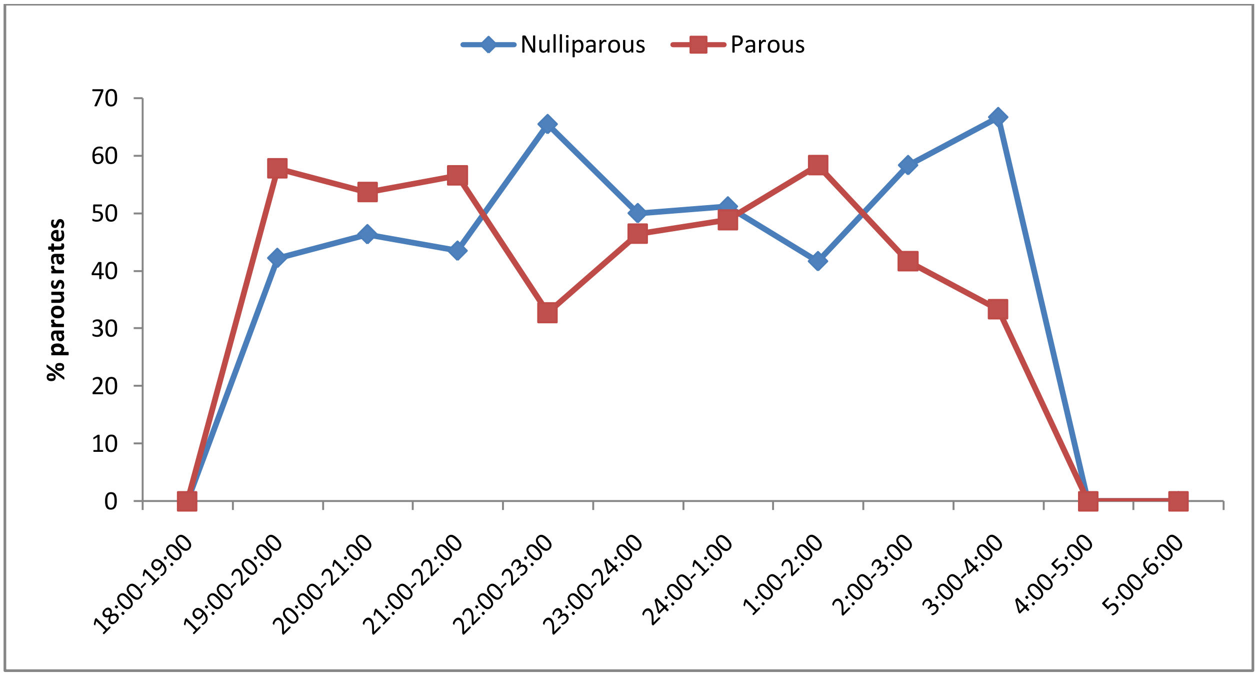

Figure 21. Hourly proportions of parous and nulliparous females *P. orientalis* in Libo-Kemkem (January –May 2012)

6.3.3. Nocturnal activity pattern of *P. orientalis* in Metema

During each collection night, the male:female ratio varied with the hour of collection, females becoming more frequent as the night progressed. The overall ratio and range was 1.2 and 0.04-9. The hourly activity pattern of *P. orientalis,* mean temperature and relative humidity are depicted in Fig 22. The activity of males of *P. orientalis* started soon after sunset and continued throughout the night ($\chi^2 = 17.6$, df=11, $P > 0.05$) with slight population peak between 22:00 and 23:00 hours. Males predominate over the females till their peak of activity at 22:00-23:00 hours (2.6 ± 1.0/LT/hour). In contrast, the activity of females started one hour later than males, and were inferior till after 23:00hours during which they predominated the males with their highest peak of activity at 3:00-4:00hours (3.21±1.4/LT/hour). The hourly activity pattern of females of *P. orientalis* was significantly different ($\chi^2 = 21.06$, df=11, $P < 0.05$) with a peak between 3:00 and 4:00hours, similar to the total population. The activity of females of *P. orientalis* continued until 6:00 hour, but the activity of males decreased drastically after 5:00 hour in the morning. Like the activity of males, the activity of total *P. orientalis* was also continuous ($P > 0.05$). There was a significant difference in the activity of females before and after midnight (F= 0.007, df=10, P=0.02) as the activity was more pronounced after midnight, but such difference was not observed on the activity of male sandflies and total population of *P. orientalis* ($P > 0.05$). During the peak activity hours of the species the mean temperature and relative humidity were, 26.85 ± 0.28^0C and 43 ± 8.5 (%), respectively (Fig 22).

Attempt was made to dissect *P. orientalis* to determine hourly parity rate and parasite detection in Metema, however due to the unfavorable (hot) weather conditions and lack of laboratory space, only a few (n=40) *P. orientalis* could be dissected and none was infected. Of these, 33 were unfed and 21 (64%) of them were parous. Due to the low number of dissected flies, determination of hourly parous rate was not possible.

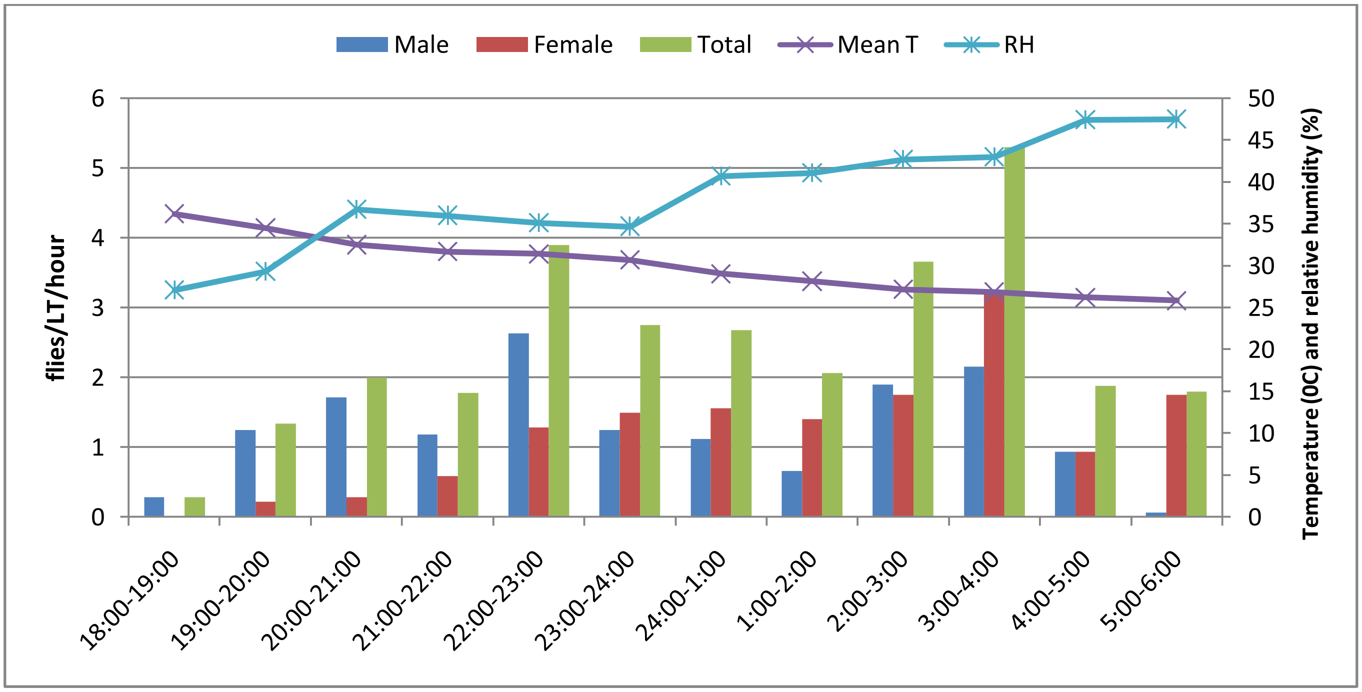

Figure 22. Nocturnal activity pattern of *P. orientalis* and hourly recorded mean temperature and relative humidity in Metema district (March-June 2013)

6.3.4. Effect of weather variables on nocturnal activity of *P. orientalis*

The Spearman correlation coefficient analysis between nocturnal activity pattern of *P. orientalis* and hourly recorded mean temperature and relative humidity of both districts is depicted in Table 14. In Libo, hourly mean temperature had significant positive association with density of male, female and total density of *P. orientalis*, whereas relative humidity had negative association with male, female and total density of *P. orientalis* though not statistically significant. In contrast to Libo, in Metema, the hourly mean temperature had significant negative association with density of female *P. orientalis* but statistically insignificant negative association with densities of male and total *P. orientalis*. Unlike temperature, the relative humidity had positive and statistically significant association with densities of female and total densities of *P. orientalis*.

Table 14. Correlation of hourly mean temperature and relative humidity with the density of *P. orientalis* in Libo-Kemkem and Metema

Species	Libo-Kemkem				Metema			
	Mean Temperature		Relative humidity		Mean Temperature		Relative humidity	
P. orientalis	ρ	P-value	ρ	P-value	ρ	P-value	ρ	P-value
Male	0.389**	0.002	-0.24	0.068	-0.091	0.54	0.167	0.256
Female	0.45**	0.000	-0.252	0.052	-0.304*	0.036	0.410**	0.004
Total (M+F)	0.44**	0.000	-0.234	0.07	-0.199	0.174	0.293*	0.043

**Correlation is significant at the 0.01 level; *Correlation is significant at the 0.05 level; ρ- is spearman correlation coefficients

6.3.5. Monthly variation in nocturnal activity pattern of *P. orientalis*

In Libo, peak nocturnal activity of *P. orientalis* showed monthly variation (Fig 23). In January and February, the peak periods of activity were about early evening 19:00-22:00hours whereas in March to May the peak was generally around midnight (24:00-1:00hours in March, 22:00-24:00hours in April and May). Like the population of Libo, the activity pattern of the Metema population also showed monthly variation (Fig 24). There were one to three peaks of activities in different months. Thus, in March and April, two peaks of activity were apparent around midnight, but the highest was at 3:00-4:00hours in March (7.5/LT/hr) while it was around 22:00-23:00hours in April (5.8/LT/hr). In May, however, there were three peaks of activity, two before midnight and another one after midnight, of which most activity occurred at 22:00-23:00hours (6.13/LT/hr). In June however, peaked period of activity shifted to much later part of the night (3:00-5:00hours).

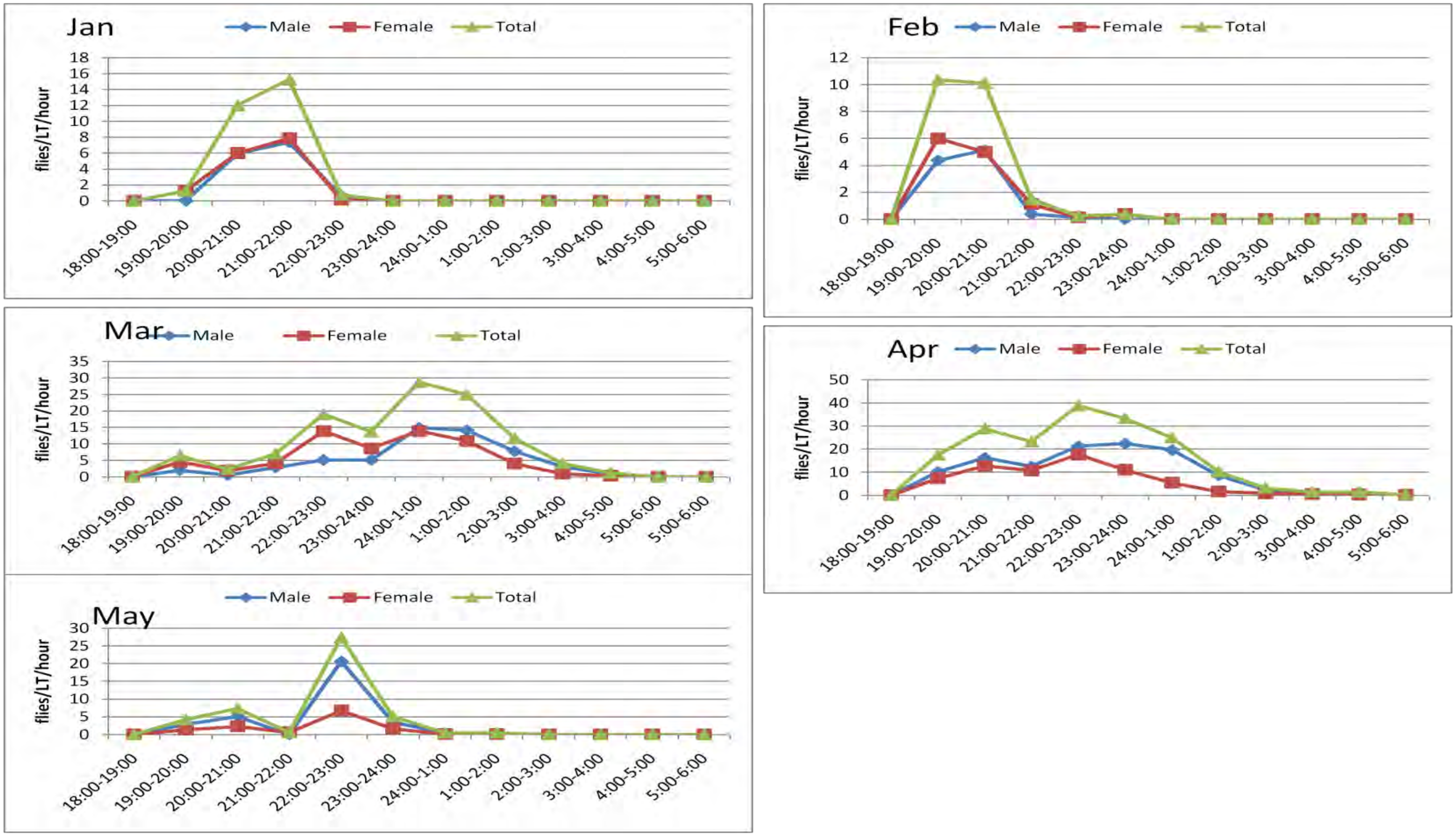

Figure 23. Monthly variation of nocturnal activity of *P. orientalis* in Libo-Kemkem (January –May 2012)

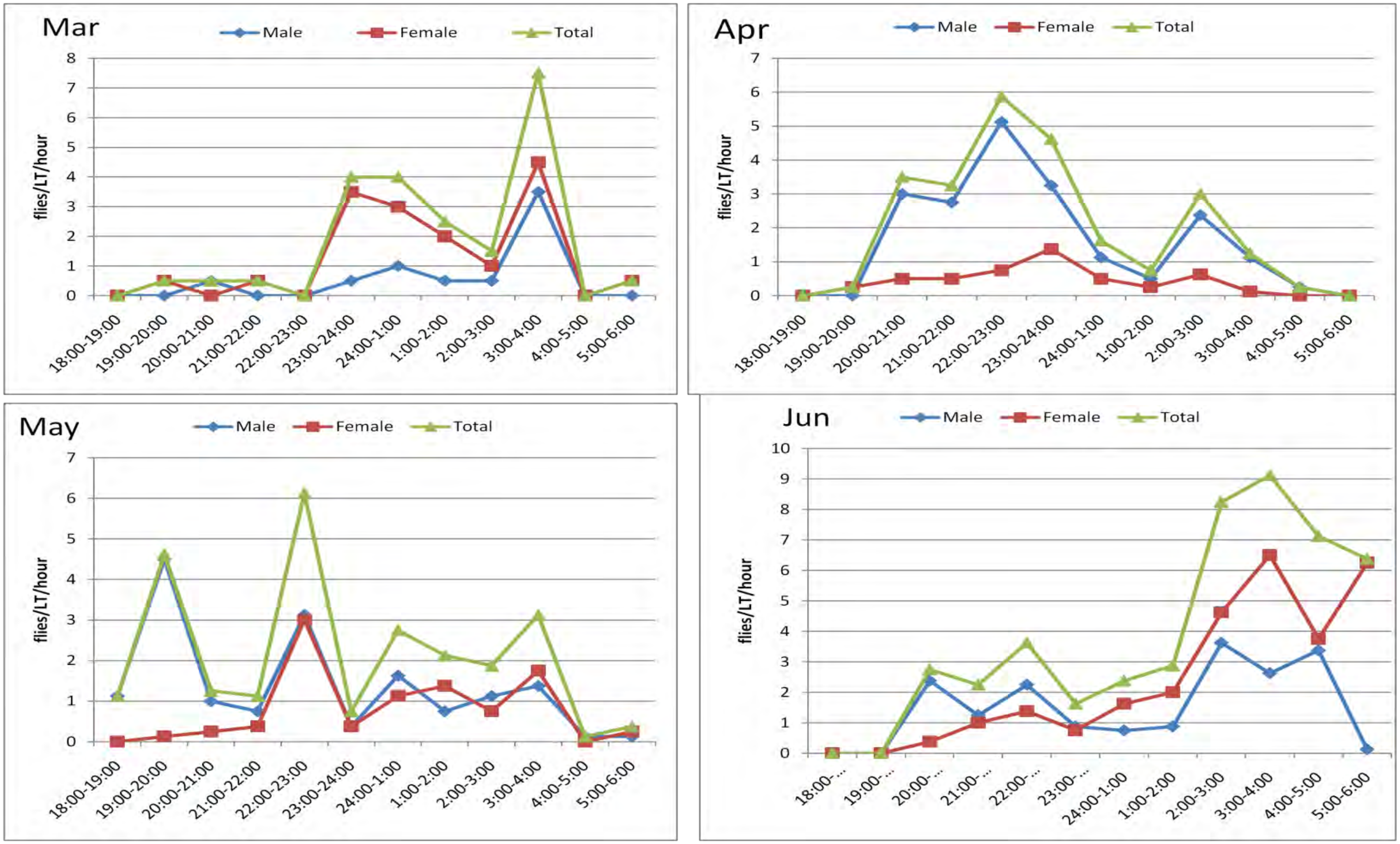

Figure 24. Monthly variation of the nocturnal activity of *P. orientalis* in Metema district (March –June 2013)

6.4. Discussion

The activity pattern of phlebotomine sandflies is either crepuscular or nocturnal (Lewis, 1971; Killick-Kendrick, 1999), however, such behavioral pattern greatly shows variation between different species in the same locality or within the same species in different localities. In the present study, the hourly activity pattern of highland and lowland populations of *P. orientalis* was determined. The activity of *P. orientalis* in the highland area was mainly restricted in the first half of the night with a peak between 22:00 and 23:00hours. After midnight the density of this species started to subside drastically. In contrast, *P. orientalis* in the lowland area was active throughout the night (from dusk to dawn) with a peak density later in the night between 3:00 and 4:00hours. Such difference in peak activity periods between the two populations may be due to highly contrasting weather and ecological variations between the two studied areas. Moreover, the sampling periods (months and years) in the two geographical areas were different. Previous studies on a peak nocturnal activity of the species reported contrasting results. Quate (1964) in Sudan found a large number of *P. orientalis* between 21:30 and 22:00 hours using human biting collection. In the same country using the same collection method, Schorscher and Goris (1992) reported that biting activity of *P. orientalis* occurred throughout the night till sunrise. In Ethiopia, Ashford *et al.* (1973a) indicated the peak biting activity of *P. orientalis* shortly after sunset in the highland area of Belessa. In Kafta-Humera (northwestern Ethiopia), Lemma *et al.* (2014b) reported the peak activity period of this species between 24:00 and 1:00hours around animal shelter. In Sheraro (northern Ethiopia), Gebresilassie *et al.* (2015) showed similar activity pattern to Metema population in which most *P. orientalis* females were active after midnight with a peak in density was observed between 24:00 and 3:00hours, while males were most active before midnight.

The difference in nocturnal activity pattern of *P. orientalis* in the highland and lowland areas has its own epidemiological implications in the respective areas. In the highland area, the peak activity period of the species coincides with the outdoor activities of the local population who are at great risk of acquiring the disease because of lack of protective means from the bite of the flies. Unlike the highlanders, both the permanent residents and seasonal migrant workers in the lowland area are at risk of being infected with *L. donovani* by the bite of *P. orientalis* throughout the night as the majority of the people sleep outdoors for various reasons: to keep their cattle from theft, to work on the farms and due to the hot weather conditions without using any protective means. This behavior is no different to the observations made in Kafta Humera and Sheraro lowlands in northwest and northern Ethiopia, respectively (Lemma *et al*., 2014b; Gebresilassie *et al*., 2015; Yared, unpublished data).

In the current study, the peak nocturnal activity periods of *P. orientalis* in both study areas showed monthly variation. In the highland area for instance, the peak activity hours of the species shifted from early evening 19:00-22:00hours in January to 24:00-1:00hours in March. In the same manner in lowland area, the peak activity of this species shifted from 3:00-4:00hours in March to 22:00-23:00hours in May. Such variation might be due to changes in weather variables (such as temperature, relative humidity and others) between the months. Similarly, Coleman *et al.* (2007) in Iraq observed shifting in peak activity periods of sandflies (*P. alexandri, P. papatasi, P. sergenti* and *Sergentomyia* spp.) from early in the evening in April and October to later in the night in May and June. Also, Guernaoui *et al*. (2006a) reported activity difference for *P. sergenti* between August (markedly crepuscular) and October (peak in activity between 20:00 and 22:00hours) in Morocco.

The correlation analysis between hourly activity patterns of *P. orientalis* and hourly mean temperature and relative humidity showed contrasting results between the highland and lowland areas. In the highland, mean temperature had positive and statistically significant association with the density of *P. orientalis*, whereas the relative humidity had negative correlation. Similarly, Ashford *et al*. (1973a) noted the association of temperature with biting activity of this species in another highland area, Belessa. He noticed that the activity of the species was considerably reduced when the temperature was below 16^0C, which is comparable to the present study. As the temperature dropped from 17.9 ± 2.4^0C (the mean temperature at the time of peak density of *P. orientalis*), the activity of the species concomitantly started to reduce. In Morocco, Guernaoui *et al*. (2006a) reported the direct association between temperature and hourly abundance of *P. sergenti* and *P. perniciosus*. In the highland area, temperature appears to be the determining factor for the nocturnal activity of *P. orientalis*, although the role of other meteorological factors such as wind speed cannot be ruled out.

In the lowland however, mean temperature and relative humidity had negative and positive relationship with the hourly density of the species, respectively. In Metema, the most important factor affecting the nocturnal activity of *P. orientalis* seems to be relative humidity. The importance of high relative humidity for sandflies activity was reviewed by Lewis and Ward (1987). Elsewhere in Kafta Humera in northwest Ethiopia, hourly density of *P. orientalis* has also similar relationship with relative humidity (Yared pers.com). Likewise, Dinesh *et al*. (2001) pointed out the importance of high relative humidity for blood feeding habits of *P. argentipes* in India. In contrast to the present observation and previous reports, Roberts (1994) revealed that nocturnal activity of *P. alexandri* was greatest on the nights with low humidity in Oman.

In the lowland area, females and males of *P. orientalis* did not have similar periodicities like the highland population. The hourly activity pattern of males commenced one hour earlier than females. This could be related with mating behavior of males of *P. orientalis* as this behavior also previously observed in Neotropical sandflies by Morrison *et al*. (1995). They observed males of *Lu. longipalpis* arrived at animal pens two hours earlier than females. The other important difference between the two sexes of *P. orientalis* in the present study is that the activity of males was minimal between 5:00 and 6:00hours however females were active during these periods. This may indicate that females exhibit both nocturnal and crepuscular behaviors, whereas males are clearly nocturnal in the area.

Determination of hourly parous rate of *P. orientalis* in the highland area indicated that the majority (75.5%) of the parous population were active before midnight mainly between 19:00 and 23:00hours. Similarly, Gebre-Michael and Lane (1996) collected more parous females of *P. martini* and *P. celiae* between 20:00 and 22:00hours in southern Ethiopia. The abundance of these epidemiologically dangerous parous female in the early part of the night might be a risk factor for inhabitants working on the farms in the evening or for people sleeping late outside their houses. In contrast to the present observation, Roberts (1994) in Oman encountered more parous females of *P. alexanderi* and *S. clydei* in the later part of the night. In addition to determination of hourly parous rates, the activity of nulliparous females was also observed. These newly emerged females had two activity peaks throughout the night, the first one was between 22:00 and 23:00hours and the second one was 3:00 and 4:00hours. The former one coincides with the peak activity of males and this might be explained by mating behavior of the species. Such activity of nulliparous females of *P. orientalis* also observed by Gebresilassie *et al*. (2015). A similar attempt was made in the lowland population of *P. orientalis*; however, due to unfavorable weather condition enough number of sandflies was not dissected to elucidate the hourly parous rates.

In conclusion, the present study showed that *P. orientalis* populations in the highland and lowland areas have different activity patterns with a peak in the early part of the night in the highland and in the latter part of the night in the lowland. Monthly variations in nocturnal periodicities were also observed in both areas. Furthermore, the activity of the sandflies appears to be governed by temperature in the highland and relative humidity in the lowland although the role of other factors cannot be ruled out.

Chapter Seven. General Discussion, Conclusions and Recommendations

7.1. General Discussion

Visceral leishmaniasis has been known as a major health problem in the Metema lowland of northwestern Ethiopia for a long time (Hailu *et al*., 2006a), and has recently spread to the highlands of the region mainly in Libo-Kemkem district (Alvar *et al*., 2007), both in the Amhara regional state. A number of entomological studies have been conducted in both of these ecologically distinct foci of the region (Gemetchu *et al*., 1983; Gebre-Michael *et al*., 2007:2010). However, such studies were based on brief observations and lacked detail information on many aspects of phlebotomine sandflies of the two endemic areas. Hence, the present study was aimed to provide comparative data base on phlebotomine sandflies of the two areas.

The studies presented in Chapter three of this dissertation determined and compared the sandfly fauna of the two areas as this information is a base for any vector borne disease control and incriminating of vector (s) species in the VL endemic area. The two study sites showed a difference in sandfly diversity, evenness and richness of sandfly fauna. The lowland area of the present study was more diverse than the highland area, which is in agreement with the previous study that showed species richness and diversity decrease with an increase in altitude (Guernaoui *et al*., 2006b). Collection of phlebotomine sandflies in the highland area revealed the presence of five species of the genus *Sergentomyia* and two species of the genus *Phlebotomus* (*P. orientalis* and a single *P. rodhaini*). Of these species, *P. orientalis*, the likely vector of VL in northwestern Ethiopia, was the most abundant species and accounted for 86.6% of the total sandflies and 99.9% of the genus *Phlebotomus* collected. This result is consistent with Gebre-Michael *et al*. (2007). On the other hand, 13 species of the genus *Sergentomyia* and six species of the genus *Phlebotomus* were collected in the lowland area. Among the species of the genus *Phlebotomus*, *P. orientalis* was also the dominant species (93.7%) which is also in agreement with Gebre-Michael *et al*. (2010).

Studies on the population dynamics of a vector species for an extended period of time are extremely useful in planning a control strategy. The number of population peaks determines the most effective time and means to mount an attack (Killick-Kendrick, 1978). Thus, population dynamics of the likely vector and the most abundant species in the genus *Phlebotomus* of the two study areas were studied and presented in chapter four. *Phlebotomus orientalis* exhibits seasonality, being at its highest during the low precipitation months of the year (November-June) in both of the study areas. Such heightened abundance and activity during the dry season was also reported in the neighboring Humera district, Sheraro (northern Ethiopia) (Lemma *et al*., 2014a; Gebresilassie, unpub data; Yared, unpub. data) and in the neighboring Sudan (Quate, 1964; Elnaiem *et al*., 1997). Although *P. orientalis* was generally a dry season species, the peak population density showed differences between the highland and lowland areas. The highest population peak of *P. orientalis* in the highland area was noted in May/April whereas in the lowland it was in June, which is just the onset of rainy season. On the other hand, seasonal variations in sandfly population might vary between years, even in the same locality since weather and other ecological changes are expected to occur from one year to another affecting the overall dynamics of sandflies at any one time.

Furthermore, habitat preference of *P. orientalis* was examined in both study areas where large numbers of *P. orientalis* were collected from the farm field habitat than in mixed forest or indoor habitats using STs. This is in conformity with previous observations (Lemma *et al*., 2014a) showing black cotton soil as the most likely breeding and resting sites in both of the study areas as previously reported (Moncaz *et al.*, 2014). The low abundance or absence of *P. orientalis* in houses has been observed by several investigators in Ethiopia (Gemetchu *et al.*, 1975; Gebre-Michael *et al.*, 2007:2010) or Sudan (Hoogstraal and Heyneman, 1969; Elnaeim *et al.*, 1997) showing transmission of the disease in both areas is mainly outdoors.

A study was also conducted on the age structure, host preferences and detection of natural infection with *Leishmania* parasites towards incriminating the vector of VL in the highland and lowland of the study areas. Determination of the parous rate of vector population has epidemiological significance as these flies must survive at least one oviposition cycle to be potentially infective with *Leishmania* spp. parasites (Lewis, 1971). The overall parous rates were higher in Metema population (66.2%) than Libo-Kemkem (45.6%), but the parity rates in both areas are higher than that of previously reported in the same study areas (Gebre-Michael *et al.*, 2007:2010). None of 1379 *P. orientalis* (1087 from Libo-Kemkem and 292 from Metema) and 53 other *Phlebotomus* spp. dissected and examined microscopically revealed any natural infection in their guts. Similar result was obtained when 347 *P. orientalis* females collected in Libo-Kemkem were processed using PCR. In general, the number processed (dissection and molecular) for detection of natural infection in both study areas was relatively small. A total of 2230 *P. orientalis* from Libo and 1131 from Metema remained unprocessed because of technical problems (breakdown of the UV reader). However, the absence of natural infection in the present study might be due to low prevalence of the disease in the study areas during the time of investigation, although previous attempts in this species in both endemic areas failed to detect any natural infection with *L. donovani* (Gebre-Michael *et al.*, 2007:2010). Though natural infection was not detected in the current observations, *P. orientalis* is considered as a vector of kala-azar in both areas due to its abundance, as it has already been incriminated in neighboring Sudan (Ashford *et al*., 1992; Elnaiem *et al*., 1998). Furthermore, an experimental infection study with *P. orientalis* colony originating from Libo has demonstrated its susceptibility to *L. donovani* allowing full development of the parasite (Seblova *et al*., 2013).

Results of blood meal analysis for determination host preferences of the presumed vector of kala-azar in the study areas using both molecular (cyt *b* PCR- RLB) and serological (ELISA) methods showed that *P. orientalis* in the areas have a strong inclination to feeding on bovids (zoophilic) (68% for Libo and 49.8% for Metema). The HBI was lower in both study areas: 17.3% in Libo and 12% in Metema, but was higher than an earlier report by Gebre-Michael *et al*. (2010). The predominantly cattle feeding behavior of *P. orientalis* has earlier been shown in Metema (Gebre-Michael *et al*., 2010) and recently in neighboring Humera and Sheraro districts (Lemma *et al*., 2014b; Yared and Gebresilassie, unpub data).

Studies on nocturnal activity revealed that the two populations of *P. orientalis* showed variation in peak activity period. In Libo, much of the activity of *P. orientalis* (both sexes) occurred before midnight and peaked between 22:00 and 23:00hours, similar with the observation in Belessa, a highland Valley in northern Ethiopia (Ashford *et al*., 1973a). In the lowland Metema, activity of females started gradually and resurged as the night progressed to form one major peak after midnight (3:00-4:00 hours), with slight decrease till 5:00-6:00 hours in the morning. However, the activity of males started earlier than the females and remained dominant till midnight peaking at 22:00-23:00 hours, after which its dominance was replaced by the females with a second smaller peak coinciding with the females (3:00-4:00 hours), which then subsided. In addition, both populations of *P. orientalis* showed monthly variation in peak activity periods. Furthermore, sex based variation in activity of the species also noted.

7.2. Conclusions

- This study showed differences in sandfly diversity and richness between the highland and lowland areas. It also revealed the occurrence of five species of the genus *Sergentomyia* and two species of the genus *Phlebotomus* in the highland area and 13 species of the genus *Sergentomyia* and six species of the genus *Phlebotomus* in the lowland area. In both areas, *P. orientalis* is the most abundant species in the genus *Phlebotomus*. Thus, based on abundance and occurrence of this species in these areas, it can be concluded that kala-azar transmission in the study areas is maintained by *P. orientalis,* although the criteria set out by WHO (2010) are not yet fulfilled for the areas.
- The study also revealed that *P. orientalis* exhibits predominately exophilic and exophagic behaviors in both areas making it difficult to control with the conventional control tools (e.g. ITNs and IRS)
- The study also showed that *P. orientalis* is a dry season species (mainly between January and early June) and absent or occurred in negligible numbers during the wet season (late June-October) in both areas. Therefore, kala-azar transmission in both areas is most probably during the dry season or just on the onset of the rainy season.
- Large numbers of *P. orientalis* were collected in farm field habitat with deep cracking black cotton soil using STs in both areas, than other habitats. This habitat might be used as breeding and resting sites for the species, therefore the habitat might be difficult to target for control.
- The result of blood meal analysis based on ELISA and PCR indicated that *P. orientalis* has a low HBI and mainly uses cattle as main source of blood meal (zoophilic) in both

study areas. Such high preference for cattle could be exploited to control *P. orientalis* by use of topical application of insecticides or systematic insecticides such as ivermectin.

- In the study areas, no natural infection was detected either by dissection or by molecular means in 1379 *P. orientalis* and other 53 *Phlebotomus* spp. processed. This might be due to the relatively small numbers of *P. orientalis* dissected or processed by PCR. It could as well be due the low prevalence of the disease in both areas during the time of investigation. Although natural infection was not detected in the present study, *P. orientalis* as the most abundant species is strongly implicated as a vector of the disease in both the highland and lowland areas.
- The overall parous rate of *P. orientalis* was 45.6% in Libo-Kemkem, and 66.2% in Metema lowlands showing the probability of *P. orientalis* being infected with *L. donovani* is higher in the lowland (Metema) than its counterpart in the highland (Libo). The parous rates showed seasonal variation in both study areas.
- Both populations of *P. orientalis* exhibited marked nocturnal periodicities with a peak between 22:00 and 23:00 hours in the highland and between 3:00 and 4:00 hours in the lowland. Thus, the risk of VL infection in Libo-Kemkem is probably higher before midnight, whereas it is probably after midnight in the lowland (Metema).

7.3. Recommendations

Based on the present study the following recommendations are forwarded for future research

- The role of other *Phlebotomus* spp. (e.g. *P. rodhaini*) as possible vectors of VL, particularly in the Metema lowland need to be explored since it is already suspected in neighboring Sudan.
- The presence of zoonotic cutaneous leishmaniasis (ZCL) caused by *L. major* in Metema lowland and the roles of *P. papatasi* and its closely related species (*P. duboscqi* and *P. bergeroti*) in ZCL epidemiology deserves investigations.
- The role of cattle in the epidemiology of VL whether they are associated with increased risk of VL or with a zooprophylactic effect needs to be carefully investigated.
- The potential for cattle treatment with topical insecticide application (as practiced in the control of tsetse or acarines) or systemic insecticides (e.g. ivermectin) towards the control of the sandfly vector need to be evaluated.
- Based on the predominant outdoor and nocturnal periodicities of the vector of VL, the local people in both areas should take appropriate protective measures. In this regard, adequate provision of ITNs and health education by the concerned health authorities for proper utilization of ITNs even if they sleep outdoors is necessary. Research wise, the potential of natural or botanical repellents such as neem is warranted as the available synthetic repellents (e.g. DEET) are expensive.
- The exact outdoor day time resting sites of *P. orientalis* should be investigated to reduce the density of the species.

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APPENDICES

Appendix 1. Monthly maximum temperature of Libo-Kemkem district from 1997 to 2012

MONTH	JAN	FEB	MAR	APR	MAY	JUN	JUL	AUG	SEP	OCT	NOV	DEC
1997	27.4	30.5	30.9	31.3	30.2	28.3	25.8	25.7	27.7	36.4	27.4	27.1
1998	27.9	28.8	29.6	28.9	29.1	28.6	27.7	24.1	26.8	29.8	27.9	28.3
1999	28.9	29.4	30.3	28.6	30.8	28.2	23.9	24.0	26.0	28.0	28.5	29.9
2000	29.8	30.1	31.0	28.3	32.5	28.1	27.2	30.1	30.6	30.2	29.7	29.7
2001	31.1	31.6	30.9	30.2	29.5	25.3	23.5	24.1	25.2	26.6	27.4	28.3
2002	28.8	32.2	30.7	30.7	31.4	26.8	25.4	25.4	26.6	29.4	29.9	29.7
2003	30.1	32.4	31.9	0.0	34.7	27.9	24.4	24.1	26.3	29.1	30.1	30.5
2004	31.0	32.4	33.5	32.0	32.8	28.5	25.3	25.4	27.7	28.6	29.4	29.1
2005	29.4	33.4	32.5	28.3	31.1	28.2	24.3	25.9	27.3	29.0	30.3	30.6
2006	32.2	33.3	33.2	33.4	31.1	27.5	25.9	25.1	26.7	29.3	30.1	30.1
2007	30.8	32.7	34.8	33.6	32.4	27.6	25.7	25.2	27.0	29.7	30.4	31.0
2008	31.7	32.7	43.9	32.1	29.6	26.8	26.1	26.4	28.4	29.6	30.0	29.9
2009	30.4	32.3	34.0			31.3	26.7	23.7	30.1	29.3	28.9	29.4
2010	31.5	34.4	35.0	34.5	34.1	32.7	26.0	22.9	0.0	31.9	31.6	43.4
2011	33.4	33.2	33.3	35.0	32.7	29.6	29.2	25.5	29.3	33.0	31.6	34.1
2012	34.0	34.9	36.1	35.6	34.4	25.5	24.7	27.0	32.5	32.0	32.5	33.2

Appendix 2. Monthly minimum temperature of Libo-Kemkem district from 1997 to 2012

MONTH	JAN	FEB	MAR	APR	MAY	JUN	JUL	AUG	SEP	OCT	NOV	DEC
1997	11.0	10.6	11.5	11.6	13.2	14.2	14.7	14.8	12.9	11.1	10.2	9.3
1998	10.3	12.1	11.1	10.2	13.1	13.0	13.6	13.0	11.5	10.9	9.3	10.9
1999	10.7	11.4	11.5	11.8	12.8	13.9	11.5	11.9	13.1	11.8	11.2	8.9
2000	9.2	10.3	10.5	13.3	12.0	9.6	9.1	15.1	11.1	10.9	11.3	11.4
2001	11.9	11.9	11.0	10.7	10.6	12.0	10.8	10.9	11.5	11.7	12.6	12.6
2002	12.8	12.6	14.7	15.7	15.9	15.1	13.6	13.7	13.3	13.2	12.2	8.3
2003	9.4	10.4	11.4	13.0	12.2	9.8	8.1	8.2	7.5	5.7	4.4	3.8
2004	4.2	5.6	7.4	9.2	9.0	9.1	8.0	7.3	7.7	4.8	5.3	8.3
2005	3.8	5.8	8.0	13.1	8.0	13.0	8.0	8.6	8.1	4.8	3.9	3.7
2006	5.6	7.6	8.4	9.1	12.9	13.7	13.5	14.4	13.7	12.5	10.3	10.0
2007	9.7	10.9	13.7	14.5	14.7	14.2	14.4	14.1	14.1	10.9	9.3	8.1
2008	10.7	10.4	11.7	24.3	13.9	14.1	13.8	14.0	13.3	15.6	8.9	8.2
2009	8.7	12.2	14.4	12.7		13.1	14.4	16.0	16.5	11.9	8.7	6.8
2010	9.9	10.7	12.1	15.3	15.1	14.7	16.3	18.5	0.0	12.7	9.3	8.8
2011	13.9	13.2	13.9	13.7	14.7	15.7	14.2	13.3	14.8	11.8	9.3	8.8
2012	8.9	13.4	14.4	13.7	15.0	15.0	13.5	13.3	12.1	11.2	7.9	8.0

Appendix 3. Monthly total rainfall of Libo-Kemkem district from 1997 to 2012

MONTH	JAN	FEB	MAR	APR	MAY	JUN	JUL	AUG	SEP	OCT	NOV	DEC
1997	0.0	0.0	4.7	26.2	97.0	118.3	337.1	49.4	33.7	56.9	5.0	0.0
1998	0.0	0.0	0.0	11.1	32.4	107.5	325.5	192.0	19.9	1.2	0.0	0.0
1999	0.0	0.0	0.0	0.0	0.0	127.6	409.7	349.1	162.1	107.0	0.0	0.0
2000	0.0	0.0	0.0	71.8	24.3	166.1	598.7	511.7	150.2	41.6	24.0	0.0
2001	0.0	0.0	0.0	16.6	71.2	200.0	147.4	212.2	36.2	14.0	0.0	0.0
2002	0.0	0.0	22.4	8.8	13.0	270.3	405.1	459.3	150.9	2.0	0.0	0.0
2003	0.0	0.0	0.0	0.0	0.0	182.2	411.3	338.5	183.9	7.9	0.0	0.0
2004	0.0	7.5	0.0	50.5	20.0	121.5	444.0	315.8	135.6	47.2	44.6	0.0
2005	0.0	0.0	50.0	0.0	36.9	0.0	397.8	319.1	248.7	0.0	2.0	0.0
2006	0.0	0.0	2.4	2.5	113.7	221.3	513.2	321.0	143.7	124.4	0.0	2.5
2007	0.0	0.0	0.0	6.0	60.6	258.3	287.3	350.9	208.4	6.9	15.3	0.0
2008	0.0	0.0	0.0	108.3	212.3	247.2	519.1	486.6	292.2	7.5	15.0	0.0
2009	0.0	0.0	3.0	0.0	0.0	105.1	673.4	492.8	118.3	71.2	0.0	0.0
2010	0.0	0.0	10.0	42.0	31.3	277.1	535.5	919.9	0.0	20.8	26.4	0.0
2011	0.0	0.0	0.0	0.0	108.0	319.3	644.1	738.3	591.7	0.0	45.4	0.0
2012	0.0	0.0	0.0	0.0								

Appendix 4. Monthly maximum temperature of Metema district from 1999 to 2013

year	JAN	FEB	MAR	APR	MAY	JUN	JUL	AUG	SEP	OCT	NOV	DEC
1999	37.4	40.9	39.6	40.2	37.8	35.5	29.6	29.6	31.1	31.7	36.6	36.4
2000	37.3	38.7	52.0	38.9	51.0	34.3	30.4	29.6	32.0	33.4	36.6	36.4
2001	35.7	38.4	39.3	41.3	39.2	33.1	29.4	29.3	31.0	33.3	36.7	38.5
2002	35.7	39.0	38.9	41.1	40.0	34.3	31.0	30.3	32.0	35.9	37.4	35.6
2003	37.3	39.6	40.7	41.1	40.4	34.6	30.7	29.9	31.8	35.0	36.9	35.9
2004	35.1	35.7	38.5	38.6	38.4	33.4	30.8	30.2	32.2	35.1	36.4	37.2
2005	35.0	38.7	38.9	41.5	39.9	34.5	30.6	30.7	31.7	34.7	38.2	38.6
2006	39.3	40.2	39.8	40.5	37.1	33.8	31.3	30.1	29.3	32.5	34.2	33.6
2007	34.7	36.6	40.3	50.5	39.2	35.4	31.4	29.1	31.0	33.3	36.2	37.0
2008	35.0	36.8	40.1	38.4	38.7	35.4	30.6	30.1	31.8	33.1	36.2	36.3
2009	36.8	38.3	40.2	40.4	39.8	36.4	30.3	31.1	33.7	36.0	37.6	36.5
2010	37.2	38.6	39.6	42.0	38.5	35.4	30.4	30.2	31.4	33.6	37.4	36.4
2011	35.7	35.5	39.5	39.2	39.0	33.4	31.6	30.4	31.8	35.1	36.7	36.3
2012	36.4	40.4	40.5	39.1	39.6	34.1	30.4	30.6	31.7	36.3	36.9	36.6
2013	37.5	39.9	40.8	41.3	39.2	34.7	31.2	29.4	32.3	34.1	37.1	36.0

Appendix 5. Monthly minimum temperature of Metema district from 1999 to 2013

year	JAN	FEB	MAR	APR	MAY	JUN	JUL	AUG	SEP	OCT	NOV	DEC
1999	14.7	18.3	17.5	23.2	21.9	20.5	20.3	19.6	18.9	18.8	15.8	15.7
2000	16.1	17.6	21.5	23.2	22.6	20.7	19.7	19.2	18.7	18.6	16.1	14.9
2001	13.5	17.9	20.4	24	23	20.5	20	20.2	19.7	19.9	16	15.6
2002	14.9	18.6	22.3	25.1	24.2	22	20.6	19.9	19.2	19.2	17.7	15.2
2003	15.6	20.6	21.9	18.4	18.8	17.1	19.1	19.7	19.2	19.2	16.7	15.4
2004	15.7	18.8	21.3	23.2	22.6	20.6	19.9	19.5	19.1	18.5	17.9	16.3
2005	15.7	19.8	21.1	25.6	23.2	21.2	20.3	20.4	19.1	19	16.7	15.5
2006	17	19.1	20.4	23.4	21.7	21	20.6	20.1	19	20.3	16.3	15.7
2007	15.2	16.2	21.7	23.3	23.8	21.3	27.9	19.9	19.5	19.1	17.3	14.6
2008	16.6	18.6	21.1	24.2	22.8	21.2	20	19.2	19.4	18.9	15.7	15.2
2009	15.3	19.8	22.2	23.7	23.6	21.7	20.2	20.4	19.6	19.4	17.7	16.3
2010	17.3	19.8	21.8	26	24.9	21.8	21	20.2	19.6	19.6	17	16.5
2011	16	17.9	21.7	20.2	22.9	20.4	19.4	19.8	19.3	19.4	17	15.8
2012	16.8	19.5	21.5	21.2	21	18.6	17.8	19.3	19.5	19.2	19	17.2
2013	16.3	18.1	21.2	23.2	22.9	20.6	20.2	19.3	19.8	19.3	18.1	17.6

Appendix 6. Monthly total rainfall of Metema district from 1999 to 2013

Year	JAN	FEB	MAR	APR	MAY	JUN	JUL	AUG	SEP	OCT	NOV	DEC
1999	0	0	0	0.3	77	143.8	265.5	251	223.2	164.6	0	0
2000	0	0	0	25.1	133.3	327.6	202.6	127	163.7	86.5	0	0
2001	0	0	0	0.2	5.1	161.4	331.1	321.5	138.5	23	0.1	0
2002	0	0	0	10.9	35.1	240.7	158.1	245.3	178.6	34.8	3	0
2003	0	0.3	0	0.8	38.1	310.3	263.1	279.4	185.9	3.4	0	0
2004	0	0	0	0	28.6	160.4	119.9	119.8	144.6	85.4	4.2	0
2005	0	0	4.7	3.2	64.9	213.3	271.8	260.9	243	66.2	0	0
2006	0	0	0	18.6	177.6	171.8	203.4	297.9	207.8	27.2	0	0
2007	0	0	0	22.5	43	142.1	320.5	332.5	254.2	52.7	25.5	0
2008	0	0	0	61	103.6	163.7	370.8	287.1	326.6	78.4	0	0
2009	0	0	0	0	64.4	163.4	201.2	233.5	109.7	15.4	0	0
2010	0	0	0	0	104.2	165.7	269	266.8	215.3	51	0	0
2011	0	0	0	0	69.9	93.7	122.5	217.4	138.2	22.6	0	0
2012	0	0	0	0	55.9	216.5	237.5	327.7	381.6	0.7	0.8	0
2013	0	0	0	1.2	47.8	196.5	216.8	352.7	150.4	51.3	0	0

Appendix 7. Monthly relative humidity of Metema district from 2004 to 2013

year	JAN	FEB	MAR	APR	MAY	JUN	JUL	AUG	SEP	OCT	NOV	DEC
2004	39	36.8	30	32.6	31.7	*	68.9	75.7	68.6	55.4	45.5	45.4
2005	27.2	32	*	27.1	*	*	*	53	*	65.5	47.9	41
2006	42.8	35.6	31.7	34	55.5	62.8	74.9	53.9	42.9	33.3	16.9	27.5
2007	31.5	23.6	0	0	0	0	0	0	0	0	0	0
2008	0	0	*	*	*	*	*	*	*	*	*	*
2009	0	24.5	*	*	*	*	0	*	*	*	*	*
2010	28	*	*	67.4	*	*	*	*	*	*	*	*
2011	35.1	*	*	*	*	79.8	85.8	85.5	83.9	77.4	69.2	40.4
2012	26.3	*	*	15.2	48	77.1	84.1	87.6	83.7	56.5	52	45.2
2013	42	29	33.6	29.5	34.8	54.9	77	80.3	76.5	66.7	49.6	40.3

Appendix 8. Lists of Plates

Plate 1. Deployment of CDC light trap in compound habitat for collection of phlebotomine sandflies

Plate 2. Collection of phlebotomine sandflies using sticky traps deployed in agricultural farm field

Plate 3. Collection of trapped *Phlebotomus* spp. using aspirator for dissection

Plate 4. Dissection of female *Phlebotomus* spp. for age determination and detection of natural infection

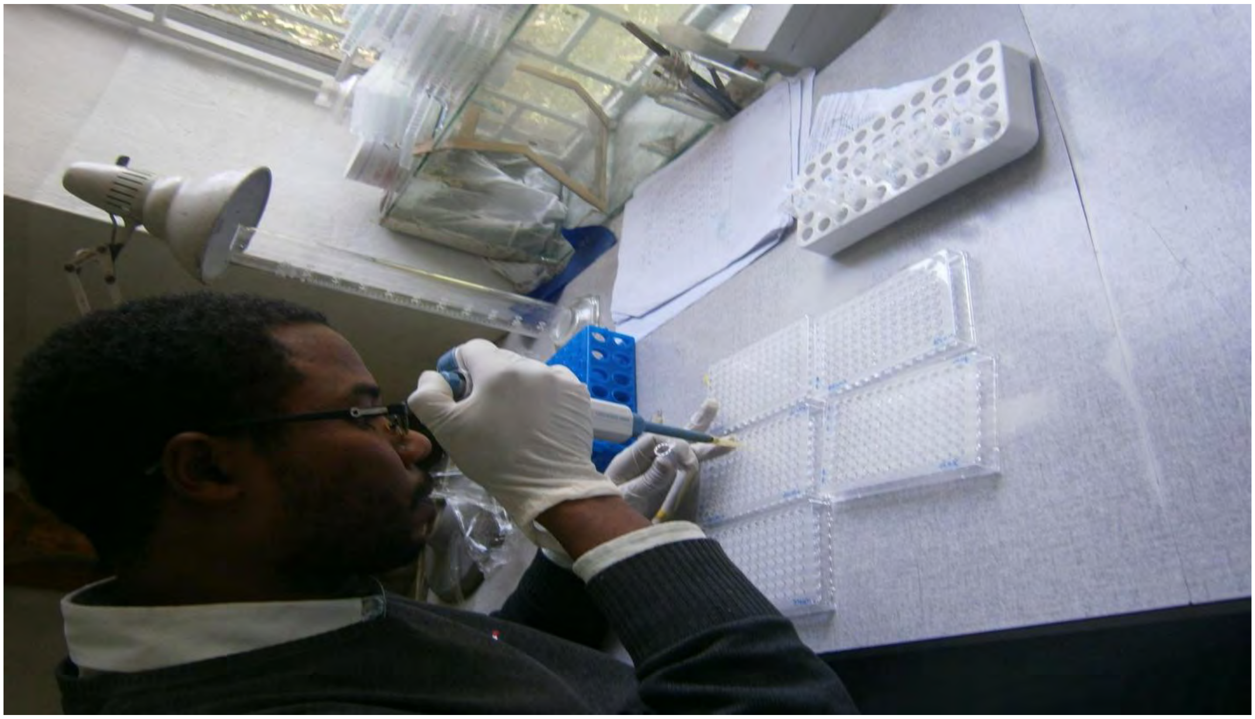

Plate 5. Blood meal identification using ELISA