Addis Ababa University

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Studies on the Bionomics and Behavior of Phlebotomine Sandflies (Diptera: Psychodidae) in Visceral Leishmaniasis foci in Tahtay Adiyabo District, Northern Ethiopia

By

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By

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Abstract

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Araya Gebresilassie

Addis Ababa University, 2015

Visceral leishmaniasis (VL) caused by *Leishmania donovani* is widespread in Ethiopia, particularly in the north and north-west of the country. Studies to investigate the ecology and behavior of sandflies and their epidemiological significance in the transmission of VL were conducted in Tahtay Adiyabo district, northern Ethiopia. Entomological studies were undertaken in three villages of the district between May 2011 and April 2012 to identify the sandfly fauna and determine the bionomics of *Phlebotomus orientalis*. Collections of sandflies were done using CDC light traps, sticky traps, and pyrethrum spray catches inside residential huts. The vectorial role of sandfly vector (s) was determined by the detection of *Leishmania* parasites by microscopy and *Leishmania* specific PCR. The host preferences of *P. orientalis* was examined using host choice experiments and bloodmeal analysis using cytochrome (cyt) *b*-PCR and reverse line blotting as well as enzyme linked immunosorbent assay. Nocturnal periodicity of *P. orientalis* was also investigated using light traps by replacing the collection bags at hourly intervals throughout the night. The attractiveness of nine plant species for *P. orientalis* was also done under field settings using unlit CDC traps. The effects of lunar phases and lunar periodicity on the performance of light traps in collecting *P*. *orientalis* were studied by sampling sandflies among the four lunar phases for seven months. In total, 100,772 sandflies, belonging to 25 species were recorded. *Sergentomyia africana* and *P. orientalis* made up 59.1% and 23.5% of the collected sandflies, respectively. The outdoor to indoor index for *P. orientalis* was 138:1 on sticky traps, exhibiting its pronounced exophilic behavior. *P. orientalis* showed marked fluctuations in seasonal density, which peaked during the months of March and April. A sharp decrease in abundance of *P. orientalis* was also observed from July to December. The parous rate in the unfed females was 34.05% in peri-domestic and 35.35% in agricultural fields. Out of 921 females of *P. orientalis* dissected, one specimen (0.11%) was found naturally infected with *Leishmania* promastigotes. Five pools (25 females) of unfed *P. orientalis* had DNA of *Leishmania* spp. Markedly higher mean numbers of female *P. orientalis* were attracted to donkey-baited tent traps than traps-baited with cow, human, dog, goat, sheep or chicken, respectively. Among the small wild animals

tested, ground squirrels attracted significantly (P<0.05) more female *P. orientalis* followed by the hares, gerbils, and the spiny rats. Bloodmeal analysis also revealed that *P. orientalis* females feed on a range of hosts with predominant preference for bovines followed by donkeys, humans, goats, sheep, dogs, and camels. Results on diel periodicity showed that the activity of *P. orientalis* females increased from 18:00 to 24:00 hrs, with a peak after midnight (24:00-03:00 hrs). Four of the plant species tested in the field (*Balanites aegyptiaca*, *Acacia seyal*, *A. sieberiana,* and *Ziziphus spina-christi*) was preferred by both sexes of the sandfly. Results revealed that lunar phases had significant effects in the trapping efficiency of CDC light traps for capturing *P*. *orientalis* (ANOVA, P<0.05). The mean density of *P. orientalis* collected in light traps during moonlit nights was around 25% of the catch during dark nights. The findings of the present study signified that *P. orientalis* is the principal vector of VL, which peaks in population abundance during the dry months of March and April in the current study area. As well, this vector species exhibits predominant preference to feed on bovine outdoors with peak activities in the late night.

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Chapter One

General Introduction

1.1. Description and classification of sandflies

Phlebotomines are usually named as sandflies, a label arising from the phlebotomines-leishmaniasis associations studied extensively in the drier regions of the Mediterranean and Middle East (Killick-Kendrick, 1999; Alten, 2010). However, this name is sometimes confused with sandflies of the family Ceratopogidae, and Siimuliidae, families with very different behaviors and vector-disease associations. Phlebotomine sandflies are the major biting members of the dipteran family Psychodidae, where they are presumed vectors of various etiologic agents. These flies are small (usually 1.5-2 mm body length), grayish-yellow to brown and are found throughout the tropical and subtropical regions. Sandflies are recognized by their lanceolate and densely haired wings, long legs, slender body, and a head nearly at right angles to the body (Lewis, 1982; Killick-Kendrick, 1990; 1999). Likewise, their normal flight pattern is very characteristic and consists of a series of short, erratic hopes, in which they seldom move farther from breeding sites (Lane, 1993).

Taxonomists have suggested several systems of the classification of sandflies, but uniformity has not been achieved and therefore the higher classification of sandflies below the suborder is still in controversy (Lewis *et al*., 1977; Lane, 1993). Some workers considered the phlebotomine sandflies as a subfamily of the Psychodidae (Theodor, 1958; Lewis *et al.,* 1977); others proposed to have separate family status (Abonnenc and Leger, 1976; Williams, 1993). However, the widely accepted classification of sandflies is to group them in the family

Psychodidae and subfamily Phlebotominae (Lewis *et al*., 1977; Lane, 1993; Munstermann, 2004). The family is classified in the heterogeneous infra-order Psychodomorpha within the suborder Nematocera, the other dipteran suborder being Brachycera (Hennig, 1981). Sandflies share the family Psychodidae with the non-vector and non-biting moth flies (subfamily: Psychodinae), often seen around shower drains.

The family Psychodidae is also further subdivided into six subfamilies that differ in size and appearance (Wagner, 1997). In terms of taxonomic characters, the family Psychodidae is very old and maintains some of the most ancient of dipteran characters (Lewis *et al*., 1977). Dense covering of narrow scales on head, thorax, legs, and wings, and the presence of a characteristic wing venation are the major morphological characters distinguishing members of the family (Triplehorn and Johnson, 2005). The presence of an elongate and more fragile structure along with piercing mouthparts capable of taking blood is characteristic of the subfamily Phlebotominae (Munstermann, 2004).

Lewis *et al*. (1977) proposed a stable classification of the phlebotomine sandflies, which is mainly the modification of Theodor's system, based on practical criteria and defined five genera with a number of subgenera. Later on, another genus *Chinius*, from China was added (Leng, 1987). Three of the genera (*Phlebotomus*, *Sergentomyia* and *Chinius*) exist in the Old World and the other three (*Lutzomyia, Brumptomyia* and *Warileya*) in the New world. However, a recent phylogenetic analysis of morphological characters led to the conclusion that the Old World species should be classified in seven genera (*Chinius*, *Phlebotomus*, *Australophlebotomus*, *Idiophlebotomus*, *Spelaeophlebotomus*, *Sergentomyia* and

Spelaeomyia), although the monophyly of *Sergentomyia* was questioned (Rispail and Leger, 1998a, b; Aransay *et al*., 2000a). Only two genera (*Phlebotomus* and *Lutzomyia)* have public health and veterinary importance. In the genus *Phlebotomus*, Lewis (1982) has recognized 11 subgenera, 96 species, and 17 subspecies.

The subgenera included *Larroussius*, *Idiophlebolomus*, *Spelaeophlebolomus*, *Australophlebotomus*, *Phlebotomus, Paraphlebolomus*, *Synphlebotomus*, *Anaphlebotomus*, *Euphlebotomus*, *Kasaulius*, *Adlerius* and *Transphlebotomus* was later created from-*Larroussius* (Artemiev and Neronov, 1984). However, some authors have given generic rank to *Spelaeophlebotomus*, *Idiophlebotomus,* and *Australophlebotomus* (Rispail and Leger, 1998a). Moreover, the taxonomic position of the subgenus *Parvidens* is the most controversial of all taxonomic questions relating to sandflies (Ashford, 1991; Rispail and Leger, 1998a). Species in the genus *Phlebotomus* are man-and mammal-bitters, and represent all the known Old World vectors of various pathogens to humans (Killick-Kendrick, 1990; Lane, 1993). The huge genus of *Sergentomyia* is distributed throughout the Old World and sandflies in this group primarily feed on reptiles and amphibians (Lewis, 1971). This genus contains no known vectors of mammalian leishmaniasis.

To date more than 900 species of phlebotomine sandflies are known to science, out of which only 98 species belonging to *Phlebotomus* and *Lutzomyia* are involved in the transmission of disease pathogens like viruses, bacterium, and most importantly the protozoan pathogens (*Leishmania* spp.) to man (Sharma and Singh, 2008; Maroli *et al*., 2013).

1.2. Phlebotomine sandflies and disease transmission

Phlebotomine sandflies are vectors of pathological agents causing bartonellosis, sandfly fever, and different forms of leishmaniasis in tropical and subtropical areas of the world.

1.2.1. Sandfly fever

Sandflies are involved in the transmission of several viral pathogens, among which the most important are grouped into the genus *Phlebovirus* (Family: Bunyaviridae), which includes the sandfly fever Sicilian and Toscana viruses, and the *Vesiculovirus* genus (Family: Rhabdoviridae), which includes vesicular stomatitis, and the Chandipura and Isfahan viruses (Tesh, 1988; Depaquit *et al*., 2010). In the New World, more than 30 serotypes of the genus *Phlebovirus* have been identified, but their medical importance is not fully known (Tesh *et al*, 1989). In the Old World, however, three sandfly fever serotypes in the genus *Phlebovirus* are known and these are Sicilian virus (SFSV), Naples virus (SFNV), and Toscana virus (TOSV) (Depaquit *et al*., 2010; Maroli *et al*., 2013). Sandfly fever (also named as papataci fever and three-day fever) results in acute febrile illness in man, lasting two to four days and sometimes for much longer periods (Tesh *et al*, 1989).

Human infections with Sandfly Fever Sicilian Virus (SFSV) have been confirmed in the Mediterranean basin, Africa, Middle East and Central Asia (Batieha *et al*., 2000; Papa *et al*., 2006). *Phlebotomus papatasi* has been confirmed as the vector of the disease. Toscana virus has been found in many countries around the Mediterranean basin and some North African countries (Peyrefitte *et al*., 2005). Two sandfly species have been incriminated as vectors of Toscana virus: *P. perniciosus* and *P. perfiliewi* (Charrel *et al*., 2005). In Africa, two distinct

viruses, namely Sicilian virus and Naples virus (Depaquit et al., 2010), cause sandfly fever. Naples virus is distributed in Ethiopia, Morocco, and Sudan (Tesh *et al*., 1976). More recently (August 2011), outbreak of acute febrile illness was reported in the Afar region of Ethiopia, in which the etiologic agent of this disease was later identified as SFSV (Abyot Bekele *et al*., 2014).

1.2.2. Bartonellosis (Carrión's Disease)

Bartonellosis is a bacterial infection caused by *Bartonella bacilliformis*, a motile, aerobic, and Gram-negative bacterium, which occurs in the Andes Mountains in parts of Peru, Ecuador, and south-west Colombia (Maguina *et al*., 2001; Xu and Chai, 2002). This disease is endemic in valleys between 750 and 2,700 m above sea level, apparently being altitudinally restricted by ecological requirements of the vectors (Lane, 1993). However, recent epidemics have been reported in previously non-endemic elevations of the Amazon basin, which suggests that the endemic range of the disease is expanding (Maroli *et al*., 2013). The bacterium produces a disease known as Carrion's disease, with two clinically distinct phases: an acute or hematic phase, known as Oroya fever, and an eruptive or tissue phase, known as Peruvian Wart (Huarcaya *et al.*, 2004). Any infected person can experience either one or both phases, which can occur once or more than once during a lifetime. Oroyo fever, the acute stage, occurs after an incubation period of about 3 weeks. On the other hand, the Verruga peruana are chronic, lasting from several months to years, and contain large numbers of *B. bacilliformis* (Xu and Chai, 2002). The only proven or identified vectors of *B. bacilliformis* are sandflies of the genus *Lutzomyia*, which includes *Lu. verrucarum* and *Lu*. *columbiana* (Alexander, 1995; Munstermann, 2004).

1.2.3. Leishmaniases

The most devastating of the sandfly-transmitted diseases are the leishmaniases, causing substantial morbidity and mortality in much of the world. The leishmaniases are a group of parasitic diseases caused by morphologically similar parasites in the genus *Leishmania* (Order: Kinetoplastida, Family: Trypanosomatidae). About 21 species and subspecies of *Leishmania* are responsible for the disease in human beings (Herwaldt, 1999; Chappuis *et al*., 2007). Within the *Leishmania*, there are three subgenera: *Leishmania*, *Viannia*, and *Sauroleishmania* (Shaw, 1994; Bates, 2007). The subgenus *Sauroleishmania* comprises species that cause leishmaniasis of reptiles, which is transmitted by the sandfly members of the genus *Sergentomyia*. The two subgenera, *Leishmania* and *Viannia*, are separated based on their location in the vector's gut (Lainson and Shaw, 1987). The subgenus *Leishmania* occurs in both the New World (Neotropical and southern Nearctic) and the Old World (Palaearctic, African and Oriental). The subgenus includes pathogenic species such as *Leishmania tropica, L. aethiopica, L. major, L. infantum,* and *L. donovani* (WHO, 1990; Kerr, 2000; WHO, 2010). The other subgenus, *Viannia*, is restricted to the Neotropical region. The most important species include *L. braziliensis, L. chagasi*, *L. guyanensis, L. panamensis* and *L. peruviana*, all of which cause human diseases (WHO, 1990; WHO, 2010).

Leishmaniasis remains a severe public health problem in most parts of the world being endemic in large areas of the tropics, subtropics and the Mediterranean basin (Herwaldt, 1999; Gramiccia and Gradoni, 2005). In terms of global disease burden, it is the third most important vector-borne disease after malaria and lymphatic filariasis (WHO, 2010). In a general, there are 12 million people currently infected with leishmaniasis and 350 million

people at risk worldwide, causing the loss of 2.4 million disability-adjusted life years (DALYs) and about 60,000 deaths in 2001 (Desjeux, 2001; WHO, 2010). Over the past 20 years, the number of human leishmaniasis cases has dramatically increased with a trend that shows no sign of abating (Alvar *et al*., 2012). This increase might be due to the establishment of the *Leishmania* transmission cycle to peri-domestic environments as a result of deforestation and urbanization and the fact that leishmaniasis is a common opportunistic infection in HIV-infected persons (Arias *et al*., 1996; Desjeux, 2004). The leishmaniases are characterized by a spectrum of clinical manifestations, including ulcerative skin lesions developing at the site of the sandfly bite (localized cutaneous leishmaniasis (LCL); multiple non-ulcerative nodules or diffuse cutaneous leishmaniasis (DCL); destructive mucosal inflammation (MCL); and disseminated visceral leishmaniasis (VL) (Reithinger *et al*., 2007; Kumar, 2013).

1.2.3.1. Visceral leishmaniasis

Visceral leishmaniasis (VL) is one of the most important protozoan vector-borne diseases affecting humans and animals. VL is a life threatening disease which mainly affects the poorest of the poor communities in 79 countries with an estimated annual incidence of 0.4 million new cases (Alvar *et al*., 2012). Epidemiologically, the disease occurs in two different forms as anthroponotic and zoonotic leishmaniasis. *Leishmania infantum* in Europe, the Middle East and North Africa and *L. chagasi* in Latin America are transmitted zoonotically with dogs and some foxes serving as reservoir hosts (Chappuis *et al*., 2007). The anthroponotic leishmaniasis is caused by *L. donovani* complex and is restricted to the Indian subcontinent and East Africa (Maroli *et al*., 2013). If untreated, the disease is usually fatal, but with appropriate treatment, the case fatality rate drops to 10% or less (Veeken *et al*.,

2000). Visceral leishmaniasis mainly affects the visceral organs, including spleen, liver, the mucosa of small intestine and bone marrow. Typically, patients with VL present with fever, weight loss, cough, abdominal pain, diarrhoea, weakness, hepatomegaly, lymphadenopathy, and splenomegaly.

Like other tropical diseases, epidemiological data on VL are incomplete, and official figures are likely to reduce grossly the real prevalence of the disease (Guerin *et al*., 2002; Singh *et al*., 2006; Alvar *et al*., 2012). Both the number of recorded cases and the geographical areas affected have grown in the past two decades. Over 90% of cases of VL occur in poor rural and suburban areas of seven countries: India, Bangladesh, Nepal, Sudan, South Sudan, Ethiopia, and Brazil (Desjeux, 2004; Chappuis *et al*., 2007).

Easter Africa region has the second highest number of VL cases, after the Indian Subcontinent, and the disease is endemic in parts of Eritrea, Ethiopia, Kenya, Somalia, Sudan, South Sudan, and Uganda (Chappuis *et al*., 2007). Population displacement because of civil unrest and hazards, lack of control measures, rural-urban migration, and HIV–VL co-infection are factors contributing to the resurgence of the disease (Boelaert *et al*., 2000; Desjeux, 2004). For instance, in the 1980s, a kala-azar epidemic in a relatively small area in the Western Upper Nile Province in South Sudan claimed the lives of approximately 100,000 people of a total population of 300,000, largely attributed to population displacement because of war and famine (Seaman *et al*., 1996).

1.2.3.2. Visceral leishmaniasis in Ethiopia

Nearly 70 years had passed since the first report of VL cases in Ethiopia by Cole *et al*. (1942) near Lake Turkana on the Kenya border, albeit the exact burden of disease, distribution, and environmental determinants are poorly understood. Since then, it has been a growing public health problem in six Regional States (Tigray, Amhara, Oromyia, Southern Nations and Nationalities People's Region, Somali, and Afar), and serologically positive cases were detected from Gambela and Benshangul Gumuz Regional States (Asrat Hailu *et al*., 1996; Malaria Consortium, 2010; Teshome Tsegaw *et al*., 2013). Reports indicate that every year 3,700 to 7,400 individuals suffer from VL caused by protozoan parasites of the *L. donovani* complex (Alvar *et al*., 2012). The worst affected region is northwest Ethiopia close to the border with Sudan, which accounts for more than 60% of the reported VL cases that are frequently associated with HIV/AIDS (Lyons *et al*., 2003). VL mainly affects adult males due to the number of people migrating from highlands to the lowlands for seasonal work.

The other most important VL foci in Ethiopia are in south Ethiopia near the border with Kenya, where 20% of cases, rarely associated with HIV/AIDS, occur (Asrat Hailu, 2008 cited in Tesfaye Gelanew *et al*., 2010). The main endemic foci of VL include the Humera and Metema plains in the northwest (Asrat Hailu *et al*., 2006), Omo plains, Aba Roba focus, and Weyto River Valley in the southwest (Teklemariam Ayele and Ahmed Ali, 1984; Teklemariam Ayele *et al*., 1988).

VL cases were also reported in the southeastern semi-arid lowlands of the Afder and Liben (Marlet *et al*., 2003) and from Gode and Afder zones from Somali Regional State (Malaria

Consortium, 2010), where the majority of cases were children of the pastoralist community, suggesting autochthonous transmission. Cases of VL in the vast lowlands of the northeastern Rift Valley of Ethiopia (Awash Valley) were also reported to occur only sporadically and in association with HIV co-infection (Asrat Hailu *et al*., 2006).

The northwestern VL focus in Ethiopia covers the semi-arid Metema and Humera plains in the Tigray and Amhara Regional States that border Sudan and Eritrea at altitude of 500–700 m. As of the 1970s the number of disease outbreaks in Humera and its surrounding region has increased, which appears to correspond to an extensive program of agricultural development with its annual influx of migrant workers from the non-endemic highlands (Mallede Maru, 1979; Nega Berhe *et al*. 2001). In addition, the recent relocation of settlers from the highland areas to Humera, Tsegede and Armachiho districts resulted to the dramatic increase in VL cases documented by Medicines Sans Frontiers (MSF) during 2002 and 2003 (Lyons *et al*., 2003; Malaria Consortium, 2010). For instance, an outbreak that had occurred in 1995 claimed the lives of about 100-200 temporary farm laborers mainly from Maykadra village in Humera in just five months (Asrat Hailu *et al*., 2006).

More recently, a new VL outbreak was identified in the districts of Libo Kemkem and Fogera in the highlands of Amhara Regional State, where VL had never been reported until May 2005 when more than 2,500 primary cases were treated and 120 deaths occurred (Alvar *et al*., 2007; Seife Bashaye *et al*., 2009). Migrant laborers coming back from endemic neighboring areas (border of Sudan) is one of the hypotheses for the introduction of VL in

the region (Sordo *et al*., 2012). The spread of the disease in the endemic or new foci of VL in Ethiopia depends on the presence of appropriate sandfly vectors in the locality.

1.2.3.3. Vectors of visceral leishmaniasis in Ethiopia

Several entomological studies have been conducted to determine the sandfly fauna and vectors responsible for the transmission of *Leishmania* in Ethiopia. Those studies have indicated the presence of at least 22 *Phlebotomus* species, representing seven subgenera (*Larroussius, Synphlebotomus, Phlebotomus, Paraphlebotomus, Anaphlebotomus, Adlerius,* and *Parvidens* (Abonnenc and Minter, 1965; Lewis, 1982; Minter, 1990). The *Parvidens* group, having both characters of *Phlebotomus* and *Sergentomyia*, remain unresolved (Lewis *et al*., 1977; Ashford, 1991; Rispail and Leger, 1998a). However, in this thesis they are treated as *Phlebotomus* because of several morphological characters shared with it (Abbonenc and Minter, 1965; Dr. Teshome Gebre-Michael., pers. comm.).

Sandflies belonging to *Larroussius*, *Phlebotomus*, *Synphlebotomus* and *Paraphlebotomus* have been incriminated as vectors of *Leishmania* spp. causing VL and CL. Sandfly species included in these subgenera are widely distributed in the endemic regions of the country (Teshome Gebre-Michael and Lane, 1996; Teshome Gebre-Michael *et al*., 2007; 2010). Different sandfly species implicated in the transmission of different forms of leishmaniasis include: *P. (Synphlebtomus*) *martini*, *P*. *(Syn.) celiae*, *P*. (*Larroussius*) *orientalis*, *P. (Lar.) pedifer*, *P. (Lar.) longipes*, *P. (Paraphlebotomus) sergenti*, *P. (Pa.) saevus,* and *P. (Phlebotomus) duboscqi* (Ashford *et al*., 1973; Teshome Gebre-Michael *et al*., 1993; Asrat

Hailu *et al*., 1995; Teshome Gebre-Michael and Lane, 1996; Meshesha Balkew *et al*., 2002; Teshome Gebre-Michael *et al*., 2004).

In south and south-west of Ethiopia where VL is endemic, several species of *Phlebotomus* have been documented (Teshome Gebre-Michael *et al*., 1986; Teshome Gebre-Michael and Lane, 1996; Meshesha Balkew *et al*., 1999; Teshome Gebre-Michael *et al*., 2013). These are *P. orientalis*, *P. alexandri, P. duboscqi, P*. *martini, P. celiae, P. vansomerenae*, *P. saevus, P. sergenti*, and *P. rodhaini*. Of these, *P. martini* and its close relative *P. celiae* have been incriminated as vectors of the disease in southern Ethiopia (Aba Roba focus) (Teklemariam Ayele and Mutinga, 1989; Teshome Gebre-Michael and Lane, 1996). Both species are also suspected vectors of VL in the Gelana and Weyto Valleys, though other species *P. alexandri* may also be involved in the Weyto Valleys (Asrat Hailu *et al*., 2006). *Phlebotomus orientalis* has also been implicated as vector of VL in the lower Omo plains in the south-west, where *L. donovani* was detected by a DNA probe in 1 out of 70 dissected *P. orientalis* (Asrat Hailu *et al*., 1995).

Various attempts to identify the sandfly vector(s) of VL in north-west Ethiopia was started by Tekle *et al*. (1970) and have continued since then (Ashford *et al*., 1973; Teferi Gemetchu, 1983; Teshome Gebre-Michael *et al*., 2010; Kirstein *et al*., 2013; Wossenseged Lemma *et al*., 2014a). Based on these investigations, seven *Phlebotomus* species, including *P. orientalis*, *P. papatasi*, *P. bergeroti, P. duboscqi, P. rodhaini*, *P*. *martini*, and *P*. *alexandri* have been recorded from the region. *P. orientalis* is not only the predominant species in most of the collections, but also the species implicated as the most likely vector of VL in the region since

it has already been established as a proven vector in the neighboring Sudan (Hooogstraal and Heyneman, 1969; Elnaiem *et al*., 1998a; Hassan *et al*., 2008) and South Sudan (Ashford *et al*., 1992).

1.3. Life history and breeding sites

Like all true flies (Order: Diptera), sandflies undergo complete metamorphosis and exhibit four life stages: egg, larva (four instars), pupa and the adult. Adult female sandflies usually lay their eggs in a suitable habitat rich in organic content, such as animal excreta and soil, which provides the newly emerged larvae with shelter, nutrition, and moisture. The number of eggs laid by a single female at one time varies greatly by species and by factors such as bloodmeal source or ambient temperature, but typically is between 40 to 70 eggs (Young and Duncan, 1994; Hanafi *et al*., 1999). Eggs (0.3–0.5 mm in length) are initially white or light grey in color, but often turn dark brown or black within a few hours of oviposition. After 7-10 days under optimum conditions, the eggs hatch into a caterpillar-like larvae with caudal bristles (Claborn, 2010; Volf and Volfova, 2011).

The mature larva is 3-6 mm long and has well-defined black head, which is provided with a pair of small mandibles and small leaf-like antennae. They also have usually two pairs of long caudal setae that can help in their identification as sandfly larvae, though these are not usually used in species identification because larvae are rarely encountered in nature (Feliciangeli, 2004). The larvae move small distance from the oviposition site (Maroli *et al*., 2013). Most previous attempts to identify the preferred microhabitats for the oviposition of sandflies have produced disappointing yields, resulting in a small number of positive soil

samples and immature forms (Dhiman *et al*., 1983; Mutinga *et al*., 1989; Feliciangeli, 2004; Singh *et al*., 2008). However, caves, crevices, animal burrows, termite mounds, cracks in the soil, domestic animal shelters, cracked walls, tree-holes, birds' nests and leaf litter are mainly postulated to afford suitable environments for sandfly breeding (Feliciangeli, 2004; Singh *et al*., 2008).

After the female has taken a bloodmeal and completed oviposition, first-instar larvae usually emerge in 7-10 days (Killick-Kendrick, 1999; Volf and Volfova, 2011). The duration of the larval instars varies greatly, both between and within species regulated mainly by temperature and availability of food (Ward, 1972; Hanafi *et al*., 1999). In temperate regions, species overwinter as diapausing fully grown larvae, often occurring in the fourth instar (Tesh *et al*., 1992; Ready, 2013). Prior to pupation the larva assumes an almost erect position in the habitat, the skin then splits open and the pupa wriggles out (Maroli *et al*., 1987).

Pupae resemble a small butterfly chrysalis except that the fourth stage larval exuvium is attached to one end to a solid substrate (Claborn, 2010). The inactive pupae usually hatch within five to ten days. The period from oviposition to adult emergence is 30-60 days, but extends to several months in diapausing species (Killick-Kendrick, 1999; Volf and Volfova, 2011). In males, the terminalia of genital organ rotate through 180^0 during the 24 hours immediately after emergence (Davis, 1967; Lane, 1993; Killlick-Kendrick, 1999).

Adult sandflies are small and seldom exceed 3.5 mm in length (Molyneux and Ashford, 1983). Their body and the small wings are covered with dense hairs and when at rest they hold their wings upright in a "V" shape over their backs. Adults range in color from almost

white to black. The legs are very long and delicate. The eyes are large and dark. The antennae are long and filiform, with 16 segments. The mouthparts are short, dagger-shaped and oriented downward (El-Hossary, 2006). The thorax is distinctively humped, pushing the head below the upper surface of the thorax.

1.4. Physiological age of adult sandflies

Physiological age grading was first reported by Russian workers and received worldwide attention because of its potentialities in providing us with some accuracy the age of individuals and therefore populations of insect vectors in a given time and locality. It has indeed provided the avenue for more detailed studies on parity, longevity, mortality, and survival, which are indicators of vector efficiency (Ferro *et al*., 1995; Hayes and Wall, 1999). It is desirable to be able to establish or estimate the age of individual vectors to assess the epidemiological significance of insect vectors of pathogens, evaluate vector control measures, and study the dynamics of field populations (Añez and Tang, 1997). Recognition of parous from nulliparous female sandflies is important because flies must survive at least one oviposition to be potentially infective. However, this aspect of the study of sandflies is limited by the small size of the ovaries and the difficulty of tracing evidence of more than one oviposition (Lewis *et al*., 1970), except some species (Wilkes and Rioux, 1980; Ready *et al*., 1984).

The methods commonly employed for physiological age grading of sandflies include the detection of residual secretions in the accessory glands of older females, and the yellowish appearance and differentiation of parous ovarioles (Lewis *et al*., 1970; Magnarelli *et al*.,

1984; Añez and Tang, 1997). Examination of the accessory glands of ovaries is a useful method for distinguishing parous females in several Old World species in the genus *Phlebotomus* (Adler and Theodor, 1957; Lane, 1993). However, the usefulness of this method to the New World genus *Lutzomyia* depends on the sandfly species (Takaoka *et al*., 1989; Hayes and Wall, 1999). The accessory glands of the oviduct produce a secretion containing numerous granules. In the newly hatched female, the glands are nearly empty and no granules are discernible. Females with no or few granules in the glands are newly hatched individuals, while females without a trace of blood in the stomach, with eggs in the early stages of development, but with granules in the accessory glands are individuals which have digested a bloodmeal, laid a batch of eggs, and are ready for a re-feeding (Adler and Theodor, 1957). A quantitative study that is carried out throughout a season on the absence or presence of these granules will provide valuable information on the population dynamics of sandflies in leishmaniasis endemic areas.

1.5. Suspected and proven sandfly vectors

Of 900 sandfly species described in various parts of the world, less than 10% are proven or probable vectors of leishmaniasis. In the Old World, 42 species are either proven or probable vectors, of these 20 are implicated in the transmission of *Leishmania infantum*, 6 for *L. donovani*, 7 for *L. major*, 7 for *L. tropica* and 3 for *L. aethiopica* (WHO, 2010; Maroli *et al*., 2013; Table 1.1). Non-vector species do not support parasite development and/or lack natural contact with humans and/or reservoirs (WHO, 2010). The main factors that affect the vectorial potential of a given species of sandfly to act as a competent vector, include sandfly species may never bite humans and their geographic distribution is different from

that of the leishmaniasis or a possible reservoir host for that particular disease (Killick-Kendrick, 1999). Moreover, sandflies can be carriers of the parasite and not vectors, due to the inability of the parasite to carry out metacyclogenesis (Bates, 2008). Recent advances in the incrimination of vectors of leishmaniasis are often slow due to the difficulty of finding naturally infected flies in the wild and the lack of suitably trained and experienced staff (Killick-Kendrick, 1999).

The history of experiments leading to the incrimination of the vector of kala-azar dates back into the first half of the 20th century. The literature identifies five criteria necessary to incriminate a sandfly as a vector of *Leishmania* spp. (Killick-Kendrick, 1990, 1999; WHO, 2010; Maroli *et al*., 2013). Among the essential criteria are: (1) the vector must be anthropophilic; (2) in zoonotic entities of leishmaniasis, the vector must also bite the reservoir host(s); (3) the vector must be infected in nature with the same *Leishmania* species as occurs in humans, and this must be ascertained by comparison of isolates using isoenzymes or DNA; (4) the vector must support the complete development of the parasite after the infecting bloodmeal has been digested; and (5) the vector must be able to transmit the parasite by bite to a susceptible host while taking a bloodmeal.

However, since it has already been recognized how difficult to satisfy all these criteria, the most important criteria now are to fulfill one and three above (Killick-Kendrick; 1999; WHO, 2010). More recently, two criteria were incorporated to demonstrate that a species was actually a biomedically important vector in a specific focus, by modeling its role and showing the direct effect of control on transmission (Ready, 2013).

For the detection of natural infections in the guts of sandflies, individual sandflies need to be examined for *Leishmania* promastigotes by dissection under a microscope. This process often requires freshly caught individual sandflies and culture of parasites found in the sandfly gut (Killick-Kendrick, 1999). However, this technique is time-consuming, needs dissecting expertise and a large number of specimens, since the *Leishmania* infection rates in sandflies are usually very low even in endemic areas (Sharma and Singh, 2008). Currently, a highly sensitive polymerase chain reaction (PCR)-based techniques are increasingly employed in epidemiological studies to detect infection and to characterize *Leishmania* parasites in sandfly vectors (Aransay *et al*., 2000b, Kato *et al*., 2007; Es-Sette *et al*., 2014).

In Ethiopian situation, particularly in the northern endemic foci of VL (viz. Metema-Humera lowlands, Tahtay Adiyabo, Libo-kemkem, Belessa, and the rest), none of the potential vectors have been incriminated as vectors of VL following essential criteria needed to incriminate the sandfly vectors. However, *P. orientalis* is regarded as the likely vector of *L. donovani* in the northern endemic areas including Libo-Kemkem and Metema-Humera plains (Teferi Gemechu *et al*., 1975; Teshome Gebre-Michael *et al*., 2007; 2010). Unlike the northern endemic areas, *P*. *martini* and its close relative *P. celiae* have been incriminated as the vectors of VL in southern Ethiopia (Aba Roba focus) (Teshome Gebre-Michael and Lane, 1996). The former species is also a vector in Kenya (Perkins *et al*., 1988).

Table 1.1. Phlebotomine sandfly species of the genus *Phlebotomus* that act as vectors of Old World *Leishmania* spp. (based on WHO, 2010; Maroli *et al*., 2013).

***Leishmania* Parasite**	**Clinical Association**	**Proven or Suspected vectors**	**Geographical Distributions**
L. donovani	AVL; PKDL	*P. (Paraphlebotomus) alexandri, P. (Larroussius) longiductus*	China, Iraq, Turkey
		P. (*Euphlebotomus*) *argentipes**	Indian subcontinent (India, Nepal, Bangladesh)
		P. (*La.*) *orientalis**	Sudan, Ethiopia, Yemen; Saudi Arabia
		*P. (Synphlebotomus) martini**, *P. (Syn.) celiae**, *P. (Syn.) vansomerenae*	Kenya, Ethiopia
L. infantum	ZVL; ZCL	*P.(Pa) alexandri*	Central Asia, China
		*P. (La.) ariasi**	Western Mediterranean
		*P. (Adlerius) balcanicus**	Armenia
		*P. (Ad.) chinensis**	China
		P. (Ad.) halepensis	Syria, Azerbaijan, Georgia
		P. (La.) galilaeus	Syria
		*P. (La.) kandelakii**	Transcaucasia; Iran; Afghanistan
		*P. (La.) langeroni**	Egypt; Tunisia, Spain
		P. (La.) longicuspis	North Africa
		*P. (La.) longiductus**	Central Asia, China
		P. (La.) major s.l*	Iran
		*P. (La.) neglectus**	Albania, Cyprus, Croatia, Greece
		*P. (La.) perfiliewi**	Italy; E. Mediterranean; North Africa
		*P. (La.) perniciosus**	W. and C. Mediterranean, N. Africa
		*P. (La.) smirnovi,** *P. (La.) syriacus*	China; Kazakhstan
			W. Mediterranean
		*P. (La.) tobbi**	E. Mediterranean; Sicily
		*P. (La.) transcaucasicus**	Azerbaijan
		*P. (La.) wui**	China
		P. (*Ad*) *turanicus**	Turkmenistan
L. tropica	ACL; LR	*P. (Pa.) sergenti*; P.* (*Pa.) saevus**	North Africa; Middle East; Afghanistan; Iran; Transcaucasus; E. Mediterranean

	ZCL	*P. (La.) guggisbergi*; P. (La.) aculeatus; P. (Ad.) arabicus**; *P. (Syn.). chabaudi*, *P. (Syn.) rossi**	Kenya, Israel, Ethiopia, Morocco, Tunisia, Namibia
L. major	ZCL	*P.(Phlebotomus) duboscqi**	West Africa; Kenya; Ethiopia; Yemen
	ZCL, DCL	*P. (P.) papatasi**	N. Africa; Sudan; Central Asia; Middle East; Indian Subcontinent
		*P. (P.) salehi**	North-west India; Iran, Pakistan
		*P.(P.) caucasicus**	Iran; Central Asia
		P. (Pa) alexandri	Turkmenistan
		P. P. (*Syn.*) *ansarii*	Iran
		P. (Pa.) mongolensis	Kazakhstan
L. aethiopica	DCL; MCL	*P. (La.) longipes*; P. (Pa.) sergenti*	Ethiopia
		*P. (La.) pedifer**	Kenya; Ethiopia

AVL: anthroponotic visceral leishmaniasis; PKDL: post kala-azar dermal leishmaniasis; ZVL: zoonotic visceral leishmaniasis; ZCL: zoonotic cutaneous leishmaniasis; DCL: diffused cutaneous leishmaniasis; ACL: anthroponotic leishmaniasis; MCL: Mucotaneous leishmaniasis; LR, leishmaniasis recidivans; ***** Incriminated vectors

1.6. Ecology and behavior of *Phlebotomus* species

1.6.1. Dispersal patterns

Adult sandflies are feeble fliers and hardly ever disperse far from their emergence sites. Low dispersal ranges of sandflies have offered important insights into larval habitat, species distributions, diversity and speciation, and disease epidemiology. Munstermann (2004) noted that field captures of adults could permit to suppose a nearby larval habitat. Sandflies have a hopping type of flight where they practice several short flights before the females land on their host. Sandflies normally disperse from their resting/breeding sites for host seeking, sugar feeding and mating. Mostly host-seeking females typically travel a few kilometers, whereas males rarely disperse more than a few hundred meters and remain near

their emergence and mating sites (Yuval and Schlein, 1986; Yuval *et al*., 1988; Ready, 2013). Moderate winds and light rain significantly reduce flight activity (Quate, 1964; Sawalha *et al*., 2003; Colacicco-Mayhugh *et al*., 2011).

Mark-release-recapture studies have indicated sandflies may travel from 50 to 2,000 meters during the nighttime (Quate, 1964; Foster, 1972; Killick-Kendrick *et al*., 1984; Morrison *et al*., 1995). For instance, mark-release-recapture study by Mutinga *et al*. (1992) in Kenya noted that *P. martini* was recaptured within a radius of 50 m from their location of release.

However, Quate (1964) in Sudan has recorded *P. orientalis* at a maximum distance of 730 m from the release points. Similarly, a single female of *P*. *ariasi* has been shown to move further than 2 km (Killick-Kendrick *et al*., 1984). Flight range evidently differs greatly from species to species and from locality to locality (Yuval *et al*., 1988). Generally, information on the dispersion pattern of sandflies in nature can be used to evaluate the potential rate of *Leishmania* dissemination and to implement surveillance and control strategies against the vectors.

1.6.2. Resting sites

The types of resting sites used by adult sandflies vary according to season, availability of microhabitat, amount of moisture present, and species of sandfly. Sandflies are found in a variety of micro-habitats such as caves, tree holes, tree trunks, cracks in rocks and cavities between boulders, fissures in the ground, buildings, organic waste, in a plowed field, termite hills and animal burrows (Lane, 1993; Killick-Kendrick, 1999; Müller *et al*., 2011a). Many of these sites are resting places where the flies take shelter during adverse weather conditions.

In addition, a large proportion of these habitats provide favorable environment for breeding and feeding (Asimeng, 1992; Singh *et al*., 2008). In northern Ethiopia in Tahtay Adiyabo district of VL focus, reduced desiccation from narrow deep cracks maintains elevated relative humidity in and around root systems of vegetation promoting ideal conditions for *P. orientalis* breeding/resting (Moncaz *et al*., 2014).

Different species of sandfly are associated with different habitats. Termite hills provide microenvironments, which are particularly suitable for sandflies throughout large parts of the lowland zone of eastern Africa (Minter, 1964). Many species of sandfly rest in the airshafts of termite hills, and *P. martini*, which occupies old shaded termitaria, is the vector of "termite hill kala-azar" in Kenya (Minter, 1964) and southern Ethiopia (Gebre-Michael and Lane, 1996). These habitats also harbor closely related species and secondary vectors, *P. vansomerenae* and *P. celiae* in southern Ethiopia and Kenya (Minter, 1964; Teshome Gebre-Michael and Lane, 1996; Teshome Gebre-Michael *et al*., 2013). *Phlebotomus orientalis* was also found resting in a porcupine burrow in northwest Ethiopia (Ashford, 1974). It was also observed resting in tree cavities (Quate, 1964) and in the mounds made by the termite, *Macrotermes herus* (Elnaiem *et al*., 1997). Recently, Muller *et al*. (2011a) found that large numbers of *P. papatasi* populations rest in the trunks of palm trees, large piles of organic waste and a plowed field. Such microhabitats can lead to much localization of sandflies and consequently to micro-foci of VL.

Man-biting species of phlebotomine sandflies also occur in certain types of man-made or natural caves. These caves are frequently shelters of bats, lizards and snakes, in which these

animals could be source of bloodmeal for sandflies inhabiting caves. Sandflies, for instance, can live throughout the year in some Central Asian caves, where they are part of a biocoenosis that includes the porcupine, a host of *L. donovani* (Lewis, 1971). Animal burrows are another group of biotopes, which provide suitable ecological settings for a widespread of *Phlebotomus* species. Inside these biotopes, a moderate temperature and high humidity create conducive environment for breeding and feeding. Ashford *et al*. (1973) found moderate numbers of *P. orientalis* and small number of *P. rodhaini* in porcupine holes in Belassa in northwest Ethiopia. In the Marigat area of Kenya, Basimike *et al*. (1992a) reported that the relative density of *P. martini* was four times greater in rodent burrows than in termite hills.

Phlebotomus orientalis occurs among patches of *Acacia* in the former southern Sudan (Hoogstraal and Dietlein, 1963) and sometimes rests in certain evergreen trees. Elnaiem *et al*. (1999a) also studied the association of *P. orientalis* to different vegetation types in the kala-azar focus in Sudan. This species is usually confined to *Acacia-Balanites* tree holes during the daytime. Specific association of *P*. *orientalis* with *Acacia-Balanites* woodland may be due to the presence of sugar diet or suitable resting site (Elnaiem *et al*., 1999a). Likewise, in Ethiopia, *P. orientalis* was detected to rest in holes of fig trees (Ashford, 1974). Besides tree holes, *P. orientalis* commonly rest in deeply cracking 'black cotton clay' soils, which create essential micro-habitats for the vector (Moncaz *et al*., 2014).

In general, understanding sandfly species resting sites is very crucial in determining the extent of host and sandfly interaction. This is also important to control the transmission of leishmaniasis from infected sandflies to the human or animal hosts.

1.6.3. Seasonal abundance

Climatic factors appear to have important effects on the gross distribution and local abundance of sandflies. Some species are widely distributed and found throughout the year, tolerating a wide range of conditions; others appear to be more sensitive, having a limited distribution and a strictly seasonal incidence.

Oscillations in population abundance and seasonality of sandflies in the Mediterranean region and Arabian Peninsula largely depend on ecological and bio-climatic factors. The seasonality of *P. tobbi* was studied in Turkey and was indicated that the species population rise in abundance through June and July, with the highest peak in August, and decreased through September and October (Kasap *et al*., 2009). Likewise, Sawalha *et al*. (2003) found that *P. tobbi* reached a peak during the driest month (July), whereas *P*. *perfiliewi* numbers peaked during August in the West Bank, Palestine. In Jordan, peak monthly abundance of *P. papatasi* occurred in September and October and then declined sharply by late November (Janini *et al*., 1995). The seasonal abundance of *P. perniciosus*, *P. neglectus*, *P. perfiliewi*, *P. papatasi* and *S. minuta* in the urban and peri-urban areas of southern Italy was determined for two years, where large number of most sandflies was recorded during July and August, when the mean temperature was high and the mean relative humidity was low (Tarallo *et al*., 2010). Indeed, the risk of being bitten by sandflies is higher during summer, when there

is also an increased movement of tourists to southern Italy and other countries in the Mediterranean region. In eastern Spain, the seasonal dynamics of *P. perniciosus* ranged from the end of March to the middle of December (Morillas-Márquez *et al*., 1983). A sharp increase in the number of this species between June and August was partly related to the hottest and driest season when the average temperature was comparatively high and the relative humidity was low.

Different species of sandflies display marked seasonality in their abundance in various parts of Morocco in response to a wide array of bio-climatic conditions (Boussaa *et al*., 2005). In a rural district of Burg El-Arab, in Egypt, the seasonal pattern of *P. papatasi* was bimodal, with one peak in July and the second one in October (El-Shazly *et al*., 2012).

Dinesh *et al*. (2001) investigated the seasonal abundance of *P. argentipes* in Patna district of India, where they reported that the mean number of *P. argentipes* showed seasonal variation, being higher during the summer than during the rainy season or winter, and apparently inversely correlated with rainfall. The most intense transmission of *L. donovani* to humans in the area also occurs at these times, as there are many adult female *P. argentipes*, which are potentially infective (Palit *et al*., 1990). Recently, Picado *et al*. (2010) studied the seasonality of *P. argentipes* in India and Nepal and determined that the species has two annual peaks around May and October.

Minter (1964) studied the seasonal dynamics of the rainy-season and perennial species in Kenya and he reported that *P. martini* and *P. celiae* showed seasonal variation in abundance with a peak in April in Kauriro village. Basimike *et al*. (1992b) also investigated the seasonal

variations of different species of sandflies including, *P. martini* in various habitats. *Phlebotomus martini* showed two peaks of abundance, the first in December, corresponding to the fourth month of the dry period, and the second in April, a month after the onset of the rainy season. Similar seasonal fluctuations have been noted for *P. martini* and *P. celiae* in southern Ethiopia (Teshome Gebre-Michael and Lane, 1996).

In parts of Sudan, most sandfly species become abundant only in the dry season, probably because their breeding places are flooded by rain; however, certain species are prevalent throughout the year (Quate, 1964). Investigation on the seasonality of *P. orientalis* in two VL endemic areas of eastern Sudan revealed a clear seasonal variation in abundance, which was closely related with the mean monthly temperature and relative humidity of the area (Elnaiem *et al*., 1997). This species appeared to increase in abundance at the beginning of the rainy season. Quate (1964), in contrast, working in the Paloich area of southern Sudan, found *P. orientalis* to be most abundant at the end of the dry season and to disappear at the onset of the rainy season. These conflicting results may be due to environmental differences between the two areas, where the field trials were carried out. Similarly, Dietlein (1964) detected a peak abundance of *P. orientalis* in April in Tir village, Upper Nile Province of Sudan. The earliest year-round study so far made in north-west Ethiopia (Humera lowlands) by Teferi Gemechu *et al*. (1975) revealed that *P. orientalis* peaked in May and June (towards the end of the dry season).

Population dynamics studies on sandfly vectors are essential steps for better understanding the transmission dynamic of leishmaniasis and planning prevention and control of it,

particularly in the areas where the risk of transmission is significant. It has also been suggested that knowing the seasonal fluctuations of sandfly vectors helps to incriminate *Leishmania* vectors (Ready, 2013).

1.6.4. Nocturnal activity and biting rhythms

Sandflies exhibit periodic activities of sugar and blood feeding, host seeking, mating and oviposition (Forattini, 1973). As in other insects (Saunders, 2002), the rhythmic activities of sandflies are probably controlled by an internal biological clock. However, they are also modulated by daily changes in light intensity and other abiotic factors such as temperature, relative humidity, cloud cover, rain, and wind velocity (Quate 1964, Morrison *et al*., 1995; Guernaoui *et al*., 2006).

With few exceptions, most adult sandflies are crepuscular or nocturnal in their biting activity, but species-specific differences are observed in the peak activities, which can influence the vectorial capacity of different species (Forattini, 1973; Morrison *et al*., 1995). Adults are mainly active in the early morning, evening and at night although they can bite during the day in caves or if disturbed (Killick-Kendrick, 1999).

In Morocco, during autumn, *P. perniciosus* has greater activity during twilight, when the humidity is low (40-50%) and the temperature is high (>20°C), while *P. ariasi* is more active only after midnight when humidity is high (70-80%) and temperature is low (15°C) (Guernaoui *et al*., 2006).

Field studies conducted in different regions of India revealed that the nocturnal periodicity in the landing/feeding activity of *P. argentipes* differ to varying extents. In northeastern India,

for example, landing on human bait peaked between 21:00 and 03:00 hours and was greatest during May (Hati *et al*., 1981; Ghosh *et al*., 1982). In Tamil Nadu, southern India, peak activity was observed between 21:00 and 01:00 hours with the largest collections being made between April and October (Rahman *et al*., 1986). Similarly, the biting activity of *P. argentipes* in Bahapur was shown to peak and subside between 22:00 and 02:00 hours (Dinesh *et al*., 2001). Lane *et al*. (1990) in Sri Lanka also observed that most *P. argentipes* landing/biting takes place in midnight, from 01:00 hours onwards. It is unclear why the time of night when landing/biting by *P. argentipes* peaks should vary with season. Probably, it reflects the seasonal changes in the sleeping patterns of the villagers.

In Ethiopia, the biting cycles of *P. martini* and *P. celiae* were determined from all-night man-landing catches from December 1988 to June 1989 (Teshome Gebre-Michael and Lane, 1996). As a nocturnal species, both vectors showed night-biting peak activity between 20:00 and 22:00 hrs, around termite hills and in dwelling compounds, respectively.

Like the rest of sandflies, *P. orientalis* shows marked difference in nocturnal activity rhythm over periods across different regions of Sudan and Ethiopia. In Sudan, the man-biting activity of *P. orientalis* continues throughout the night with an increase at 18:30 and 20:30 and often bites until full sunlight at 07:00 (Quate, 1964). A field study carried out in eastern Sudan near the Ethiopian border revealed that *P. orientalis* exhibit peak man-biting activity between 20:00 and 22:00 hours (Elnaiem *et al*., 1997). The peak time of biting activity of this vector in northern (Belessa Valley) Ethiopia was indicated to begin shortly after dark though some flies came throughout the night to bite (Ashford, 1974). Field-tests of repellent activities of

plant oils against wild populations of *P. orientalis* in northeast of Ethiopia illustrated the peak time of nocturnal periodicity between 20:00 and 21:00 hours (Yosef Kebede *et al*., 2010). Thus, knowledge about the peak biting times of a vector helps to prevent the likely risky time of the night in contracting the disease.

1.6.5. Feeding behaviors

1.6.5.1. Sugar feeding

Adult male and female sandflies obtain energy by ingesting sugars in their natural environment. Sugar meals can be obtained from a variety of sources, including the sap of plants and honeydew from aphids and coccids to sustain their daily activities (Schlein and Warburg, 1986, Killick-Kendrick and Killick-Kendrick 1987; Junnila *et al*., 2011). Plant sugars are either acquired passively by feeding from floral and extra-floral nectars, ripe fruits, fallen fruits etc. or after active piercing of leaves and stems (Schlein and Yuval, 1987). A sandfly's search for sugar sources is apparently guided by order of preferences to attractants (Müller and Schlein, 2004). Previous study by Chaniotis (1974) revealed that sandflies feed preferentially on sugars commonly found in nectars and ripe fruits. A similar phenomenon was seen with a laboratory-bred *P. papatasi* of both sexes, where they pierced leaves, or stems of some plants for sugars caged with variety of plants (Schlein and Warburg, 1986). These authors observed that the behavior was selective, with sap taken from only eight of eighteen species of plants offered. Nevertheless, Ashford (1974) in Ethiopia noticed *P. orientalis* males and females apparently feeding fresh leaves of wide variety of plants without detectable preferences. He observed the flies to probe plants with noxious sap including *Ficus* and *Euphorbia* spp. More recently, Müller *et al*. (2011b) compared the

relative attractiveness of 56 flowering plants to *P. papatasi* in Israeli dessert and they found that the plants showed various degree of attractiveness.

Another important source of sugar for sandflies is honeydew from aphids and coccids. Honeydew is the phloem sap of plants having passed through sucking aphids, in which plant sugars undergo enzymatic transformations resulting in the formation of oligosaccharides (Dixon, 1985; MacVicker *et al*., 1990). These oligosaccharides formed by transferases were detected in the guts of *P. perfiliewi* and *P. perniciosus* (MacVicker *et al*., 1990). Fructose or fructose-containing carbohydrates were also identified from wild caught *P. ariasi* by Van Handel test (Young *et al*., 1980). Similarly, *P. ariasi* was shown to take readily the honeydew of *Lachnus roboris*, an aphid of oak trees, and the presence of honeydew sugars in wild-caught *P. ariasi* from the Cevennes, southern France (Moore *et al*., 1987).

The sugar feeding behavior of sandflies influences their widespread biological activities, including longevity and fecundity, dispersal, host seeking behavior and ultimately blood feeding and disease transmission (Schlein and Yuval, 1987, Müller and Schlein, 2004). Earlier studies have suggested that sugar meals may also play an important role in the development of *Leishmania* parasites in sandfly guts and subsequent transmission of leishmaniasis to susceptible hosts (Schlein and Warburg, 1986; Schlein and Jacobson, 1994). Sugars are important nutrients for the development of leishmanial parasites in the foregut of the fly. Young *et al*. (1980) asserted that sugars passing from the mouthparts of the fly to the diverticulum are the only obvious nutrients available to the parasites. Experimental infection of *P. argentipes* by *L. donovani* demonstrated that the sugar meals affect the development

of *Leishmania* in sandfly vectors (Shortt, 1945 cited in Schlein and Warburg, 1986). In these experiments, sandflies fed only on blood failed to transmit the disease, whereas those that were given raisins infected hamsters and humans. Schlein and Warburg (1986) further claimed that sugar meals might indirectly affect both the sandflies and *Leishmania* by introducing contaminants into the gut. Sandfly guts are normally sterile (Lewis and Domoney, 1966), and bacteria or fungi in the meals may cause infections that eliminate *Leishmania* and lead to sandfly death.

Considering the importance of sugar feeding to sandflies, it is necessary to identify specific sources of sugars and the relative attractiveness of plants found in the natural habitats of sandflies. This in turn provides important insight to develop more selective, environmentally friendly and less expensive sandfly control methods such as attractive toxic sugar baits (ATSB), which provided highly effective local mosquito control (Müller *et al*., 2010).

1.6.5.2. Host preference patterns and techniques for the determination of bloodmeal sources

Sandflies are known to take bloodmeal from different host species, which makes them vectors of various groups of pathogens. Only females need to feed on blood of vertebrate hosts including domestic animals, urban and wild rodents, reptiles, amphibians, and humans. The preference of sandfly vectors for different vertebrate hosts varies according to species and to the availability of hosts, but may also vary in the same species in different regions.

Feeding takes place on exposed parts of the body by thrusting the tiny mouthparts into the skin and (with the minutely toothed mandibles used in a scissors-like manner) creating a

small pool from which the blood is sucked. An extremely potent vasodilating peptide is injected into the wound to induce formation of an extra-vascular pool of blood (Ribeiro *et al*., 1989). Blood taken in this manner is directed into the mid-gut. Bloodmeals are necessary for ovarian development in many species, but few species are autogeneous (able to lay eggs without a bloodmeal) and some of these include *P. papatasi*, *P. bergeroti,* and *Lu. gomezi* (Killick-Kendrick, 1978; Hanafi and Fryauff, 1991; Hanafi *et al*., 1999; Dr. Teshome Gebre-Michael, pers. comm.).

Species vary in taking of bloodmeals during a gonotrophic cycle. Some will take more than one bloodmeal on different days, whereas others feed only one time for each batch of eggs (Ready, 2013). Suitable combinations of air movement, ambient temperature, light intensity, relative humidity, and other exogenous factors stimulate hungry sandflies to search for bloodmeals (Young and Lawyer, 1987). Frequent bloodmeals increase the contact between vertebrates and vectors, creating possible situation for the transmission of leishmaniasis.

Female sandflies have apparently no preference for any particular part of the host body and bite indiscriminately, but usually aggregate on specific parts of individual hosts, often where it is safer and hair is short (Lane, 1993; Ready, 2013). For instance, sandflies were observed biting adult Sudanese on all exposed parts from the feet to the face, except the hairy parts of the body (Quate, 1964). Most anthropophilic species feed from evening to dawn, when temperature drops and relative humidity rises. However, some forest dwelling species like *Lu. wellcomei*, *Lu. carrerai* and *Lu. pesoana* attack during daytime if their habitat is disturbed (Lawyer and Perkins, 2004).

Blood-feeding behavior studies have been critically important for estimating the efficiency of pathogen transmission and assessing the relative human disease risk. The proportion of bloodmeals from humans is a key component to estimate the vectorial capacity, or the efficiency of transmission of certain species, expressed as the number of new infections disseminated per day by each vector fed on an infective host (Kent, 2009).

Studies on host attraction and feeding habits of sandflies indicate that New as well as Old World sandfly species display varying degrees of host preference, but in general most are opportunistic feeders (Morrison *et al*., 1993; Montoya-Lerma and Lane, 1996; Palit *et al*., 2005; Hassan *et al*., 2009). For instance, *P. papatasi* is considered a highly anthropophilic species by workers in India (Dhanda and Gill, 1982), but it has been observed biting several other animal species, in the same country and Egypt (El Sawaf *et al*., 1989). In Sri Lanka, *P. argentipes* is predominantly zoophilic in lowland areas, and yet it prefers humans in the highlands (Lane *et al*., 1990). Bloodmeal analysis of *P. argentipes* population from various areas of India revealed their zoophilic and anthropophilic nature, depending on the biotopes where fed specimens were collected (Ghosh *et al*., 1990; Palit *et al*., 2005). Similarly, *P. perniciosus* and *P. perfiliewi* in Italy appear to be opportunistic feeders rather than exhibiting preferences for any specific animal (Bongiorno *et al*., 2003).

Phlebotomus martini displays wide differences in host preferences, depending on host availability (Mutinga *et al*., 1986). This species showed highest preferences to chicken, goat, dog and rats in that order. Recently, Hassan *et al*. (2009) compared host attractiveness of *P. orientalis* and other sandflies to different groups of animals in eastern Sudan and reported

the greater preference of this species to domestic dog over mongoose, genet, and Nile rat. Teshome Gebre-Michael *et al*. (2010) also reported the preference of *P. orientalis* to feed on bovine blood than human in Humera-Metema plains. Similar feeding habits are also known for other *Leishmania* vectors, both in the Old and New World (Ngumbi *et al*., 1992; Hassen Mamo, 1999; Afonso *et al*., 2005; Palit *et al*., 2005; Garlapati *et al*., 2012).

Bloodmeal source identification in sandfly vectors to date has largely depended on serological techniques such as the precipitin test (Dhiman *et al*., 1984; Morrison *et al*., 1993; Ogusuku *et al*., 1994; Afonso *et al*., 2005), counter current immuno-electrophoresis (Morsy *et al*., 1993; Hassen Mamo, 1999), agarose gel diffusion (Ghosh *et al*., 1990; Srinivasan and Panicker, 1992; Palit *et al*., 2005), and the enzyme-linked immunosorbent assay (ELISA) (Ngumbi *et al*., 1992, Bongiorno *et al*., 2003, Svobodova *et al*., 2003; Teshome Gebre-Michael *et al*., 2010). To some extent, a more laborious histological technique has been used (Guzman *et al*., 1994). Despite these techniques have and continue to provide valuable, insightful information on the identity of the vertebrate hosts of blood feeding sandflies, they have some inherent limitations such as the need to produce species-specific antibodies against each potential animal host, the requirement for relatively fresh blood specimens, time-consuming and lack of sensitivity (Blackwell *et al*., 1994; Kent, 2009).

Consequently, PCR-based identification of vertebrate host bloodmeals is a potentially convenient alternative, which has already been performed on different species of sandfly vectors (Abassi *et al*., 2008; Maleki-Ravasan *et al*., 2009; Garlapati *et al*., 2012; Tiwananthagorn *et al*., 2012). Different molecular methods used for bloodmeal analysis in

sandflies involve the amplification of mitochondrial DNA (cytochrome *b* gene) by PCR followed by species identification using restriction digestion (PCR-RFLP) (Maleki-Ravasan *et al*., 2009; Oshaghi *et al*., 2009), sequencing (Berdjane-Brouk *et al*., 2012; Tiwananthagorn *et al*., 2012), and reverse line blotting (RLB) (Abassi *et al*., 2008; Garlapati *et al*., 2012). More recently, Valinsky *et al*. (2014) applied the amplification of mitochondrial rRNA genes by PCR and identification of host species by sequencing. Cyt *b* and other diagnostic markers have proven utility for identifying sandfly bloodmeals due to high copy number as a mitochondrial gene and sufficient genetic variation at the primary sequence level among vertebrate taxa for reliable identification (Abassi *et al*., 2008; Valinsky *et al*., 2014).

Overall, appropriate characterization of the blood feeding habits of sandflies is vital for identifying the natural transmission cycles of leishmaniasis and for designing possible control strategies in vector control programs.

1.7. Rationale of the study

Visceral leishmaniasis is a systemic parasitic illness, transmitted primarily by the sandfly vectors from animal or human reservoirs. This disease is endemic in Ethiopia, with a patchy distribution in the southern and northwestern lowlands (WHO, 1996; Asrat Hailu *et al*., 2006). Over the past few years, VL has become a growing public health threat in various parts, with an upsurge year after year in new cases as well as new foci. Importantly, a more recent VL cases have been reported in Tahtay Adiyabo district of Tigray Regional State, an area where it had not been known for VL before (Tigray Health Bureau unpublished data; Abbasi *et al*., 2013). As part of a cohort study aimed at understanding transmission dynamics

of VL in northern Ethiopia, around 4,757 individuals living in 18 villages of Tahtay Adiyabo were screened for infection or exposure to *L. donovani* by physical and laboratory tests, including Leishmanin Skin Test (LST), Direct Agglutination Test (DAT) and kDNA / RT-PCR (Abbasi *et al*., 2013). Out of these, 680 (14.3%) dried blood samples tested by Real time-PCR were found positive for *Leishmania* k-DNA (Asrat Hailu *et al*., in preparation). Additionally, a total of 209 VL and 3 PKDL cases were treated in the district from 2006 to 2011 (Desjeux *et al*., 2013).

In spite of such magnitude of VL in the area, no thorough entomological investigations to demonstrate the respective role and importance of the infected and non-infected phlebotomine sandflies, especially for *Phlebotomus* species have been conducted in the area. The current study was intended to look at the magnitude of potential infectivity of phlebotomine sandflies in the area with the following objectives:

1.8. Objectives of the study

1.8.1. General objective

The general objective of this study was to investigate the species composition, ecology, behavior and population dynamics of sandfly vector (s) in relation to transmission and management of VL in the Tahtay Adiyabo district, northern Ethiopia.

1.8.2. Specific objectives

1. To determine the sandfly fauna, relative abundance and seasonal variation of *Phlebotomus* species
2. To characterize the physiological age structure and identify the potential vectors of VL in the area
3. To determine the host preference patterns of *P. orientalis*
4. To study the nocturnal activity patterns of *P. orientalis*
5. To investigate the attractiveness of some common plant species to *P. orientalis* under field condition
6. To investigate the effect of moon light and lunar periodicity on the performance of light traps in collecting *P. orientalis*

Chapter Two

General Materials and Methods

2.1. Study area

2.1.1. Descriptions of the study area and sampling villages

Tahtay Adiyabo is a third-order administrative division and is located in Tigray Regional State, northern Ethiopia (Fig. 2.1). Tahtay Adiyabo is bordered on the south by the Asigede Tsimbela and the Tekezé River, which separates Tahtay Adiyabo from Wolqayt and Kafta Humera on the southwest, on the north by Eritrea, and on the east by Laelay Adiyabo. The topography is predominantly flat plain in the east and mountainous in the southwest. Sheraro, the administrative center of the district, is 1,028 meters above sea level, with latitude of 14°23'41" N, and longitude of 37°46'15" E. The town is also located about 1,117 km north of Addis Ababa and 402 km northwest of Mekelle, the capital of the Tigray Regional State.

The climate is generally characterized as tropical semi-arid with an extended dry period of nine to ten months. The area has uni-modal rainfall pattern (June to late-September) with a mean annual precipitation of about 600 mm (Ethiopian National Meteorological Agency). During the rainy season, there is frequent cloud cover and thunder. Vertisols also become excessively muddy and even flooded during the rainy seasons. Around the last week of September the rain ceases and there begins a period marked by clear skies and cool winds which continue with gradual warming to February. March to May is the hottest part of the year with an average temperature of 39°C at noon and January is the coldest one with an average temperature of 14.2 °C at night. Under the relentless sun, the vertisols dry forming

large cracks as deep as 1m and with finer fissures below (Moncaz *et al*., 2014). The visible moisture line recedes to the bottom of the cracks.

Three villages namely Ademeyti (14°21’31.53’’ N; 37°41’37.89’’ E; 1,060 m a.s.l), Lemlem (14°22’15.27’’ N; 37°44’35.96’’ E; 1,068 m a.s.l), and Mentebteb (14°19’37.78’’ N; 37°44’15.56’’ E; 1,079m a.s.l) were selected for longitudinal entomological studies (Fig. 2.1). The selections of the villages in the district were based on the degree of VL burden, species richness, and abundance of sandflies and accessible to transport. The villages of Ademeyti and Lemlem are situated approximately 17 and 6 kms northwest and west of Sheraro town, respectively. The third village, Mentebteb is located about 13 km southwest of Sheraro town. The distance between the three major study villages is about 8-12 km.

2.1.2. Socio-economic characteristics

Based on the 2007 national census, the district has a total population of 90,144, of whom 45,834 are men and 44,310 women; 6,377 (7.07%) are urban inhabitants. With an area of 3,841.51 square kilometers, Tahtay Adiyabo has a population density of 23.47 people per square kilometer, which is less than the Zone average of 40.21. The three largest ethnic groups reported in the 2007 census were Tigrean (71.36%), Kunama (1.41%), Eritrean residents (26.23%) and all other ethnic groups made up 1% of the population (Central Statistical Agency of Ethiopia, 2007). In terms of religion, 95.59% of the populations in the area practice Ethiopian Orthodox Christianity while 3.15% do Islam.

Agriculture and allied activities are the most important source of subsistence for the majority of the population, which is largely rain fed. The main crops grown for consumption

and source of income are sorghum, finger millet, maize, and chickpea. Surplus sorghum and millet are destined for sell to nearby towns. Sesame is the main cash crop and is mainly produced for export. Its production is also an important source of agricultural labor, especially for weeding and harvesting activities.

Livestock production is a major component of the livelihood system in the area. The main livestock types are cattle, sheep, goats, and chicken. Livestock are kept partly as capital, which can be turned into cash when required, but, most importantly, cattle are kept as breeding stocks to provide draft power for agricultural operations. Donkeys also serve as the principal pack animals complemented by camels. In some instances, however, these pack animals are also used for ploughing. Crop residues are the major livestock feed source in the region followed by grazing lands, browse and crop aftermath, respectively.

2.1.3. Housing types

The villages are situated on sandy clay loam soil surrounded by large fields many of which are in vertisols and red clay soil. The human dwellings comprise stonewalls and wooden roofs covered with flat stones and sandy soil, mud-plastered walls and thatched roofs and few houses are made of stone wall and thatched roofs or corrugated iron roofs. Houses are built close to animal enclosures and are situated approximately 50-200 meters from farm fields many of which are in vertisols, which crack during the dry season. The majority of the rural populations live in single roomed houses made of mud-plastered walls with earthen floors and clay soil roofs. The inside walls and floor of these houses are leveled with alluvial soil and plastered with mud frequently.

2.1.4. Vegetation composition

The vegetations of Tahtay Adiyabo lowlands could be described as the remnants of *Acacia-Balanites-Zyzpus-Combretum* savannah woodlands (Emiru Birhane *et al*., 2011). The clearing of forests to expand agricultural land and tree cutting for fuel wood, timber, and agricultural implements were the causes that have reduced the vegetation cover of the region to scattered and mixed trees mainly of *Balanites spp., Ziziphus spp. Acacia* spp., and some scrub vegetation. An increase in livestock population and the free grazing system that prevails in the region have also had a considerable influence on the degradation of vegetation resources. However, the common trees and shrubs in Tahtay Adiyabo areas are *Acacia seyal, A. abyssinica, A. sieberiana, A*. *polyacantha*, *Acacia spp.*, *Balanites aegyiptiaca, Ziziphus spina-christi, Tamarindus indica, Terminalia brownii*, *Combretum fragrans, Combretum sp., Dichrostachys cinerea, Diospyros mespiliformis*, *Azadrichta indica*, *Anogeissus leiocarpus*, *Dalbergia melanoxylon*, *Boscia angustifolia*, *Delonix regia*, and *Ficus* spp. (Emiru Birhane *et al*., 2011).

Figure 2.1. Map of Tahtay Adiyabo district (modified based on GIS of Ethiopia); red, green, and yellow colors show study villages.

2.2. Sandfly collection and processing

In each of those study villages, three sandfly sampling habitats were considered: indoor (inside tukuls), peri-domestic habitats (homesteads shared with animal shelters), and agricultural fields on the periphery of human residence with scattered and mixed trees mainly *Acacia-Balanites-Zyzpus-Combretum* trees and some scrub vegetation. An average distance between peri-domestic and agricultural field was around 60-70 meters. Whereas the mean distance between indoor and peri-domestic was approximately 10-15 meters. From those mentioned biotopes sandflies were sampled to demonstrate various bionomic and behavior of *P. orientalis* sandfly populations.

CDC light traps: CDC miniature light traps (John W. Hock, Gainesville, FL) were used for sampling sandflies from peri-domestic and agricultural fields in different periods of the study (Alexander, 2000). CDC light traps were deployed in representative sites (i.e., near cracked walls, a stone pile, compound of human dwellings, and animal enclosures) of peri-domestic habitats. Likewise, light traps in each village were operated to sample sandflies in agricultural fields, where they were suspended under trees/ bushes, in open fields, dry riverbeds, and the edge of farmlands. CDC light traps were suspended with the fan 40-50 cm above the ground level. The traps were set 1 h before sunset and collected at dawn the next morning. Then, traps containing sandflies were transported to the field laboratory, where sandflies were sorted by sex and genus and were preserved in 70% ethanol for later identification to species level. *Phlebotomus* species were sorted out from *Sergentomyia* spp. by the presence of many erect hairs on tergites 2-6 and cibarium without row of teeth. In order to avoid the significant impacts of the moonlight on the collection of sandflies using

CDC light traps, sampling of sandflies for population dynamics and nocturnal activity rhythm studies have always been planned according to the phases of the moon when moon light was completely absent during the whole or for most of the night.

Sticky trap collections: A4-sized (21×29 cm) white of polypropylene sheets coated with sesame oil were used for capturing sandflies from all sampling habitats (Alexander, 2000). The fifty sticky traps were divided into 10 sets each having five sheets tied together on nylon string about 50 cm apart. Each morning, sandflies from sticky traps were removed using forceps and stored in 96% ethyl alcohol in labeled vials for later processing and identification.

Pyrethrum spray catches: Indoor resting sandflies were sampled in the morning (6:00 to 9:00) from ten randomly selected houses by the application of a pyrethrum spray catch method (Kent *et al*., 2007). Prior to spraying in each house, all food items, water, and small animals were removed; the openings and eaves of windows and doors were filled with pieces of cloth, and the floor was entirely covered with white plastic sheets. The door was closed and the pyrethrum aerosol (Roach killer, M/S Kafr EI Zayat, Egypt) was sprayed for about three to five minutes. After about ten minutes of waiting outside post spraying, the knocked down sandflies were collected from the white sheets using fine forceps or fine camel hairbrushes and placed in vials containing 70% ethanol for latter processing and identification.

2.3. Mounting and identification of sandflies

Sandflies collected during the study were mounted on microscope slides in Hoyer's medium with their heads separate from thoraces and abdomens. Sandflies were identified to the species level based on the morphology of the external genitalia of males and the pharynx, antennal features and spermathecae of females, using different keys, (Quate, 1964; Abonnenc and Minter, 1965) and other publications (Lane and Fritz, 1986; Teshome Gebre-Michael and Girmay Medhin, 1997).

2.4. Meteorological records

Meteorological data on maximum and minimum temperatures, average relative humidity, and rainfall of Tahtay Adiyabo district during May 2011 to April 2012 were obtained from the National Meteorology Agency of Ethiopia to determine the effect of local weather elements on the seasonal dynamics of sandflies (Annex 1).

Chapter Three

Species composition of phlebotomine sandflies and bionomics of *Phlebotomus orientalis* (Diptera: Psychodidae)

3.1. Introduction

Visceral leishmaniasis, caused by *L. donovani* complex, is an important public health problem in several regions of Ethiopia, with an estimated annual incidence of 3,700 to 7,400 cases (Alvar *et al*., 2012). This systemic disease has been reported from at least 40 areas, with the most important endemic foci being the arid southwest and the northwest lowlands of the country bordering Kenya and Sudan, respectively (Lyons *et al*., 2003; Hailu *et al*., 2006). In recent years, reports have described increasing numbers of VL cases as well as new foci of disease in the semi-arid lowlands of Tigray Regional State, northern Ethiopia (Abbasi *et al*., 2013; Tigray state Health Bureau, unpublished data). For instance, between 2006 and 2011, 209 VL cases were treated in Tahtay Adiyabo district (Desjeux *et al*., 2013).

Of the estimated 900 phlebotomine sandfly species known to exist, only 98 species of *Phlebotomus* and *Lutzomyia* genera are currently proven or suspected vectors of human leishmaniasis in the Old and the New World, respectively (Maroli *et al*., 2013; Ready, 2013). Among *Phlebotomus*, 42 species are proven or suspected vectors of *Leishmania* spp. and of these 26 species are implicated in VL transmission caused by *L. donovani* and *L. infantum* in the Old World (Alexander and Maroli, 2003; Maroli *et al*., 2013).

So far, 22 species of *Phlebotomus* have been reported in Ethiopia. Of these, the incriminated vectors of VL, from which parasites have been detected and/or isolated and identified, include *P. martini*, *P*. *celiae,* and *P. orientalis* for *L. donovani* from the south and southwest foci (Asrat Hailu *et al*., 1995; Teshome Gebre-Michael and Lane, 1996). However, detailed studies on the bionomics of sandflies in north and northwest Ethiopia in general and Tahtay Adiyabo district in particular, are very limited since the occurrence of the disease. Knowledge on the distribution, population dynamics, and behavior of sandfly vectors contributes to understanding of where, when, and how humans become infected with *L. donovani*. Moreover, determining the abundance and seasonal dynamics of vector species are crucial for recommending sound vector management methods towards the control of VL transmission in the area. Because of the limited information available about the sandfly vector (s) involved, an extensive entomological study aimed at identifying the sandfly fauna and bionomics of *P. orientalis*, the vector of the disease, was initiated in the rural community of Tahtay Adiyabo district.

3.2. Materials and Methods

Longitudinal entomological studies were undertaken in three different villages (Ademeyti, Lemlem, and Mentebteb) of the district between May 2011 and April 2012. For this activity, sandflies were trapped from three permanent sampling habitats (i.e., indoor, peri-domestic and agricultural fields).

3.2.1. Sandfly trapping

Sandflies were collected using CDC miniature light traps from peri-domestic and agricultural fields for 12 nights per month. However, no light trap was used indoors as light traps have a tendency to attract flies from outside, as a result, the collection may not represent the true endophagic/endophilic species. Five CDC light traps were deployed in representative sites (i.e., near cracked walls, a stone pile, compound of human dwellings, and animal enclosures) of peri-domestic habitats (Fig. 3.1). Likewise, another five light traps in each village were operated to sample sandflies in agricultural fields, where they were suspended under trees/ bushes, in open fields, dry riverbeds, and the edge of farmlands (Fig 3.2). The traps were set 1 h before sunset and collected at dawn the next morning. The next morning, traps containing sandflies were processed, where sandflies were sorted by sex and genus (*Phlebotomus* or *Sergentomyia* spp.). Female *Phlebotomus* were further processed for other purposes (bloodmeal analysis, dissection for parous rates and promastigote detection, and preservation of un-dissected females for PCR detection of *Leishmania*), results of which are reported elsewhere (Chapters 4 and 5). The remaining specimens were preserved in 70% ethanol for later identification to species level (see section 2.3).

Secondly, sticky traps were used for capturing sandflies from all sampling habitats for 18 trapping nights. Indoor, the 10 sets of sticky traps were placed inside ten different houses in each village to intercept and capture any endophilic sandflies (Fig. 3.3). Similarly, these tens sets of sticky traps were also suspended randomly on cracked walls, stone piles, and animal enclosures in the peri-domestic environment. Another 50 sticky traps were also deployed in agricultural fields. In this habitat, five sets of sticky traps (5 A4 sized sheets/set) were hung

vertically in a row 30 cm above the ground supported by metal or wooden pegs (Fig. 3.4-A). Simultaneously, another five sets of sticky traps were placed horizontally on the cracks of agriculture fields (Fig. 3.4-B). Each morning, sandflies from sticky traps were processed as indicated 2.2.

Indoor resting sandflies were sampled in the morning (6:00 to 9:00) from ten randomly selected houses by the application of a pyrethrum spray catch method (Kent *et al*., 2007). The knocked down sandflies were preserved in vials containing 70% ethanol for latter processing and identification.

Figure 3.1. CDC light traps deployed for sampling sandflies from peri-domestic habitats

Figure 3.2. CDC light traps deployed in agricultural fields

Figure 3.3. Sticky traps deployed inside huts

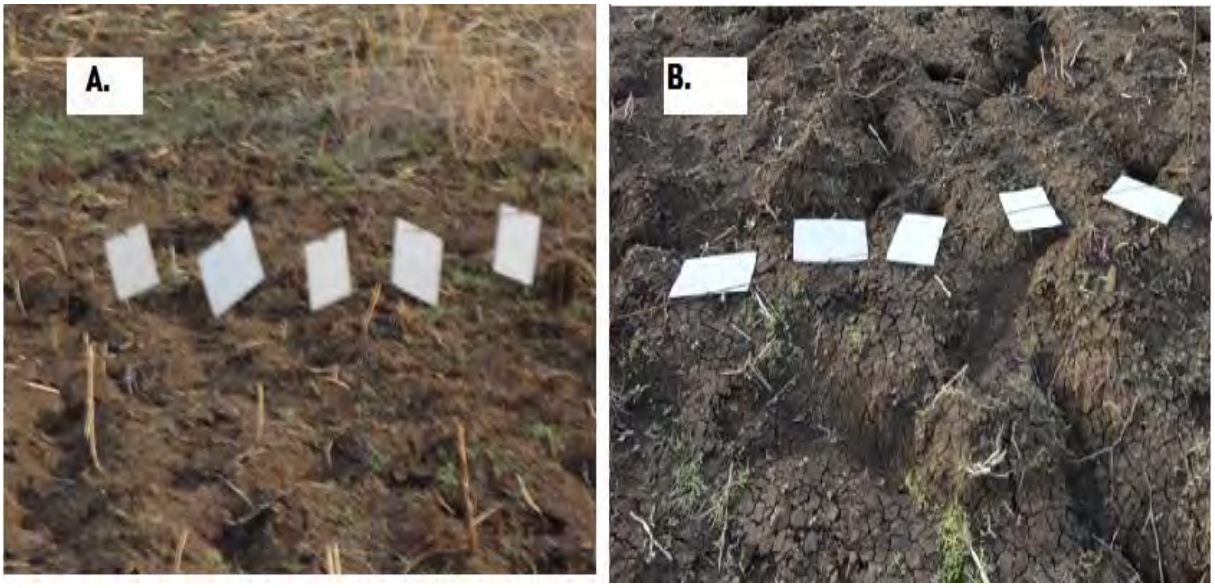

Figure 3.4. Sticky traps deployed for sampling sandflies from agricultural field. **A**: Vertically placed sticky traps. **B**: Horizontally placed sticky traps.

3.2.2. Age grading of wild-caught male sandflies

Age grading of wild-caught males of *P. orientalis* was conducted based on the orientation of their genitalia. The external genitalia of male sandflies rotate around the longitudinal body axis through 180° during the initial 24 hours (within 1 day) of adult life to assume their mature (= rotated) position (Davis, 1967; Moncaz *et al*., 2012, 2014). In order to make use of this easily discernable physical characteristic to identify young males, *P. orientalis* males were mounted on slides. Males with un-rotated or partially rotated external genitalia were considered active for the first night of their adult life.

3.3. Data analysis

Prior to data analysis, checked for normality by Shapiro-Wilk test and sandfly numbers were log-transformed [log (n+1)] to fit normal distribution. When trapping data did not conform to the normal distribution, the non-parametric equivalent tests of Kruskal-Wallis and Mann-

Whitney-*U* were applied. Kruskal-Wallis test (K-W) was followed to compare the mean numbers of *P. orientalis* collected in the three sampling villages using CDC-LTs and STs. For non-parametric comparisons, multiple-Mann-Whitney *U*-test was used and, *P*-values were adjusted with the Bonferroni correction to adjust for the inflation of type I errors when several Mann-Whitney tests are performed (Dytham, 2011). For habitat preference comparison, Mann-Whitney test (*U)* was used for CDC light trap collection and Kruskal-Wallis for sticky trap captures. Seasonal abundance of *P. orientalis* was analyzed using Kruskal-Wallis-test (K-W). Pearson correlation analysis was also used to compare the effects of mean monthly temperature, relative humidity, and rainfall on the mean number of *P. orientalis* caught per trap-night. Mann-Whitney test (*U*) was also used for comparing the number and proportions of *P. orientalis* indoor and outdoor abundance, age-grading, sex ratio in trapping methods and comparative efficacy of different arrangements of sticky traps. Statistical analysis were considered significant when $P<0.05$ unless stated. Statistical analyses were carried out using IBM SPSS statistics, version 20 for Windows (SPSS Inc., Chicago, IL, USA), and Microsoft® Office Excel 2007.

3.4. Results

3.4.1. Species composition and relative abundance of sandflies

In total, 100,772 sandfly specimens, representing twenty-five species (nine *Phlebotomus* and sixteen *Sergentomyia)* were recorded (Table 3.1). The genus *Phlebotomus* represented six subgenera while four subgenera were identified in *Sergentomyia*. Sandfly species identified in the present study consist of *Phlebotomus (Larroussius) orientalis, P. (Anaphlebotomus*) *rodhaini, P.* (*Synphlebotomus) martini, P. (Phlebotomus) bergeroti*, *P. (P.) papatasi, P. (P.) duboscqi, P.* (*Paraphlebotomus*) *alexandri, P.* (*Parvidens*) *lesleyae*, *P.* (*Parv.*) *heischi*, *Sergentomyia* (*Parrotomyia*) *africana, S.* (*Sergentomyia*) *schwetzi, S.* (*S.*) *antennata, S. (S.) bedfordi group, S.* (*S.*) *dubia,* S. *(Sintonius) clydei, S. (Sin.) adleri, S.* (*Sin.*) *calcarata, S.* (*Sin.*) *subtilis, S.* (*Sin.*) *adami, S.* (*Sin.*) *satti, S.* (*Sin.*) *christophersi, S.* (*Sin.*) *capensis, S. (Sin.) thomsoni, S.* (*Sin.*) *affinis*, and *S.* (*Grassomyia*).

The relative abundance of these species is shown in Table 3.1. The most abundant species of *Phlebotomus* was *P. orientalis* (23.5%) followed by *P. lesleyae* (0.15%), while the other *Phlebotomus* species constituted only 0.31% of the entire sandfly collection. Among the genus *Sergentomyia*, *S. africana* (59.1%) was the most prevalent species followed by *S. schwetzi* (5%).

Table 3.1. Relative abundance, sex ratio, and species composition of sandflies collected from three villages of Tahtay Adiyabo district, May 2011 to April 2012

Species	CDC light traps		Sticky traps		Indoor space sprays		All methods combined		
	M/F	Sex ratio	M/F	Sex ratio	M/F	Sex ratio	Total number	%	Sex ratio
Phlebotomus orientalis	5,360/2,606	2.06	14,547/1,150	12.65	26/22	1.18	23,711	23.53	5.28
P. rodhaini	17/13	1.31	29/33	0.88	0/0	NA	92	0.09	1.00
P. bergeroti	26/20	1.30	15/13	1.15	2/3	0.67	79	0.08	1.19
P. martini	19/11	1.73	11/10	1.10	3/3	1.0	57	0.06	1.40
P. papatasi	14/16	0.88	6/4	1.50	0/3	NA	43	0.04	0.87
P. duboscqi	5/9	0.56	1/6	0.17	1/5	0.2	27	0.03	0.35
P. alexandri	0/2	NA	0/0	NA	0/0	NA	2	0.002	NA
P. lesleyae	8/85	0.09	21/33	0.64	0/0	NA	147	0.15	0.25
P. heischi	2/2	1.00	0/2	NA	0/0	NA	6	0.006	0.50
Sergentomyia africana	19,373/1,7812	1.09	10,301/10,423	0.99	406/1204	0.34	59,519	59.06	1.02
S. schwetzi	774/1,255	0.62	1,191/1,358	0.88	97/365	0.27	5,040	5.00	0.69
S. clydei	1,059/1,631	0.65	446/721	0.62	60/111	0.54	4,028	3.99	0.64
S. antennata	*346/276	1.25	*1398/1097	1.27	*74/344	0.22	3,535	3.51	1.06
S. bedfordi	270/388	0.7	421/402	1.05	13/32	0.41	1,526	1.51	0.86
S. dubia	*-/198	NA	*-/894	NA	*-/75	NA	1,167	1.16	NA
S. squamiplueris	146/278	0.53	129/64	2.02	6/6	1.0	629	0.62	0.81
S. adleri	132/168	0.79	105/122	0.86	36/48	0.75	611	0.61	0.81
S. calcarata	27/102	0.26	38/211	0.18	6/91	0.07	475	0.47	0.18
S. subtilis	4/11	0.36	11/18	0.61	0/0	NA	44	0.04	0.52
S. adami	4/5	0.8	3/2	1.50	6/7	0.86	27	0.03	0.93
S. satti	2/0	NA	0/0	NA	0/0	NA	2	0.002	NA
S. christophersi	0/0	NA	1/1	1.00	0/0	NA	2	0.002	1.00
S. capensis	0/0	NA	1/1	1.00	0/0	NA	2	0.002	1.00
S. thomsoni	1/0	NA	0/0	NA	0/0	NA	1	0.001	NA
S. affinis	1/0	NA	0/0	NA	0/0	NA	1	0.001	NA
Total	27590/24888	1.11	28675/16565	1.73	736/2319	0.32	100,772	100	1.30

*NA= Not applicable; *Males of S. antennata and S. dubia are morphologically difficult to distinguish with certainty (Abonnenc and Minter, 1965).*

3.4.2. Comparison of the three villages for *P. orientalis* abundance

Mean numbers of *P. orientalis* collected from the three sampling villages (Ademeyti, Lemlem, and Mentebteb) using CDC light traps and sticky traps during the entire collection period are illustrated in Table 3.2. Significant differences were recorded in the mean number of *P. orientalis* captured per CDC trap/night in the three sampling villages ($\chi^2_{K\text{-}W}$= 8.00, df = 2, *P*<0.05). Likewise, the sampling villages differed significantly in their sandfly density on sticky trap collections ($\chi^2_{K\text{-}W}$= 9.05, df =2, *P*<0.05). In both trapping methods, Ademeyti and Lemlem were the most productive sampling villages for *P. orientalis* compared to Mentebteb (Table 3.2).

Table 3.2. Mean numbers of *P. orientalis* collected by CDC light traps and sticky traps from three different sampling villages, May 2011 to April 2012

	Mean No. ± SE of *P. orientalis*/trap/night	
Sampling villages	**CDC light traps**	**Sticky traps**
Ademeyti	6.57 ± 1.57[a]	1.18 ± 0.38[a]
Lemlem	4.53 ±1.02[a]	1.05 ± 0.35[a]
Mentebteb	2.85 ±0.58[b]	0.54 ± 0.19[b]

Mean values followed by the different letters in the same column are significantly different (Multiple-Mann Whitney *U*-test, *P*< 0.016).

3.4.3. Population dynamics of *P. orientalis*

The annual mean maximum and minimum temperatures were 38.4 ^{0}C in March and 15.8 ^{0}C in January, while relative humidity was 63.5% in August and between 38-39% during the months of January to April 2012, respectively. The main rainy period was between July and

September. During the period of sandfly sampling, the maximum precipitation recorded was 287.5 mm in August 2011.

The mean monthly density of *P. orientalis* in the villages of Ademeyti and Lemlem using CDC light traps and sticky traps had significant difference (Kruskal-Wallis test, $P<0.05$). In Ademeyti and Lemlem, the peak density of *P. orientalis* in CDC light traps appeared to be in March while the lowest was during the rainy period from July to September (Fig. 3.5-A). The highest and the lowest mean monthly density of *P. orientalis* on sticky trap collection in the two villages (Ademeyti and Lemlem) were found in April and during July–November, respectively (Fig. 3.5-B). In Mentebteb, however, the mean monthly density of *P. orientalis* both in CDC light traps and sticky traps was low and did not have any significant differences among sampling periods (Kruskal-Wallis test, $P>0.05$) (Fig. 3.5-A and B).

Data from each village were pooled to assess the overall seasonal changes in density of the *P. orientalis* in the district collected by means of CDC light traps and sticky traps. There were significant mean monthly fluctuations in the numbers of *P. orientalis* caught using light traps and sticky traps from different biotopes over the twelve months of trapping period (Kruskal-Wallis test, $P<0.05$). *Phlebotomus orientalis* showed distinct seasonality, with the greatest overall density between January and June, reaching its peak density in March (6.18±2.43/trap-night) when the average temperature was also high (30.4°C). From July to December, including the rainy season (July-September) and shortly after that (October-December), there was a sharp decrease in abundance of *P. orientalis* (Fig. 3.6-A) with an increase in relative humidity and decrease in average temperature (Fig. 3.7). The lowest

mean density of *P. orientalis*/trap/night was found in the period from August to October in the range of 0.07±0.04 to 0.09±0.03.

The highest mean monthly density of *P. orientalis* determined from sticky trap collections was in April (1.10±0.50/trap/night) while the lowest was during August to October, which ranged from 0.008 to 0.03/trap/night (Fig. 3.6-B).

In light trap catches, monthly density of *P. orientalis* had a significant positive correlation with temperature (r=0.76; P=0.004; Annex 2). In contrast, the mean monthly density of *P. orientalis* was correlated negatively with relative humidity (r=-0.80; P=0.03) and rainfall (r= -0.47), respectively, though the relationship with the latter was not significantly different (P=0.12). Mean monthly density of *P. orientalis* on sticky traps had a positive correlation with temperature (r=0.87; *P=0.00)*, and was associated negatively with relative humidity (r= -0.780; P=0.003) and rainfall (r=-0.47; P=0.16; Annex 2).

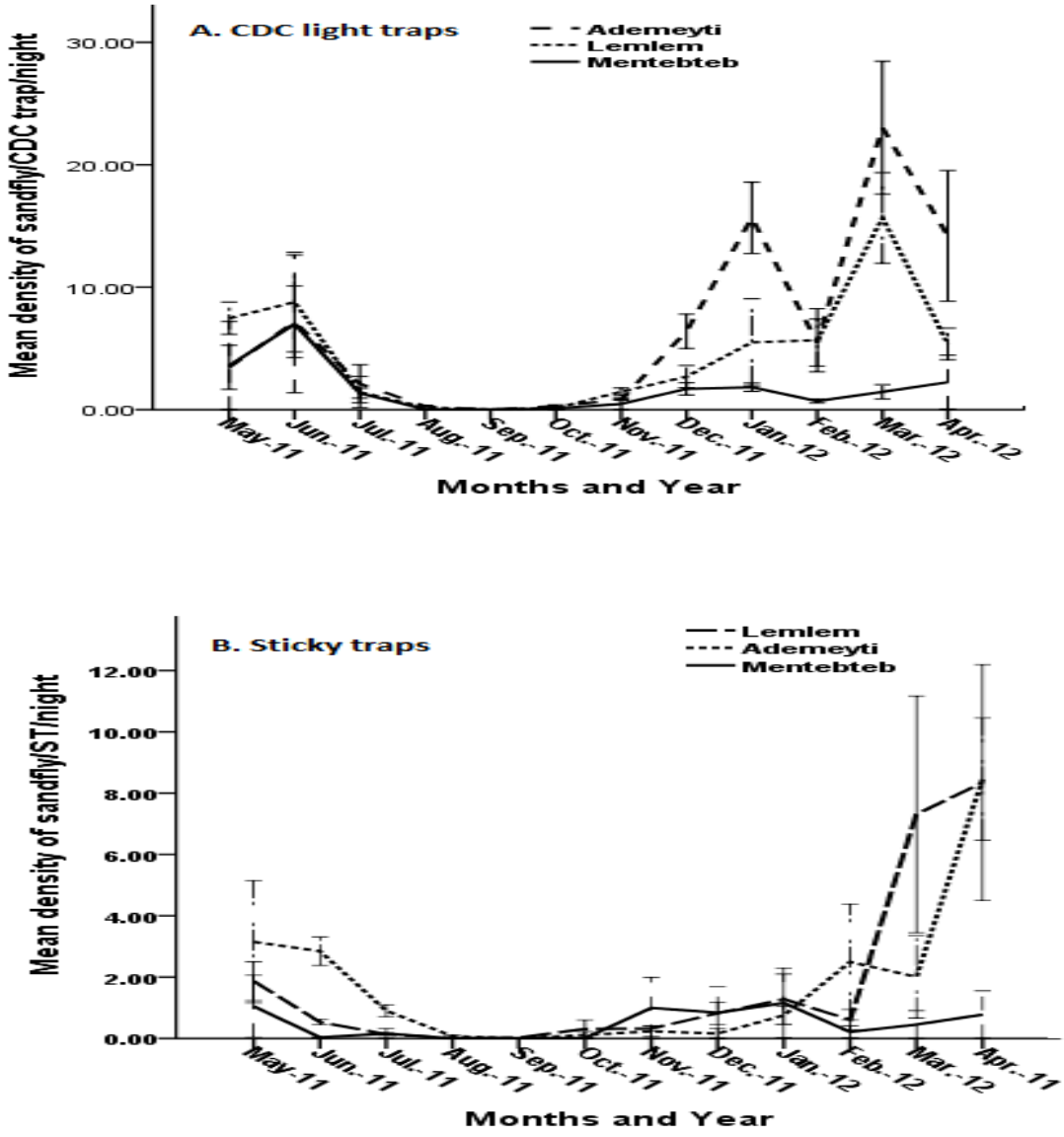

Figure 3.5. Mean monthly density of *P. orientalis* in the three different sampling villages (Ademeyti, Lemlem and Mentebteb), May 2011 to April 2012. **A**: monitored by CDC light traps (specimen/trap/night). **B**: monitored by sticky traps (specimen/trap/night).

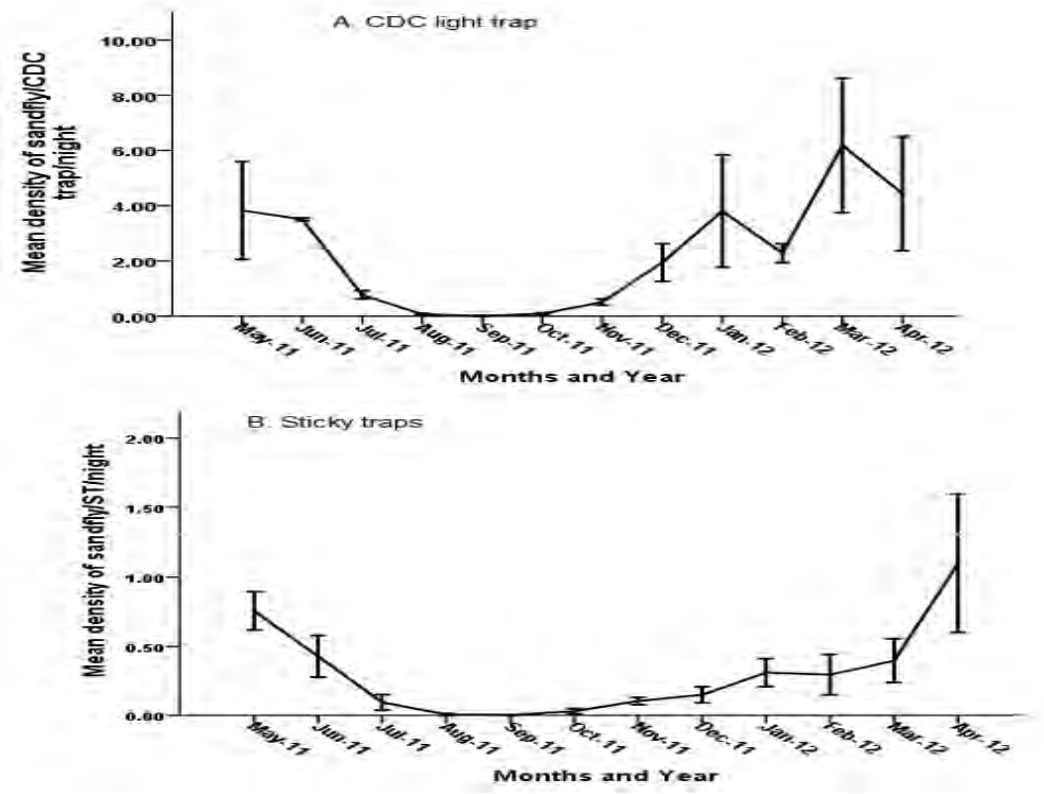

Figure 3.6. Seasonal density of *P. orientalis* trapped from three villages of Tahtay Adiyabo district, May 2011 to April 2012. **A**: collected by CDC light traps (specimen/trap/night). **B**: collected by sticky traps (specimen/trap/night).

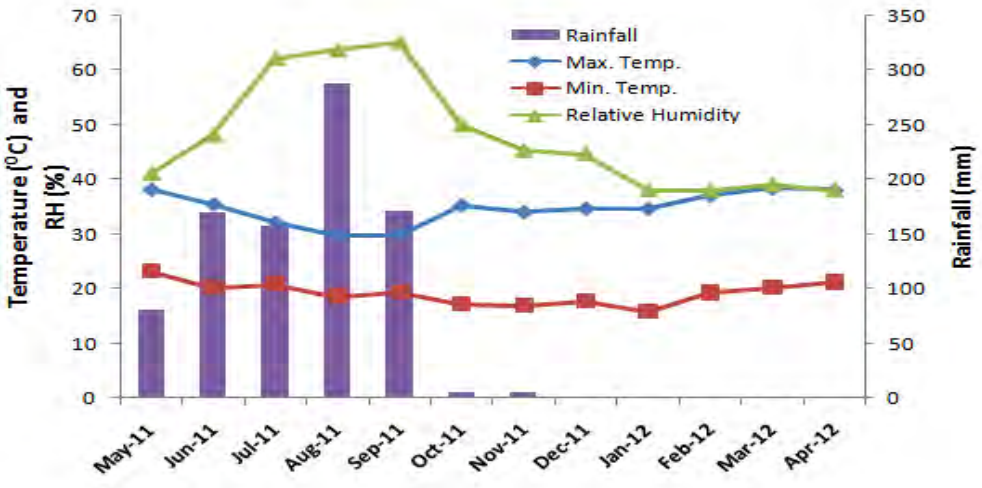

Figure 3.7. Seasonal fluctuations in the mean monthly maximum and minimum temperatures, relative humidity and rainfall in the study area, May 2011 to April 2012

3.4.4. Habitat preference of *P. orientalis*

Higher mean number of *P. orientalis* per CDC light trap/night was caught in agricultural fields than in peri-domestic habitats, although this number in the two habitats was not significantly different ($U(1) = 62.00$, $Z = -0.56$, $P>0.05$; Fig 3.8-A). The mean number of *P. orientalis* caught per CDC light trap/night in peri-domestic and agricultural fields were 1.48 ± 0.45 and 2.36±0.82 per trap/night, respectively.

Unlike light trap collections, habitat types had significant effects on the density of *P. orientalis* on sticky trap collections ($\chi^2_{K\text{-}W}= 20.38$, df = 2, $P<0.05$). On sticky traps, mean density of *P. orientalis* indoor, peri-domestic and agricultural field was 0.003±0.001, 0.18±0.07 and 0.88±0.24/trap/night, respectively (Fig 3.8-B).

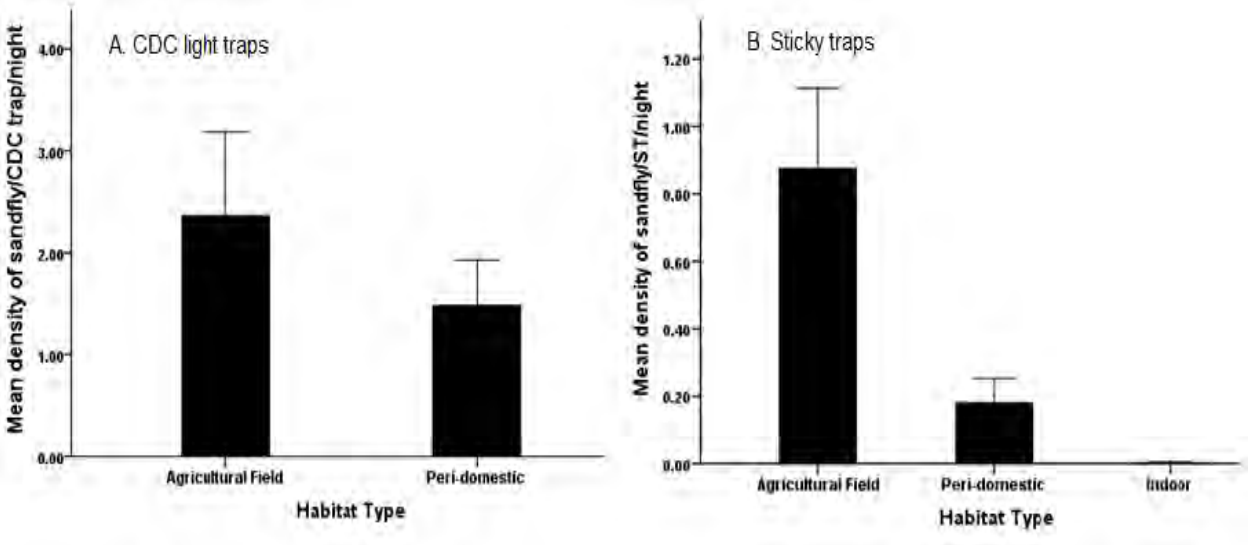

Figure 3.8. Mean number (±SE) of *P. orientalis* collected per trap/night from different habitats from May 2011 to April 2012. **A**: CDC light traps (specimen/trap/night). **B**: Sticky traps (specimen/trap/night).

3.4.5. Indoor and outdoor density of *P. orientalis*

In total, 52 (29 males and 23 females) from indoors and 15,901 (14,519 male; 1,382 female) *P. orientalis* from outdoors were captured on sticky traps. Statistically significant differences were observed in the abundance of *P. orientalis* between indoor and outdoor habitats ($U(1) = 72.00$, $Z = -6.59$, $P<0.05$; Table 3.3). *Phlebotomus orientalis* was common outdoors and scarce indoors with a ratio of 138:1 on sticky trap captures (Table 3.3). Moreover, of 3,054 indoor resting sandflies captured in the pyrethrum spray collections during 578 house visits, 48 *P. orientalis* were found resting inside houses. The proportion of *P. orientalis* was low compared to other sandfly species, which accounted only 1.57% of the total captures (Table 3.1).

Table 3.3. Indoor and outdoor mean number of *P. orientalis* determined by sticky traps in Tahtay Adiyabo district, May 2011 to April 2012

Habitat types	No. collected (M/F)	Males/trap/night ± SE)	Females/trap/night ± SE)
Indoor	52(29/23)	$(0.006 \pm 0.002)^a$	$(0.004 \pm 0.001)^a$
Outdoor*	15,901 (14,519/ 1,382)	$(1.25 \pm 0.29)^b$	$(0.13 \pm 0.02)^b$

Mean values followed by different letters in the same columns are significantly different (U, $P<0.05$); M=male; F=female

*Outdoor (combined collections of peri-domestic and agricultural field).

3.4.6. Sex ratios

Sex ratios (males: females) for different sandfly species demonstrated that males caught by all methods was higher than that of females (57,001 male: 43,771 female), with an overall sex ratio of 1.3:1 (Table 3.1). For *P. orientalis*, the sex ratio in light traps was 2.1:1, which did

not show any significant difference between sexes ($U_{(1)}$= 634.00, Z=-0.16, P>0.05) as opposed to a very high ratio of male to female (12.65:1) on the sticky traps that was clearly significant ($U_{(1)}$ = 330.00, Z= -3.59, P<0.05).

3.4.7. Age grading of wild-caught male sandflies

Sticky traps intercepted 655 *P. orientalis* males with un-rotated or partially rotated genitalia during the study period (recently emerged young males) (Table 3.4). Of these, 187 and 468 were captured in peri-domestic and agricultural field habitats, respectively. The difference in the proportion of *P. orientalis* immature males with un-rotated or partially rotated genitalia captured in agricultural field versus peri-domestic habitat was significant (U(1)=30.00, Z=-2.44, P=0.015). However, no freshly emerged *P. orientalis* males were recorded with other collection methods.

Table 3.4. *Phlebotomus orientalis* young males (=un-rotated genitalia) caught over 12 months on sticky traps that were placed in peri-domestic and agricultural field.

Habitat type	No. collected (%)	Mean no. ± SE/sticky traps /month
Peri-domestic	187 (28.55)	15.58 ± 6.75[a]
Agricultural field	468 (71.45)	39.00 ± 4.98[b]
Total	655	

Mean values followed by different letters in the same column are significantly different (U-test, P<0.05).

3.4.8. Comparative efficacy of sticky traps deployed horizontally versus vertically

Significant difference was observed in the mean density of *P. orientalis* captured between horizontally (HSTs) and vertically (VSTs) placed sticky traps ($U(1) = 432.00$, $Z = -2.44$, $P<0.05$). A relatively higher mean density of *P. orientalis* was found on horizontally placed sticky traps (mean= 3.71±0.89/trap/night) than vertically deployed (mean= 0.79±0.22/trap/night, Fig. 3.9).

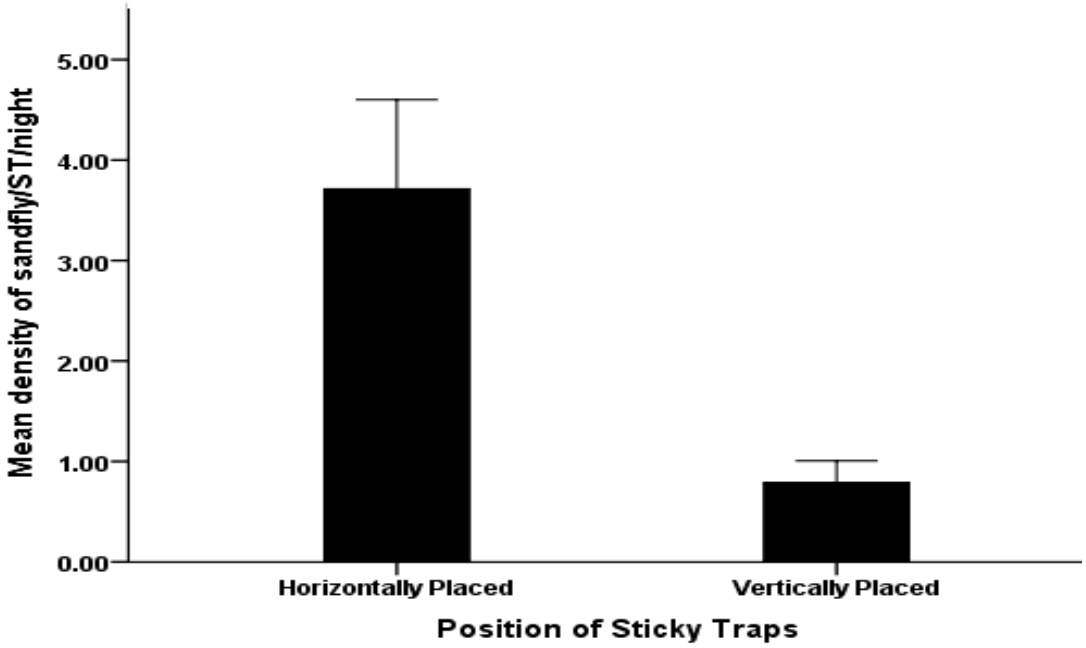

Figure 3.9. Comparison of efficacy of sticky traps deployed in different positions for trapping *P. orientalis* in agriculture field, May 2011 to April 2012.

3.5. Discussion

In the remote rural villages of Tahtay Adiyabo district, longitudinal entomological studies revealed the presence of twenty five species of phlebotomine sandflies, including nine species of *Phlebotomus* (six subgenera) and sixteen species of *Sergentomyia* (four subgenera). The sandfly fauna in the area is composed of the Afrotropical elements (Quate, 1964; Buttiker and Lewis, 1983; Lane, 1986).

Out of the nine species of *Phlebotomus* caught, *P. orientalis* was the most prevalent, accounting for more than 98%. This species is the proven vector of *L. donovani* in Sudan, South Sudan, southwestern Ethiopia (Ashford *et al*., 1992; Asrat Hailu *et al*., 1995; Elnaiem *et al*., 1998a) and the presumed vector in northern Ethiopia (Gebre-Michael et *al*., 2010). The preponderance of this species in sandfly catches was also noted in previous studies in north-west Ethiopia (Teferi Gemechu *et al*., 1975; Teferi Gemetchu, 1983; Teshome Gebre-Michael *et al*., 2010; Wossenseged Lemma *et al*., 2014a) as well as in the same district (Kirstein *et al*., 2013; Moncaz *et al*., 2013, 2014). *Phlebotomus martini* and *P. rodhaini* were also recorded in the present study: the former being the major vector of VL in southern Ethiopia (Teshome Gebre-Michael and Lane, 1996) and the latter was implied as possible vector *of L. donovani* between animal reservoir hosts in Sudan (Elnaiem *et al*., 2011a). The three sympatric species (*P. papatasi, P. bergeroti* and *P. duboscqi*) (Teshome Gebre-Michael *et al*., 2010) were also identified, where their epidemiological role as possible vectors of zoonotic CL would be minor despite *L. major* from blood samples was reported in this area (Abbasi *et al*., 2013).

Among the *Sergentomyia* spp., *S*. *africana* was found to be the predominant species (77.5%) followed by *S. schwetzi* (6.6%). Some of these (*S. schwetzi*, *S*. *adleri* and *S. clydei*) are known to occasionally bite humans (Quate, 1964; Teshome Gebre-Michael and Lane, 1996), but none of these or any other *Sergentomyia* spp. has ever been demonstrated to transmit mammalian leishmaniasis (Sadlova *et al*., 2013). In general, the species composition of phlebotomine sandflies encountered in the present study concord with previous reports in other parts of Ethiopia (Teferi Gemetchu, 1983; Meshesha Balkew *et al*., 1999, 2002; Teshome Gebre-Michael *et al*., 2010; Kirstein *et al*., 2013).

The mean density of *P. orientalis* differed between the three study villages, with Ademeyti and Lemlem being the most productive villages while Mentebteb was the least. Ademeyti and Lemlem villages are similar with respect to their ecosystem and the entire areas are surrounded with vertisols fields. Moreover, most of the houses are made of mud-plastered walls with cracks and crevices. Possibly, these factors have contributed to the moderately abundant number of *P*. *orientalis* in the two villages. Mentebteb is relatively urbanized and largely surrounded by rocky soils that do not form cracks and hence this could be less favorable for the resting/breeding habitat of *P. orientalis* (Moncaz *et al*., 2014). These results could possibly explain the relatively high rates of k-DNA PCR, DAT, and LST positive cases among the residents of Lemlem and Ademeyti compared with Mentebteb (Asrat Hailu *et al*., unpublished data).

The peak density of *P. orientalis* was during the hot-dry period of March to April. A similar trend was also reported in Sudan, where the numbers of *P. orientalis* captured using sticky

traps and CDC light traps remained low in the early dry season (January and February) and increased highly between March and May, after the hot and dry weather has begun (Quate, 1964; Hoogstraal and Heyeneman, 1969; Lambert *et al*., 2002). Likewise, it was found that *P. orientalis* appeared to reach its peak during the driest months of March and April in Kafta-Humera, north-west Ethiopia (Teferi Gemetchu *et al*., 1975; Wossenseged Lemma *et al*., 2014a). Importantly, this sandfly period also tends to be the most likely period of VL transmission in humans in the area. The current study also demonstrated that adults of *P. orientalis* diminish and disappear as the rain commences. This disappearance was also observed in preceding studies in South Sudan (Quate, 1964). However, these findings did not concur with previous studies in north-west Ethiopia (Ashford *et al*., 1973) and in eastern Sudan (Elnaiem *et al*., 1997).

Sandfly population dynamics is largely regulated by a complex interplay between the biotic potential of the vector species and meteorological-environmental conditions (Ibrahim and Abdoon, 2005; Belen and Alten, 2009; Elnaiem, 2011b). In the current study, temperature and relative humidity showed significant correlations with *P. orientalis* abundance. Sharp increases in abundance of *P. orientalis* from March to April coincide with an increase in temperature and a reduction in relative humidity in the area. However, a drop in abundance of adult populations of *P. orientalis* through July to October presumably attributed to an increased amount of rainfall that completely floods the surface and seals the deep cracks of vertisols, leading to a micro-climate change in the breeding/resting sites of the fly population. Moreover, changes in the monthly temperature and relative humidity during those periods might have resulted in a decrease in population abundance. During the rainy

season, *P. orientalis* seems to undergo diapause as larval stage, an adaptation that has been recorded in the fourth larval stage of a laboratory colony (Seblova *et al*., 2013). The diapause is possibly broken by the end of rainy season as temperature increases re-opening of soil cracks.

Habitat preferences of most sandfly species is associated with biotopes, harboring high levels of stable mild temperatures and high humidity, and that contain decaying organic matter allowing better breeding sites and more suitable diurnal resting shelters (Elnaiem *et al*., 1998b; Feliciangeli, 2004). More *P. orientalis* were trapped in agricultural fields than other habitats. Agricultural fields are mostly vertisols, which are characterized by high contents of clay minerals that enhance swelling when hydrated and shrinkage upon desiccation, thereby, causing extensive cracking during the dry season (Moncaz *et al*., 2014). The combination of high humidity and stable temperatures maintained throughout the dry season, and the availability of organic matter that provide food for larval development in the deeper layers of cracked vertisols (Moncaz *et al*., 2014), possibly explains the relative increased abundance of *P. orientalis* in cultivated fields than other habitats observed. Secondly, the presence of trees and scrub vegetations provide shade and source of sugar for adults (Elnaiem *et al*., 1999a). Earlier studies in various parts of Sudan also stressed that *P. orientalis* is mainly associated to forest area with large expanses of vertisols (Quate, 1964; Hoogstraal and Heyeneman, 1969; Elnaiem *et al*., 1997) and rarely associated with human dwellings.

Exophilic and endophilic behaviors of sandflies are important from the control point of view and determining where transmission takes place. It is evident from the study that *P. orientalis* exhibited pronounced exophilic behavior expressed in outdoor/indoor index of 138:1 on sticky traps. Supportive of the sticky trap indoor collections, less proportion of *P. orientalis* females was found resting indoors in 578 house visits, indicating lower rates of endophily. Elnaiem *et al*. (1997) reported the same behavior in Umsalala village in eastern Sudan. The yield from indoor collections of *P. orientalis* in some villages of northwest Ethiopia was also small (Essayas Aklilu, unpublished data). As the weather condition, in Tahtay Adiyabo is typically warm from January through May, the people usually sleep outdoors in their compounds during these months. Farmers also usually keep domestic animals in the yard overnight often within a few meters of the sleeping area, a practice that contributes to an increased abundance of *P. orientalis* females. Therefore, these activities expose people to the bite of *P. orientalis* leading to an outdoor transmission of *L. donovani*.

As for the sex ratio of sandflies throughout the collections, *P. orientalis* had higher proportion of male to female both in CDC light traps and sticky traps, though the mean number ratio of male to female for the former was not significant. This male biased sex ratio was also observed for other sandfly species (Kamhawi *et al*., 1995; Ibrahim and Abdoon, 2005; Kaspa *et al*., 2009). For instance, Kaspa *et al*. (2009) reported that male sandflies composed 80% of their collections. Given a normal sex ratio of 1, then skewed ratios in favour of males might be related to the fact that the traps were placed near emergence sites and/or sugar feeding sources, where males are generally abundant.

The natural breeding habitats of sandflies could be determined from sticky trap catches as these traps usually capture sandflies by passive interception rather than attraction in their diurnal/breeding habitats (Alexander, 2000). In the present study, sticky traps deployed in vertisols caught higher percentages of recently emerged young males of *P. orientalis*. This habitat constituted more than 71% of *P. orientalis* immature males emerged within the first 24 hours. More accurate recording of the condition of males with un-rotated external genitalia can indicate the proximity of immature sites (Ready, 2013). Moreover, males apparently are more sedentary than females (Yuval *et al*., 1988; Janini *et al*., 1995; Orshan *et al*., 2010). In this study area, deeply cracked vertisols in open cultivated fields and tree-related habitats were also identified as breeding sites for *P. orientalis* (Moncaz *et al*., 2014). Accordingly, it can be conceived that a relatively high catch with large proportion of recently emerged immature males in this study indicates a proximity to breeding sites.

Higher mean density of *P. orientalis* was recorded on horizontally placed (HSTs) sticky traps than those vertically (VSTs) deployed. Both sexes of *P. orientalis* were caught on HSTs, albeit twenty-two times as many males as females were trapped. Interestingly, deploying sticky traps horizontally is preferable for monitoring sandfly populations, particularly in flat plains with crevices, although it remains to be determined in other sandfly habitats. The higher proportion of male *P. orientalis* on HSTs in the present study could be due to the aggregation behavior of this vector species on the horizontal surface, establishing mating swarms (Ashford, 1974; Moncaz *et al.*, 2013). The alternative could be the difference in the flight angle of the species.

In conclusion, the study demonstrated that *P. orientalis* was found to be the most abundant *Phlebotomus* species, showing distinct seasonality that mainly peaks during the dry season (March to April) within the entire study area. Furthermore, this study depicts the exophilic behavior of *P. orientalis*. This behavior is of practical importance because it apparently makes the species less vulnerable to insecticide residual spraying (IRS) or long lasting insecticidal nets indoor conditions. However, the small number of *P. orientalis* collected in sticky traps and pyrethrum spray catch indoors may still be of epidemiological significance. Therefore, control programs designed to contain VL transmission in different villages of Tahtay Adiyabo should focus mainly on targeting *P. orientalis* in outdoors without ignoring its minor endophilic behavior.

Chapter Four

Physiological age structure and *Leishmania* spp. detection in *Phlebotomus orientalis* and other sandflies

4.1. Introduction

In Ethiopia, the first case of VL was identified in the 1940s in the lower Omo plains, the southwestern part of the country (Cole *et al*., 1942). Subsequently, the disease has been known to be endemic in many parts of the country. The main endemic areas of VL in the country are in the north and south-west (Ashford *et al*., 1973; Teklemariam Ayele and Ahmed Ali, 1984; Ahmed Ali and Ashford, 1994; Asrat Hailu *et al*., 2006). However, recently increasing VL cases have been reported from previously non-endemic regions such as Libo Kemkem district of Amhara and Tahtay Adiyabo district in Tigray, northern Ethiopia (Alvar *et al*., 2007; Abbasi *et al*., 2013), causing high mortality and morbidity among the local residents.

The distribution of VL depends on the presence of competent vectors and reservoir hosts in a particular area. Detection of natural infection with *Leishmania* and determination of the physiological age structure of field vector populations are of prime importance in vectorial and epidemiological studies of leishmaniasis (Ferro *et al*., 1995). The infection of competent vectors with *Leishmania* promastigotes has been determined by dissection of individual sandflies under a microscope. However, the use of this method to determine infection rate is difficult because promastigote infections in competent vectors is generally low and estimation of infection rate requires the examination of a large number of specimens.

Previous microscopical surveys on different species of sandflies in different regions showed infection rates ranging from 1.7% to 10.7% (Bettini *et al*., 1986; Maroli *et al*. 1994; Teshome Gebre-Michael and Lane, 1996; Elnaiem *et al*., 1998a). Recently, polymerase chain reaction (PCR)-based techniques have been adapted to detect *Leishmania* spp. in sandflies (Aransay *et al*., 2000b; Pandey *et al*., 2008; Berdjane-Brouk *et al*., 2012).

In different parts of Ethiopia, *P. martini*, *P. celiae,* and *P. orientalis* have been found infected with *L. donovani* and implicated as vectors of VL (Hailu *et al*., 1995; Gebre-Michael and Lane, 1996). In particular, *P. orientalis* has been suspected as the vector of VL in the north and northwest of Ethiopia (Teferi Gemetchu *et al*., 1975; Teshome Gebre-Michael *et al*., 2010), but it has already been incriminated in the adjacent endemic regions of Sudan (Elnaiem *et al*., 1998a; Hassan *et al*., 2004; WHO 2010). However, data on the rate of naturally infected sandflies with *Leishmania* parasites and physiological age characterization of *Phlebotomus* species specifically from the VL endemic area of Tahtay Adiyabo is lacking. Therefore, the current study was carried out with the aim of determining the physiological age structure and detection of *L. donovani* from *Phlebotomus* spp. towards incriminating the vector of VL in the area.

4.2. Methods and Materials

4.2.1. Sandfly collection

Sandfly specimens collected for the determination of species composition and bionomics (Chapter 3) were used for this section of the study.

4.2.2. Determination of abdominal status, parous rates, and infection rates of *Phlebotomus* sandflies

Sandflies captured in CDC light traps from various sampling habitats (peri-domestic and agricultural fields) were transported to a field station laboratory and females were transferred to test tubes. They were then knocked down using chloroform and emptied on petri dishes for sorting into different categories (genera, sex, and abdominal status) under a dissecting microscope. *Phlebotomus* females were categorized into unfed, semi-gravid, gravid, and fresh fed. The fresh-fed females were preserved in ethanol and/or silica gel grain for bloodmeal analysis and parasite detection by PCR. Unfed, gravid, and semi-gravid female sandflies were washed in 2% savlon/saline solution followed by sterile physiological saline, and dissected in sterile physiological saline for the detection of promastigotes (Teshome Gebre-Michael *et al*., 1993). After dissection, the ovaries of unfed *Phlebotomus* females was drawn out together with the guts to determine their parous state (parous or nulliparous). Parity was determined based on the presence of granules in the accessory glands (Añez and Tang, 1997) and the states of the ovaries (Teshome Gebre-Michael *et al*., 1993). The gut contents of parous, gravid, and semi-gravid females were examined under a phase contrast microscope at 40x10 magnifications for the presence of *Leishmania* promastigotes. When promastigotes were observed in dissected females, the gut with its content was kept in 70% ethanol and stored at -20^{0}C for later PCR detection of *Leishmania*. Unfortunately, the promastigote positive guts could not be cultured into NNN media due to the scarcity of this media during the time of dissection.

Un-dissected females of *P. orientalis* were also preserved in 70% ethanol and stored at -20^{0}C for later PCR detection of *Leishmania.* The head and abdominal tips from the above process were mounted on glass slides using Hoyer medium and identified using the identification keys as described in section 2.3.

4.2.3. Detection of *Leishmania* parasites by PCR

DNA Extraction: *Phlebotomus orientalis* females with abdominal status of unfed, half-gravid, and gravid were first grouped into 115 pools according to sampling date, collection site, and abdominal conditions with up to five specimens in each pool. Following this, DNA was extracted in pools from these specimens using phenol and chloroform method (Abbasi *et al*., 2013). At the same time, DNA was processed individually from blood-fed females. Briefly, ethanol-preserved sandflies were incubated in a microfuge tube with 200 µl of lysis buffer (50 mM NaCl, 10 mM EDTA, 50 mM Tris–HCl pH 7.4, 1% triton X-100, and 200 µg/ml of proteinase K) at 65°C for 2 hours. Equal volumes of TE-saturated phenol (pH 8) were added to the aqueous solution, the mixture was vortexed for few seconds and then centrifuged for 2 minutes at 14,000 rpm. The upper aqueous layer was transferred to a new micro centrifuge tube and the DNA was precipitated by adding NaCl to a concentration of 0.2 M (addition of 8 µl of 5M NaCl to 200 µl aqueous solution) and 2.5 volumes of 100% cold ethanol. DNA was incubated at −20°C overnight and centrifuged at 14,000 rpm for 10 minutes. The supernatant was discarded and the DNA pellet was dried in speed-vac. The DNA pellet was suspended with double distilled water and stored at -20 °C until use for PCR.

ITS polymerase chain reaction (PCR): PCR reactions were carried out in a volume of 25 µl using ready mix PCR tubes (Syntezza, Jerusalem, Israel). For each reaction, 20 p moles of the two 320 bp *Leishmania* specific ribosomal internal transcribed spacer 1 (ITS1) region using the primers LITSR (5'-CTG GAT CAT TTT CCG ATG-3') and L5.8S (5'-TGA TAC CAC TTA TCG CACTT-3') were added followed by 5 µl of the template DNA (El Tai *et al*., 2000). Double distilled water along with primers and DNA from promastigotes of *L. donovani*, *L. major*, *L. aethiopica*, and *L. tropica* were used as negative and positive controls, respectively. The thermal profile comprised 5 min at 95°C, followed by 35 cycles starting at 95°C for 30 seconds, 56°C for 30 seconds, and 72°C for 1 min, a final elongation step at 72°C for 10 min.

Gel Electrophoresis: Amplified products (7 µL) were run on 2% agarose gel containing ethidium bromide for 1 h at 80 V. The gel products were visualized under ultraviolet (UV) transilluminator and then digital photographs were prepared. *Leishmania* infections were identified by comparison of PCR products of specimens with the reference strains and molecular weight markers.

DNA sequencing: The amplified ITS1 PCR products that demonstrated a moderate to strong ITS1 bands were sequenced by-automated fluorescent DNA sequencing using ABI PRISM 377 sequencer (PE Biosystems, Foster City, California). The sequences obtained were compared for their homology to known sequences in the GenBank database using BLAST online service provided through the PubMed /US National Institute of Health (http://www.ncbi.nlm.nih.gov/BLAST).

4.3. Results

4.3.1. Abdominal status and parous rates

Tables 4.1 and 4.2 show the abdominal categories of different *Phlebotomus* spp. collected from different study villages. In Ademeyti, the number of unfed, freshly fed, half-gravid, and gravid *P. orientalis* females from peri-domestic habitat was 180, 163, 17, and 2, respectively (Table 4.1). Similarly, more number of unfed (n=197) and engorged (n=133) *P. orientalis* were found in peri-domestic sites of Lemlem village. Of the 60 female *P. orientalis* obtained from Mentebteb, 40 were unfed, 17 freshly fed, and 3 gravid and half-gravid.

Out of the total 524 females of *P. orientalis* dissected from agricultural fields, 430, 48, 32, and 14 were unfed, freshly fed, half-gravid and gravid females, respectively (Table 4.2). Unlike the peri-domestic sites of Ademeyti and Lemlem, less numbers of blood-fed females were found in agricultural fields. No blood fed female was detected in agricultural fields of Mentebteb. More (n=32) half-gravid females were recorded in Lemlem than the two villages.

Table 4.1. Abdominal status of female *Phlebotomus* captured from peri-domestic locations in three different villages.

Species	No. Dissected			No. Unfed			No. Freshly fed			No. Half-gravid			No. Gravid			Total
	Adm	Lem	Men	Adm	Lem	Men	Adm	Lem	Men	Adm	Lem	Men	Adm	Lem	Men	
P. orientalis	362	334	60	180	197	40	163	133	17	17	2	1	2	2	2	756
P. bergeroti	3	10	1	1	3	0	2	0	0	1	2	0	1	3	1	14
P. martini	0	0	2	0	0	0	0	0	0	0	0	0	2	0	0	2
P. papatasi	2	3	0	2	3	0	0	0	0	0	0	0	0	0	0	5
P. duboscqi	2	1	0	2	1	0	0	0	0	0	0	0	0	0	0	3
P. lesleyae	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1
Total	370	348	63	186	204	40	165	133	17	18	4	1	5	5	3	781

Abbreviations: Adm= Ademeyti; Lem= Lemlem, and Men= Mentebteb

Table 4.2. Abdominal status of female *Phlebotomus* captured from agricultural fields in three different villages

Species	No. dissected			No. Unfed			No. Freshly fed			No. Half-gravid			No. Gravid			Total
	Adm	Lem	Men	Adm	Lem	Men	Adm	Lem	Men	Adm	Lem	Men	Adm	Lem	Men	
P. orientalis	173	264	87	144	200	86	24	24	0	0	31	1	5	9	0	524
P. bergeroti	0	2	2	0	1	1	0	0	0	0	0	0	0	1	1	4
P. martini	2	1	0	1	0	0	0	0	0	1	1	0	0	0	0	3
P. rodhaini	1	3	0	1	3	0	0	0	0	0	0	0	0	0	0	4
P. papatasi	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	1
P. duboscqi	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
P. lesleyae	2	31	0	2	31	0	0	0	0	0	0	0	0	0	0	33
P. heischi	0	2	0	0	2	0	0	0	0	0	0	0	0	0	0	2
Total	178	304	89	148	238	87	24	24	0	1	32	1	5	10	1	571

Abbreviations: Adm= Ademeyti; Lem= Lemlem and Men= Mentebteb

In total, 847 unfed *P. orientalis* were dissected and examined for parous state based on the appearance of accessory glands and condition of ovaries (Table 4.3). Of 417 *P. orientalis* dissected in peri-domestic, 142 (34.05%) were parous: 33.33%, 30.96% and 52.5% in Ademeyti, Lemlem and Mentebteb villages, respectively while the parous rate in agricultural fields in Ademeyti, Lemlem, and Mentebteb was 43.8%, 31.0%, and 31.4%, respectively (Table 4.3).

Table 4.3. Parous rates of *P. orientalis* females trapped using CDC light traps

	Habitat type			
	No. Dissected	**Peri-domestic**	**No. Dissected**	**Agricultural field**
Villages		**No. parous (%)**		**No. parous (%)**
Ademeyti	180	60 (33.3)	144	63 (43.8)
Lemlem	197	61 (30.9)	200	62 (31.0)
Mentebteb	40	21 (52.5)	86	27 (31.4)
Total	417	142 (34.1)	430	153 (35.6)

4.3.2. Natural infection rates with promastigotes

Among the 921 *P. orientalis* females dissected for parasite detection, one female was found infected with *Leishmania* promastigotes. The infected specimen was collected from agricultural field in Lemlem village during July. However, none of the other seven species of *Phlebotomus* was found infected with *Leishmania* promastigote. The number of dissected female sandflies include: 34 *P. lesleyae* (1 from peri-domestic and 33 from agricultural

fields), 16 *P. bergeroti* (12 from peri-domestic and 4 from agricultural field), 6 *P. papatasi* (5 from peri-domestic and 1 from agricultural field), 4 *P. rodhaini* (all from agriculture field), 3 *P. duboscqi* (all from peri-domestic), 5 *P. martini* (2 from the peri-domestic and 3 from agricultural field), and 2 *P. heischi* (all from agricultural field).

4.3.3. *Leishmania* DNA detection in sandflies

In total, 575 *P. orientalis* females, which were divided into 115 pools, were submitted to ITS1-PCR testing for *Leishmania*. Five pools were observed to possess the characteristic DNA band of *Leishmania* spp. However, acceptable sequence could not be obtained during DNA sequencing due to the weak amplification of the *Leishmania* DNA. Moreover, 200 blood-engorged sandflies (198 *P. orientalis*, 1 *P. bergeroti,* and 1 *Sergentomyia africana*) were analyzed. *Leishmania* DNA was detected by PCR in 22 female specimens (11%). In the direct sequencing of the 22 PCR products, only three specimens produced a complete genomic sequence of *L. donovani* in *P. orientalis*, *P. bergeroti,* and *S. africana* females, respectively (Fig. 4.1). Engorged specimen of *S. africana* was accidentally included in the analysis.

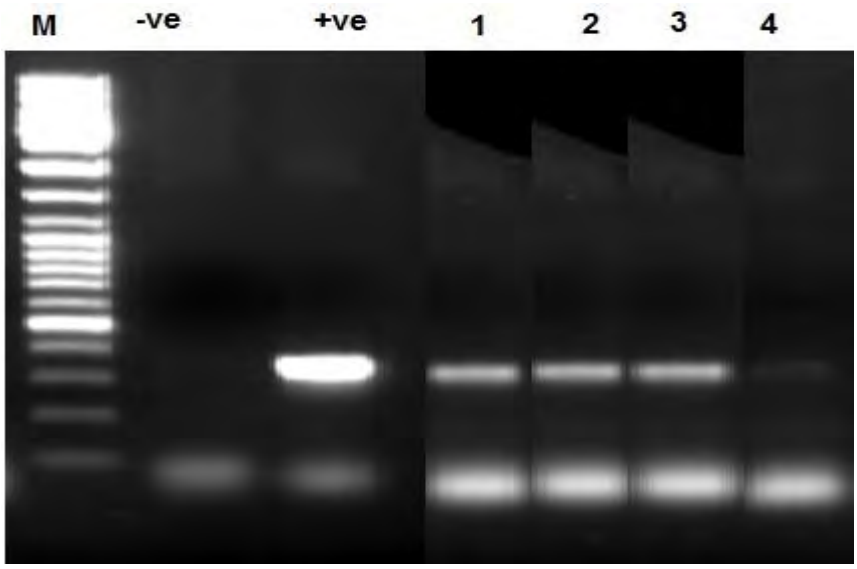

Figure 4.1. PCR of *Leishmania* internal transcribed spacer 1 (ITS1) region amplified from female sandflies. M: 100 bp size marker; -ve: negative control and +ve: reference *Leishmania* spp.; Lanes 1 and 2: *L. donovani* DNAs in *P. orientalis* and *P. bergeroti* females, respectively; Lane 3: *L. donovani* in *S. africana*; Lane 4: *Leishmania* DNA in *P. orientalis*, but failed to be sequenced.

4.4. Discussion

Visceral leishmaniasis is affecting several people in various endemic areas of north and northwest Ethiopia. In particular, in this study area, 14.3% (680/4,757) of individuals were found positive for *Leishmania* k-DNA by Real-time (RT)-PCR (Abbasi *et al*., 2013). In addition, 209 VL and 3 PKDL cases were identified in the district in five years (2006-2011) (Desjeux *et al*., 2013).

Parous rates of *P. orientalis* dissected from peri-domestic (34.05%) and agricultural field habitats (35.35%) were relatively low, which is comparable with those observed for the same species in Addis Zemen (Teshome Gebre-Michael *et al*., 2007) and the Humera-Metema low lands (Teshome Gebre-Michael *et al*., 2010), northern Ethiopia and for *P. martini* in Aba Roba, southern Ethiopia (Teshome Gebre-Michael and Lane, 1996). The relatively small percentage of parous flies caught during the study period could indicate that females are short-lived, and that few survive long enough to obtain more than one bloodmeal during their lives. This might also partly explain why low infection was observed in *P. orientalis*.

Regarding abdominal stages of *P. orientalis*, large numbers of blood-fed females were sampled in peri-domestic habitats of the study villages compared with agricultural fields. This high number of blood-fed females in peri-domestic environment is mainly attributed to the accessibility of large numbers of bloodmeal sources to questing females of *P. orientalis* (See chapter 5). On the other hand, relatively moderate numbers of gravid and semi-gravid females were found in agricultural fields, suggesting that older populations tend to remain in their breeding/resting sites until full development of ovules (Yuval and Schlein, 1986).

Incrimination of a certain type of sandfly vector of *Leishmania* involves a number of criteria that include abundance in a leishmaniasis focus, high feeding habits on human, or reservoir host if the disease is zoonotic, demonstration of natural infections and the ability to harbor, develop, and transmit the parasite to a susceptible host (Killick-Kendrick, 1999; Ready, 2013). Quite often, however, fulfilling all these criteria is exceedingly difficult (WHO, 2010; Ready, 2013).

Usually, a sandfly species is suspected as a vector when it is predominant and proved to have anthropophilic behavior. In this area, *P. orientalis* was found to be the preponderant (98%) and human biting *Phlebotomus* species (see chapters 3 & 5). Importantly, this species has already been incriminated as the vector of *L. donovani* in the adjacent endemic regions of Sudan (Elnaiem *et al*., 1998a; Hassan *et al*., 2004; 2008). The present study also detected *Leishmania* DNA by PCR in 20 blood-fed and 5 pools of unfed *P*. *orientalis* females. Specifically, one *P. orientalis* female was positive for *L. donovani* after sequencing, although it was in a recently engorged female. In addition, PCR detection of *Leishmania* DNA in *P. orientalis* females devoid of blood (i.e. parous) is also suggestive, though sequencing of the PCR product failed to determine the exact identity of the parasites. Furthermore, promastigotes were detected in one of 921 *P. orientalis* wild caught females dissected, resulting in 0.11% natural infection. The promastigotes were observed in the abdominal and thoracic midgut of the fly but with low density. Despite the positive sample yielded a characteristic DNA band of *Leishmania* in the PCR test, direct sequencing of this PCR product failed to give complete genomic sequence.

A definitive substantiation can also be drawn from the results of earlier xenodiagnosis experiment, which was carried out to demonstrate the role of asymptomatic VL persons infectious to *P. orientalis* sand flies in the area (Teshome Gebre-Michael and Asrat Hailu, unpublished). In that xenodiagnosis study, laboratory bred-females of *P. orientalis* from the study area were allowed to feed on a VL-HIV co-infected patient and females were positive for promastigotes after 6-7 days of feeding. As well, the promastigotes were observed flourishing in the midgut with anterior migration to the foregut.

In addition, engorged females of *P. bergeroti* and *S. africana*, collected from Lemlem village in peri-domestic habitat during January, were positive for *L. donovani* DNA. *Phlebotomus bergeroti* bites human readily and have been suspected as a vector of *L. major* in the Sahara (Seccombe *et al*., 1993) and around Mecca in Saudi Arabia (Lewis and Biittiker, 1980). Different *Sergentomyia* spp. were found to be PCR positive for DNA of human pathogenic *Leishmania* species: *L. major* DNA was found in *S. darling* in Mali (Berdjane-Brouk *et al*., 2012), and *S. sintoni* in Iran (Parvizi and Amirkhani, 2008) while *L. donovani* DNA was detected in *S. babu* in the Indian VL foci (Mukherjee *et al*., 1997). However, the observations might not necessarily reflect their involvement in the transmission of the parasites to humans since *Sergentomyia* spp. do not support successful development of mammalian *Leishmania* in their guts, albeit they can be found to be PCR positive for DNA of human pathogenic *Leishmania* species (Minter and Wijers, 1963; Lawyer *et al*., 1990; Sadlova *et al*., 2013).

Therefore, the predominant abundance of *P*. *orientalis*, its attraction and blood feeding habits on humans, the detection of *L. donovani* and *Leishmania* in wild-caught females of *P*. *orientalis* coupled with the results of xenodiagnosis on a patient strongly suggests that *P*. *orientalis* is the principal natural vector of VL in Tahtay Adiyabo district. Other species (e.g. *P. martini* and *P. rodhaini*) are probably involved as secondary vectors, although they are scarce in abundance. The former being the proven vector of VL in southern Ethiopia (Gebre-Michael and Lane, 1996) while the later was implied as possible vector in Sudan (Elnaiem *et al*., 2011a).

Chapter Five

Host preferences of *Phlebotomus orientalis* and other sandflies

5.1. Introduction

Ninety-eight countries and three territories on five continents of the world are endemic with either of the two major forms of leishmaniasis: CL, a disfiguring and stigmatizing disease, and VL, which is fatal if untreated (Desjeux, 2004; Alvar *et al*., 2012). In Ethiopia VL is considered an emerging disease with an estimated number of 3,700 to 7,400 cases per year (Alvar *et al*., 2012). The known VL endemic foci are in the south-west and the Humera-Metema lowlands in the north-west of the country bordering Kenya and Sudan, respectively (Lyons *et al.*, 2003; Hailu *et al*., 2006).

The various forms of leishmaniasis, including VL are transmitted by the bite of infected female sandflies of the genus *Phlebotomus* in the Old World and *Lutzomyia* in the New World (Killick-Kendrick, 1999; Ready, 2013). Transmission of VL occurs when a sandfly acquires infection by feeding on an infected host and transmits the parasite during subsequent feedings after completion of the gonotrophic cycle, during which the parasite takes full development in the gut.

The host preferences of several sandfly species have been investigated mostly through the identification of the sources of bloodmeals using serological (e.g. Ngumbi *et al*., 1992; Bongiorno *et al*., 2003) or molecular assays (Abassi *et al*., 2008; Garlapati *et al*., 2012) and host attractiveness experiments (Johnson *et al.*, 1993; Montoya-Lerma and Lane, 1996). Previously, the host preference of *P. orientalis* in Sudan and Ethiopia was determined via the

identification of bloodmeal sources by ELISA (Teshome Gebre-Michael *et al*., 2010) or quantifying the host preferences using different animal baits (Turner and Hoogstraal, 1965; Hassan *et al*., 2009). In eastern Sudan, it has been shown that *P*. *orientalis* is largely attracted to dogs, which is a suspected reservoir host for domestic transmission of VL in the area (Hassan *et al*., 2009). In the Humera-Metema plains, *P. orientalis* exhibits zoophilic behavior by predominantly feeding on bovine blood (Teshome Gebre-Michael *et al*., 2010). These limited studies in East Africa indicated *P. orientalis* to be an opportunistic feeder; however, detailed studies are needed to understand the natural host preference profile of *P. orientalis* and other sandfly species and the possible epidemiological significance of both domestic and wild animals in the transmission dynamics of VL.

The possible role of some domestic and wild animals in VL transmission had been under discussion for quite some time. For example, ownership of cattle in Nepal and density in Bangladesh were found to be protective (Bern *et al*., 2005). In contrast, increased risk of VL was indicated to be associated with the density of cattle or ownership in India (Barnett *et al*., 2005). In Sudan, Dereure *et al*. (2000) demonstrated natural infection of dogs while Mukhtar *et al*. (2000) were able to detect the presence of anti-*Leishmania* antibodies in donkeys, cows, and goats. A recent study in Nepal detected the presence of *Leishmania* DNA in domestic animals such as goats, cattle, and buffaloes, several months after the active transmission season (Bhattarai *et al*., 2010). *Leishmania donovani* DNA was also detected in cattle, donkeys, sheep, and goats from two VL endemic districts of northern Ethiopia (Rhoussova *et al*., in preparation). However, the parasites have not yet been detected microscopically or in cultures. Concerning the role of wild animals in VL transmission in East

Africa, investigations using the conventional parasitological methods have been extensive, but only a few species of mammals have been found infected with the parasite (Hoogstraal and Heyenman, 1969; Elnaiem *et al*., 2001; Asrat Hailu *et al*., unpublished data).

Taking into account the fragmentary information available on the feeding habit of *P. orientalis* in the VL endemic areas of northern Ethiopia, an experiment was designed to determine the attraction and feeding success of *P. orientalis* and other sandflies on domestic and small wild mammals. Moreover, to complement the host choice study, bloodmeal source identification of wild caught *P. orientalis* was conducted using cytochrome (cyt) *b*-PCR and reverse-line blotting (RLB) as well as enzyme linked immunosorbent assay (ELISA).

5.2. Materials and Methods

5.2.1. Animal baits for host choice experiments

Different domestic animals (cattle, sheep, goat, donkey, dog and chicken) and a volunteer human were used in the first host choice experiment.

Trapping small animals: Sherman-live traps and Tomahawk collapsible traps were used for trapping small animals needed for conducting host choice experiments. Traps baited with peanut butter were set near rock crevices, farm fields, rodent burrows and visible animal paths at night and at day time, and were checked for catches in the morning and in the evening, respectively. The only animals captured in this way were ground squirrel (*Xerus rutilus*), gerbil (*Tatera robusta*), and Cairo spiny mouse (*Acomys cahirinus*). Hares (*Lepus* sp.) were captured by chasing them from their hidings in the bush. The animals captured were identified to their respective species by Dr. Jan Votýpka (Pers. comm.)

5.2.2. Experimental designs for host attractiveness

The two independent host choice studies were conducted under field conditions between March and April 2013 in rotational experimental design in which wild sandflies were given choice of different animal baits. Experiments were undertaken in an open agricultural field, where there were no any potential bloodmeal source animals (cattle, sheep, goats, donkeys, camels, dogs, chickens and other small wild animals) in the vicinity of the test traps for at least 250-300 meters.

Experiment I: The experiment was conducted using wild sandflies, where they were given the choice of seven baits (human, cow, sheep, goat, donkey, dog and chicken) and control (without baits) assigned in tent traps. Each tent trap (dimension: 2.5m×2.5m×2.5m) was constructed from transparent sandfly-proof netting supported with four rectangular metal frames and four metal poles to firmly fix them to the ground when installed (Fig. 5.1-A). The tent was also raised a few centimeters above the ground to allow entry of host seeking sandflies. One side of the tent had a long zipped slit (top to bottom) to enable entry and exit of host plus the participant to aspirate sandflies from inside the trap. The tent trap design was based on a prototype used previously to assess sandfly attraction behavior in Colombia (Montoya-Lerma and Lane, 1996).

The animals were tethered in the center of the tent while the human volunteer slept on a cot protected by untreated sandfly-proof mosquito net (also locally made) inside the tent trap. Baited tent traps and un-baited control traps were placed in a circular manner at a distance of about 30 meters apart from each other (Fig. 5.1-B). The baits and un-baited traps

rotated every night between different positions to minimize possible differences in abundance of sandflies due to locations. Each baited trap was randomly placed on a site for the first night, and then repeated the following nights until each host and the control was placed in each of the eight sites. The experiment was conducted on two complete rounds, totaling 16 nights. During the trapping nights, some of the animals were provided with grass and straw in the tents to calm them down for the nightlong session.

Collection of trapped sandflies from the interior walls and the roof of the tent traps was performed early in the mornings (06:00-08:00 hours) by three to four trained and experienced collectors (one person/net) using mouth aspirators while the animals were still inside the net to prevent sandflies from escaping. The collected sandflies were placed in small Barraud cages, each labeled corresponding to the host until processing in the field laboratory. Blood-fed females were separately sorted out and all sandfly specimens were preserved in 70% ethanol for latter identification to species as indicated in section 2.3

***Experiment II*:** Square box traps (30cmX30cmx30cm) with entrance brass screen cones on the three sides, locally constructed on the design of Turner and Hoogstraal (1965), and baited with the above mentioned animals (each restrained in a cylindrical wire mesh cage) were used for the experiment, but failed to catch sandflies on several occasions. Therefore, as an alternative approach, the host choice experiment was carried out by placing modified CDC traps in an up-draft position after removing the light bulbs. These were set up in caged animals and a blank cage as control (Fig. 5.1C-D). The unlit CDC traps were suspended with their opening 5 cm above each animal. The respective sizes of the rectangular metal wire

cages used for the hare and ground squirrel were 40 × 20 × 20 cm^3 and 12 × 10 × 8 cm^3, respectively. The smaller rodents were kept inside a cylindrical wire mesh cage (18 cm x 6 cm) with a metal lid at both ends. Like experiment one, caged animal baits and the blank control were placed in a circular manner at a distance of 30 m from each other. Experimental sessions started 1 h before sunset and terminated 1 h after sunrise, the following morning. The experiment was repeated 10 times totaling 10 trapping nights per bait and the animals were rotated between the different positions as above. In the morning, sandfly specimens caught in the traps were collected using mouth aspirators, placed in separate Barraoud cages and transported to the field laboratory. Females were separated into fed and unfed, and preserved in 70% ethanol for latter identification to species.

5.2.3. Ethical Considerations

The experiments involving human volunteers and animals, described in this thesis, were ethically approved by the ethical review committee at the Medical Faculty, Addis Ababa University, and the National Research Ethics Review Committee (NRERC) at the Ethiopian Ministry of Science and Technology. Similarly, informed consent was obtained from all human volunteers who participated in host attraction study. Moreover, experimental work carried out on host attractiveness of domestic and small wild animals conformed to the International Guiding Principles for Biomedical Research Involving Animals developed by the Council for International Organizations of Medical Sciences and with the Standards for Human Care and Use of Laboratory Animals. Importantly, verbal informed consent was obtained from heads of households selected for sampling sandflies from inside houses.

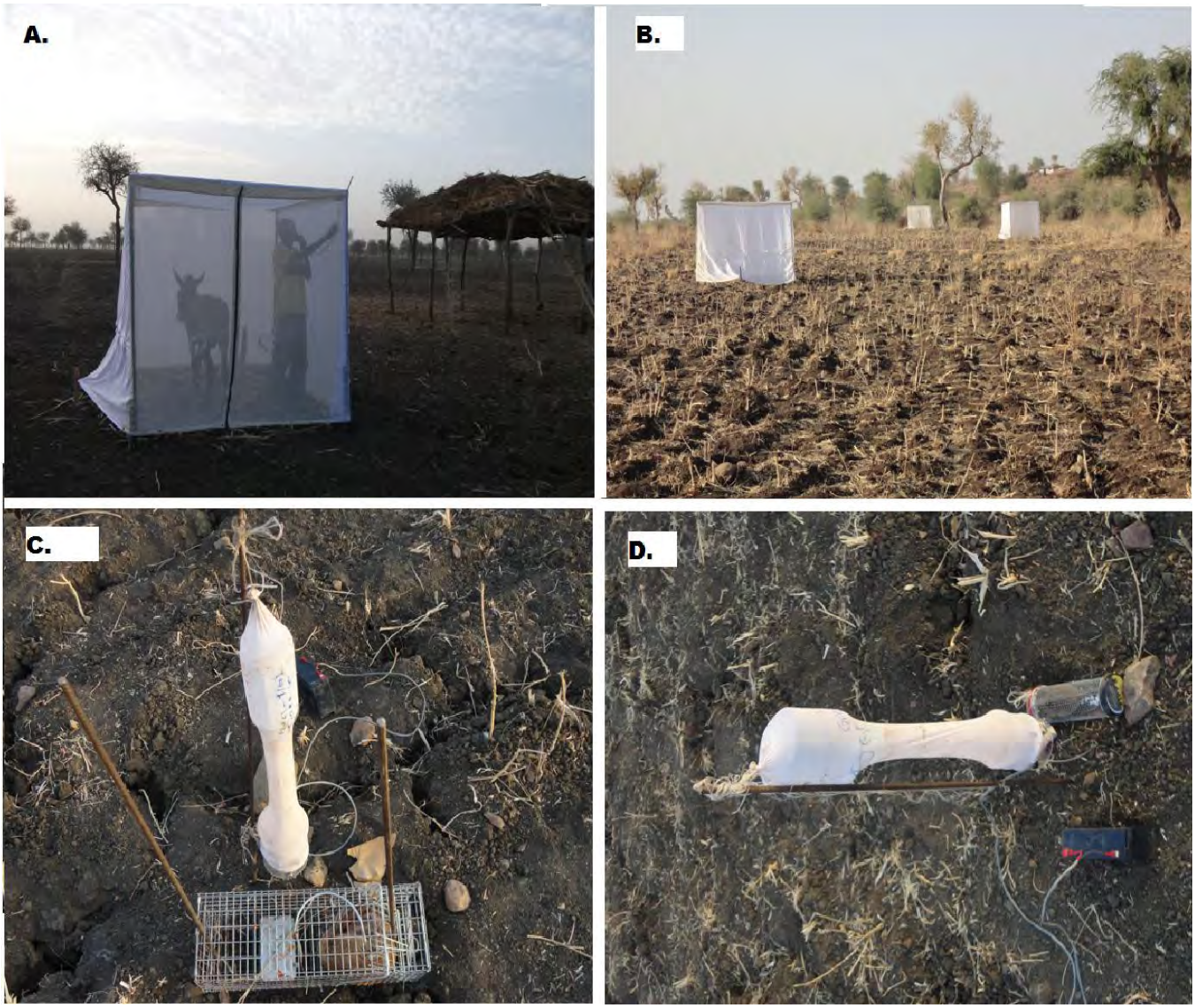

Figure 5.1. Host Attractiveness Experiments. **A**: Tent trap with animal bait. **B**: Arrangement of animal baits in the field. **C**: Cage trap baited with ground squirrel. **D**: Cage baited with rodent species.

5.2.4. Collection of blood-fed sandflies and bloodmeal analysis

Wild-caught females of *P. orientalis* and other *Phlebotomus* spp. with fresh bloodmeals in the collection for bionomic studies (chapter 3) were preserved for later bloodmeal analysis. For this purpose, the head of each fed female was carefully separated from the thorax and slide-mounted for later species identification as indicated in section 2.3. The remaining body parts (thorax and abdomen) were individually placed in empty antibiotic capsules with silica gel grains and cotton pads inside. Similarly, some of blood-fed females were preserved in 70% ethanol for DNA extraction. In the laboratory, the specimens were stored at -20°C for latter bloodmeal analysis using ELISA (Beier *et al*., 1988) and Cyt *b* PCR and RLB (Abbasi *et al*., 2008).

1.8.2.1. Cytochrome *b* PCR and reverse line blotting

DNA extraction: DNA was extracted individually from blood-fed females of sandflies by digestion in a total volume of 200 µL of lysis buffer (50 mM NaCl, 10 mM ethylenediaminetetraacetic acid [EDTA], 50 mM Tris-HCl pH 7.4, 1% triton X-100, and 200 µg/mL of proteinase K). This was followed by extraction with phenol-chloroform and precipitation using ethanol. The precipitated DNA was suspended in Tris-EDTA (TE, 10 mM Tris-HCl pH 7.4, 1 mM EDTA) buffer at a concentration of 50 µL.

PCR amplification of the mtDNA cyt *b* gene: A 344 bp sequence of the conserved region of the mitochondria cyt *b* gene was amplified using bio-tinilated universal primers designed by Abbasi *et al*. (2008). The sequences of the primers used were Cyto1: 5'-CCA TCA AAC ATC TCA GCA TGA TGA AA-3' (forward primer) and Cyto2: 5'-CCC CTC AGA ATG ATA TTT GTC CTC-

3’ (reverse primer). The cyt *b* region was amplified in a total reaction volume of 50 µL consisting of 25 µL Hot start taq Master mix (1.5 mM $MgCl_2$, 200 µL each deoxyribonucleotide triphosphates (dNTP) and 75 mM KCl, 10 mM Tris HCl pH8.8) and 0.5 µM of each primer and 5 µL of genomic DNA. The thermo cyclic conditions consisted of 95°C for 5 min, 35 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min; followed by elongation step at 72°C for 10 min. Cow blood was used as positive control and double distilled water as negative control. The amplified PCR products were used as probes in RLB hybridization reactions followed by chromogenic detection. The methods used by Abbasi *et al*. (2008) were followed for immobilization, hybridization, and detection.

Species-Specific Probes, Immobilization, Hybridization, and Detection: Species-specific 5'-amino-linked oligonucleotide probes for human, cow, sheep, goat, camel, donkey, dog, mice, brown rat, chickens and a general avian probe developed by Abbasi *et al*. (2008) were used in the current study.

The synthetic 5'-end amino modified oligonucleotide probes was covalently linked to nylon membranes through the formation of amide bonds between the carboxyl groups on the nylon and the amino groups linked to the oligonucleotides. Biodyne C (Pall Biomedical, Fajardo, Puerto Rico) nylon membrane were activated in 0.1 N HCl for 5 min, rinsed with DH_2O and soaked in 10% 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide (EDC) (Sigma, St. Louis, MO, USA) for 15 minutes. The membranes were rinsed in DH_2O water and air-dried. Species-specific, 5'-end amino modified oligonucleotides were diluted to 5 p moles/ µL and applied to the membrane in line format using a manifold blotting apparatus.

The nylon membrane sheets with the above-mentioned probes were cut at a right angle to the direction of the blotting so that each strip contained a section of each probe. Strips were incubated in prehybridization solution (2X SSC [0.15 M NaC1, 0.015 M sodium citrate], 0.1% sodium dodecyl sulphate [SDS]) for 30 min at 45^0C with gentle shaking. Biotinylated PCR products were denatured by boiling for 5 min and applied to the membrane strips. Hybridization was performed at 46^0C for 1 h followed by a single wash with 0.7X SSC, 0.1% SDS for 20 minutes. Hybridized biotinylated DNA was detected by incubating the strips in streptavidin-horse radish peroxidase (HRP; diluted in 2X SSC, 0.1% SDS) for 30 min at room temperature. Strips were washed briefly 3 times in 2X SSC, 0.1% SDS. For chromogenic detection, a freshly prepared solution containing 0.1 mg/mL of 3,3'5,5' tetramethylbezidine (Sigma), 0.003% H_2O_2 in 0.1 M sodium citrate (pH 5.0) was added. Enhanced Chemiluminescence (ECL) detection was performed immediately after streptavidin-HRP incubation and washing steps using EZ-ECL detection kit (Biological Industries, Beit Haemek, Israel).

1.8.2.2. Serologic analysis

Bloodmeal origins of freshly fed sandflies were also determined using a direct enzyme-linked immunosorbent assay (ELISA) following the method of Beier *et al*. (1988) and Burniston *et al*. (2010). The abdomen and thorax of each blood-fed sandfly was individually triturated in 2 ml eppendorf tubes with micro-tissue grinders to which 50 µl of 0.01 M phosphate buffered saline (PBS), pH 7.2 was added. Samples were then mixed with PBS to desired dilutions and kept in the refrigerator (4°C) until tested. Sandfly triturate (50 µl) was diluted in PBS (3:50) and then 50 µl of the sample was added to wells of polyvinyl chloride, U-shaped, 96-well

micro titer plates (Dynatech Laboratories, Inc., Alexandria), which were covered and incubated at 4°C overnight. Plates were washed three times with phosphate-buffered saline, Tween 20, pH 7.2. The plate was blocked using 200 µl of bovine serum albumin/carbonate-bicarbonate buffer (200 mg of bovine serum albumin in 20 ml of carbonate-bicarbonate buffer), and left to incubate for 2 h at room temperature, washed three times with phosphate-buffered saline, Tween 20, pH 7.2. This was followed by the dilution of host-specific peroxidase-conjugated anti-IgG antibodies (anti-human, anti-bovine, anti-donkey, anti-dog, anti-goat and anti-sheep) diluted at 1:2,000; 1:250 1:5000, 1:5000, 1:5000 and 1:5000 in 0.5% boiled casein containing 0.025% Tween 20, respectively. Cross-reactions were noted between anti-goat and anti-sheep with the blood samples of cow, human, and donkey. Thus, mixed bloodmeals of these antibodies were excluded. The boiled casein was prepared by dissolving 5 g casein in 100 ml of 0.1 N NaOH by boiling, adding 900 ml PBS, adjusting pH to 7.2 adding 0.1 g Thimerosal (Sodium ethylmercurithiosalicylate) and 0.02 gm phenol red. After 1 h incubation, wells were washed three times with 200 µl PBS Tween 20, and then 100 µl of ABTS (2, 2-azino-di- [3-ethyl benzthiazoline sulfonate]) peroxidase substrate (Kirkegaad and Perry Laboratories, Inc.) was added to each well.

Negative controls were prepared using unfed laboratory-reared female *P. orientalis* while positive serum controls were done by making host serum: PBS dilutions of 3:50 (Ngumbi *et al.*, 1992). Each plate contained a positive control of host species; four negative controls and test samples (1:50 dilution in PBS for all cases). Results were visually assessed, and absorbance was measured with an ELISA reader at 405 nm approximately 30 minutes after

addition of substrate solution. Test samples were considered positive if absorbance values exceeded the mean plus three times the standard deviation of four negative controls.

5.3. Data analysis

Prior to data analysis, sandfly numbers were checked for normality by Shapiro-Wilk test and were log-transformed [log (n+1)] to fit normal distribution. One-way ANOVA was used to compare the mean number of *P. orientalis* attracted to different animal baits and control traps. Tukey's Studentized test post hoc analysis was utilized to ascertain the extent of the difference among the groups in cases where ANOVA was significant. The Kruskal-Wallis test was used when trapping data did not conform to the normal distribution. For nonparametric post hoc comparisons, multiple-Mann-Whitney *U*-test was used and, *P*-values were adjusted with the Bonferroni correction (Dytham, 2011). Statistical analysis were considered significant when $P<0.05$ unless stated.

5.4. Results

5.4.1. Host attractiveness of *P. orientalis* and other sandflies

In the experiment one involving domestic animals and human, 21,189 sandfly specimens, belonging to six species of *Phlebotomus* and seven of S*ergentomyia* were attracted (Table 5.1). Of these, 13,764 were males and 7,425 females. The most abundant species was *P. orientalis* (54.50%) followed by *S. africana* (25.18%).

There were significant differences in the mean numbers of sandfly specimens caught by different baited traps (ANOVA, $F_{(df=7)} = 67.93$, $P<0.05$, Table 5.2). All hosts, except chicken, attracted considerably more sandflies than controls (Table 5.2). Highest attractions in decreasing order of magnitude were found in cow, donkey, and human-baited tents. The cow- and donkey-baited tent traps had significantly higher attractions than all the rest ($P<0.05$).

Table 5.1. Sandfly species captured in tent traps baited with different domestic animals and human in agricultural fields at Tahtay Adiyabo district.

Sandfly Species	**Animal baits**								
	Cow	**Donkey**	**Human**	**Sheep**	**Goat**	**Dog**	**Chicken**	**Control**	**Total**
	M/F	**M/F**	**M/F**	**M/F**	**M/F**	**M/F**	**M/F**	**M/F**	
P. orientalis	5753/855	1661/1323	458/ 595	344/80	213/56	67/39	20/60	16/7	11,547
P. rodhani	0/0	0/0	0/ 0	0/0	4/0	0/0	0/0	0/0	4
P. lesleyae	1/4	1/4	0/9	0/2	5/5	0/1	0/2	1/1	36
P. bergeroti	0/0	0/0	0/0	0/0	4/0	0/0	0/0	0/0	4
P. martini	0/0	0/0	11/11	0/0	4/0	0/0	0/0	0/0	26
P. heischi	0/1	0/0	0/0	0/0	4/0	0/0	0/0	0/0	5
S. africana	305/198	278/203	190/258	568/286	507/257	599/250	553/155	650/79	5,336
S. schwetzi	367/526	433/756	112/105	204/181	109/290	64/173	21/37	7/30	3,415
S. clydei	27/97	23/114	4/5	12/76	23/90	14/45	1/1	3/3	538
S. bedfordi group	10/8	12/10	5/28	6/6	5/9	15/16	2/3	1/0	136
S. antennata group	11/12	12/16	7/6	14/9	4/9	5/15	6/2	5/5	138
S. calcarata	0/0	0/0)	0/0	0/0	0/0	0/1	0/0	0/0	1
S. squamipleuris	0/0	0/0	0/0	1/0	0/0	0/0	1/0	1/0	3
Total	6474/1701	2420/2426	787/1017	1149/640	882/716	764/540	604/260	684/125	21,189

M=Male; F= Female

Table 5.2. Mean numbers (± SE) of sandfly specimens captured in tent traps baited with different domestic animals and human host in agricultural fields at Tahtay Adiyabo district.

Bait types	Mean number ±SE of sandflies collected/tent trap/night
Cow	510.93±75.87[a]
Donkey	302.94±45.74[b]
Human	112. 81±9.60[c]
Sheep	111.81±20.94[c]
Goat	99.88±11.52[c]
Dog	81.50±20.15[c]
Chicken	51.25±10.96[d]
Control	50.50±8.61[d]

Mean values followed by different letters in the same column are significantly different (Tukey's Studentized test; $P<0.05$).

Animal baits differed substantially in their attractiveness to female and male *P*. *orientalis* (Table 5.3, $\chi^2_{K\text{-}W}= 88.79$, df = 7, $P<0.05$). Cow-baited traps collected notably higher mean number of *P*. *orientalis* (mean=413 flies/tent trap) than other baits and control traps. However, donkey-baited traps attracted the highest mean number of *P*. *orientalis* females (mean=82.69) (Table 5.3), though it was not significantly different from cow (multiple-Mann Whitney *U*-test, $P>0.01$). Human bait was the third most effective attractant for collecting large numbers of *P*. *orientalis* females followed by sheep and goat, respectively. Dog and chicken-baited traps were the least attractive to *P*. *orientalis* females with no statistical

difference between them and the control (Multiple-Mann Whitney *U*-test, *P*>0.01) (Table 5.3).

Table 5.3. Mean numbers (±SE) of female and male *P. orientalis* collected and sex ratio of sandflies attracted to tent traps baited with different domestic animals and human.

	Mean number ± SE of sandflies collected/tent trap/night		
Bait types	**Female**	**Male**	**Sex ratio (F/M)**
Cow	53.44±3.19AB	359.56±54.25A	0.15
Donkey	82.69±10.81A	103.81±21.16B	0.8
Human	37.19±2.82C	28.63±3.24C	1.3
Sheep	5.00±1.12D	21.50±4.79CD	0.23
Goat	3.5±1.19DE	13.31±3.34D	0.26
Dog	2.44±0.99EF	4.19±1.52E	0.58
Chicken	0.38±0.25F	1.25±0.61E	0.3
Control	0.44±0.12F	1.00±0.45E	0.44

Mean values followed by different letters in the same column are significantly different (Multiple-Mann Whitney *U*-test; *P*<0.01).

In experiment two, comparing the attractiveness of small wild animals, in total 9,015 sandfly specimens (males: 3,831; females: 5,184), representing 11 species in two genera were captured (Table 5.4). As in experiment one, *P. orientalis* was the dominant species comprising 81.8% of the catch followed by *S. africana* (10.1%). There was a significant difference between the baits and the control in attracting sandfly specimens (ANOVA, $F_{(df=4)}$ = 23.16, *P*<0.05; Fig. 5.2). The mean number of sandflies attracted to ground squirrel was higher than that attracted to hare-baited traps, though these differences were not significantly different (*P*>0.05). There was no statistically significant (*P*>0.05) differential sandfly attraction between the spiny mouse (*A. cahirinus*) and gerbil (*T. robusta*).

Table 5.4. Number of sandfly species attracted to different small wild mammals in agricultural fields at Tahtay Adiyabo district

Sandfly Species	Animal baits										Total
	Squirrel		Hare		Gerbil		Spiny mouse		Control		
	M	F	M	F	M	F	M	F	M	F	
P. orientalis	955	2054	917	906	394	854	523	634	48	89	7374
P. rodhaini	5	5	11	11	6	7	2	3	0	2	52
P. lesleyae	2	2	0	1	0	2	3	2	0	1	13
P. martini	1	0	0	0	0	0	0	0	0	0	1
P. heischi	1	2	3	2	0	0	1	1	0	0	10
S. africana	109	28	243	27	188	26	98	54	112	26	911
S. schwetzi	8	18	11	28	7	14	18	20	6	65	195
S. clydei	26	149	15	57	12	37	18	20	5	5	344
S. bedfordi group	6	1	6	0	11	0	3	3	4	2	36
S. antennata group	8	3	7	7	22	2	9	9	7	4	78
S. adleri	0	0	0	0	0	1	0	0	0	0	1
Total	1,121	2,262	1,213	1,039	640	943	675	746	182	194	9,015

M=Male; F=Female

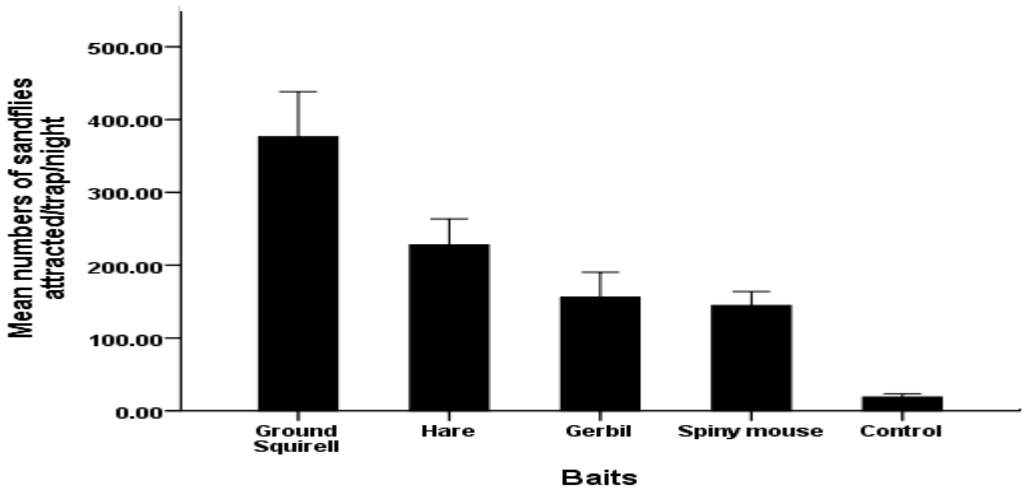

Figure 5.2. Mean numbers (± SE) of sandfly specimens captured in traps baited with different wild small mammals and control traps.

Analysis of variance (ANOVA) revealed that caged-animals differed in their attractiveness to both female and male *P*. *orientalis* ($F_{(df=4)}$ =35.19; *P*<0.05; Table 5.5). Higher mean numbers of *P*. *orientalis* females were attracted to ground squirrel than other baits and control traps (*P*<0.05). The hare was the second attractive animal to *P*. *orientalis* females, followed by *T. robusta* and *A. cahirinus* with insignificant differences in their mean numbers.

Table 5.5. Mean numbers (±SE) of female and male *P*. *orientalis* collected and sex ratio of sandflies attracted to traps baited with small wild mammals

Bait types	Mean number ± SE of sandflies captured/CDC trap/night		
	Female	**Male**	**Sex ratio (F/M)**
Ground squirrel	256.75±44.91A	119.38±19.25A	2.15
Hare	113.25±18.72B	114.63±18.87AB	0.99
Spiny mouse	79.25±14.05B	65.37±7.77BC	1.21
Gerbil	106.75±28.26B	49.25±11.75C	2.17
Control	12.75±2.91C	6.00±1.53D	1.85

Mean values followed by different letters in the same column are significantly different (Tukey's Studentized test; *P*<0.05).

5.4.2. Engorgement rates of *P. orientalis* on baited animals

In experiment one, of the total 2,359 host-seeking *P*. *orientalis* females trapped in baited-tent traps excluding human, 80.57% were freshly engorged. The mean numbers of blood engorged sandfly specimens differed among the other six hosts (Kruskal-Wallis test, $P<0.05$, Table 5.6). *P. orientalis* fed most successfully on donkey (Mean=78.56 engorged flies). Cow was the second preferred host for *P. orientalis*. Conversely, this species fed less successfully on goat, sheep, dog and chicken in decreasing order with no significant difference (multiple-Mann Whitney *U*-test, $P>0.01$).

In experiment two involving small wild animals, only 1.08% (48/4,448 flies) were found with bloodmeals. Although the number of engorged *P*. *orientalis* was small, there were significant differences in the mean numbers of engorged females of sandfly specimens between the four bait species (ANOVA, $F_{(df=3)}= 5.37$; $P=0.005$, Fig. 5.3). Ground squirrel and hare were the preferred hosts over the two rodent species.

Table 5.6. Number and percentage of female sandflies attracted and engorged on different domestic animal baits.

Sandfly Species	Percentage of blood-fed females					
	Cow	Donkey	Sheep	Goat	Dog	Chicken
	% fed	% fed	% fed	% fed	% fed	% fed
P. orientalis	72.8(855)	92.6(1323)	27.5(80)	44.6 (56)	18 (39)	66.7 (6)
P. rodhaini	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
P. lesleyae	0 (4)	0 (4)	0 (2)	0 (5)	0 (1)	0 (2)
P. bergeroti	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
S. africana	0 (198)	0 (203)	0 (286)	0 (257)	0 (250)	0 (155)
S. schwetzi	1.9 (526)	0.7 (756)	0 (181)	0.7 (290)	0 (173)	0 (37)
S. clydei	3.1 (97)	0 (114)	0 (76)	2.2 (90)	0 (45)	0 (1)
S. bedfordi group	0 (8)	0 (10)	0 (6)	0 (9)	0 (16)	0 (13)
S. antennata group	0 (12)	0 (16)	0 (9)	0(9)	0 (15)	0 (2)

* Figures in brackets denote actual number of sandfly females caught in various animal baited tent traps

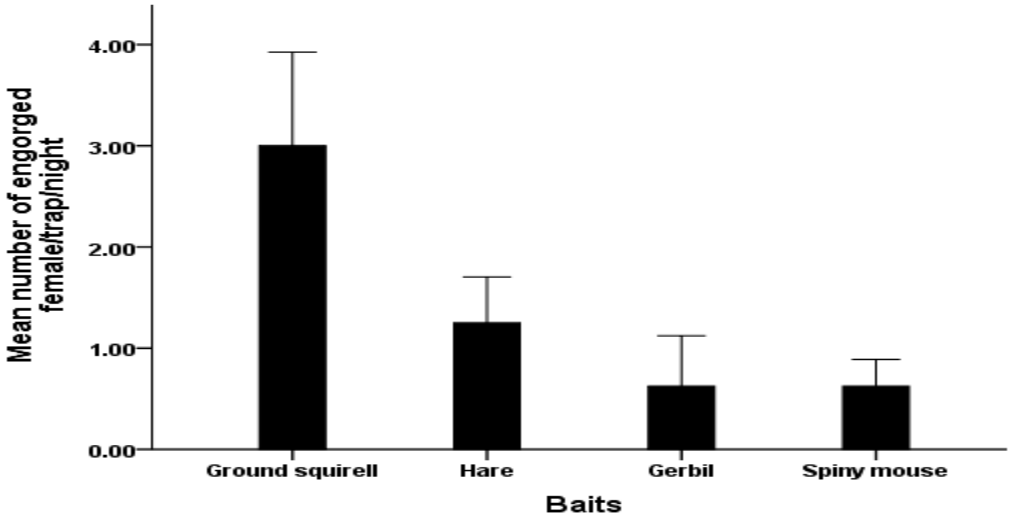

Figure 5.3. Mean numbers (± SE) of engorged female *P. orientalis* on different species of wild small mammals.

5.4.3. Sex ratio

In experiment I, the total number of *P. orientalis* females caught by animal baited traps and control traps combined was smaller than that of males (2,961 female: 8,532 male). The overall female/male sex ratio for *P. orientalis* was 0.54, which was substantially in favour of males (ANOVA, $F_{(df=7)}$= 35.23; P<0.05). It was only in human-baited traps that the female/male ratio (=1.3) was inclined in favour of females (Table 5.3).

In experiment II, the overall female/male sex ratio of *P. orientalis* attracted to host species and control traps was 1.6, which was significantly different (ANOVA, $F_{(df=3)}$= 3.66; P=0.024), showing predominantly female sandfly attractiveness by all hosts except hare baited traps (Table 5.5).

5.4.4. Identification of host animals from bloodmeals of sandflies

In all, 824 blood-fed females were collected (820 *P. orientalis*, 1 *P. papatasi*, 1 *P. bergeroti*, and 2 *P. martini*) and 641 were randomly selected for bloodmeal analysis (Table 5.7). One hundred eighty three of those randomly drawn blood-fed samples were analyzed by cyt *b* PCR-RLB (Table 5.7). On the other hand, the remaining 458 samples were processed by ELISA.

Table 5.7. Number of blood-fed sandflies tested, listed by species, location, and method

Species	Villages						Total
	Ademeyti		Lemlem		Mentebteb		
	ELSA	RLB	ELSA	RLB	ELSA	RLB	
P. orientalis	220	87	230	68	7	25	637
P. papatasi	0	0	0	1	0	0	1
P. bergeroti	0	0	0	1	0	0	1
P. martini	0	1	0	0	1	0	2

Cyt *b* PCR-RLB

A total of 183 blood-fed *Phlebotomus* spp. (180 *P*. *orientalis*, 1 *P. papatasi*, 1 *P. bergeroti*, and 1 *P*. *martini*) were analyzed for bloodmeal identification and 168 (91.8%) were positive to cyt *b* PCR (Fig. 5.4). The remaining 15-bloodmeal samples of *P. orientalis* did not produce distinctive bands for cyt *b* amplifications. All the PCR-positive samples were used for the identification of bloodmeals imbibed in female sandflies using RLB (Table 5.8; Fig. 5.5). Successful identification of the host from bloodmeals was achieved in 137/165, 1/1, 1/1 and 1/1 of *P. orientalis*, *P. papatasi*, *P. martini* and *P. bergeroti*, respectively. However, some of the samples (i.e., 28) which were positive for cyt *b* PCR amplification did not produce bands on the RLB analysis (Table 5.8).

A high proportion of *P. orientalis* was found to have fed on bovine blood in all the three study villages (Table 5.8). In Ademeyti, out of 87 bloodmeals tested 41 (51.25%), 10 (12.5%), 3 (3.75%), 1 (1.25%) and 10 (12.5%) contained blood of bovine, human, goat, sheep and mixed hosts, respectively. In Lemlem, 58.3% and 13.3 % of *P. orientalis* fed on bovine and human blood respectively while the remaining 31.67% fed on the blood of other hosts. The

proportions of *P. orientalis* found to be positive to bovine, human and goat in Mentebteb were 64%, 16%, and 2%, respectively. Moreover, large proportions of unidentified bloodmeals were found in Ademeyti and Lemlem villages compared to Mentebteb. One specimen of *P. martini* was positive to human blood in Ademeyti. Similarly, *P. papatasi* and *P. bergeroti* each with one specimen contained human blood.

In terms of collection habitats, human blood proportions for *P. orientalis* were 25.0%, 6.54% and 26.32% indoor, per-domestic and agricultural field, respectively (Table 5.9). For bovines, however, it was 20.0% indoor, 66.36% in peri-domestic and 44.74% in agricultural fields. A single bloodmeal source of sheep and camel was detected in *P. orientalis*. Mixed bloodmeals in *P*. *orientalis* females, including human-cow, human-cow-goat-camel, human-cow-goat, cow-goat, and cow-sheep were detected using the PCR-RLB technique (Tables 5.8 and 5.9; Fig. 5.5).

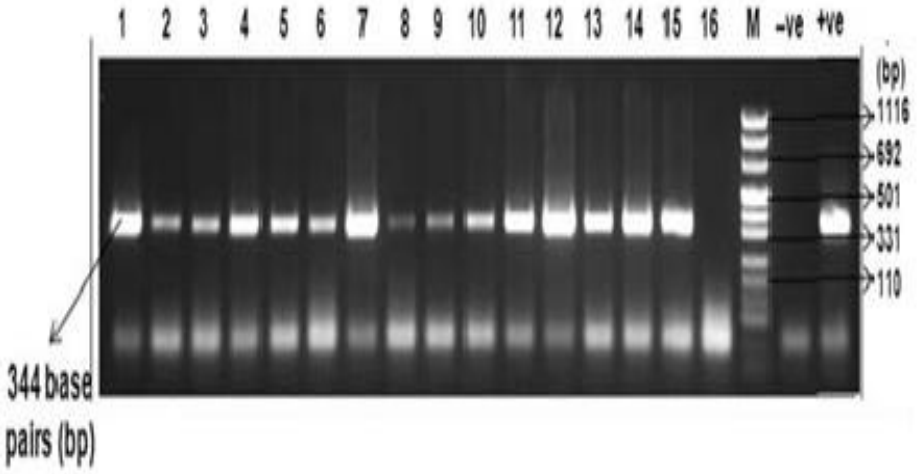

Figure 5.4. Gel image of cyt *b* PCR reaction targeting DNA extracted from wild caught blood fed sandflies. Lanes 1 to 16 PCR product of blood fed *P. orientalis* amplified for cyt *b* region. **M**: is DNA ladder. **–ve**: control (pure water). **+ve**: positive control (cow).

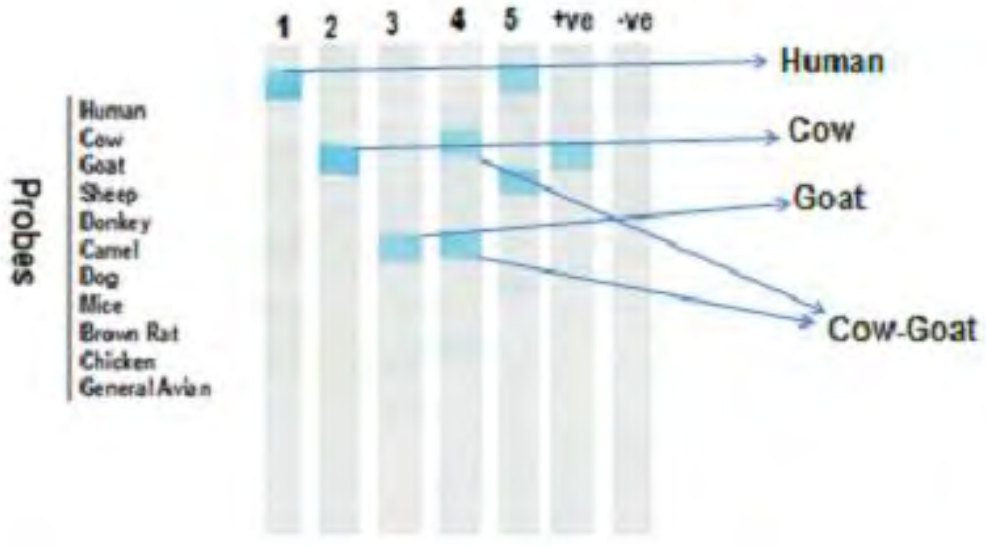

Figure 5.5. Representative RLB results of cyt *b* PCR products from blood-fed, *P. orientalis*. Sample number 1: human blood. Sample 2: cow blood. Sample 3: goat blood. Sample 4: cow and goat. Sample 5: human and cow blood. +ve sample is cow blood. -ve sample with no PCR product.

Table 5.8. Bloodmeal sources of *P. orientalis* captured from three different villages and identified using cyt *b* PCR and RLB

Sources of bloodmeal	Number (%) of blood-fed sandfly specimens			
	Ademeyti	**Lemlem**	**Mentebteb**	**Total (%)**
Bovine	41 (51.25)	35 (58.33)	16 (64.0)	92 (55.76)
Human	10 (12.5)	8 (13.33)	4 (16.0)	22 (13.33)
Goat	3 (3.75)	3 (5.0)	2 (8.0)	8 (4.85)
Sheep	1 (1.25)	0	0	1 (0.61)
Camel	0	0	1 (4.00)	1 (0.61)
Human-Bovine	4 (5.0)	2 (3.33)	0	6 (3.64)
Human-Bovine-Goat	3 (3.75)	0	0	3 (1.82)
Human-Bovine-Goat-Camel	1 (1.25)	0	0	1 (0.61)
Bovine-Goat	1 (1.25)	1 (1.67)	0	2 (1.21)
Bovine-Sheep	1 (1.25)	0	0	1 (0.61)
Unidentified	15 (18.75)	11 (18.33)	2 (8.0)	28 (16.97)
Total (+cyt *b* PCR)	80	60	25	165
Negative	7	8	0	15
Total (tested)	87	68	25	180

Table 5.9. Number and percentage of bloodmeal sources of *P*. *orientalis* collected from different habitats and detected by Cyt *b* PCR-RLB

Sources of bloodmeal	Number (%) of blood-fed sandfly specimens		
	Indoor	**Peri-domestic**	**Agricultural field**
Bovine	4(20.0)	71(66.36)	17(44.74)
Human	5(25.0)	7(6.54)	10 (26.32)
Goat	2(10.0)	1(0.93)	5(13.16)
Sheep	0	0	1(2.63)
Camel	0	0	1(2.63)
Human-Bovine	3(15.0)	4 (3.74)	0
Human-Bovine-Goat	3(15.0)	0	0
Human-Bovine-Goat-Camel	1(5.0)	0	0
Bovine-Goat	0	1(0.93)	0
Bovine-Sheep	0	0	1(2.63)
Unidentified	2(10.0)	23(21.5)	3(7.89)
Total (+cyt *b* PCR)	20	107	38
Negative	0	12	3
Total (tested)	20	119	41

ELISA assay

Table 5.10 shows the bloodmeal origins of *P. orientalis* from different sampling villages. In this assay, 458 blood-fed females of *P. orientalis* (n=457) and *P. martini* (n=1) were analyzed, and a general reactivity index of 92.1% was obtained against antibodies of test animals (human, bovine, donkey, dog, and goat/sheep). *Phlebotomus orientalis* had fed on five hosts, with feeding patterns of: bovine (46.6%), donkey (9.63%), human (6.78%), goat/sheep

(5.03%), and dog (1.75%) (Table 5.10). Additionally, 103 (22.54%) of *P. orientalis* had bloodmeals of mixed origin (Table 5.10). Hosts for the remaining 7.66% of the blood samples, could not be identified. One specimen of *P. martini* collected from Mentebteb also had mixed bloodmeal of bovine-donkey-dog.

Further classifications of the bloodmeals by sampling villages and habitat types are presented in Tables 5.10 and 5.11. In Ademeyti and Lemlem, the highest proportion of bloodmeal index was for bovine, constituting 51.36% and 42.61%, respectively. Whereas in Mentebteb, the highest bloodmeal index was for donkey (42.86%) followed by bovine and human was 28.57% for each.

The bloodmeal analysis also indicated that the host preference of *P. orientalis* differed depending on sampling habitats. *Phlebotomus orientalis* females caught indoor had bloodmeal origin in the following order: 34.78%, 26.09%, 17.39%, and 8.7% for bovine, human, donkey, and goat/sheep, respectively (Table 5.11). In peri-domestic habitat, the highest host preference was for bovine (51.76%) followed by donkey (10.0%), human (6.47%), dog (2.35%) and goat/sheep (2.35%). Similarly, the rate of host feeding patterns of *P. orientalis* on different hosts in agricultural field ranged from 30.85% of bovine to 3.19% of human.

Table 5.10. Results of ELISA assays on bloodmeals of *P. orientalis* collected from different study villages of Tahtay Adiyabo district

Bloodmeal origins	Number (%) of blood-fed sandfly specimens			
	Ademeyti	**Lemlem**	**Mentebteb**	**Total**
Bovine	113 (51.36)	98 (42.61)	2 (28.57)	213 (46.61)
Donkey	16 (7.27)	25 (10.87)	3 (42.86)	44 (9.63)
Human	12 (5.45)	17 (7.39)	2 (28.57)	31 (6.78)
Goat/sheep	12 (5.45)	11 (4.78)	0	23 (5.03)
Dog	3 (1.36)	5 (2.17)	0	8 (1.75)
Bovine-Donkey-Dog	10 (4,55	19 (8.26)	0	29 (6.35)
Bovine-Dog	12 (5.45)	8 (3.48)	0	20 (4.38)
Donkey-Dog	4 (1.82)	6 (2.61)	0	10 (2.19)
Human-Bovine-Dog	2 (0.92)	0 (0)	0	2 (0.44)
Human-Bovine	2 (0.92)	7 (3.04)	0	9 (1.97)
Human-Bovine-Donkey	11 (5)	10 (4.35)	0	21 (4.60)
Human-Donkey	0	5 (2.17)	0	5 (1.10)
Human-Dog	1 (0.45)	2 (0.87)	0	3 (0.66)
Human-Donkey-Dog	1 (0.45)	2 (0.87)	0	3 (0.66)
Unidentified	20 (9.09)	15 (6.52)	0	35 (7.66)
Total	220	230	7	457

Table 5.11. Bloodmeal origins of *P. orientalis* collected indoors, peri-domestic and agricultural field as determined by ELISA assay

Bloodmeal origins	Number (%) of blood-fed sandfly specimens		
	Indoor	**Peri-domestic**	**Agricultural field**
Bovine	8(34.78)	176(51.76)	29(30.85)
Donkey	4(17.39)	34(10.0)	6(6.38)
Human	6(26.09)	22(6.47)	3(3.19)
Goat/sheep	2(8.70)	8(2.35)	13(13.83)
Dog	0	8(2.35)	0
Bovine-Donkey-Dog	2(8.70)	14(4.12)	13(13.83)
Bovine-Dog	0	18(5.29)	2(2.13)
Bovine-Donkey	0	1(0.29)	0
Donkey-Dog	0	6(1.76)	4(4.25)
Human-Bovine-Dog	0	2(0.59)	0
Human-Bovine	0	8(2.35)	1(1.06)
Human-Bovine-Donkey	1 (4.35)	17(5.0)	3 (9.57)
Human-Donkey	0	4(1.18)	1(1.06)
Human-Dog	0	2(0.59)	1(1.06)
Human-Donkey-Dog	0	2(0.59)	1(1.06)
Unidentified	0	18(5.29)	17(18.09)
Total	23	340	94

5.5. Discussion

Host preferences of insect vectors represent an important aspect of the bionomics of vector-borne disease dynamics, directly affecting the magnitude of disease transmission. The current study focused on analyzing the host preference of *P. orientalis* through host choice experiment and bloodmeal source determination of wild caught females. The host choice experiment described here indicated that females of *P. orientalis* were attracted and engorged more frequently upon certain hosts than others. Host attractiveness to sandflies varies temporally and spatially; phenomena which could be associated with host body surface area, dose-specific responses to ubiquitous cues such as CO_2 and host-specific odors (Quinnell *et al*., 1992; Hamilton and Ramsoondar, 1994; Adler *et al*., 2003).

In the experiment involving domestic animals and humans, large numbers of *P. orientalis* females were attracted and engorged on donkey and cow than other hosts. Similar results have been previously recorded for Old as well as New World vectors (Mutinga *et al*., 1986; Quinnell *et al*., 1992; Teshome Gebre-Michael *et al*., 2010). Prominently, cattle constitute favored bloodmeal sources for female *P. orientalis* as demonstrated in direct bloodmeal analysis by PCR-RLB and ELISA with the proportion of 55.76% and 46.61% bovine blood index, respectively.

Large proportions of *P. orientalis* contained bloodmeals of cattle, which could be related to their availability and abundance as well greater release of kairomones, compared to other animal hosts in the area (Quinnell *et al*., 1992; Hamilton and Ramsoondar, 1994). Secondly, this could also be associated with capturing of most engorged *P. orientalis* females from

peri-domestic habitats. In the study area, cattle are raised in large numbers by villagers and are usually kept in enclosures close to dwellings. The accessibility of bovine blood hosts to questing *P. orientalis* females in the peri-domestic habitats may provide zooprophylactic barrier potentially reducing human-vector contact, or it may aggravate the risk of VL infection. Studies in Nepal (Bern *et al*., 2005) showed that ownership or proximity of livestock was associated with significant protection of VL infection, whereas in India VL appeared to increase for those living in close proximity to cattle (Barnett *et al*., 2005). Therefore, the putative role of cattle in the epidemiology of VL in this focus requires detailed and thorough investigation.

Although *P. orientalis* females were found attracted and engorged avidly on donkey during the host choice study, less proportion (9.6%) of donkey blood origin was detected in the bloodmeal analysis. Discrepancy between the two methods could be attributed to the lesser abundance of donkeys in these study villages, thereby reducing their accessibility to sandfly bite. The role of donkeys in the epidemiology of VL is a subject of further study.

The current as well as a previous study from Sudan has shown that humans are attractive hosts to *P. orientalis* (Elnaiem *et al*., 1999b). Besides, 8.5% (both methods combined) of the wild collected *P. orientalis* females contained human blood origin in the bloodmeal identification. This finding supports the likelihood that *P. orientalis* is the vector of VL in these parts of East Africa since attraction to humans by a sandfly vector is a minimum requirement for disease transmission (Campbell-Lendrum *et al*., 1999).

Relatively few *P. orientalis* females were attracted to and engorged on hosts of sheep, goat, dog, or chicken. In support of this finding, bloodmeal analyses of engorged wild-caught females revealed that only a small proportion had fed upon these hosts. Thus, the results of the current study do not support a role of goats, sheep, dogs, and chickens as food source for *P. orientalis* and *P. martini* as suggested in previous studies from Kenya (Mutinga *et al*. 1986; Johnson *et al.*, 1993) and Sudan (Hassan *et al*., 2009). These variations might be due to differences in the innate behavior of the sandfly species involved, and the experimental design used. Importantly, parasitological studies in Kenya also confirmed that sheep could not support the infection of *L. donovani* (Anjili *et al*., 1988; Anjili *et al*., 2012). Chickens are refractory to infection by *Leishmania* and their blood is less nutritious than that of mammals (Alexander *et al*., 2002; Bruce *et al*., 2002).

In the experiment using small mammals, *P. orientalis* was more attracted to ground squirrels (*X. rutilus*), followed by the hares (*Lepus* sp.), gerbils *(T. robusta*), and the spiny mice (*A. cahirinus*). However, the blood feeding rates in all cases were very low compared with baited-tent traps above, probably because the attracted sandflies in this case were trapped before they had sufficient time to feed on the hosts. Rejection does not seem to be the case, since these small mammals are the common animals that *P. orientalis* would encounter in the wild including fissures in vertisols and fields, where humans and domestic animals are absent. It was previously observed that wild-caught *P. orientalis*, *P. martini* and other sandfly vectors had fed upon squirrels and rodents (Ngumbi *et al*., 1992; Hassen Mamo, 1999; Svobodová *et al*., 2003). Concomitantly, different species of rodents are known as the reservoir hosts of *Leishmania* spp. in the various parts of the world (De Lima *et al*., 2002;

Mehrabani *et al*., 2007; WHO, 2010) and hares (*L. granatensis*) were recently incriminated as reservoir hosts of *L. infantum* in Spain (Molina *et al,* 2012*;* Moreno *et al*., 2013). The exact role of these animals in the epidemiology of VL in the study area remains to be explored.

The apparent presence of multiple bloodmeals from a single specimen in some females is a strong evidence of the eclectic diet of *P*. *orientalis*. This behavior is a common phenomenon in sandflies and therefore it may be a result of the difficulties sandflies face in freely engorging on a single host due to host defensive mechanisms, little or no exposed host skin or the difficulty to locate adequate skin blood capillaries (Bongiorno *et al*., 2003). For instance, *P. orientalis* females analyzed for bloodmeal in northeast Ethiopia had 54.3% multiple bloodmeals from available vertebrate hosts (Hassen Mamo, 1999), the predominant being cattle-camel hosts (60%). This host-feeding behavior can influence pathogen transmission through increased frequency of vector-human contact, or possibly reduce vector-human contact if some bloodmeals are taken from alternative mammalian hosts.

Prominently, 17% in PCR-RLB and 7.7% in ELISA assays of the bloodmeal samples were not from any of the eleven oligonucleotide probes or antibodies tested. These unidentified bloodmeals could belong to different species of wild animals found in the area; in particular, squirrels, rodents, hares and other small carnivores, which are highly attractive to *P. orientalis* in the host choice experiment. Alternatively, in ELISA assays it could have resulted from enzymatic degradation of the blood. Therefore, when there are large numbers of

potential hosts it may be appropriate to consider applying direct nucleotide sequencing when conducting bloodmeal analyses.

Male sandflies predominated in the tent traps-baited with large domestic animals, indicating that mating occurs on the host. Swarms of mating sandflies often form close to the animals used as bloodmeal source by females (Palit *et al*., 1993). This suggests that males of *P. orientalis* exhibit some sort of aggregation behavior on hosts, which has been observed in *P. argentipes* (Lane *et al*., 1990) and *Lu. longipalpis* (Jarvis and Rutledge, 1992). Unlike the larger domestic animals, however, the sex ratio in small wild animals was female biased except for hares. This variation in sex ratio between the larger and smaller animals may be associated with the differences in body posture of the bait animals or the design of trappings used in both experiments.

In conclusion, the results of host choice and bloodmeal analysis demonstrated that *P. orientalis* is primarily zoophilic in its host preference with feeding habits that may vary depending on the availability of hosts. In addition, increased predilection of *P. orientalis* to bite cattle, the predominant domestic animal in this particular area, may have a protective or increased exposure to VL, which requires further investigations. This zoophilic behavior can, however, be exploited for killing sandflies using insecticide treated animals (Warburg and Faiman, 2011). Furthermore, detailed parasitological and xenodiagnostic studies on cattle, donkeys, and the small rodents and hares, may shed some light on the epidemiology of kala-azar, facilitating the implementation of effective control strategies.

Chapter Six

Nocturnal activity rhythms of *Phlebotomus orientalis*

6.1. Introduction

Phlebotomine sandflies have considerable public health importance in the tropics and subtropics attributed mainly to their role as potent vectors of the various forms of leishmaniasis (visceral and dermal), bartonellosis, and 3-day fever (papataci fever). These diseases are transmitted by the bite of infected female sandflies of the genus *Phlebotomus* in the Old World and *Lutzomyia* in the New World when taking repetitive bloodmeals (Tesh, 1988; Killick-Kendrick, 1999; Maroli *et al*., 2013).

Most sandflies from the Old World are active during the night (Killick-Kendrick, 1999; Dinesh *et al*., 2001; Kravchenko *et al*., 2004; Gaglio *et al*., 2014) for sugar feeding, host seeking, blood feeding, mating, and oviposition (Forattini 1973; Yuval and Schlein, 1986; Morrison *et al*., 1995; Ngumbi *et al*., 2012). However, species-specific differences are observed in the peak activity, which can influence the vectorial capacity of different species (Rahman *et al*., 1986; Dinesh *et al*., 2001; Souza *et al*., 2005). Yet, little is known why the nocturnal activity of most sandflies varies with season and time.

Information on the nocturnal activity and biting rhythms of *P. orientalis* is available from Sudan and two works from north-west Ethiopia (Quate, 1964; Ashford, 1974; Elnaiem *et al*., 1997; Lemma *et al*., 2014). In Sudan, Quate (1964) indicated that peak biting activity of *P. orientalis* took place between 18:30 and 20:30 hours. Recently, Elnaiem (2011b) reviewed that in Dinder National Park in eastern Sudan, the hourly light trap and human-landing

collections of *P. orientalis* continued until late in the night. Earlier, Gebre-Michael and Lane (1996) studied the nocturnal periodicity of *P. martini* and *P. celiae* in southern Ethiopia. Nevertheless, this has not been systematically investigated on populations of *P. orientalis* in northern Ethiopia.

Knowledge on the sandfly nocturnal activity is noteworthy because it indicates the time when a person is most likely to be bitten by the sandfly vector and possibly get leishmaniasis. It also reveals the best possible time to collect and monitor the adults. As well, information on the peak activity period of sandfly vectors can be used to schedule outdoor activities to avoid peak exposure periods. In this perspective, the current study was designed to observe the nocturnal activity patterns of *P. orientalis* in an endemic focus of VL in Tahtay Adiyabo district, northern Ethiopia. Effects of variations in hourly nighttime temperature and relative humidity on the nocturnal activity patterns of the vector species were also studied.

6.2. Materials and Methods

6.2.1. Sandfly trapping

The study was conducted in the locality of Geza Adura within Lemlem village and sandflies were sampled using two CDC light traps from outdoors (viz., peri-domestic and agricultural fields) twice a month for 6 months (between January 2013 and June 2013), when the sandfly abundance was high. CDC light traps were set and their collecting cages were replaced at hourly intervals starting before sunset till after sunrise (18:00-07:00 hrs). The removed collection cages were replaced by another set of bags for the next one-hour. The next morning, captured sandflies were transported to the laboratory where female *P. orientalis*

were sorted out from males and the rest of *Phlebotomus* and *Sergentomyia* spp. Male *Phlebotomus* and *Sergentomyia* spp. were preserved in 70% ethanol for later species identification as indicated in section 2.3.

6.2.2. Determination of abdominal status and parous rates of female *P. orientalis*

Females of *P. orientalis* were examined for abdominal status and the numbers of unfed, freshly fed, half-gravid and gravid sandflies were recorded. After that, representative samples of unfed females of *P. orientalis* were dissected under a microscope to determine parity (reproductive history) and gonotrophic states as described above (Chapter 4).

6.2.3. Meteorological data recording

Variations in hourly temperature and relative humidity (RH) were recorded using data loggers (HOBO Micro Station©) on an hourly basis during the collection nights.

6.3. Data analysis

Prior to data analysis, sandfly numbers were checked for normality by Shapiro-Wilk test. One-way ANOVA was used to compare the overall (male and female) hourly activity patterns of *P. orientalis* during the night. Tukey's Studentized test post hoc analysis was utilized for mean separation where ANOVA was significant. The Kruskal-Wallis test was used to compare the hourly activity of individual male and female *P*. *orientalis*. Similarly, to assess differences in parity and blood feeding rates among collection intervals, a Kruskal-Wallis test was followed. For non-parametric comparisons, multiple-Mann-Whitney test (*U*) was used and, *p*-values were adjusted with the Bonferroni correction (Dytham, 2011). Spearman rank-

correlation (r_s) analysis (P<0.05) was also used to compare the effects of average nighttime temperature and humidity on the number of sandflies captured per hour. Statistical analysis were considered significant when P<0.05.

6.4. Results

6.4.1. Nocturnal activity rhythms

In total, 21,716 nocturnally active sandfly specimens, which belong to two genera were collected and identified. Of those, 14,158 (65.20%) and 7,558 (34.80%) were males and females, respectively. In the collection, *P. orientalis*, the dominant species in the genus *Phlebotomus*, constituted 33.79% while *Sergentomyia* spp. comprised 65.44% (Table 6.1).

Table 6.1. Nocturnally active sandfly species captured using CDC light traps in Tahtay Adiyabo district, January-June 2013.

Sandfly Species	Number of sandflies collected		
	Male	**Female**	**Total (%)**
P. orientalis	5,343	1,995	7,338 (33.79)
P. bergeroti	54	17	71 (0.33)
P. lesleyae	9	20	29 (0.13)
P. rodhaini	6	15	21 (0.09)
P. heischi	6	11	17 (0.08)
P. duboscqi	7	8	15 (0.07)
P. martini	3	6	9 (0.04)
P. papatasi	1	2	3 (0.01)
P. alexandri	0	1	1 (0.005)
Sergentomyia spp.	8,729	5,483	14,212 (65.44)
Total	14,158	7,558	21,716 (100)

The overall hourly activity patterns of *P. orientalis* were significantly different among collection intervals (ANOVA, $F_{(df=12)}$ =8.04; *P*=0.000, Fig. 6.1) with a peak nocturnal activity (21.5 flies/trap/hr) before midnight (22:00-23:00 hrs). Increased *P. orientalis* activity

continued after midnight until a smaller peak towards dawn (04:00-05:00 hrs) and sharp decline afterwards (Fig. 6.1). The Kruskal-Wallis test also indicated a significant difference in the mean number of male ($\chi^2_{K\text{-}W}$= 40.63, df =12, *P*=0.000) and female ($\chi^2_{K\text{-}W}$= 54.19, df= 12, *P*=0.000) *P. orientalis* caught in each hour (Fig. 6.1). Females of *P. orientalis* were lower in number than the males throughout the night, but tended to increase slowly as the night progressed with a peak at just after midnight (24:00-03:00 hrs), after which decreased progressively (Fig. 6.1). *Phlebotomus orientalis* males had a single peak at 22:00-23:00 hrs, though continued to be moderately active until 05:00 hrs (Fig. 6.1).

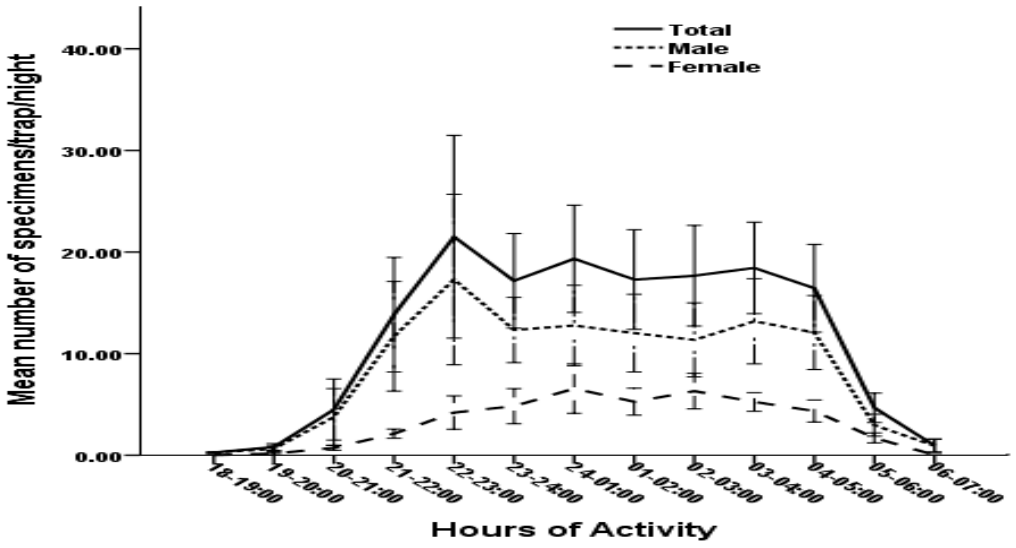

Figure 6.1. Nocturnal activity patterns of female and male *P. orientalis*

The exact timing of the peak activity of the females varied with months (Fig. 6.2A-F). The nocturnal activity rhythm of *P. orientalis* females in January, February, April, and June was similar, showing maximal activity in the second half of the night. On the other hand, the peak nocturnal periodicity of females in March and May was in the first half of the midnight (Fig. 6.2C and D).

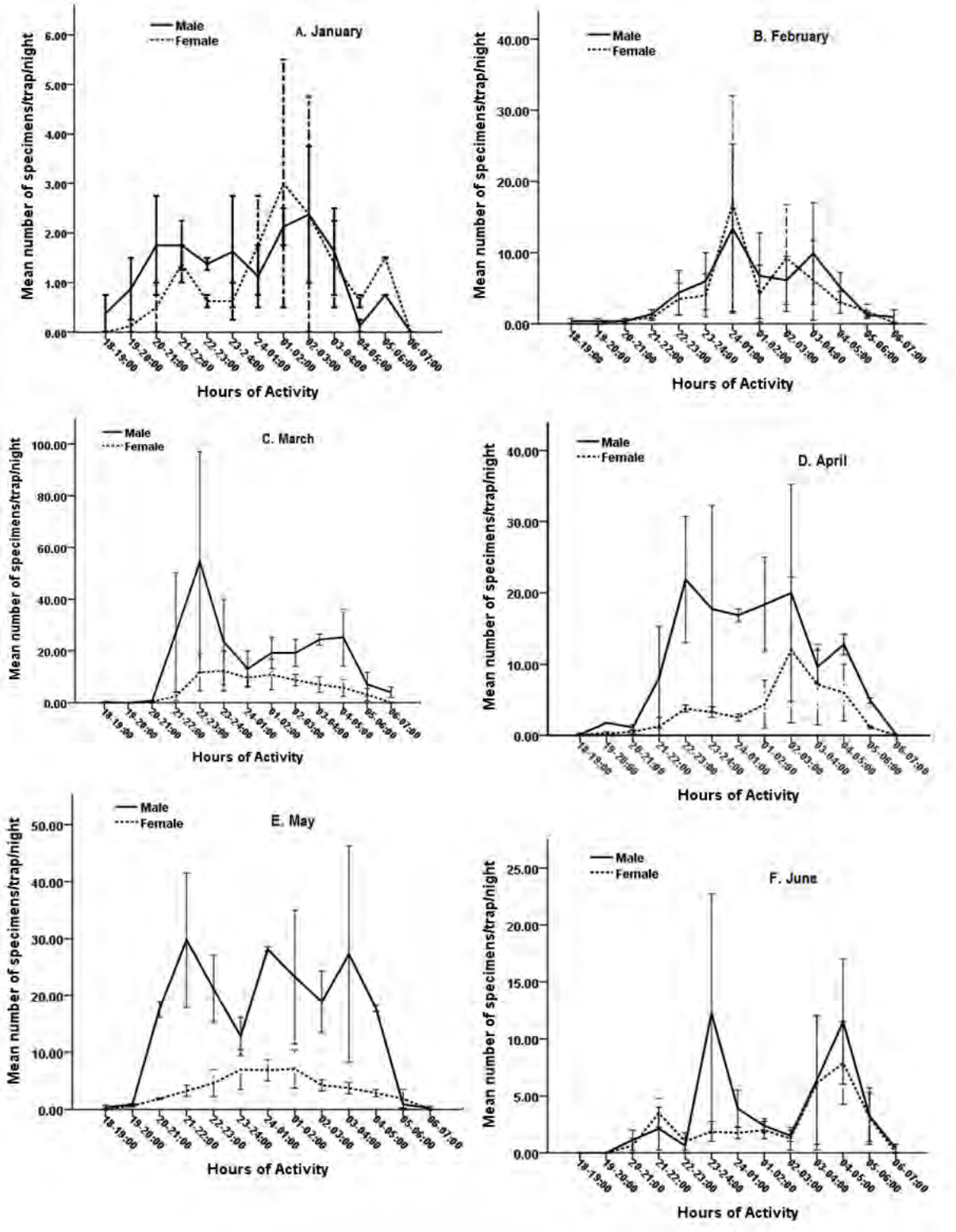

Figure 6.2. Hourly nocturnal periodicity of *P. orientalis* male and females in each month

6.4.2. Abdominal status and parous rates

Abdominal categories for nocturnally active *P. orientalis* females are shown in Table 6.2. Of 1,995 *P. orientalis* females trapped, 1,630 (81.70%) were unfed, 305 (15.29%) freshly blood-fed, 14 (0.70%) semi-gravid and 46 (2.31%) gravid. Significant differences in the hourly nighttime blood feeding pattern of *P. orientalis* females were observed ($\chi^2_{K\text{-}W}$= 23.01, df =12, *P*<0.05; Table 6.2). Higher proportions (62.29%) of freshly blood-fed females were caught after midnight with peak at 24:00-01:00 hr. However, blood-feeding activity of this species steadily declined after 05:00 hr.

Table 6.2. Abdominal status of nocturnally active females of *P. orientalis* during January-June 2013

Hours of Activity	Abdominal status				
	Unfed	Freshly blood-fed	Semi-gravid	Gravid	Total
18:00-19:00	1	0	0	0	1
19:00-20:00	7	1	0	0	8
20:00-21:00	29	4	1	1	35
21:00-22:00	61	27	2	12	102
22:00-23:00	157	38	1	6	202
23:00-24:00	177	45	0	5	227
24:00-1:00	265	50	1	4	320
1:00-2:00	205	46	0	2	253
2:00-3:00	264	32	3	4	303
3:00-4:00	220	26	5	1	252
4:00-5:00	172	27	0	10	209
5:00-6:00	71	9	1	1	82
6:00-7:00	1	0	0	0	1
Total	1,630 (81.70%)	305 (15.28%)	14 (0.70%)	46 (2.31%)	1,995

Out of 236 unfed females dissected to determine parity rate, 80 (33.90%) were found to be parous. There was significant difference in the hourly proportion of parous females caught, where the highest (55.90%) being after midnight (24-01:00) ($\chi^2_{K\text{-}W}$= 28.59, df =12, P<0.05, Fig. 6.3). Nulliparous females; however, had bimodal peak activity period: the first between 22:00 and 23:00 hrs and the rest of 02:00-03:00 hrs (Fig. 6.3).

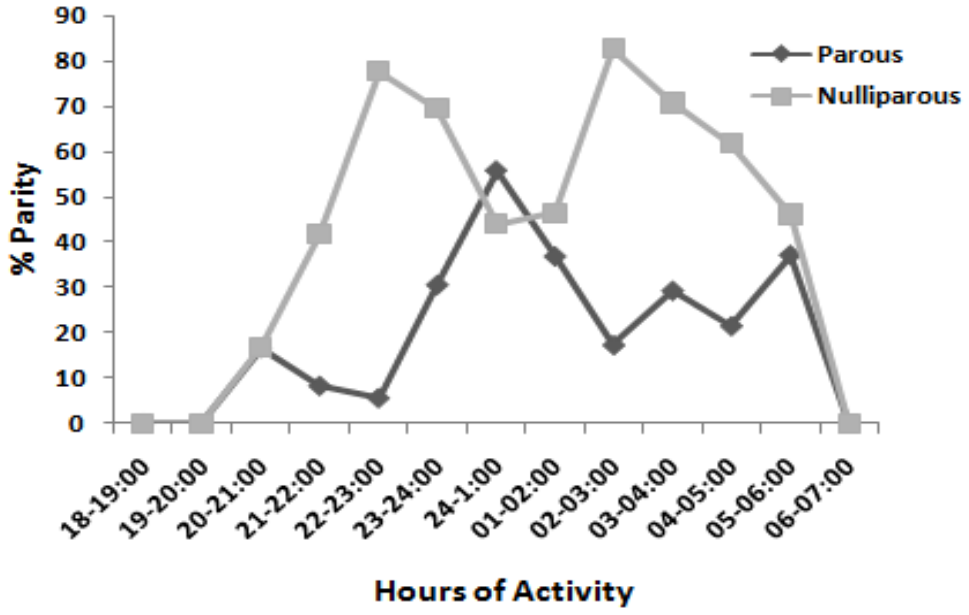

Figure 6.3. Hourly proportion of nulliparous and parous *P. orientalis* females

6.4.3. Effects of temperature and relative humidity on nocturnal activity

The relationship between the activity of *P. orientalis* males and females and weather variables (temperature and relative humidity) is illustrated in Fig. 6.5 and Annex 3. Temperature decreased and relative humidity increased after sunset through the night. There was no significant correlation between the number of male *P. orientalis* caught at hourly intervals with the hourly night temperature (r_s=-0.129; P=0.259) and relative humidity (r_s =0.032, P=0.783). Whereas, the nocturnal activity of female *P. orientalis* had a weak

negative significant correlation with temperature (r_s =-0.229, P=0.044) and a weak non-significant positive correlation with relative humidity (r_s =0.173, P=0.129).

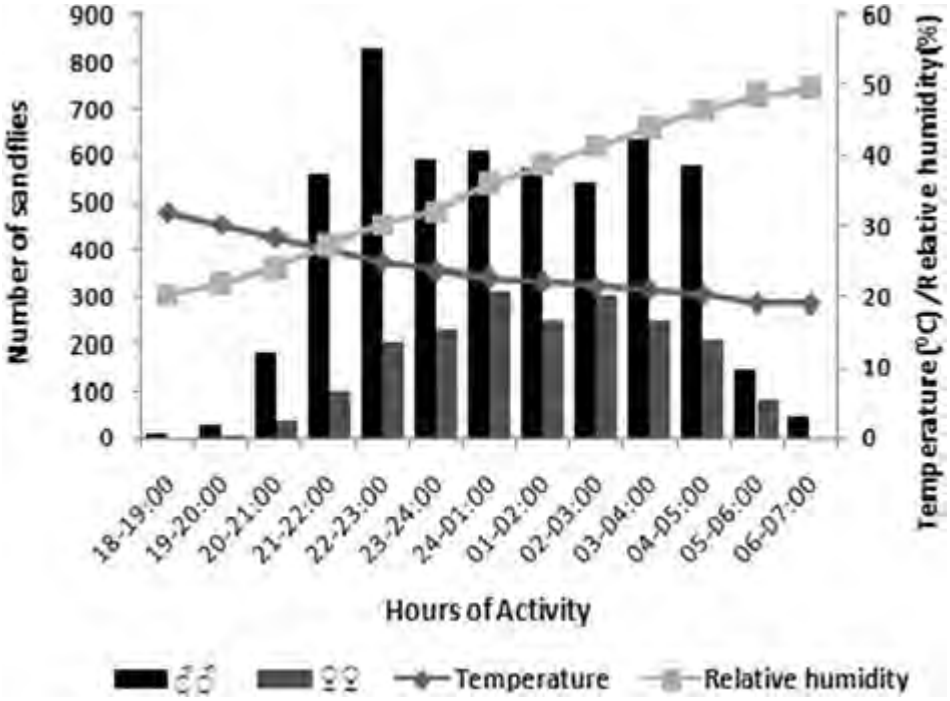

Figure 6.4. Nocturnal activity rhythms of male and female *P. orientalis* relative to average temperature and relative humidity at different hours of night, January-June 2013.

6.5. Discussion

Different studies have indicated that sandflies are either crepuscular or nocturnal in their diel periodicity, but species-specific differences are observed in the peak activity, which can influence the vectorial capacity of different species (Forattini, 1973; Souza *et al*., 2005; Coleman *et al*., 2007).

In this study, both sexes of *P. orientalis* were found to have nocturnal activity with different patterns. Females appeared to have increased activity during midnight, which corresponded to the relatively low temperature. Thereafter, activity patterns subsided progressively towards dawn before it stopped immediately after sunrise. These observations are in accordance with the reports of Wossenseged Lemma *et al*. (2014b) who used CDC light traps and found that the peak the nocturnal activities of female *P. orientalis* were between 24:00 and 1:00 close to animal shelters in Kafta Humera, north-west Ethiopia.

However, the nocturnal activity patterns reported for this species based on human landing collections elsewhere in Sudan and Ethiopia differed to varying extents. Quate (1964) reported that in the Upper Nile region, biting activity of *P. orientalis* took place between 18:30 and 20:30. In the same way, *P. orientalis* exhibited peak man-biting activity between 20:00 and 22:00 hours in eastern Sudan (Elnaiem *et al*., 1997) and shortly after dark in Arbaya, a highland valley in north-west Ethiopia (Ashford, 1974). Possible explanation for this difference in the pattern of activity peaks are the regional difference in environmental and other endogenous factors related to strain variation, where they were illustrated as factors to govern sandfly nocturnal activities (Guernaoui *et al*., 2006).

The data also showed that females display variations in peak nocturnal activity with different months, which is consistent with observations of Dinesh *et al*. (2001) on *P. argentipes* in India and Coleman *et al*. (2007) on *P. alexandri* in Southern Iraq. The important factor affecting female nocturnal activity in this study appeared to be the average hourly temperature, though the interaction was not strong. As the temperature decreased from 32 to 24^0C, the activity pattern increased rapidly and reached maximum when temperature value was between 23 and 22^0C, then, decreased steadily when the temperature was below 19^0C. Therefore, detailed studies on the possible effects of cloud cover and wind velocity on the nocturnal activity of sandflies may improve our understanding on those variations.

Males of *P. orientalis* were more active earlier than females and this could be associated with mating behavior. Similar adaptive behaviors were also recognized for males of *Lu. longipalpis* in Colombia (Morrison *et al*., 1995) and *P. argentipes* in India (Dinesh *et al*., 2001). The adaptive significance of this early landing of male *P. orientalis* could be for lekking purposes, thereby increasing their chances of mating with female flies that will be attracted to the hosts (Lane *et al*., 1990; Palit *et al*., 1993; Morrison *et al*., 1995; Killick-Kendrick, 1999).

Nulliparous females of *P. orientalis* had a bimodal peak activity period. The first batch of nulliparous females arrived earlier than parous flies, which corresponded to the period when male abundance was greatest. This implies an overlap in periodicity for mating between the male and newly emerged female populations. Other activities of nulliparous females might be related to attending their physiological demands such as search for sugar and bloodmeal.

However, large proportions of parous females of *P. orientalis* were caught between midnight and dawn. Equally, more numbers of blood-fed individuals were collected after midnight with a peak between 24:00 and 02:00 hours. This might be because females feed soon after oviposition, which was also observed in *Lu. longipalpis* females (Ferro *et al*., 1995). However, Ngumbi *et al*. (2012) in Kenya noted that more than 58% of blood-fed *S. schwetzi* was caught before midnight, which was not the case with this study.

Successful bloodmeal acquisition by biting insects requires that their active periods overlap with periods of host availability, predator inactivity, and suitable environmental conditions (Barrozo *et al*., 2004; Fritz *et al*., 2014). Hence, physiological age structure and blood feeding rhythm differences of *P. orientalis* before and after midnight might have epidemiological implications. During the dry season, almost all villagers in the study area sleep outside the house and often go to sleep after 22:00 hr. This sleeping period is not only the time that corresponds to the higher *P. orientalis* female biting rhythms, but also to the time that the human hosts become inactive and less defensive for sandfly blood feeding. At the same time, a consistent use of insecticide treated bed nets among residents who are sleeping outside houses is less (Araya Gebresilassie *et al*., unpublished data), increasing the possibility of people bite to sandfly vectors. The presence of large numbers of parous females after midnight could exacerbate the risk of acquiring VL infection.

In conclusion, *P. orientalis* males and females showed marked nocturnal periodicity, with a peak between 22:00 and 23:00 hrs and 24:00 and 03:00 hrs, respectively. Female activities were also much lower than the males. Likewise, the epidemiologically dangerous parous

females generally were more active after midnight. While these observations are important as a general precaution against sandfly exposure, the exact activity patterns of host seeking females on human hosts remain to be determined whether it corresponds to the present observation based on light trap catches. The results of this study provide insights to protect better the individual or the community from sandfly bites by the use of repellents or insecticide treated nets (ITNs).

Chapter Seven

The relative attractiveness of some local plants to *Phlebotomus orientalis* under field condition

7.1. Introduction

Sugar feeding is a fundamental characteristic of adult phlebotomine sandflies' life. Sugar is the only nutrient consumed by males and additional dietary supplement of blood for females. Overall, the sugar feeding behavior of sandflies have multiple effects on longevity and fecundity, dispersal, mate, host seeking behavior and ultimately blood feeding and disease transmission (Schlein and Yuval, 1987; Yuval *et al*., 1988, Killick-Kendrick, 1999; Müller and Schlein, 2004).

Sandflies normally obtain sugar meals from honeydew excreted on plants by aphids and coccids as indicated by the detection of melezitose in their gut (Moore *et al*., 1987; MacVicker *et al*., 1990) and by feeding directly on plant parts (Schlein and Warburg 1986; Schlein and Muller, 1995; Müller and Schlein 2004). Laboratory and field experiments demonstrated that sandflies feed on various plant organs including stems, leaves, and flowers (Schlein and Warburg 1986; Schlein and Müller 1995; Muller *et al*., 2011b) and even fruits (Hamilton and Elnaiem, 2000; Junnila *et al*., 2011).

Little is known about the sugar feeding habits of *P. orientalis,* as there are only two studies. The first one was based on preliminary field observation in north-west Ethiopia (Ashford, 1974). The other was reported from Sudan showing *P. orientalis* obtaining its sugar meals from fruits of *Balanites aegyptiaca* and *Combretum kordofanum* or some aphid and coccid

secretions (Hamilton and Elnaiem, 2000). However, studies that employ the field techniques for studying the attractiveness of local plant species to *P. orientalis* have not been conducted in Ethiopia or anywhere in East Africa. Taking into account the importance of sugar feeding to sandflies, it is necessary to identify specific sources of sugars and the attractiveness of plant species found in the natural habitats of sandflies. This in turn provides important insight to develop more selective, environmentally friendly and less expensive sandfly control methods such as attractive toxic sugar baits (ATSB) (Müller *et al*., 2010), which provided highly effective local mosquito control. Therefore, the present study was designed to determine the relative attractiveness of some local plant species in Tahtay Adiyabo district to *P. orientalis* under field condition.

7.2. Materials and Method

7.2.1. Experimental setup for plant attraction

The experimental site was open and leveled fallow field, which was 200 x 250 m, in the locality of Geza Adura. At the time of the experiment (during April 2013), there were no potential sugar meal sources near the plant baited-traps for at least 80-90 m. In this habitat, *P. orientalis* populations breed and rest in large numbers (Moncaz *et al*., 2014).

Nine different types of local plants, identified to species level, were investigated for their attractiveness to *P. orientalis* and other sandflies in the field experiment following the methods of Schlein and Yuval (1987) and Müller *et al*. (2011b) with some modifications. The tested plant species were collected in the study village within 2-3 km radius from the test site (Table 7.1). Attractiveness of these plants was conducted by placing modified CDC traps

from which the light bulbs were removed in an up-draft position over a small bucket, containing plant baits (Fig. 7.1). About 0.75 kg of fresh cut and washed branches (50-60 cm in length) of each test plant were placed inside a small bucket containing water. The unlit CDC traps were placed with their opening 8-10 cm above each bucket, containing plant baits so that the branches were under the metal covering of the trap. Concurrently, control CDC traps baited with water soaked sponges (1-liter total) were operating the night along the treatments. Plant-baited traps and un-baited control trap were arranged in a circular manner at a distance of about 25 meters from each other. The position of the control trap and plant-baited traps was changed each night in Latin-square design to avoid spatial bias. The experiment was conducted for eleven consecutive nights during April 2013.

Experimental sessions started 1 hour before sunset and terminated 1 hour after sunrise, the following morning. Each morning, sandflies caught in the traps were collected using mouth aspirators, placed in separate Barraoud cages and transported to the field laboratory, where they were processed for latter species identification as described in section 2.3. The water as well as the plant parts was changed daily.

Table 7.1. List of plants tested for attractiveness to different sandfly species

Family Name	Species	Local Name
Zygophyllaceae	*Balanites aegyptiaca*	Mekie
Rhamnaceae	*Ziziphus spina-christi*	Gaba
Fabaceae	*Acacia seyal*	Keih Chea
Fabaceae	*Acacia sieberiana*	Tseada Chea
Fabaceae	*Acacia polyacantha*	Gomoro
Fabaceae	*Dichrostachys cinerea*	Gonok
Combretaceae	*Terminalia brownii*	Weyba
Combretaceae	*Anogeissus leiocarpus*	Hanse
Combretaceae	*Combretum fragrans*	Tenkeleba

Figure 7.1. An up-draft position of CDC trap for collecting sandflies attracted to plant baits in the field.

7.3. Data analysis

Prior to data analysis, sandfly numbers were checked for normality by Shapiro-Wilk test. The non-parametric (Kruskal-Wallis) test was used to compare the number of male and female *P. orientalis* attracted by each plant species compared to the water soaked sponge control. For non-parametric post hoc comparisons, multiple-Mann-Whitney *U*-test was used and, *P*-values were adjusted with the Bonferroni correction (Dytham, 2011). Statistical analysis were considered significant when $P<0.05$ unless stated. Plants were ranked by being assigned an attraction index which was calculated by taking the mean catch with the plant bait (AP) divided by the average catch with the water soaked sponge control (AC); AP/AC = Attraction Index (Bradbury and Bennett, 1974).

7.4. Results

Table 7.2 summarizes the number of sandfly species attracted to different plant species tested in the field experiment. In total, (males=3,125; females=2,362) sandfly specimens, belonging to ten species were collected. The most attracted sandfly species to plant baits was *S. africana* (37.27%) followed by *P. orientalis* (31.73%) (Table 7.2).

Field-tested plant species differed significantly in their attractiveness to female ($\chi^2_{K\text{-}W}$= 51.19, df = 9, *P*<0.05) and male *P*. *orientalis* ($\chi^2_{K\text{-}W}$= 69.29, df = 9, *P*<0.05; Tables 7.3 and 7.4). For females, five of the nine tested plants were significantly attractive compared to water soaked sponge control trap. *Balanites aegyptiaca* was the most attractive plant followed by *Z. spina-christi*, *A. seyal*, *A. sieberiana* and *T. brownii* in their decreasing rank in terms of attraction indices (Table 7.3). Males of *P. orientalis* were also significantly attracted to the first four that were attractive to females. The highest attraction index for males was 6.67 for *B. aegyptiaca*, which was not statistically different from the three most preferred plant baits (*Z. spina-christi*, *A. seyal* and *A. sieberiana*) (Table 7.4). However, the remaining four plant species viz., *D. cinerea*, *A*. *polyacantha*, *C. fragrans* and *A. leiocarpus* were less attractive to both sexes of *P*. *orientalis* compared to control traps (*P*>005).

Table 7.2. Sandflies species attracted to traps baited with different plants species

Plant species	Sandfly species attracted										Total
	P. orientalis	*P. rodhaini*	*P. martini*	*P. lesleyae*	*P. heischi*	*S. africana*	*S. schwetzi*	*S. antennata* group	*S. clydei*	*S. bedfordi* group	
	M/F	M/F	M/F	M/F	M/F	M/F	M/F	M/F	M/F	M/F	M/F
Balanites aegyptiaca	260/187	1/0	0/1	0/1	0/0	181/104	8/32	17/5	11/5	7/6	485/341
Ziziphus spina-christi	134/102	2/1	0/0	2/0	0/0	211/89	12/24	30/9	13/2	8/10	412/237
Acacia seyal	205/94	5/0	0/0	1/0	0/0	217/50	29/77	28/11	13/5	10/8	508/245
A. sieberiana	165/89	1/2	0/0	2/2	0/2	212/70	50/598	23/6	8/3	15/4	476/776
Combretum fragrans	62/48	2/5	0/0	2/2	1/0	82/32	45/21	7/3	8/3	8/2	217/116
A. polyacantha	55/52	0/0	0/0	0/0	0/0	174/50	15/23	38/9	12/1	12/12	306/147
Terminalia brownii	49/65	0/0	0/0	0/0	0/0	150/75	17/30	17/9	3/3	14/2	250/184
Dichrostachys cinerea	35/26	0/0	0/0	0/0	0/0	147/32	7/25	24/8	8/2	26/0	247/93
Anogeissus leiocarpus	45/39	0/0	0/0	0/0	0/0	5/51	15/33	19/10	2/6	14/4	100/143
Water soaked sponge	18/11	1/0	0/0	0/1	0/0	94/19	2/23	4/11	1/5	4/10	124/80
Total	1,028/713	12/8	0/1	7/6	1/2	1,473/572	200/886	207/81	79/35	118/58	3,125/2,362

Table 7.3. Mean numbers of *P. orientalis* females attracted by different plant species per trap/night

Plant species	Mean ± SE	Attraction index
B. aegyptiaca	17.00 ± 2.26a	6.68
Z. spina-christi	9.27 ± 0.86b	3.64
A. seyal	8.55 ± 1.14bc	3.36
A. sieberiana	8.09 ± 1.89bc	3.18
T. brownii	5.91 ± 1.16bc	1.71
D. cinerea	5.18 ± 0.71cd	1.86
A. polyacantha	4.73 ± 0.41cd	2.32
C. fragrans	4.36 ± 0.75cd	2.04
A. leiocarpus	3.54 ± 0.71cd	1.39
Water soaked sponge	2.55± 0.31d	-

Mean values followed by different letters in the same column are significantly different (P< 0.005; Multiple-Mann Whitney U-test).

Table 7.4. Mean number of male *P. orientalis* attracted by different plant per trap/night

Plant species	Mean ± SE	Attraction index
B. aegyptiaca	23.64 ± 2.42a	6.67
A. seyal	18.64 ± 3.94ab	5.26
A. sieberiana	15.00 ± 2.12ab	4.23
Z. spina-christi	12.18 ± 1.84bc	3.44
C. fragrans	5.64 ± 1.07cd	1.59
A. polyacantha	5.00 ± 0.74d	1.41
T. brownii	4.45 ± 1.22d	1.26
A. leiocarpus	4.09 ± 0.73d	1.15
D. cinerea	3.18 ± 0.58d	0.9
Water soaked sponge	3.55 ± 0.80d	-

Mean values followed by different letters in the same column are significantly different (P< 0.005; Multiple-Mann Whitney U-test).

7.5. Discussion

Presence of sugar feeding sources in the environment are important determinants of sandfly ecology and sandfly-*Leishmania* interactions, and hence the transmission of leishmaniasis (Schlein and Jacobson, 1994; Schlein and Jacobson, 1999; Jacobson *et al*., 2007).

The results of this study imply how *P. orientalis* is attracted to many types of plant species in the natural environment of Tahtay Adiyabo district. Five of the field-tested plant species (*B. aegyptiaca*, *Z. spina-christi*, *A. seyal*, *A. sieberiana*, and *T. brownii*) were preferred most by *P*. *orientalis* than the other four plants. This increased variation in attraction tendency highlights how available plant species vary in their overall importance as possible sources of sugar diets for the vector species in the area. The preference of *P. orientalis* to certain plant species in the current study well fits with earlier observations in Sudan where the abundance, distribution, and activity patterns of *P. orientalis* was strongly associated with the availability of specific vegetation types (Elnaiem *et al*., 1999a). The authors indicated that *P. orientalis* and other sandfly species were identified to associate with *A. seyal*, *B. aegyptiaca*, *C. kordofanum* or riverine bushes of *Z. spina-christi* that provide sources of sugar feeding. Similarly, the dependence of *P*. *orientalis* on fruits of *B*. *aegyptiaca* and *C. kordofanum* for sugar meals was demonstrated in Sudan (Hamilton and Elnaiem, 2000). Recently, a field experiment in southern Israel also identified the attractiveness of *B. aegyptiaca* and *Z. spina-christi* to *P. papatasi* (Muller *et al*., 2011b).

There were also some differences in the number and order of plant preferences by male and female *P. orientalis* when the plant baits are arranged by mean number of capture. This observation may show that males and females have separate nutritional requirements in the sugar feeding habits in their life cycle. Presumably, this can also be related to differences in the total energy requirements between the two sexes. Sugar content analysis in the gut of male and female *P. orientalis* in Sudan also showed that females take sugar meals from more than one source (Hamilton and Elnaiem, 2000). Nevertheless, previous studies in Israel pointed out that male and female sandflies and mosquitoes were either attracted or fed on different plant parts in similar numbers with no significant difference (Schlein and Müller 1995, Schlein and Jacobson 1999; Junnila *et al*., 2010; Muller *et al*., 2011b).

Studies have demonstrated that several factors are involved in the attraction and orientation of sandflies to vascular plants in nature. Some of the common plant stimuli involved in controlling differential attraction of insects to plants, include specific floral volatile compounds eliciting olfactory receptors (Foster, 2008; Nyasembe *et al*., 2014), or an insect's ability to detect CO_2 emissions (Schlein and Yuval, 1987; Schlein and Jacobson, 2000, 2008). Plants normally release CO_2 at night as by-products of respiration (Golding *et al*., 1988), which is also a key attractant that female haematophagous insects use to locate their vertebrate hosts (Bowen, 1991, Pinto *et al*., 2001). Therefore, the combined effect between CO_2 and plant odors might explain the differential preference of *P. orientalis* to certain plant baits in the current study.

Importantly, all of the five attractive plant species identified in the current study are available throughout the year and often remain green most of the dry season, which could make them to be continuous and readily available source of carbohydrate for sandflies in the area. Pertinently, this phenomenon might be used for developing attractive taxa-specific lures that could be applied for controlling *P. orientalis* populations in the VL endemic areas. Nevertheless, one limitation of this study is that field-testing did not cover all existing plant flora in the area due to logistic problems. Moreover, as the present experiment on the attractiveness of plant species was based on small cut branches and at ground level, further studies are required on the vertical forage of *P. orientalis* or other *Phlebotomus* species on these naturally occurring trees, which are mostly quite tall for their sugar questing activities. Additional studies should also try to include other plant species, which could be possible sources of sugar feeding for sandflies in the area.

Results in this initial study demonstrated that both sexes of *P. orientalis* were discriminately attracted to plant species tested in the field set up. This information gives an insight into the ecology of sugar feeding behavior of *P. orientalis* in the area. Further studies on the ingestion of these attractive plants by *P. orientalis* as source of sugar meals are required. Moreover, it would be necessary to identify and isolate the specific attractive compounds that elicit attraction behavior and test the responses of *P. orientalis* in the laboratory experiments to be followed by field experiments using blends of the most attractive semiochemicals.

Chapter Eight

The influence of moonlight and lunar periodicity on the efficacy of CDC light trap in sampling *Phlebotomus orientalis*

8.1. Introduction

Sandflies are small, fragile, nocturnally active nematoceran insects with weak flight capabilities. In the Old World, females of the genus *Phlebotomus* have considerable public health importance as vectors of the leishmaniases, and sandfly fever viruses (Tesh, 1988; Killick-Kendrick, 1999; Ready, 2013). In addition, sandfly bites cause allergic reactions and substantial irritation in sensitive people.

In order to understand sandfly bionomics, it is imperative to sample sandflies in their different habitats. Commonly used techniques for monitoring sandfly populations are CDC light traps and sticky traps (Davies *et al*., 1995; Alexander, 2000; Alexander and Maroli, 2003). However, their trapping efficiency is greatly influenced by various environmental factors such as weather (wind speed, temperature, rainfall, relative humidity, night-length) and lunar illumination (Colacicco-Mayhugh *et al*., 2011; Gaglio *et al.*, 2014; Hesam-Mohammadi *et al*., 2014).

The lunar phase is known to influence adult flight behavior of many insects including those of the order Diptera, particularly Culicidae (Bidlingmayer, 1964; Neumann, 1995). Charlwood *et al*. (1986) reported that moonlight variations could directly influence mosquito activity. However, Guimarães *et al*. (2000) did not find a direct influence of the

lunar cycle on Brazilian populations of mosquitoes. Results on the effect of lunar phases on sandflies activity are contradictory. In Brazil, Aguiar *et al*. (1985) noted that fewer sandflies were attracted to light traps during a full moon. Carvalho *et al*. (2000) also reported that *Lu. intermedia, Lu. migonei*, and *Lu. fischeri* had higher abundance in light traps during the new and half moon phases. However, a recent study in Italy (Gaglio *et al*., 2014) indicated that *P*. *perniciosus* and *S. minuta* were mainly collected during the full moon phases, while no significant differences in the capturing of sandflies was observed among lunar phases in Kenya (Kasili *et al*., 2010).

In Ethiopia, it has frequently been observed that CDC light trap catches of sandflies (both *Phlebotomus* spp. and *Sergentomyia* spp.) were almost empty during full or partial moonlit nights, although they have never been properly evaluated (Teshome Gebre-Michael and Meshesha Balkew, unpublished data). As a result, field visits for sampling of sandflies have always been planned according to the phases of the moon when moon light is completely absent during the whole or for most of the night. Therefore, the present investigation was carried out to elucidate quantitatively the effect of moon light and lunar periodicity on the performance of light traps in collecting *P*. *orientalis* during the active periods in northern Ethiopia where VL is becoming an emerging disease.

8.2. Materials and Methods

8.2.1. Sandfly sampling methods

For sandfly collections, two sampling sites were selected: peri-domestic habitats and agricultural fields in Geza Adura village. Sandfly trapping was conducted for 7 months

between December 2012 and June 2013. Throughout the trapping periods, collections of sandfly were carried out for four nights per month, totaling 28 sampling nights. Sampling nights were categorized into four nights so as to coincide with the four lunar phases (viz., first quarter, third quarter, new and full moon) distributed in each month.

Two CDC light traps/night/lunar phase were deployed in representative sites of peri-domestic habitats throughout the sampling seasons. Simultaneously, another two light traps were operated in agricultural fields in similar way as peri-domestic habitat. In all those sampling periods, the CDC light traps were suspended in two habitats in open spaces, which were devoid of objects that could potentially shield the exposure of traps to moonlight source.

Five sticky traps were also randomly installed horizontally: on cracked walls (2 STs), a stone pile (1 ST), and animal enclosures (2 STs) in the peri-domestic environment. At the same time, another five STs were placed horizontally: over cracked vertisols (2 STs), dry riverbed (1 ST), and branches of scrub vegetation (2 STs). Traps were set up for four nights every month divided among the four lunar phases in each habitat.

Deployment and processing of collection traps in this study was done as indicated in section 2.2. In field laboratory, sandflies sorted by sex and genus (*Phlebotomus* or *Sergentomyia* spp.) were kept in 70% ethanol in labeled vials for later processing and identification to species level as indicated in section 2.3.

8.2.2. Data on moon phases and percent illumination

Timings of moonrise and moonset, tables of moon-phases, and the percent illumination of the moon corresponding to each night of moon phase were downloaded from the Astronomical Applications Department of the US Naval Observatory: http://aa.usno.navy.mil/cgi-bin/aap/ap.pl and was adjusted to Standard Time.

8.3. Data analysis

The sandfly trap-yields captured in different lunar phases and habitats by CDC light traps and sticky traps were tested for normality by Shapiro-Wilk test and log-transformed [log(X+1)] to fit normal distribution. As a result, this allowed the application of parametric tests. One-way Analysis of variance (ANOVA) was used to compare the mean number of *P. orientalis* collected using CDC light traps during the four lunar phases. Similarly, the mean numbers of other *Phlebotomus* spp. captured in CDC light traps and on sticky traps were analyzed using one-way ANOVA. Tukey's Studentized test post hoc analysis was utilized for mean separation where ANOVA was significant. Linear correlation analysis was also applied to determine the relationship between mean number of *P. orientalis* /light trap/night and the percentage of moonlight available for the corresponding day.

Kruskal-Wallis (K-W) and Mann-Whitney-test (*U* were used when trapping data did not conform to the normal distribution. K-W test was used to compare the mean number of *P. orientalis* caught on sticky traps among the four lunar phases. Statistical tests were considered significant if $P<0.05$. Though log-transformed values were used for the analyses, actual values are reported in the text, figures, and tables.

8.4. Results

8.4.1. Total number of *Phlebotomus* spp.

In total, 13,533 sandfly specimens belonging to eight species of the genus *Phlebotomus* were collected: 11,667 in light traps and 1,866 on sticky traps (Table 8.1). The most abundantly collected species was *P. orientalis* (97.78%) followed by *P. bergeroti* (0.75%). The other species constituted less than 1.5% of the total collection. Additionally, the total number of *P. orientalis* males caught by both CDC light traps and sticky traps in the two collection sites was higher than that of females (9,663 males: 3571 females) (Fig. 8.1). The male/female sex ratio for *P. orientalis* was 2.55 and 4.18 for CDC light traps and sticky traps, respectively (Fig. 8.1).

Table 8.1. *Phlebotomus* species captured using CDC light traps and sticky traps in peri-domestic and agricultural fields.

	Collection sites				
Sandfly species	**Peri-domestic**		**Agricultural Field**		
	CDC Traps	**Sticky Traps**	**CDC Traps**	**Sticky Traps**	**Total (%)**
P. orientalis	5,943	1,175	5,546	569	13,233 (97.78)
P. bergeroti	72	9	11	9	101 (0.75)
P. rodhaini	9	8	41	8	66 (0.49)
P. duboscqi	7	1	3	0	11 (0.08)
P. papatasi	3	2	2	2	9 (0.07)
P. martini	0	0	3	0	3 (0.02)
P. lesleyae	11	21	9	57	98 (0.72)
P. heischi	2	2	5	3	12 (0.09)
Total	6,047	1,218	5,620	648	13,533 (100)

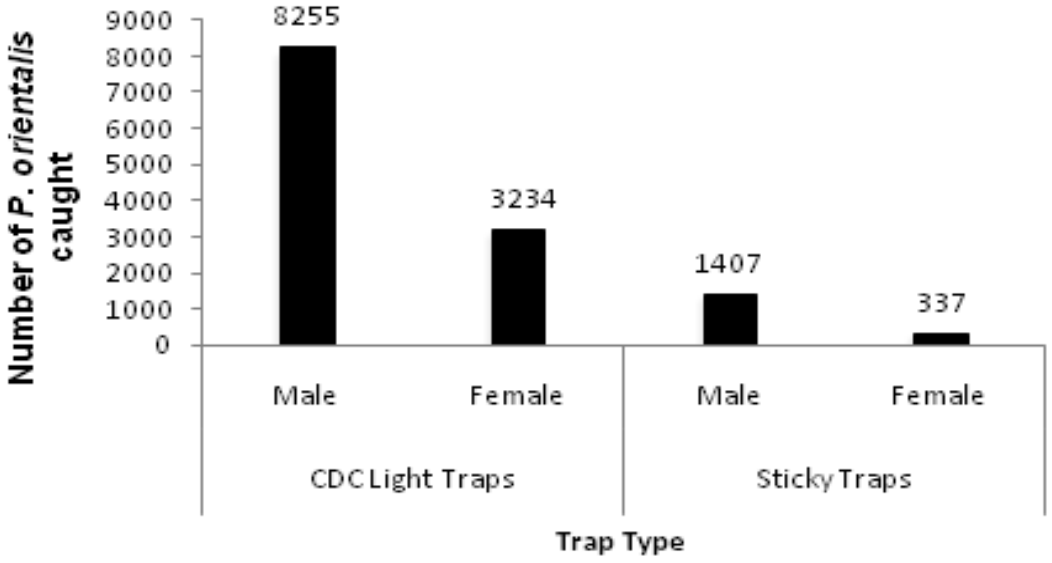

Figure 8.1. *Phlebotomus orientalis* male and female sandflies trapped by CDC light traps and sticky traps (December 2012 to June 2013).

8.4.2. Effect of lunar phases on the trap-yield for capturing *P. orientalis*

The analysis of the data of light trap catches showed a highly significant difference in the attraction response of *P*. *orientalis* in different lunar phases (ANOVA, $F_{(df=3)} = 13.96$; $P<0.05$, Fig. 8.2). The abundance of *P*. *orientalis* was significantly higher during the new moon phase with a mean of 231.13±36.27 flies/trap. The mean number of *P*. *orientalis* (60.64±13.72 flies/trap) collected in CDC light traps on moonlit nights was around 25% of the catch during a non-moon phase. There was no significant mean number difference among the first quarter, third quarter and full moon phases ($P>0.05$) (Fig. 8.2).

There was a significant difference between the mean numbers of *P*. *orientalis* females captured in the four lunar phases (ANOVA, $F_{(df=3)} = 4.86$, $P<0.05$; Fig. 8.2). The mean

number of *P*. *orientalis* females captured during new moon phases was higher than other lunar cycles. In particular, the density of female *P*. *orientalis* was substantially reduced during the moonlit nights around the full moon (Fig. 8.2).

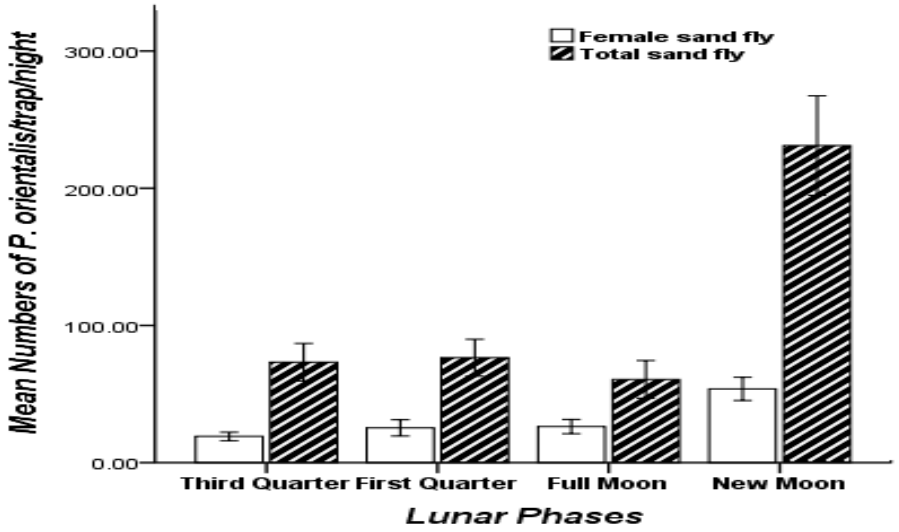

Figure 8.2. Mean numbers (±SE) of total and female *P*. *orientalis*/trap/night captured during different lunar phases with CDC light traps

Contrary to CDC light traps, different lunar phases had no significant effect on the mean numbers of *P. orientalis* intercepted by sticky traps ($\chi^2_{K\text{-}W}$= 6.41, df= 3, *P*>0.05, Fig. 8.3). The mean numbers of *P. orientalis* captured during new, third quarter, first quarter, and full moon phases were 11.0±4.25, 6.27±1.7, 2.85±1.04, and 3.87±0.65/trap/night, respectively. Likewise, non-significant differences ($\chi^2_{K\text{-}W}$= 0.91, df= 3, *P*>0.05) were observed in the mean numbers/trap/night of female *P*. *orientalis* intercepted by STs during the four lunar cycles. The mean density of *P. orientalis* females caught during the four lunar phases ranged from 1.01 to 1.47/trap/night (Fig. 8.3).

Figure 8.3. Mean numbers (±SE) of total and female *P*. *orientalis*/ST/night captured in different lunar phases

8.4.3. Effect of lunar phases on the trap-yield for capturing other *Phlebotomus* spp.

The effect of moon light on catches of other *Phlebotomus* spp. (i.e., *P*. *bergeroti*, *P. rodhaini*, *P. duboscqi, P. papatasi*, *P. martini*, *P*. *lesleyae,* and *P. heischi*) pooled was also analysed since catches of each species was low in density. Thus, the four lunar phases had a significant effect on the mean numbers of the pooled *Phlebotomus* spp., which were captured by CDC light traps (ANOVA, $F_{(df=3)} = 50.19$; $P<0.05$, Fig. 8.4). Nearly twice the mean number of *Phlebotomus* species/trap was collected during the new moon phase than the other three phases. Nonetheless, the difference between the total numbers of sandflies collected using sticky traps during the four lunar phases was not significant for *Phlebotomus* species (ANOVA, $F_{(df=3)} = 0.305$; $P>0.05$). Mean numbers of *Phlebotomus*

sandfly specimens captured on sticky traps during the four lunar phases were small, which ranged from 0.56 for new moon phase to 2.07/trap/night for full moon phase.

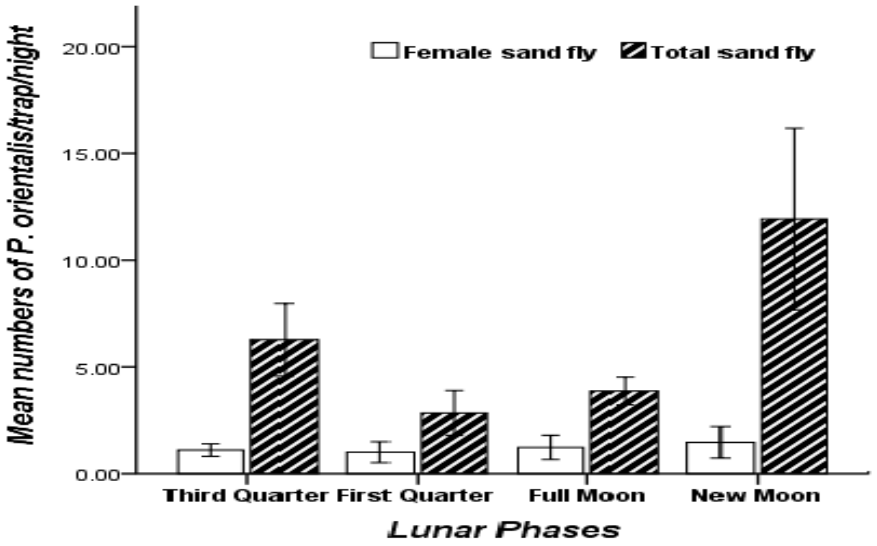

Figure 8.4. The mean (± SE) number of pooled *Phlebotomus* species caught in Tahtay Adiyabo district during four lunar phases per trap per night

8.4.4. Relationship between moonlight and light trap catches

Regression analysis revealed a highly significant inverse linear relationship between the percentage of moonlight illumination and CDC light trap catches of *P*. *orientalis* (R^2 = 0.560, df= 27, *P* <0.05) (Fig. 9.5). The number of *P. orientalis* collected by CDC light traps decreased linearly as the percentage of moon illumination increased.

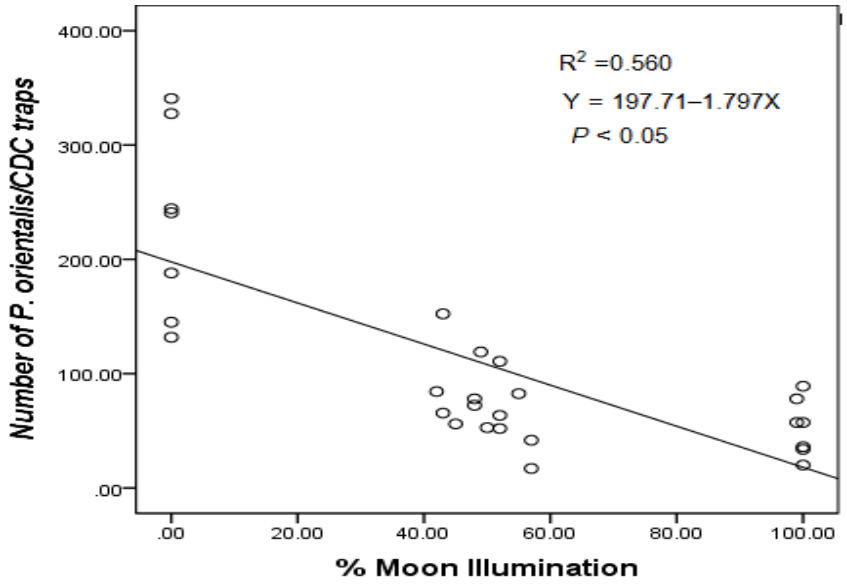

Figure 8.5. A linear decrease in the number of *P. orientalis* collected/light trap/night with the increase in the percentage of moonlight.

8.5. Discussion

The current study assessed the possible influence of moonlight and lunar periodicity on the efficacy of CDC light traps for sampling *P*. *orientalis* and other *Phlebotomus* species in northern Ethiopia during different moon phases. During 28 trapping nights, 13,533 specimens of *Phlebotomus* spp. were found using CDC light traps and sticky traps.

The results of the present study, which is the first of its kind in Ethiopia, demonstrated the significant effects of lunar phases and fractionation of moon illumination on the efficacy of CDC light traps for sampling *P. orientalis* while the effect on sticky trap collections was non-significant. As fullness of the moon increased, the attraction response of *P*. *orientalis* to light traps significantly decreased. Moreover, the mean number of *P*. *orientalis* females caught was twice as high during phases with no moon than with a full moon. Prominently, regression analysis ascertained that the intensity of moon illumination had the strongest influence on the mean density of *P*. *orientalis* caught by CDC light traps. The steeper slope in the figure revealed that with increased percentage of moon surface illumination there would be increased ambient light leading to decreased numbers of sandflies collected (Fig. 8.5). The results clearly imply that lunar phases and illuminations have an adverse effect on the trapping efficiency of light traps for sampling disease vectors in the field.

As phototrophic insects, sandflies exhibit positive phototaxis and are, therefore, attracted to light traps (Killick-Kendrick *et al*., 1985; Mann *et al*., 2009), which may be adversely affected by increased intensity of moon illumination (Nowinszky and Puskas, 2011). Santos-de Marco *et al*. (2002) found that the attractiveness of light traps toward *Lu. intermedia*

was decreased during the brightest (gibbous and full moon) phases of the moon than the dark phases (new and crescent). Studies in Brazil (Souza *et al*., 2005) and in Iraq (Colacicco-Mayhugh *et al*., 2011) also reported similar significant negative correlation between moonlight intensity and number of sandflies collected in CDC light traps as shown in the present observation on *P*. *orientalis* and other *Phlebotomus* spp.

Contrary to the present findings in this work and the above-mentioned reports, other investigators reported different results on the role of lunar cycles on the trapping performance of various light traps. Light trapping in Colombia (Morrison *et al*., 1995) resulted in increased abundance of *Lu*. *longipalpis* in moon nights as compared to dark nights. A recent study in Italy by Gaglio *et al.* (2014) also indicated that *P*. *perniciosus* and *S*. *minuta* were mainly collected during the full moon phases. However, no differences in the number of *Phlebotomus* spp. and *Sergentomyia* spp. caught in CDC light traps were observed among lunar phases in Kenya (Kasili *et al*., 2010). Such differences of observations could partly be explained by the variation in the response of sandfly species to light sources in the lunar phases and the experimental procedures followed by different investigators (Souza *et al*., 2005).

Decreased flight activity and diminishing of collecting distance as a cause for a drop in the efficiency of light trappings due to moon light are proposed (Nowinszky and Puskas, 2010). For increased moon illumination in the environment, there is increased ambient light that could compete with the light from the trap, thereby reducing the number of sandflies that will pick up the visual cue from the light trap and be attracted to it (Colacicco-Mayhugh *et*

al., 2011). Similarly, the observation that ambient moonlight competes with light traps is supported by the effect of cloud cover on the number of individual noctuid moths caught (Yela and Holyoak, 1997).

In other sampling techniques such as sticky traps and landing/biting catches that do not rely on a light source, the possible impact of lunar illumination on the trapping efficacy and sandfly activity is minimal. For example, *P*. *papatasi* collection in Egypt using sticky traps was not significantly affected by lunar phases (Kassem *et al*., 2009), which is comparable with the present observations on sticky trap collections.

In conclusion, results of the current study indicated that during the full moon, the trapping efficiency of light traps was minimal, but as the fraction of moon illumination decreased, the mean number of sandflies caught increased with peak around the new moon. In contrast, the total number of *P. orientalis* collected in sticky traps appeared to be unaffected by the lunar cycles. Therefore, it would appear that the lunar phase is a factor that should be taken into account when sandfly sampling using light traps is planned.

Chapter Nine

General discussion, Conclusions and Recommendations

9.1. General Discussion

Visceral leishmaniasis is becoming a growing health problem in Ethiopia, with endemic areas that are continually spreading. Over the last two decades, almost all cases and outbreaks of VL were reported from arid and semi-arid parts of the country; however, recent reports revealed the introduction of this disease into previously non-endemic areas (Alvar *et al*., 2007; Abbasi *et al*., 2013). In the north and north-west Ethiopia in general and Tahtay Adiyabo district in particular, VL is prevalent and affecting several people.

An important step in vector incrimination is to determine the diversity and distribution of populations of sandflies in different habitats in the *Leishmania* endemic foci (Lewis and Ward, 1987). This is achieved by sampling phlebotomine sandflies using a variety of methods. Therefore, from the faunal checklist obtained we can predict one or more species likely to be the vector(s) of disease. Among the 25 species of sandflies collected and identified in the present study area district, *S. africana* and *P. orientalis* were the dominant species, with an overall relative abundance of 59.1% and 23.5%, respectively. The identified species were also reported in many parts of the country (Teferi Gemetchu, 1983; Meshesha Balkew *et al*., 2002; Teshome Gebre-Michael *et al*., 2010; Kirstein *et al*., 2013) and neighboring Sudan (Quate, 1964; Hoogstraal and Heyeneman, 1969; Lambert *et al*., 2002; Widaa *et al*., 2012). Of the collected species, *P. orientalis* and *P. martini* reported here include the most important vectors of VL in East Africa, which also contribute to the

persistence of VL transmission cycle in the south, south-west, north and north-west Ethiopia.

The population dynamics of *P. orientalis* in Tahtay Adiyabo were greatly affected by seasonal variations. *P. orientalis* showed distinct seasonal fluctuations in abundance, with the greatest overall density between January and June, reaching its peak between March and April, associated with the hot dry months when the mean temperature was high. Following the onset of rainy period from July to late September and shortly after that (October-December), there was a sharp decrease in abundance of *P. orientalis*. This decrease in abundance during these periods could be related to the prevailing cold weather condition and the complete flooding and sealing of deep cracks of vertisols because of heavy rain. Such pattern of seasonal abundance of *P. orientalis* in different parts of Sudan and neighboring districts of northwest Ethiopia was observed (Quate, 1964; Teferi Gemetchu *et al*., 1975; Lambert *et al*., 2002; Wossenseged Lemma *et al*., 2014; Essayas Aklilu *et al*., in preparation). Identifying the seasonal abundance of the collected species is of importance for prediction of the period of maximum risk for leishmaniasis transmission and for the successful implementation of control program.

The parity rate is an essential parameter for estimating the vectorial capacity of a given species of sandfly (Lewis *et al*., 1970). Parous rates of *P. orientalis* dissected from peri-domestic (34.05%) and agricultural field habitats (35.35%) were relatively low, which is in accordance with earlier observations on the parous rate of the same species from neighboring districts (Teshome Gebre-Michael *et al*., 2007; 2010). *Phlebotomus orientalis* is

strongly implied as a vector of VL in the present study because of its preference to feed on humans as indicated in the bloodmeal analysis, its overlapping distribution with VL caused by *L. donovani*, detection of *Leishmania* spp. from unfed females, evidence from xenodiagnosis studies using the local strain of *P. orientalis* (Gebre-Michael and Hailu, unpublished data). As well, it is the proven vector in Sudan and South Sudan (Ashford *et al*., 1992, Elnaeim *et al*., 1998a; Hassan *et al*., 2008).

Data on the host attractiveness experiment indicated that *P. orientalis* females are attracted and engorged more upon certain hosts. In the experiment involving domestic animals and volunteer human host, donkey and cow were the most attractive hosts for engorging females. In support of this, bloodmeal analyses on wild captured blood-fed samples revealed that *P. orientalis* mostly prefer bovines followed by donkey and humans as source of bloodmeal. The attractiveness of these animals and their importance as source of bloodmeal for various sandfly vectors was evaluated in different areas of the world (Mutinga *et al*., 1986; Quinnell *et al*., 1992; Ngumbi *et al*., 1992; Hassen Mamo, 1999; Teshome Gebre-Michael et *al*., 2010; Garlapati *et al*., 2012).

Relatively low number of *P. orientalis* females were attracted and engorged on sheep, goat, dog, and chicken. The bloodmeal analysis also reinforces this observation, where less numbers of *P. orientalis* females were found with bloodmeal origins of these hosts. Therefore, the results of this study throw doubt on the possible role of goats, sheep, dog, and chicken in the epidemiology of VL in this region.

P. *orientalis* was also more attracted to ground squirrels, followed by hares, gerbils, and spiny mice in the experiment that involved small mammals. Some of these wild animals are common in the fissures of vertisols and the edge of farm fields where *P. orientalis* is usually abundant. This habitat association between these mammals and *P. orientalis* could provide a good source of bloodmeal. Prominently, these animal hosts serve as the reservoir hosts of *Leishmania* spp. in the various parts of the world (Mehrabani *et al*., 2007; WHO, 2010; Moreno *et al*., 2013). Therefore, their possible roles in the epidemiology of VL in the area need further research.

Observations on the nocturnal activity of *P. orientalis* revealed that the maximum diel periodicity of *P. orientalis* females occurs during midnight contrary to the reports of Quate (1964) and Elnaiem *et al*. (1997) in Sudan, where they reported the peak biting activity to be before midnight. This disparity may account to regional differences, which in turn contribute to variations in environmental situations.

The data analysis in the present study also indicated that females of *P. orientalis* display variations in peak nocturnal activity depending on gonotrophic stages. Parous females had increased activity between midnight and dawn. In the same way, more numbers of blood-fed individuals were collected after midnight with peak between 24:00 and 02:00 hrs. Increased activity of large proportions of parous females after midnight possibly implies higher risk of VL infection during this period, which coincides with peak time of retiring to bed by the residents in the village.

Sugars are important nutrient source of sandflies (Killick-Kendrick, 1999) and it enhances the chances of an infected fly transmitting *Leishmania* (Schlein and Jacobson, 1994). Sugar sources include natural and cultivated plant parts (Cameron *et al*., 1994; Müller and Schlein, 2004) as well as honeydew excreted on plants by aphids and coccids (Moore *et al*., 1987; Killick-Kendrick and Killick-Kendrick 1987; MacVicker *et al*., 1990). The current study demonstrated that both sexes of *P*. *orientalis* are attracted to particular plants that generate mixtures of volatile chemicals. Light-less CDC traps baited with cut branches of *B. aegyptiaca*, *Z. spina-christi*, *A. seyal*, *A. sieberiana,* and *T. brownii* were highly effective in attracting *P*. *orientalis* adults in the field set-ups. The fact that adults of *P. orientalis* are selectively attracted to sugar source plants in the VL endemic areas has practical implications to control. Once, the most attractive plant species has been identified in a particular sandfly ecology it would be technically easier to apply an effective, cheap, and environmentally friendly control method such as attractive toxic sugar bait (ATSB) plant-spraying methods against leishmaniasis vectors.

Light traps are one of extensively used tools for sampling sandfly species in field studies for research purposes and evaluation of vector control programs (Alexander, 2000). Indeed, their trapping efficiency is greatly influenced by various environmental factors. Lunar phases and fractionation of moon illumination has a considerable impact on the mean number of *Phlebotomus* species captured in light traps while the effect on sticky trap collections was non-significant. Consequently, this study provides evidence that the trapping efficiency of CDC light traps be largely affected by the presence of moon

illumination, which is in agreement with the observations of Santos-de Marco *et al*. (2002) and Colacicco-Mayhugh *et al*. (2011).

9.2. Conclusions

The results of the current study demonstrated the presence of 25 species of phlebotomine sandflies, belonging to two genera: *Phlebotomus* and *Sergentomyia*. Of all the sandfly species collected and identified, *S. africana* was found to be the predominant species, constituting more than 59% followed by *P. orientalis* (23.5%). Moreover, ecological analysis of *P. orientalis*, demonstrated that it has distinct seasonality in abundance that mainly peaks during the dry season (March to April). *P. orientalis* exhibits increased exophilic behavior.

Based on different evidences, including results from xenodiagnostic studies it is concluded that *P. orientalis* is the principal natural vector of VL in Tahtay Adiyabo district.

Host choice and bloodmeal analysis demonstrated that *P. orientalis* is primarily zoophilic in its host preference with eclectic feeding habits, having wide range of mammals as bloodmeal sources depending on the available host.

The results on the nocturnal activity showed that the peak activity period of *P. orientalis* females is after midnight (24:00-03:00 hrs). This is also a period where humans are at risk of *L. donovani* infections through the bite of *P. orientalis*.

Five of the plant species (*B. aegyptiaca*, *Z. spina-christi*, *A. seyal*, *A. sieberiana,* and *T. brownii*) tested was found highly attractive to adult *P. orientalis* populations.

The study on the possible effect of lunar phases on the trapping efficacy of CDC light traps and sticky traps for sampling *P. orientalis* highlighted the significant effect of lunar phases and the fractionation of moon illumination in the trapping efficiency of CDC light traps while the effect on sticky trap collections was non-significant.

9.3. Recommendations

The current entomological research presented here attempted to make some inroads into the overall understanding of the biology and ecology of sandfly vectors in the northern Ethiopia with a focus on the *P. orientalis* the most common vector species trapped in the VL endemic district of Tahtay Adiyabo. Based on the outputs of the study, the following control implications and research priority goals for *P. orientalis* and other sandfly vectors in the area are directed:

- The study has shown that *P. orientalis* in the area is seasonal and exhibits exophilic habits. Thus, this knowledge is important in the selection and application of effective vector control interventions, and in this regard, programs should be designed by taking the exophilic behavior of *P. orientalis* in to consideration.
- Further studies should be conducted to reveal the exact infection rates of *P. orientalis* in the current study area and other VL endemic foci in north Ethiopia.
- Increased predilection of *P. orientalis* to bite cattle, might have either a zooprophylactic barrier or aggravating effect on man-vector contact, demanding further clarification. However, this predominant zoophilic behavior may be exploited for controlling sandfly vectors using insecticide treated animals.
- In order to understand better the role of cattle, donkey, small rodents and hares in the epidemiology of VL in the area, further research on xenodiagnosis is essential.

- Activity of female sandflies after midnight increases and this indicates that the risk of VL transmission is elevated when many of the local inhabitants sleep. In such situations, applications of personal protection methods such as repellents against adult *P. orientalis* and other sandflies would be best later in the evening when females are most active.
- Five of the plant species found to be attractive to *P. orientalis* under natural settings could be utilized to develop plant-based attractants. These plant-based attractants can be successfully used to monitor and control sandfly vectors, either by the use of attractive blend mixtures to lure them to kill stations, or by using toxic baits that the sandflies can take up during normal sugar feeding. However, the vertical foraging activities of *P. orientalis* on these plants need to be investigated, as most of the plants are quite tall trees.
- The strong effects of lunar phases and illumination in the trapping efficacy of CDC light traps for sandfly surveillance provide evidence that sole reliance on the use of this method might not be the most effective approach. Sampling of sandfly species in field studies for research purposes and evaluation of vector control programs need to employ a more comprehensive strategy where possible to provide more accurate field data. To make that possible, research must continue to explore new and modified surveillance equipment and methods.

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11. Appendices

Annex 1: Summary of a raw meteorological data on average maximum and minimum temperatures, average relative humidity, and rainfall of Tahtay Adiyabo district during May 2011 to April 2012 were obtained from the National Meteorology Agency of Ethiopia

Average maximum temperature (^{0}C)												
Year	**January**	**February**	**March**	**April**	**May**	**June**	**July**	**August**	**September**	**October**	**November**	**December**
2009	34.6	29.4	38.3	39.2	38.6	36.9	30.9	29.6	33.7	35.9	35.8	34.2
2010	34.7	36.1	NA	39.2	38.6	35.8	29.6	29.1	31	35.3	35.2	33.8
2011	33.5	36.6	36.8	39.5	38.2	35.5	32.1	29.77145	29.9	35.3	34	34.65
2012	34.6	37.1	38.4	38.1	38.5	35.1	30.9	29.5	33.4	35.3	34.9	35.1

Average minimum temperature (^{0}C)												
Year	**January**	**February**	**March**	**April**	**May**	**June**	**July**	**August**	**September**	**October**	**November**	**December**
2009	15.9	16.5	20.1	22.7	24.3	21.7	19.6	20.2	24	25.2	24.4	19.8
2010	17.1	19.4	22.1	24.8	23.9	18.7	16	17	14.3	22.5	18.3	16.5
2011	15.7	17.6	17.7	21.9	23.1	20.1	20.8	18.6	19.3	17.1	16.8	17.7
2012	15.8	19.2	20.1	21.1	20.65	20.2	20.2	19.3	19	25.1	19.2	16.8

Average relative humidity (%)												
Year	**January**	**February**	**March**	**April**	**May**	**June**	**July**	**August**	**September**	**October**	**November**	**December**
2009	38	39	39	36	33	43	64	73	57	39	35	36
2010	37	35	NA	36	40	40	73	81	73	53	45	43
2011	44	36	40	35	41	48	62	63.5	65	49.95	45.2	44.65
2012	38	38	39	38	38	68	73	79	69	47	48	48

Total rainfall (Mm)												
Year	**January**	**February**	**March**	**April**	**May**	**June**	**July**	**August**	**September**	**October**	**November**	**December**
2009	0	0	24.8	8.1	0	129	286.8	332.8	64.7	41.6	1.8	0
2010	19	0	60.5	36.4	53.1	148	246.2	289.1	265.8	30.8	0	0
2011	5	0	10	14.1	80.9	169	156.7	287.5	171.4	5.7	5.1	0
2012	0	0	0	0	44.6	54.2	119.6	297.6	123.6	5.3	10	0

Annex 2. Summary table for Pearson correlation analysis for comparing the effects of mean monthly temperature, relative humidity, and rainfall on the mean number of *P. orientalis* caught per trap-night.

Correlations				
		Average temperature (^{0}C)	Relative humidity (%)	Rainfall (mm)
Mean # *P. orientalis*/ CDC/trap-night	Pearson Correlation	0.762	-0.803	-0.467
	Sig. (2-tailed)	0.004	0.002	0.126
	N	12	12	12
Mean # *P. orientalis*/ Sticky trap/trap-night	Pearson Correlation	0.867	-0.780	-0.430
	Sig. (2-tailed)	0.000	0.003	0.163
	N	12	12	12
Correlation is significant at the *P*<0.05 level (2-tailed).				

#-Number

Annex 3. Summary table for Spearman rank-correlation analysis for comparing the effects of hourly night temperature and relative humidity on the number of *P. orientalis* caught at hourly intervals in CDC light traps.

Correlations			
		Temperature (^{0}C)	Relative humidity (%)
# Male *P. orientalis*/trap-night	Correlation Coefficient	-0.129	0.032
	Sig. (2-tailed)	0.259	0.783
	N	78	78
# Female *P. orientalis*/trap-night	Correlation Coefficient	-0.229	0.173
	Sig. (2-tailed)	0.044	0.129
	N	78	78
Temperature (^{0}C)	Correlation Coefficient	1.000	-0.871
	Sig. (2-tailed)	.	0.000
	N	78	78
Relative humidity (%)	Correlation Coefficient	-0.871	1.000
	Sig. (2-tailed)	0.000	.
	N	78	78
Correlation is significant at the *P*< 0.05 level (2-tailed).			

#-Number

DECLARATION

I declare that *Studies on the Bionomics and Behavior of Phlebotomine Sandflies (Diptera: Psychodidae) in the Visceral Leishmaniasis foci in Tahtay Adiyabo District, Northern Ethiopia* is my original thesis work, that it has not been submitted for any degree or examination in any other University, and that all the sources I have used or quoted have been indicated and duly acknowledged as complete references.

Full name_____

Date_____

Signature_____