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Addis Ababa University

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This is to certify that the thesis prepared by Wossenseged Lemma, entitled: Study on the ecology of sand fly vectors and reservoir hosts of visceral leishmaniasis in extra-domestic habitats of Kafta-Humera lowlands, Northwest Ethiopia and submitted in fulfillment of the requirements for Deggree of Doctor of Philosophy (Zological sciences) complies with the regulations of the University and meets the accepted standards with respect to origionality and quality.

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Declaration

I declare that this thesis is submitted to the School of Graduate Studies of Addis Ababa University for the degree of Doctor of Philosophy (PhD) in Biology (Insect Science). I would like to attest through my signature affixed below that it is my own independent work. All authors in the references cited in the present study are duly acknowledged.

Signed on June 9, 2015 _____

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List of abbreviations

ANOVA= analysis of variance

CL = cutaneous leishmaniasis

DAT = Direct Agglutination Test

DCL = diffused cutaneous leishmaniais

DNA =Deoxyribonucleic acid

ELISA = Enzyme Linked Immuno-sorbent Assay

ITS1 = internal transcribed spacer 1

HIV = Human immunodeficiency virus

kDNA = kinetoplast DNA

kDa = killo dalton

LCL = localized cutaneous leishmaniais

LST = Leishmanin Skin Test

MCL= Mucocutaneous Leishmaniasis

MLEE = Multilocus enzyme electrophoresis

NNN = Novy-McNeal Nicolle

PBSS = proline Balanced Salts Solution

PCR = Polymerase Chain Reactions

PKDL= post kala-azar dermal leishmaniasis

RLB = Reverse Line Blot

RT-PCR= real time PCR

SPSS = statistical package of social sciences

VL = visceral leishmaniasis

Abstract

Kala-azar (visceral leishmaniasis; VL), due to *Leishmania donovani*, is transmitted by *Phlebotomus orientalis* in Metema Humera lowlands where this disease is a major health problem. The aims of this study were to determine seasonal dynamics, habitat preferences, nocturnal activities, host preferences, and *Leishmania* infections of *P. orientalis* in addition to study on sero-prevalence of *L. donovani* infection in migrant labourers with associated risk factors and the role of rodents as reservoir hosts of VL. Centers for Disease Control and Prevention miniature light traps (CDC traps) (John W. Hock, USA) and/or sticky paper traps were used for sand fly collections. The blood meal sources for *P. orientalis* were detected by reverse line blot (RLB) of cytochrome *b* polymerase chain reaction (PCR) amplification products using 11 probes for domestic animals. Polymerase chain reaction amplification of internal transcribed spacer 1 (ITS1) and kinetoplast DNA (kDNA) markers were used to detect *Leishmania* infections in *P. orientalis*. Blood for direct agglutination test (DAT) was sampled, randomly, from migrant labourers involved in sesame harvest to study sero-prevalence of *L. donovani* infection and entomological risk factors. Sherman collapsible rodent traps were used to capture rodents. The rodents were anaesthetized before sacrificed for tissue biopsies from liver, spleen, bone marrow and tip of the nose (skin) in addition to blood for screening of *Leishmania* infections. The species of the rodents were identified by their morphological characters. The tissues from liver, spleen and skin were macerated in Locke's solution before transferring them into Novy-McNeal Nicolle (NNN) medium for *Leishmania*cultivation. Blood and touch smears of liver, spleen, skin and bone marrow were made on microscope slide and allowed to air dry before fixing using methanol and

staining by Giemsa stain for microscopy. Polymerase chain reaction technique was also used to identify *Leishmania* infection in the tissues of the rodents. The studies were conducted from May, 2011 to January, 2014. During study on bionomics of *P.orientalis*, a total of 376, 441 sand flies using CDC light trap (n=955) and sticky traps (n=5, 551) were collected from May 17, 2011 to June 6, 2012 from agriculture fields, thickets of *Acacia seyal* and dense mixed forest. Of these sand flies, 313, 055 (80.45%) were *Sergentomyia* species. The highest mean monthly density of *P. orientalis* trapped by CDC light traps was found in thickets of *Acacia seyal* in March (64.11 $\pm$ 75.87). The corresponding highest mean monthly density of *P. orientalis* trapped by sicky traps was found in April (58.69 $\pm$ 85.20) in agricultural field. No *P. orientalis* were caught in September using CDC traps and July - October using sticky traps. The overall mean monthly density of *P. orientalis* (female and male) trapped by CDC light traps was 15.78 $\pm$ 28.93 (n=320) in agricultural field, 19.57 $\pm$ 36.42 (n=255) in thickets of *A. seyal*, and 3.81 $\pm$ 6.45 (n=380) in dense mixed forest. For sticky traps, the overall mean monthly density of *P. orientalis* was 14.76 ± 38.78 (n=2378) in agricultural fields, 11.45 ± 15.56 (n=1500) in the thickets of *A. seyal* and 0.95 ± 2.16 (n=1168) in dense mixed forest. Analysis of variance (ANOVA) result has showed statistically significant mean difference (p=0.000) for different habitats. *Phlebotomus orientalis*, *P. papatasi*, *P. duboscqi*, *P. bergeroti*, *P. rodhaini*, *P. martini* and *P. alexandri* were the *Phlebotomus* species found in the area. *Phlebotomus orientalis* was the dominant species (99%) in the extra-domestic study sites. During January to May, 2013 collection of *P. orientalis* at hourly intervals using 22 CDC light traps, the peak activities of *P. orientalis* were at 1.00 hr (134.0 ± 7.21) near animal shelters, 3.00 hr (66.33 ± 46.40) in agricultural fields and

21:00 hr (40.6 ± 30.06) in thickets of *A. seyal*. This species was not attracted to the different species of rodents in trials carried out in March and April, 2013. Riverse line blot PCR identified 7 human (28%), 9 mixed (human and cattle) (36%) and 2 cattle (8%) blood meals while 7 were unknown (28%). Molecular screening of 30 pools (1 pool = 5 individual *P. orientalis*) from dense mixed forest in July (rainy season), 2011, for *Leishmania* infection, was performed by targeting kDNA in a PCR assay. Five pools (5/30, 16.7%) were positive for *Leishmania* kDNA PCR. For March–May, 2013 dry season collections, 9/15 pools (60%) were ITS1 PCR positive. Of the total 359 labour migrants screened during October – November (2013), using DAT, 45(12.5%) were seropositive (≥1:800) for *L. donovani* infections with risk of VL development in 3 (0.8%) individuals who had very high titer (1:12800). *Leishmania donovani* infection in labour migrants seemed to correlate more with relatively higher density of *P. orientalis* during the June – August weeding season than the September – October harvest season. Staying in the areas both in the weeding and harvesting seasons (p=0.035; odds ratio (OR) = 2.83) and sleeping in the agricultural fields (p=0.01; OR=15.096) were positively correlated with *L. donovani* infection. Night harvest (p=0.028; OR=0.133) and knowledge about sign or symptoms (p=0.042; OR=0.383) were negatively associated with this infection. A total of 128 rodents such as *Arvicanthis niloticus* (n=68), *Acomys cahirinus* (n=25), *Tatera* (*Gerbilliscus*) *robustus* (n=21), *Mastomys erythroleucus* (n=3), *Mylomys albipes* (n=2), *Rattus rattus* (n=5), *Paraechimus aethiopicus* (Hedgehog) (n=2) and Xerus *erythropus* (striped ground squirrel) (n=2) were trapped and screened for *Leishmania* infections by parasitological, serological and PCR techniques. Of 91 rodents collected from extra-domestic habitats of Beaker and Gelanzeraf (Kafta-Humera district) and

analyzed by ITS1 PCR using skin, spleen, liver and bone marrow samples, 6/54 (11.1%) of *Arvicanthis nilothicus* were positive compared to the infection rates in *Acomys cahirinus* (3/17 or 17.6%) and *Tarera* (*G*) *robustus* (2/16 or 12.5%). Almost all the PCR infections were found from bone marrow samples (8/48 or 16.7%) compared with 1/91(1.1%) liver, 2/87(2.2%) spleen and 0/87 (0%) skin. Different organs on the same rodent were not found infected. These rodents were negative with NNN-medium, microscopy (Giemsa stains) and direct agglutination tests (DAT) except 2 *Arvicanthis niloticus* NNN-medium positives spleen samples from Baeker. The remaining 37 *Arvicanthis niloticus,Acomys cahirinus, Tatera (G) robustus*, *Mastomys erythroleucus, Mylomys albipes,Paraechimus aethiopicus* (Hodge hoge), *Rattus rattus* and *Xerus erythropus* (striped ground squirrel) collected from Baeker, Ademiti, Mayhas and Adijamus (western Tigray)screening using NNN-medium, Giemsa stain and DAT were negative. Agricultural fields and thickets of *A. seyal* habitats are the breeding sites for *P. orientalis* in extra-domestic habitats of Kafta Humera lowlands where female *P. orientalis* can bite humans at any hour of the night with peak biting after mid night. Sleeping in open agricultural fields was related to *L. donovani* infections in labour migrants. *Arvicanthis niloticus, Acomys cahirinus* and *Tarera* (*G*) *robustus* might play important role in the transmission cycle of zoonotic VL in endemic lowlands areas of Ethiopia. Further studies are required for *L. donovani* isolation from rodents in the endemic areas in addition to experimental infection for xenodiagnosis before considering these rodents as reservoir hosts of *L. donovani* conclusively. Access and proper use of bed nets, especially during crop growing season, are required for reducing the incidence of the infection.

Key words:Visceral leishmaniasis; *L. donovani*; *P. orientalis*; migrant labourers; rodents; agriculture fields; thickets of *A. seyal*; dense mixed forest.

Chapter 1

Introduction

Leishmaniasis is a disease caused by *Leishmania* parasites (Kinetoplastida: Trypanosomatidae), protozoa, that are characterized by the presence of an evident mitochondrial organelle called kinetoplast (Barker *et al*., 1986). The *Leishmania* species have a general life cycle involving a mammalian reservoir hosts and Phlebotomine sand fly vectors. More than 20 different species of the genus *Leishmania* that cause human leishmaniasis are transmitted by nineteen proven Phlebotomine vectors, eleven *Phlebotomus* species in old world and eight *Lutzomyia* species in new world (Killick-Kendrick, 1990).

Human *Leishmania* parasites multiply and develop to the infective stage in different parts of thesand fly midgut. After ingesting infected blood meal by *Phlebotomus* sand flies (suprapylarian), peritrophic membrane develops around the blood in mid gut where *Leishmania* amastigotes transform first to procyclics then to nectomonad forms of promastigotes. After blood digestion is completed and peritrophic membrane is ruptured, nectomonads migrate to the anterior cardia (thoracic midgut) where they transform to haptomonads which divides to form the metacyclic promastigotes (Barker *et al*., 1986; Sacks, 2000; Seblova *et al*., 2013). Abundant metacyclic promastigotes colonize the stomodeal valvesas early as 5 days in *P. orientalis*, the known vector of kala-azar in east Africa (Seblova *et al*., 2013). Metacyclic promastigotes, inoculated during blood feeding, initiate infection in the mammalian host (Sacks, 2000).

Depending mainly on the species of *Leishmania*, human infection can display three clinical forms: (i) visceral leishmaniasis (VL), (ii) cutaneous leishmaniasis (CL), and (iii) mucocutaneous leishmaniasis (MCL). Visceral leishmaniasis is the most severe form, which is fatal, if left untreated (Hailu and Formmel, 1993; Hailu, *et al.,* 2006; Chappuis, *et al*., 2007). There are an estimated 12 million cases of leishmaniasis in the world with 1.5 to 2 million new leishmaniasis cases (1-1.5 million CL; O.5 million VL) every year from 350 million people at risk in 88 endemic countries (Desjeux, 2001; Desjeux, 2004). Of 0.5 million cases, more than 50,000 deaths from VL was reported annually worldwide (Desjeux, 2004). More than 90% of VL cases in the world occur in Bangladish, India, Nepal, Sudan, Ethiopia and Brazil (Chappuis *et al*., 2007) where 310 million people are at risk of infection. In East Africa and the Indian subcontinent, VL is caused by the *L. donovani* complex, unlike Europe, North Africa and Latin America where the agent is *L. infantum* (Mauricio *et al*., 2000; Lukes *et al.,* 2007).

In East Africa (Sudan, Ethiopia, Uganda and Kenya), VL causes at least 4, 000 deaths annually and a loss of approximately 385, 000 disability-adjusted life years (Reithinger *et al*., 2007). Reported incidence of VL in Kenya, Sudan and Ethiopia were 2/1000, 40/1000 and 14/1000 persons respectively (Schaefer *et al*., 1995; Zijlstra *et al*., 1994; Ali and Ashford, 1994). A devastating epidemic of visceral leishmaniasis occurred in Sudan from 1984 to 1994 where the disease caused 100, 000 deaths in a population of around 300, 000 in the western upper Nile area (Seaman *et al*., 1996). In East Africa, transmission of *L. donovani* is considered to be anthroponotic (Zijlstra and El-Hassan, 2001; Dereure *et al*., 2003) or zoonotic (Hoogstraal and Heynemann, 1969; Elnaiem *et al*., 1997; Elnaiem

et al., 2001; Elnaiem *et al.*, 2003; Hassan *et al*., 2009). Some consider the transmission as anthroponotic during epidemic situation and zoonotic during normal endemic situation (Alvar *et al.*, 2007; Kolaczinski *et al*., 2008). In Ethiopia, the estimated annual incidence of VL ranges from around 4, 000 to 7, 000 (Hailu *et al*., 2009; Alvar *et al*., 2012) in 3.2 million people at risk (Tsegaw *et al*., 2013). During the major epidemics in 2005 and 2006 in the Libo Kemkem district (Addis Zemen areas) of Amhara region in Ethiopia, 1, 841 VL cases were treated (Alvar *et al*., 2007).

Phlebotomus orientalis is the suspected vector of VL in southwestern and northwestern Ethiopia (Gemetchu *et al*., 1975; Gemetchu *et al*., 1976; Hailu *et al*., 1995; Gebre-michael *et al*., 2010). *Phlebotomus martini* and *P. celiae* are the principal and secondary vectors of *L. (L.) donovani* in Kenya and southern Ethiopia (Minter *et al*., 1962; Gebre-michael *et al*., 1996). Visceral leishmaniasis foci of Metema-Humera lowlands (northwestern Ethiopia) accounted for 60% of all VL cases in Ethiopia (Hailu *et al*., 2006). Kafta Humera lowland (Western Tigray region) is the highly endemic VL focus in Metema-Humera lowlands.

An average, 200, 000 labour migrants visit the Kafta Humera lowlands, mainly from the surrounding Amhara and Tigray highland areas, for weeding and harvest of sesame, sorghum and cotton cultivated for commercial purpose during June – October season(Tigray Regional Report, 2007). Permanent residents and labour migrants involved in agricultural activities were reported to be at high risk of acquiring kala-azar infection

(Fuller *et al*., 1976; Rithijer *et al*., 2006; Argaw *et al*., 2013; Yared *et al*., 2014; Lemma *et al*., 2015).

In northwestern Ethiopia, like other East African countries, little knowledge exists regarding the transmission dynamics of VL. Lack of enough information about ecology of *P. orientalis* prevented the implementations of effective VL control measures. The need for further study on the zoonotic visceral leishmaniasis in extra-domestic habitats was emphasized (Elnaiem, 2011a; Wurburge and Haiman, 2011). This study was conducted in extra-domestic habitats of Kafta Humera lowlands such as agricultural fields, thickets of *A. seyal* and dense mixed forests to achieve five objectives described below.

1.1. Objectives

1.1.1. General Objectives

The main aim of the study was to describe the ecology of *P. orientalis* in extra-domestic habitats of Kafta Humera lowlands, and to examine infections of *L. donovani* suspected animal reservoirs of VL, i.e., rodents. Ecological descriptions of the vector included studies on seasonal population dynamics, host preferences, and resting/breeding site identifications. The study also had a secondary aim of identifying risk factors of human exposure to *L. donovani* infection.

1.1.2. Specific Objectives

I. To describe the seasonal dynamics and habitat preferences of *P. orientalis* and *Sergentomyia* species in extra-domestic habitats.

II. To conduct studies on the nocturnal activities of *P. orientalis* and their host preferences for blood meal.

III. To isolate *Leishmania* parasites from the gut of *P. orientalis* by dissection method as well as using Polymerase Chain Reactions (PCR).

IV. To determine the seroprevalence rate (DAT positivity and sero-reaction) of *L. donovani* in labour migrants andentomological risk factors in the extra-domestic habitats.

V. To investigate the role of rodents as reservoir hosts of *L. donovani* in northwestern Ethiopia by using parasitological and molecular techniques.

1.2. Structure of the thesis

After general introduction (chapter 1) and literature review (chapter 2), I describe the seasonal bionomics of *P. orientalis* and *Sergentomyia* species in relation to possible resting and breeding sites (chapter 3). The second and third research problems such as nocturnal activities and habitat preferences and *Leishmania* infections of *P. orientalis* areaddressed in Chapter 4 and 5 respectively. The other research problems on sero-prevalence of *Leishmania* infections in labour migrants in relation to bionomics of *P. orientalis* and the role of rodents as reservoir hosts of VL are covered in chapter 6 and chapter 7, respectively. Finally, general discussion and conclusion are given in the last chapter (chapter 8).

Chapter 2

Literature Review

2.1. Systematics of Phlebotomine sand flies

Sandflies are small flying insects belonging to the order Diptera and family Psychodidae. The classification of sand flies as a family (the family Phlebotomidae) was a matter of some controversy (Lewis *et al*., 1977). Some authors considered sand flies to be a separate family (Abonnenc and Leger, 1976) while others retained the sub family status (Phlebotominae) (Lewis, 1978; Lewis, 1982) in family Psychodidae (Theodor, 1965). The subfamily Phlebotominae, which include vectors of leishmaniasis, was separated from other sand fly subfamilies (*Tricho*myiinae and *Bruchomyinae*) based on their haematophagous habits (Theodor, 1948; Theodor, 1965; Lewis *et al*., 1977; Lewis, 1978, Lewis, 1982).

Family Psychodidae contains six genera of sand flies: *Phlebotomus*, *Sergentomyia*, *Lutzomyia*, *Brumptomyia*, *Warileya* and *Chinius* (Lewis, 1978, Lewis, 1982). *Phlebotomus*, *Sergentomyia* and *Chinius* are Old World genera while *Lutzomyia*, *Warileya*and*Brumptomyia*are restricted to the New World. *Anaphlebotomus*, *Euphlebotomus,Adlerius, Idiophlebotomus,Larroussius, Paraphlebotomus, Phlebotomus, Synphlebotomus, Kasaulius, Transphlebotomus* and *Chinius* are the 11 recognized sub genera in the genus *Phlebotomus* (Lewis, 1982; Rispail and Léger, 1998a, Rispail and Léger, 1998b). The genus *Sergentomyia*, on the other hand, is grouped as the subgenera *Neophlebotomus*, *Sintonius*, *Sergentomyia*, *Parrotomyia*, *Grassomyia, Nicinic, Parvidens* and *Rondanomyia* (Artemiev, 1978). The genera *Phlebotomus* and *Sergentomyia* in the

Old World and genus *Lutzomyia*in in the New World include all the habitual animal-biters that transmit *leishmaniasis* (Killick-Kendrick, 1990).

Phlebotomine sand flies (*Phlebotomus* and *Lutzomyia spp.*) are responsible for transmission of leishmaniasis in human and domestic animals (Killick-Kendrick, 1990; Pessoa *et al.*, 2007). Most sand fly vectors of Old World VL belong to the subgenera *Synphlebotomus*, *Larroussius*, *Adierius*, and *Euphlebolomus*. In East Africa, *P.* (*Larroussius*) *orientalis* is the established and suspected vector of VL in Sudan and northwest and southwest Ethiopia, respectively (Hoogstraal and Heyneman, 1969; Gemetchu *et al*., 1975; Hailu *et al.*, 1995; Elnaiem *et al*., 1997). Two species of the subgenus *Synphlebotomus*, (*P. martini* and *P. celiae*) are incriminated or probable vectors of *L. donovani* in Kenya and southern and southwestern Ethiopia and probableother VL endemic areas of Uganda and Somalia (Gebre-michael *et al*., 1996; Minter *et al*.,1962; Marle *et al*., 2003).

2.1.1. Genus *Phlebotomus*

Unlike genus *Sergentomyia*, genus *Phlebotomus* is characterized by lack of cibarium in females most of the time but often having a group of spicules. Pigment patch is usually absent and antenna segment 3 is usually long with two ascoids. Abdominal tergites are 2-6 with many erect hairs. The paramere often is complex while the style is with three to five spines, only one or two terminally. Phlebotomine Species are often relatively large and pale and their females have segmented spermathecae most of the time. All subgenera in New world have 3 spines while the subgenera in Old world have 4 spines (*Paraphlebotomus*, and *Anaphlebotomus*) or 5 (*Euphlebotomus*, *Phlebotomus*, *Kasaulius*,

Larroussius, Synphlebotomus and *Adlerius* (Abonnec and minter, 1965; Quate, 1964, Lewis, 1982).

Five spined style and a lobed paramer is the character of subgenus *Phlebotomus.* The characters of paramers such as tri-lobed with sub-equal (*P. duboscqi*) or unequal size (*P. bergeroti* and *P. papatasi*) are important for intra-species identification of phlebotomus males. Sub-terminal 2 proximal spines with about 7 long satea at the distal end of the coxite characterize *P* (*P.*) *bergeroti* while 2 sub median proximal spines on style and 13 long saetae at the distal end of the coxite characterize *P.* (*P.*) *papatasi* (Abonnes and Minter, 1965; Lewis, 1982). The relative length of the ascoid on the 4th antennal segment was found as a reliable character in distinguishing both sympatric and allopatric populations of *P. papatasi* and *P. bergeroti* females (Lane and Fritz, 1986). Similarly, the distances from sockets of the longest ascoids on segment 3 and 4 to distal margin of the segment were used to separate females of *P. duboscqi* and *P. bergeroti* (Gebre-michael and Medhin, 1997).

Sympatric female *P. papatasi* and *P. duboscqi* could not be identified (Quate, 1964), unless the ultra-structural differences of the pharyngeal armature and the genital atria are used (Madulo-Lellond *et al*., 1994). However, the females of *P. duboscoqi* and *P. papatasi* can also be distinguished on the basis of the ratio of the length of ascoid on segment 4 to the length of the segment (Gebre-michael and Medhin, 1997). Recently, Khalid *et al*.(2012) developed a molecular method to separate the 3 species. *Phlebotomus papatasi*, *P. bergeroti* and *P. duboscqi* were reported to exist together in Metema-

Humera lowlands (Gemetchu *et al*., 1975; Gemetch, 1983; Gebre-Michael *et al.,* 2010; Lemma *et al*., 2014a, b) as in several foci in Sudan (Quate, 1962; Hoogstraal and Heyneman, 1969; Widaa *et al*., 2012).

2.1.2. Genus *Sergentomyia*

Subgenus *Sergentomyia* (*S. bedfordi*, *S. antanata, S. Schwetzi*) is charachterized by finger shaped penis (aedeagus) sheath while subgenus *Sintonius* (*S. christophersi*, *S. clydei* and *S. adleri*) has sharply pointed end. *Grassomyia* (*S. squamipleuris*) has long conical aedeagus with gradually tapering end and mesanepisternum with two groups of hairs and antennae 3 without ascoid (Abonnenc and Minter, 1965; Quate, 1964). Genus *Grassomyia* appears to form a distinct major clade and unique with auto-apomorphy of convex cibarial tooth row and rounded spermatheca in the female and genital filaments with extended tips. On the other hand, Subgenus *Rondanomyia* andsubgenus *Parrotomyia* have blunt ended aedeagus sheath and differ by the presence of hairy tubercule on the paramer of the former subgenus while the later lack this character (Abonnenc and Minter, 1965).

2.2. *Leishmania* parasite detection methods

Initially, the classification of *Leishmanai* was based on geographical distribution, clinical manifestation, ecobiological criteria such as vectors, and antigenic properties (Bray, 1974; Lainson and Shaw, 1987). Chance *et al.* (1974) has started biochemical analysis based on iso-enzyme systems. Further isoenzyme analysis has defined species complexes within the subgenera *Leishmania* (Le Blancq *et al*., 1986; Rioux *et al.*, 1990). Currently, the use of molecular techniques has increased sensitivity and specificity of *Leishmania*

identification (Ferroglio *et al*., 2006; Tintaya *et al*., 2004; Marfurt *et al*., 2003; Schonian *et al*., 2003; Schonian *et al*., 2008; Lemma *et al*., 2009; Dalit *et al*., 2010).

2.2.1. Impression smears or touch smears

Impression smear or touch smear can be prepared on a slide from tissues such as skin scrapping, lymph nodes and liver biopsy and bone marrow aspirate. A slide with the tissue smear then fixed with absolute methanol and then stained with Giemsa or *Leishman* stain for microscopic observation of the amastigotes stages. Although amastigote identification upon microscopic examination is routinely used in the laboratory, it has low sensitivity. The sensitivity of the bone marrow smear is about 60 to 85% compared to splenic aspirate with a sensitivity exceeding 95% for visceral leishmaniasis (Sundar *et al.,* 2002). The classical ways of isolation of *Leishmania* in sand fly gut involves dissection of guts in physiological saline to search for parasite using 400x microscope magnification (Hailu *et al.,* 1995). When promastigotes are observed, the preparation is stained with Giemsa stain in addition to the use of NNN-medium culture.

2.2.2. Culture

Diaphasic NNN-mediumis often used with Giemsa stain of tissue biopsies to increase sensitivity during *Leishmania* diagnosis. These two techniques are the gold standard to confirm positivity in routine clinical practice. But, culturing parasites is expensive and time consuming and requires expertise and costly equipment that severely restrict its use in all laboratories. To obtain a sufficient number of *Leishmania* parasites to use as an antigen for immune-diagnosis, or for inoculating to susceptible experimental animals or

for iso-enzyme and molecular analysis, they are often grown in different culture media. The following culture media are commonly used.

2.2.3. Animal inoculation

Animal inoculation is not usually employed as a diagnostic test, since several months may be required to obtain a positive result. Golden hamster is the animal of choice for maintaining *L. aethiopica* (Lemma *et al*., 2009) or *L. donovani* (Sundra *et al*., 1998). Both amastigotes and promastigotes can infect the animal. After inoculation, the animal is examined weekly for signs of infection, such as cutaneous lesions, hepatosplenomegaly, or metastatic lesions. Amastigotes can be harvested by biopsy from the spleen and the liver of the animal for *L. donovani* or from skin lesion for *L. aethiopica* after it has been anesthetized.

2.2.4. Immunodiagnosis

2.2.4.1. Antigen detection (Latex agglutination test (KAtex)

Two decads ago, De Colmenares *et al*.(1995) from Spain have reported two polypeptide fractions of 72-75 killo dalton (kDa) and 123 kDa in the urine of VL patients for diagnosis. The sensitivities of the 72-75-kDa fractions were 96%, and the specificities were 100%. These antigens were not detectabled within 3 weeks of anti-VL treatment in VL patients, suggesting this test to have a very good prognostic value (De Colmenares, *et al*., 1995; Sundar *et al*., 2005). Antigen detection is more specific than antibody-basedimmunodiagnostic tests (De Colmenares, *et al*., 1995,Vinayak, *et al*., 1994). Antibody detection (Immunochromatographic strip test based onrK39) has considerably low sensitivity (57.4%) in those coinfected with human immune - deficiency virus (HIV) (Sundar *et al*., 2002). Latex agglutination test (KAtex) reported as

a useful in the diagnosis of disease in cases where there is deficient antibody production (as in AIDS patients)(Sundar *et al*., 2005).In latex agglutination test, boiled urine is mixed latex particles (800-nm diameter polystyrene particles coated with polyclonal antibodies against the leishmanial antigen) on a reusable black glass slide with four equal circles (reaction zone). *Leishmania* culture supernatant diluted in saline is used as a positive control while buffered saline as a negative control (Sundar *et al*., 2005)

2.2.4.2. Direct agglutination test (DAT)

In direct agglutination test (DAT), the trypsinized whole promastigotes are formalin fixed and stained with Coomasie brilliant blue. Serum from a patient is then incubated with the antigen and agglutination is observed the next day. Use of a 0.8% concentration of 0.1 M 2-mercaptoethanol in the sample diluent further improves its performance (Shiddo *et. al*., 1995). Freeze-dried antigens developed in Belgian and Dutch laboratories has improved by the incorporation Specificity and sensitivity of this test (Boelaert, *et al*., 1999). DAT is a user friendly with one-step dilution and reduced incubation time, its field use in endemic areas is unquestionable (Harith *et al.,* 1988). Like most antibody-based tests, DAT may yield positive results for a long time after complete cure and thus does not have much prognostic value (Harith *et al*., 1988). Previous studies on DAT as screening tool for VL patients in VL endemic areas in Ethiopia were highly promising (Hailu and Berhe, 2002; Diro *et al*., 2002). For parasitological confirmed canine leishmaniasis, 100% sensitivity and 98.9% specificity for DAT were reported (Harith *et al*., 1989).

2.2.4.3. Enzyme linked immuno-sorbent assay (ELISA)

Crude soluble antigen (CSA) of *Leishmania* promastigotes is a commonly used antigen in ELISA. It is prepared by repeated freezing and thawing (four to six cycles) of a

suspension of promastigotes in phosphate-buffered saline, followed by cold centrifugation. The supernatant is used as soluble antigen and is used to coat ELISA plates after estimation of protein content (100 to 5,000 ng/ml). The other antigens used in ELISA include 116 kDa, 72 kDa, and 66 kDa and glucose-mannose ligand (De Colmenares, *et al.,* 1995).

2.2.4.4. Immunochromatographic strip test based on rK39

A recombinant antigen, rK39, has been shown to be specific for antibodies in patients with VL caused by members of the *L. donovani* complex (ter Horst *et al*., 2009). The recombinant antigen is immobilized on a small rectangular piece of nitrocellulose membrane in a band form including goat anti-protein A (protein A colloidal gold the conjugate) which is attached to the membrane above the antigen band. After the finger is pricked, half a drop of blood is smeared at the tip of the strip, and the lower end of the strip is allowed to soak in 4 to 5 drops of phosphate-buffered saline that is placed on a clean glass slide or tube. Blood from VL patients will produce two pinkish lines on the nitrocellulose strips (the upper pinkish band serves as a procedural control). It is found to be 100% sensitive and 98% specific in India (Sundra *et al*., 1998). The sensitivity of rk39-based rapid diagnostic test in African VL patients is low, and thus, patients with a clinical diagnosis of VL require a second test, e.g. DAT (Hailu et al., 2009).

2.2.7. Isoenzyme Analysis: Multilocus enzyme electrophoresis (MLEE)

Since species identification cannot be achieved by microscopic examination and culture cultivation of *Leishmania*, MLEE is considered the ‘gold standard’ and used as reference method for identification of species of *Leishmania* (Le Blancq et *al*., 1986; Rioux *et al.,* 1990). Multilocus enzyme electrophoresis detects different alleles of housekeeping

genes(species specific alleles) by scoring the electrophoresis mobility of the enzymes they encode.Mobility differences on starch or acetate gel usually reflect differences in the charge or sequence of amino acids of proteins, and thus nucleotide differences in the genes encoding them. A panel between 10 and 20 iso-enzymes is utilized. The commonly used enzymes are: 6-phosphogluconate dehydrogenase (PGD), phosphoglucomutase (PGM), glutamate-oxaloacetate transaminase (GOT); glucose-phosphate isomerase (GPI); malic enzyme (ME), glucose-6-phosphate dehydrogenase (GPD); nucleoside purine phosphorylase (NP); malate dehydrogenase (MDH), mannose phosphate isomerase (MPI); isocitrate dehydrogenase (ICD); diaphorase nicotinamide adenine dinucleotide (DIA); glutamate dehydrogenase (GLUD); Fumarate hydratase (FH).

This technique has several disadvantages. First, it requires mass culture of *Leishmania* and use of a large amount of protein. Second, it is very time consuming, labour intensive, costly and technically demanding. Third, it has relatively poor discriminatory power since nucleotide substitutions that do not change the amino acid composition remain undetected, as do changes in the amino acid composition that do not modify the electrophoretic mobility. This short coming of iso-enzyme analysis was later solved by DNA detection techniques.

2.2.8. *Leishmania* DNA detection

2.2.8.1. Squash-blotted techniques (Dot-blot hybridization)

Squash-blotted techniques or Dot-blot hybridization involves squashing sand flies on nitrocelluloase membrane and hybridization of *Leishmania* DNA, mostly kinetoplast DNA (kDNA) in the gut of the sand flies using appropriate probes. It has been

successfully employed to detect *L. braziliensis* in sand flies (*Lutzomyia* species)using kDNA probes in Brazil (Rogers *et al.,* 1988). Similarly, different sand flies in VL endemic foci of Ethiopia and Sudan were screened for *L. donovani* using L-met2, DK-20 and 7-059 DNA probes (Gebre-Michael *et al.* 1996; Hailu *et al*., 1995; El-naim *et al.*, 1998a). These techniques have proved useful for epidemiological field studies because large number of samples can be handled at the same time. However, due to the low sensitivity, it has not been used by many investigators.

2.2.8.2. Polymerase chain reaction (PCR)

The correct identification of *Leishmania* parasites in sand fly vectors is crucial for epidemiological studies. Identification of these flagellates usually involves the dissection of the digestive tract and microscopic observation of the contents. But, this method has been proven operationally inadequate and with poor diagnostic specificity since female sand flies are also hosts for other flagellates. Recently, due to the efficiency and specificity of some DNA marker sequences, many investigators preferred to use PCR for *Leishmania* identification in sand flies (Ferroglio *et al*., 2006; Dalit *et al*., 2010).

Polymerase chain reaction based molecular techniques has almost replaced the MLEE. Experimental infection and vector competence studies in sand flies require parasite load determination. Estimation of the number of parasites in the gut of sand flies using microscope is very tedious, subjective and requires an experienced worker. It is also difficult to reveal the part of the gut colonized manually unless the recent PCR techniques including real time PCR (RT-PCR) are used (Dalit *et al*., 2010; Seblova *et al*., 2013). Many different PCR-based methods targeting different marker genes have been evaluated

for *Leishmania* parasite species identification: gp63 coding sequences (Victoir *et al*., 1998; Mauricio *et al*., 2001), kDNA (Ferroglio *et al*., 2006), the cysteine proteinase B gene (cpb) (Tintaya *et al*., 2004), miniexon (Marfurt *et al*., 2003) and ITS-1 (Schonian *et al*., 2003; Schonian *et al*., 2008; Lemma *et al*., 2009; Dalit *et al*., 2010).

2.2.9. Blood meal analysis by Reverse Line Blot assay

Reverse Line Blots (RLB) of PCR amplification of the mitochondrial cytochrome *b* gene products from blood of fresh fed sand flies have been used for identification of the mammalian species which serve as source of blood meal for sand flies (Abbasi *et al.*, 2008). In this method, 5′-amino linked oligonucleotide probes, that identifies the sequence of cytochrome b gene of animals, which could serve as source of blood for the sand flies, are synthesized and covalently attached to Biodyn C nylon membrane. The probes for the different animals are added on the membrane ($15cm^2$) in parallel lines using a blotter apparatus (Immunetics, Cambridge, MA). Biotinylated PCR products were, then, applied to the membrane strips. Hybridization is visualized using a peroxidase labeled streptavidine, which interacts with the biotin of the PCR product, followed by chemiluminescence detection. RLB is a highly reproducible technique.

2.3. Leishmaniasis in Ethiopia

The descriptions of cutaneous leishmaniasis (CL) and sand fly studies in Ethiopia have a long history. Parrot and Martin (1939a, b) surveyed the Phlebotomine sand fly fauna in Ethiopia that included the first description of biology of *P. longipes* in Addis Ababa and successful rearing of this species in the laboratory. The first VL cases in Ethiopia were reported from lower Omo areas where 136 cases were found from east African troops deployed in this area during the Second World War (Anderson, 1943). Until late 1960's,

no attempts were made to describe vectors of both CL and VL. It was in 1969, *P. longipes* proved to be a vector of CL in highlands of Ethiopia (Lemma *et al*., 1969; Ashford *et al*., 1973a). At about the same time, *P. orientalis* was incriminated as a vector of VL in Paloich area in eastern Sudan (near Ethiopia Border) with the description of the disease ecology and the sand fly behaviour (Quate, 1964; Hoogstraal and Heyneman, 1969).

In 1970, Tekle and his colleagues diagnosed VL from migrant workers of the agriculture schemes in Metema-Humera lowlands (Tekle *et al.,* 1970). Then, several studies were conducted in several VL endemic foci in different part of Ethiopia (Tekle *et al*., 1970; Fuller *et al*., 1974; Fuller *et al*., 1976; Mengesha and Abohay, 1978; Fuller *et al*., 1979; Lindtjorn and Olaffson, 1983; Hailu *et al*., 2006; Hailu *et al*., 2009).

2.3.1. Cutaneous leishmaniasis (CL)

Leishmaniasis (cutaneous and visceral) is one of the most important vector-borne diseases in Ethiopia (Hailu *et al*., 2006). Cutaneous leishmaniasis is restricted to highland areas (1700 - 2700 m) with rainfall above 800 mm (Lemma *et al.,* 1969; Ashford, 1977). It is a zoonotic disease with *P. longipes* and *P. pedifer*as vectors and hyraxes (*Pracavia capenensis* and *Heterohyrax brucei*) as reservoir hosts (Lemma *et al.,* 1969; Ashford *et al.,* 1973; Ashford, 1977; Lemma *et al.,* 2009). *Leishmania aethiopica* is the agent causing almost all cutaneouse leishmaniasis in highland areas (Hailu *et al*., 2006) which causes localized cutaneous leishmaniais (LCL), mucocutaneous leishmaniais (MCL) and diffused cutaneous leishmaniais (DCL) (Bray, 1973; Hailu *et al*., 2006). Unlike self–healing LCL, the diffused forms are not always responsive to treatments and can lead to

non-healing skin or mucosal lesions (Hailu *et al*., 2006). The existence of CL in Ethiopian highlands is always associated with the existence of hyraxes in pre-domestic areas (Ashfors *et al*., 1973a; Lemma *et al*., 2009).

2.3.2. Visceral leishmaniais (VL)

Most VL foci in Ethiopia are located in lowlands (400m – 1800m a.s.l) of southwest (Gelana, near lack Abaya; Segen valley in Konso district and Omo river plains), southern (Moyale, Wadera and Dawa valley) and northwest Ethiopia (Metema-Humera lowlands and Libo kemkem district, south Gondar) (Fuller *et al.,* 1976; Lindtjorn and Olaffson, 1983; Ayele and Ali, 1984; Hailu and Formmel, 1993; Hailu *et al*. 2006; Alvar *et al*., 2007).

2.3.2.1. Ecology of Visceral Leishmaniasis

Visceral leishmaniasis distribution and incidence are greatly influenced by environmental factors (altitude, black cracking soil, termite mounds, *Acacia seyal – Balanites aegyptiaca* woodlands and so on) and intrusion into VL foci where *L. donovani* transmission is maintained between sand fly vectors and unknown reservoir hosts (Anderson, 1943, Ali and Ashford, 1994; Hailu *et al.,* 2006; Hailu *et al.,* 2009). The two known ecological settings of VL in East Africa are *Acacia seyal-Balanites aegyptiaca* forest with black cotton soil (Ecology of *P. orientalis*) and termite mounds (ecology of *P. martini* and *P. celiae*) (Gemetchu *et al*., 1975; Fuller *et al*., 1979; Hailu *et al*., 1995; Gebre-Michael *et al.,* 2004).

The type of soil in a particular area affects sand flies in many ways. Black cracking vertisol soil is believed to be one of the important ecological determinants for distribution

of the *P. orientalis* in northwest and southwest Ethiopia (Ashford *et al*., 1973b; Fuller *et al*., 1976; Gemetchu and Fuller, 1976; Fuller *et al*., 1979; Hailu *et al*., 2006; Gebre-Michael *et al*., 2004; 1995; Moncaz *et al*., 2014a), and Sudan (Ashford and Bettini, 1987; El-naiem *et al*., 1998; Thomson *et al*., 1999; El-naiem *et al*., 2003).

Phlebotomus martini, *P. celiae* and *P. vansomerenae* are the three East African species associated with various types of macrotermes termite mounds in Kenya and Ethiopia.In these foci, *P. martini* is a principal vector of VL, while the two remaining are the suggested secondary vectors (Wijers and Minter, 1962; Minter, 1963a; Minter, 1963b; Minter and Wijers 1963; Gebre - Michael and Lane, 1996; Gebre-Michael *et al.*, 2013). The termite mounds are believed to provide ideal breeding and resting habitats for the main vector, *P. martini* and its close relatives (Minter, 1964; Gebre-Michael and Lane, 1996).

Annual rainfall appeared to be the most important environmental factor affecting both the presence and incidence of CL and VL. Cutaneous leishmaniasis is restricted to highland areas with rainfall above 800 mm (Ashford *et al.*, 1973a). The November – May dry season and June - October wet (rainy) season are the two seasons in the year in Metema - Humera lowlands. *Phlebotomus* sand flies are found abundantly, mainly, during the dry season (Lemma *et al*., 2014a). The rain occurs usually from June to October at a level of 500 - 800 mm per year (Fuller *et al*., 1976; Maru, 1979; Lemma *et al*., 2014a). The two rainy seasons in southern Ethiopia are believed to have effect on populations of sand fly and possible reservoir hosts of VL (Fuller *et al*., 1979).

The distribution of cutaneous leishmaniasis and the vectors (*P. longipes* and *P. pedifer*) in Ethiopia are restricted in highlands (1800-2800m) (Ashford *et al*., 1977). All other *Phlebotomus* species are found at an altitude less than 1800m a.s.l. In lower Omo basin, leishmanin skin test positivity was found to be inversely correlated with altitude: highest positivity for tribes living around 500m, intermediate for 500-1000m and lowest for 1400m (Fuller *et al*., 1979).

Temperature is also another important environmental factor affecting VL prevalence and sand flies distribution. Temperature is known to affect survival and the speed of development of the different stages in the life cycle. Tropical species like *P. orientalis* require 20 - 30 ^{0}C constant temperature for their survival and development (Ward, 1989). During the study of environmental determinants affecting the distribution of *P. orientalis* and VL cases in Sudan, the positive sites for *P. orientalis* were characterized by higher mean maximum(36.08 ^{0}C) and minimum(20.36^{0}C) daily temperature compared with mean maximum (33.87^{0}C) and minimum (18.39^{0}C) of the negative sites (Elnaiem *et al*., 1998). Rise in temperature accelerates the insect's metabolic rates, increases egg production and makes blood feeding more frequent. It also shortens the time period required for the development of pathogens within insects (Rioux *et al.*, 1985; Oshaghi *et al.*, 2009).

Most VL patients in Ethiopia occur among rural communities who visit the lowlands,during agricultural activities and guarding animals, where *L. donovani* cycle is maintained between the vectors and unknown reservoir hosts (Fuller et al., 1976; Hailu *et*

al., 2006; Hailu *et al*., 2009; Argaw *et al*., 2013; Yared *et al*., 2014). Generally, VL patients have low income, are malnourished; do not use bed nets, and live in poor housing conditions (Fuller, 1976; Mengesha and Abohay, 1978; Alvar *et al*, 2007; Bashaye *et al*., 2012; Argaw *et al*., 2013). Some of these conditions were associated with high incidence of VL. The high influx of migrant workers attracts large commercial sex workers and consequently the possibility of HIV infection, especially in northwest Ethiopia. In Humera focus, 20–30% of patients of VL are also infected with HIV, the highest VL-HIV co-infection rate in the world (Lyones *et al*., 2003).

2.3.2.2. Vectors of visceral leishmaniasis in Ethiopia

Belonging to 6 genera, phlebotomine sand flies of Ethiopia are *P. (La.) orientalis, P.* (*Sy.*) *martini, P.* (*Ph.*) *duboscqi, P.* (*La.*) *fentalensis, P.*(*Pa.*) *saevus, P.*(*Pa.*) *sergenti, P.*(*Pa.*) *gametchi, P.* (*Pa.*) *alexandri, P.*(*Ph.*) *duboscqi, P.*(*Ad.*) *arabicus, P.* (*An.*) *rodhaini,P.* (*La.*) *longipes, P.* (*La.*) *pedifer, P.* (*La.*) *ashfordi, P.* (*La.*) *gibenensis, P. (S.) celiae* and *P.* (*La.*) *aculeatus* (Ashford, 1973b; Ashford *et al.,* 1974; Gemetchu *et al.,* 1975; Gebre-Michael *et al*., 2003; Lemma *at al*., 2014a). Of these species, *P.*(*Ph.*) *duboscqi, P.* (*La.*) *orientalis, P.* (*Sy.*) *martini, P.* (*Pa.*) *sergenti, P.* (*Pa.*) *alexandri, P.* (*Ph.*) *duboscqi, P. (An.). rodhaini* and *P.* (*La.*) *longipes* were found in northwest Ethiophia (Ashford *et al*.,1973a; Ashford *et al*., 1974; Gemetchu *et al*., 1983; Hailu *et al*., 2006; Gebre-Michael *et al*., 2007; Gebre-Michael *et al.,* 2010; Lemma *et al*., 2014a). Only *P. orientalis*is considered as vector of VL in northwestern Ethiopia as it was the most abundant man biting species exist (Gemetchu *et al*., 1975; Gebre-Michael *et al*., 2007; Gebre-Michael *et al*., 2010; Lemma *et al*., 2014a).

2.3.2.2. 1.*Phlebotomus orientalis* and visceral leishmaniais incidence

The study on the behavior of *P. orientalis* has several consequences on the transmission and possible VL control measures in northwest Ethiopia and Sudan. In VL endemic foci of Ethiopia, people are permanently settled on black cracking soil. Previous attempts to collect *P. orientalis* from VL endemic villages in northwestern Ethiopia and Sudan were not successful (Tekle *et al*., 1970; Zeese and Frank, 1987) indicating the incidence of the disease might be related with involvements in the extra-domestic environments. An epidemiological study using Leishmanin Skin Test (LST) (Fuller *et al.,* 1976) and a case control study (Yared *et al*., 2014; Argaw *et al*. 2013) in Humera have showed most infections were acquired in extra-domestic habitats. Similar study (Lemma *et al*., 2015) also showed that the risk of kala-azar infection was higher when labour migrants sleep in open agricultural fields compared to inside huts in the camps.

It is believed that in the crouded villages of Libo Kemkem(Addis Zemen) districts(South Gondar),the peridomestic *P. orientalis* was responsible for the anthroponothic transmission of VL that resulted in the 2005/6 epidemic in the area where labour migrants returning from Metema-Humera lowlands were serving as the reservoir hosts (Alvar *et al*., 2007).

Phlebotomus orientalis is a seasonal species with peak abundance in March and April in Kafta Humera lowlands (Lemma *et al*., 20014a). The report of non-seasonality of *P. orientalis*, during September – April, in Arbaya might be related to the sampling technique which entirely depended on Human bait (Ashford *et al*., 1973). Thus, *P.*

orientalis might be a vector of zoonotic VL in extra domestic habitats (Elnaiem *et al*., 1997; Elnaiem *et al*., 2001) in addition to the domestic anthroponotic transmission during epidemic outbreaks (man - *P. orientalis* - man) (Lambert *et al*., 2002; Alvar *et al*., 2007). In Awash valley, the existence of *P. orientalis* did not correlate with VL prevalence (Gebre-Michael *et al*. 2004).

2.3.2.2.1.*Phlebotomus martini* **and visceral leishmaniais incidence**

Phlebotomus (*Sy.*) *martini, P.* (*Sy.*) *celiae* and *P.* (*Sy.*) *vansormerenae* are the three species associated with various types of *Macrotermes* termite mounds found in Kenya, Ethiopia, Somalia and South Sudan **(**Wijers and Minter, 1962, Minter, 1963a, Minter and Wijers, 1963, Abonnenc, 1972, Ashford and Bettini, 1987, Perkins *etal.,* 1988, Gebre-Michael and Lane, 1996; Gebre-Michael *et al.,* 2013**).**Females of these three species can not be individually distinguished (Abonnenc, 1972, Gebre-Michael *et al.*, 2013)**,** particularly when the three are sympatric as in Melka Guba(Dawa River Valley) of Liben district, south eastern Ethiopia. Based on comparisons of the infection rates (0.7% rin *P. martini* and 0.3% in *P. celiae*) and abundance of these sand flies, Gebre-Michael and Lane (1996) concluded that *P. martini* and *P. celiae* are the principal and secondary vectors of *L. donovani* in the Aba Roba area of southern Ethiopia. The three species of subgenus *Synphlebotomus* were found to be wet season species in Ethiopia and Kenya with peak night-biting activity between 07:00 and 10:00 p.m. in Ethiopia (Minter, 1964, Gebre-Michael and Lane, 1996).

Although, strong association between kala-azar incidence and existence of termite mound was reported in Kenya and Abaroba (Ethiopia) (Wijers and minter, 1962; Gebre-Michael

and Lane, 1996; Gebre-Michael *et al*., 2004), neither *P. orientalis* nor *P. martini* were found from the termite mounds in lower Omo basin (Fuller *et al*., 1979). *Phlebotomus orientalis,* however, was found in black cracking soil around the termite mounds (Fuller *et al*., 1979; Hailu *et al*., 1995). Sometimes, factors like seasonality and trapping methods could affect sand fly collection (Gebre-Michael *et al*., 2004). In the termite mound habitat, *P. orientalis* is either absent or rare. Conversely, *P. martini* is usually absent or rare in *P. orientalis* black cracking soil habitat (Gebre-Michael *et al*., 2004). Human infections in southern Ethiopiais related to visits or settlements in the Segen and Omo valleys as well asto Negele Borena lowlands where there could be exposed to the bite of *P. martini* (Gebre-Michael *et al*., 1996; Gebre-Michael *et al*., 2013).

2.3.2.3. Reservoir hosts of visceral leishmaniasis

Based on circumstantial evidence and incidental observations, rodents are among the main suspected animals that possibly are serving as natural hosts of VL in Ethiopia. Among the many wild animals, *Arvicanthis* species is a widely distributed rodent that has been suspected as reservoir hosts of VL, along with *Acomys* spp, *Tetera*, *Mastomys* and small carnivores (Hoogstraal and Heineman, 1969; Haile and Lemma, 1977; Githure *et al.,* 1984; Kassahun *et al*., 2015). Stauber *et al*. (1996) conducted experimental *L. donovani* infection using *A. nilothicus*, confirming its susceptibility.However, serological screening of *Arvicanthis* by DAT in in this study did not reveal any reaction for *L. donovani*.

2.3.2. 4. Visceral leishmaniasis (VL) control

Unlike Southeast Asian VL foci, where the ecology of the disease is well understood, in East Africa, little knowledge exists about zoonotic transmission cycles and ecology of the

vector (Elnaiem, 2011; Wurburg and Faiman, 2011). In this region, search for breeding sites and ecology of the vectors has become a research priority (Wurburg and Faiman, 2011). As opposed to *P. argentipes*, vector of *L. donovani* in the Indian subcontinent, feasible and sustainable control methods for *P. orientalis*are not yet available (Elnaiem, 2011). *Phlebotomus orientalis* is primarily a sylvatic species (Quate 1964; Lemma *et al*., 2014a, b) with extraordinarily low abundance reported in and around human habitations in endemic areas in northwest Ethiopia and Sudan (Zeese and Franke, 1987). Use of insecticide fogging to eliminate adults in the agricultural fields and tickets of *A. seyal* in extra-domestic habitat would not be cost effective (Elnaiem et al., 1999a; Elnaiem *et al*., 2011).

Chapter 3

Population dynamics and habitat preferences of *Phlebotomus orientalis* and *Sergentomyia* species

3.1. Introduction

Acacia seyal - *Balanites aegyptiaca* forest and deeply cracking soil are described as habitats of *Phlebotomus orientalis* within eastern Sudan and northwestern Ethiopia (Quate, 1964; Hoogstraal and Heyneman, 1969; Gemetchu *et al.*, 1975; Fuller *et al.*, 1979; Zeese and Frank, 1987; Ashford and Thomson, 1991; Schorscher and Goris, 1992; Elnaiem *et al.*, 1997; Elnaiem *et al.*, 1998; Elnaiem *et al.*, 1999a; Thomson *et al.*, 1999; Widaa *et al.*, 2012).

The sugar feeding habits and diapauses of this species have not been described (Quate, 1964; Hoogstraal and Heyneman, 1969) except Elnaim *et al*. (1999b) who described *P. orientalis* as forest species with special preference to dense *A. seyal* than *B. aegyptiaca*, *Combretum kordofanum*, *Hyphaena* or *Zizipus* trees. The smooth surface of *A. seyal*, however, lacks cracks, fissures or cavities to serve as resting site during wet rainy season as opposed to *B. aegyptiaca*, or other trees in the forest (Quate, 1964). *Phlebotomus orientalis* is believed to depend on the black cracking soil as day resting and breeding site in dry season (Quate, 1964; Hoogstraal and Heyneman, 1969). Elnaim *et al*. (1997) found *P. orientalis* resting site is termite mound but these sites were not considered as principal day resting site for *P. orientalis* in Dinder National Park. Extensiveeffort to finds breeding sites of *P. orientalis* were not successful (Quate, 1964).

In the highlands of Belessa valley in Ethiopia, the population of *P. orientalis* was reported to show no significant variation from September (rainy season) to April (dry season) (Ashford *et al*., 1973). In contrast, in Sudan, the numbers of *P. orientalis* captured using sticky traps were reported to be few in the early dry season (January and February) and increased significantly in March until it reached peak number in April. The population of *P. orientalis* declined in May and June when rain commenced (Quate, 1964, Hoogstraal and Heyneman, 1969). During the study of sand flies in the Dinder National Park, next to Ethiopian border, *P. orientalis* showed a slight peak from December 1994 to February 1995 and then dropped in March – May, and then peaked suddenly in June 1995 in thickets of *A. seyal* (Elnaim *et al.,* 1997).

Bionomics of *P. orientalis* has not been conducted in northwestern Ethiopia. Seasonal population study of *P. orientalis,* however, is important to determine time of the year when people are exposed to *P. orientalis* bites or VL infection. Thus, the aim of this study was to describe the seasonal dynamics of *P. orientalis* and habitat preferences in extra-domestic habitats of the Kafta Humera lowlands.

3.2. Material and methods

3.2.1. Study area

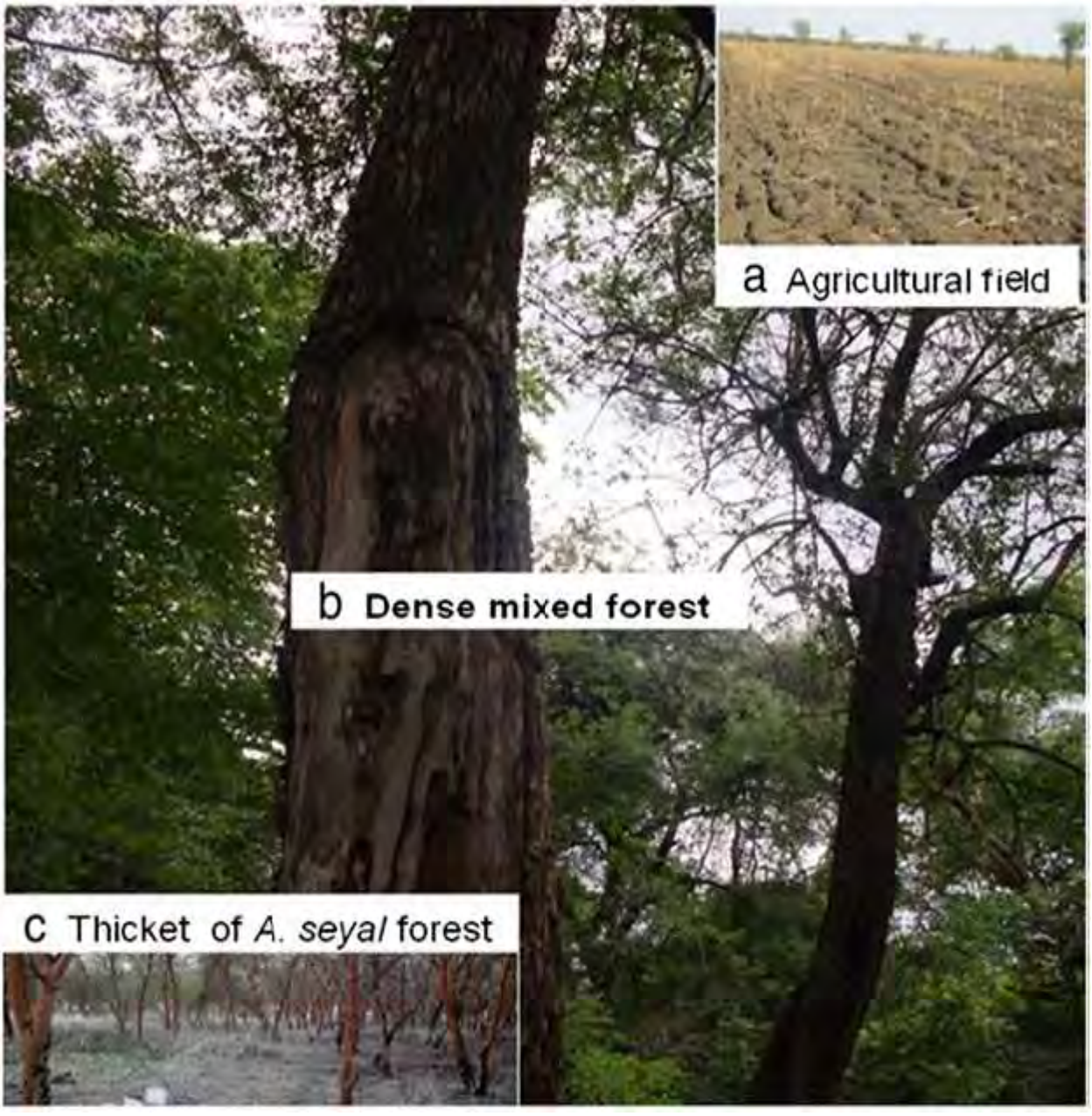
Kafta Humera district (wereda) is found in Western Tigray Zone which includes Welkait and Tsegede districts (Figure 1). Humera town is the administrative center of the district. It has latitude 14°17'N and longitude 036°39'E at an altitude of 637 m above sea level. The smaller towns around Humera include Rawyan (14°17' 19"N , 036°37' 18"E, 600 m ASL), May Kadra (14°08' N , 036°34' E, 612 m ASL), and Adebay town (14°17' 22 "N , 036°38'E, 625 m ASL). Rawyan is found between MayKadra and Humera at 10 km

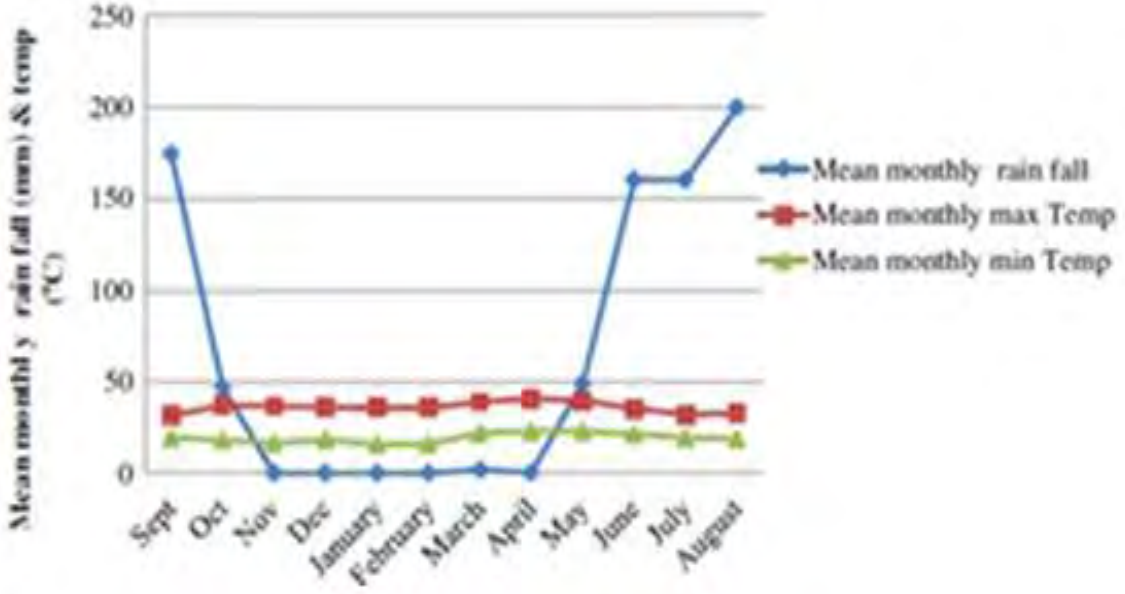

Eritrea
Sudan
Tigray
Amhara
Afar
Beneshangul
Addis Ababa
Ethiopia
Dire Dawa
Oromiyia
Gambella
Southern Nations
Somali
Kafta Humera
Welkait
Tsegede
Legend:
Towns
Sample sites
HU = Humera town
AD = Adebay
BA = Barker
RA = Rawyan
HT = Hay Kadita
KA = Kafta
S1 = Sample site 1
S2 = Sample site 2
S3 = Sample site 3
S4 = Sample site 4

3.3.2. Study sites and Habitats

A preliminary survey was conducted during field trip in May 15 – 28, 2010 to identify the habitats in the study area by the research team from Addis Ababa and Hebrew Universities. Sand fly sampling sites were selected from extra-domestic areas around the Adebay (Site-1), Baeker (Site 2), Rawyan (Site 3) and May Kadra (Site 4) towns (Figure 2). The sampling sites were at least 10 kms away from these towns. Site 4 was located 23 km southwest of the May Kadra in an area called Gelanzeraf closer to the Sudan border (13°59'N and 036°31'E). Each site had agricultural field, thicket(s) of *A. seyal* and dense mixed forest habitats (Figure 3.2) for sand fly sampling except Adebay and Rawyan where thicket of *A. seyal* and dense mixed forest were missing respectively.

The agricultural fields that were used as sampling sites were those which have been used for cultivation of sesame. Thickets of *A. seyal* sampling sites were found in the depression where water floods during rainy season, next to agricultural fields. The thickets of *A. seyal* around Rawyian town were dense (>20 trees/100m^2) compared to the sparse forests (10-20 trees/100m^2) around the Baeker town. Both thick and sparse forests were found in Gelanzeraf area. The two ends of dense mixed forest (>20 trees/100m^2) extending between Adebay and Beaker towns (about 45 km) were used as extra-domestic sample sites around Adebay (site 1) and Baeker (site 2). This forest is part of the Kafta Shiraro National Park - the largest park in Ethiopia. Another dense mixed forest with rocky and black soil was also used as sampling site in Gelanzeraf area. The common trees and shrubs in Kafta Humera areas are *A. seyal, A. mellifera, B. aegyptiaca*, *Combretum spp, Terminalia spp*., *Boswellia papyrifera*, *Ficus sycomorus*, *Sclerocarya birrea*,


a Agricultural field
b Dense mixed forest
c Thicket of A. seyal forest


Mean monthly rain fall (mm) & temp (°C)
250
200
150
100
50
0
Sept
Oct
Nov
Dec
January
February
March
April
May
June
July
August
Mean monthly rain fall
Mean monthly max Temp
Mean monthly min Temp

3.2.4. Sand flies collection and identification

At least 12 CDC (Model512, Hock and Co., USA) - light traps/month/habitat were used to collect sand flies from agricultural fields, thickets of *A. seyal* and dense mixed forests at the four localities (sample sites). The number of CDC used per day was ranged from one to eight. Similarly, 20 – 388 sticky traps/month/habitat were used to collect sand flies from the three habitats. CDC light traps were set at 6 p.m., hanged at about 0.5 meter above ground level, and left overnight till 6 a.m. Sesame oiled sticky traps were randomly placed horizontally on the ground at about 5 m interval. Sand flies from sticky traps were collected in 95% alcohol and transferred to saline containing detergent for washing, sorting and counting. Similarly, sand flies collected using CDC traps were sorted and counted. Sand flies were dissected in saline and mounted in Hoyer's medium, after the head is separated and turned upside down before placing cover slip. The last segment of the abdomen was also removed for visualizing the spermathecae in female sand flies. Species identification was carried out using the appropriate keys (Quate, 1964; Abonnenc and Minter, 1964; lewis, 1982).

3.2.5. Study on habitat preference and Bionomics

The sand fly species, sex, habitat, numbers, date and type of trap used were documented. Mean monthly density of trapped sand flies was determined by total counts divided by number of traps used. Comparisons of density of *P. orientalis* in different habitats were used for study of habitat preference of this vector. Similarly, density of *P. orientalis* at different months was used to determine the seasonality of the vector. Resting sites of *P. orientalis* in the dense mixed forest during rainy season were determined by comparing

the results of *P. orientalis* catched using the sticky traps placed on the ground and the emergence traps deployed on tree trunks. Hand-held torch light battery was also used for making observations of sand flies resting sites. The bionomics and habitat preferences of the different species of *Sergentomyia*in agricultural fields and dense mixed forests, which were distinctly separated from each other, were estimated from relative abundance or relative percentage.

3.2.6. Statistical Analysis

The density of sand flies calculated as average numbers of male and female sand flies per trap per day were entered into statistical package of social sciences (SPSS) version 16 for analysis of data using descriptive statistics (Mean $\pm$ SD), analysis of variance (ANOVA) and Post hoc Tukey Honestly Significant Difference (HSD) tests so that seasonal dynamics and habitat preferences of *P. orientalis* and *Sergentomyia* species could be studied. Tests of normality (normal distribution of the data) were cheked by the results of the Kolmogorov-Smirnov statistics. P-values less than 0.05 were considered as statistically significant differences.

3.3. Results

3.3.1.Bionomics and habitat preferences of *Phlebotomus orientalis*

A total of 376, 441 sand flies were collected using CDC light traps (n=955) and sticky traps (n=5, 551) from agricultural fields, thickets of *A.seyal* and dense mixed forests during the study period. Of the total sand flies collected using CDC and sticky traps from the three types of habitats, 62, 733 (5, 546 females and 57,187 males) or 16.7% were *P. orientalis* (Table 3.1).

Table 3.1. Total sand flies collected from agricultural fields, thickets of *Acacia seyal* and dense mixed forest using CDC and sticky traps from May 17, 2011 to July 6, 2012.

Species	CDC (No. traps; n=955)			Sticky traps(n=5, 551)		
	Female	Male	Total	Female	Male	Total
Phlebotomus orientalis	3, 876	8, 512	12, 388	1, 670	48, 675	50, 345
P. papatasi	88	189	277	16	224	240
P. duboscqi	7	24	31	0	0	0
P. bergeroti	0	21	21	0	0	0
P. rodhaini	30	6	36	0	27	27
P. martini	0	10	10	0	0	0
P. alexandri	0	11	11	0	0	0
Sergentomyia species	90, 078	74, 314	164, 392	92, 383	56,280	148, 663
Total	94, 078	83, 087	177, 166	94, 069	105, 206	199, 275

There was statistically significant difference between mean values of *P. orientalis*/traps in males and females ($p = 0.001$). The Mean of *P. orientalis*/CDC in all habitats was 8.03 $\pm$ 20.77 (range: 0-144.33) for males and 3.62 $\pm$ 7.01 (range: 0-49) for females. Similarly, the overall mean density of *P. orientalis*/sticky was 9.57 $\pm$ 27.07 (range: 0-295) for males and 0.32 $\pm$ 0.92 (range:0-11) for females. Almost all (99%) *Phlebotomus* species found in the extra-domestic study sites were *P. orientalis* (Table 3.1).

For CDC traps, the highest mean monthly density of *P. orientalis*/CDC light trapwas found in thickets of *A. seyal* in March (64.11 $\pm$ 75.87) and lowest was found in September (0 $\pm$ 0) in agricultural fields. The highest *P. orientalis*/sticky was found in

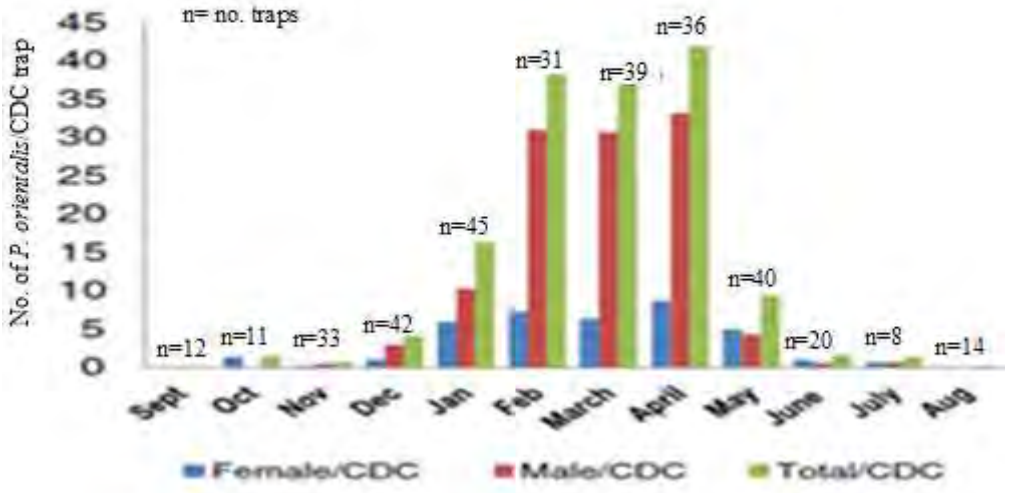
April (58.69 ± 85.20) in the agricultural fields; and the lowest (0 ± 0) was found during July–October wet and rainy season in all habitats (Figure 4). When post hoc (HSD) tests were performed, mean *P. orientalis/*CDC light trap for April was not statistically significantly differently different from January (p=0.384), February (p=0.980) and March (p=0.783) as opposed to the other months (p< 0.05). During rainy time, mean density of *P. orientalis/*CDC trap sharply decreased in June and July both in agricultural fields and thickets of *A. seyal* compared to dense mixed forest which showed the highest mean density of *P. orientalis/*CDC trap (7.94 ± 10.50) in July with female: male ratio of 5.47:1(Figure 3.4).

Dense mixed forest yielded 13.2% of the total *P. orientalis* collected from all habitats after using 392 CDC traps (39.4%) of the total CDC traps. Of all *P. orientalis* (1, 577) collected from the dense mixed forest, 57.9% (772 female and 141 male) were trapped in July (7.9 *P. orientalis*/CDC trap) using 104 CDC traps (27.36%) (Figure 3.4). The overall mean monthly density of *P. orientalis* (female and male) trapped by CDC light traps was 15.78 ± 28.93 (n=320) in agricultural field, 19.57 ± 36.42 (n=255) in thickets of *A. seyal*, and 3.81 ± 6.45 (n=380) in dense mixed forest. For sticky traps, the overall mean monthly density of *P. orientalis* was 14.76 ± 38.78 (n=2, 378) in agricultural fields, 11.45 ± 15.56 (n=1, 500) in the thickets of *A. seyal* and 0.95 ± 2.16 (n=1, 168) in dense mixed forest. Analysis of variance has showed statistically significant mean difference (p=0.000) for different habitats. However, similar habitats in different localities did not show statistically significant difference for the mean monthly density of *P. orientalis* trapped by CDC light traps (p=0.117) and sticky traps (p=0.134). During the August rains, habitat with more protection such as dense mixed forest harbored more number of

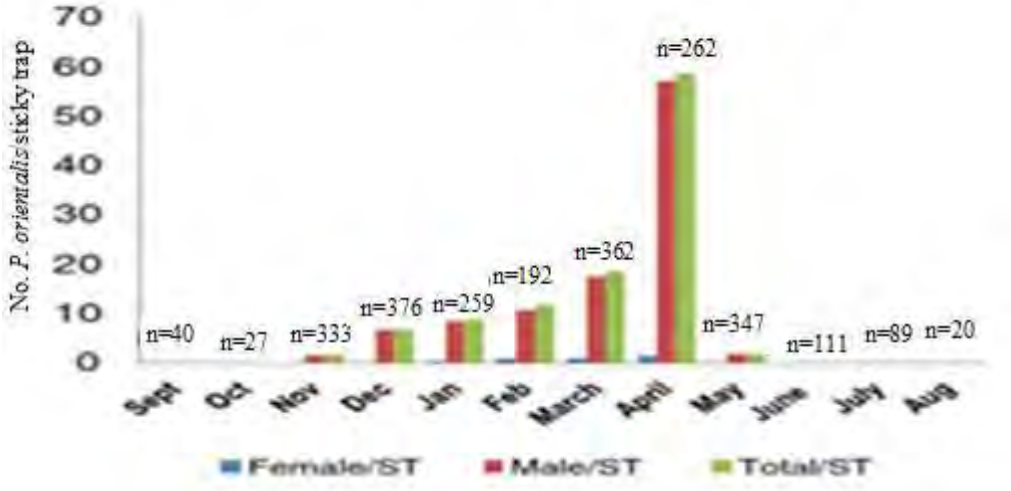
*P. orientalis/*CDC light trap (1.04 ± 1.2) compared to agricultural fields (0.24 ± 0.42) and thickets of *A. seyal* (0.2 ± 0.44). Sticky traps placed on the ground in July and August did not collect sand flies when compared to emergence traps deployed on the tree trunks in the dense mixed forests. A total of 44 (32 female, 12 male) *P. orientalis* and 8, 115 *Sergentomyia* species (3, 736 female, 4, 379 male) were collected using 77 emergence traps.

Sparse thickets of *A. seyal* (counts of 29.37 ± 49.41) in Beaker and Gelanzeraf (with counts of 28.00 ± 41.33) were ranked first and second compared to the different habitats in different sites based on mean number of *P. orientalis*/CDC trap during the study period. The other habitats with higher mean *P. orientalis*/CDC trap values were agricultural fields in Baeker (counts of 22.47 ± 33.24) and Adebay (counts of 21.11± 34.07). Analysis of variance for mean number *P. orientalis*/CDC trap between sparse thickets of *A. seyal* in Beaker and sparse thickets of *A. seyal* in Gelanzeraf showed no statistically significant difference ($p>0.05$). Similarly, ANOVA for mean number *P. orientalis*/CDC trap in Adabay and Beaker agricultural fields showed no difference ($p>0.05$).

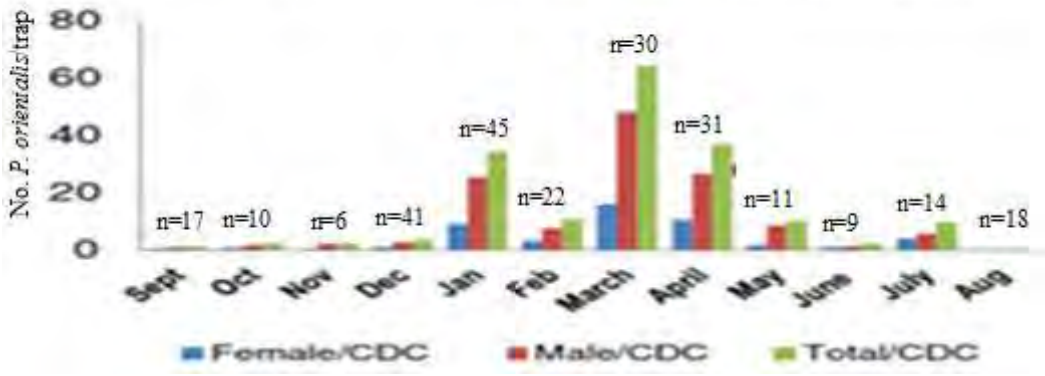
A. *Phlebotomus orientalis* collected from agriculture fiels using CDC light traps



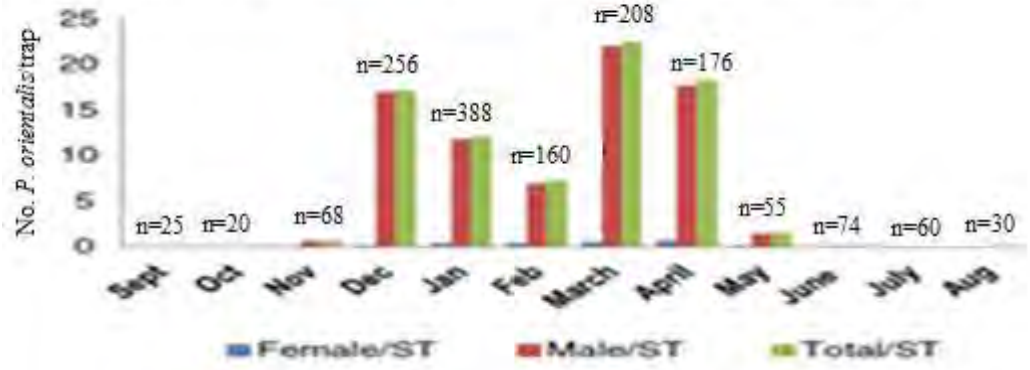
B. *Phlebotomus orientalis* collected from agricultural fields using stickytraps



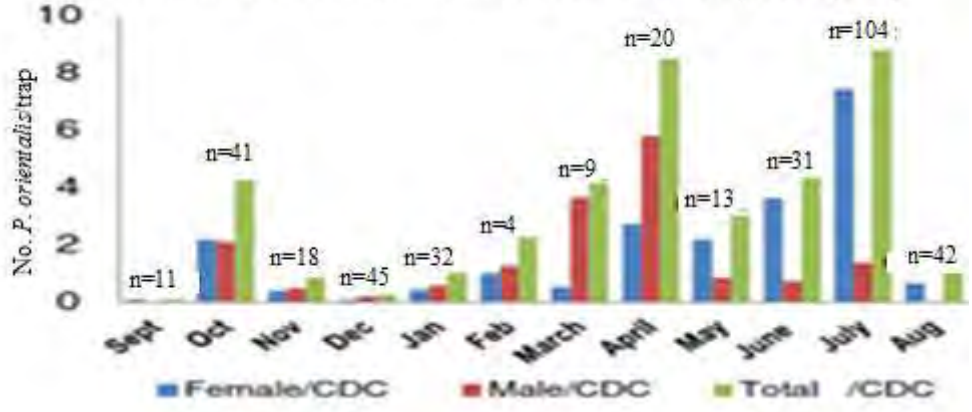
C. *Phlebotomus orientalis* collected from thickets of *Acacia seyal* using CDC light traps



D. *Phlebotomus orientalis* collected from thickets of *Acacia seyal* using sticky traps



E. *Phlebotomus orientalis* collected from dense mixed forest using CDC light traps



F. *Phlebotomus orientalis* collected from dense mixed forest using sticky traps

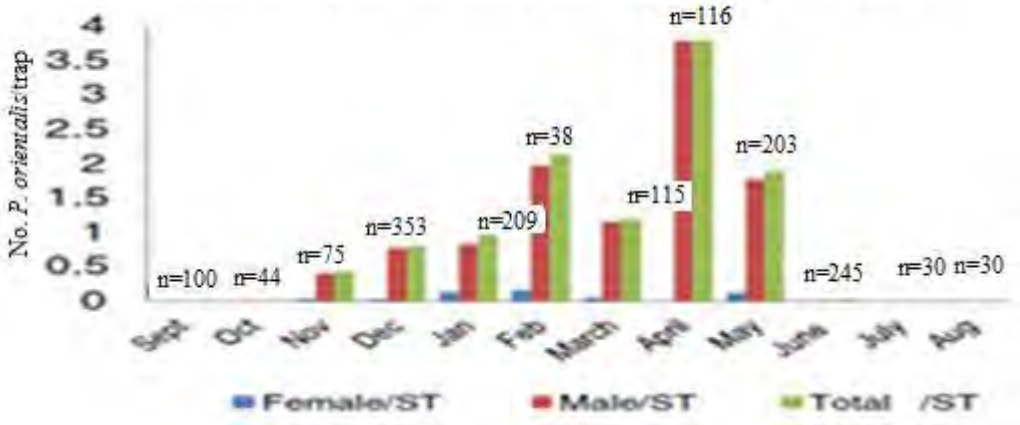


Table 3.2. *Phlebotomus orientalis* abundance in different sampling sites in relation to specific habitats, i.e., agriculture fields, thickets of *Acacia seyal* and mixed forest using CDC traps.

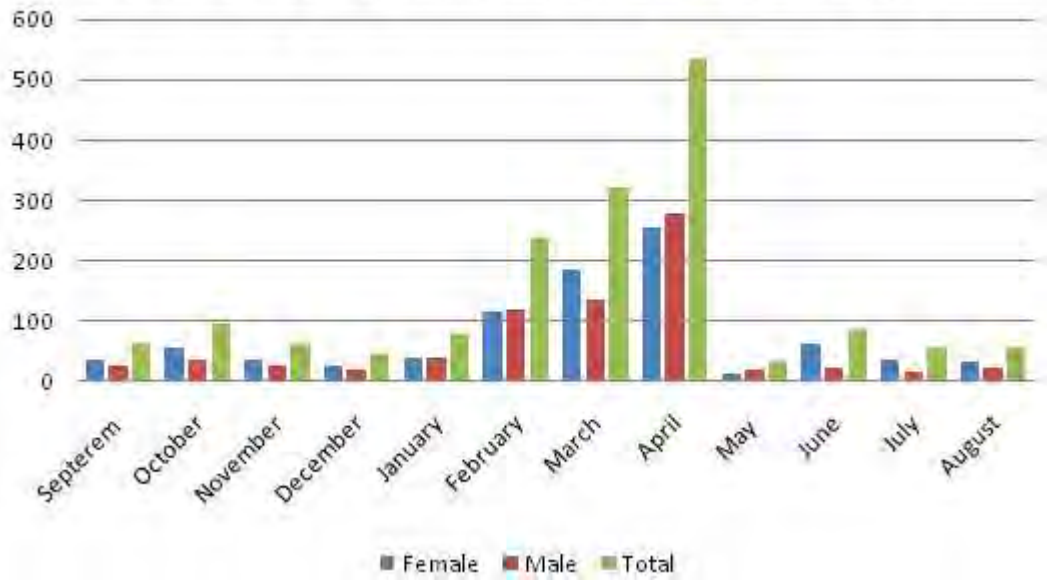
	Agricultural field			Thickets of Acacia seyal			Dense mixed forest		
Locality	*P. orientalis*/CDC trap			*P. orientalis*/CDC trap			*P. orientalis*/CDC trap		
	CDC	Mean	Std. D	CDC	Mean	Std. D	CDC	Mean	Std. D
Adebay extra-domestic site	110	25.99	36.98	-	-	-	92	4.75	6.03
Baeker extra-domestic site	78	22.47	33.24	31	29.37	49.41	149	4.60	8.14
Gelanzeraf extra-domestic site	85	5	9.57	140	28	41.33	116	141	2.09
Rawiyan extra-domestic site	47	1.57	3.09	77	2.74	3.64	-	-	-
Total	320	15.78	28.93	255	19.37	36.42	380	381	6.45

When mean *P. orientalis*/CDC trap for all thickets (19.37±36.42, n=248) and dense mixed forest (3.81±6.45) were compared, there was statistically significant difference between these values (p=000). But, no difference (p=0.55) was observed between dense *A. seyal* in Rawyan (2. 86±3.69, n=83) and the dense mixed forests. Post hoc Tukey HSD test has showed sparse Gelanzeraf thickets of *A. seyal* had almost the same mean number *P. orientalis*/CDC with thickets of Beaker Site 2 (p=0.994) but different mean counts when compared to dense Rawyan thickets of *A. seyal* (p=0.036).

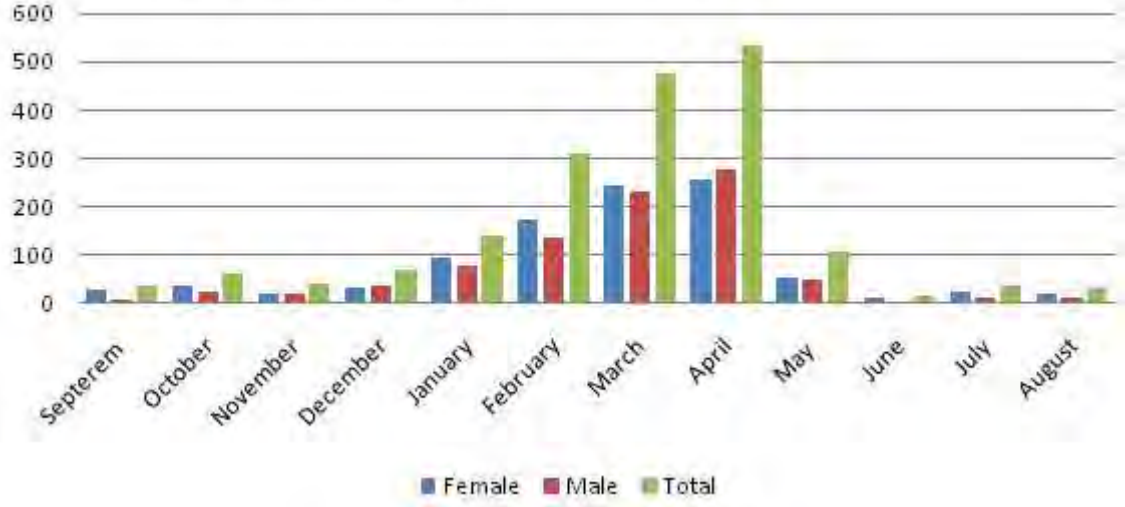
3.3.2. Population dynamics and habitat preferences of *Sergentomyia* species

A total of 164, 392 *Sergentomyia* species were collected from agricultural fields (30, 537 females, 28, 384 males), dense mixed forest (22, 158 female, 16, 974 male) and thickets of *Acacia seyal* (37, 383 females, 28, 956 males) from May, 2011 to June, 2012

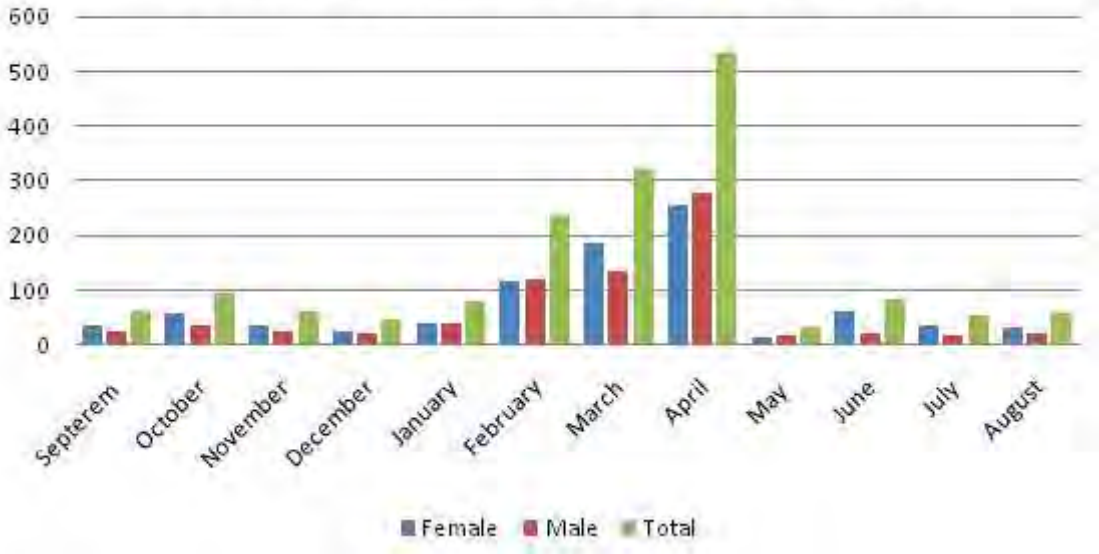
A. Mean no. of Sergentomyia species in Densemixed forest

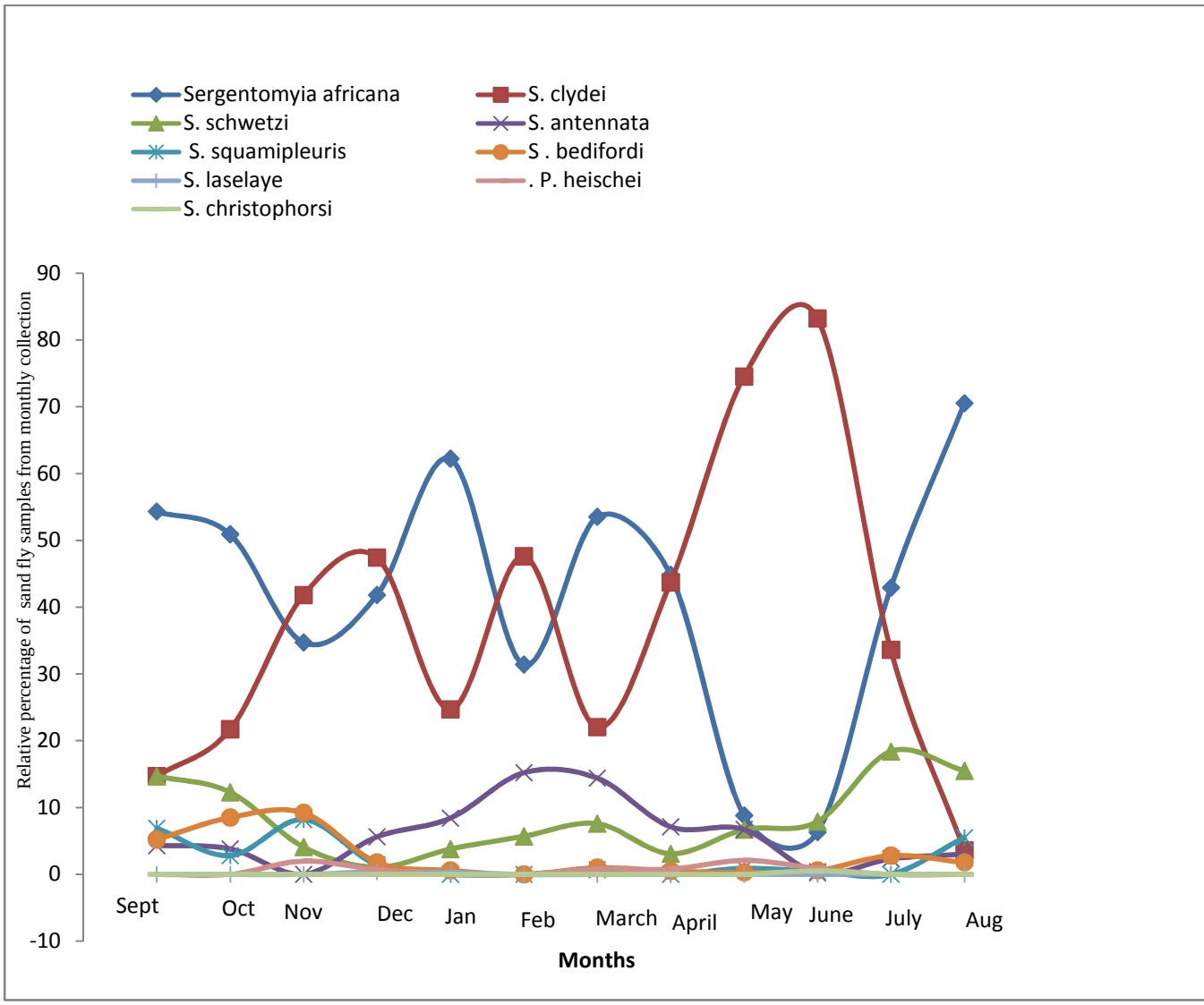


B. Mean no. of *Sergentomyia* spp in Agricultural fields



C. Mean no. of *Sergentomyia spp* in *Acacia seyal* thickets




Sergentomyia africana
S. clydei
S. schwetzi
S. antennata
S. squamipleuris
S . bedifordi
S. laselaye
. P. heischei
S. christophorsi
Relative percentage of sand fly samples from monthly collection
90
80
70
60
50
40
30
20
10
0
-10
Sept
Oct
Nov
Dec
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Feb
March
April
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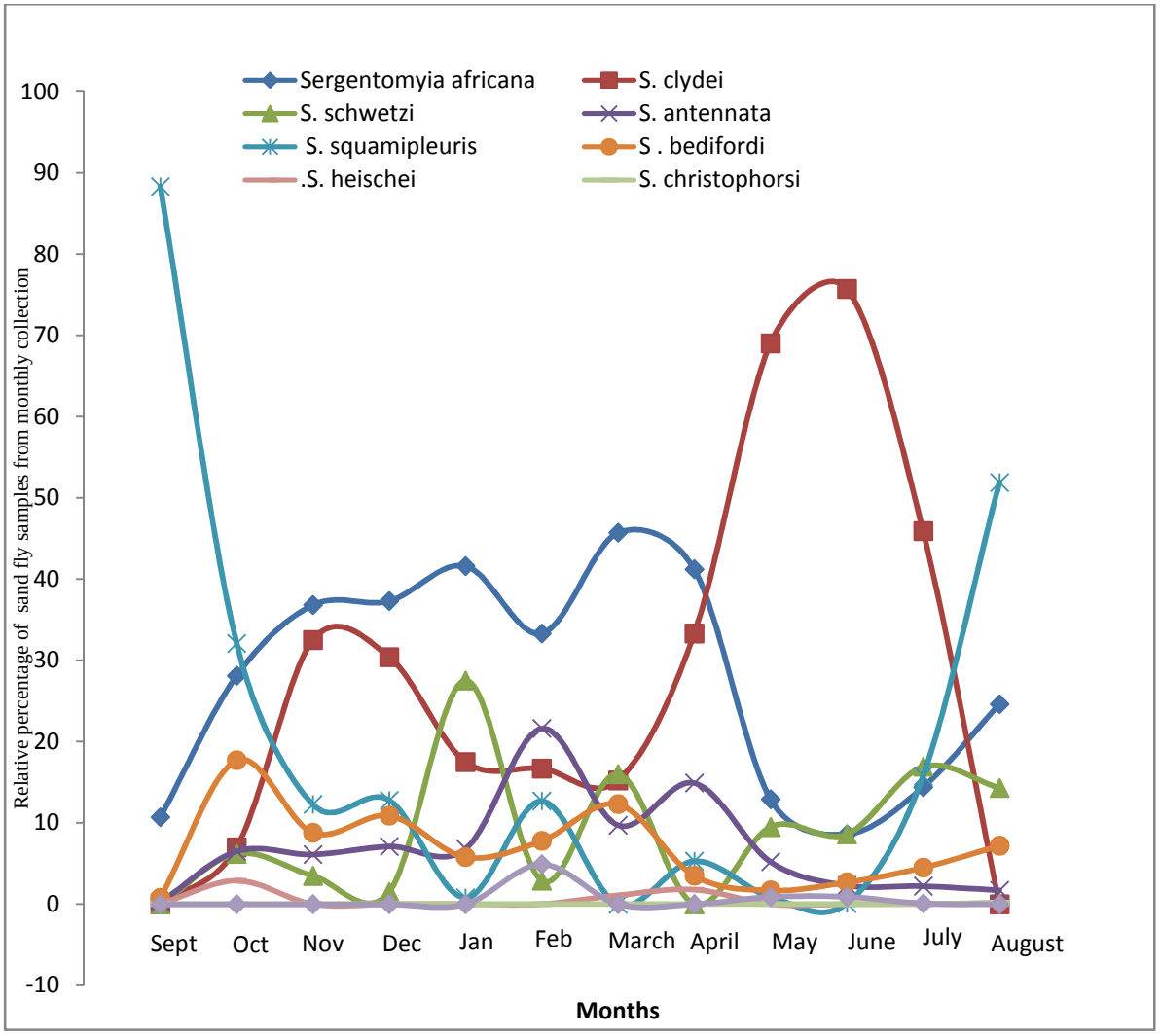
June
July
Aug
Months


Sergentomyia africana
S. clydei
S. schwetzi
S. antennata
S. squamipleuris
S . bedifordi
.S. heischei
S. christophorsi
Relative percentage of sand fly samples from monthly collection
100
90
80
70
60
50
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June
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August
Months

Three decades ago, the vegetations of Metema – Humera lowlands were described as *Acaciaseyal* - *Balanites aegyptiaca* forest and *Argeissus* - *Combretum* savannah woodlands (Gemetchu, *et al.,* 1983), but now it has been converted mainly into big mechanized agricultural fields, especially in Kafta-Humera district. Absence of *Acacia seyal* - *Balanites aegyptiaca* forests and termite mounds in the study areas in this district gave advantage to analyze fewer habitats such as agricultural fields and thickets of *A.seyal* in addition to dense mixed forests in the periphery of Kafta - Shiraro National Park. These habitats are distinctly separated from each other as opposed to habitats studied previously that had no demarcation between agricultural fields and the different types of forests (Quate, 1964; Elnaiem *et al*., 1997; Elnaiem *et al*., 1999).

3.4.1. *Phlebotomus orientalis* **population dynamics and habitat preferences**

In this study, *P. orientalis* reached peak mean numbers in March and April with sudden drop in May (Figure 3.4). The effect of wind on sand fly collection in May and June should not be underestimated. The trend of seasonal dynamics of *P. orientalis* in dense mixed forest did not fully match the patterns in agricultural field and thickets of *A. seyal* (Figure 3. 4). Generally, mean monthly density in dense mixed forest was low compared to other habitats, but it showed an increase during dry season. In July, mean monthly density reached its peak value in dense mixed forest unlike other habitats where *P. orientalis* population declined. These results show that dense mixed forests may not the preferred breeding site for *P. orientalis*. The seasonal changes in *P. orientalis* population in dense mixed forest might be due to the inter-habitat shift of this vector. During rainy and wind stress season (May - June), *P. orientalis* from neighboring agricultural fields and thickets of *A. seyal* might have shifted to the dense mixed forests where cracks and

burrows in tree trunks keep them protected. The cooler temperature inside the forest, which has prevented soil crack formation, might have played a significant role for dense forests not to act as breeding site. Most previous studies (Quate, 1964; Hoogstraal and Heyneman, 1969; Elnaiem *et al.,* 1997) have considered *P. orientalis* as forest species without comparisons among different types of forests (thickets of *A. seyal*, *Acacia seyal-Balanites aegyptiaca* and dense mixed forests) and the exact habitats of *P. orientalis* were not identified.

The overall mean collections of *P. orientalis* usin*g* sticky traps from the three different habitats such as 11.50/sticky traps (n=2, 418) from agriculture fields, 12.17/sticky (n=1, 500) from thickets of *A. seyal* and 1.08/sticky (n=1, 633) from dense mixed forest in Kafta-Humera lowlands could be compared with similar study in Sudan (Quate, 1964). The overall result of mean *P. orientalis*/sticky from the different forests in the Paloich areas in South Sudan 0.195/sticky trap (n=100) (Quate, 1964) was similar with the result obtained from dense mixed forest in Kafta-Humera district. These results were lower than the overall results obtained from agricultural fields and thickets of *A. seyal* and might also suggest dense forests not to be a prefered site for *P. orientalis*.

Habitats with cracks during dry season such as agricultural fields and less dense thickets of *A. seyal* might be breeding sites of *P. orientalis.* Males of *P. orientalis* collected from thickets of *A. seyal* and agricultural fields were found with unrotated genitalia (data not shown) indicating these habitats as places where this vector emerged from larvae. A mere absence of *P. orientalis* in CDC light traps set in forest habitats of Paloich area (Sudan)

could not have been exclusively due to the failure of *P. orientalis* not being attracted to light as already described (Quate, 1964). The absence of cracking type black soil or other breeding sites of *P. orientalis* were decisive. Dense forests could be devoid of soil cracks as already reported (Hoogstraal and Heyneman, 1969) and unsuitable for *P. orientalis* breeding.

Habitats of *P. orientalis* were reported to have persistently lower normalized difference vegetation index (NDVI) value during the dry season and experienced more extreme dry and wet seasons than *P. orientalis* negative sites (Elnaiem, *et al*., 1998). Our field observations in northwest Ethiopia concur with this. Typically, agricultural fields and thickets of *A. seyal* (sparse or thin), where *P. orientalis* was caught in abundance appeared to experience full exposure of sun during dry season whereas being covered with vegetation during rainy season. Agricultural fields and sparse thickets of *A. seyal*, including *Acacia seyal-Balanites aegyptiaca* wood lands, could be a target for future control of kala-azar in the northwest Ethiopia and Sudan.

3.4.2. Bionomics and habitat preferences of *Sergentomyia* ***Species***

Sergentomyia africana, *S. clydei*, *S. schwetzi* and *S. antennata* were non-seasonal species found more or less uniformly in dense mixed forests and agricultural fields as reported before (Quate, 1964; Hoogstraal and Heyneman, 1969). *Sergentomyia heischi* and *S. laselaye* was a rare species in the study areas.

A study of breeding sites of *Sergentomyia* species in agricultural fields of Humera in November, 2011 using emergence traps (Moncaz *et al.*, 2014a) revealed that *S squamipleuris* was the predominant species (54,29.8%), followed by *S. clydei* (40, 22*%),*

S. antennata (35, 19%) and *S. africana* (27, 15%). This study found almost similar result with the study by Moncaz *et al.*, (2014) except for *S. squamipleuris* which was found as seasonal dense mixed forest species with peak abundance in August and September (Figure 3.6; Figure 3.7). In Sudan, *S. squamipleuris was* also reported as non-seasonal species which flourished in humid environments like tropical forests and rare in villages (Quate, 1964; Hoogstraal and Heyneman, 1969). The highest number of *S. antennata* was found in February and gradually decreased in March and June (Dietlein, 1964; Quate, 1964).

Chapter 4

Nocturnal activities and host preferences of *Phlebotomus orientalis*

4.1. Introduction

Phlebotomus orientalis exhibits anthropophilic behavior and it transmits VL from man to man and/or to dog and vice versa (Lambert *et al.,* 2002; Dereure*et al.,* 2003). The dog was considered as an intermediate host between a possible sylvatic and the anthroponotic cycles (Dereure *et al.*, 2003). The large numbers of patients with post kala-azar dermal leishmaniasis (PKDL) in heavily affected villages in Sudan might also indicate a possible human reservoir and anthroponotic transmission (Zijlstra and El-Hassan, 2001). *Phlebotomus orientalis* was also reported to be exophagic/exophilic (zoonotic VL) (Dereure *et al*., 2003; Zijlstra and El-Hassan, 2001; Schorscher and Goris, 1992; Elnaiem *et al.,* 1998; Hassan *et al*., 2009). Zoonotic transmission of VL in East Africa was also suggested based on evidence of VL outbreaks among people that camped in uninhabited areas of eastern and south Sudan in addition to isolation of *L. donovani* from wild animals in the uninhabited Dinder National Park (Kirk, 1956; Ibrahim *et al*., 1999; Elnaiem *et al*., 2001).

Experimental study on host preferences of *P. orientalis* and the isolation of *L. donovani* in East Africa have indicated that *Canis familiaris* (dogs), *Herpeistes ichneumon* (mongoose), *Genetta genetta* (genet cat), *Acomys* spp. and *Arvicanthis* spp. are preferred hosts for source of blood meals (Hoogstraal and Heyneman, 1969; Hassan *et al*., 2009; Haile and Lemma, 1977; Mutinga *et al.,* 1980). The result of *P. orientalis* from human bait also showed humans to be preferred hosts (Elnaiem *et al*., 1997; Ashford *et al*.,

1973b; Hailu *et al*., 1995; Kolaczinski *et al*., 2008). Attraction of *P. orientalis* towards cattle, sheep, goat, mule, donkey and horse has not been defined in East Africa as a whole, even though *Leishmania* sero-prevalence rate ranging from 8.5% to 68.7% has been reported for these domestic animals in eastern Sudan (Mukhtar *et al*., 2000) and 92% in cattle innorthwest Ethiopia (Gebre-Michael *et al*., 2010).

In the Metema-Humera lowlands, seasonal labour migrants who stay in agricultural fields for weeding and harvest of sesame, sorghum and cotton are exposed to *L. donovani* infections from June to October (Fuller, 1976; Lemma *et al*., 2015). In these areas, the nocturnal activities (diel periodicity) and the peak biting hours of *P. orientalis* were not studied. Adequate knowledge on these aspects helps to reduce kala-azar incidence by avoiding contact with sand flies at that particular period. Thus the aim of this study was to elucidate the nocturnal activities and host preferences of *P. orientalis* in Kafta – Humera lowlands.

4.2. Materials and Methods

4.2.1. Study area

The study was conducted in two selected extra-domestic sites. These were near Baeker town (Site 1) and Gelanzeraf (Site 2). Baeker extra-domestic sand fly sampling Site 1 (14°01’N, 36°59’E, 651 m) is located 33 km from Humera town close the main road between Humera and Gondar towns. Gelanzeraf is a locality southwest of Humera, near the Sudan border. Gelanzeraf extra-domestic sampling Site 2 (13°59’N, 036°31’E) is located at 38 km from Humera town near the new asphalt road leading to Sudan, at about

20 km from the Sudan border (Figure 1). In both sites, there were closely placed huts (tukuls) in the camps on the agricultural fields.

Domestic animal shelters are located at about 200 m in Site 1 and 250 m in Site 2 from the huts. *Acacia seyal* in Site 1 is located about 2 km from the huts compared with 4 km in Site 2. In Site 1, the two vacant huts were giving service only during agricultural rainy season. The 4 guards near telecommunication tower and 4 animal keepers near the animal shelters were the only persons near the huts in Site 1 in addition to 3 individuals working on irrigation of vegetables, using ground water in a gorge found slightly far away from the huts. One individual, of those working in the irrigation, left the area following VL infection in September, 2011, after completing treatment in Humera Kahsaye Abera hospital. In Site 2, the camp has 3 huts and a big store with 5 individuals who take care of the area and the animals.

Goats and sheep are browsing and grazing in agricultural fields and in the sparse thickets of *A. seyal* including the dense mixed forest (Part of Kafta-Shirao park in Baeker at about 7 km from Site 1) during the day and shelter near the camps at night unlike cattle which graze at night during dry season (October - June). In agricultural rainy season, however, animals are guarded in the dense mixed forest and rocky uncultivated areas. Permanent locations on agricultural fields, animal shelter and sparse thickets of *A. seyal* were selected for the repeated sand fly collections during studies of nocturnal activities and host preference towards rodents. The sampling points on agricultural fields and animal

Any freshly bloodfed female *P. orientalis* encountered during previous sand fly collection from thickets of *A. seyal* (March, 2011) and dense mixed forest (July, 2011) in Baeker, was preserved for molecular identification of animal species that served as source of blood for this vector using Reverse Line Blot (RLB) of cytochrome *b* PCR product. For this purpose, the head of each fed female was mounted on the slides by Hoyer's medium for later species identification. The remaining body (thorax and abdomen) was individually placed in empty sterile eppendorf tube with silica gel grains and cotton pads inside, bearing a corresponding label with the mounted specimen on the slide. They were stored at −20 °C until molecular blood meal analysis was conducted in August 2012 in the Department of Microbiology & Molecular Genetics, Hadassah Medical School, Hebrew University, Israel.

4. 2.4.2 DNA extraction

The thorax and abdomen of freshlyfed *P. orientalis* from each tube with silica gel were placed on filter paper and transferred to a test tube for digestion of tissues using lysis buffer (200 µl) and proteinase K (10 µl). Each *P. orientalis* was homogenized using sterile wooden sticks and incubated in water bath (65°C) for 2 hours. Phenol extraction of DNA was made by adding 180 µl phenol before centrifugation at maximum speed (1, 400 r.p.m) for 2.5 minutes. For ethanol DNA extraction, the aqueous part (150 µl) was transferred into a test tube containing sodium chloride solution (8 µl) before 400 µl cold (−20°C) ethanol was added and placed in refrigerator (−20°C) for 2 hours and cold centrifugation at maximum speed (1, 400 r.p.m) for 10 minute that precipitated DNA. Finally, pure DNA was obtained by removing the ethanol and suspending in 50 µl double distilled water (Abbasi *et al.*, 2008).

4.2.4.3. Species-specific probes

This study described a blood meal identification approach based on PCR amplification of the mitochondrial cytochrome *b* gene followed by RLB analysis as already described (Abbasi *et al.,* 2008). The species-specific oligonucleotide probes designed by Abassi *et al*. (36) for human (ATG CAC TAC TCA CCA GAC GC), cattle (ATT ATG GGT CTT ACA CTT T), sheep (TCC TAT TTG CGA CAA TAG CTT CCT), goat (ATA CAT ATC GGA CGA GGT CTA), camel (CGT TGG AAT TGT TTT ATT), donkey (CTA CTT TTC ACA GTT TAG CTA CA), dog (CAG ATT CTA ACA GGT TTA), mouse (TGG AGT ACT TCT ACT GTT CGC AGT), rat (CAG TCA CCC ACA TCT GC), chicken (CAT CCG GAA TCT CCA C) and avian (TAC ACA GCA GAC AC) were used which adsorbed on the 3 mm strips of membrane in the following order from the top to bottom: Human, cattle, donkey, sheep, goat, camel, dog, mouse, rat, chicken and avian. One probe was found effective in identification of all avian species (Abbasi *et al.,* 2008).

4.2.4.4. Polymerase chain reaction

To make 25 µl of final volume for PCR reaction, 20 µl master mix (1 µl forward primer, 1 µl backward primer and 18 ddH_2O) and 5 µl DNA sample were mixed. PCR amplification of the mitochondrial cytochrome *b* gene was made using primers Cyto1 (5'-CCA TCA AAC ATC TCA GCA TGA TGA AA-3') and Cyto2 (5'- CCC CTC AGA ATG ATA TTT GTC CTC-3') before gel electrophoresis at 120 V in 1× TAE buffer in 1.5% agarose gels. The DNA fragments were visualized by UV light for determination of the sizes. The PCR product left from gel electrophoresis (18 µl) was used for RLB hybridization.

4.2.4.5. Reverse line blot hybridization

The species-specific 5′-amine-labeled oligonucleotide probes from the 11 species were bounded on 5.5 × 15 cm negatively charged biodyne C membrane (Gelman, USA) following activation with 10% (wt/vol) 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC) solution for 30 minute at room temperature as described previously (Abbasi *et al.,* 2008). The activated membrane was rinsed with distilled water and placed in a miniblotter (MN45; Immunetics, Cambridge, MA). The slots of the Miniblotter were filled with 150 µl of each of the diluted oligonucleotide solutions (5 pmoles/μL). The membrane from the Miniblotter was eemoved and incubated in 250 ml 0.1 M NaOH for 9 min on rocking platform to inactivate the membrane. Then, briefly wash the membrane in a plastic container on the rocking platform in 250 ml 2 × SSPE and incubated in 250 ml pre warmed 2 × SSPE, 0.1% SDS for 5 min at 60 °C in hybridization oven with rocking. The membrane sheets with the oligonucleotide probes were cut into strips at a right angle to the direction of the blots so that every strip contained a section with each probe. Strips were incubated in prehybridization solution (2xSSC [0.15 M NaC1, 0.015 M sodium citrate], 0.1% sodium dodecyl sulphate [SDS]) for 30 min at 45^{o}C with gentle shaking. Biotinylated PCR products were denatured by boiling for 5 min and applied to the membrane strips. Hybridization was performed at 43°C for 1 h followed by a single wash with 0.7xSSC, 0.1% SDS for 20 minutes. Hybridized biotinylated DNA was detected by incubating the strips in streptavidin horseradish peroxidase (HRP; diluted in 2x SSC, 0.1% SDS) for 30 min at room temperature. Strips were washed briefly 3 times in 2_ 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. ECL detection was performed immediately after streptavidin-HRP incubation and washing steps using EZ-ECL detection kit (Biological Industries, Beit Haemek, Israel).

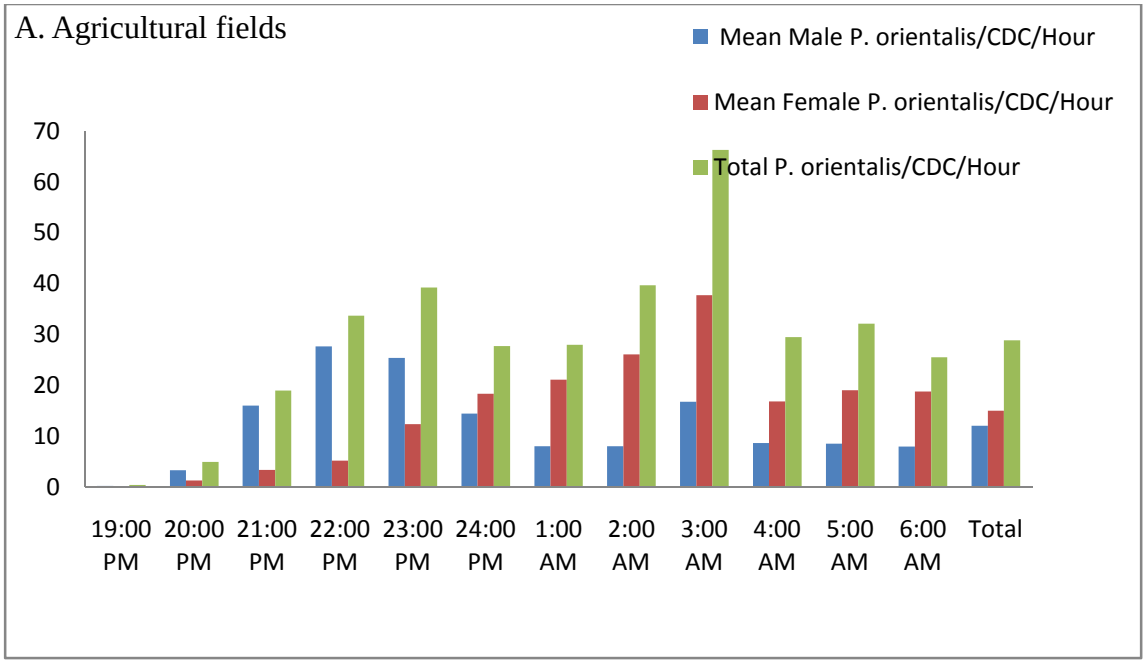
4.2.6. Statistical analysis

The nocturnal activities of *P. orientalis* during 12 hours of the night were estimated from mean number *P. orientalis*/hour/CDC. All statistical analyses were performed using the Statistical Package of Social Science (SPSS), version 16. Comparisons of nocturnal activities during the night hours were analyzed using one-way analysis of variance (ANOVA). P-values less than 0.05 for mean number *P. orientalis*/hour/CDC were considered as significantly different.

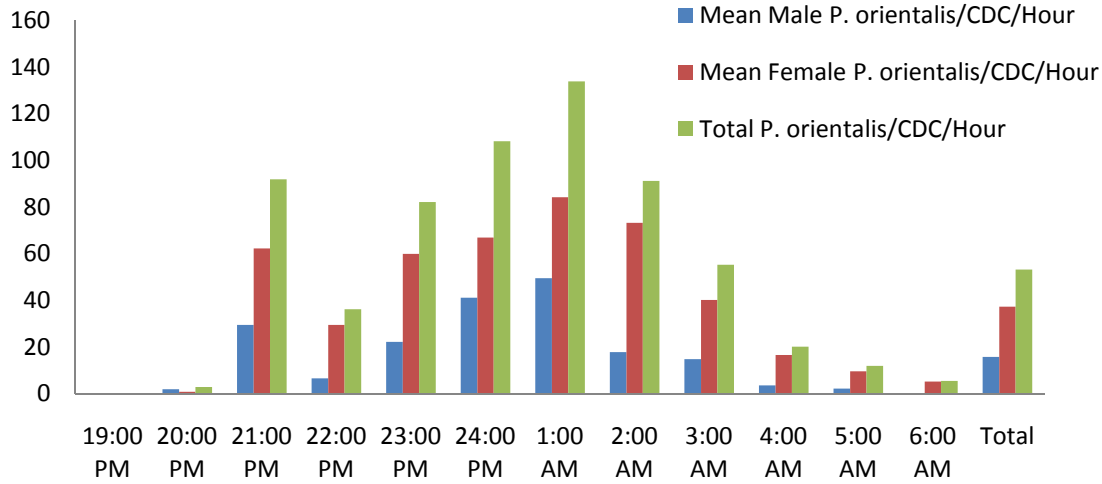
4.3. Results

4.3.1. Nocturnal activities of *Phlebotomus orientalis*

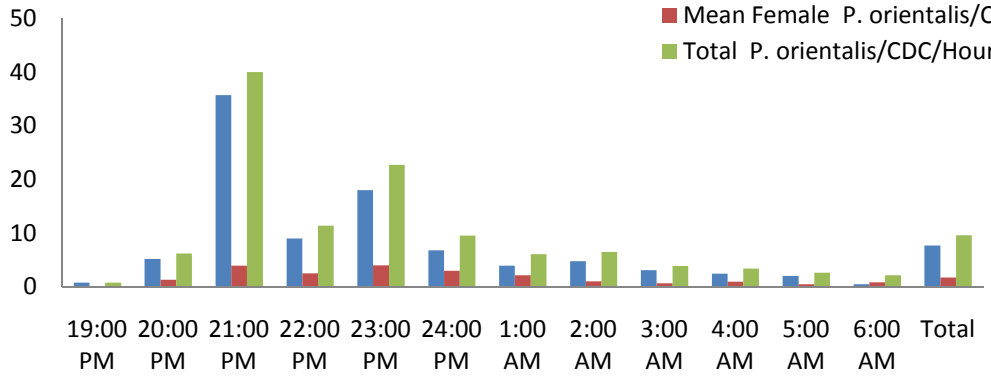
A total of 6,083 *P. orientalis* (3,381 females and 2,702 males) was collected at one hour interval for 18 nights using a total of 22 CDC traps from agricultural fields near the camps (1,989 females and 1,614 males; 11 CDC traps), sparse thickets of *A. seyal* (167 females and 738 males; 8 CDC traps) and near sheep and goat shelters (1,349 females and 573 males; 3 CDC traps) during the study period. *Phlebotomus papatasi* (n=18), *P. duboscqi* (n=9), *P. begeroti* (n=7) and *P. rodhaini* (n=6) were other species of *Phlebotomus* collected with the same traps. Overall 20, 236 (10, 211 males and 10, 025 females) *Sergentomyia spp* were collected. The highest overall mean number of *P. orientalis*/hour/CDC trap was collected from animal shelters (53.37 ± 45.80) and followed by agricultural fields (28.87 ± 23.45). The lowest mean number of *P.*


A. Agricultural fields
Mean Male P. orientalis/CDC/Hour
Mean Female P. orientalis/CDC/Hour
Total P. orientalis/CDC/Hour
70
60
50
40
30
20
10
0
19:00 PM
20:00 PM
21:00 PM
22:00 PM
23:00 PM
24:00 PM
1:00 AM
2:00 AM
3:00 AM
4:00 AM
5:00 AM
6:00 AM
Total

B. Animal shelters



C. Sparse thickets of *A. seyal*



4.3.3. Host preference study of *Phlebotomus orientalis* based on molecular analysis of blood meals

Of the 25 blood-fed *P. orientalis* analyzed using cytochrome *b* PCR and RLB (Figure 4.2), 7 (28%) were human, 9 mixed human and cattle (36%) and 2 cattle (8%). There were 7 samples (28%) with bands on agarose gel but not in nitrocellulose membrane indicating the existence of other animals outside the 11 probes.

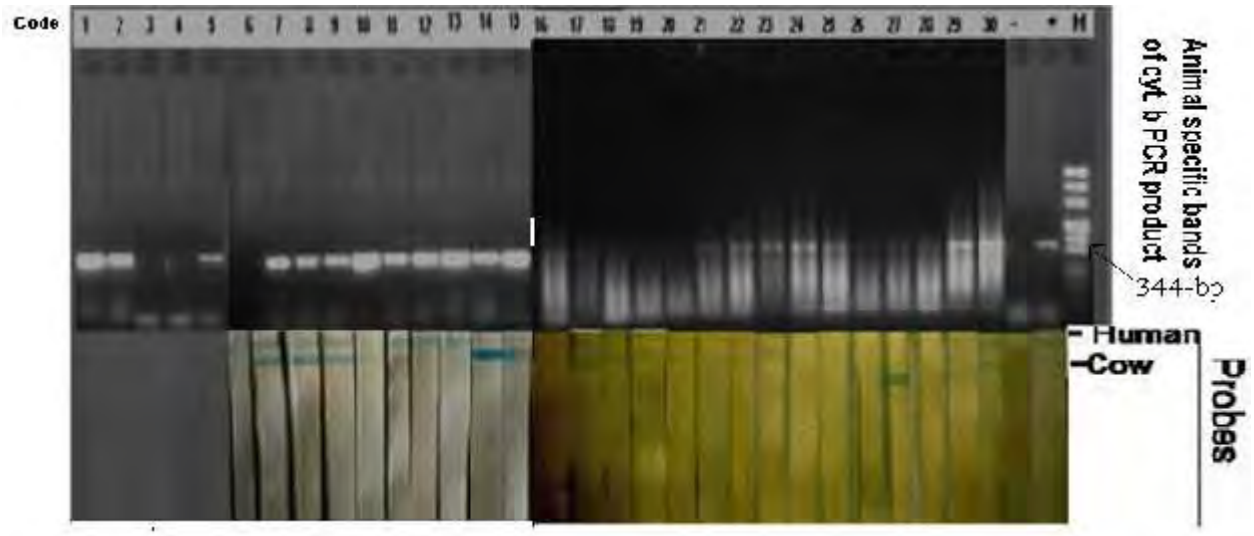


Figure 4.2. Animal's specific bands of cytochrome *b* PCR products (344 bp) obtained from blood meals of *Phlebotomus orientalis* on gel-electrophoresis after visualized by UV light (Top) and species specific hybridization of the PCR products obtained from different fresh fed *P. orientalis* using the different probes on nitrocellulose (bottom). Only Human and cattle were found as source of blood meal for *P. orientalis* collected from *Acacia seyal* (March, 2012) and dense mixed forest (July, 2011) in the beaker site 1.

4.4. Discussion and conclusion

So far no effective VL control measures have been conducted in Sudan and northwest Ethiopia, mainly due to the sylvatic nature of adult *P. orientalis* and the lack of enough knowledge on transmission dynamics of *L. donovani* between unknown reservoir hosts and humans (Elnaiem, 2011). Studies on nocturnal and feeding activities of adult *P. orientalis* could give an insight about the behavior of this vector which is an essential component of the kala-azar control strategies. The results on nocturnal activities from hourly CDC traps showed a risk of *P. orientalis* bite throughout the night as already reported (Schorscher and Goris, 1992).

The fact that the highest mean value of *P. orientalis* collected near animal shelters might indicate that goats and sheep are also sources of blood meals. The role of goats and sheep in the epidemiology of kala-azar is a subject of further study. Only one serological study in eastern Sudan indicated 8.5% *L. donovani* infection rate in goats using DAT (Mukhtar *et al.,* 2000). In Kenya, *P. duboscqi* and *P. martini* were reported to be attracted to goats (Mutinga, *et al*., 1986). Recently, RLB of cytochrome *b* PCR products of blood meal collected from *P. orientalis* in domestic areas in Kafta-Humera district showed goats and sheep as a source of blood meal for this vector in addition to cattle (Yared *et al.*, personal communication). This study has shown that cattle are important sources of blood for *P. orientalis* in extra-domestic habitats where 72% of this species was found to have human and/or cattle blood-meal source. Mixed (human and cattle) blood meal was found to be very high (36%) which might indicate that *P. orientalis* feeds interruptedly on both hosts. Interruptions might have occurred due to inherent behavior of the female fly or due to the physical disturbance by the human or animal hosts during the probing of the fly.

Previous study has shown that cattle serve as the main source of blood for *P. orientalis* in domestic areas (Gebre-Michael *et al.,* 2010). The reason why more cattle (92%) served as source of blood than human (2.2%) in domestic and predomestic areas using ELISA (Gebre-Michael *et al.,* 2010) in the area of Metema compared with this study is difficult to explain. Further investigation is needed.

The bands of DNA fragments (cytochrome b) on agarose gel (28%), that were not captured by the 11 probes used, may represent wild animals in the area. The probes that were not used include wild rodents and small carnivores. The existences of small carnivores such as genet cats and mongoose were confirmed during our study on reservoirs in these areas especially in the dense mixed forest (unpub. data). Either rodents or small carnivores or both could be the unknown source of blood for *P. orientalis*. Attractiveness towards mongoose (63.9 ± 12.1/CDC/night), genet cat (17.4 ± 3.72/CDC/night) and Nile rat (2.6 ± 0.56/CDC/night) compared to the control (0.4 ± 0.16/CDC/night) were reported in eastern Sudan (Hassan *et al*., 2009). No *P. orientalis* or other sand fllies, except 5 *Sergentomyia schwetzi*, were attracted to the rodents from all Turner and Hoogstraal box traps in this study. Similar study using CDC or sticky traps is required before reaching a conclusion.

The results of this study are in complete agreement with other observations (Hoogstraal *et al.,* 1962; Schorscher and Goris, 1992) showing night-long human bite of *P. orientalis.* The nocturnal activities and human biting behavior was described as wave-like,exhibiting non-uniformity of biting rate at different hours of the night (Schorscher and Goris, 1992). Such wave-like biting was due to the effect of wind. There were different trends of winds

according to the observations during this study. Sometimes it began at 19:00 hrs and ended at 21:00 hrs or started at 21:00 hrs and came down at 23:00 or 24:00 hrs. In a few instances, strong wind persisted until 24:00 hrs. *Phlebotomus orientalis* could bite humans at different hours of the night and there is a risk of kala-azar infection in agricultural fields, thickets of *A. seyal* and in the camps. It is also attracted to domestic animals in the extra-domestic habitats for blood meal. *Phlebotomus orientalis* is most probably catholic in its feeding habits and have wide range of mammals as blood meal sources depending on the available host in space and time.

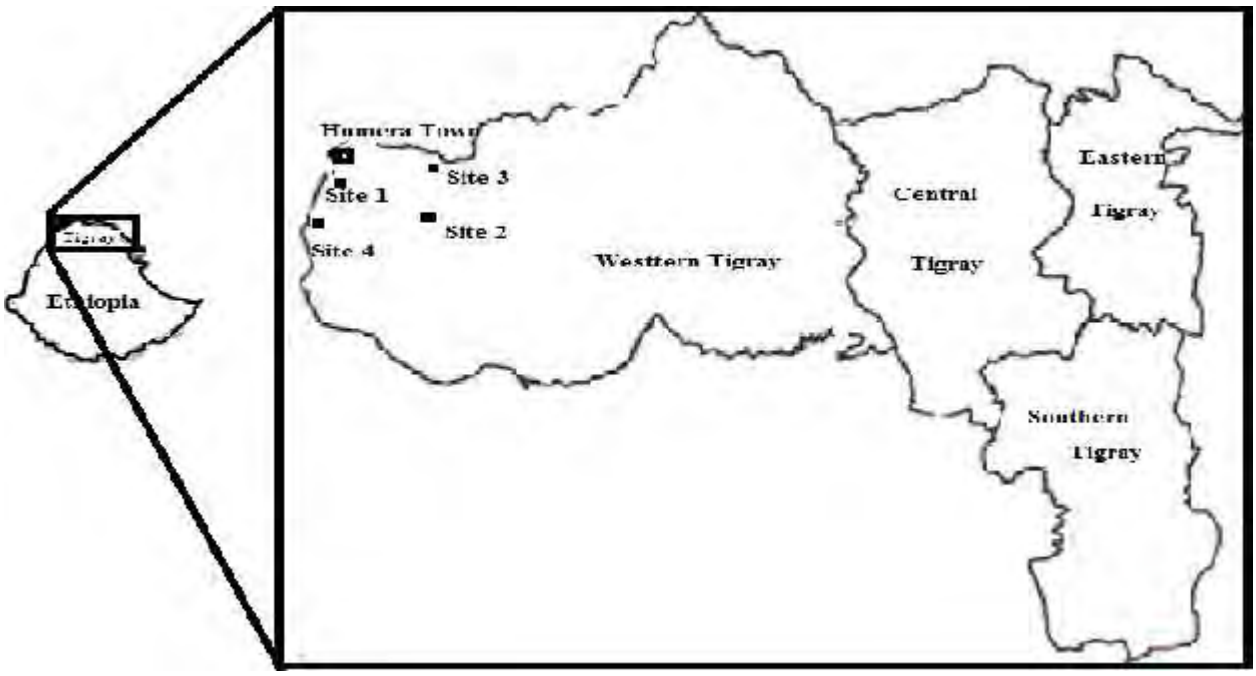
Chapter 5

Leishmania parasite infections in *Phlebotomus orientalis*

5.2. Introduction

Due to population growth and expansion of arable lands, people are attracted more to the black fertile soil especially in northwestern Ethiopia (Gemetchu *et al.*, 1983; Gebre-Michael *et al*., 2010). Travel history to Kafta Humera lowlands during agricultural season was the main reason for seasonal migrant labourers to contract VL (Haile and Andersonm, 2006; Alvar *et al*., 2007; Argaw *et al*., 2013).

Phlebotomus orientalis was suspected as the vector of VL in Kafta-Humera lowlands mainly based on observations of disease distribution and abundance of this man biting species (Gemetchu *et al*., 1975; Gebre-Michael *et al*., 2010). So far, no naturally infected *P. orientalis* was found in Ethiopia, except 1/70 (1.4%) infection rate reported from the lower Omo plain, southwestern Ethiopia with black cracking soil (Hailu *et al*., 1995). Previous attempts in northwestern Ethiopia did not succeed in *Leishmania* parasite isolation from the suspected vector (Ashford, 1973b; Gemetchu *et al.*, 1975; Gebre-Michael *et al*., 2007; Gebre-Michael *et al.*, 2010). High infection rates of *P. orientalis* (6.9% - 18%) were reported from neighboring eastern Sudan (Hoogstraal and Heynemann, 1969; El-naim *et al*., 1998a). The aim of this study was to detect *Leishmania* parasites from the gut of *P. orientalis*by using microscopy and PCR techniques so as to confirm its role as the vector of *L. donovani* in the study area.


Ethiopia
Humera Town
Site 1
Site 3
Site 2
Site 4
Westtern Tigray
Central
Tigray
Eastern
Tigray
Southern
Tigray

5.2.2. Sand fly collection, Sorting and dissection

CDC light traps were set at 6 p.m, hanged at about 0.5 meter above ground level, and left overnight till 6 a.m. The sand flies were kept in the refrigerator as soon as possible until dissected within about 12 hours. All female *P. orientalis* were withdrawn step by step from the refrigerator for dissection to isolate *Leishmania* parasites and to determine their gut status as already described (Hailu *et al*., 1995). In brief, female *P. orientalis* were first washed in 2% savlon saline solution and sterile physiological saline three times before dissection in the saline. Parity was determined by presence of granules in the accessory glands. The gut was examined under 10x and 40x objectives. The gut contents of the parous and gravid including those with suspected *Leishmana* parasites were transferred to absolute alcohol (95%) under sterile conditions individually for PCR detections. The heads and last segment of the abdomens of all *P. orientalis* were mounted in Hoyer's medium, after the head was turned upside down for species identification using the appropriate keys (Quate, 1964; Abonnenc and Minter, 1965; Lewis, 1982).

5.2.3. Molecular analysis of *Leishmania* infection

5.2.3.1. Extraction of DNA

Five *P. orientalis* from absolute alcohol were placed on filter paper to dry out before pooling in a test tube (one pool). *Phlebotomus orientalis* were crushed using wooden sticks and incubated in water bath (65^0C) for 2 hours after addition of 200 ul lysis buffer and 10ul of proteinase K. Phenol extractions of DNA was performed by adding 180 µl phenol before centrifugation at maximum speed (1400 r.p.m) for 2.5 minute. For ethanol DNA extraction, the aqueous part (150 µl) was transferred into a test tube containing sodium chloride solution (8 µl) before 400 µl cold (-20^0C) ethanol was added and placed

in refrigerator (-20^{o}C) for 2 hours and subsequently centrifugated for 10m minutes at maximum speed of 1400 r.p.m. Finally, pure DNA was obtained by removing the ethanol and suspending in 50 µl double distilled water.

5.2.3.2. PCR analysis

The kinetoplast DNA (kDNA) and internal transcribed spacer 1 (ITS1) are the two known markers used in PCR for identification of *Leishmania* species with 98.7% and 91.0% sensitivity respectively. Unlike 100% specific ITS1 PCR, kDNA PCR is less specific and detect false positives (Bensousan, *et al.,* 2006). In this study, ITS1 PCR was used for detection of *Leishmania* species from 15 pools collected from agriculture fields and thickets of *Acacia seyal* in dry season and kDNA PCR was used for detection of *Lieshmania* species in 30 pools of *P. orientalis* collected from dense mixed forest in July rainy season.

To make 25 µl of final volume for PCR reaction, 20 µl master mix (1 µl forward primer, 1 µl backward primer, dNTP and 18 ddH_2O) and 5 µl DNA sample were mixed. Polymerase chain reaction amplification of ITS 1 gene was made for 15 pooled *P. orientalis* using primers LITSR (5′-CTG GAT CAT TTT CCG ATG-3′) and L5.8S (5′-TGA TAC CAC TTA TCG CAC TT-3′) for 35 cycles (Schonian *et al*., 2003). Another 30 pooled *P. orientalis* were screened by kDNA PCR using the primers 13A (5′-GTG GGG GAG GGG CGT TCT-3′) and 13B (5′-ATT TTC CAC CAA CCC CCA GTT-3) (Bensousan, *et al.,* 2006). The PCR amplification productes were electrophoresed at 120 V in 1× TAE buffer in 1.5% agarose gels. The DNA fragments were visualized by UV light for determination of Leishmania specific bands. A PCR result was considered positive for genus *Leishmania* when a band of the expected size was obtained: for

13A/13B primers (~120 bp) for kDNA PCR while ~300-350 bp for ITS1 PCR (Bensoussan *et al*., 2006; Nasereddin *et al*., 2008).The molecular analysis of *P. orientalis* for screening *Leishmania* parasites using PCR was conducted in the Department of Microbiology and Molecular Genetics, Hadassah Medical School, Hebrew University, Israel.

5.3.Results

5.3.1. Number of *P. orientalis* processed

A total of 18, 416 sand flies (n = 91 CDC light traps) were collected from dense mixed forest, agricultural fields and sparse thickets of *A. seyal* (Table 5.1). A total of 867 *P. orientalis* (762 females; 105 males) were collected from dense mixed forest using 56 CDC light traps in July, 2011 (rainy season) in addition to 766 *P. orientalis* (304 females; 462 males) during March – May, 2012 from agricultural fields (19 CDC traps) and sparse thickets of *Acacia seyal* (n= 16 CDC traps). The other *Phlebotomus* species from all the traps were *P. papatasi* (4 females; 5 males), *Phlebotomus duboscqi* (2 males), *P. bergeroti* (2 males) and *P. rodehaini* (2 females; 4 males).The *Sergentomyia* species identified during the study period included *Sergentomyia africana*, *S. clydei*, *S. schwetzi*, S. *antenata*, *S. squamipleuris*, *S. heischi* and *S. bedifordi* (the result not shown).

Table 5.1. Female (F) and Male (M) sand flies collected from study areas during July 2011 and March –May, 2012.

Month	Habit	CDC traps			*P. orientalis*			other *Phlebotomus* spp.			*Sergentomyia* spp.
		F	M	TF	M	TF	M	T			
March	Agricultural fields	8	38	40	78	3	2	5	893	827	1, 720
April	Agricultural fields	7	45	128	173	1	2	3	1, 782	1, 478	3, 260
	Thickets of A. seyal	12	177	158	335	0	3	3	3124	2947	6, 071
May	Agricultural fields	4	38	46	84	2	4	6	713	628	1, 341
	Thickets of A. seyal	4	6	90	96	0	0	0	392	436	828
July	Dense mixed forest	56	762	105	867	0	2	2	2, 450	1, 094	3, 544
Total		91	1066	567	1633	6	13	19	9, 354	7, 410	16, 764

5.3.2. Microscopic examination

None of the 762 female *P. orientalis* from the dense mixed forest were examined microscopically. The abdominal status of 304 *P.orientalis* collected from agricultural field were unfed (244), gravid (40), and fresh fed (20). Of 244 unfed females, 155 were parous and 89 were nulliparous. Only 2 *P. orientalis* from gravid guts were found infected with *Leishmania* flagellates during microscopic examination (Table 5.2).

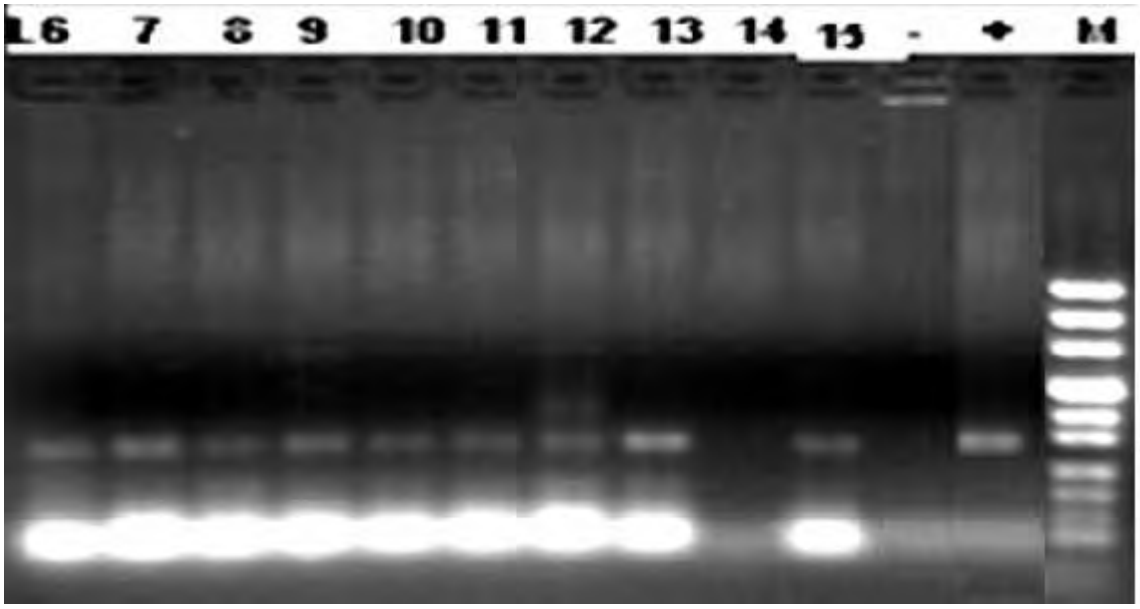
Table 5.2. The gut status and *Leishmania* infection of female *P. orientalis* examined microscopically and using PCR reactions (ITS1 and kDNA).

Sampling localities	CDC traps	P	G	NP	FF	Total	gut +ve Micro-scopy	Positive PCR ITS1	Positive PCR kDNA
Adebay agriculture field	8	23	5	8	2	38	0	0/5 pools	-
Adebay agriculture field	7	25	9	7	4	45	2	2/2 pools	-
Baeker *Acacia seyal*	4	47	14	27	9	97	0	4/4 pools	-
Gelanzeraf *Acacia seyal*	8	33	9	34	4	80	0	1/2pools	-
Gelanzeraf *Acacia seyal*	4	5	1	0	0	6	0	1/1 pool	-
Adebay agriculture field	4	2 2	2	13	1	38	0	1/1 pool	-
Adebay dense forest	24	-	-	-	-	235	-	-	0/8 pools
Baeker dense forest	32	-	-	-	-	527	-	-	4/22 pools
Total	91	155	40	89	20	1066	2/304	9/15	4/30 pools

Key: P = parous; G = gravid; NP = nulliparous; FF = fresh fed; - = not done

5.3.3. PCR analysis

Of the total 15 pools analysed from agriculture fields and thickets of *A. seyal* using ITS1, during the dry season, 9 were positive for *Leishmania* species (60%). A total of 4 pools were positive from 30 pools (13.3%) of *P. orientalis* collected from dense mixed forest and analysed for *Leishmania* infection using kDNA PCR (Table 5.2). None of the kDNA and ITS1 PCR *Leishmania* positive pools were sequenced for identification of *Leishmania* species.


L6
7
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9
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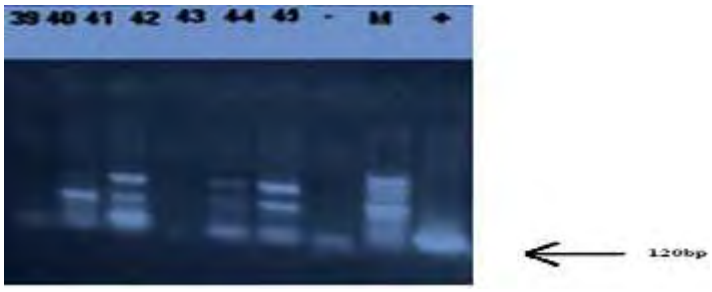

39 40 41 42 43 44 45
-
M
+
120bp

from extra-domestic habitats of Adebay and Beaker dense mixed forest. Lane M: MW marker; Lanes 39 and 43: *Leishmania* negative *P. orientalis* from Beaker and Adebay dense mixed forest; Lanes 41, 42, 44, 45: *Leishmania* positive *P. orientalis* from Beaker dense mixed forest; Lane +: positive control (DNA of *L donovani*); Lane - : negative control.

5.4. **Discussion**

Previous study in Metema-Humera lowlands (Gebre-Michael *et al*., 2010) did not isolate *Leishmania* parasites from *P. orientalis* fromdomestic and predomestic collections.Extra-domesticKafta Humera lowlands are areas where migrant labourers are believed to be exposed to *L. donovani* infections (Lemma *et al*., 2015).Finding naturally infected *L. donovani*wild caught *P. orientalis* in these areas helps to incriminate this sand fly as a proven vector. Although in this study we found *Leishmania*positive pools in *P. orientalis*, the species of the*Leishmania* were not identified by sequencing or by furtherrestriction enzyme digestion. The *Leishmania* positive pools can be assumed as *L. donovani* as the vectors of other *Leishmania* species are rare in the extra-domestic habitats of Kafta – Humera lowlands.

In this study, the rate of *Leishmania* infection in the gut of *P. orientalis* determined by microscopy (2/304 or 0.7%), is relatively small as compared to parous reports. In Upper Nile Province in Sudan, Ashford *et al.* (1992) found 5 *L. donovani* positive *P. orientalis* from the total 48 females dissected (9.6%). March-April dry season, *P. orientalis* was reported to have higher in *L. donovani* infectionrate (6.9% - 7.1%) compared to 3.5% in

June-October (rainy season) (Elnaiem *et al*., 1998a). Such seasonal variation in infection rate was assumed to be related with parity rates of the local *P. orientalis* (Guilvard *et al.*, 1984). Similarly, in this study, the use of kDNA (highly sensitive marker) detected 13.3% *Leishmania* infection rates for the pools of *P. orientalis* from dense mixed forest in rainy season and 60% ITS1 PCR positives for March–May dry season collections. But, it was not possible to compare the infection rates obtained due to the use of different PCR methods during the different seasons.

Of all the 7 *P. orientalis* pools screened from Adebay agricultural fields using ITS marker, 2 were positive (2/7 or 28.6%) compared to 6 positive from the 7 pools (6/7 or 85.7%) in sparse thickets of *Acacia seyal* during March – May, 2012 (Table 6.2). The least infection rate for pools was found in the dense mixed forest (4/30 or 13.3%). It seemed thickets of *A. seyal* might be the habitat where more*P. orientalis* is found with *Leishmania* infection. Difference in *Leishmania* infections ofthe different habitats in dry season seemed to be related to the difference in density of reservoir hosts. Elnaiem *et al.* (1998a) has showed thickets of *A. seyal* as important habitats where *P. orientalis* were found infected in higher number.

Chapter 6

Sero-prevalence of *Leishmania donovani* infection in labour migrants and entomological risk factors

6.1. Introduction

Visceral leishmaniasis (VL) or kala-azar is a fatal systemic disease if left untreated (Hailu *et al.,* 2006; Chappuis *et al.,* 2007). There is high incidence of kala-azar in East Africa (Zijlstra *et al.,* 1994; Ali *et al.,* 1994; Schaefer *et al.,* 1995); the second annual incidence in the world, next to the Indian subcontinent (Alvar *et al.,* 2012). Kala-azar distribution and incidence in East Africa are greatly influenced by environmental, behavioral and socio-economic factors in addition to the HIV co-infection and genetic susceptibility (Seaman *et al.,* 1996; Thomson *et al.,* 1999; Bucheton *et al.,* 2002; Elnaiem *et al.,* 2003; Gebre-Michael *et al.,* 2004; Kolaczinski *et al.,* 2008). In East Africa and the Indian subcontinent, VL is caused by the *L. donovani* complex, unlike Europe, North Africa and Latin America where the agent is *L. infantum* (Mauricio *et al.,* 2000; Lukes*et al.,* 2007). Ethiopia has second largest number of annual VL cases (4000–7000) in Africa, next to Sudan (Hailu *et al.,* 2009). In endemic areas of VL, *L. donovani* infection does not necessarily mean clinical illness (Chappuis *et al.,* 2007). Due to reasons not well understood, *L. donovani* infections remain asymptomatic in certain subjects and cause a lethal disease in others. The ratio of the incident of asymptomatic infections to incident of clinical cases in Ethiopia is 5.6:1 (Ali *et al.,* 1994) compared to the range from 1:2.6 to 11:1 in Sudan (Zijlstra *et al.,* 1994) and 4:1 in Kenya (Schaefer *et al.,* 1995). Leishmanin skin test (LST) and direct agglutination test (DAT) are among the immunoassays widely used in kala-azar endemic areas to determine *L.donovani* infection rates (Zijlstra *et*

al.,1994, Ali *et al.,* 1994, Schaefer *et al.,* 1995). But, kala-azar patients will not show LST positive result until 3-6 months incubation phase becoming less useful as early diagnostic tool for infection detection during VL outbreaks (Zijlstra *et al.,* 1993). Furthermore, asymptomatic subjects may have to be repeatedly exposed to the parasite before they undergo LST conversion (Schaefer *et al.,* 1995). Of the several serological tests, DAT appears to be a simple and economical test with high sensitivity and specificity (Harith *et al.,* 1988). However, it cannot differentiate among past kala-azar, subclinical infection, and active disease (Zijlstra *et al.*, 1991).

The largest kala-azar focus in Ethiopia is found in the Metema–Humera lowlands where Kafta-Humera is located (Hailu *et al.,* 2006). The fertile black clay soil in the area is used for growing sesame, sorghum and cotton in a commercial scale. The agricultural activities (weeding and harvest) attract around 200,000 seasonal labour migrants annually, mainly from the surrounding Amhara and Tigray highland areas (Argaw *et al.,* 2013). In this region, VL particularly affects migrant workers (Fuller *et al.,* 1976; Hailu *et al.,* 2006; Haile and Anderson, 2006; Argaw *et al.,* 2013)in addition to residents involved in agricultural activities (Fuller *et al.,* 1976; Yared *et al.,* 2014). The population of Humera that were involved in agricultural activities were 45.6% positive for leishmanin skin test compared to 8.3% in non-farmers (urban and farm-owning population) with annual sero-conversion rate of 7% and less than 1%, respectively (Fuller *et al.,* 1976).

Kala-azar is a very important public health problem and causes high mortality and morbidity, especially among labor migrants. A total of 1, 258 VL cases were treated from 2009 – 2011 in Kasaye Abera Hospitals in Humera (Argaw *et al.*, 2013). The aim of this study was to investigate the seroprevalence rate (DAT positivity and sero-reaction) of *L. donovani* infection in labour migrants and associated entomological risk factors in the extra-domestic habitats of the Kafta–Humera lowlands.

6.2. Materials and Methods

6.2.1. Study area

Studies on bionomics of *P. orientalis* was conducted in extra–domestic areas (agricultural fields and associated thickets of *A. seyal*) around (>10km) Adebay (site 1), Rawyan (site 2) and May Kadra (Gelanzeraf and Mysegen-Mehari areas) (site 3) from May, 2011 to June, 2012). In the typical agricultural fields in Kafta-Humera lowlands, there are *Balanites aegiptiaca* trees at about 25 m intervals in any direction. The clear spaces between these trees are usually used for growing sesame.

After the land was ploughed and seeded in the mid and late June, labour migrants engage in removing weeds from sesame seedlings, mostly after establishing themselves in the agricultural fields. Weeding of the sesame fields is repeated during the flowering stage around August. Mostly, the same labour migrants perform the harvest and separation of the seed from the plant in October-November (harvest season), before they return to their home, mostly in the surrounding highland areas of Amhara and Tigray regions. But, some labour migrants return to their home at the end of August to take care of their own farm activities at home. Yet, there are some labour migrants, who would come to the lowlands

for October–November harvest. Only the seeds are removed from the agriculture fields. The agricultural leftovers are left on the field which serves as food for animals (cattle, sheep and goats) during dry season.

Almost always, there are low lying areas (depressions), next to the agricultural fields, where water floods during the rainy season. These depressions are the areas where thickets of *A. seyal* are found. Animals also graze inside these thickets. Labour migrants often go to these thickets for collecting fire woods. During the agricultural activities, all movements of the labour migrants are limited in the agricultural fields and thickets of *A. seyal*. The labour migrants who are stationed in the agricultural fields are mostly supplied with water and raw materials to make their own food. They cook and sleep under the shade of *B. aegiptiaca* trees.The big cracks in the agricultual fields and sparse thickets of *A. sayal* are the breeding habiatats of *P. orientalis* (Moncaz *et al.*, 2014, Lemma *et al.*, 2014a). Rarely, labour migrants come from areas outside Amhara and Tigray region.

6.2.2. Study design

Since all study subjects were not using bed nets, the effect of using bed net could not be evaluated. Only labour migrants with seasonal visit to the study area from June to November agricultural season were included in the study. Individuals with past VL cases were excluded. During sampling, the research team patrolled through the different agricultural fields of the study sites for sampling of blood from volunteer migrant labour workers involved in harvest of sesame. Oral consent was obtained to obtain blood for testing by DAT, with a pre-planned sample size of 359.

Sero-prevalence of *L. donovani* infection for labour migrants was obtained from DAT which would be correlated with the possible risk factors (Age intervals, address, number of visits, knowledge about the transmission of kala-azar, weeding and harvest stays, sleeping in the farm and night harvest) for *L. donovani* of infection by calculating the odds ratio in logistic regression analysis. The season for *L. donovani* infection or *P. orientalis* bite was estimated from the study of bionomics of *P. orientalis* in areas where the blood was sampled. Attempt to reach Mysegen-Mehari areas in October, 2012, where big farms are located, failed as the road for the car was out of use. As a result, blood sampling was conducted in all study areas including Mysegen-Mehari areas during the next season (October and November, 2013), after the reconstruction of the road.

6.2.3. Popuation dynamics of *Phlebotomus orientalis* in agriculture fields and associated sparse thickets of *Acacia seyal*

CDC light traps were set at 6 p.m, hanged at about 0.5 meter above ground level, and left overnight till 6 a.m. Sand flies from traps were kept in 95% alcohol before transferring to physiological saline containing detergent for washing, sorting into genus *Sergentomyia* and *Phlebotomus* and counting before mounting in Hoyer's medium. Species identification was carried out using the appropriate keys (Abonnenc and Minter, 1965; Quate, 1964; Lewis *et al.,* 1982). The mean monthly density of *P. orientalis* was determined by total counts divided by the number of CDC light traps.

6.2.4. Blood sampling

Blood samples were randomly obtained from labour migrants involved in harvest of sesame in the study area after oral consents were obtained. Blood samples were obtained by skilled laboratory technician using sterile needle and transferred to vacutainer tube

which was allowed to clot at room temperature (25°C). Serum was separated by centrifugation (1200 cycle per minute) before it was stored at - 20°C. The cold serum box containing each serum sample was transported to the Department of Microbiology, Immunology and Microbiology in Medical faculty of the Addis Ababa University, where DAT was performed.

6.2.5. Direct agglutination test (DAT)

Serum samples were diluted in a dilution solution containing 0.9% NaCl solution, 1.0% (wt/vol) Fetal Calf Serum and 0.2 M 2-mercaptoethanol. A two fold dilution series of the sera was made, starting at a dilution of 1:100 until a maximum dilution of 1:12800. Prior to its use, aliquots of FD antigen (Royal Tropical institute *L. donovani* promastigote) (The Netherlands) were reconstituted in 5 ml of normal saline (0.9% [wt/vol] NaCl). Reconstituted antigen (50 ml) was added to each well of the microwell plate containing 50 ml of diluted serum. A 24 h incubation period, at 18 to 20^{o}C employed, before the reading of the DAT was conducted. The DAT titers were grouped as negative (< 1:100), reactive (1:100 to <1: 800) and infected with *L. donovani* (1:800 - 1:12800).

6.2.6. Ethical considerations

The study protocols were approved by the ethical review committee of the Department of Zoological Sciences, Addis Ababa University and the Tigray Regional State Health Bureau. Each participant was involved after oral consent was given. The study subjects with DAT positive results were informed to go to the nearest kala-azar treatment center in Axum, Gondar or Humera for check up for any signs and symptoms of kala-azar during the follow up period (first 6 months) via their telephone numbers.

6.2.7. Statistical analysis

Descriptive statistics was used to calculate the Mean and standard deviation of the socio-demographic characters and mean monthly density of *P. orientalis.* Logistic regression analysis (bivariate) was used to study the risk factors associated with *L. donovani* infections. For each of the study factors, risk was estimated by calculating the odds ratio as an approximation of the relative risk with 95% confidence intervals using statistical package of social science (SPSS) version 20.

6.3. Results

6.3.1. Population dynamics of *Phlebotomus orientalis*

A total of 7, 443 (1, 748 females and 5, 695 males) *P. orientalis* was collected from agricultural fields (859 females and 2, 593 males; 3, 452 total) and thickets of *A. seyal* (889 females and 3, 102 males; 3, 991 total) in Adebay, Rawiyan, Gelanzeraf and Mysegen – Mehari using 461 CDC light trap nights. In addition, *P. papatasi* (n=158), *P. duboscqi*, (n=42), *P. bergeroti* (n=11), *P. rodhaini* (n=24) and *Sergentomyia spp.* (n=91, 292) were collected. There were statistically significant differences (P=000) among the mean densities of *P. orientalis* for the agricultural fields and thickets of *A. seyal* in different seasons and months.

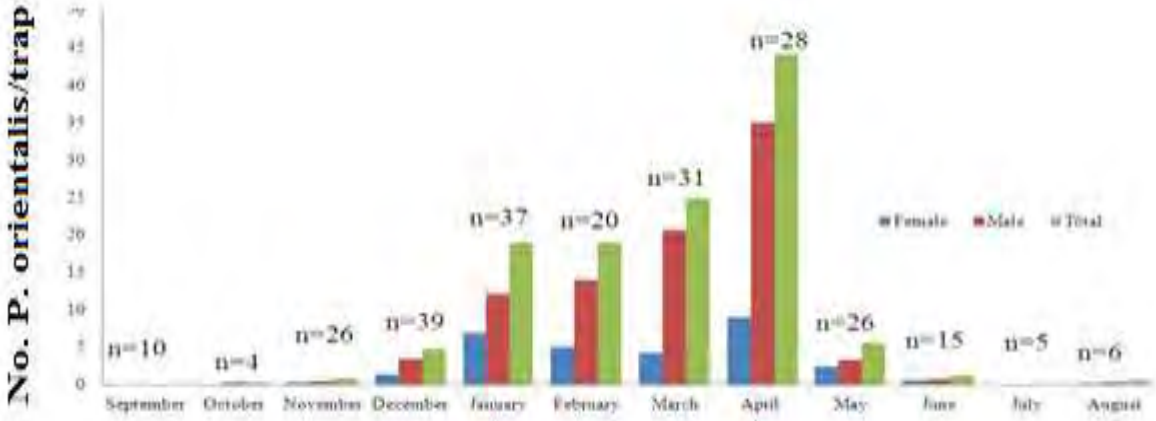
Following the heaviest rain in August, the population of *P. orientalis* drops almost to zero. The number of *P. orientalis* remained low until January, the time for the beginning of high temperature and heavy cracking black soil (Figure 6.1; Figure 6.2). The mean density of *P. orientalis* during the November – May dry season was 11.39 ± 22.98 in

agricultural fields which was lower than 25.30 ± 40.06 in thickets of *A. seyal*. March is the month with the highest overall mean number of *P. orientalis* in thickets of *A. seyal* (46.88 ± 71.46).

Table 6.1. Total number of sand flies collected from Adebay, Rawiyan, Gelanzeraf and Mysegen – Mehari fields and thickets of *Acacia seyal* (May 2011 to June 2012).

Species	Agriculture fields No. CDC-traps(n) =247			Thickets of Acacia seyal No. CDC-traps(n) =214		
	Female	Male	Total	Female	Male	Total
Phlebotomus orientalis	859	2593	3452	889	3102	3991
P. papatasi	34	67	101	18	39	57
P. duboscqi	2	27	29	0	13	13
P. bergeroti	0	6	6	0	5	5
P. rodhaini	17	3	20	3	1	4
Sergentomyia species	25, 419	22, 631	48, 050	16, 337	26, 905	43, 242

Higher mean number of *P. orientalis* wasalso collected from agricultural fields in April (43.89 ±61.57). The very low mean number of *P. orientalis* in agricultural fields (0.03±0.08) and tickets of *A. seyal* (1.97±1.81) during June-August weeding season might have also been attributed to the strong wind, especially during May and June. Lower mean number of *P. orientalis* was obtained during September - October harvest season in agricultural fields (0.66 ± 0.65) and thickets of *A. seyal* (3.92 ± 7.71).


No. P. orientalis/trap
n=10
n=4
n=26
n=39
n=37
n=20
n=31
n=28
n=26
n=15
n=5
n=6
Female
Male
Total
September
October
November
December
January
February
March
April
May
June
July
August

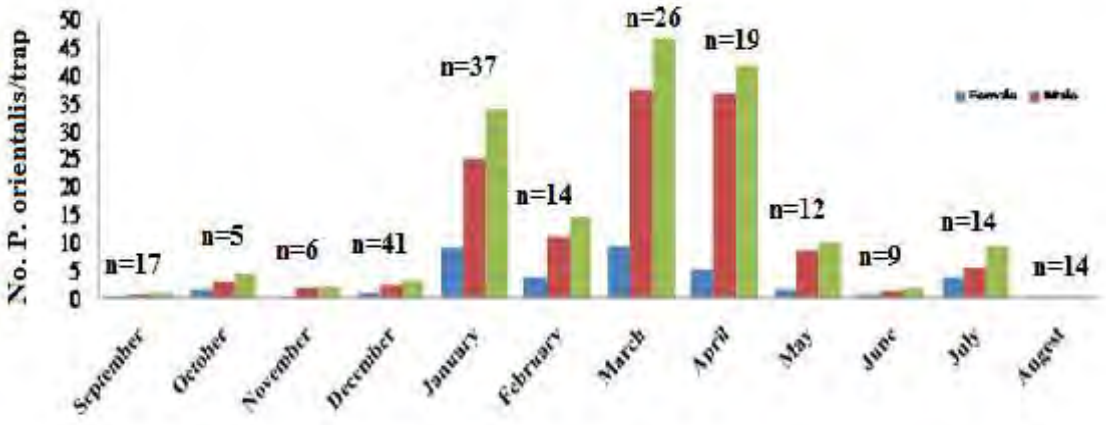

No. P. orientalis/trap
50
45
40
35
30
25
20
15
10
5
0
n=17
n=5
n=6
n=41
n=37
n=14
n=26
n=19
n=12
n=9
n=14
n=14
September
October
November
December
January
February
March
April
May
June
July
August

Table 6.2. Socio-demographic data and Knowledge, Attitude and Practice (KAP) of labour migrants related to kala-azar infection in the Kafta – Humera lowlands.

Variables	Characterstics	Frequency	Percent
1. Sample sites	Adebay	54	15
	Rawiyan	24	6.7
	Gelanzeraf	180	50.1
	Mysegen- mehari	101	28.1
	Total	359	100
2. Visit to Humera	First time	65	18.1
	Second time	79	22
	Third time	83	23.1
	>three times	132	36.8
	Total	359	100
3. Knows at least a symptom of Kala-azar	no	185	51.5
	yes	174	48.5
	Total	359	100
4. Knows agent causing kala-azar	no	358	99.7
	yes	1	0.3
	Total	359	100
5. Knows at least a method to prevent kala-azar	no	290	80.8
	yes	69	19.2
	Total	359	100
6. Knows kala-azaris avector-borne disease	no	345	96.1
	yes	14	3.9
	Total	359	100
7. Stayed throughweeding and harvest season	no	101	28.1
	yes	258	71.9
	Total	359	100
8. Involved in night harvest activities	no	56	15.6
	yes	303	84.4
	Total	359	100
9. Sleep in the farm	no	68	18.9
	yes	291	81.1
	Total	359	100
10. DAT Positive (Direct agglutination test +ve)	no	314	87.5
	yes	45	12.5
	Total	359	100
11. Seropositive	no	243	67.7
	yes	116	32.3

Until sesame was harvested, domestic animals were guarded in dense mixed forest far away from the farm. There were no animals in the farm including dogs during June - November.The strongest predictors for reporting DAT positivity and sero-reaction in labour migrants were sleeping in the farm under *B. aegiptiaca*, recording odds ratios of 15.096 and 6.63 respectively. This indicated that labour migrants who sleep in the farm were 15 times more likely to have DAT positive (*L. donovani* infection) or 6.63 times more likely to become sero-reactive than those who sleep in the camp, controlling all other factors in the model. Similarly, staying both in the harvest and weeding season in Humera was 2.83 times more likely to have DAT positive (kala-azar infection) or 4.43 times sero-reactive than when labour migrants stayed in Humera only during the harvest time following heavy rain season in September – November (Table 6.3). The Odds ratio of 0.133 for DAT positive and 0.169 for sero-reactivity for night harvest was less than 1, indicating that laborers engaged in night harvest were 0.133 times less likely to have DAT positive or 0.17 times less likely to become sero-reactive. Involving in night harvest during September – October might not be risky to have *L. donovani* infections as *P. orientalis* population is the lowest in these months.

Table 6.3. Results from logistic regression analysis to evaluate factors that affect the incidence of *Leishmania donovani* infection in the Kafta - Humera lowlands.

Dependant Variables	B	S.E.	Wald	df	Sig	Exp (B)	95.0% C.I.for EXP(B)	
							Lower	Upper
A) DAT Positive								
- Stayed both weeding and harvest season	1.04	0.492	4.463	1	0.035	2.83	1.078	7.428
- Involved in night harvest activities	-2.014	0.918	4.816	1	0.028	0.133	0.022	0.806
- Sleep in the farm	2.714	1.048	6.71	1	0.01	15.096	1.936	117.716
- Age intervals	-0.263	0.226	1.358	1	0.244	0.769	0.494	1.197
- Address of migrant workers	0.92	0.42	4.785	1	0.029	2.508	1.1	5.717
- Number of visit to Humera	0.181	0.176	1.064	1	0.302	1.198	0.85	1.691
- Knows, at least, a sign or symptom	-0.961	0.473	4.13	1	0.042	0.383	0.151	0.966
- Knows agent causing VL	-18.573	40193	0	1	1	0	0	
- Knows, at least, a method to prevent VL	0.475	0.565	0.709	1	0.4	1.609	0.532	4.864
- Knows VL is a vector born disease	-0.468	1.146	0.167	1	0.683	0.626	0.066	5.916
- Constant	-4.664	1.264	13.627	1	0	0.009		
B) Sero-reaction								
- Stayed both weeding and harvest season	1.513	0.348	18.889	1	0.00	4.542	2.295	8.988
- Involved in night harvest activities	-1.778	0.767	5.375	1	0.02	0.169	0.038	0.76
- Sleep in the farm	1.892	0.779	5.901	1	0.015	6.635	1.441	30.541
- Age intervals of migrant workers	-0.304	0.158	3.679	1	0.055	0.738	0.541	1.007
- Adress of migrant workers	1.067	0.321	11.021	1	0.001	2.907	1.548	5.459
- Number of visit to Humera	0.248	0.131	3.588	1	0.058	1.281	0.991	1.655
- Knows, at least, a sign or symptom	-1.084	0.318	11.622	1	0.001	0.338	0.181	0.631
- Knows agent causing VL	24.004	40193	0	1	1	2.66	0	
- Knows, at least, a method to prevent VL	-0.248	0.418	0.352	1	0.553	0.78	0.344	1.77
- Knows VL is a vector born disease	-1.367	1.096	1.555	1	0.212	0.255	0.03	2.185
- Constant	-3.336	0.865	14.888	1	0	0.036		

6.4. Discussion and conclusion

Labour migrants have little knowledge about the agent, vector and ways to prevent kala-azar (P> 0.05). These results indicated the need for public health awareness. Furthermore, knowledge of signs and symptoms has showed negative correlation to DAT positivity or sero-reaction (p< 0.05) indicating a possible contribution of knowledge to personal protection. Where there is no utilization of bed nets, sleeping in the open farm or camp, labour migrants could be easy targets for *P. orientalis*. Sleeping in the farm was found 15 times more likely to have DAT positive or 6.63 more likely to have sero-reaction than those sleeping in the camp. The risk of kala-azar infection in labour migrants might have been exacerbated by poor dietary condition and lower educational attainment (Argaw *et al.*, 2013). In general, the number of visits did not show association with DAT positive (p=0.302) result or sero-reaction (p=0.058). But, immunological naive labor migrants with first visit were expected to have a high risk of getting kala-azar.

Evidence has showed shift of *P. orientalis* from black cracking soil in agricultural fields and thickets of *A. seyal* to hollows in tree trunks in dense mixed forest near farms in July before the heaviest rain in August wiped out this vector (Lemma *et al.,* 2014a). This result could also suggest the shift of *P. orientalis* from their breeding habitats to possible shelters like grass huts of labour migrants in the camp which might have increased the chance of *P. orientalis* bites or *L. donovani* infection during the weeding season. Labour migrants and permanent residents who lived in tukuls with grass wall were found to be 4.5 times more likely to be exposed to *L. donovani* infection than those living in houses with mud-plastered walls (Argaw *et al.,* 2013).

In this study, regression analysis has showed neither staying during September – October harvest nor night harvest were associated with*L. donovani* infection (Table 6.3). Thus, labour migrants could be exposed to *P. orientalis* bites in June - August weeding season before the heavy rain destroys *P. orientalis* in August. Successful *P. orientalis* collections in May and June have been indicated (Elnaiem *et al.*, 1997) with the *P. orientalis* abundance increasing 20-fold from March-April to June in 1995/96 in Sudan.

Peak monthly VL cases in permanently settled farmers in Shiraro (northern Ethiopia) was reported in January (39/223 or 17.49%) during study period lasted from August, 2010 to July, 2011 (Hailu *et al*., unpub. data). Probably, July - August might be the season when these farmers, also, get the infection after possible 2-6 months incubation period suggested earlier (Zijlstra *et al.*, 1994). The relative risk of *P. orientalis* bite in this habitat might have increased in July - August rainy season after the cracks were sealed off and *P. orientalis* shifted to the villages for protection. In eastern Sudan, transmission most likely occurs during the dry season (March to May) and illnesses often occur in October and November with incubation period from 2 and 6 months (Zijlstra *et al.*, 1994). This might be true for pastoral communities who expose themselves to highest possible *P. orientalis* bites during their stay in *Acacia - Balanites* woodlands for guarding livestocks. Labour migrants in the Metema-Humera lowlands are not exposed to the highest rates of *P. orientalis* bites expected in March-May as the majority normally arrives in the farms later in June or July. Migrant Labourers are most probably exposed to kala-azar infection during June - August weeding season. Staying during September –

October harvest season or involving in night harvest might not associate with the risk of *L. donovani* infection due to the reduction of *P. orientalis* density.

Chapter 7

Study on reservoir hosts of kala-azar in endemic lowland areas of Ethiopia: Preliminary data on fauna and *Leishmania* infections in rodents

7.1. Introduction

Ethiopia is a country in the horn region of east Africa covering an area of 1,100,000 km^2 comprising the northern and central plateaus, divided by the Great Rift Valley, and surrounded by the lowlands areas in the east and west. The diverse ecological environments in Ethiopia have allowed the existence of about 70 rodent species with 21.4% (15/70) endemicity (Hillman, 1993; Yalden & Largen, 1992 and Yalden *et al.*, 1996; Bekele and Corti, 1997). Besides their role as pests (Bekele and Leirs, 1997), rodents harbor pathogens causing disease in man acting as reservoir hosts(Ashford, 1986). There could be the possibility of rodents acting as reservoir hosts of VL in Ethiopia where an estimated 3.2 million people are at risk of getting this infection with annual incidence of around 4,000 – 7,000 (Hailu *et al*., 2009; Alvar *et al*., 2012). Kala-azar in Ethiopia is believed to be a zoonotic disease except during epidemics situation (Ali and Ashford, 1994; Hailu *et al*., 2006; Alvar *et al*., 2012). Migrant labourers arethe risk groups for VL in Metema-Humera lowlands (Argaw *et al*., 2013; Lemma *et al*., 2015) who were believed to be the source of anthroponotic transmission in Libo Kemkem district during the 2005-2006 epidemics (Alvar *et al*., 2007).

Zoonotic diseases require one or more vectors and mammalian reservoir hosts that exist abundantly in the same area for infectious agents to be sustained persistently (Ashford, 1997). Reservoirs are natural hosts and are not affected by the parasitic agent. Morbidity and mortality result when the vectors transmit the disease to humans. Intrusion into a

zoonotic cycle in extra-domestic environments have been indicated as the source VL infections in Ethiopia (Ali and Ashford, 1994; Hailu *et al*., 2006; Hailu *et al*., 2009; Yared *et al*., 2014). But, the reservoir hosts of kala-azar have not been found conclusively. Based on isolation of *Leishmania* parasites from different animals, a study suggested Egyptian mongoose as probable reservoir host (Elnaiem*et al.*, 2001) while others considered rodents (Hoogstraal and Heineman, 1969; Haile and Lemma, 1977, Githure *et al.*, 1984; Kassahun *et al*., 2015). Based on host preference studies for blood source of *P. orientalis*, vector of VL in eastern Sudan, rodents were found as preferred hosts compared to small carnivores, i.e.,Aegyptian mongoose (*Herpeistes ichneumon*)and genet cat (*Genetta genetta*) (Quate *et al*.,1964). A similar study, however, showed dogs to be the most favored for blood meal, followed by small carnivores (mongoose and Genet cat) and rodents (Nile rat) respectively (Hassen *et al.*, 2009). Cattle are the most preferred blood meal sources for *P. orientalis* in domestic and predomestic areas (Gebre-Michael *et al*., 2010) compared to human, sheep and goats in extra-domestic habitats in Metema-Humera lowlands (Lemma *et al*., 2014b).

Experimental *L. donovani* infection in *Arvicanthis niloticus* in Sudan indicated the susceptibility of this species to *Leishmania* infection; parasitemia deacreased with time indicating that rodents may not be the reservoir host of VL (Stauber *et al*., 1966). Hoogstraal and Heyneman (1969), on the other hand, found chronic natural infection in *A. niloticus* and considered this rodent species as the most probable reservoir host of VL in Sudan where the *Leishmania* parasites isolated from this rodent was found to be identical with parasites from VL patients and *P. orientalis* vectors. *Leishmania donovani* complex was detected from spleen samples of *Arvicanthis* spp., *Gerbilliscus nigricaudus*,

and *Mastomys erythroleucus* and confirmed by real time PCR using kinetoplast (k)DNA and internal transcribed spacer 1 (ITS1) *Leishmania* markers in addition to sequencing the amplicons (Kassahun *et al.*, 2015).

Preliminary ecological surveys in areas where VL occurred showed that presence of different species of rodents was associated with spatial distribution of cases in the endemic lowland areas of Ethiopia. Thus, it was tempting to incriminate rodents as natural reservoir hosts of VL in Ethiopia. The correct identification of rodents is crucial in the considerations of the investigations towards incrimination of rodents as reservoir host of VL. Thus, the aim of this study was to describe the rodent fauna and investigate their *Leishmania* infections in kala-azarendemic lowland areas of Kafta Humera.

7.2. Materials and methods

7.2.1. The study area

In Western Tigray zone (Kafta Humera district), extra-domestic agricultural fields and tickets of *Acaciaseyal* near Baeker (14°01'N, 36°59'E) and May Kadra towns (Gelanzeraf) (13°59'N, 036°31'E) were used for trapping the rodents in addition to agriculture fields in Adijamus village in Welkit districts. In Tahtay Adiyabo district (around Shiraro town; Western Tigray Zone), rodents were sampled from Ademiti and Mayhas villages.

7.2.2. Study design and period

The rodent sampling sites in villages and extra-domestic habitats were selected based on document obtained from kala-azar patients treated in treatment centers in Humera and Gondar Hospitals. Rodents were trapped for screening of *Leishmania* infection by parasitological, serological and molecular techniques before the species of the rodents were identified by morphological characters. Wild animal fauna exploration in the study sites, before the onset of the research, has indicated rodents as the most abundant animals in areas surveyed for source of human VL infections. Carnivores were rare in these open lowland areas, with high human disturbance rates, due to the involvements of man in extensive agricultural activities. Rodents were sampled from sampling sites in March, April, September and December, 2013/14.

7.2.3. Climate

In Baeker and Humera towns (Kafta Humera district), the annual mean maximum temperature varied from 29.1 to 41.2 ^{0}C, while the monthly mean minimum ranges from 13.50 to 25.40^{0}C. March, April and May were the hottest months with mean maximum temperature ranging from 38.9 to 40.83^{o}C. The rainy season starts in May and extends up to October with the highest rainfall occurring in August. Average annual rainfall received by the area in the period 2011 - 2013 was 791 mm (Lemma *et al.*, 2014a).

7.2.4. Rodent trapping and tissue sampling techniques

Rodents were captured by Sherman live traps baited with peanut butter and placed overnight in the sampling sites. The rodents were anaesthetized for tissue biopsies from liver, spleen, bone marrow and tip of the nose (skin) for screening of *Leishmania* infections. Ketamine (Clorketam Veterinary) was used to anaesthesize the rodents

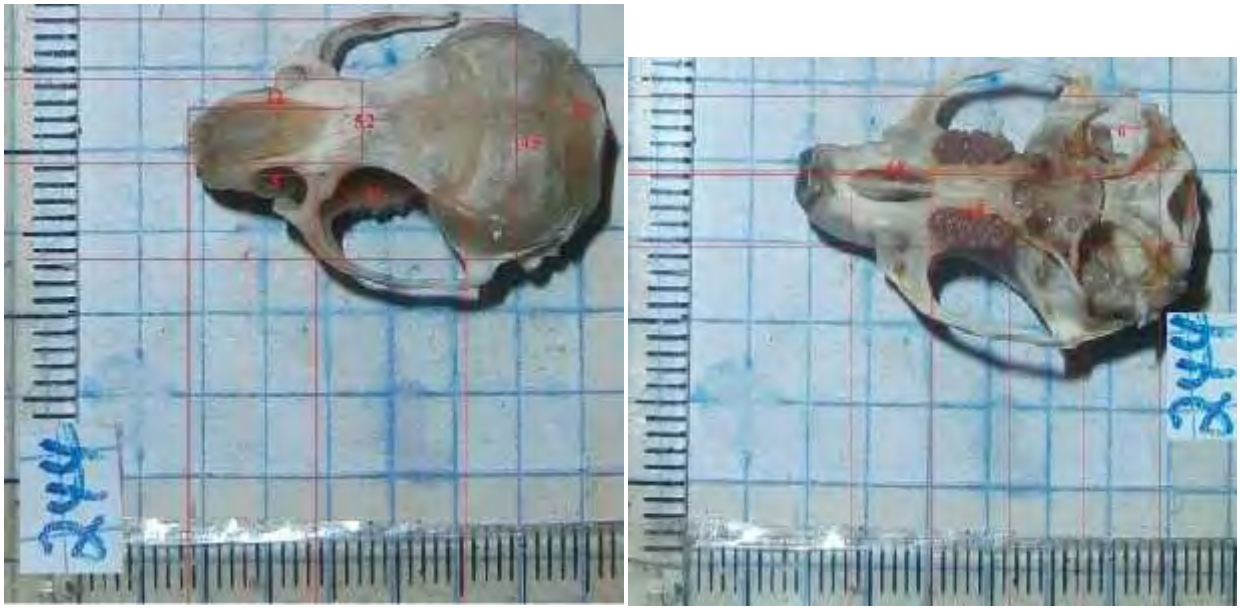
according the prescribed dosage (average 2 mg/kg for intra-venous route). The blood obtained using sterile needle was dropped into sterile filter paper and allowed to air dry before sealing in plastic bags. The tissues from liver, spleen and skin were macerated in Locke's solution before transferring them into NNN medium. Blood and touch smears of liver, spleen, skin and bone marrow were made on microscope slide and allowed to air dry before fixing using methanol and staining by geimsa stain for microscopy. Rodents were trapped from the sampling sites after permission was obtained from Ethiopian Wildlife Conservation Authority (EWCA), Government of Ethiopia.

Diphasic Novy-McNeal Nicolle medium (NNN medium)

Solid phase is prepared by boiling 1.4 gm agar, 0.6 gm Nacl in 90 ml distilled water in a flask which will be transferred into a screwed-topped bottle for autoclaving (121^{o}C for 15 min) before cooling to 50^{o}C and 10ml of defibrinated rabbit blood is added. Then the tube will be sloped until the agar is solidified. Classically the liquid phase is the water that condenses at the bottom of the slopes. In practice, however, most workers add additional liquid phase such as Proline Balanced Salts Solution (PBSS), Locke's solution or Roswell Park Memorial Institute medium (RPMI 1640) (Gibco BRL, Grand Island, N.Y.).

7.2.5. Morphological data

Morphological data relevant to the systematics of rodents such as pelage colour, length of body, head, tail, soles of the feet and ear size were obtained when the rodents were anaesthetizedfor tissue biopsies. Each rodent has been photographed before dissection and the head was preserved in 95% ethanol. Each head sample was individually boiled to



7.2.6. Serological test of the blood samples on filter paper

Five mm disks from the dried blood samples on the filter papers were punched out before eluting in 125µl DAT buffer in each well of a micro-plate row B which was incubated for overnight at 4°C. Fifty micro-liter DAT diluent (physiological saline (0.9% NaCl) containing 0.78% β–mercaptoethanol) was dispensed into every well of the vertical rows A, B, C, D, E, F, G and H except into row B. Freeze dried serum diluted in PBS (100 µl) was used as positive control and dispensed into well 12C while 100µl Freeze dried solution of bovine albumin was added (negative control) in 11C. Serial dilution, using a multi-channel pipette, was performed from Row B until Row H by transferring 50 µl and mixing five times. Mixed and re-suspended DAT-antigen (50 µl) was pipetted to every well except row B. Finally, the micro-plate was sealed with an adhesive plate sealer and shacked gently and incubated for overnight. The end point titre was determined by visual inspection of the agglutination reactions with reference of the negative controls. The cut-off value of the DAT was set at >1:800 so as to increase sensitivity of the test in detecting leishmania infection.

7.2.7. DNA extraction and PCR

DNA was extracted from spleen, liver, bone marrow and skin according to Abassi *et al.* (2013). In brief, tissue samples 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 60°C for 2 hours (spleen) or 24 hours (skin, liver, bone marrow). 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 removed to a new microcentrifuge 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.

For PCR reaction, 20 µl master mix (1 µl forward primer, 1 µl backward primer, dNTPs and 18 ddH_2O) and 5 µl DNA sample were mixed to make 25 µl final volumes. PCR amplification of ITS 1 gene was made using primers LITSR (5′-CTG GAT CAT TTT CCG ATG-3′) and L5.8S (5′-TGA TAC CAC TTA TCG CAC TT-3′) for 35 cycles. The thermal profile of the PCR 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. ITS1 PCR product, sized ca 330 bp, was analyzed by gel electrophoresis at 120 V in 1× TAE buffer in 1.5% agarose gels to find *Leishmania* spp. specific bands. The fragments were visualized using ethidium bromide up on exposure to UV light. The molecular analysis of blood and tissue samples from the rodents for screening *Leishmania* parasites using PCR was conducted in the Department of Microbiology and Molecular Genetics, Hadassah Medical School, Hebrew University, Israel.

7.2.8. Statistical analysis

The length of different morphological characters were entered into Statistical Package of Social Sciences (SPSS) version 20 for analysis of data using descriptive statistics (Mean $\pm$ SD) and analysis of variance (ANOVA) for statistically significant mean differences

among different species. P-values less than 0.05 were considered as statistically significant differences.

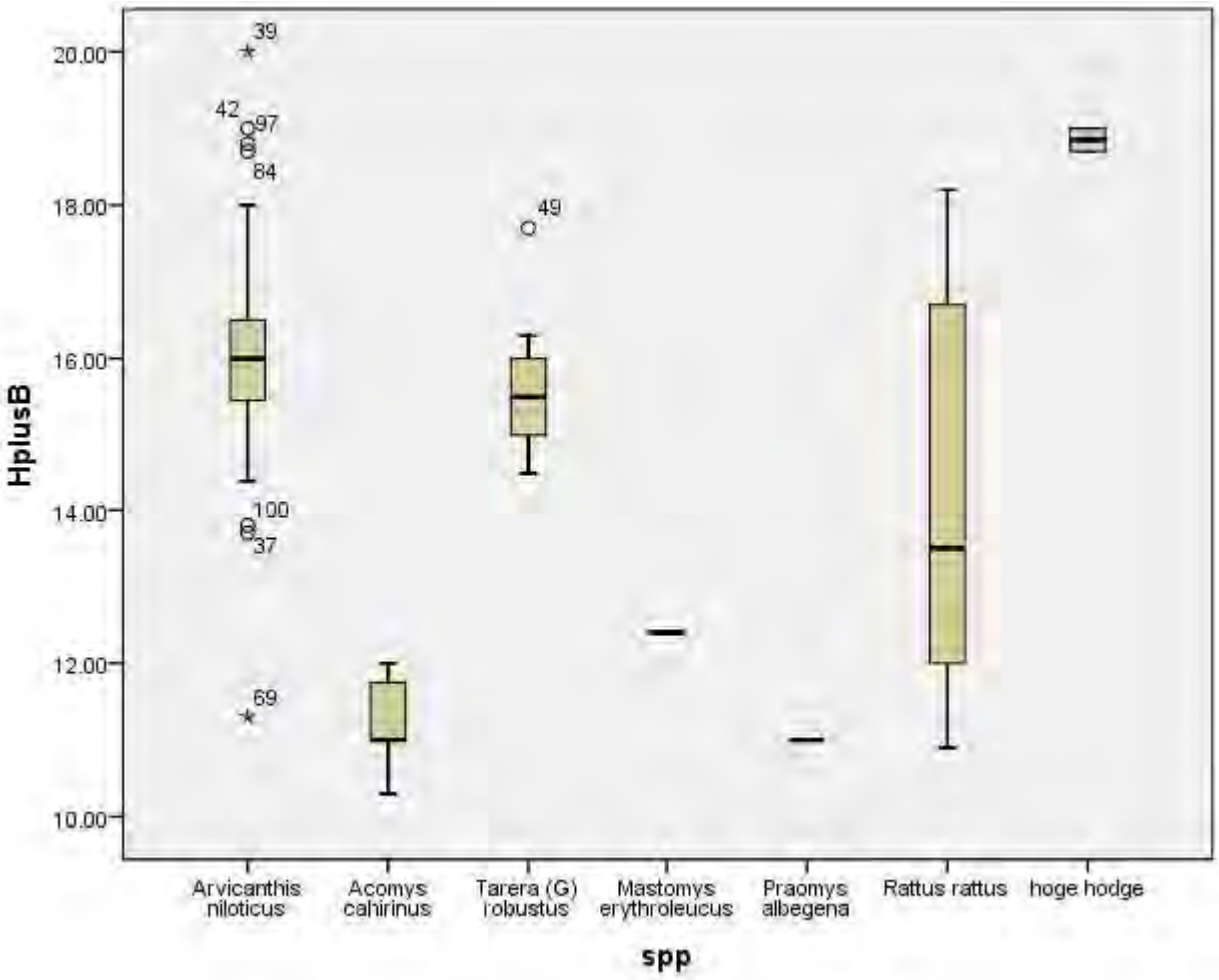
7.3. Results

A total of 128 rodents were trapped from Baeker (86), Gelanzeraf (16), Ademiti (9) Mayhas (9) and Adijamus (8) Rodents were found in predomestic and extra domestic areas. Fences and bushes around houses in predomestic areas and agricultural fields and grasses in sparse tickets of *Acacia seyal* in extra-domestic habitats were habitats for the rodents. *Arvicanthis niloticus andAcomys cahirinus* were the rodent species found abundantly in the study areas. *Tatera* (*Gerbilliscus*) *robustus* was the third most abundant species in the area.

Table 7.1. The number of different species of rodents sampled from the different sampling sites

Species	Baeker	Gelanzeraf	Ademiti	Mayhas	Welkit (Adijamus)	Total
Arvicanthis niloticus	55	5	0	5	3	68
Acomys cahirinus	13	4	6	0	2	25
Tatera (G) robustus	16	4	1	0	0	21
Mastomys erythroleucus	0	0	0	0	3	3
Mylomys albipes	2	0	0	0	0	2
Rattus rattus	0	2	0	3	0	5
Paraechimus aethiopicus (Hodgehog)	0	2	0	0	0	2
Xerus erythropus (Striped ground squirrel)	0	0	2	0	0	2
Total	86	17	9	8	8	128

Weight
600.00
500.00
400.00
300.00
200.00
100.00
.00
65
39
82
spp
Arvicanthis niloticus
Acomys cahirinus
Tarera (G) robustus
Mastomys erythroleucus
Praomys albegena
Rattus rattus
Xerus erythropus
hoge hodge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Arvicanthis niloticus
Acomys cahirinus
Tarera (G) robustus
Mastomys erythroleucus
Praomys albegena
Rattus rattus
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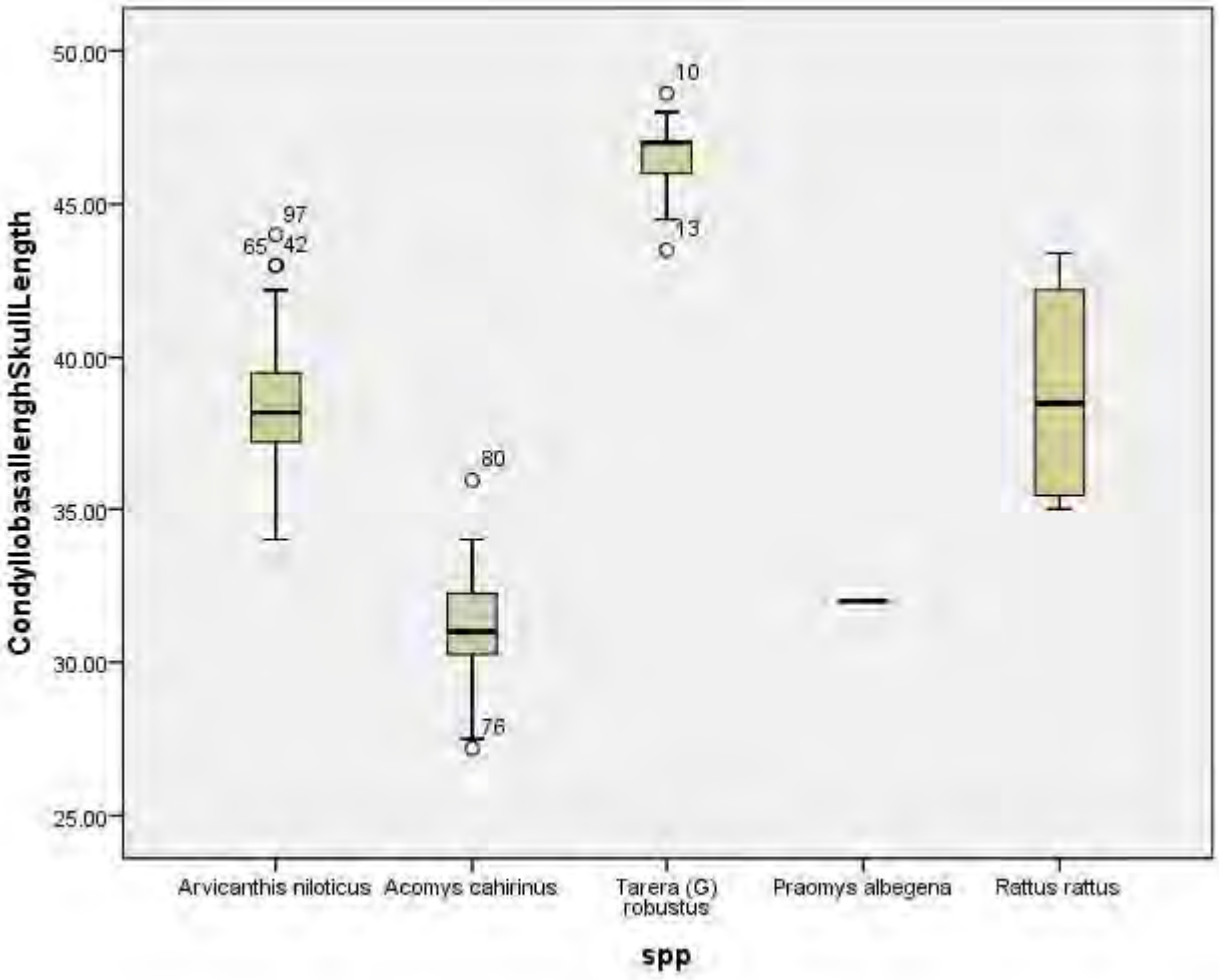
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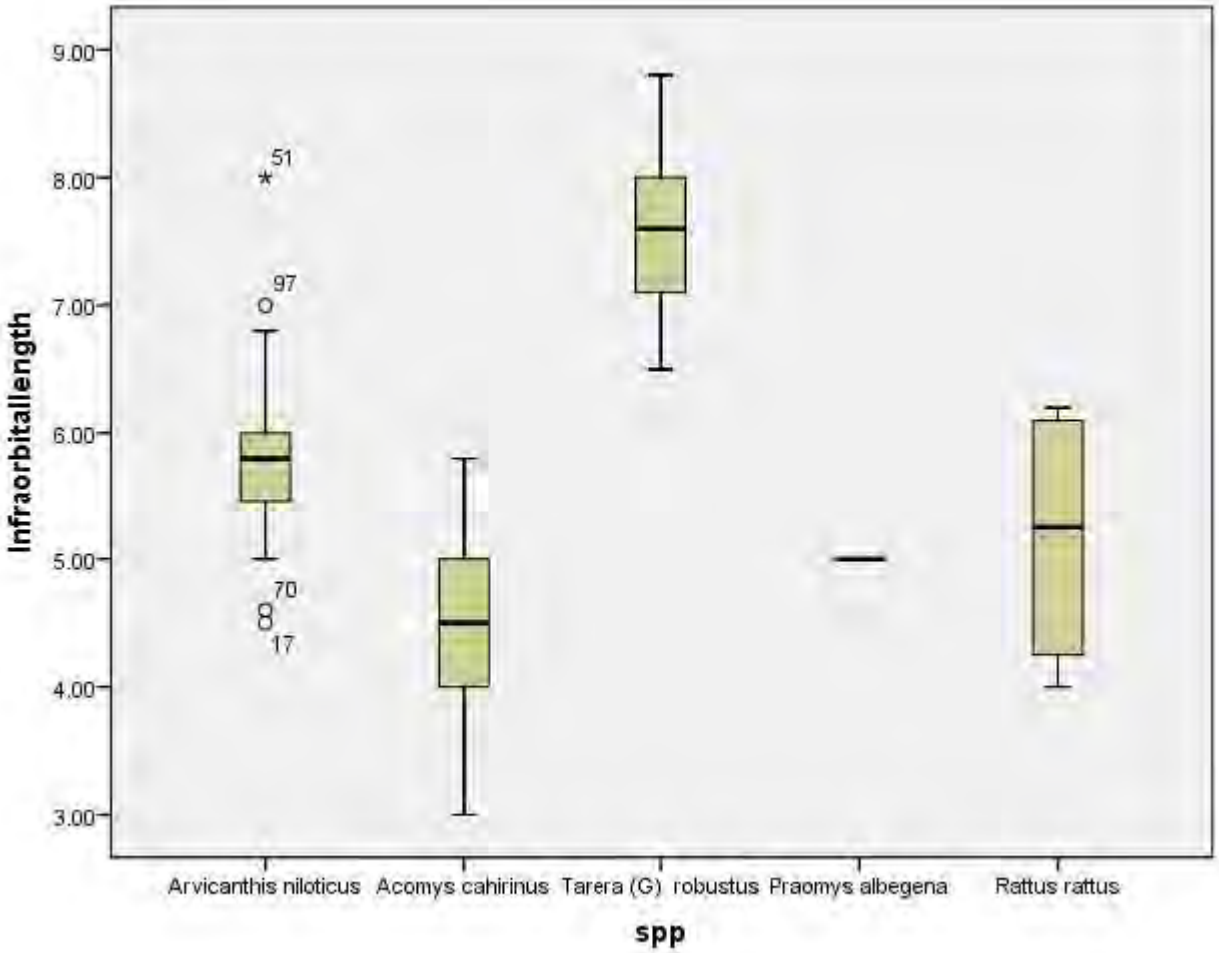
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Arvicanthis niloticus
Acomys cahirinus
Tarera (G) robustus
Praomys albegena
Rattus rattus
spp

significant differences for the morphological characters listed below: Weight (P=.7), Head plus body (p=0.4), CBL(p=0.6), OCN (p=0.97), IOL(P=0.9), INF(p=0.6), LIL(p=0.5), BBC(p=0.2), LLJ(p=0.5) and WFM (p=0.2). These morphometric characters did not show significant differences (p>0.05) for the *Tarera (G) robustus* collected from Baeker and Gelanzeraf.

7.3.2. Breeding season and nocturnal activities

No Juveniles was found in March and April dry sampling season in Humera – Shiraro lowlands where, probably, only one breeding season exists during June-August rainy season. Only one juvenile *A. niloticus* was found during September with body weight of 30 gram. The mean ± Std D of weight for the adult of this species was 108.39 ± 21.99

7.3.3. *Leishmania* infection

Of the total 91 rodents (*Arvicanthis niloticus*(*n*=54), *Tarera* (*G*) *robustus* (*n*=16), *Acomys cahirinus* (n=17), *Mylomys albegena* (n=2), *Xerus erythropus* (n=2) collected from extra-domestic habitats of Baeker and Gelanzeraf (Kafta Humera district) in March and April, 2013 and analyzed by ITS1 PCR using skin, spleen, liver and bone marrow samples, 6/54 (11.1%) of *Arvicanthis nilothicus* were positive compared to infection rates in *Acomys cahirinus*(3/17 or 17.6%) and *Tarera* (*G*) *robustus* (2/16 or 12.5%). Almost all these rodents were screened using samples from skin (87), liver (91) and spleen (87) except bone marrow samples. Only 48 bone marrow samples were found enough for DNA extraction to run PCR. Almost all the PCR infections were found from these bone marrow samples (8/48 or 16.7%) compared with 1/91(1.1%) liver, 2/87(2.2%) spleen and 0/87 (0%) skin. The genus specific bands obtained around 331 bp were not further sequenced to identify the species *Leishmania.*Different organs of the same rodent

were not found infected. Direct agglutination tests (DAT) of blood on the filter papers for all the 91 rodents were negative. Microscopy, NNN medium and DAT were negative for these rodents except 2 spleen samples NNN medium positive *Arvicanthis niloticus.* Bone marrow samples were not used for NNN medium.The other 27 rodents collected from Baeker, Gelanzeraf, Adamiti and Adijamus were negative for NNN medium, Giemsa stain and DAT.

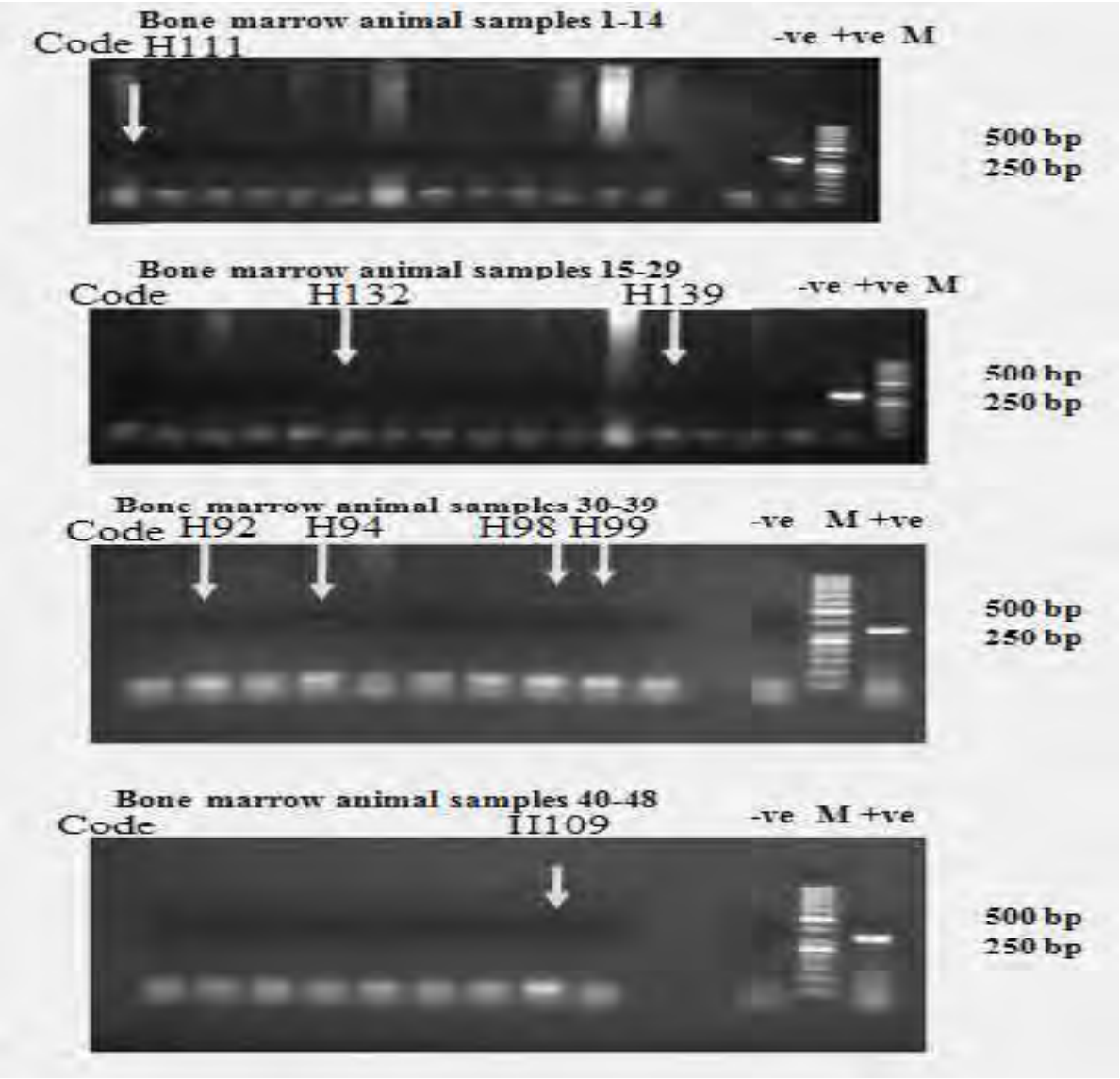

Figure 7.4. Representative samples of PCR amplifications of ITS1 of *Leishmania* parasites from bone marrow samples from rodents collected in March and April, 2013 in extra-domestic habitats of Baeker and Gelan zeraf areas in Kaft – Humera district. Lane M: MW marker; Lanes 1 and 4(code 111and 109): are *Tarera* (*G*) *robustus* collected from Gelanzeraf in April; Lanes 20 and 27 (Code H132 and H139): are *Acomys cahirinus* collected from Baeker and Gelanzeraf; Lanes 31, 33, 37 and 38 (Code H92, H94, H98 and H99): are *Arvicanthis niloticus* from Baeker.

7.4. Discussion

The genus *Arvicanthis* contains seven currently recognized species (*Arvicanthis abyssinicus, A. ansorgei, A. blicki, A. nairobae, A. neumanni,A. niloticus* and *A. rufinus*) (Musser and Carleton, 2005). In this grouping, the small-bodied *A. somalicus* is grouped in *A. neumanni,* the species endemic to East Africa which exhibits significant geographic variation. Four species of *Arvicanthis* were reported in Ethiopia, namely, *A. abyssinicus*, *A. dembeensis*, *A. blicki* and *A. somalicus* (Yalden *et al.,* 1976). *Arvicanthis abyssinicus* and *A. dembeensis* are considered synonyms (Musser and Carleton, 2005). Morphological analysis of *Arvicanthis* species in Ethiopia, however, showed *A. abyssinicus* and *A. dembeensis* to be different species (Bekele *et al*., 1993). Corti and Fadda (1996) considered *A. abyssinicus* as highland form (2000 m a.s.l. to 3400 m a.s.l.) and *A. dembeensis* as a lowland form (0 - 2000 m a.s.l).

The *Arvicanthis* species from Omo valley were identified as *A. somalicus* and *A. niloticus* (Hubert, 1976). The species identified in Omo and Mago parks, however, were *A. dembeensis* and *A. somalicus* (Yalden *et al*., 1996). The *A. dembeensis* inthe lowlands has been considered as synonym for *A. niloticus* (Musser and Carleton, 2005).*Arvicanthis*

abyssinicus, A. blicki and *A. somalicus* were the species of *Arvicanthis* from southern and southwestern Ethiopia (Yalden *et al*., 1996) whereas *A. dembeensis* is found in central Ethiopia (Bekele' and Leirs, 1997). Habtamu and Bekele (2008) reported *A. dembeensis* and *A. niloticus* as the species found in Alatish Park (northwestern Ethiopia). *Arvicanthis abyssinicus* and *A. blicki* are endemic to the Ethiopian Plateau and easily diagnosed and recognizable by morphology, ecological and geographic distribution (Dorst, 1972). The species of *Arvicanthis* in the kala-azar endemic lowlands of northern Ethiopia was *A. niloticus* which is distinct from *A. abyssinicus* or *A. dembeensis* found in the surroundings of Dembyia plain near Lake Tana (Bekele and Leirs, 1997) in having whitish dorsal pelage. Relative small size could identify *A. somalicus* insouthern Ethiopia as priviously described (Musser and Carleton, 2005).

The genus *Tatera* (*Gerbilliscus)* has been described as a widespread rodent in open arid habitats of Sub-Saharan Africa where 11 species exist (Colangelo *et al*., 2009; Muser and Carleton, 2005). *Gerbilliscus nigricaudus, G. robustus, G. phillipsi* and *G. leucogaster* are the four species found in east Africa (Colangelo *et al*., 2009). *Tatera* (*Gerbilliscus*) *robustus* was the species found in the Baeker and Gelanzeraf but not Myhas and Adijamus. Lack of enough samples might be the reason for their absence.

Cracks of black soil were shared between rodents and *P. orientalis* as habitats. The behavior of the rodents such as diurnal or nocturnal might have no effect on contact as long as they are sharing a habitat. But, rodents were not seen attracted during trial experiments using Turner's box traps (Lemma *et al*., 2014b). After the identification of

Leishmania donovani from *Phlebotomus rodhaini*, with rodent preferred blood meal source (Elnaiem *et al*., 2011), infections in rodents could be assumed due to this *Phlebotomus rodhaini*-rodent system. Infection of rodents with *Leishmania donovani* (Kassahune *et al*., 2015) and this study may not be useful in control of kala-azar unless it is related with the established vectors of the disease (*P. orientalis*) or human cases. Neverthless, our field observations, showed the association of human VL cases with the existence of *Arvicanthis nilothicus, Tarera (G) robustus* and *Acomys cahirinus.* These rodent species were found with high infection rate (12.5% - 17.6%) in Kafta Humera area. *Mastomys erythroleucus* was also found infected in southern endemic lowlands of Ethiopia in addition to these rodents (Kassahune *et al*., 2015). This implies the need for more investigations before full discriptions of the role of rodents in epidemiology of Kala-azar in endemic lowlands of Ethiopia.

Cutaneous leishmaniasis due to *L. major* seems absent in Kafta – Humera lowlands. *Phlebotomus papatasi, P. duboscqi and P. bergeroti* were rare in extra-domesic habitats in this area (Gebre-Michael et al., 2010; Lemma *et al*., 2014a; Lemma, *et al*., 2014b). The *Leishmania* species from the rodents in this study most probably was *Leishmania donovani*. Recent investigation on the role of rodents in leishmaniasis in Ethiopia identified *Leishmania donovani* complex from spleen samples of *Arvicanthis* spp., *Gerbilliscus nigricaudus*, and *Mastomys erythroleucu*from southern Kala-azar endemic lowlands of Ethiopia, using real time PCR (Kassahun *et al.*, 2015).

Infection rates obtained in this study using ordinary PCR ranged from 0 to 2.2% for skin, liver and spleen samples compared with 16.7% in bone marrow. Kassahun *et al.* (2015)

did not use bone marrow samples, all the infections were obtained from spleen samples using RT-PCR. Bone marrow samples were not used for NNN-medium in this study. Only spleen samples (4) were positive by NNN-medium from the total 335 rodents from kala-azar endemic lowlands of the country. Future study should take into considerations the type of techniques and tissue samples used during the screening of infections in rodents. Unlike the common tought of the sequestration of *L. donovani* amastigotes in the spleen (Sundar and Rai, 2002), bone marrow has also been shown, in this study, as a major source of *L. donovani* inection. Our finding was supported with previous experiment on BALB/c mice which confirmed bone marrow as site of persistent infection in addition to a striking similarity with the spleen in the time of onset of rapid amastigote accumulaton (Cotterell *et al*., 2000).

Arvicanthis niloticus, Acomys cahirinus, Gerbilliscus nigricaudus and *Mastomys erythroleucus* could play a role in the transmission cycle of zoonotic kala-azar infection due to *L. donovani* in endemic areas of Ethiopia. Further studies are required for *L. donovani* isolation from rodents in the endemic areas. Additional evidences relating to infectivity of sand flies using xenodiagnosis should be sought before considering these rodents as potential reservoir hosts of *L. donovani*.

Chapter 8

General discussion and conclusions

The present study established that VL is endemic in Metema-Humera lowlands with a prevalence of 12.5% in migrant labourers. The major phlebotomine sand fly vectors of VL in the area are *Phlebotomus orientalis.* CDC light trap and sticky trap collections in this study showed that population densities of *P. orientalis* increased sharply from September to April and declined in May - July and attained low levels in August and September. *Phlebotomus orientalis* was found in higher density in agriculture fields and sparse thickets of *A. seyal* compared to dense mixed forest. The trend of seasonal dynamics of *P. orientalis* in dense mixed forest did not fully match the patterns in agricultural field and thickets of *A. seyal* (Figure 3.4). Unlike the dense mixed forest without big cracks in black soil, agricultural fields and sparse thickets of *A. seyal* might be breeding sites of *P. orientalis.* In July rainy season, mean monthly density reached its peak number in dense mixed forest unlike other habitats where *P. orientalis* population declined. During rainy season (May - June), *P. orientalis* from neighboring agricultural fields and thickets of *A. seyal* might have shifted to the dense mixed forests where cracks and burrows in tree trunks keep them protected.

The highest mean number of *P. orientalis* was collected from goats and sheep shelters (chapter 4) which might indicate these animals to be important sources of blood meals for this vector. Reverse line blot of PCR cytochrome b products confirmed sheep and goat to be among the sources of blood meal for *P. orientalis* at the peripheries of Adebay and

Rawiyan towns in Hafta Humera district (Yared *et al*., pers. comm.). Reverse line blot of PCR cytochrome b products in this study has also shown cattle and human to be important sources of blood for *P. orientalis* in extra-domestic habitats. The bands of DNA fragments (cytochrome b) on agarose gel (28%), that were not captured by the 11 probes used, might represent wild rodents and small carnivores in the area.

During the study of nocturnal activities based on hourly collection, mean numbers of males and females*P. orientalis* did not show statistically significant difference in agricultural fields ($P>0.05$). But, the mean numbers of males and females collected near animal shelters and thickets of *Acacia seyal* were significantly different ($P<0.05$). Except for thickets of *Acacia seyal,* the peak activities of *P. orientalis* were found after mid-night. Peak hour for thickets of *Acacia seyal* was found at 21 hour where *P. orientalis* collections were male biased (4.42 male: 1 female) compared 1.00 hr peak hour collection near animal (goats and sheep) shelters where collections were female biased (1 male: 2.35 female) (Figure 4.1). These results seemed related to the feeding habits of *P. orientalis.* Males most probably attracted to red *Acacia seyal* trees (probable sugar source) while females were attracted to animal shelters where they could get blood meal. When the overall activities of *P. orientalis* analyzed, there is a risk of *P. orientalis* bite or kala-azar infection throughout the night (chapter 4) as already described by the observations in Sudan (Schorscher and Goris, 1992).

Of the total 45 *P. orientalis* pools (groups of 5 individuals) screened using PCR, 14 were positive for *Leishmania* infections (14/45) from all the habitats (3/8 agricultural fields; 6/7 in thickets of *Acacia seyal* and 5/30 dense mixed forest). Difference in *Leishmania* infection rate seemed related with the difference in density of the rodents, the probable reservoir hosts. Rodents were found more in thickets of *Acacia seyal* than agricultural fields in dry season. More *P. orientalis* PCR positive infections was in March-May dry season collection (9/15 or 60%) than July rainy season (5/30 or 16.7%) and might be due to high parous rate. The high density of *P. orientalis*might increase parasite circulation due to repeated bites from infected reservoir host. The agriculture practice in Kafta Humera lowlands, where migrant labourers visit the extra-domestic habitats in June – October, avoid the exposure to highly repeated and infectious bites of *P. orientalis* during dry season.

Migrants labourers have little knowledge about the agent, vector and ways to prevent kala-azar and positively correlated with *Leishmania* infection ($P > 0.05$). These results indicated the need for public health awareness. But, knowledge of signs and symptoms has showed negative correlation to DAT positivity or sero-reaction ($p < 0.05$) indicating a possible contribution of knowledge to personal protection. Where there is no utilization of bed nets, sleeping in the open farm or camp, migrants labourers could be easy targets for *P. orientalis*. Sleeping in the farm was found 15 times more likely to have DAT positive or 6.63 more likely to have sero-reaction than those sleeping in the camp. The risk of kala-azar infection in migrant labours might have been increased by poor dietary condition and lower educational attainment (Argaw *et al.*, 2013). In general, the number

of visits did not show association with DAT positive (p=0.302) result or sero-reaction (p=0.058). But, immunological naive migrant laborers with first visit were expected to have a high risk of getting kala-azar. Regression analysis has also shown neither staying during September – October harvest nor night harvest were associated to *L. donovani* infection (Table 6. 3). Thus, labour migrants could be exposed to *P. orientalis* bites in June - August weeding season before the heavy rain destroys *P. orientalis* in August.

The findings of this thesis (chapter 7) together with recent study on rodents (Kassahun *et al*., 2015) provided evidence to suggest rodents as reservoir hosts of kala-azar in Ethiopia. *Arvicanthis niloticus, A. somalicus, Acomys cahirinus, Tarera (G) robustus,Gerbilliscus nigricaudus* and *Mastomys erythroleucus* that were found infected with *Leishmania* parasites in this study (chapter 7) and Kassahun *et al*. (2015) are found abundantly in VL endemic lowlands of Ethiopia. These rodents, which found infected with *Leishmania* parasites, share habitats (cracks of black soil or termite mounds) with *P. orientalis* and *P. martini* in addition to their existence in abundance to maintain the persistent parasite circulation. Thus, rodents satisfy most of the requirements (Ashford, 1997) to be considered as a reservoir hosts. More investigations for additional evidences are required for concluding the rodent species as reservoir hosts in kala-azar endemic lowlands of Ethiopia.

*Phlebotomus rodhaini*was a rare species in Kafta Humera lowlands (chapter 3) and Sudan (Quate, 1964). Neither *P. rodhaini* nor *P. orientalis*were found during our study (chapter 4) on host preferences of *P. orientalis.Phlebotomus rodhaini,* which feed on rodents

preferentially, were found infected with *Leishmania donovani* in Sudan (Elnaiem *et al*., 2011) suggesting the possibility of *Leishmaniadonovani* circulation among the rodents by this sand fly. Human biting habitof *P. rodhaini* is not clearly known in Sudan or Ethiopia. The status of *P. rodhaini* in the epidemiology of kala-azar requires further investigation.

The comparison of infection rates of *Arvicanthis niloticus, Tarera (G) robustus* and *Acomys cahirinus* indicated almost all infections were found from PCR technique. Of 128 rodents screened using DAT, NNN medium and microscopy, only 2 NNN medium were found positives. Detection ofparasites inrodents using low sensitive techniques such as NNN medium, DAT and microscopy was cumbersome. It is necessary to use highly sensitive PCR techniques.

8.2. Conclusions and recommendations

Knowledge about the bionomics and habitats of *P. orientalis* is important to design appropriate kala-azar control measures based on vector management. Information about human behavior which increases the chances of *P. orientalis* bite or incidence of kala-azar is also useful to control the disease by awareness creation. Control option based on reservoir hosts, on the other hand, requires the identification of the reservoir hosts.

8.2.1. Conclusions

- Agricultural fields and sparse thickets of *A. seyal* in extra-domestic habitats in which the cracking black soil (vertisol) are possible breeding sites of *P. orientalis* in Kafta Humera district
- *Phlebotomus orientalis* is a seasonal species increasing in abundance during the dry seasons and reaches its peak in March and April

- Peak activities of *P. orientalis* near animal shelter is after mid night (1 hr) and before midnight (21 hr) in sparse thickets of *A. seyal*
- *Phlebotomus orientalis* collectionsusing CDC traps are female biased near animal shelter and male biased in sparse thickets of *Acaciaseyal*
- Seasonal labour migrants may be exposed to infected sandfly bites during the weeding months of June and July when *P.orientalis* temporarly shelters in the dense mixed forest and other resting habitats
- Blood meal anlysis showed that *Phlebotomus orientalis* feeds on cattle and human
- Sleeping in open agriculture field has higher risk of kala-azar infection compared to sleeping in camps
- Rodents may serve as probable reservoir hosts of kala-azar in Kafta Humera lowlands although the identity of *Leishmania* is not yet confrmed

8.2.2. Recommendations

- The role of *P.orientalis* as a definitive vector of *L.donovani* needs further investigation by employing different methods such as isolation of the parasite, molecular characterization and experimental infection
- Studies are required to investigate the role of *A. seyal* and other plants as sugar sources for*P. orientalis* as a potential to exploit control of the presumed vector..
- Future vector control of kala-azar should mainly target agricultural fields, sparse thickets of *Acacia seyal* and *Balanites aegyptiaca-Acacia seyal* wood lands with black cracking soil.

- The disapperance of *P.orientalis* during the main rainy season and reappearance during the dry season needs a through study so as to understand how it survives during the wet conditions.
- Creating public awareness onthe habitats and habits of *P. orientalis* in addition to kala-azar for labuor migrants and permanent residents in Kafta Humera lowlands is required.
- Access and proper use of bed nets, especially during the weeding period, are required to prevent infection.
- Further studies are required on the role of the different species of rodents as reservoir hosts of kala-azar in the endemic lowlands of Ethiopia.

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9. **Annexes**

9.1. Questioner to collected data about different risk factors of VL.

1. Personal information, Behavioral factors and Serological result

-Name _____

-Sex _____

-Age_____

-Site_____

-Address_____

-Previous visit : For the first time ; For the second time ;For third time ; For more than three time

-VL history : yes No

-Knowledge Atitude practice (KAP)= ;? symptom & ?mode. Very good(2/2); good (1/2) ; bad (0/2)

Agent: yes No Method to prevent VL: yes No Vector-borne disease yes No

Stayed both weeding and harvest season: yes No Sleep: – Outdoor(farm) ; In camp Work at night ; day DAT Positive +ve -ve Seropositive +ve -ve

2.Environmental factors: -Forest close to sleeping or working sites: Yes ; No

- Risk of P. orientalis bite:- Yes ; No

3. Socio economic factors:-

- Income:- - < 500birr ; 500 – 1000birr ;1000 -1500 birr ; > 1500 birr

- Protein diet: - N0 ; Once in a week ; twice in a week ; more than twice

4. Climatic data

-June: - Temp. - monthly Maximum____; Monthly minimum_____;Average_____. Average maonthly rain fall:- _____;Soil cracks:- Widely open ; Partially opened ; Sealed

-July: - Temp. - monthly Maximum____; Monthly minimum_____; Average_____. Average maonthly rain fall:- _____;Soil cracks:- Widely open ; Partially opened ; Sealed

-August Temp. - monthly Maximum____; Monthly minimum_____; Average_____. Average maonthly rain fall:- _____;Soil cracks:- Widely open ; Partially opened ; Sealed

-September Temp.- monthlyMaximum_____; Monthly minimum____; Average_____. Average maonthly rain fall:-_____;Soil cracks:- Widely open ; Partially opened ; Sealed

-October: Temp. - monthly Maximum_____; Monthly minimum_____; Average_____. Average maonthly rain fall:-_____;Soil cracks:- Widely open ;Partially opened ; Sealed

-NovemberTemp.- monthly Maximum_____; Monthly minimum_____; Average_____. Average maonthly rain fall:-____;Soil cracks:- Widely open ;Partially opened ; Sealed

Name _____ signature _____Date_____