Leishmania infection in sand flies in a Cutaneous Leishmaniasis focus in Ghana.

By

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A Thesis submitted to the Department of Theoretical and Applied Biology, Kwame Nkrumah University of Science and Technology in partial fulfillment of the requirements for the degree of

MASTER OF PHILOSOPHY

In

Entomology

JUNE 2015

DECLARATION

I hereby declare that this submission is my own work towards the MPhil degree and that, to the best of my knowledge, it contains no materials previously published by another person nor materials which has been accepted for the award of any other degree of the university, except where due acknowledgement has been made in the text.

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DEDICATION

This work is dedicated to the Almighty God and to my parents, Mr. And Mrs Mosore who have supported me all through my MPhil Programme. This is also to my awesome siblings, Boapok and Mosorezor who have wished me well in my studies, without their prayers I would not come this far. I thank you all for your immense support and contribution to my achievement.

ACKNOWLEDGEMENT

I want to express my appreciation to my supervisors Prof. Daniel Boakye and Dr. Philip Kweku Baidoo for their support and guidance offered me through my study. I thank Profs Daniel Boakye and Paul Bates who gave me the opportunity to participate in training relevant to completing my study. I thank Prof. Michael Wilson, Prof. Maxwell A. Appawu and Dr. Dadzie who have served as mentors in my research career and pursuit of academic excellence.

I want to specially acknowledge staff and members of the Department of Parasitology (Noguchi Memorial Institute for medical Research and US. Naval Research Unit. #3 for their support. My deepest appreciation to Mrs. Naiki Puplampu Attram and Shirley Nimo-Paintsil for their immense support and contribution towards this study. To you both I say the Almighty God bless you.

I am also very grateful to Andy Asafu-Adjaye, Emmanuel Kwame Amoako (Sawa), Seth Addo, Sylvester Nyarko, Mawuli Agbodzi, Caleb Sinclair, Clara Yeboah, Samuel Abbey, Janice Tagoe, Eric Behene and Selassie kumordjie for their contributions towards this study.

I also want to acknowledge the support of the Authorities of Noguchi Memorial Institute for Medical Research and US Naval Medical Research Unit #3 Ghana detachment, for giving me the opportunity to do this work in their laboratories.

Finally, I say a big thank you to my family and loved ones for their prayer and support.

ABSTRACT

Leishmaniasis is a parasitic disease caused by various species of the genus *Leishmania*, a protozoan parasite transmitted through the bite of an infected female Phlebotomine sand fly. There are three forms of the disease namely, visceral leishmaniasis, cutaneous leishmaniasis and muco-cutaneous leishmaniasis. Cutaneous leishmaniasis is the most common form of leishmaniasis, characterized by sores on the skin that usually enlarge with time and develop into an ulcer. The disease is caused by various *Leishmania sp*. and depending on whether human beings are the main reservoir or other small mammals, transmission is usually considered anthroponothic or zoonotic. The distribution of *leishmania* infection in sand flies was examined in some endemic cutaneous leishmaniasis (CL) communities of the Volta Region, Ghana. CL was first reported in 1999 in these communities by the Ghana Health Service in the Ho, Hohoe and kpando municipality. Since the first outbreak of the disease, there have been increasing reports of the disease in various villages in the Volta Region of Ghana. The present study therefore, conducted to identify natural infection by *Leishmania sp*. in insect vectors of CL. Entomological survey was conducted in three endemic communities (Dodome Awuiasu, Dodome Dogblome and Lume Atsiame) in the Volta Region. From October 2012 to February 2013, a total of 4219 female sand flies were captured with Center for Disease Control (CDC) light traps and dissected for studies on the head and last three abdominal segments for identification. It was observed that twenty (0.5%) female sandflies were identified from the genus *Phlebotomus* and 4199 (99.5%) belong to *Sergentomyia*. To determine *leismania* infection in female sandlies, DNA was extracted from pools of sand fly species ranging from 1 to 25 dissected females. This was done using a Qiagen DNA extraction Kit. In considering the pools of individual sandfly species, *Leishmania sp*. infection of 0.0384% (95% CI, 0.00119-0.197) was detected in a pool of 7 (5.7%). *S. africana* female sand flies out of 122 pools using PCR. The infection was detected in

sand flies collected from Dodome Dogblome. This is the first report of natural infection by *Leishmania sp*. in *S. africana* in Ghana. This observation that *S. africana* is naturally infected by *Leishmania sp*., suggested that the sand fly species might play a role in the transmission of cutaneous Leishmaniasis in that part of the Volta Region of Ghana. The control of Leishmaniasis in endemic areas requires understanding of *Leishmania* ecology and the epidemiology of the disease. Finding naturally infected sand flies is important in identifying species of sand flies as a vector(s) of *Leishmania* in studying infection rates and estimating the prevalence of the disease in endemic communities that experience occasional outbreaks of Cutaneous Leishmaniasis.

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CHAPTER ONE

INTRODUCTION

1.1 Background

Leishmaniasis is a parasitic disease caused by various species of the genus *Leishmania*, a protozoan parasite transmitted through the bite of an infected female *Phlebotomine* Sand fly. There are over 30 species of sand flies that are proven *leishmania* vectors (Desjeux, 2001). Recent studies, however, have found ceratopogonide midges to be probable vectors among marsupials (Dougall *et. al.*, 2011) About 30 species of *Leishmania* have been described to date, of which 20 are known to be pathogenic to numerous mammals including humans. There are two subgroups under the genus *Leishmania*, *Leishmania* and *Vianna*. Parasites under these subgenera are classified depending on which part of the sand fly gut they colonize (Lainson *et al.*, 1977).

Geographically, the disease is grouped into New world or Old world Leishmaniasis. New world Leishmaniasis are endemic from Texas through South America, whereas the Old world group is endemic in Africa, Asia, the Middle East and the Mediterranean (Mitropoulos *et al.*, 2010). In humans, different forms of the disease are described depending on the associated species of *Leishmania;* different forms of the disease are; visceral (VL), cutaneous (CL), muco-cutaneous (MCL) and Post-Kala-azar dermal leishmaniasis (PKDL) which is a complication of visceral leishmaniasis (VL) in areas where *L. donovani* is endemic(Dawit, Girma, & Simenew, 2012)

Among the forms, VL is the most severe and almost always fatal, if untreated. The annual incidence of the disease is estimated at half million VL cases worldwide with over 90% occurring in just six countries; India, Bangladesh, Nepal, Sudan, Brazil and Ethiopia

(Desjeux, 2004). CL is the most common form of leishmaniasis globally. It is characterized by sores on the skin that usually enlarge with time and develop into an ulcer. CL is rarely fatal and heals on its own and it takes between three to eighteen months (Piscopo & Azzopardi, 2007) .

The *Leishmania,* a zoonotic multi-host parasite are maintained by several mammal species including rodents, other small mammals and dogs. Human host infections are usually considered accidental (Dantas-torres, 2007; Roberts *et al.*, 2000). *Leishmania* undergoes two host life cycles; intracellular parasites inhabits man and other mammals as amastigotes or flagellated promastigotes inside the midgut of the sand fly vector.

Cutaneous leishmaniasis (CL) is caused by various *Leishmania* species. Depending on whether human beings are the main reservoir host or animal, transmission is either considered as anthroponotic or zoonotic. Sand flies of the genus *Phlebotomus* (Old world) and *Lutzomyia* (New world) are the primary vectors responsible for the disease transmission. Clinical manifestation of the disease is varied ranging from a localized papule to the classic deep ulceration lesion with raised borders. The ulcer is usually selflimiting and can heal within three to six months leaving a disfiguring scar on the skin. Cutaneous leishmaniasis is usually painless; however the ulcer can be secondarily invaded by opportunistic bacteria causing pain and joint aches if the lesion occurs around the joints. Though the lesions are self-limiting, the rate of healing varies with different *Leishmania* species. The annual incidence of CL in the old world is estimated to be between 1 to 1.5 million cases with a greater percentage occurring in the Middle
East (Desjeux, 2004).

Over the past decade, increase in the number of CL cases worldwide indicates an increase in case reporting and improved diagnoses of the disease (Singh, 2006). Though the World Health Organisation (WHO) network for leishmaniasis surveillance aims at early diagnosis and treatment including co-infection with HIV, control of sand fly populations using indoor residual insecticide spraying in homes and insecticideimpregnated bed nets (Desta *et al.*, 2005) , a greater number of infections are asymptomatic or misdiagnosed underestimating the global burden of CL (Escobar *et al.*, 1992). This also implies the existence of poor vector and reservoir control interventions. In addition, ecological changes e.g. deforestation, urbanization and socio economic activities such as migration and farmland cultivation are variables contributing to the expansion of CL globally. Man-made changes have led to destabilization of the range and density of the vectors and reservoirs, spreading human exposure to infected sand flies in previously non-endemic areas (Assimina *et al.*, 2008; Desjeux, 2001). In the absence of the natural host as a result of human activities sand flies are forced to feed on humans, with transmission facilitated by individuals and families sleeping outside protective clothing during the dry, hot and humid seasons (Desta *et al.,* 2005). Human movement into areas endemic for cutaneous leishmaniasis also serves as a channel for disease transmission for individuals who are immune-naive. Poor nutrition is considered to increase susceptibility to CL by exposing populations in endemic regions to secondary opportunistic infectious diseases (Reithinger *et al.*, 2007). Changes in rainfall patterns, atmospheric temperature and humidity have been suggested to be associated with CL transmission, although the nature of the association may be different depending on the area (Analysis, 2010).

Globally, of the 1.5 million incidence of CL reported, about 90 percent occur in Iran, Afghanistan, Algeria, Syria, Saudi Arabia, Brazil and Peru (Choi & Lerner, 2001;

Desjeux, 2004). In Africa, countries bordering the leishmaniasis belt of West Africa, North, Central, East and the Horn of Africa are mostly endemic to leishmaniasis *(*Fryauff *et al*., 2006) . Amongst the North African countries, *L. major* distribution lays a region from Morocco, Algeria, Libya, through to Egypt with most of these cases transmitted by the vector *P. papatasi*. Similarly *L. tropica* is distributed across the North from the Canary Islands to Egypt (Kimutai *et al.*, 2009). In East Africa, CL occurs in a few areas in Kenya, North Sudan and Ethiopia with *L. aethiopica* being the prevalent parasite (Analysis, 2010).

In West Africa the main cause of CL is *L. major*; reports of CL seems to appear in epidemic proportion in various countries e.g. .Mauritania, Gambia and Senegal to the west of Nigeria and Cameroon to the east (Kimutai *et al.*, 2009). The frequency of the disease increases over a period of time and reduces drastically in the number of reported cases (Boakye *et al*., 2005)

1.2 Problem Statement

In comparing Leishmaniasis in West Africa to the rest of the world, very few studies have been done, probably because of misdiagnoses or underreported cases. Within the West African sub-region only a few published reports addressed the epidemiological and entomological status of leishmaniasis. However, although MCL forms are uncommon, recent reports of CL with mucous membrane involvement have been found in Senegal and Mali (Strobel *et al.*, 1987). While VL has been identified in Togo, Burkina Faso and Gambia (Boakye *et al.,* 2005). This raises the suspicion of the presence of various forms of Leishmaniasis within the West African region and establishes the need for a comprehensive study to identify cases, reservoir hosts and vector in order to access the

risk of sporadic epidemic outbreaks. In Ghana, an increasing number of suspected cutaneous leishmaniasis cases have been reported within the Ho municipality of the Volta Region in a primarily moist semi-deciduous forest ecosystem (Fryauff *et al.*, 2006). Typically, cases of CL are expected to occur in the northern, arid, Sahel Savanna regions of Ghana bordering Burkina Faso which lies on the CL belt of West Africa, however this is not the case (Boakye *et al.*, 2005), Rather reports of CL have been identified within the semi-deciduous forest of the Volta Region of Ghana (Kweku *et al*., 2011).

Since the outbreak of CL in 1999 within the Ho municipality, a total of 2,426 suspected CL case have been recorded by the Ghana Health Service (GHS) in the Ho, Hohoe and Kpando districts between 1999 and 2002 (Fryauff *et al.*, 2006). The number of suspected cases rose to 6,450 in 2003 with 116 communities affected (Fryauff *et al.*, 2006; Kweku *et al.*, 2011). Polymerase chain reaction (PCR) testing on skin biopsies collected from the original outbreak revealed the presence of *L. major* and an uncharacterized species (Villinski *et al.*, 2008).

In 2011 after an active surveillance in the Volta region, three villages all in the Ho municipality were identified with CL cases indicating the persistence of active CL transmission. Sand fly collections, both indoors and outdoors in CL endemic areas in the Ho municipality yielded mostly *Sergentomyia* species (Fryauff *et al.*, 2006). Although *P. duboscqui* is principal vector for *L. major* in West Africa, *P. duboscqui* and *P. rodhaini* were least abundant of 17 different sand flies collected *(Boakye et al., 2005; Fryauff et al.*, 2006). These two species made up 0.4% of the total sand flies captured, but these numbers were so low their role in CL transmission is uncertain.

1.3 JUSTIFICATION

Currently the vector(s) transmitting CL infections in the Volta region of Ghana is not clearly determined. Sand fly collections between March 2004 and May 2005 yielded 17 species, with majority being the man-biting *Sergentomyia* species and considered a nonhuman vector (Fryauff *et al*., 2006). However, the man-biting *Phlebotomus* species, *P. duboscqui* and *P. rodhaini* were collected in very low numbers and their vectorial role in

CL transmission remains uncertain. Recent studies in Senegal have suggested *Sergentomyia* species as a possible vector for the transmission of human *Leishmania* (Senghor *et al.*, 2011). The possibility of *Sergentomyia* being a competent vector in Ghana cannot be over emphasized considering the fact that some CL are caused by newly identified species of *Leishmania* (Hitakarun *et al.,* 2014) This further reiterates the need to study this area and examine all possible vector(s) responsible for the transmission of cutaneous leishmaniasis in the Volta region of Ghana.

1.4 OBJECTIVES

1.4.1 Main objectives

The main objective of the study was to:

- Determine and characterize the species of sand fly involved in the transmission of cutaneous leishmaniasis and identify the *Leishmania* parasite responsible for the disease in the Volta Region of Ghana.

1.4.2 Specific Objectives

The specific Objectives were to:

- Collect and identify sand flies caught in the study area using morphological keys
- Detect cutaneous leishmaniasis parasites in sand fly samples caught using PCR**.**

CHAPTER TWO

LITERATURE REVIEW

Leishmaniasis is an emerging vector-borne disease that is adapting to changing environment and affecting new geographic areas. The disease is considered the second important protozoan causing disease affecting humans after malaria (Lawyer , 2004). About 2 million new cases occur every year in more than 80 countries, with 350 million people considered at risk (World Health Organization, 2010). The disease burden of leishmaniasis has been increased and covered a wider geographical distribution pattern and has become a growing public health concern. The worldwide increase of its incidence is mainly attributed to several risk factors that are man-made e.g. deforestation, urbanization, migration and agriculture, immunosuppression, treatment failure and malnutrition (Desjeux, 2001).

2.1 Distribution of Leishmaniasis

Leishmaniasis is widely distributed around the world. *Leishmania* has been reported on every continent apart from Antarctica (Fever, 2012). The disease ranges over the intertropical zones of America, Africa and extends into the temperate regions of South America, Southern Europe and Asia (Figure.1). The extension limits are latitude 45º North and 32º South. The geographic distribution of the disease depends on the sand fly species acting as a vector, their ecology and conditions of internal development of the parasite (Desjeux, 2001). New world CL is found in Mexico, Central America, and South America from Northern Argentina to Southern Texas and southern Europe (Bari and Rahman, 2008) meanwhile in Old world CL it occurs in Asia, Middle East, and Africa. *L. major* and *L. aethiopica* cause zoonotic cutaneous leishmaniasis in the Old world (Dawit *et al.*, 2012).

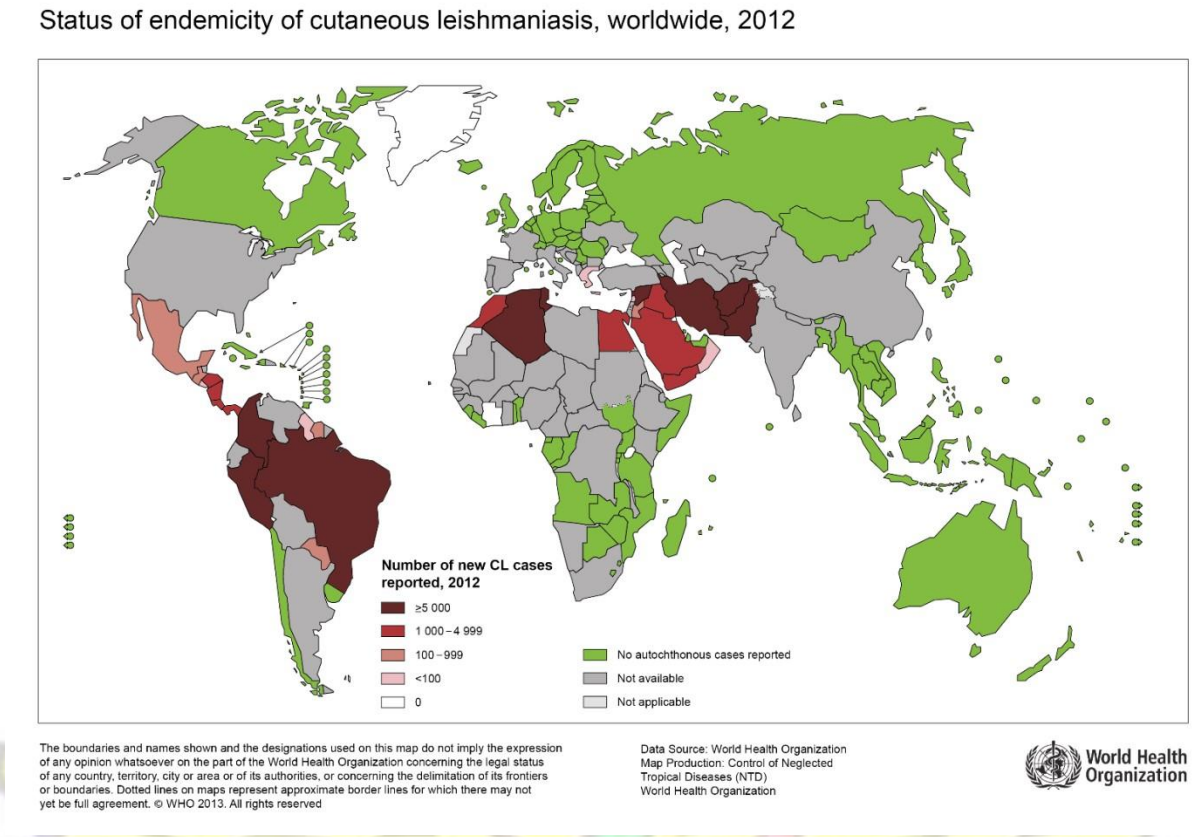

Figure 1: World Map showing the distribution and endemicity of Cutaneous Leishmaniasis. Status of endemicity of Cutaneous Leishmaniasis, worldwide. (Source: WHO Leishmaniasis Control Programme, Annual Country Reports, 2012)

CL is an environmental disease, climatic changes in temperature, rainfall and humidity can affect breeding of sand flies and reservoir host by altering their distribution, survival and population size. Population movement resulting from drought, famine, food and agriculture lead to displacement and migration of people to areas with transmission of

leishmania which may result in increase in human exposure to infected sand flies (Assimina, 2008).

2.2 The Disease Cutaneous Leishmaniasis

CL usually occurs as a single lesion on the skin or may result in multiple lesions as a result of several bites from infected sand flies. In the Old world, localized CL is caused by *L. major*, *L. tropica*, and *L. aethiopica*, which are all members of the *L. tropica* complex (Fever, 2012). *L. major* lesion may take between 2 - 4 months to heal (Murray *et al*., 2005). Nevertheless, *L. tropica* infections are strictly human related and lesions observed usually last for 6 - 15 months. *L. infantum* is the most common species reported in domesticated animals and Human infections due to *L. infantum* are less commonly observed.

In the New world, *L. peruviana*, *L. guyanensis*, *L. braziliensis* or *L. mexicana* infections cause localised CL (Richard Reithinger et al., 2007). Diffused cutaneous leishmaniasis infections are caused by *L. aethiopica* in Africa, and *L. amazonensis* in South America. Diffused cutaneous leishmaniasis, however is associated with immunosupressed patients. Most Old world and New world species only cause lesions on the skin, but New world species *L. braziliensis* and *L. guyanensis* may cause either CL or MCL.

2.3 The Vector

Phlebotomus sand flies are blood feeding arthropods of the family Psychodidae in the Order Diptera. Approximately 800 sand fly species have been recorded, namely *Phlebotomus, Sergentomyia, Lutzomyia*, *Brumptomyia* and *Warileya*, however only some are known to be medically important and are proven vector species of *Leishmania* protozoa (Killick-Kendrick, 1987; Morrison *et al.,* 2004). Moreover, only about 30 species have been demonstrated to have vectorial capacity with less than 10% of sand flies

implicated as vector species for leishmaniasis (Bates, 2007). Different vectors of *leishmania* are responsible for transmission of the disease in different geographical region, e.g. *L. infantum* is transmitted by *Lutzomyia Longipalpis* in South America, *P. perniciosis* in spain, and *P. ariasi* in France. *P. duboscqui* is the main vector for *L. major* in Africa and *P. papatasi* in Asia.

2.4 Appearance and Behaviour of *Phlebotomine* Sand flies

Phlebotomus sand flies are small, silver-grey to almost black hairy flies that are identified by the presence of erect narrow wings covered with hair (Service, 1980). They have a body length approximately 2-3mm and hold their wings in an upright V-shape when at rest (**Plate. 1)**. Unlike mosquitoes, they are silent, fly short distances and attack their host using a characteristic hopping type of flight (Killick-Kendrick 1987). Adults are weak fliers and do not usually disperse more than a few hundred meters from their breeding places. The activities of sand flies are nocturnal, although a few species bite during the day.

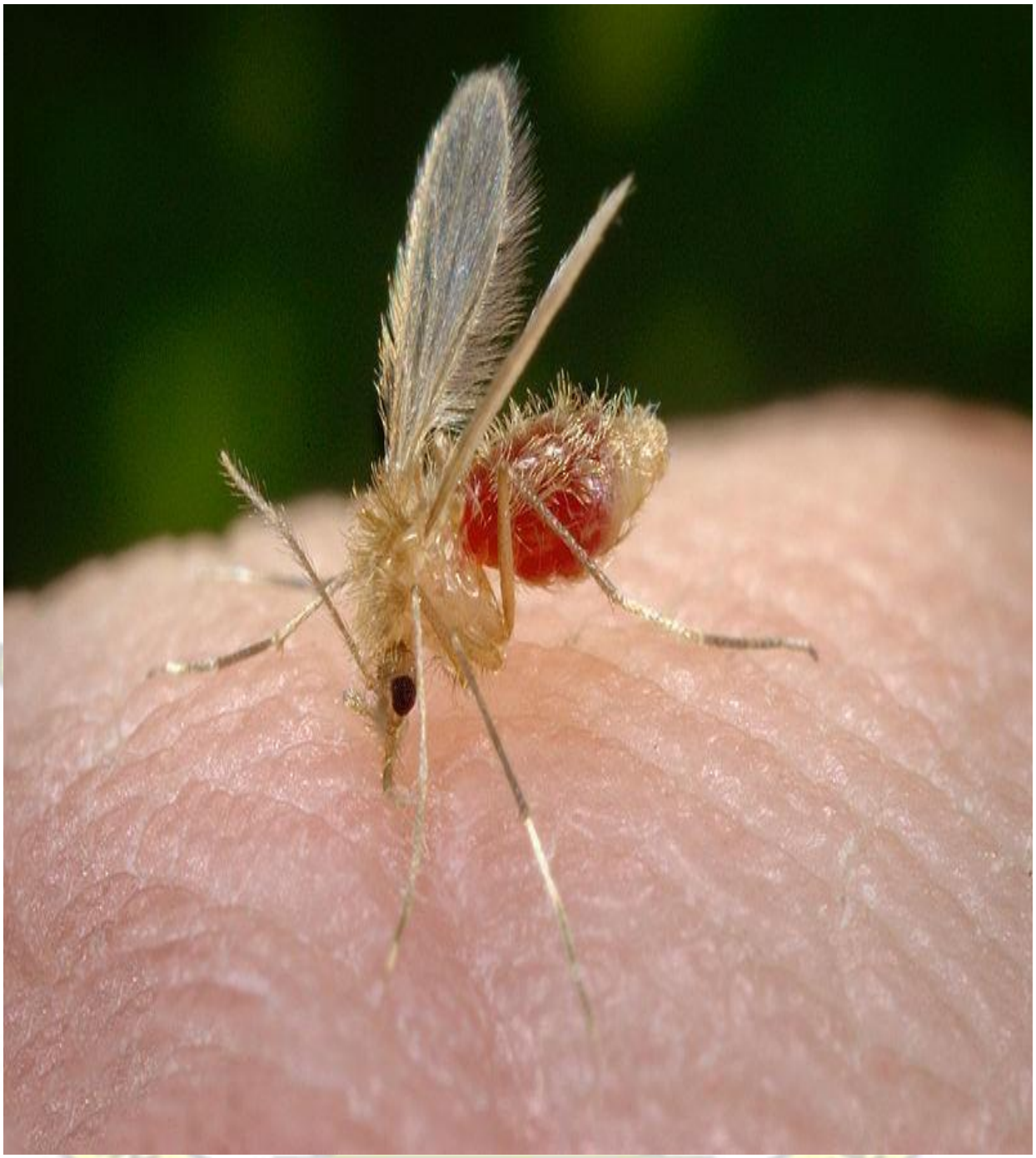

***Plate1: Female Phlebotomine Sand fly taking blood meal. Source: courtesy Prof. Bates,* University of Lancaster, 2011.**

2.5 Breeding and Resting Sites

Sand flies breed in a confined microclimate that may be provided by the wild mammalian reservoir. They are found in highest densities in potential larval habitats, in soil and feces mixed to form a conditioned, organically enriched environment (Alten, 2010). Subsoils water also contributes to breeding. A high water table level is suitable for the development of the larvae of sand flies (Bari and Rahman, 2008). Adult sand flies have been collected from varied resting sites in rural and forest habitats including animal burrows, shelters and tree epiphytes, away from light and with high humidity (Alten, 2010). In most forest areas, cavities in trees and buttress roots are common dwelling places for sand flies (Alten, 2010; Killick-Kendrick *et al.,* 1986). Mating times for female sand flies varies amongst species in relation to blood feeding. Mating may occur either before, during or after taking a blood meal and again for subsequent gonotrophic cycles (Guilvard *et al.,* 1985; Wardl & Newstead, 1977), *Lu. longipalpis* and *P. argentipes* mate on the host where the males first arrive to establish individual territorial boundaries and lurk around before the females arrive to take a blood meal (Jarvis and Rutledge, 1992; Lane *et al*., 1990). *P. dubosqui* female agree to mate when she has the male riding on her back for a variable time (Valenta *et al.,* 2000) Other species e.g. *Lutzomyia*, the males attract females with pheromones from abdominal glands and sounds (Maingon *et al.,* 2003). After mating, the period from a blood meal to maturation of eggs ranges from about 4 to 8 days. Eggs usually take 7 to 10 days to hatch. Sand flies lay their eggs in detritus, identifying special markers which provide optimal condition for oviposition (Killick-Kendrick 1987). Gravid females of *Lu. longipalpis* are also attracted by hexanol and 2-methyl-2-butanol in chicken or rabbit feces. Choice of oviposition site is by physical and chemical constituents of the substrate and they are stimulated to oviposite by pheromones of conspecific eggs (Dougherty *et al.,*

1995; Elnaiem and Ward, 1992). The larval stages pass through four instars before pupation and adult emergence. The larvae of sand flies are terrestrial and the rate of development is shortened by increased temperature or lengthened by decreased temperature. Sand flies diapause in the larval stages and this is often due to changes in day length and temperature. Larval development is slow and can take at least 3 weeks before pupation. Adult flies emerge from the pupae after 10 days with males emerging first.

2.6 Food Source of Phlebotomine Sand flies

Adult sand flies feed on natural sources of plant sugar. The female in addition suck blood from a variety of hosts which provides nutrition for the production of egg (Schlein and Warburg, 1986). A few species are able to produce the first batch of eggs without a blood meal (autogeny) and they can be found in caves and other enclosed habitats (ElKammah, 1973; Lewis, 1974). This behavior reduces the number of alternate feeding and laying of eggs (Gonotrophic cycle), human contact, and vectorial capacity of the sand fly. Female sand flies, like mosquitoes locate their host by making zig zag flights upwind until they locate a host odor plume along which they can then fly (KillickKendrick and Rioux, 2002; Killick-Kendrick *et al.,* 1986). Some other species may use visual cues in starlight conditions as done by *Lu. longipalpis* (Mellor & Hamilton, 2007). Host preferences by sand flies often depend on host availability and size. However landing and biting may depend on certain factors such as carbon dioxide, odour, and host behavior such as grooming and activity pattern (Bray and Hamilton, 2007; Campbell-Lendrum *et al.*, 1999). In some studies, blood meal analysis of sand flies collected in the wild have shown that several *Phlebotomus* and *Lutzomyia*, and some

Sergentomyia species will bite a wide range of mammals and birds (KillickKendrick and Rioux, 2002; Rossi *et al.,* 2008). Species differ in the number of blood meals taken during a gonotrophic cycle. Some species (*Lu. longipalpis and P. papatasi*) will take multiple blood meal on different days as a result of *Leishmania* manipulation (Perfil'ev, 1968), whereas others feed only once for each batch of eggs. The efficiency of *Leishmani*a transmission from infected sand flies to host is increased by the frequency of blood meal uptake. Studies have demonstrated that infected flies probe several times when biting (Killick-Kendrick *et al.,* 1977). Behavioral persistence to feed is manipulated by the protozoan parasite *Leishmania* (Rogers and Bates, 2007). This manipulative adaptive behavior by infected sand flies elicit the production of filamentous proteophosphoglycan that impairs the functioning of sand fly gut mechanoreceptors that detect blood flow, and explains the blockage and reduced ability to take a blood meal (Jenni *et al.,* 1980; Molyneux and Jefferies, 2009). The sand fly experiences increased starvation and the persistence to feed, or alternatively, increases the threshold blood volume at which blood-seeking behavior is inhibited. More importantly, sand fly manipulation directly increase the fitness of the parasite through enhanced transmission (Rogers and Bates, 2007).

2.7 Biology and Life Cycle of *Leishmania*

Leishmania parasites belong to the family trypanosomatidae. The life cycle of a *Leishmania* begins when an infected female sand fly takes a blood meal from the vertebrate host. During the process of blood uptake, the sand fly introduces its mouthparts in the skin tissues and the salivary gland content is injected together with *Leishmania* promastigotes into the host's skin (Andrade *et al.,* 2007). *Leishmania* parasite exists in two main morphological forms, either as amastigotes inside the phagocytes of the

vertebrate host or as promastigotes within the guts of the *Phlebotomine* sand fly. The amastigotes are small round to oval bodies which measures about 3-5µm in diameter without a flagellum (Kakarsulemankhel, 2011; Azevedo *et al.* 2012; Brandão-Filho *et al.* 2003). They are colourless, have a homogenous cytoplasm and surrounded by a pellicle (Singh, 2006; Siqueira-Neto *et al.*, 2012). The promastigote forms are seen in the midgut of the sand fly, until the parasite develops and reaches the buccal cavity, then becomes the insect vector of the parasite. They are motile, slender organisms measuring about 10 to 15µm in length, with a single anterior flagellum (Hide *et al.,* 2007). Amastigotes lack the flagellum, but a short flagellum may be seen arising from the kinetosome (Bari and Rahman, 2008; Singh, 2006). The parasite has two basic life stages, one extracellular stage within the invertebrate host and one intracellular stage in the vertebrate, the promastigotes are then phagocytosed by the host's macrophages and consequently transforms into amastigotes with then multiply in cells of various tissues (figure 2). The parasite evolves into amastigote forms, spherical, intracellular form without flagellum. They replicate by binary fission. The multiplication of the parasites occurs inside the microphages, which are the main targets. The macrophages lyse and the cycle continues when other host's phagocytes are being infected (Bañuls *et al.,* 2007; Desjeux, 2004).

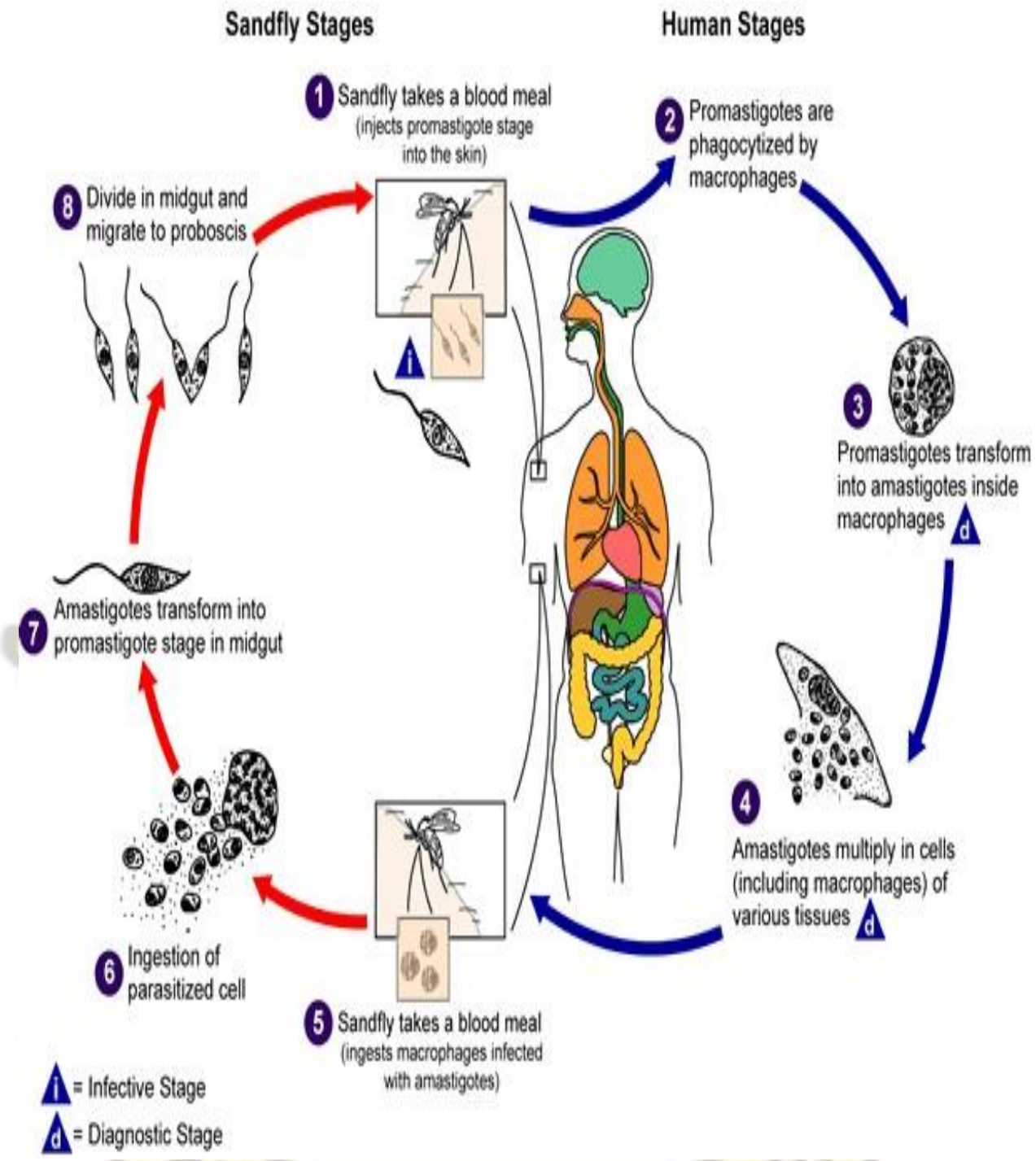

Figure 2: Life cycle of human Cutaneous Leishmaniasis. Source CDC, 2013

2.8 Mode of Transmission of Human Cutaneous Leishmaniasis

The disease is transmitted to a vertebrate host by the bite of a female *Phlebotomine* sand fly. Flies from the genera *Phlebotomus* and *Lutzomyia* are the known biological vectors of the disease (Azevedo *et al.*, 2012; Siqueira-Neto *et al.*, 2012). The parasite is usually indirectly transmitted between hosts by the sand fly vector. Female sand flies are hematophagous. Like mosquitoes, sand flies take up blood meals for the production of their eggs (Assimina *et al*., 2008). Some ticks and canine fleas may serve as mechanical vectors (Paz *et al*., 2013) . Transmission in humans and dogs is possible through blood transfusion and by trans-placental transmission in dogs, mice and humans (Fever, 2012)

2.8.1 Vector Competence

Vector competence refers to the ability of an individual in a population of arthropods to acquire, maintain, and transmit a given strain of pathogen. Establishing vector competence is one of the requirements for vector incrimination. Almost any blood feeding arthropod which feeds upon an infected vertebrate host can obtain and retain the disease agent for a while, but does not necessarily mean that the arthropod is a competent vector (Barnett, 1962). Some sand fly vectors support the full development and successfully transmit several *Leishmania* species (permission vectors), whiles others are just vectors of one particular species (specific or restrictive vectors), even if other species coexist in the same environment (Kamhawi *et al*., 2000; Sacks, 2001). In a review by Killick-Kendrick, a vector must go through critical steps to be a competent vector.

1. Vector (*Phlebotomus papatasi* and *Phlebotomus sergenti*) must support the full development of only their specific parasites, whereas others (*L. Longipalpis*) are susceptible to many parasites of the subgenera *Leishmania* and *Vianna.*

2. Parasites of different developmental stages must survive a proteolytic enzyme within the blood meal in the female fly's midgut,

3. Escape the peritophic membrane

4. Prevent excretion with digested blood by inhibiting peristalsis and adhering to midgut epithelia or hindgut

5. Avoid competition from gut microbiota

6. Find nutrients for morphogenesis and migration to anterior midgut before they transmit to host at a later blood meal (Sacks and Kamhawi, 2001). Vectorial competence must be accompanied by frequent biting of reservoirs and human host within a favourable environment (Palatnik-de-Sousa, 2012).

2.8.2 Vectorial Capacity

Vectorial capacity is defined quantitatively and it is influenced by vectorial density and longevity in nature (Beerntsen *et al.,* 2000). A competent vector will become epidemiologically important when it has a critical vectorial capacity (Ready, 2013).

2.8.3 Vector Incrimination

The majority of sand fly species play no role in the transmission of leishmaniasis. This may be due to several reasons; they are not man biting; their distribution may be different from that of their reservoir host; their feeding preference may not include a reservoir host; or they may be unable to support all the developmental stages of the parasite. The incrimination of a vector is based on a series of generally accepted observations (Killick-Kendrick 1987), namely,

1. demonstrate that members of a suspected arthropod species feed upon a vertebrate host, or otherwise makes effective contact with the host under natural conditions.
2. demonstration of a convincing biological association in time and/or space between the suspected vector species and clinical or subclinical infections in vertebrate hosts.
3. repeated demonstration that the suspected vector species, collected under natural conditions, harbors identifiable, infective stage of the infectious agent indistinguishable from isolates from patients.
4. demonstration of efficient transmission of identified infectious agent by the suspected vector under experimental conditions.

It is important to satisfy the above requirements for vector incrimination since the presence of the pathogen in an arthropod does not necessarily indicate a vectorial status.

2.8.4 Reservoir Host of Leishmaniasis

Leishmania parasites are hosted by a wide range of vertebrate animals called the wild host. The most common reservoirs are sloth, opossum, and small forest rodents such as the hydrax and peri-domestic dogs (Fever, 2012). Each species of *Leishmania* adapts to one or more animal reservoir host. In most parts of central Asia, Middle East and North Africa, *Rhombomys opimus, Meriones spp*. and *Psammomys obesus* are the three major reservoir species of rodents that maintain infection

2.9 Detection of *Leishmania*

Current existing methods of *Leishmania* identification in blood and other body tissues include isoenzyme characterization, microscopy, detection of antibodies (indirect

fluorescent Antibody test and direct Agglutination test) and detection of DNA (Polymerase chain reaction).

2.9.1 Microscopy

Microscopy is used to detect *Leishmania* amastigotes in relevant tissues aspirates or biopsy, such as bone marrow, spleen, lymph nodes, liver and skin split smears. Observation of amastigotes is readily seen in smears or touch preparation of infected tissues stained with Giemsa stain (Singh, 2006). Identification of *Leishmania* parasites could be done in *Leishmania* vectors using a dissecting microscope. Parasite isolates from the midgut of the sand fly can be cultured, however some species can be difficult to isolate and culture and some species will grow only in certain media. For parasite isolation, Novy-MacNeil-Nicole medium, Grace's medium, Schineider"s Drosophila medium might be used initially, but for parasite culture maintenance M199 is used.

2.9.2 Indirect Fluorescent Antibody Test (IFA)

IFA is a commonly used test to detect anti-leishmanial Antibodies using fixed promastigotes. Detection of antibodies is demonstrated in the everyday stage of infection, and is undetectable six to nine months after cure. Lower sensitivity of test can be overcome by using *Leishmania* amastigotes as antigen instead of promastigotes. IFA is ideal for diagnosing CL, MCL and Post-Kala-azar dermal leishmaniasis (PKDL) (Assimina *et al.*, 2008).

2.9.3 Leishmanin skin test

Delayed hypersensitivity is an important feature of Cutaneous forms of human leishmaniasis and can be measured by the leishmanin skin test, also known as the

Montenegro reaction (Singh, 2006). Leishmanin Skin test is useful for determining the distribution of human infections, distinguishing immune from nonimmune cases.

2.9.4 Polymerase Chain Reaction (PCR)

Amongst the molecular methods used for diagnosis, PCR have proved to be the most sensitive and specific technique. Conserved region target specificity makes PCR suitable for specific gene amplification needs. Compared to traditional techniques, PCR has several advantages, it is highly sensitive, rapid and the ability to perform with a broad range of clinical specimen (Rose *et al.,* 2004; Tavares *et al.,* 2003). Several studies have reported that PCR assays could detect parasiteamia a few weeks before the appearance of clinical signs (Singh, 2006). Singh *et al., 2006* have shown that a modified form of PCR such as nested PCR have proved its predictive values in diagnosis of PKDL. Parasite load analysis can also be determined quantitatively or qualitatively using a realtime PCR, as the fluorescence emitted is directly proportional to the number of amplicons(Bell & Ranford-Cartwright, 2002; Bossolasco *et al.*, 2003; R. Reithinger & Dujardin, 2006). In detection of mixed infections, such as in suspected AIDS patients multiplex PCR could be used (World Health Organization, 2010; Singh, 2006))

2.9.5 Sampling Method for Sand flies

Sand flies can be collected by several methods, either while foraging at night or resting during the day. Collection techniques for adults Sand flies include use of human landing collections, sticky papers, aspirators and Center for Disease Control (CDC) light trap collection (Killick-Kendrick, 1987). Human landing collection often attract the largest number of sand flies (Hanafi *et al.* 2007), however catches depend on the skill and attractiveness of the individual and often expose collectors to an increased risk of

Leishmania infection. Light traps are extensively used in field studies of sand flies. They are simple to use and less labour intensive than other methods (Davies *et al.*, 1995). The battery-operated CDC light trap is fitted with a tungsten bulb and a fine-mesh collecting net. It is frequently used at night to collect photo-tropic species. CDC light traps are particularly useful in the wet, forest environments where unbaited traps are unproductive (Bernier *et al.*, 2008). Carbon dioxide or a small caged animal may be used to increase the trap attractiveness to non-phototrophic species. Sticky traps are interceptive traps that capture sand flies as they fly in search of sugar or a blood meal, mate, or a resting or oviposition site. It consists of a piece of white paper soaked in castor oil and placed in a potential resting place, or mounted on sticks in areas where sand flies might be expected to fly or breed. These traps are generally inexpensive and easy to make in large numbers. They can be prepared in large numbers and stored until required. Sticky traps can be used as exit-entrance trap to capture sand flies as they fly in and out of animal burrows, tree holes, termitaria or small cavities used as diurnal resting or breeding sites by many species. Diurnal collections of sand flies may be made with a mouth or battery-powered, hand-held aspirator with the aid of a flashlight or head lamp.

2.10 Transportation and Preservation of Sand fly specimen

The method used in preservation and transportation of field collected sand flies depends on the focus of the study. Samples to be used for taxonomic studies can be preserved in layers of tissue paper in petri dishes or vials with silica gel before being cleared with a clearing medium for identification (Lewis, 1982). Sand flies can also be stored in vials containing 70% ethanol. Storage in ethanol better maintains the specimen, however long time preservation of the sample discolours the samples and hardens the muscles of the

insect hindering the observation of internal organs used for identification (Alexander, 2003). Captured sand flies to be observed for natural *Leishmania* infections should be freeze- killed by dry ice or by direct exposure to sunglight and soaked in a dilute detergent (1.0% of soap and distilled water) to reduce the hydrostatic force and to remove the seta from the body of the flies. The flies should be moved to a petri-dish containing 1X phosphate-buffered saline (PBS) and transferred to cryovials containing 10% dimethyl sulphoxide (DMSO). The vials containing the insect specimen are transferred into a thermo cool box and then placed in a dry shipper prior to total immersion (Killick-Kendrick 1987).

2.11 Morphological Identification of Sand flies

Sand fly species maybe identified based on their morphology, mainly internal structures such as spermatheca, cibarium and pharynx in females and genitalia in males (Hanafi, 2005). The head and terminal segments of the sand fly maybe detached and placed in a drop of clearing medium and subsequently mounted on a slide with a drop of Puris medium for identification under a microscope (Lewis, 1982). This morphological technique is laborious and time consuming when large numbers of specimen have to be examined to obtain informative data, since the infection rate of sand flies with

Leishmania is generally very low (0.01-1%) even in endemic areas (Hashiguchi and Gomez, 1991; Kato *et al.*, 2007)

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study Site

The study was carried out in the Ho District, one of the twelve districts in the Volta region of Ghana. It is bordered to the North by the Hohoe District and to the East by the Ghana-Togo border. The Volta Lake borders the region to the west and Atlantic Ocean to the south (figure 3). It has an estimated population of 271,881 people with an annual growth rate of 2.5% in 2010 (Statistical Service of Ghana, 2010). The town of Ho doubles as the District capital (of the Ho District) and the Regional Capital (of the Volta Region). The northern zone of the District is mountainous and is covered with forest while the southern zone is a mix of savanna and grassland with some marshy areas**.** There are two main seasons; the wet and dry. The wet season encompasses the major rainy season from May

to August, and the minor rainy season during October and November. The remainder of the year is relatively dry. The main occupations of the population are farming and livestock rearing. The outbreak of CL occurred early in the year 1999 in the areas within the moist semi-deciduous forest, an ecotype that is not typically associated with leishmaniasis (Fryauff *et al.*, 2006). The villages in the Ho- district were actively surveyed by disease control officers who performed site visitations seeking individuals with cases of CL.

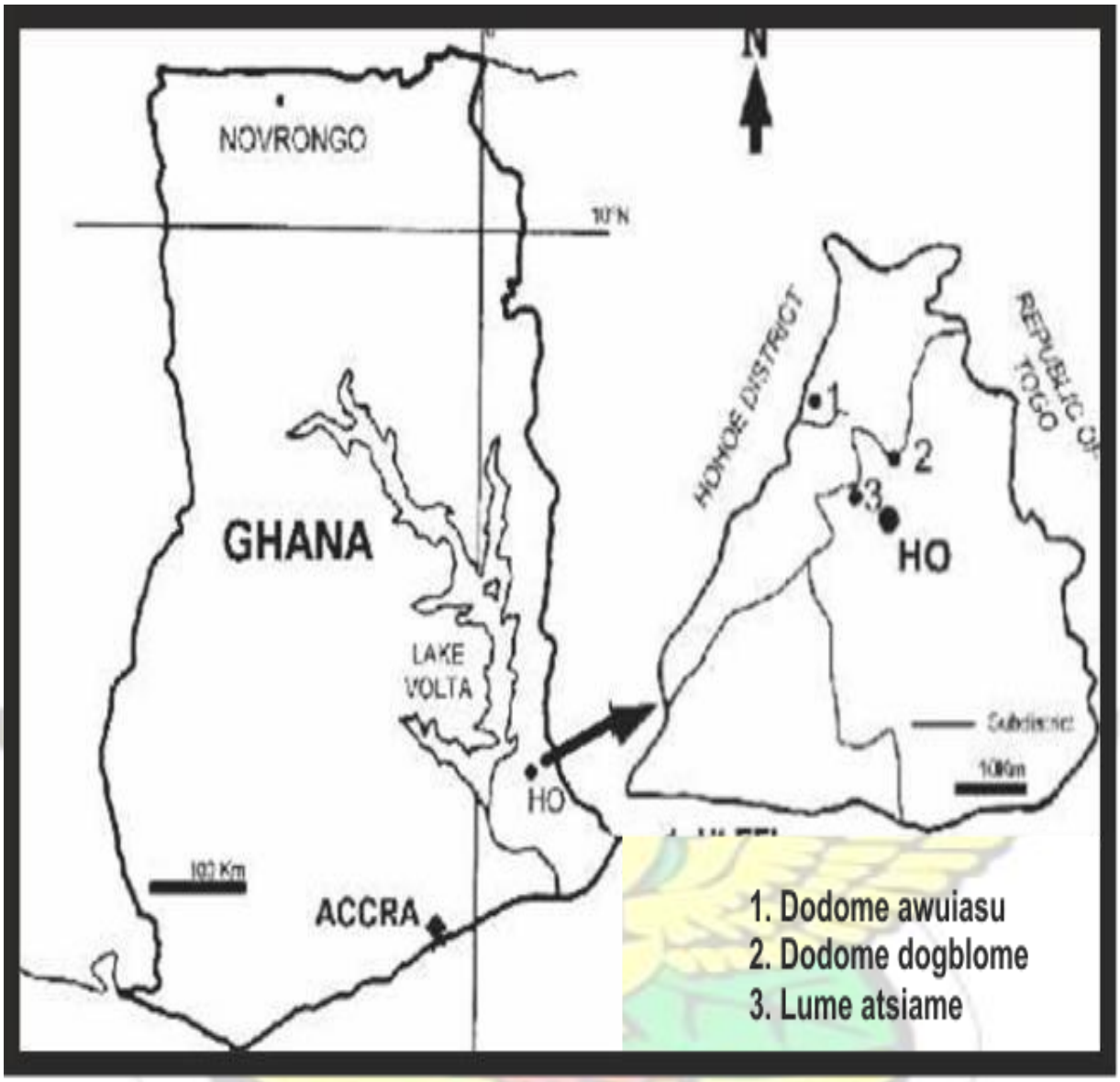

Figure 3: Map of the three study villages, Awuiasu, Dodome Dogblome and Lume Atsiame, in the Ho District of Ghana from where CL cases were identified in 2013.

3.2 Sand fly Sampling

Five (5) months of sand fly collections (October 2013 - February 2014) were conducted from 2013 to 2014 in three communities, namely Awuiasu (N 06˚46. 056’ E 000˚30. 971’), Dodome Dogblome (N 06˚45. 734’ E 000˚30. 895’) and Lume Atsiame (N 06 ˚

45. 487’ E 000˚30. 967’) all in the Ho municipality. These communities represent areas in the district where cases of CL were reported by the Ho District Directorate of Health Services in 2013. CDC light traps were randomly placed to collect flies from human and animal dwellings upon the consent of the household. Traps were set at dusk and flies collected at dawn (approximately 12hr). A total of 20 traps were set each day in each community. Sampling was carried out for three (3) consecutive nights in each month. Field collected sand flies were freeze-killed at -20º C and sorted out into labeled 1.5ml eppendorf tubes containing silica gel for dry preservation. The tubes were secured in sealed Ziploc bags and transported to the entomology Laboratory at Noguchi Memorial Institute for Medical Research (NMIMR), University of Ghana, Legon-Accra.

3.3 Sand fly Dissections and Morphological Identification

All sand flies were separated into either male or female on the basis of morphology of their reproductive organ observed under a stereomicroscope (Olympus SZ60). Each sand fly was dissected in a drop of sterile normal saline (PBS) using a pair of dissecting pins. The junction between the head and thorax was held down on a glass slide and the other hand was used to cut off the thorax and drawn away from the immobilized head. The mid-section of the fly was held down with dissecting pins while the abdomen was leased away at the fourth abdominal section. The head and the last three abdominal segments were placed on a labeled glass slide and about 2-3 drops of clearing medium made up of chloral hydrate and phenol was added to clear overnight (Appendix IV). The thorax and the upper abdominal segments were kept in a complementary 0.2 ml sterile eppendorf micro tube with same labeling as the head and last abdominal segments on the slide. A drop of mounting medium (Appendix IV) was placed on the head and last three abdominal

sections of the fly and covered with a glass coverslip, making sure that the abdomen was placed laterally and the head placed with proboscis facing upward. The slide with the fixed fly was placed on a heating block at 80ºC (C.S. & E. Slide warmer No. 26020; Clinical Scientific Equipment Co.; Melrose Park Illinois, U.S.A) and allowed to clear for three (3) weeks and then observed under a stereomicroscope for species identification.

Sand flies were differentiated using taxonomic keys (Abonnenc., 1972; Lewis, 1982). Morphological characters such as the presence of cibarium, cibarial teeth, pharynx, pharyngeal armature, spermatheca, palpal and ascoid formula were used for the identification of species. Images of the various species were taken with the aid of an Olympus BH-2 mounted camera connected to a monitor-utilizing software

PLATE 2: Sampling, sorting and packing of adult sand flies

A is assembling of CDC light traps for trapping in the communities, **B** setting up of CDC light traps **C and D** Sorting and labeling of sand flies from individual traps into 1.5ml eppendorf tubes

3.4 DNA Extraction

DNA was extracted from the dissected thorax and attached anterior abdomen of same species in pools of 10 or less in a 1.5 ml eppendorf micro-tubes. The DNA extraction was

undertaken using the Qiagen Kit, 180µl of buffer ATL was added to each pool of flies; the flies were macerated using a plastic pestle on a battery powered rotor, 20 µl proteinase K was then added to denature proteins and the mixed thoroughly by vortexing using a vortexer (Standard Mini Vortexer, VWR Scientific Products). The mixture was incubated at 56 ºC for 10mins. Each micro-centrifuge tube was vortexed briefly for 15 seconds and 200 µl buffer AL was added to each of the tubes and mixed thoroughly by vortexing. 200 µl of absolute ethanol (96-100%) was added and the solution mixed thoroughly by vortexing. The mixture was transferred into a DNeasy Mini spin column placed in a 2 ml collection tube. This was placed in a centrifuge (eppendorf centrifuge 5415D) and spun at ≥ 6000 x g (8,000 rpm) for 1 min; the flow-through and the collection tubes were discarded. The DNeasy Mini spin columns were each placed in new 2ml collection tubes and a volume of 500µl buffer AW1 was added and centrifuged at ≥ 6,000 x g(8,000 rpm) for 1 min. The flow-through and the collection tubes were again discarded. The DNeasy Mini spin columns were again placed in new 2ml collection tubes. 500 µl of buffer AW2 was added to each spin column and centrifuged for 3 minutes at 20,000 x g (14,000 rpm) to dry the DNeasy membrane. The flowthrough and the collection tube were again discarded. The DNeasy Mini spin columns were each finally placed in 1.5 ml eppendorf micro-centrifuge tubes. 100µl buffer AE was added to the DNeasy membrane of each spin column and incubated at room temperature for 1 min. The purified DNA extracts were eluted by centrifuging at ≥ 6,000 x g (8,000 rpm) for 1 min.

3.5 Identification of *Leishmania* Infection in Pools of Sand flies Using Conventional PCR.

DNA extracts from sand fly were used in a 25µL PCR reaction mix. The PCR mix constituted by 1X Green GoTaq (Flexi buffer), 25mM $MgCl_2$, 0.2mM of each dNTPs and 1.25 Unit of GoTaq DNA polymerase. Three sets of PCR assays were used to detect *leishmania*. Different parts of *leishmania* ribosomal repeats of the ssu rRNA gene were amplified using primers R221 and R332 and ribosomal internal transcribed spacer 1 (ITS1) separating the gene coding for ssu rRNA and 5.8S rRNA using LITSR and L5.8S primers (El Tai *et al.* 2000). The third PCR assay used amplified conserved regions of *leishamania* species minicircle DNA of the parasite kinetoplast using mincr2 and mincr3 primers. (Table 3 and Appendix II). DNA extracts from *L. major* and *L. tropica* culture were used as PCR positive controls. Deionised water was used as negative control in a reaction well; 5 µL of DNA template was added to the PCR mix.

The PCR runs were done using the Gene Amp PCR system 9700 (Applied Biosystems).

Three sets of primers were used; Mincr2/Mincr3, L5.8S/ LITSR and R222/R333. The Mincr2/Mincr3 primers are derived from the conserved region of *leishmania* species minicircle DNA of the parasite kinetoplast and generate a band of 120 base pairs. L5.8S/LITSR and R222/R333 both amplifies a range of 300-350 base pair and 603 base pair respectively of the ITS 18SrRNA conserved region of *leishmania*. Cross contamination was monitored during sample extraction and PCR.

3.6 Agarose gel electrophoresis

100 ml of 1X Tris Acetate EDTA (TAE) buffer was measured into a conical flask. Two grams of agarose powder was weighed and added to the buffer. The suspension was heated

(80˚C-90˚C) in a microwave to get a uniform solution. The solution was allowed to cool and transferred into a gel cast system with combs having appropriate protrutrions inserted into it to form wells. The gel was left for about 20 min to solidify. The cast gel was then transferred into a gel tank filled with 1XTAE buffer. 10µL of amplicons were loaded into wells of the 2% agarose gel for electrophoresis and then allowed to range for 45 minutes. The gel was stained in a 3X GelRed 50ml staining solution after the electrophoresis, for about 30 minutes. The stained gel was viewed over a UV Transilluminator (Model TM-20) and photographed.

3.7 Molecular Identification of *Leishmania* species Using Restriction Fragment Length Polymorphism (RFLP)

The PCR product obtained from the above procedure was used as template in a restriction fragment length polymorphism (RFLP) analysis for distinguishing between Old and New world agents of CL in Africa; 16µL of the PCR product was placed into a clean 1.5ml micro-centrifuge tube. In the same tube 2µL of 10x restriction buffer and 1 µL of *Hae*III (Sigma-Aldrich, St. Louis, MO) restriction enzyme was added. The mixture was incubated at 37ºC for 3 hours. The digested product was run on a 2% agarose gel to visualize the different band size fragments with gelRed over a uv-light.

The expected band sizes are 171 and 172 bp (Schönian *et al.*, 2003).

3.8 Data Analysis

All the laboratory studies were documented using Microsoft word, Excel 2007 and PoolScreen 2.0. Software Version 2.0.1 January 2002. The PoolScreening software provides estimates of *leishmania* infection in the vector population based upon a selected confidence interval, pool size, the number of pools examined and the number of negative

pools (Katholi & Unnasch, 2006). The frequencies of infected sand flies were determined using 95% confidence interval.

CHAPTER FOUR

RESULTS

4.1 Sand fly Collection

A total of 5,888 sand flies were collected during the five (5) month sampling period from the three study communities. Females constituted 71.7% (4,219/5,888) of the flies collected, whereas males constituted 28.3% (1,669/5,888), (Figure 4). February recorded the largest number of sand flies 1,647, followed by November 1,406 and January 1,350, October and December recorded the least numbers, 785 and 556 respectively (Figure 5).

4.2 Sand fly Distribution in Communities

S. africana were the most predominant species distributed across all three communities. *S. africana* comprised 1666/2607 (65.3%), 292/471 (69.8%) and 796/1141 (69.8%) from Dodome Dogblome, Dodome Awuiasu and Lume-Atsiame respectively. The second highest distributed species were *S. simillima*, 349/2607 (13.4%) in Dodome Dogblome, 24/471 (5.1%) in Dodome Awuiasu and 80/1141 (7.0%) in Lume-Atsiame accordingly. The least distributed species amongst the communities were *P. rodhaini*, *S. inermis* and *S. squamipleuris* (Table 1).

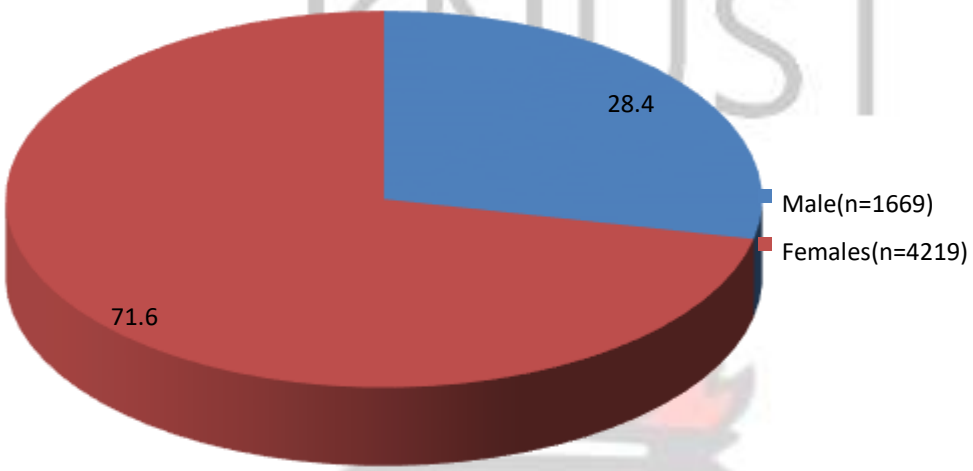

Figure 4: Percentage of male and female sand flies

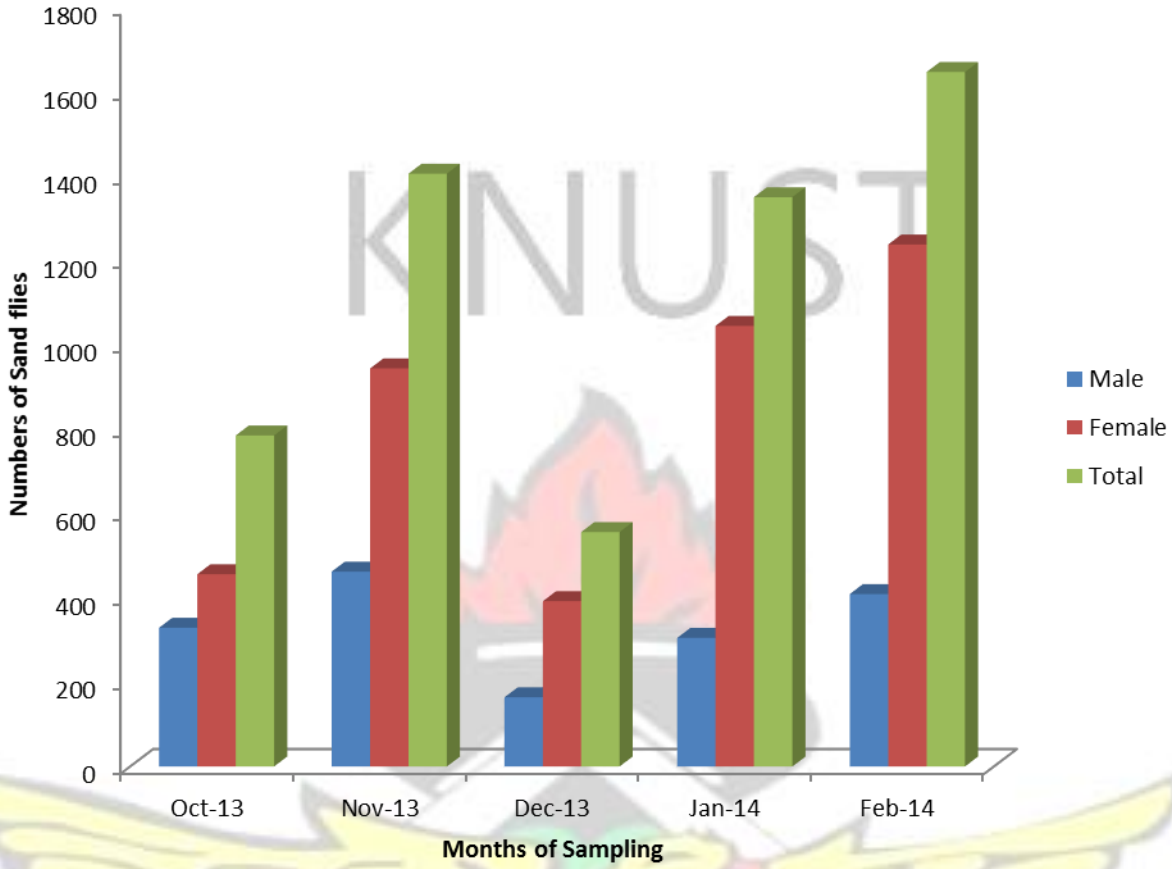

Figure 5: Monthly distribution of male and female sand flies Table 1: Distribution of Female Sand flies in Villages

	Villages			
Sand fly Species	Dodome-Dogblome (%)	Dodome-Awuiasu (%)	Lume-Atsiame (%)	Total (%)
P. rodhaini	7(0.3)	4(0.8)	9(0.8)	20(0.5)
S. ghesqueiri	76(2.9)	25(5.3)	29(2.5)	130(3.1)
S.inermis	2(0.1)	1(0.2)	0(0.0)	3(0.1)
S. squamipleuris	0.(0.0)	0(0.0)	1(0.1)	1(0.0)
S. collarti	105(4.0)	11(2.3)	16(1.4)	132(3.1)
S. dureni	40(1.5)	17(3.6)	15(1.3)	72(1.7)
S. ingrami	172(6.6)	16(3.4)	84(7.4)	272(6.4)
S. africana	1666(63.9)	292(62.0)	796(69.8)	2754(65.3)
S. antennata	109(4.2)	40(8.5)	55(4.8)	204(4.8)
S. buxtoni	16(0.6)	3(0.6)	15(1.3)	34(0.8)

S. schwetzi	56(2.1)	36(7.6)	41(3.6)	133(3.2)
S. hamoni	9(0.3)	2(0.4)	0(0.0)	11(0.3)
S. simillima	349(13.4)	24(5.1)	80(7.0)	453(10.7)

*Numbers in parenthesis represent percentages

4.3 Sand fly Identification and Composition

A total of 4,219 female sand flies were collected and identified using taxonomic keys from the three communities (Abonnenc., 1972). After identification, 20(0.5%) female sandflies were identified to be from the genus *Phlebotomus*, the rest of the sandflies were from the genus *Sergentomyia.* It was observed that a total of 2754 (65.30%) were *S. africana*, the highest recorded in the collection. *P. rodhaini, S. inermis* and *S. squamipleuris* were the least recorded species representing 20(0.5%), 3(0.1%) and 1(0.0%) respectively (Figure 6)

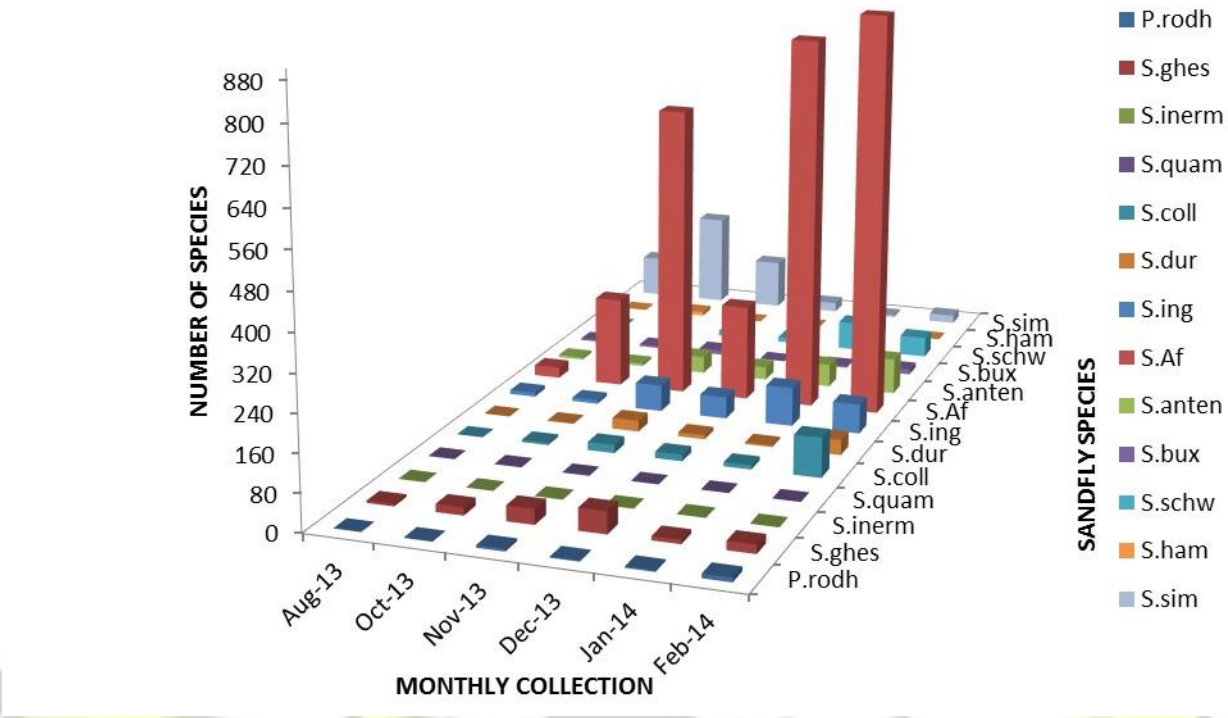

Figure 6: Individual Composition of Sand fly Species

4.4 Seasonal Distribution of Sand flies

The dry season recorded the highest abundance of female sand flies. *S. africana* were the dorminant species, 1896/2675 (70.9%) in the dry season as compared to 858/1544 (55.60%) in the wet season. *S. simillima* was the only species with high numbers, 410/1544 (26.60%) in the wet season compared to 43/2675 (1.60%) in the dry season (Appendix III and Figure 7)

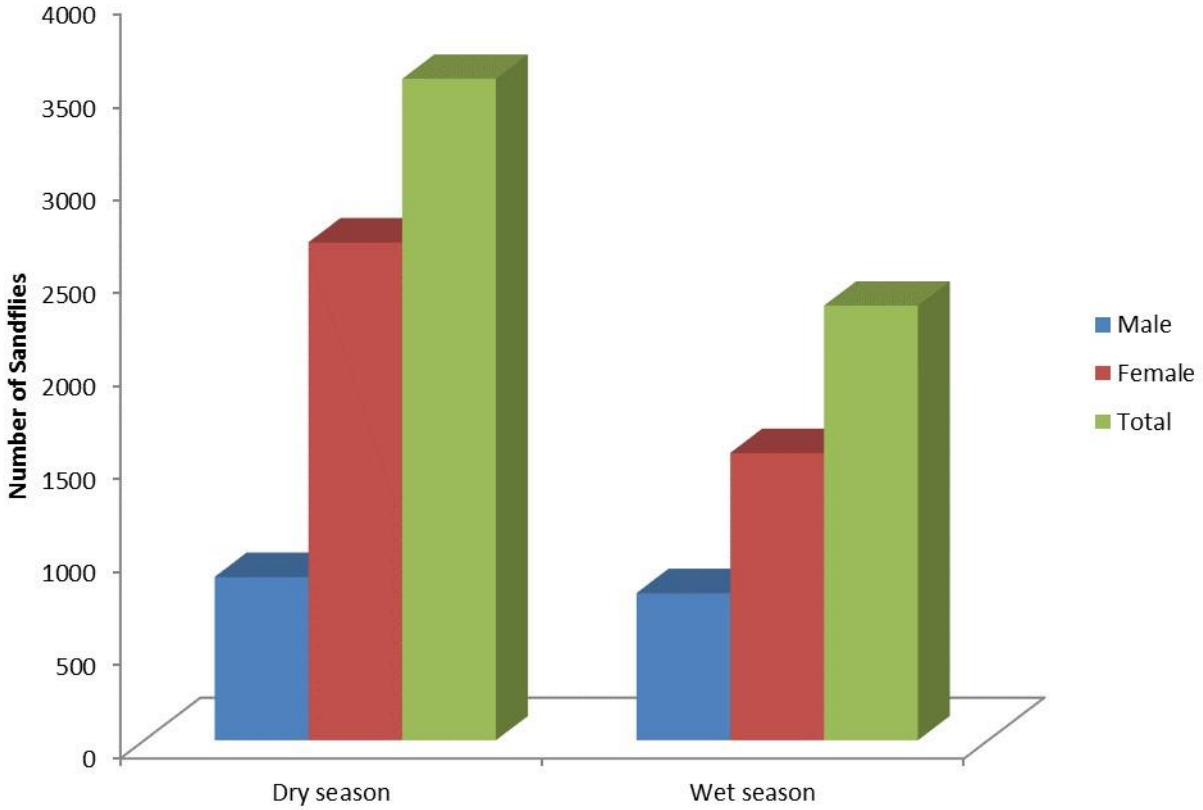

Figure 7: Seasonal distribution of Male and Female sand flies

Several morphorlogical characters were used in the identification of sandflies but only key characters such as the pharynx, cibarium and spermathecae were considered during the identification process. The pharynx consists of three plates enclosing a cavity which is triangular in cross-section. The base of the pharynx can have spicules, scales, teeth, tooth lines, or be unarmed. All these spicules and teeth together are named the pharyngeal armature. The shape of the pharynx is usually bottle or lamp-glass shaped which varies considerably between species as to the size, shape, and distribution of the teeth on the

posterior portion of the pharynx. Typically, *Phlebotomus* are usually larger bodied with lighter intergument whiles *Sergentomyia* are smaller with a darker cuticle. In *Phlebotomus* the Cibarium is without teeth and a pigment patch, whereas in *Sergentomyia* the cibarium has one or more rows of teeth and the pigment patch is usually present.

Phlebotomus rodhaini

Female *Phlebotomus rodhaini* lack the presence of rows of teeth in its cibarial armature with the shape of the posterior pharynx weakly distended and a strong pharyngeal armature (Figure 8). The spermathecae duct are separated from each other with the spermathecae tubular in shape. The head of the spermathecae is rounded and sessile (Figure 8).

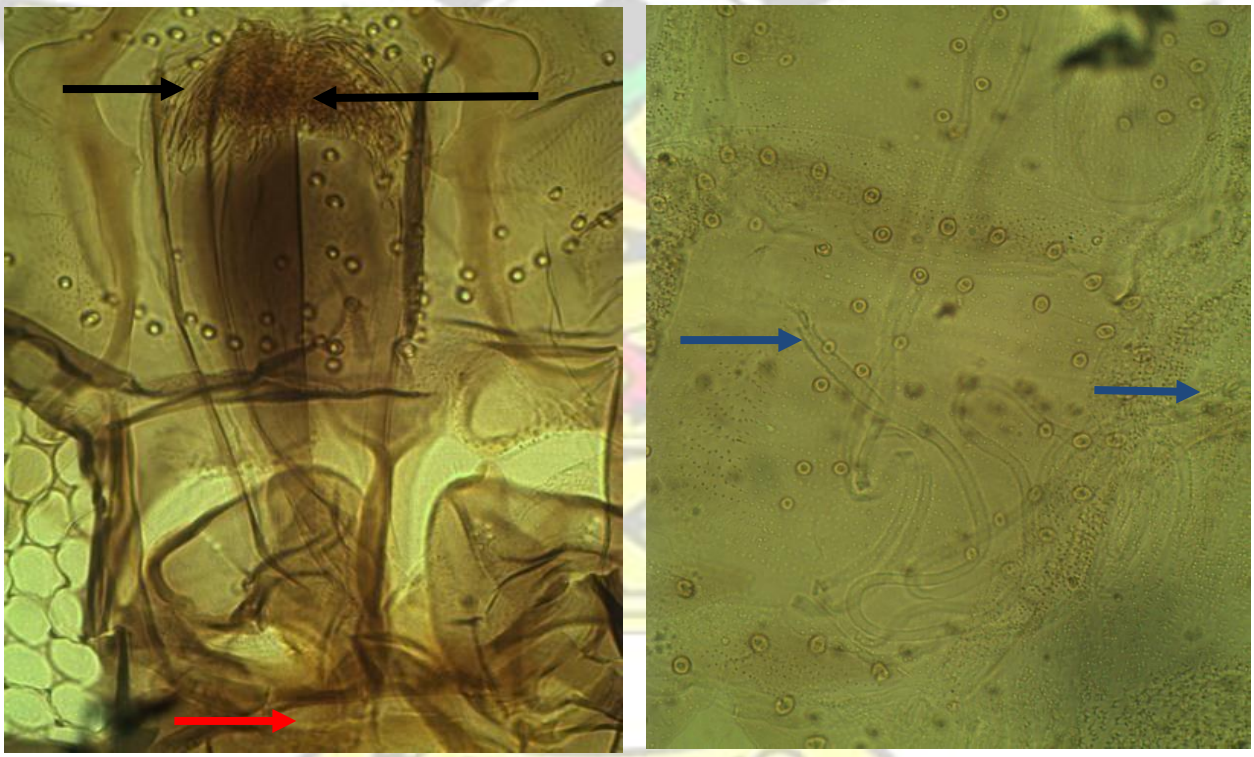

Figure 8. Cibarium (red) and pharyngeal (black) armature; Spermatheca (blue) of *P. rodhaini*

Sergentomyia africana

Female *S. africana* has a unique hour-glass shaped pharynx and a well-developed set of teeth about 55 to 79 in the cibarium armature on a posterior concave pigment patch (Figure 9). It has a weakly armed posterior pharyngeal armature. The spermatheca duct of *S. africana* is separate with the spermathecae tubular in shape and double walled. The inner wall of *S.africana* has a distinct wave-like lining (Figure 9).

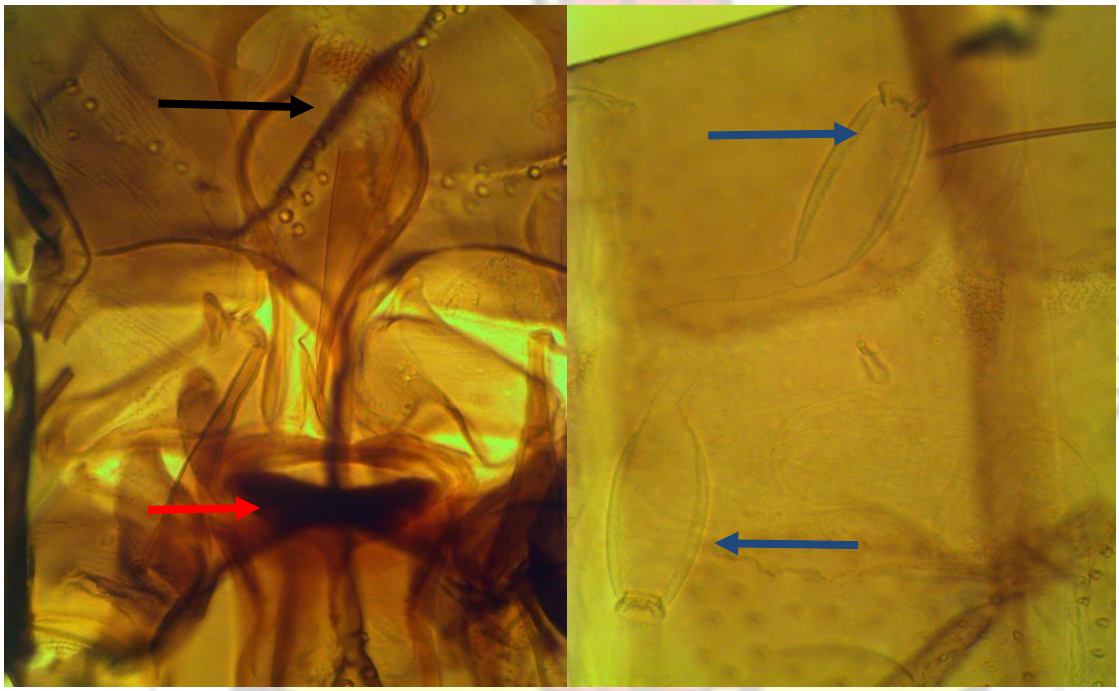

Figure 9. Cibarium (red) and pharyngeal (black) armature; Spermatheca (blue) of *S. africana* .

Sergentomyia ingrami

S. ingrami has a narrow shaped posterior pharynx (Figure 10) with a strong pharyngeal armature. The cibarium of *S. ingrami* is well developed with two sets of teeth. The first row of teeth numbers about 20 or 21, and appears in a median tightly arranged set and a

lateral set different from the former. Two rows of reduced teeth called denticles can be found below the first set of teeth. The spermatheca of *S. ingrami* is an elongated capsule with the head of the spermatheca sessile and inverted (Figure 10).

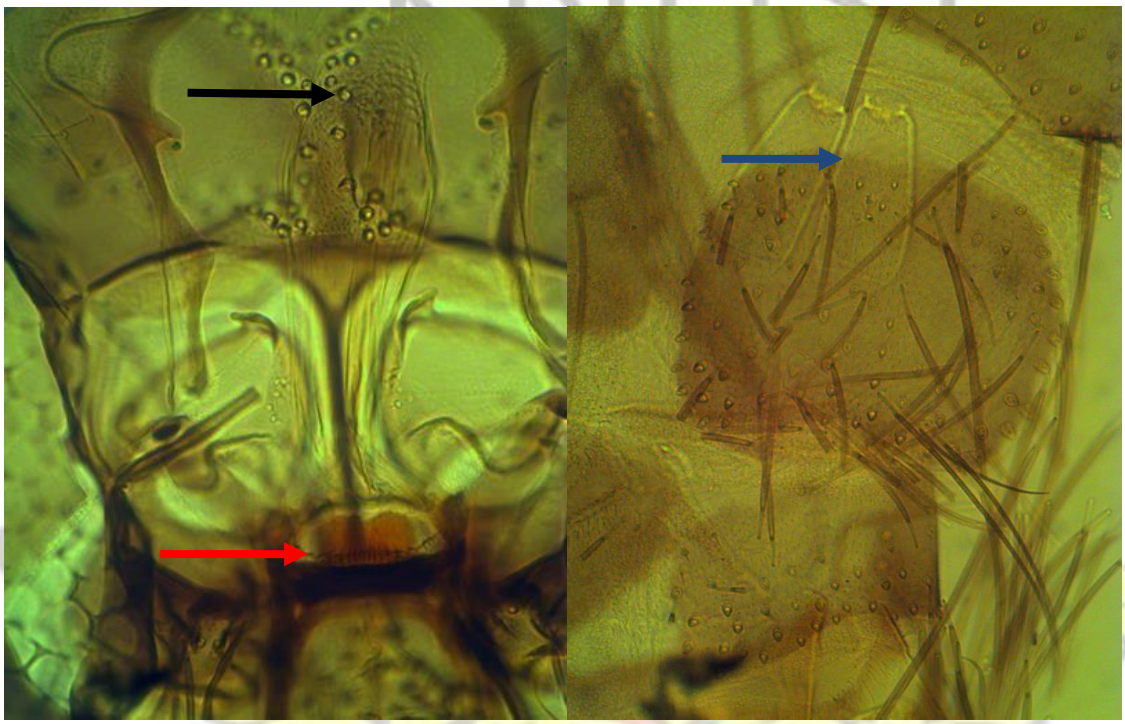

Figure 10. Cibarium (red) and pharyngeal (black) armature; Spermatheca (blue) of *S. ingrami*

Sergentomyia collarti

S. collarti has a characteristically even-spaced fence-like cibarial teeth alignment. The number of cibarial teeth ranges from 11-20. *S. collarti* has a narrow posterior pharynx and a rudimentary pharyngeal armature (Figure 11). The spermatheca is elongated tubular capsule with a sessile head. The base of the individual spermatheca duct is separated (Figure 11).

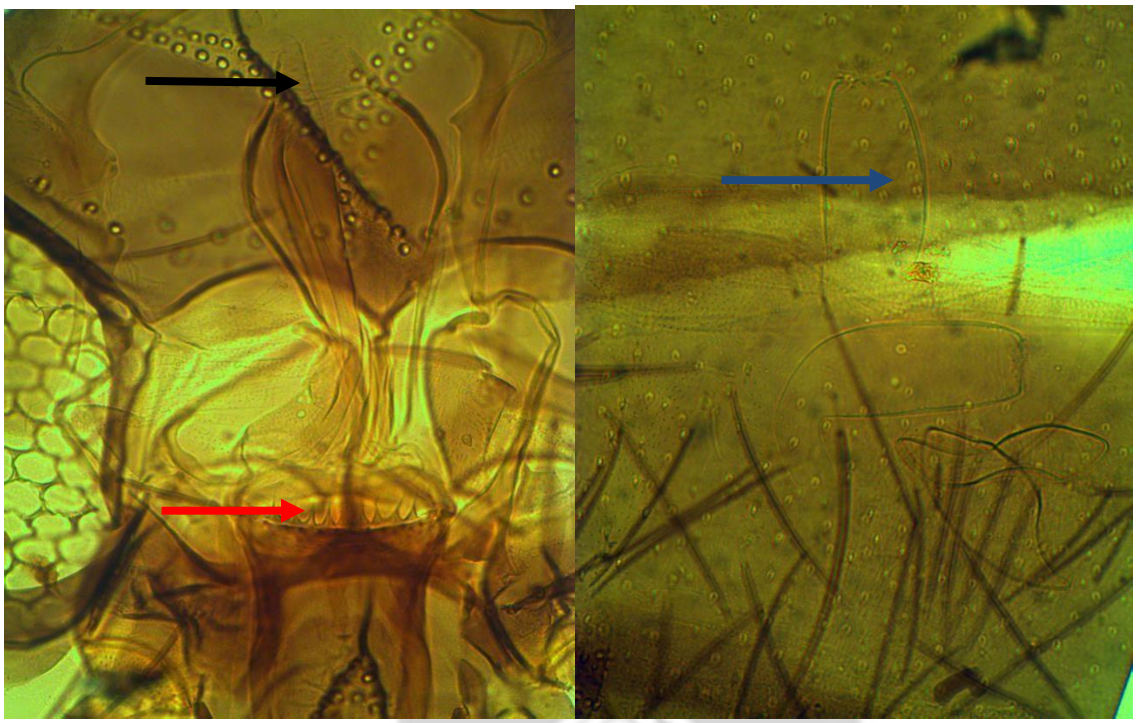

Figure 11. Cibarium (red) and pharyngeal (black) armature; Spermatheca (blue) of *S. collarti*.

Sergentomyia buxtoni

S. buxtoni has a peculiar large mushroom shaped cibarium patch in the middle of the cibarium. The cibarium armature is well developed with sharp pointed teeth aligned in a fence-like pattern. The pharyngeal armature is armed with a typical diamond shape. *S. buxtoni* have a wide tubular spermatheca with the spermatheca duct joined at the base (Figure 12).

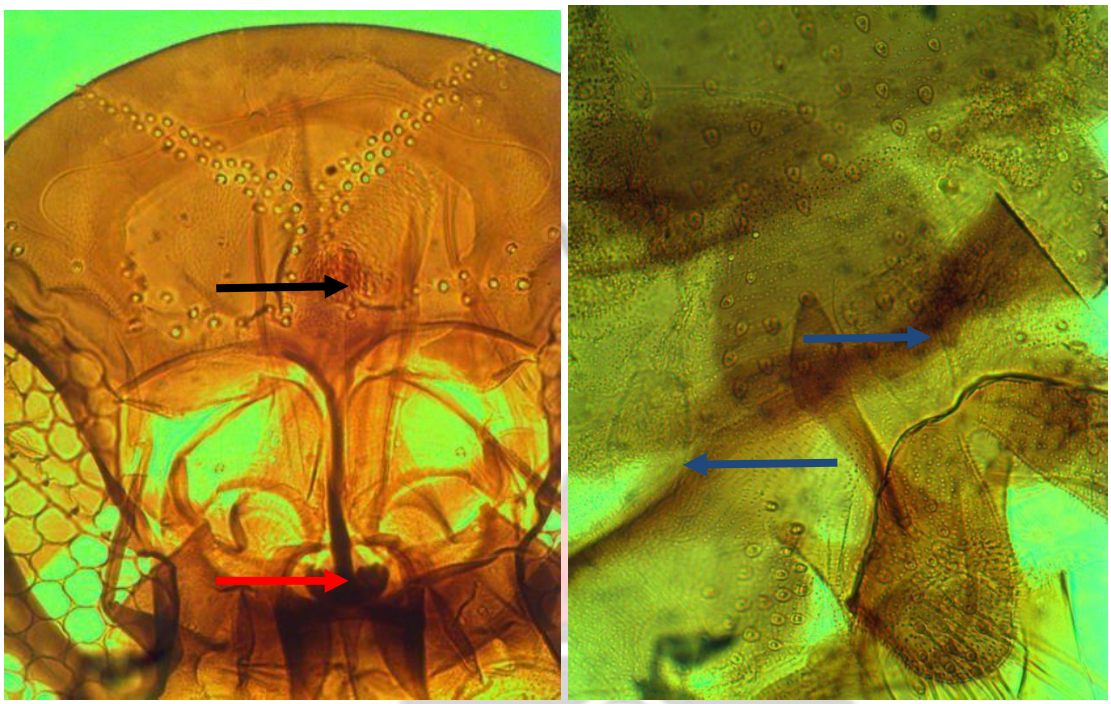

Figure. 12. Cibarium(red) and pharyngeal (black) amature; Spermatheca (blue) of *S. buxtoni*

Sergentomyia schwetzi

Female *S. schwetzi* is characterized by a very close and tightly packed median teeth bordered by large row of lateral teeth. The pharynx is distended posteriorly and strong armed with pharyngeal spines. The spermatheca of *S. schwetzi* is tubular and joined at the base (Figure 13).

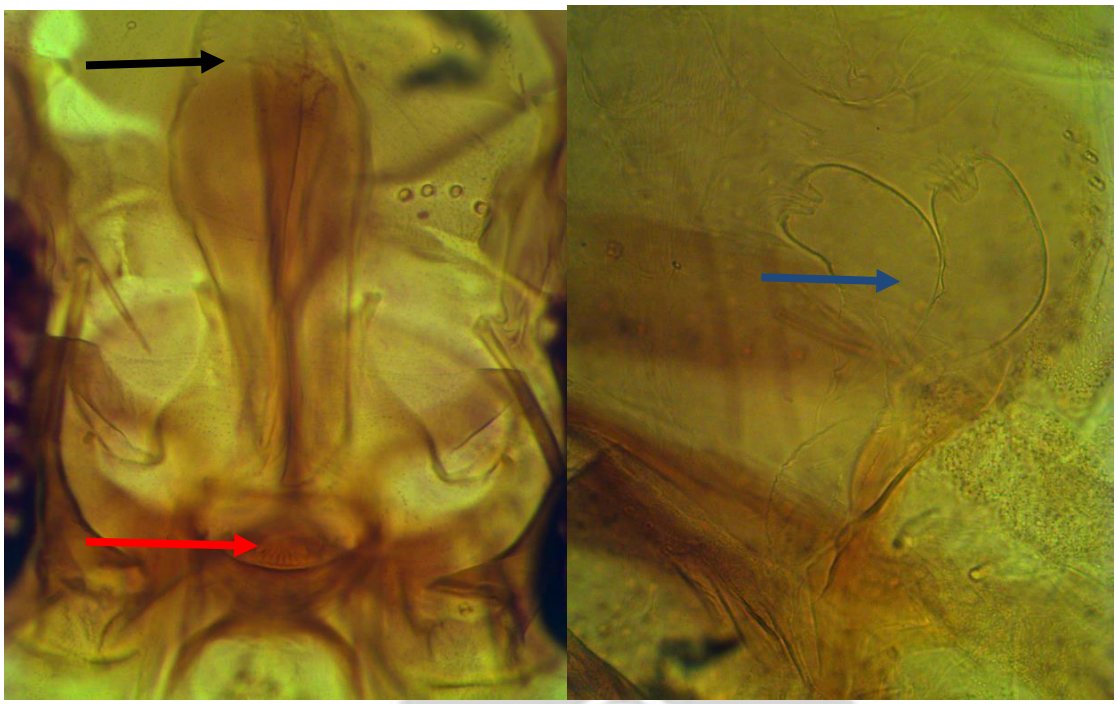

Figure 13. Cibarium(red) and pharyngeal (black) armature; Spermatheca (blue) of *S. schwetzi*

Sergentomyia simillima

S. simillima has a characteristic heart shaped posterior pharynx with strong pharyngeal spines and scales. The teeth of *S.simillima* are equal sized, pointed and fence-like. The row of teeth ranges from 55-72. *S. simillima* has a tubular spermatheca with ducts separated at the base **(**Figure 14).

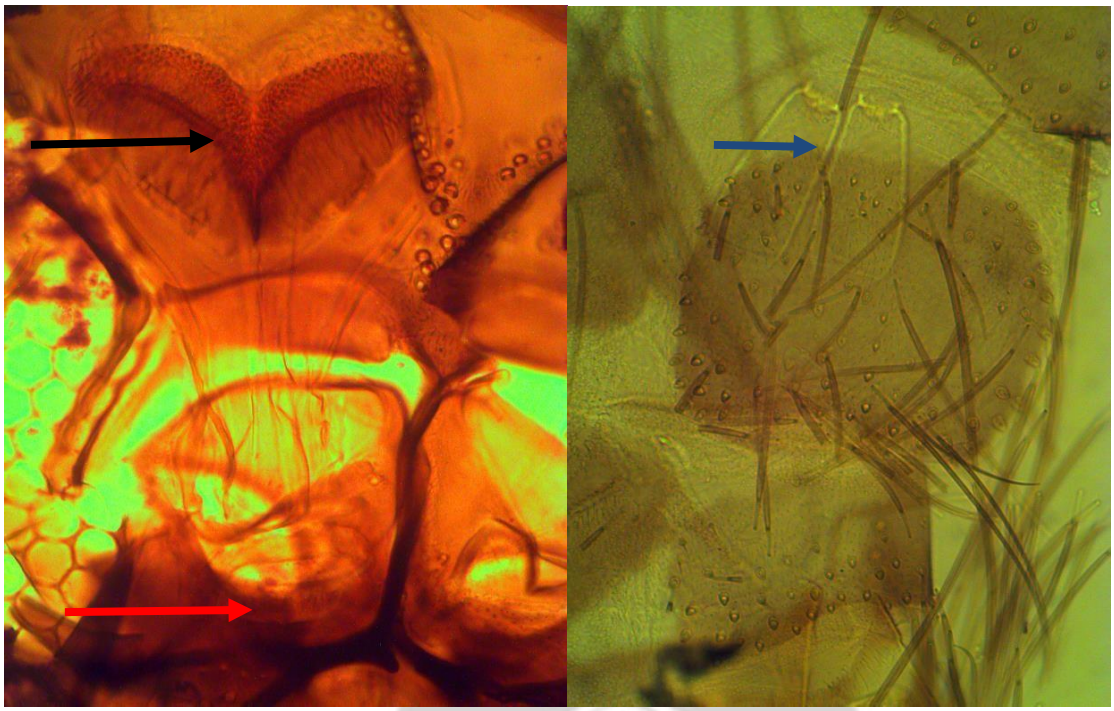

Figure 14. Cibarium (red) and pharyngeal (black) armature; Spermatheca (blue) of *S. simillima*

Sergentomyia harmoni

S. harmoni has a distinct broad heart shaped pharynx (Figure 15).

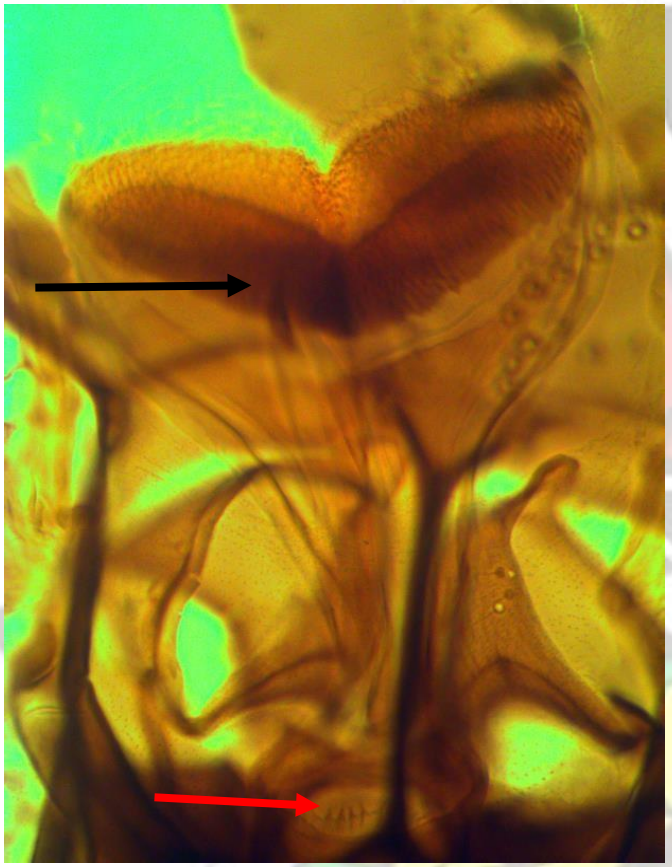

Figure 15. Cibarium (red) and pharyngeal Armature (black) of *S. hamoni*

4.5 Molecular Identification of *Leishmania*

The dissected female sand flies were grouped into 25 per tube for each species for DNA extraction. The number of some of the sand flies species obtained during the study collection was not enough to give a pool size of 25 and therefore resulted in pools of less than 25 per tube per species. The extracted DNA was used for the detection of *leishmania*. (Appendix I).

4.6 Detection of *Leishmania* Using Conventional Polymerase Chain Reaction

Amongst the primers used the Mincr2/Mincr3 primer amplified *leishmania* infection in a single incomplete pool out of the two hundred and ninety five (295) sand fly pools (**Plate 3**). The L5.8S/LITSR and R222/R333 primers could not identify any *leishmania* infection in the sand fly pools screened. Cross contamination was monitored during sample extraction and PCR. All were negative.

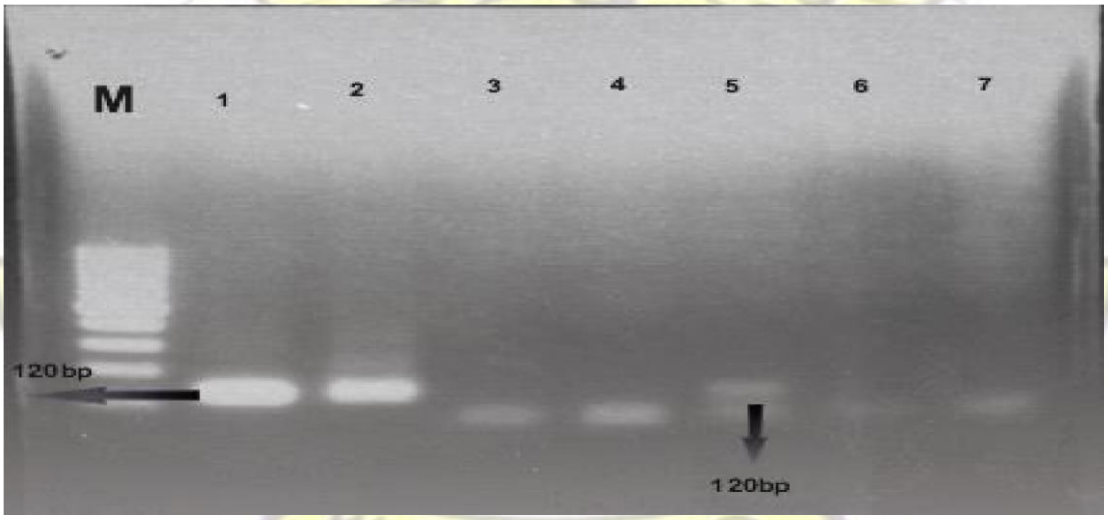

Plate 3: PCR gel image showing the result of positive leishmania sp. infection in S. africana. (Lane M, 100bp Marker; lane 1 and 2, positive controls- L. major and L. tropica; lane 3 and 4, Sergentomyia sp.; lane 5, S. africana and lane 6, negative control.)

4.7 Infection Rate in Sand flies

The infection rate is the ratio of the number of sand flies with *Leishmania* parasite to the total number of sand flies examined. This is usually expressed as a percentage.

The *Leishmania* specific minicircle DNA mincr2/minr3 primer confirmed that a single pool containing a total of seven (7) *S. africana* were infected with *Leishmania sp.* out of a total of 122 pools of *S. africana* screened. The number of female *S. africana* in a pool varied from 1 to 25. According to the minimum and maximum infection rates, the prevalence of *Leishmania* infection in female *S. africana* was 0.0384% (95% CI) with a lower limit of 0.00119% and an upper limit of 0.197%. *S. hamoni* was the least screened. No infection was detected in the other species of sand flies screened.

Table: 2 Infection Rates of *Leishmania sp*. in female sand flies

Sandfly Speicies	Number of Pools	Pool size	Pool size	Number Positive	Minimum Infection rate (%)	Lower limit (%)	Upper Maximum Limit (%)
S. africana	122	1	25	1	0.0384	0.00119	0.197
P. rodhaini	8	1	4	0	0	0	0
S. antennata	22	1	25	0	0	0	0
S. buxtoni	8	1	6	0	0	0	0
S. collarti	15	1	25	0	0	0	0
S. dureni	16	1	9	0	0	0	0
S. ghesquierei	17	1	25	0	0	0	0
S. hamoni	5	1	6	0	0	0	0
S. ingrami	27	1	25	0	0	0	0
S. schwetzi	21	1	25	0	0	0	0

S. similima	33	1	25	0	0	0	0

CHAPTER FIVE

Discussion

The control of leishmaniasis in areas of endemicity requires a thorough knowledge of *leishmania* ecology and epidemiology. There is a major problem for epidemiologist in both the identification of the reservoir host and in the detection of vectors. Finding naturally infected sandflies is essential in identifying the species which serves as a vector of *leishmania*. Despite the increasing incidence of cutaneous leishmaniasis disease worldwide, very little attention has been given to the disease because of the selflimiting nature of the disease. Prevention and control strategies have mainly aimed at treatment of the disease rather than the elimination of reservoir or the reduction of human-vector contact.(Alexander and Maroli, 2003). As a result CL is one of the most neglected tropical diseases in terms of the few tools available for control and the lack of clear cut criteria for methods of control (Alvar, Yactayo, & Bern, 2006). In West Africa, because very little attention has been given to leishmaniasis, information on the disease is very limited. Though there have been advocacy for the control of *leishmania* vectors with insecticides and its potential to reduce incidence of the disease, chemotherapy is only administered to individuals showing symptoms of the disease with a rather poor and disorganized control efforts (Boakye *et al.*, 2005). For most countries endemic to leishmaniasis, vector control seems unlikely to become a major component of disease control except where sand fly distribution overlaps with other vectors or where the use of personal protection measures can be more widely emphasized (Reithinger *et al.*, 2007). Usually, the vectorial capacity of sand fly species is epidemiologically important in a leishmaniasis focus when the species is abundant and proved anthropophilic behavior. The suspicion is strengthened when the same sandfly species is found infected with promastigotes of the same

leishmania parasite species isolated within humans and potential animal reservoir hosts. Transmission of *leishmania* by the vector to humans, however is demonstrated experimentally by the bite of a sand fly (Killick-Kendrick, 1990).

In this study, to determine the potential vectors of *leishmania*, a total of 11 different sand fly species were trapped using CDC light traps. The eleven sandfly species collected in this study had previously been described out of 17 sand fly species that were collected within the Volta Region of Ghana (Fryauff *et al.*, 2006). About 95% of the sand flies collected were from the genus *Sergentomyia*, the distribution of sand fly species in this present study was similar to reports on the distribution of *Phlebotomus* sandflies in vector studies conducted in West Africa. Previous studies have reported 99.5% species composition by Fryauff *et al.* 2006, in the Ho district of Ghana. These may suggest the involvement of the genus in the transmission of cutaneous leishmanisasis. In the present study, *S. africana* (65.5%) was the predominantly identified species which correlates with previous vector studies by (Fryauff *et. al.*, 2006) in the Ho district. Most of the *P. rodhaini* is considered a suspected vector in the transmission of *L. donovani* in Sudan (Elnaiem *et al*., 2011). Despite the wide distribution of *P. rodhaini* in most leishmaniasis endemic foci, it is considered a rare species and therefore it's ignored as a possible vector of leishmaniasis parasites. In this study, a 0.5% of *P. rodhaini* was identified in the species composition of the collections. Though *P. rodhaini* was collected in small numbers it is a known vector for *L. major* elsewhere in West Africa (Anderson et al., 2011). Even though *P. rodhaini* has not been implicated as a vector this observation will have an important implication in the understanding of the epidemiology of leishmaniasis in this endemic area.

In this study, most female sand flies were collected in the dry season as compared to the wet season. The seasonality is a likely determinant of the number of each species to be caught in the various communities. The large numbers of sand flies caught in the dry season was due to the breeding ecology of sand flies in dry, arid and humid environments (Killick-Kendrick, 1990). This data suggest evidence of the possible CL transmission in the dry months in most of the endemic communities in the Volta Region of Ghana. Humid weather conditions compel locals to dress half naked e.g. on farms and bedtime, exposing their upper bodies to the bite of female sand flies seeking blood meal. In the Volta Region, CL lesions are usually observed starting from the month of June until end of year (Personal communication and Observation).

In this study, the detection of *leishmania* DNA in *S. africana* goes to question the vectorial capacity of sand flies from the genus *Sergentomyia* and their role in leishmaniasis transmission. This finding is consistent with previous studies conducted in the same region (Nzelu *et al.,* 2014). They detected *leishmania* DNA in three species of *sergentomyia* sandflies, *S. hamoni*, *S. ingrami* and *S. africana*. When the *leishmania* amplicons were subjected to restriction fragment length polymorphism analysis using *HaeIII*, the restriction enzyme revealed fragments characteristic of *Leishmania major*.

Sergentomyia species were rarely considered as vectors of medically important *leishmania* since they are known to prefer animal blood and transmit *sauroleishmania* among lizards(Kanjanopas *et al.,* 2013). However, in some studies, *Sergentomyia schwetzi*, *Sergentomyia gamhani* and *Sintonius clydei* bite humans (Abonnenc, 1972). Others studies have reported the presence *leishmania* DNA in other *Sergentomyia* species and considered as potential vectors of *leishmania*. These include *S. sintoni*, *S. munila*, *S.*

darling, *S. babu*, *S. garnhami* and *S. gemmea*. The vectorial role of dominant sand fly species in leishmaniasis endemic areas is epidemiologically suspected as a vector (Berdjane-Brouk *et al.*, 2012). This suspicion is strengthened when the same sand fly is found infected with the same parasite as the one found in man in the same area. The suspected vector's role is confirmed when the transmission of *leishmania* to humans is experimentally demonstrated by the bite of the sand fly (Killick-Kendrick, 1990).

PCR methods are available for the diagnosis and identification of leishmaniasis from different types of human and animal samples. Sensitivity of different PCR assays rely on the amplification of different repeated and polymorphic DNA sequences such as ribosomal internal transcribed spacer 1(ITS1), cysteine protease B, kinetoplast DNA minicircles, surface glycoprotein 63, heat shock protein 70, mini-exon and microsatellites (Richard Reithinger et al., 2007; Schönian et al., 2003). In this study, three target specific primers L5.8S/ LITSR, R222/R333 and Mincr2/Mincr3, were explored in the determination of *leishmania* DNA in sand flies in a cutaneous *leishmania* focus. An infection rate of 0.0384 %(CI=95%) was determined in *S.africana* using minicircle primers. This was detected in Sand fly collections in Dodome Dogblome. None of the ITS primers detected any infections in the sand fly pools. The ability of the minicircle primers to amplify *leishmania* DNA in sand flies makes it useful in areas of endemicity where unidentified species and identified *Leishmania* species are present.

Detecting *leishmania* DNA does not imply that a sand fly species is a vector since the assay used cannot distinguish between the presence of *Leishmania* amastigotes from an infected blood meal and promastigotes. However, future field and laboratory work will be

required to study the development of *leishmania sp*. in *S. africana* and determine the efficiency of the vector in transmission of CL in endemic communities.

CHAPTER SIX

CONCLUSION AND RECOMMENDATION

6.1 Conclusion

These studies show the diversity of sand flies and also suggest that *S. africana* might be a vector of *Leishmania sp*. in Ghana. The vector role of sand fly species in leishmaniasis focus epidemiologically suspected when the species is predominant. This suspicion is strengthened when the same sand fly is found infected with the same leishmanial parasite

as found in man in the same area. Although man may accidentally be bitten by *S. africana*, it is unlikely that this sand fly species plays a significant role in transmitting the parasite to humans. Definitive conclusions that *Sergentomyia* species are vectors of human *Leishmania* species require confirmation by demonstrating experimentally their capacity to transmit *Leishmania* parasites to mammals. The disease is under reported in Ghana and most parts of West Africa because of limited local resource. The control of leishmaniasis in endemic areas requires understanding of *Leishmania* ecology and epidermiology of the disease. Finding naturally infected sandflies is important in identifying the species of sand fly as a vector of *leishmania* in studying infection rates in endemic communities.

6.2 Recommendations

Leishmaniasis continues to be an emerging disease affecting the poorest of the poor if not controlled. In attempting to incriminate the sand fly vector transmitting cutaneous leishmaniasis in the Volta Region, the following should be under taken;

1. there should be a continuous entomological surveillance of the disease
2. Sand flies collected on the field should be dissected on the field for the presence of *leishmania* parasites.
3. Blood meals, host-preference and breeding habitats of sand flies collected from the field should be analyzed for possible reservoir studies.
4. The vector competence of *S. africana* should be further investigated if it plays a role in disease transmission.

REFERENCES

Abonnenc., E. (1972). Les phlébotomes de la région éthiopienne (Diptera, Psychodidae). *Paris : ORSTOM, 1972, 289 P. Multigr. (Mémoires ORSTOM ; 55)*.

Alexander, B., & Maroli, M. (2003). Control of phlebotomine sandflies. *Medical and Veterinary Entomology, 17*, 1–18.

Alten, B. (2010). Speciation and Dispersion Hypotheses of Phlebotomine Sandflies of the subgenus Paraphlebotomus (Diptera : Psychodidae): The Case in Turkey, *38*(3), 229–246.

Alvar, J., Yactayo, S., & Bern, C. (2006). Leishmaniasis and poverty. *Trends in Parasitology*, *22*(12), 552–557.

Analysis, G. (2010). Leishmaniasis control in eastern africa : past and present efforts and future needs Situation and Gap Analysis, (9).

Anderson, J. M., Samake, S., Jaramillo-Gutierrez, G., Sissoko, I., Coulibaly, C. a., Traoré, B., Kamhawi, S. (2011). Seasonality and prevalence of Leishmania major infection in Phlebotomus duboscqi neveu-lemaire from two neighboring villages in Central Mali. *PLoS Neglected Tropical Diseases*, *5*(5).

Andrade, B. B., de Oliveira, C. I., Brodskyn, C. I., Barral, A., & Barral-Netto, M. (2007). Role of sand fly saliva in human and experimental leishmaniasis: current insights. *Scandinavian Journal of Immunology*, *66*(2-3), 122–7.

Assimina, Z., Charilaos, K., & Fotoula, B. (2008). Leishmaniasis: an overlooked public health concern., *4*, 196–205.

Azevedo, E., Oliveira, L. T., Castro Lima, A. K., Terra, R., Dutra, P. M. L., & Salerno, V. P. (2012). Interactions between Leishmania braziliensis and Macrophages Are Dependent on the Cytoskeleton and Myosin Va. *Journal of Parasitology Research*, *2012*, 275436.

Bañuls, A.-L., Hide, M., & Prugnolle, F. (2007). Leishmania and the leishmaniases: a parasite genetic update and advances in taxonomy, epidemiology and pathogenicity in humans. *Advances in Parasitology*, *64*, 1–109.

Bari, A. U., & Rahman, S. B. (2008). Review Article Cutaneous leishmaniasis : an overview of parasitology and host-parasite-vector inter relationship, 42–48.

Barnett, H. C. (1962). The incrimination of arthropods as vectors of disease. *Int. Congr. Entomol. AND (Vienna), Proc. XI*(1), 341–345.

Bates, P. a. (2007). Transmission of Leishmania metacyclic promastigotes by phlebotomine sand flies. *International Journal for Parasitology*, *37*(10), 1097–106.

Beerntsen, B. T., James, A. A., & Christensen, B. M. (2000). Genetics of mosquito vector competence. *Microbiology and Molecular Biology Reviews : MMBR*, *64*(1), 115–37.

Bell, A. S., & Ranford-Cartwright, L. C. (2002). Real-time quantitative PCR in parasitology. *Trends in Parasitology*, *18*(8), 337–42.

Berdjane-Brouk, Z., Koné, A. K., Djimdé, A. a., Charrel, R. N., Ravel, C., Delaunay, P., Izri, A. (2012). First detection of Leishmania major DNA in Sergentomyia (Spelaeomyia) darlingi from cutaneous leishmaniasis foci in Mali. *PLoS ONE*, *7*(1), 1–5.

Bernier, U. R., Allan, S. A., Quinn, B. P., Kline, D. L., Barnard, D. R., & Clark, G. G. (2008). Volatile compounds from the integument of White Leghorn Chickens (Gallus gallus domesticus L.): Candidate attractants of ornithophilic mosquito species*. *Journal of Separation Science*, *31*(6-7), 1092–1099.

Boakye, D. a, Wilson, M., & Kweku, M. (2005). A review of leishmaniasis in west Africa. *Ghana Medical Journal*, *39*(3), 94–7.

Bossolasco, S., Gaiera, G., Olchini, D., Gulletta, M., Martello, L., Bestetti, A., … Cinque, P. (2003). Real-time PCR assay for clinical management of human immunodeficiency virus-infected patients with visceral leishmaniasis. *Journal of Clinical Microbiology*, *41*(11), 5080–4.

Brandão-Filho, S. P., Brito, M. E., Carvalho, F. G., Ishikawa, E. A., Cupolillo, E., Floeter-Winter, L., & Shaw, J. J. (2003). Wild and synanthropic hosts of Leishmania (Viannia) braziliensis in the endemic cutaneous leishmaniasis locality of Amaraji, Pernambuco State, Brazil. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, *97*(3), 291–6.

Bray, D. P., & Hamilton, J. G. C. (2007). Host odor synergizes attraction of virgin female Lutzomyia longipalpis (Diptera: Psychodidae). *Journal of Medical Entomology*, *44*(5), 779–87.

Campbell-Lendrum, D. H., Pinto, M. C., Brandão-Filho, S. P., de Souza, A. A., Ready, P. D., & Davies, C. R. (1999). Experimental comparison of anthropophily between geographically dispersed populations of Lutzomyia whitmani (Diptera: Psychodidae). *Medical and Veterinary Entomology*, *13*(3), 299–309.

Choi, C. M., & Lerner, E. A. (2001). Leishmaniasis as an emerging infection. *The Journal of Investigative Dermatology. Symposium Proceedings / the Society for Investigative Dermatology, Inc. [and] European Society for Dermatological Research*, *6*(3), 175–82.

Dantas-torres, F. (2007). The role of dogs as reservoirs of Leishmania parasites , with emphasis on Leishmania (Leishmania) infantum and Leishmania (Viannia) braziliensis, *149*, 139–146.

Davies, C. R., Lane, R. R., Villaseca, P., Pyke, S., Campos, P., & Llanos-Cuentas, A. (1995). The relationship between CDC light-trap and human-bait catches of endophagic sandflies (Diptera: Psychodidae) in the Peruvian Andes. *Medical and Veterinary Entomology*, *9*(3), 241–8.

Dawit, G., Girma, Z., & Simenew, K. (2012). A Review on Biology , Epidemiology and Public Health Significance of Leishmaniasis, *3*(3), 43–53.

Desjeux, P. (2001). The increase in risk factors for leishmaniasis worldwide. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, *95*(3), 239–43.

Desjeux, P. (2004). Leishmaniasis: current situation and new perspectives. *Comparative Immunology, Microbiology and Infectious Diseases*, *27*(5), 305–18.

Desta, A., Shiferaw, S., Kassa, A., Shimelis, T., & Dires, S. (2005). Leishmaniasis.

Dougall, A. M., Alexander, B., Holt, D. C., Harris, T., Sultan, A. H., Bates, P. a, … Walton, S. F. (2011). Evidence incriminating midges (Diptera: Ceratopogonidae) as potential vectors of Leishmania in Australia. *International Journal for Parasitology*, *41*(5), 571–9.

Doughherty, M. J., Guerin, P. M., & Ward, R. D. (1995). Identification of oviposition attractants for the sandfly Lutzomyia longipalpis (Diptera: Psychodidae) in volatiles of faeces from vertebrates. *Physiological Entomology*, *20*(1), 23–32.

El Tai, N. O., Osman, O. F., el Fari, M., Presber, W., & Schönian, G. (2000). Genetic heterogeneity of ribosomal internal transcribed spacer in clinical samples of Leishmania donovani spotted on filter paper as revealed by single-strand conformation polymorphisms and sequencing. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, *94*(5), 575–9.

El-Kammah, K. M. (1973). Studies of autogeny in Phlebotomus papatasi (Scopoli) (Diptera: Psychodidae). *Journal of Medical Entomology*, *10*(3), 261–3.

Elnaiem, D. E., & Ward, R. D. (1992). Oviposition attractants and stimulants for the sandfly Lutzomyia longipalpis (Diptera: Psychodidae). *Journal of Medical Entomology*, *29*(1), 5–12.

Elnaiem, D.-E. a, Hassan, H. K., Osman, O. F., Maingon, R. D., Killick-Kendrick, R., & Ward, R. D. (2011). A possible role for Phlebotomus (Anaphlebotomus) rodhaini (Parrot, 1930) in transmission of Leishmania donovani. *Parasites & Vectors*, *4*(Cl), 238.

Escobar, M. A., Martinez, F., Scott Smith, D., & Palma, G. I. (1992). American cutaneous and mucocutaneous leishmaniasis (tegumentary): a diagnostic challenge. *Tropical Doctor*, *22 Suppl 1*, 69–78;63–4.

Fever, B. (2012). Leishmaniasis (Cutaneous and Visceral) Leishmaniasis (cutaneous and visceral), 1–11.

Fryauff, D. J., Hanafi, H. a, Klena, J. D., Hoel, D. F., Appawu, M., Rogers, W., Boakye, D. (2006). Short report: ITS-1 DNA sequence confirmation of Leishmania major as a cause of cutaneous leishmaniasis from an outbreak focus in the Ho

district, southeastern Ghana. *The American Journal of Tropical Medicine and Hygiene*, *75*(3), 502–4.

Guilvard, E., Rioux, J. A., Jarry, D., & Moreno, G. (1985). Accouplements successifs chez Phlebotomus ariasi Tonnoir, 1921. *Annales de parasitologie humaine et comparée*, *60*(4).

Hanafi, H. A., Fryauff, D. J., Modi, G. B., Ibrahim, M. O., & Main, A. J. (2007). Bionomics of phlebotomine sandflies at a peacekeeping duty site in the north of Sinai, Egypt. *Acta Tropica*, *101*(2), 106–14.

Hashiguchi, Y., & Gómez Landires, E. A. (1991). A review of leishmaniasis in Ecuador. *Bulletin of the Pan American Health Organization*, *25*(1), 64–76.

Hide, M., B. Bucheton., S. Kawihawi., R. Bras-Goncalves., S. Sundar, J. L. L. and A. L. B. (2007). *Understanding Human Leishmaniasis: The need for an intergrated approach in ecyclopedia of infectious disease book of microbiology.* (M. Tibayrenc, Ed.). Hoboken, NJ, USA: John Wiley & Sons, Inc.

Hitakarun, A., Tan-ariya, P., Siripattanapipong, S., Mungthin, M., Piyaraj, P., Naaglor, T., Leelayoova, S. (2014). Comparison of PCR methods for detection of Leishmania siamensis infection. *Parasites & Vectors*, *7*(1), 458.

Jarvis, E. K., & Rutledge, L. C. (1992). Laboratory observations on mating and leklike aggregations in Lutzomyia longipalpis (Diptera: Psychodidae). *Journal of Medical Entomology*, *29*(2), 171–7.

Jenni, L., Molyneux, D. H., Livesey, J. L., & Galun, R. (1980). Feeding behaviour of tsetse flies infected with salivarian trypanosomes. *Nature*, *283*(5745), 383–5.

Kakarsulemankhel, J. K. (2011). Leishmaniasis in Pak-Afghan Region, 611–620.

Kamhawi, S., Modi, G. B., Pimenta, P. F., Rowton, E. & Sacks, D. L. (2000). The vectorial competence of Phlebotomus sergenti is specific for Leishmania tropica and is controlled by species - specif ic, lipophosphoglycan - mediated midgut attachment. *Parasitology*, *121*(Pt 1), 25 – 33.

Kanjanopas, K., Siripattanapipong, S., Ninsaeng, U., Hitakarun, A., Jitkaew, S., Kaewtaphaya, P., Leelayoova, S. (2013). Sergentomyia (Neophlebotomus) gemmea, a potential vector of Leishmania siamensis in southern Thailand. *BMC Infectious Diseases*, *13*(1), 333.

Katholi, C. R., & Unnasch, T. R. (2006). Important experimental parameters for determining infection rates in arthropod vectors using pool screening approaches. *American Journal of Tropical Medicine and Hygiene*, *74*(5), 779–785.

Kato, H., Uezato, H., Gomez, E. a, Terayama, Y., Calvopiña, M., Iwata, H., & Hashiguchi, Y. (2007). Establishment of a mass screening method of sand fly vectors for Leishmania infection by molecular biological methods. *The American Journal of Tropical Medicine and Hygiene*, *77*(2), 324–9.

Killick-Kendrick, R. (1987a). Methods for the study of Phlebotomine Sand flies. *In the Leishmaniases*, *1*, 473–497.

Killick-Kendrick, R. (1987b). The biology and control of phlebotomine sand flies. *Clinics in Dermatology*, *17*(3), 279–89.

Killick-Kendrick, R. (1990). Phlebotomine vectors of the leishmaniases: a review. *Medical and Veterinary Entomology*, *4*, 1–24.

Killick-Kendrick, R., Leaney, A. J., Ready, P. D., & Molyneux, D. H. (1977). Leishmania in phlebotomid sandflies. IV. The transmission of Leishmania mexicana amazonensis to hamsters by the bite of experimentally infected Lutzomyia longipalpis. *Proceedings of the Royal Society of London. Series B, Containing Papers of a Biological Character. Royal Society (Great Britain)*, *196*(1122), 105–15.

Killick-Kendrick, R., & Rioux, J. A. (2002). Mark-release-recapture of sand flies fed on leishmanial dogs: the natural life-cycle of Leishmania infantum in Phlebotomus ariasi. *Parassitologia*, *44*(1-2), 67–71.

Killick-Kendrick, R., Wilkes, T. J., Bailly, M., Bailly, I., & Righton, L. A. (1986). Preliminary field observations on the flight speed of a phlebotomine sandfly. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, *80*(1), 138–142.

Kimutai, A., Ngure, P. K., Tonui, W. K., & Gicheru, M. M. (2009). Review Paper, *3*, 14–25.

Kweku, M. A., Odoom, S., Puplampu, N., Desewu, K., Nuako, G. K., Gyan, B., Akuffo, H. (2011). An outbreak of suspected cutaneous leishmaniasis in Ghana: lessons learnt and preparation for future outbreaks.

Lainson, R., Ward, R. D., & Shaw, J. J. (1977). Leishmania in phlebotomid sandflies : VI . Importance of hindgut development in distinguishing between parasites of the Leishmania mexicana and L . braziliensis complexes, *320*, 309–320.

Lane, R. P., Pile, M. M., & Amerasinghe, F. P. (1990). Anthropophagy and aggregation behaviour of the sandfly Phlebotomus argentipes in Sri Lanka. *Medical and Veterinary Entomology*, *4*(1), 79–88.

Lawyer PG, P. P. (2004). *Medical Entomology: Leishmaniasis and trypanosomiasis*. (E. J. D. Eldridge BF, Ed.). Netherlands: Kluwer Academic Publishers.

Lewis, D. J. (1974). The biology of Phlebotomidae in relation to leishmaniasis. *Annual Review of Entomology*, *19*, 363–84.

Lewis, D. J. (1982). A taxonomic review of the genus Phlebotomus (Diptera: Psychodidae). *Bull Br Mus Nat Hist(Ent)*, (45), 121–209.

Maingon, R. D. C., Ward, R. D., Hamilton, J. G. C., Noyes, H. A., Souza, N., Kemp, S. J., & Watts, P. C. (2003). Genetic identification of two sibling species of Lutzomyia longipalpis (Diptera: Psychodidae) that produce distinct male sex pheromones in Sobral, Ceará State, Brazil. *Molecular Ecology*, *12*(7), 1879–94.

Mellor, H. E., & Hamilton, J. G. C. (2007). Navigation of Lutzomyia longipalpis (Diptera: Psychodidae) under dusk or starlight conditions. *Bulletin of Entomological Research*, *93*(04), 315–322.

Mitropoulos, P., Konidas, P., & Durkin-Konidas, M. (2010). New World cutaneous leishmaniasis: updated review of current and future diagnosis and treatment. *Journal of the American Academy of Dermatology*, *63*(2), 309–22.

Molyneux, D. H., & Jefferies, D. (2009). Feeding behaviour of pathogen-infected vectors. *Parasitology*, *92*(03), 721.

Morrison, A. C., Munstermann, L. E., Ferro, C., Pardo, R., and Torres, M. (2004). *Biology of Disease Vectors*. (W. H. Marquardt, Ed.) (second). London: Elsevier academic press.

Murray, H. W., Berman, J. D., Davies, C. R., & Saravia, N. G. (2005). Advances in leishmaniasis, *366*.

Nzelu, C. O., Kato, H., Puplampu, N., Desewu, K., Odoom, S., Wilson, M. D., … Boakye, D. a. (2014). First Detection of Leishmania tropica DNA and

Trypanosoma Species in Sergentomyia Sand Flies (Diptera: Psychodidae) from an Outbreak Area of Cutaneous Leishmaniasis in Ghana. *PLoS Neglected Tropical Diseases*, *8*(2).

Palatnik-de-Sousa, C. B. (2012). Vaccines for canine leishmaniasis. *Frontiers in Immunology*, *3*, 69.

Paz, G. F., Reis, I. a, Avelar, D. M., da Mata Ferreira, E. C., & Werneck, G. L. (2013). Ectoparasites and anti-Leishmania antibodies: association in an observational case-control study of dogs from a Brazilian endemic area. *Preventive Veterinary Medicine*, *112*(1-2), 156–9.

Perfil'ev, P. (1968). *Fauna of the U.S.S.R., Diptera*. Jerusalem: Israel Program for Scientific Transl.

Piscopo, T. V, & Mallia Azzopardi, C. (2007). Leishmaniasis. *Postgraduate Medical Journal*, *83*(976), 649–57.

Ready, P. D. (2013). Biology of phlebotomine sand flies as vectors of disease agents. *Annual Review of Entomology*, *58*, 227–50.

Reithinger, R., Dujardin, J., Louzir, H., Pirmez, C., Alexander, B., Brooker, S., … Leopold, P. (2007). Cutaneous leishmaniasis.

Reithinger, R., & Dujardin, J.-C. (2006). Molecular Diagnosis of Leishmaniasis: Current Status and Future Applications. *Journal of Clinical Microbiology*, *45*(1), 21–25.

Roberts, L. J., Handman, E., & Foote, S. J. (2000). Clinical review, *321*(9).

Rogers, M. E., & Bates, P. a. (2007). Leishmania manipulation of sand fly feeding behavior results in enhanced transmission. *PLoS Pathogens*, *3*(6),

Rose, K., Curtis, J., Baldwin, T., Mathis, A., Kumar, B., Sakthianandeswaren, A., Handman, E. (2004). Cutaneous leishmaniasis in red kangaroos: isolation and characterisation of the causative organisms. *International Journal for Parasitology*, *34*(6), 655–64.

Rossi, E., Bongiorno, G., Ciolli, E., Di Muccio, T., Scalone, A., Gramiccia, M., … Maroli, M. (2008). Seasonal phenology, host-blood feeding preferences and natural Leishmania infection of Phlebotomus perniciosus (Diptera, Psychodidae) in a high-endemic focus of canine leishmaniasis in Rome province, Italy. *Acta Tropica*, *105*(2), 158–65.

Sacks, D., & Kamhawi, S. (2001). Molecular aspects of parasite-vector and vector-host interactions in leishmaniasis. *Annual Review of Microbiology*, *55*, 453–83.

Sacks, D. L. (2001). Leishmania-sand fly interactions controlling species-specific vector competence. *Cellular Microbiology*, *3*(4), 189–96.

Schlein, Y., & Warburg, A. (1986). Phytophagy and the feeding cycle of Phlebotomus papatasi (Diptera: Psychodidae) under experimental conditions. *Journal of Medical Entomology*, *23*(1), 11–5.

Schönian, G., Nasereddin, A., Dinse, N., Schweynoch, C., Schallig, H. D. F. H., Presber, W., & Jaffe, C. L. (2003). PCR diagnosis and characterization of Leishmania in local and imported clinical samples. *Diagnostic Microbiology and Infectious Disease*, *47*(1), 349–58.

Senghor, M. W., Faye, M. N., Faye, B., Diarra, K., Elguero, E., Gaye, O., Niang, A. a. (2011). Ecology of phlebotomine sand flies in the rural community of Mont Rolland (Thiès region, Senegal): area of transmission of canine leishmaniasis. *PloS One*, *6*(3),

Service, M. W. (1980). A guide to medical entomology. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, *74*(6), 78–82.

Singh, S. (2006). New developments in diagnosis of leishmaniasis. *The Indian Journal of Medical Research*, *123*(3), 311–30.

Siqueira-Neto, J. L., Moon, S., Jang, J., Yang, G., Lee, C., Moon, H. K., FreitasJunior, L. H. (2012). An image-based high-content screening assay for compounds targeting intracellular Leishmania donovani amastigotes in human macrophages. *PLoS Neglected Tropical Diseases*, *6*(6),

Statistical Service of Ghana, 2010

Strobel, M., N'Diaye, B., Marchand, J. P., & Dedet, J. P. (1987). [2d case of cutaneous leishmaniasis with mucous involvement in Senegal]. *Bulletin de La Société de Pathologie Exotique et de Ses Filiales*, *71*(6), 423–9.

Tavares, C. A. P., Fernandes, A. P., & Melo, M. N. (2003). Molecular diagnosis of leishmaniasis. *Expert Review of Molecular Diagnostics*, *3*(5), 657–67.

Valenta, D. T., Killick-Kendrick, R., & Killick-Kendrick, M. (2000). Courtship and mating by the sandfly Phlebotomus duboscqi, a vector of zoonotic cutaneous leishmaniasis in the Afrotropical region. *Medical and Veterinary Entomology*, *14*(2), 207–12.

Villinski, J. T., Klena, J. D., Abbassy, M., Hoel, D. F., Puplampu, N., Mechta, S., … Raczniak, G. (2008). Evidence for a new species of Leishmania associated with a focal disease outbreak in Ghana. *Diagnostic Microbiology and Infectious Disease*, *60*(3), 323–7.

Wardl, B. R. D., & Newstead, L. (1977). The colonization of lutzomyia flaviscutellata (diptera : psychodidae), a vector of leishmania mexicana amazonensis in brazil .

World Health Organization. (2010). Control of the leishmaniases. *World Health Organization Technical Report Series*, (949), 22–26.

APPENDICES

Appendix I: Raw data of Sand fly collection and PCR results

NO.	VILLAGE	LOCATION/ TRAP NO.	SPECIES	POOL CODE	POOL SIZE	PCR RESULTS
1	DOGBLOME-AWIASU	LT1	*S. africana*	LT1A001	25	Negative
2	DOGBLOME-AWIASU	LT4	*S. africana*	LT4A001	25	Negative

3	DOGBLOME-AWIASU LT4	*S. africana*	LT4A002	25	Negative	
4	DOGBLOME-AWIASU LT4	*S. africana*	LT4A003	25	Negative	
5	DOGBLOME-AWIASU LT6	*S. africana*	LT6A001	25	Negative	
6	DOGBLOME-AWIASU LT7	*S. africana*	LT7A001	25	Negative	
7	DOGBLOME-AWIASU LT7	*S. africana*	LT7A002	25	Negative	
8	DODOME-DOGBLOME	LT10	*S. africana*	LT10A001	25	Negative DODOME-
9	DOGBLOME	LT10	*S. africana*	LT10A002	25	Negative DODOME-
10	DOGBLOME	LT10	*S. africana*	LT10A003	25	Negative DODOME-
11	DOGBLOME	LT10	*S. africana*	LT10A004	25	Negative DODOME-
12	DOGBLOME	LT13	*S. africana*	LT13A001	25	Negative DODOME-
13	DOGBLOME	LT13	*S. africana*	LT13A002	25	Negative DODOME-
14	DOGBLOME	LT13	*S. similima*	LT13S001	25	Negative DODOME-
15	DOGBLOME	LT14	*S. africana*	LT14A001	25	Negative DODOME-
16	DOGBLOME	LT9	*S. africana*	LT9A001	25	Negative
17	LUME-ACHIAME	LT16	*S. africana*	LT16A001	25	Negative
18	LUME-ACHIAME	LT16	*S. africana*	LT16A002	25	Negative
19	LUME-ACHIAME	LT16	*S. africana*	LT16A003	25	Negative
20	LUME-ACHIAME	LT16	*S. africana*	LT16A004	25	Negative
21	LUME-ACHIAME	LT16	*S. africana*	LT16A005	25	Negative
22	LUME-ACHIAME	LT16	*S. africana*	LT16A006	25	Negative
23	LUME-ACHIAME	LT16	*S. africana*	LT16A007	25	Negative
24	LUME-ACHIAME	LT16	*S. africana*	LT16A008	25	Negative
25	LUME-ACHIAME	LT16	*S. africana*	LT16A009	25	Negative
26	LUME-ACHIAME	LT16	*S. africana*	LT16A010	25	Negative
27	LUME-ACHIAME	LT16	*S. africana*	LT16A011	25	Negative

28	LUME-ACHIAME	LT15	*S. africana*	LT16A012	25	Negative
29	LUME-ACHIAME	LT16	*S. africana*	LT16A013	25	Negative
30	LUME-ACHIAME	LT16	*S. africana*	LT16A014	25	Negative
31	LUME-ACHIAME	LT16	*S. africana*	LT16A015	25	Negative
32	LUME-ACHIAME	LT16	*S. africana*	LT16A016	25	Negative
33	LUME-ACHIAME	LT16	*S. africana*	LT16A017	25	Negative
34	LUME-ACHIAME	LT16	*S. africana*	LT16A018	25	Negative
35	LUME-ACHIAME	LT16	*S. ingrami*	LT16I001	25	Negative
36	LUME-ACHIAME	LT18	*S. africana*	LT18A001	25	Negative
37	DODOME-DOGBLOME	AURELIA	*S. africana*	AA001	25	Negative
38	DODOME-DOGBLOME	AURELIA	*S. africana*	AA002	25	Negative
39	DODOME-DOGBLOME	AURELIA	*S. africana*	AA003	25	Negative
40	DODOME-DOGBLOME	AURELIA	*S. africana*	AA004	25	Negative
41	DODOME-DOGBLOME	AURELIA	*S. africana*	AA005	25	Negative
42	DODOME-DOGBLOME	AURELIA	*S. similima*	AS001	25	Negative
43	DODOME-DOGBLOME	AURELIA	*S. similima*	AS002	25	Negative
44	DODOME-DOGBLOME	AURELIA	*S. similima*	AS003	25	Negative
45	DODOME-DOGBLOME	PEARL	*S. africana*	PA001	25	Negative
46	DODOME-DOGBLOME	PEARL	*S. africana*	PA002	25	Negative
47	DODOME-DOGBLOME	PEARL	*S. africana*	PA003	25	Negative
48	DODOME-DOGBLOME	PEARL	*S. africana*	PA004	25	Negative
49	DODOME-DOGBLOME	PEARL	*S. africana*	PA005	25	Negative
50	DODOME-DOGBLOME	PEARL	*S. africana*	PA006	25	Negative
51	DODOME-DOGBLOME	PEARL	*S. ingrami*	PI001	25	Negative
52	DODOME-DOGBLOME	PEARL	*S. similima*	PS001	25	Negative

Negative

Negative

Negative

							Negative
							Negative
							Negative
							Negative
53	DOGBLOME	HSE1	*S. similima*	H1S001	25	Negative	
54	DODOME-DOGBLOME	LT13	*S. africana*	LT13A003	25	Negative	
55	DODOME-DOGBLOME	LT13	*S. africana*	LT13A004	25		
56	DODOME-DOGBLOME	LT13	*S. africana*	LT13A005	25		
57	DODOME-DOGBLOME	LT13	*S. africana*	LT13A006	25		
58	DODOME-DOGBLOME	LT13	*S. africana*	LT13A007	25		
59	DODOME-DOGBLOME	LT13	*S. africana*	LT13A008	25		
60	DODOME-DOGBLOME	LT13	*S. africana*	LT13A009	25		
61	DOGBLOME-AWIASU	LT1	*S. africana*	LT1A002	10		
62	DOGBLOME-AWIASU	LT1	*S. dureni*	LT1D001	5	Negative	
63	DOGBLOME-AWIASU	LT1	*S. buxtoni*	LTIB001	1	Negative	
64	DOGBLOME-AWIASU	LT1	*S. similima*	LT1S001	6	Negative	
65	DOGBLOME-AWIASU	LT1	*S. ingrami*	LT1I001	2	Negative	
66	DOGBLOME-AWIASU	LT1	*S. collarti*	LT1C001	1	Negative	
67	DOGBLOME-AWIASU	LT1	*S. ghesquierei*	LT1G001	1	Negative	
68	DOGBLOME-AWIASU	LT1	*S. antennata*	LT1AN001	6	Negative	
69	DOGBLOME-AWIASU	LT1	*S. schwetzi*	LT1SC001	8	Negative	
70	DOGBLOME-AWIASU	LT2	*S. africana*	LT2A001	7	Positive	
71	DOGBLOME-AWIASU	LT2	*S. schwetzi*	LT2SC001	3	Negative	
							Negative
							Negative
							Negative

						Negative
						Negative
						Negative
						Negative
72	AWIASU DOGBLOME-	LT2	*S. antennata*	LT2AN001	1	Negative
73	AWIASU DOGBLOME-	LT2	*S. ghesquierei*	LT2G001	1	Negative
74	AWIASU DOGBLOME-	LT2	*S. hamoni*	LT2H001	1	Negative
75	AWIASU DOGBLOME-	LT3	*S. africana*	LT3A001	6	Negative
76	AWIASU DOGBLOME-	LT3	*S. antennata*	LT3AN001	1	Negative
77	AWIASU DOGBLOME-	LT3	*S. schwetzi*	LT3SC001	8	Negative
78	AWIASU DOGBLOME-	LT3	*S. ingrami*	LT3I001	1	Negative
79	AWIASU DOGBLOME-	LT3	*S. ghesquierei*	LT3G001	4	Negative
80	AWIASU DOGBLOME-	LT3	*P. rodahaini*	LT3R001	2	Negative
81	AWIASU DOGBLOME-	LT4	*S. africana*	LT4A004	25	
82	AWIASU DOGBLOME-	LT4	*S. africana*	LT4A005	4	
83	DOGBLOME- AWIASU	LT4	*S. schwetzi*	LT4SC001	3	
84	AWIASU DOGBLOME-	LT4	*S. antennata*	LT4AN001	5	
85	AWIASU DOGBLOME-	LT4	*S. ghesquierei*	LT4G001	3	
86	AWIASU DOGBLOME-	LT4	*S. similima*	LT4S001	1	
87	AWIASU DOGBLOME-	LT4	*S. ingrami*	LT4I001	2	
88	AWIASU DOGBLOME-	LT4	*S. dureni*	LT4D001	3	Negative
89	AWIASU DOGBLOME-	LT5	*S. africana*	LT5A001	6	Negative
90	AWIASU DOGBLOME-	LT5	*S. antennata*	LT5AN001	2	Negative
91	AWIASU	LT5	*S. dureni*	LT5D001	3	Negative
						Negative
						Negative
						Negative

						Negative
						Negative
						Negative
						Negative
92	DOGBLOME-AWIASU	LT5	*S. ghesquierei*	LT5G001	5	Negative
93	DOGBLOME-AWIASU	LT5	*S. buxtoni*	LT5B001	1	Negative
94	DOGBLOME-AWIASU	LT5	*S. collarti*	LT5C001	2	Negative
95	DOGBLOME-AWIASU	LT5	*S. ingrami*	LT5I001	4	Negative
96	DOGBLOME-AWIASU	LT5	*S. schwetzi*	LT5SC001	3	Negative
97	DOGBLOME-AWIASU	LT5	*S. similima*	LT5S001	2	Negative
98	DOGBLOME-AWIASU	LT6	*S. africana*	LT6A002	2	Negative
99	DOGBLOME-AWIASU	LT6	*S. schwetzi*	LT6SC001	5	Negative
100	DOGBLOME-AWIASU	LT6	*S. dureni*	LT6D001	2	Negative
101	DOGBLOME-AWIASU	LT6	*S. collarti*	LT6C001	3	Negative
102	DOGBLOME-AWIASU	LT6	*S. similima*	LT6S001	10	Negative
103	DOGBLOME-AWIASU	LT6	*S. ghesquierei*	LT6G001	6	Negative
104	DOGBLOME-AWIASU	LT6	*S. ingrami*	LT6I001	1	Negative
105	DOGBLOME-AWIASU	LT6	*S. antennata*	LT6AN001	5	Negative
106	DOGBLOME-AWIASU	LT7	*S. africana*	LT7A001	24	
107	DOGBLOME-AWIASU	LT7	*S. antennata*	LT7AN001	17	
108	DOGBLOME-AWIASU	LT7	*S. ingrami*	LT7I001	2	
109	DOGBLOME-AWIASU	LT7	*S. collarti*	LT7C001	3	
110	DOGBLOME-AWIASU	LT7	*S. schwetzi*	LT7SC001	7	
						Negative
						Negative
						Negative

							Negative
							Negative
							Negative
							Negative
111	DOGBLOME-AWIASU	LT7	*S. dureni*	LT7D001	1		
112	DOGBLOME-AWIASU	LT7	*S. ghesquierei*	LT7G001	2		
113	DOGBLOME-AWIASU	LT7	*S. similima*	LT7S001	3	Negative	
114	DOGBLOME-AWIASU	LT7	*P. rodahaini*	LT7R001	1	Negative	
115	DODOME-DOGBLOME	LT8	*S. africana*	LT8A001	25	Negative	
116	DODOME-DOGBLOME	LT8	*S. africana*	LT8A002	11	Negative	
117	DODOME-DOGBLOME	LT8	*S. africana*	LT8A003	16	Negative	
118	DODOME-DOGBLOME	LT8	*S. schwetzi*	LT8SC001	3	Negative	
119	DODOME-DOGBLOME	LT8	*S. ingrami*	LT8I001	5	Negative	
120	DODOME-DOGBLOME	LT8	*S. dureni*	LT8D001	3	Negative	
121	DODOME-DOGBLOME	LT8	*S. similima*	LT8S001	2	Negative	
122	DODOME-DOGBLOME	LT8	*S. collarti*	LT8C001	1	Negative	
123	DODOME-DOGBLOME	LT9	*S. africana*	LT9A001	24	Negative	
124	DODOME-DOGBLOME	LT9	*S. antennata*	LT9AN001	9	Negative	
125	DODOME-DOGBLOME	LT9	*S. collarti*	LT9C001	3	Negative	
126	DODOME-DOGBLOME	LT9	*S. ghesquierei*	LT9G001	5	Negative	
127	DODOME-DOGBLOME	LT9	*S. schwetzi*	LT9SC001	7	Negative	
128	DODOME-DOGBLOME	LT9	*S. ingrami*	LT9I001	4	Negative	
129	DODOME-DOGBLOME	LT9	*S. similima*	LT9S001	2	Negative	
							Negative
							Negative
							Negative

						Negative
						Negative
						Negative
						Negative
130	DOGBLOME DODOME-	LT9	*S. dureni*	LT9D001	5	Negative
131	DOGBLOME DODOME-	LT10	*S. africana*	LT10A005	25	Negative
132	DOGBLOME DODOME-	LT10	*S. africana*	LT10A006	25	
133	DOGBLOME DODOME-	LT10	*S. africana*	LT10A007	25	
134	DODOME- DOGBLOME	LT10	*S. africana*	LT10A008	9	
135	DOGBLOME DODOME-	LT10	*S. antennata*	LT10AN00 1	7	
136	DOGBLOME DODOME-	LT10	*S. buxtoni*	LT10B001	1	
137	DOGBLOME DODOME-	LT10	*S. dureni*	LT10D001	1	
138	DOGBLOME DODOME-	LT10	*S. hamoni*	LT10H001	1	
139	DOGBLOME DODOME-	LT10	*S. similima*	LT10S001	16	Negative
140	DOGBLOME DODOME-	LT10	*S. schwetzi*	LT10SC001	5	Negative
141	DOGBLOME DODOME-	LT11	*S. africana*	LT11A001	25	Negative
142	DOGBLOME DODOME-	LT11	*S. africana*	LT11A002	25	Negative
143	DOGBLOME DODOME-	LT11	*S. africana*	LT11A003	25	Negative
144	DOGBLOME DODOME-	LT11	*S. africana*	LT11A004	25	Negative
145	DOGBLOME DODOME-	LT11	*S. africana*	LT11A005	25	Negative
146	DOGBLOME DODOME-	LT11	*S. africana*	LT11A006	5	Negative
147	DOGBLOME DODOME-	LT11	*S. antennata*	LT11AN00 1	7	Negative
148	DOGBLOME DODOME-	LT11	*S. dureni*	LT11D001	2	Negative
						Negative
						Negative
						Negative

						Negative
						Negative
						Negative
						Negative
149	DOGBLOME DODOME-	LT11	*S. ingrami*	LT11I001	25	Negative
150	DOGBLOME DODOME-	LT11	*S. ingrami*	LT11I002	1	Negative
151	DOGBLOME DODOME-	LT11	*S. collarti*	LT11C001	8	Negative
152	DOGBLOME DODOME-	LT11	*S. similima*	LT11S001	22	Negative
153	DOGBLOME DODOME-	LT11	*S. hamoni*	LT11H001	1	Negative
154	DOGBLOME DODOME-	LT11	*S. buxtoni*	LT11B001	6	Negative
155	DOGBLOME DODOME-	LT11	*S. ghesquierei*	LT11G001	7	Negative
156	DOGBLOME DODOME-	LT11	*S. schwetzi*	LT11SC001	6	Negative
157	DOGBLOME DODOME-	LT12	*S. africana*	LT12A001	25	
158	DOGBLOME DODOME-	LT12	*S. africana*	LT12A002	25	
159	DOGBLOME DODOME-	LT12	*S. africana*	LT12A003	25	
160	DOGBLOME DODOME-	LT12	*S. africana*	LT12A004	1	
161	DOGBLOME DODOME-	LT12	*S. antennata*	LT12AN001	7	
162	DOGBLOME DODOME-	LT12	*S. collarti*	LT12C001	22	
163	DOGBLOME DODOME-	LT12	*S. dureni*	LT12D001	4	
164	DOGBLOME DODOME-	LT12	*S. similima*	LT12S001	5	Negative
165	DOGBLOME DODOME-	LT12	*S. ingrami*	LT12I001	4	Negative
166	DOGBLOME DODOME-	LT12	*P. rodahaini*	LT12R001	2	Negative
167	DOGBLOME DODOME-	LT12	*S. ghesquierei*	LT12G001	7	Negative
						Negative
						Negative
						Negative

						Negative
						Negative
						Negative
						Negative
168	DOGBLOME-DODOME	LT12	*S. schwetzi*	LT12SC001	1	Negative
169	DOGBLOME-DODOME	LT13	*S. africana*	LT13A010	25	Negative
170	DOGBLOME-DODOME	LT13	*S. africana*	LT13A011	25	Negative
171	DOGBLOME-DODOME	LT13	*S. africana*	LT13A012	25	Negative
172	DOGBLOME-DODOME	LT13	*S. africana*	LT13A013	25	Negative
173	DOGBLOME-DODOME	LT13	*S. africana*	LT13A014	25	Negative
174	DOGBLOME-DODOME	LT13	*S. africana*	LT13A015	25	Negative
175	DOGBLOME-DODOME	LT13	*S. africana*	LT13A016	4	Negative
176	DOGBLOME-DODOME	LT13	*S. antennata*	LT13AN001	17	Negative
177	DOGBLOME-DODOME	LT13	*S. dureni*	LT13D001	9	Negative
178	DOGBLOME-DODOME	LT13	*S. collarti*	LT13C001	25	Negative
179	DOGBLOME-DODOME	LT13	*S. collarti*	LT13C002	22	Negative
180	DOGBLOME-DODOME	LT13	*S. ingrami*	LT13I001	7	Negative
181	DOGBLOME-DODOME	LT13	*S. hamoni*	LT13H001	6	Negative
182	DOGBLOME-DODOME	LT13	*S. ghesquierei*	LT13G001	10	Negative
183	DOGBLOME-DODOME	LT13	*S. similima*	LT13S002	1	
184	DOGBLOME-DODOME	LT13	*S. schwetzi*	LT13SC001	11	
185	DODOME-	LT13	*P. rodahaini*	LT13R001	3	
						Negative
						Negative
						Negative

						Negative
						Negative
						Negative
						Negative
	DOGBLOME					
186	DODOME-DOGBLOME	LT14	*S. africana*	LT14A001	25	
187	DODOME-DOGBLOME	LT14	*S. africana*	LT14A002	25	
188	DODOME-DOGBLOME	LT14	*S. africana*	LT14A003	25	
189	DODOME-DOGBLOME	LT14	*S. africana*	LT14A004	25	
190	DODOME-DOGBLOME	LT14	*S. africana*	LT14A005	25	Negative
191	DODOME-DOGBLOME	LT14	*S. africana*	LT14A006	25	Negative
192	DODOME-DOGBLOME	LT14	*S. africana*	LT14A007	25	Negative
193	DODOME-DOGBLOME	LT14	*S. africana*	LT14A008	25	Negative
194	DODOME-DOGBLOME	LT14	*S. africana*	LT14A009	25	Negative
195	DODOME-DOGBLOME	LT14	*S. africana*	LT14A010	25	Negative
196	DODOME-DOGBLOME	LT14	*S. africana*	LT14A011	18	Negative
197	DODOME-DOGBLOME	LT14	*S. antennata*	LT14AN001	13	Negative
198	DODOME-DOGBLOME	LT14	*S. dureni*	LT14D001	6	Negative
199	DODOME-DOGBLOME	LT14	*S. ingrami*	LT14I001	25	Negative
200	DODOME-DOGBLOME	LT14	*S. ingrami*	LT14I002	2	Negative
201	DODOME-DOGBLOME	LT14	*S. ghesquierei*	LT14G001	25	Negative
202	DODOME-DOGBLOME	LT14	*S. ghesquierei*	LT14G002	2	Negative
203	DODOME-DOGBLOME	LT14	*S. collarti*	LT14C001	13	Negative
						Negative

204	DOGBLOME	LT14	*S. schwetzi*	LT14SC001	18	Negative
205	DODOME-DOGBLOME	LT14	*S. similima*	LT14S001	24	Negative
206	DODOME-DOGBLOME	LT14	*P. rodahaini*	LT14R001	2	Negative
207	LUME-ACHIAME	LT15	*S. africana*	LT15A001	25	Negative
208	LUME-ACHIAME	LT15	*S. africana*	LT15A002	25	Negative
209	LUME-ACHIAME	LT15	*S. africana*	LT15A003	1	Negative
210	LUME-ACHIAME	LT15	*S. similima*	LT15S001	25	Negative
211	LUME-ACHIAME	LT15	*S. similima*	LT15S002	2	Negative
				LT15AN00		
212	LUME-ACHIAME	LT15	*S. antennata*	1 3	Negative	
213	LUME-ACHIAME	LT15	*S. ghesquierei*	LT15G001	8	
214	LUME-ACHIAME	LT15	*S. schwetzi*	LT15SC001	10	Negative
215	LUME-ACHIAME	LT15	*S. ingrami*	LT15I001	15	Negative
216	LUME-ACHIAME	LT15	*S. schwetzi*	LT15SC001	2	Negative
217	LUME-ACHIAME	LT15	*S. dureni*	LT15D001	7	Negative
218	LUME-ACHIAME	LT15	*P. rodahaini*	LT15R001	1	Negative
219	LUME-ACHIAME	LT15	*S. buxtoni*	LT15B001	1	Negative
220	LUME-ACHIAME	LT16	*S. africana*	LT16A019	25	Negative
221	LUME-ACHIAME	LT16	*S. africana*	LT16A020	25	Negative
222	LUME-ACHIAME	LT16	*S. africana*	LT16A021	25	Negative
223	LUME-ACHIAME	LT16	*S. africana*	LT16A022	25	Negative
224	LUME-ACHIAME	LT16	*S. africana*	LT16A023	25	Negative
225	LUME-ACHIAME	LT16	*S. africana*	LT16A024	25	Negative
226	LUME-ACHIAME	LT16	*S. africana*	LT16A025	25	Negative 227
	LUME-ACHIAME	LT16	*S. africana*	LT16A026	14	Negative
				LT16AN00		
228	LUME-ACHIAME	LT16	*S. antennata*	1 25	Negative	
229	LUME-ACHIAME	LT16	*S. schwetzi*	LT16SC001	25	Negative
230	LUME-ACHIAME	LT16	*S. schwetzi*	LT16SC002	1	Negative
231	LUME-ACHIAME	LT16	*S. ingrami*	LT16I002	25	Negative
232	LUME-ACHIAME	LT16	*S. ingrami*	LT16I003	2	Negative
233	LUME-ACHIAME	LT16	*S. buxtoni*	LT16B001	3	Negative
234	LUME-ACHIAME	LT16	*S. ghesquierei*	LT16G001	10	Negative
235	LUME-ACHIAME	LT16	*S. similima*	LT16S001	25	Negative
236	LUME-ACHIAME	LT16	*S. similima*	LT16S002	1	Negative
237	LUME-ACHIAME	LT16	*S. dureni*	LT16D001	5	Negative
238	LUME-ACHIAME	LT16	*P. rodahaini*	LT16R001	4	Negative
239	LUME-ACHIAME	LT16	*S. collarti*	LT16C001	10	Negative
240	LUME-ACHIAME	LT17	*S. africana*	LT17A001	12	Negative
241	LUME-ACHIAME	LT17	*S. collarti*	LT17C001	2	Negative
				LT17AN00		
242	LUME-ACHIAME	LT17	*S. antennata*	1 1	Negative	
243	LUME-ACHIAME	LT17	*S. schwetzi*	LT17SC001	1	Negative
244	LUME-ACHIAME	LT17	*S. similima*	LT17S001	8	Negative

Negative

					LT18AN00		
245	LUME-ACHIAME	LT18	*S. antennata*	1	5	Negative	
246	LUME-ACHIAME	LT18	*S. ghesquierei*	LT18G001	2	Negative	
247	LUME-ACHIAME	LT18	*S. similima*	LT18S001	4	Negative	
248	LUME-ACHIAME	LT18	*S. dureni*	LT18D001	1	Negative	
249	LUME-ACHIAME	LT18	*P. rodahaini*	LT18R001	1	Negative	
250	LUME-ACHIAME	LT18	*S. africana*	LT18A002	2	Negative	
251	LUME-ACHIAME	LT18	*S. collarti*	LT18C001	1	Negative	
252	LUME-ACHIAME	LT19	*S. ingrami*	LT19I001	5	Negative	
253	LUME-ACHIAME	LT19	*S. collarti*	LT19C001	1	Negative	
254	LUME-ACHIAME	LT19	*S. ghesquierei*	LT19G001	1	Negative	
255	LUME-ACHIAME	LT19	*S. similima*	LT19S001	1	Negative	
256	LUME-ACHIAME	LT19	*S. africana*	LT19A001	17	Negative	
257	LUME-ACHIAME	LT19	*S. schwetzi*	LT19SC001	1	Negative	
258	DODOME-DOGBLOME	LT10	*S. ingrami*	LT10I001	11	Negative	
259	DODOME-DOGBLOME	LT14	*S. ingrami*	LT14I001	1		
260	DODOME-DOGBLOME	AURELIA	*S. africana*	AA005	23	Negative	
261	DODOME-DOGBLOME	AURELIA	*S. buxtoni*	AB001	3	Negative	
262	DODOME-DOGBLOME	AURELIA	*S. antennata*	AAN001	11	Negative	
263	DODOME-DOGBLOME	AURELIA	*S. ghesquierei*	AG001	1	Negative	
264	DODOME-DOGBLOME	AURELIA	*S. similima*	AS004	17	Negative	
265	DODOME-DOGBLOME	AURELIA	*S. ingrami*	AI001	12	Negative	
266	DODOME-DOGBLOME	ERASMUS	*S. africana*	EA001	12	Negative	
267	DODOME-DOGBLOME	ERASMUS	*S. similima*	ES001	25	Negative	
268	DODOME-DOGBLOME	ERASMUS	*S. similima*	ES002	18	Negative	
269	DODOME-DOGBLOME	ERASMUS	*S. ingrami*	EI001	1	Negative	
270	DODOME-DOGBLOME	EMMA	*S. similima*	EMS001	2	Negative	
271	DODOME-DOGBLOME	EMMA	*S. hamoni*	EMH001	1	Negative	
272	DODOME-DOGBLOME	EDITH	*S. africana*	EDA001	12	Negative	

Negative

273	DOGBLOME	EDITH	*S. antennata*	EDAN001	1	Negative	
274	DODOME-DOGBLOME	HSE1	*S. africana*	H1A001	12	Negative	
275	DODOME-DOGBLOME	HSE1	*S. similima*	H1S002	3	Negative	
276	DODOME-DOGBLOME	HSE	*S. africana*	HSA001	1	Negative	
277	DODOME-DOGBLOME	HERMEN	*S. africana*	HA001	8	Negative	
278	DODOME-DOGBLOME	MABEL	*S. africana*	MBA001	17	Negative	
279	DODOME-DOGBLOME	MABEL	*S. antennata*	MBAN001	2	Negative	
280	DODOME-DOGBLOME	MABEL	*S. ingrami*	MBI001	4	Negative	
281	DODOME-DOGBLOME	MABEL	*S. similima*	MBS001	3	Negative	
282	DODOME-DOGBLOME	MARGARET	*S. africana*	MGA001	15	Negative	
283	DODOME-DOGBLOME	MARGARET	*S. antennata*	MGANOO1	3	Negative	
284	DODOME-DOGBLOME	MARGARET	*S. similima*	MGS001	10	Negative	
285	DODOME-	MARGARET	*S. ingrami*	MGI001	12		

Negative

	DOGBLOME					
286	DODOME-DOGBLOME	PEARL	*S. africana*	PA007	25	Negative
287	DODOME-DOGBLOME	PEARL	*S. africana*	PA008	25	Negative
288	DODOME-DOGBLOME	PEARL	*S. africana*	PA009	9	Negative
289	DODOME-DOGBLOME	PEARL	*S. buxtoni*	PB001	4	Negative
290	DODOME-DOGBLOME	PEARL	*S. ingrami*	PI002	20	Negative
291	DODOME-DOGBLOME	PEARL	*S. antennata*	PAN001	16	Negative
292	DODOME-DOGBLOME	PEARL	*S. similima*	PS002	15	Negative
293	DODOME-DOGBLOME	PEARL	*S. schwetzi*	PSC001	2	Negative
294	DODOME-DOGBLOME	PEARL	*S. dureni*	PD001	2	Negative
295	DODOME-DOGBLOME	SYLVESTER	*S. africana*	SLA001	3	Negative

Appendix II: PCR Thermal Cycler Conditions and Master Mix Concentrations

Assay	Primers	Product (bp)	Annealing tº	PCR Condition (Final Concentration)	Cycling Protocol	Cycling Number
ssu rDNA-PCR	R221: 5' GGTTCCTTTCCTGATTTACG R332: 5' GGCCGGTAAAGGCCGAATAG	603	53	250 µM dNTP's 1 µM Primers 1 U Taq Pol	annealing: 60 sec extension 120 sec	38
ITS-PCR	LITSR: 5' CTGGATCATTTTCCGATG L5.8S: 5' TGATACCACTTATCGCACTT	300-350	53	4.0 Mm MgCl2 200 µM dNTP's 500 µM Primer 2 U Taq Pol 1.5 mM MgCl2	annealing: 30 sec extension 60 sec	35
Minicircle	Mincr2 : GGGGAGGGGCGTTCTGCGAA Mincr3 : CGCCCCCTATTTTACACAACCCC	120	60	200 µM dNTP's 100 µM Primer 1.5 mM MgCl2 1 U Taq Pol	annealing: 30 sec extension 90 sec	35

Appendix III: Frequency distribution of female Species of Sandflies by season

Sandfly Species		wet	dry
P.rodh	Count	6	14
	% within season	0.40%	0.50%
S.ghes	Count	55	75
	% within season	3.60%	2.80%
S.inerm	Count	1	2
	% within season	0.10%	0.10%
S.quam	Count	1	0
	% within season	0.10%	0.00%
S.coll	Count	23	109
	% within season	1.50%	4.10%
S.dur	Count	26	46
	% within season	1.70%	1.70%
S.ing	Count	75	197
	% within season	4.90%	7.40%
S.Af	Count	858	1896
	% within season	55.60%	70.90%
S.anten	Count	48	156
	% within season	3.10%	5.80%
S.bux	Count	19	15
	% within season	1.20%	0.60%
S.schw	Count	13	120
	% within season	0.80%	4.50%
S.ham	Count	9	2

	% within season	0.60%	0.10%
S.sim	Count	410	43
	% within season	26.60%	1.60%
	Count	1544	2675
	% within season	100.00%	100.00%

S. Africana was the abundant species obtained and most of them were caught during the dry season

Appendix IV

Preparation of Clearing and Mounting Medium

Clearing Medium

Preparation of chloral hydrate and phenol

- Saturated solution of chloral hydrate(using light heat)
- Dissolve the phenol using a water bath
- Mix equal volumes of chloral hydrate and phenol together in dark bottle.

Mounting Medium

Puri's Medium

Distilled water……. 10 ml
Gum Arabic …… 8 gm
Chloral hydrate …… 70 gm
Glycerin…… 5 ml
Acetic acid …….. 3 ml

Using a water bath (80˚C), Dissolve gum in water. Add chloral hydrate while stirring. Add glycerin and acetic acid. Filter the solution into a dark bottle.