Chair of Comparative Tropical Medicine and Parasitology
Faculty of Veterinary Medicine of the Ludwig Maximilians-University Munich
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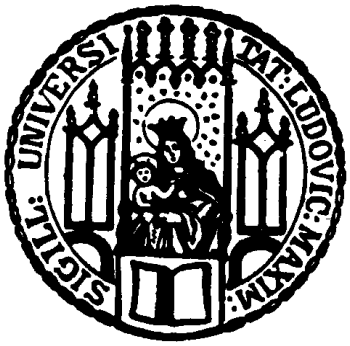
EPIDEMIOLOGY OF LEISHMANIOSIS IN SOUTHERN GERMANY WITH EMPHASIS ON THE FAMILY OF PSYCHODIDAE, PRIMARILY PHLEBOTOMINAE



INAUGURAL-DISSERTATION
for the attainment
of the title of Doctor in Veterinary Biology (Dr. rer. biol. vet.)
from the Faculty of Veterinary Medicine of the Ludwig-Maximilians-University Munich

by

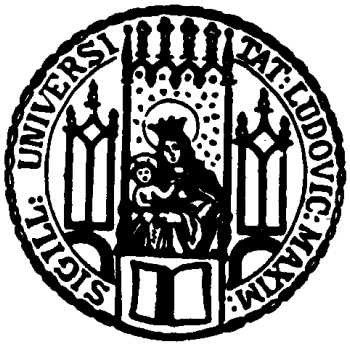
Bernadett Beran

from Cottbus, Germany

Munich 2010

Aus dem Lehrstuhl für Vergleichende Tropenmedizin und Parasitologie
der Tierärztlichen Fakultät der Ludwig Maximilians-Universität München
Vorstand: Univ.-Prof. Dr. Kurt Pfister

EPIDEMIOLOGIE DER LEISHMANIOSE IN SÜD-DEUTSCHLAND MIT FOKUS AUF DIE FAMILIE DER PSYCHODIDAE, PRIMÄR PHLEBOTOMINAE



INAUGURAL-DISSERTATION
zur Erlangung
der veterinärbiologischen Doktorwürde (Dr. rer. biol. vet.)
der Tierärztlichen Fakultät der Ludwig-Maximilians-Universität München

von

Bernadett Beran

aus Cottbus, Deutschland

München 2010

Gedruckt mit Genehmigung der Tierärztlichen Fakultät der Ludgwig-Maximilians-Universität München

Dekan: Univ.-Prof. Dr. Braun

Berichterstatter: Univ.-Prof. Dr. Pfister

Korreferent/en: Univ.-Prof. Dr. Straubinger

Tag der Promotion: 24. Juli 2010

Die vorliegende Arbeit wurde nach § 6 Abs. 2 der Promotionsordnung für die Tierärztliche Fakultät der Ludwig-Maximilians-Universität München als kumulative Dissertation gestaltet.

Meiner Mutter Ursula Beran

(1955-2001)

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1. Introduction

Phlebotomine sand flies are of considerable importance for public health. They are the principal vectors of leishmaniosis, a complex worldwide zoonotic, vector-borne disease of humans and animals (mainly dogs) with a range of clinical and epidemiological features (Lawyer and Perkins, 2004; Gramiccia and Gradoni, 2005). In terms of global disease load, leishmaniosis is the third most important vector-borne disease after malaria and lymphatic filariasis. There are 12 million people currently infected and 350 million people at risk worldwide causing the loss of 2.4 million disability-adjusted life years (DALYs) and about 60.000 deaths in 2001 (Desjeux, 2001; WHO, 2002). The disease is acquired by the bite of female phlebotomine sand flies that previously fed on an infected host and therefore transmit *Leishmania* spp., an obligate intramacrophage prototzoan parasite (Herwaldt, 1999). Leishmaniosis is endemic in 88 countries, mainly tropical and subtropical areas including southern Europe. Currently, leishmaniosis shows a wider geographical distribution and increased global incidence of human disease than previously known (Desjeux, 2001). Environmental and demographic factors contribute to a change of leishmaniosis including risk factors for zoonotic cutaneous and visceral leishmaniosis, which is lethal if untreated. To date, no effective vaccine is available despite substantial efforts by many laboratories (Parra et al., 2007). Control measures rely on chemotherapy to alleviate disease and vector control to reduce transmission (Handman, 2001; Reed, 2001; Davies et al., 2003). In Europe, the infection is widespread in the Mediterranean Basin. In endemic areas, transmission is maintained between the parasite, the vector and the host within a particular geographical ecosystem. However, the disease is no longer confined to the poor countries and prevalence is increasing in the EU countries showing a tendency of a northward spread of *Leishmania infantum* transmission with new foci (Dujardin et al., 2008; Maroli et al., 2008). Autochthonous *Leishmania* transmissions have recently been recorded in traditionally non-endemic areas (Gramiccia and Gradoni, 2005). During the last decade, sporadic cases of leishmaniosis were reported as autochthonous in Germany (Bogdan et al., 2001; Koehler et al., 2002; Kellermeier et al., 2007) and public health awareness have since paid new attention to this emerging disease.

Phlebotomine sand flies are widespread to the north of the Tropic of Cancer (Lewis, 1971). Due to rising temperatures, the maximum northern latitude for phlebotomine sand fly survival is currently moving further north, resulting in a new settlement of habitats that could become endemic (Desjeux, 2001; Gramiccia and Gradoni, 2005). Additionally, the increased

movement of infected animals, which could act as reservoirs, from endemic areas might contribute to the spread of the disease (Desjeux, 2001).

The respective role and importance of the infected and non-infected phlebotomine sand flies are not yet clearly determined in all species, especially in *Phlebotomus mascittii* (Killick-Kendrick, 1990), which has sporadically been reported in Baden-Wuerttemberg, Germany (Naucke and Pesson, 2000).

To establish whether populations of phlebotomine sand flies have become endemic in Germany, the present thesis focused on the following four objectives:

(1) Implementation of strategic entomological surveys for phlebotomine sand flies in the Southern federal states of Germany.

(2) Comparison of the applied methodology in an endemic focus in Central Italy.

(3) PCR analysis of trapped phlebotomine sand flies for detection of *Leishmania* spp. infections.

(4) Retrospective data analysis of diagnosed *Leishmania* infections in the Diagnostic laboratory.

This study will increase the data pool on the epidemiology of phlebotomine sand flies, their current distribution and abundance in Southern Germany. Thereby, this survey will be of great importance to the contribution to the knowledge of the *status quo* on the potential risk assessment of contracting leishmaniosis in Southern Germany.

2. Literature Review

2.1. Family of Psychodidae

2.1.1. Taxonomy

The family of Psychodidae is often called "moth flies" due to their conspicuous morphology. The Psychodidae belong to the suborder Nematocerca and are divided into six subfamilies (Fig. 1) that differ in size and appearance (Wagner, 1997), recognised in the "Catalogue of Palaearctic Diptera". Among these subfamilies, the subdivisions of Psychodinae are still being debated. This work is followed by Vaillant's revision in his proposal (1971-1983) "Die Fliegen der palaearktischen Region".

PHYLUM Arthropoda
CLASS Insecta
SUBORDER Nematocerca
ORDER Diptera
FAMILY Psychodidae
SUBFAMILY **Phlebotominae**
Psychodinae
Trichomyiinae
Bruchomyiinae
Sycoracinae
Horaiellinae

Fig. 1: Taxonomy of Psychodidae (adapted from Wagner, 1997).

2.1.2. Morphology

Psychodidae are small, dark, squat Diptera with an erratic flight and densely hairy bodies (Fig. 2) and oval wings, which are held roof-like or horizontally over the body at rest (Lane, 1993). Three main features distinguish the subfamilies of Psychodidae morphologically: **wing**, **antenna** and **genitalia** of male and female.

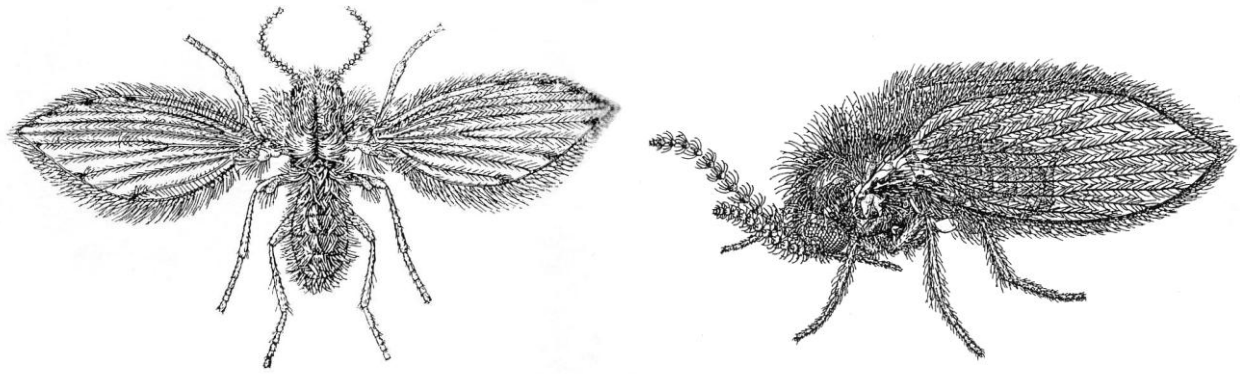

Fig. 2: Appearance of Psychodidae: (left) hairy appearances of an adult fly; (right) typical wing posture at resting position (Ordman, 1946).

The venation is characterised by 9-10 parallel, longitudinal veins. Cross-veins are almost absent. Intersection separating veins R_{2+3} and R_4 appear towards the base of the wing (Fig. 3).

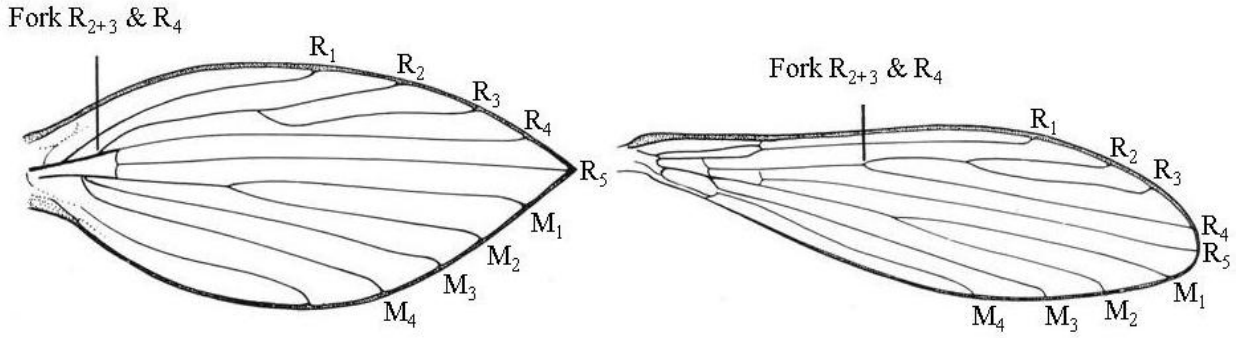

Fig. 3: Wing venation: (left) of Psychodinae (right) of Phlebotominae; R = radial vein, M = medial vein (Kettle, 1984).

Normally, the long antenna consists of scape, pedicel and 10-14 bottle- or barrel-shaped flagellomeres. Male flagellomeres are covered with ascoids (receptors) varying in shape (Y-, S-shaped). The mouthparts are not piercing because, apart from females of Phlebotominae and Sycoracinae, flies are not haematophagous. Their eyes are circular in all subfamilies except Psychodinae (eyes reniform "eye-bridge"). The abdomen with sternite I is unsclerotized or completely reduced (Psychodinae) which gives them the typical hump-backed psychodid appearance. Male genitalia supply various morphological features for species differentiation. Female genitalia show a uniform appearance, and distinction at the generic level is often impossible (Wagner, 1997).

2.1.3 Biology

Psychodid flies are barely of economic or health importance except the group of Phlebotominae. Only a few species can be found e.g. in sewage treatment plants. Psychodinae larvae in particular feed on organic matter and bacteria. Some are important in facilitating filtration at sewage plants by keeping them free from accumulated organic material. One species of Psycho-dinae, *Tinearia alternata* (Say) is a cosmopolitan filter fly and larvae can cause myiasis. Some species are found in houses, especially in toilets (Ordman, 1946; Wagner, 1997).

2.2. Subfamily of Phlebotominae

The name sand fly is a misnomer and confuses the layman since in some parts of the world midges (genus *Culicoides*) or black flies (Family Simulidae) are called the same. Vectors of leishmaniosis are correctly termed phlebotomine (literally "vein cutter") sand flies (Killick-Kendrick, 1999).

2.2.1. Taxonomy

The higher classification of sand flies is still a matter of controversy and no universally accepted system exists. Following Fairchild (1955), Lewis (1978, 1982) and Theodor (1958) sand flies retain a subfamily status within the Psychodidae. Lewis et al. (1977) proposed a conservative and stable classification for phlebotomine sand flies and defined five genera with a large number of subgenera. Later, Lenk (1987) added another genus *Chinius*. There are six genera altogehter. Three of which exist in the Old World (Europe, Africa and Asia): *Phlebotomus, Sergentomyia* and *Chinius*. The other three exist in the New World (North, Central and South America): *Lutzomyia, Brumptomyia* and *Warileya*. Only two of these genera are of medical importance. One is *Phlebotomus,* separated into 12 subgenera and the other is *Lutzomyia*, divided into 25 subgenera and species group. Out of more than 700 species that are involved in the transmission of disease to man, all proven vectors of leishmaniosis to date are among 70 species of these two genera (Lane, 1993; Killick-Kendrick, 1999).

The genus *Phlebotomus* contains almost all man- and mammal-biting sand flies, and represents the only vector of pathogen to humans in the Old World. *Lutzomyia*, the largest sand fly genus feeds on both mammals and reptiles and is distributed in Nearctic regions throughout

North and Neotropical - Central and South America. The huge genus of *Sergentomyia* is distributed throughout the Old World and primarily feeds on reptiles and amphibians (Adler and Theodor, 1957). This genus contains no known vectors of leishmaniosis. Finally, *Brumptomyia* is associated with armadillos, on which they feed predominantly (Lane, 1993; Kettle, 1984).

2.2.2. Morphology

Phlebotomine sand flies are very small, fragile, fuzzy and densely hairy with narrow bodies and a body length that seldom exceeds 3-5 mm. Their colour appearance ranges from pale to dark-gray to dusty brown and almost black (Lane, 1993; Killick-Kendrick, 1999). Typical features of recognition of phlebotomine sand flies include:

Resting position: wings are held upward above the abdomen so that costal margins form an angle of approximately 60°, "V" shaped (Fig. 4)

Movement: phlebotomines typically hop around on the host before settling down to bite

Thorax: dorsal surface is covered in long slender scales, giving the hairy appearance

Wings: lanceolate wings with numerous of fine hair, radial sector is four-branched, the intersection of R_{2+3} and R_4 occurs at about the middle of the wing (Fig. 3)

Antenna: 16 segments, pilose, no sexual dimorphism

Eye: relatively large

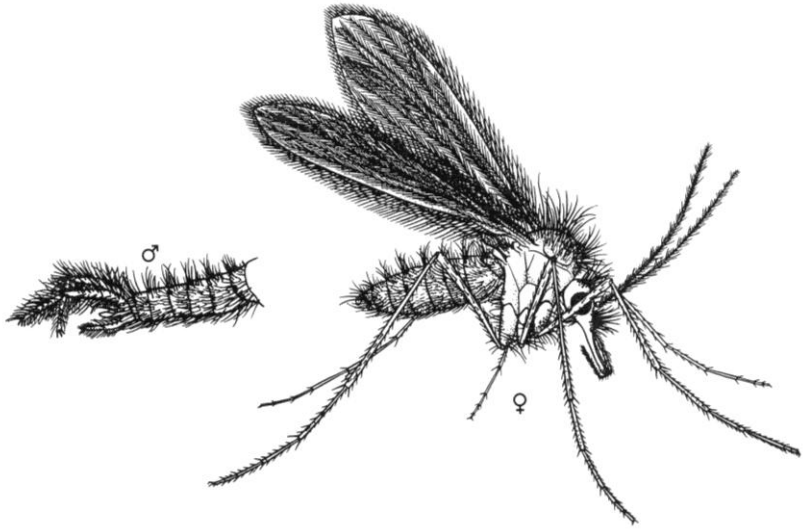

Fig. 4: Typical resting position of *Phlebotomus* spp., lateral view: (left) terminal abdominal segment of the male *P. papatasi*; (right) female of *P. papatasi* (Lane, 1993).

For the characterisation of different sand flies species, certain features have to be taken into consideration. The head contains many taxonomically important characters, especially in the cibarium, pharynx and antennae. The cibarium and pharynx are armed with teeth and other sclerotized structures (Fig. 5). The number, size and arrangement of cibarial and pharyngial teeth are of considerable importance in distinguishing genera (Lane, 1993). Adapted for blood feeding, females' mouth-parts are moderately long and compromise a fascicle of six blade-like stylet (labrum, paired mandibles, maxillae and hypopharynx). Mandibles of males are absent because they are not haematophagous.

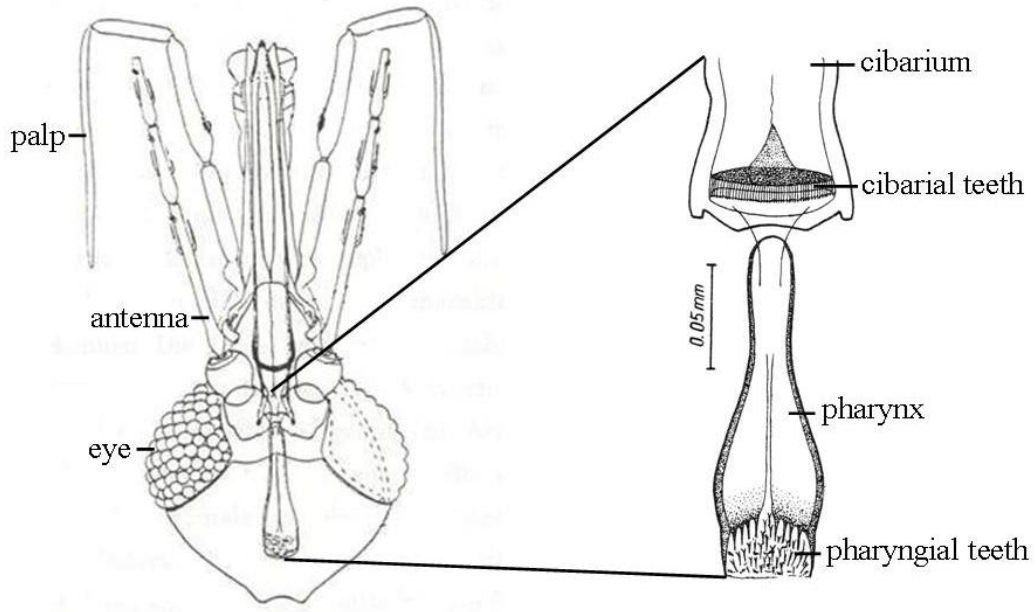

Fig. 5: Caput structures of phlebotomine sand flies (dorsal), enlargement of cibarium and pharynx (adapted after Theodor, 1958).

Wing venation is extensively used to distinguish genera and to differentiate species. In general, *Sergentomyia* has narrower and more pointed wings than *Phlebotomus* and species of *Lutzomyia*. Furthermore, length ratios in combinations of wing veins, antennal and leg segments or mouthparts are used for determination.

Females' genitalia**,** the abdomen is plumb cylindrical and appears to end with two rounded segments (cerci). Internally, there is a sclerotized genital fork (shaped like a "Y"), common or individual sperm ducts and paired spermathecae, which vary in size and shape (Fig. 6, left). The base of the common spermathecal ducts has been used to separate species of *Phlebotomus* (*Larroussius*), which were almost indistinguishable (Léger et al., 1983).

Males' genitalia feature characteristically elaborate, bilaterally symmetrical external clasping structures (coxite and style), parameres, sclerotized aedeagus and surstyle on the distal end of their abdomen (Fig. 6, right). Internally, the genital filaments are connected to a sperm-pump, which ejects the sperm in a spermatophore (Lane, 1993; Killick-Kendrick, 1999; Lawyer and Perkins, 2004). Jobling (1987) made the detailed anatomical drawings.

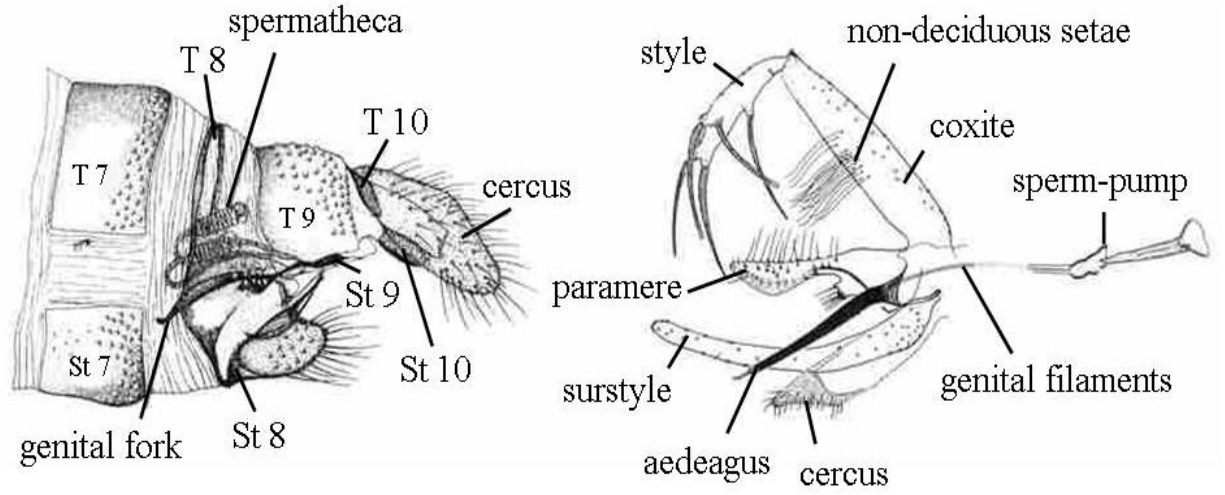

Fig. 6: Lateral view of genitalia of phlebotomine sand flies: (left) position of spermathecae relative to other features of the terminal segments of the female abdomen; (right) male genitalia; St = sternite, T = tergite (Lane, 1993).

2.2.3. Distribution and Habitat

Sand flies are generally found in warm parts of the world: Central and South America, Africa, Australia, Asia and southern Europe, with a few species ranging into temperate zones of the northern (to 50°N) and southern (to 40°S) hemispheres. They are only absent from New Zealand and the Pacific islands. Distribution is limited to areas where temperatures are above 15.6°C for at least three months of the year (Lane, 1993). Sand flies occur in a wide variety of habitats from below sea level in areas surrounding the Dead Sea in Israel and Jordan, to 2800 m above sea level in the Andes, and from hot deserts, through savannas and open woodland to dense tropical rain forest. Many species require special ecological niches coincident with conditions around human and domestic animal dwellings (Lawyer and Perkins, 2004). Phlebotomines rest by day at different sites in dark cool and humid places where the microclimate is favourable for survival: houses, latrines, cellars, stables; caves, fissures in walls,

rocks or soil; dense vegetation, tree holes and buttresses; burrows of rodents and other mammals or bird's nests (Killick-Kendrick, 1999). Natural breeding sites of sand flies are poorly characterised. The first finding of a sandfly larva by Grassi (1907) was described as a new species, *Phlebotomus mascittii* Grassi, 1908 that was found in a cellar in Rome. Grassi's work is considered to be the first report of an immature stage of any phlebotomine sandfly in nature. Searching for developmental stage of sand flies in their natural biotopes is still tedious and has proven to be unproductive (Killick-Kendrick, 1987, 1999).

2.2.4. Activity and Dispersal

Phlebotomine sand flies appear to be weak fliers compared to other nematocerous biting flies (mosquitos, black flies, ceratopogonids). In nature, they are most commonly seen to make short hopping movements, but when disturbed, they move forward in a sustained flight of undetermined duration. The activity of sand flies is nocturnal or crepuscular, although some species are active during daylight. Their flight capacity is limited and sand flies tend to remain localized (Chaniotis et al., 1974). Slight air movement aids the detection of hosts along odor plumes. However, wind speeds exceeding 1.5 m/sec inhibit flight and there is no activity above 4-5 m/sec of wind speed (Lane, 1993). Most species of sand flies fly horizontally near ground level, probably in an effort to avoid higher wind speeds. Sand flies inhabiting forest also move vertically between the forest floor and canopy but rarely fly further than 200 m from their resting site (Chaniotis et al., 1974; Ready et al., 1986; Alexander, 1987). Nevertheless, Killick-Kendrick et al. (1984) discovered that their long-range movement can be up to 2.2 km in an open habitat over a period of a few days.

2.2.5. Life Cycle

Sand flies, like all Diptera, are holometabolic insects that go through a complete metamorphosis. The development includes the preimaginal stages - the embryonated egg, four larval stages, and pupa - and the imaginal stage - adult (Fig. 7).

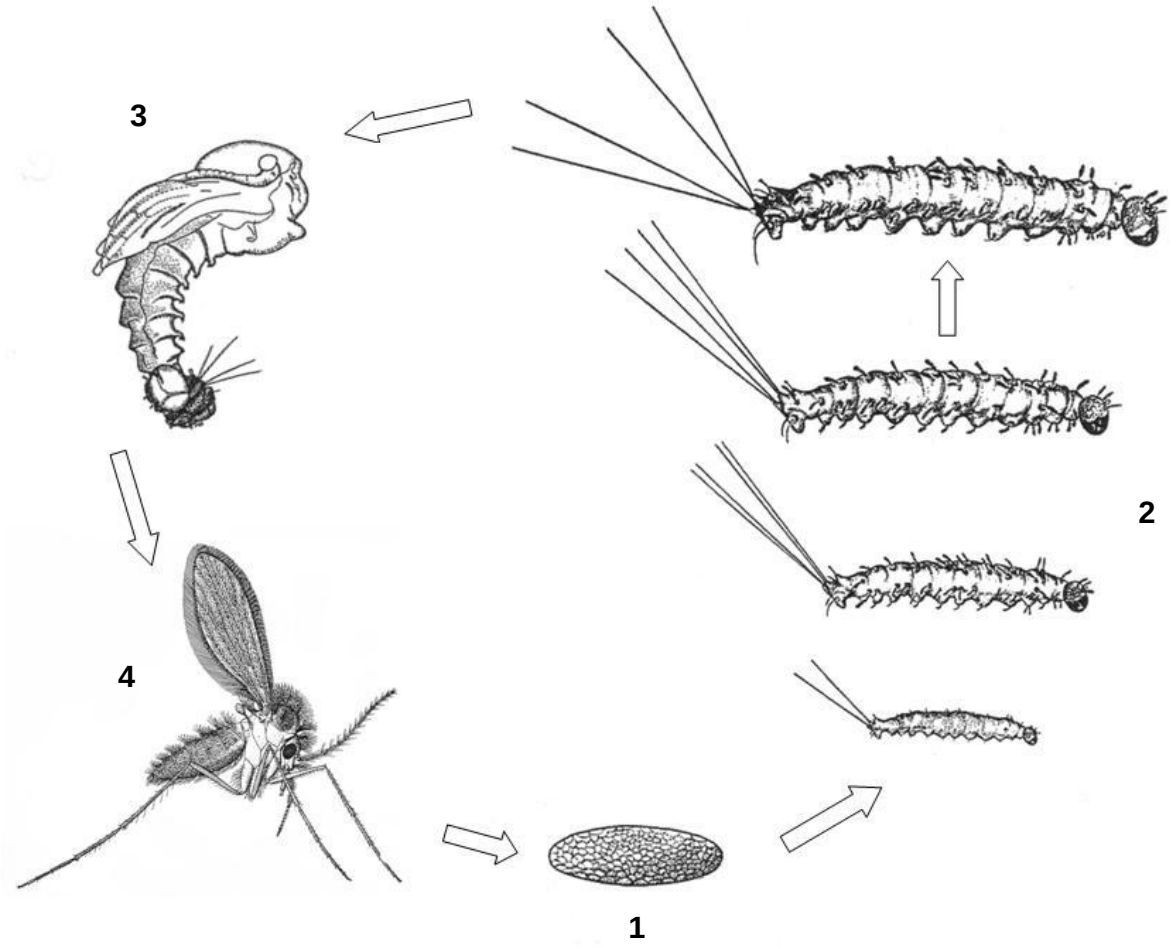

Fig. 7: Sand fly life cycle showing (1) egg, (2) four larval instars, (3) pupa and (4) adult (adapted after Lawyer and Perkins, 2004).

Females deposit eggs (banana shaped, sculptured chorion) singly or in small batches on various grounds providing moisture and a relative constant temperature of 15-26°C. These eggs usually hatch within 10 days of post-oviposition. Some sand fly species undergo diapause or quiescence triggered by environmental extremes such as hot, dry summers or cooler temperatures and shorter photoperiods (Killick-Kendrick, 1978 a; Lawyer and Young, 1991). Larvae are known to be terrestrial and pass through four instars before pupating. First stage larvae, with one pair of caudal setae, develop within 3-8 weeks and start immediately to feed on organic matter. The second to fourth instars have two pairs of setae. The fourth stage larvae stops feeding and seek a drier place to pupate. Attached to a dead leave or stone, the pupal stage lasts 7-12 days. Finally, adults emerge, males generally 24-48 hours before females (Lawyer and Perkins, 2004).

2.2.6 Feeding Behaviour

Both male and female adult sand flies require carbohydrates for energy and longevity. Therefore, they feed on natural sources of sugar (fructose, glucose, sucrose), such as sap of plants, including floral and extra-floral nectars, ripe fruits and honeydew of aphids (Killick-Kendrick and Killick-Kendrick, 1987; Schlein and Raymond, 1999). Additionally, females need to feed on blood, which provides nutrition for the production of eggs. Species vary in intake of blood meals during a gonotrophic cycle. Some will take more than one blood meal on different days, whereas others feed only one time for each batch of eggs. Few man-biting species that are autogeny, such as *Phlebotomus papatasi* and *Lutzomyia gomezi*, are exceptional due to their ability to lay eggs without a blood meal (El Kammah, 1972; Killick-Kendrick, 1978 a). Suitable combinations of air movement, ambient temperature, light intensity, relative humidity and other exogenous factors stimulate hungry sand flies to search for blood meals (Young and Lawyer, 1987). Frequent blood meals increase the contact between vertebrates and vectors and thus the possibility of transmitting leishmaniosis. Feeding takes place on exposed parts of the body and preferred areas are the ears and feet of rodents, the noses of dogs and the bellies of cattle (Lane, 1993). For pool feeders, sand fly bites are quite painful. Most anthropophilic species feed during the evening at dawn, when temperatures drop and relative humidity rises. Forest dwellers like *L. wellcomei*, *L. carrerai* and *L. pesoana* attack during daytime if their habitat is disturbed (Lawyer and Perkins, 2004).

2.2.7. Vectorial Competence

Phlebotomines are rarely present in sufficient density to reach pest proportion. Nevertheless, they are medically important vectors of various pathogens. Sand flies transmit bacteria like *Bartonella* causing bartonellosis (Oroya fever, Carrion's disease), the protozoon *Leishmania* (visceral leishmaniosis – kala azar, cutaneous leishmaniosis – oriental sore, espundia) and various types of viruses causing sandfly fevers (papataci fever) exemplary phleoboviruses, orbiviruses, vesiculoviruses (vesicular stomatitis) and flaviviruses (Comer and Tesh, 1991; Lane, 1993; Desjeux, 1996; Killick-Kendrick, 1999; Birtles, 2001). These pathogens are mostly zoonotic pathogens in which man becomes involved by entering the endemic areas. As a vector of leishmaniosis, the disease is affecting people in more than 80 countries worldwide. Sand flies transmit various *Leishmania* spp., which comprise more than 40 species of

Phlebotomus in the Old World and 30 species of *Lutzomyia* in the New World (WHO, 1990). Under natural conditions, sand flies transmit low numbers of promastigotes (100-1000), which are sufficient to induce a disease in susceptible hosts (Ferrer, 2002).

2.2.8. Phlebotomine sand flies in parts of Europe

Screening the literature until 2009, there are 25 species of phlebotomine sand flies belonging to two genera of *Phlebotomus* and *Sergentomyia* in Europe (Tab. 1). Countries involved in the survey range from Southern Europe (Portugal, Spain, Italy and Malta), Western Europe (France and Belgium), Central Europe (Germany, Switzerland, Austria, Hungary and Croatia) and South-Eastern Europe (Greece, Cyprus and the Balkan States).

Tab. 1: Occurrence of sand fly species in parts of Europe. References are provided in the last column.

Genus (Subgenus) Species	Country	Reference
Phlebotomus (*Paraphlebotomus*) *alexandri*, Sinton 1928	Cyprus, Greece, Spain	Léger et al., 2000 b; Ivović et al., 2007; Rioux et al., 1974 a
Phlebotomus (*Larroussius*) *ariasi*, Tonnoir 1921 a	France, Italy Portugal, Spain	Rioux et al., 1973; Maroli et al., 2008; Schrey et al., 1989; Guilvard et al., 1996
Phlebotomus (*Adlerius*) *balcanicus*, Theodor 1958	Greece, Serbia	Ivović et al., 2007; Miščevic et al., 1998
Phlebotomus (*Paraphlebotomus*) *chabaudi*, Croset et al. 1970	Spain	Rioux et al., 1974 b
Phlebotomus (Transphlebotomus) economidesi, Léger et al. 2000 a	Cyprus	Léger et al., 2000 a
Phlebotomus (Anaphlebotomus) *fortunatarum*, Ubeda Ontiveros et al. 1982	Canary Islands	Lane and Alexander, 1988
Phlebotomus (*Larroussius*) *galilaeus*, Theodor 1958	Cyprus	Léger et al., 2000 b
Phlebotomus (Paraphlebotomus) jacusieli, Theodor 1947	Cyprus, Greece	Léger et al., 2000 b; Depaquit et al., 1996

Phlebotomus (Larroussious) kandelakii, Shurenkova 1929	Montenegro	Ivović et al., 2004
Phlebotomus (*Larroussius*) *langeroni*, Nitzulescu 1930	Spain	Martínez-Ortega et al., 1996
Phlebotomus (*Larroussius*) *longicuspis*, Nitzulescu 1930	Spain	Martínez-Ortega et al., 1982
Phlebotomus (*Transphlebotomus*) *mascittii*, Grassi 1908	Croatia, Cyprus, Belgium, France, **Germany**, Greece, Italy, Spain, Switzerland	Bosnić et al., 2006; Léger et al., 2000 b; Depaquit et al., 2005; Rioux et al., 1984 b; Naucke and Pesson, 2000; Ivocić et al., 2007; Maroli et al., 2002; Rioux et al., 1984 b; Grimm et al., 1993
Phlebotomus (*Larroussius*) *neglectus*, Tonnoir 1921 b	Albania, Croatia, Greece, Hungary, Italy, Malta, Montenegro, Serbia	Velo et al., 2005; Bosnić et al., 2006; Ivović et al., 2007; Farkas et al., 2009; Maroli et al., 2002; Adler and Theodor, 1935; Ivović et al., 2004; Miščevic et al., 1998
Phlebotomus (*Phlebotomus*) *papatasi*, Scopoli 1786	Albania, Cyprus, France, Greece, Italy, Malta, Montenegro, Portugal, Serbia, Spain,	Velo et al., 2005; Léger et al., 2000 b; Raynal, 1954; Ivović et al., 2007; Maroli et al., 2002; Adler and Theodor, 1935; Ivović et al., 2004; Lane and Fritz, 1986; Miščevic et al., 1998; Conesa Gallego et al., 1999;
Phlebotomus (*Larroussius*) *perniciosus*, Newstead 1911	Croatia, France, **Germany**, Italy, Malta, Portugal, Spain, Switzerland	Biševac et al., 1990; Izri et al., 1992; Naucke and Schmitt, 2004; Maroli et al., 2002; Adler and Theodor, 1935; Schrey et al., 1989; Aransay et al., 2004; Grimm et al., 1993
Phlebotomus (*Larroussius*) *perfiliewi*, Parrot 1930	Albania, Croatia, Greece, Italy, Malta, Montenegro, Macedonia, Montenegro, Serbia	Kero and Xinxo, 1998; Bosnić et al., 2006; Ivović et al., 2007; Maroli et al., 2008; Adler and Theodor, 1935; Ivović et al., 2004; Miščevic et al., 1998
Phlebotomus (*Paraphlebotomus*) *riouxi*, nov. spec.[1]	Spain	Depaquit et al., 1998

Phlebotomus (*Paraphlebotomus*) *sergenti*, Parrot 1917	Canary Islands, Cyprus, France, Greece, Italy, Malta, Portugal, Spain	Lane and Alexander, 1988; Léger et.al., 2000 b; Rioux et al., 1982; Papadopoulos and Tselentis, 1998; D'Urso et al., 2004; Adler and Theodor, 1935; Schrey et al., 1989; Romera Lozano and Martínez Ortega, 2001
Phlebotomus (*Adlerius*) *simici*, Nitzulescu & Nitzulescu 1931	Bosnia, Macedonia, Serbia,	Simitch and Živković, 1956;
	Greece	Papadopoulos and Tselentis, 1998
Phlebotomus (Paraphlebotomus) similis, Perfiliewi 1963	Albania, Croatia, Greece,	Kero and Xinxo, 1998; Biševac et al., 1990; Ivović et al., 2007;
	Macedonia, Serbia	Simitch and Živković, 1956
Phlebotomus (*Larroussius*) *tobbi*, Adler et al. 1930	Albania, Croatia, Cyprus, Greece, Italy, Montenegro, Serbia,	Velo et al., 2005; Bosnić et al., 2006; Léger et al., 2000 b; Ivović et al., 2007; Lewis, 1982; Ivović et al., 2004; Miščevic and Milutinović, 1987;
	Bosnia, Macedonia, Slovenia	Simitch and Živković, 1956
Sergentomyia (Sergentomyia) azizi, Adler 1946	Cyprus	Léger et al., 2000 b
Sergentomyia (*Sergentomyia*) *dentata*, Sinton 1933	Greece, Macedonia	Ivović et al., 2007; Miščevic et al., 1998
Sergentomyia (*Sergentomyia*) *fallax*, Parrot 1921	Canary Islands, Cyprus	Lane and Alexander, 1988; Léger et al., 2000 b
Sergentomyia (*Sergentomyia*) *minuta*, Rondani 1843	Albania, Bosnia, Macedonia, Serbia,	Miščevic et al., 1998;
	Canary Islands, Croatia, Cyprus, France, Greece, Italy, Malta, Montenegro, Portugal, Spain, Switzerland	Lane and Alexander, 1988; Bosnić et al., 2006; Léger et al., 2000 b; Léger et al., 1985; Ivović et al., 2007; Maroli et al., 2002; Léger et al., 1991; Ivović et al., 2004; Schrey et al., 1989; Benito-De Martin et al., 1991; Grimm et al., 1993

[1] nova species, mentioned for the first time in the literature

A detailed literature analysis of the presence of phlebotomine sand flies in different districts of France, Spain, Portugal, Italy, Greece and the Balkan Peninsula is given by Weise (2004), including all available literature until 2004.

2.3. *Leishmania*

2.3.1. Systematic and Morphology

Leishmania are protozoan hemoflagellates (2-5µm large) belonging to the order of Kinetoplastida (Fig. 8), which includes parasites possessing a kinetoplast, a deeply staining structure close to the end of a flagellum. A single locomotory flagellum, free or attached to a pellicle as an undulating membrane, originates from the kinetoplast. All species are intracellular parasites that undergo an obligate change of host and propagate in their respective invertebrate host. *Leishmania* invade and proliferate in mononuclear phagocytic cells of the vertebrate host. Within both hosts, parasites alter their morphology (Lawyer and Perkins, 2004). Wiliam Leishman and Charles Donovan are the investigators, who independently of each other, identified the disease and its etiological agents, the protozoan *Leishmania donovani*, in splenic tissue from patients in India in 1903 (Leishman, 1903; Donovan, 1903).

Kingdom Protista
Subkingdom Protozoa

Phylum Sarcomastipophora
Subphylum Mastigophora

Class Zoomastigophora

Order Kinetoplastida
Suborder Trypanomatina

Family Trypanosomatidae

Genera **Leishmania**
Trypanosoma

Fig. 8: Classification of *Leishmania* spp. (after Molyneux and Ashford, 1983).

Species of the genus *Leishmania* are classified into two subgenera. The subgenus *Leishmania* includes pathogenic medically important vectors of the Old World species: *L. tropica*, *L. aethiopica*, *L. major*, *L. infantum* and *L. donovani*. Restricted to the New World, the medical important species of the *L. mexicana* group comprise *L. mexicana*, *L. amazonensis*, *L. venezuelensis* and *L. pifanoi*. The remaining Neotropical species have no medical importance. The other subgenus, *Viannia*, is found only in Central and South America. The most important species include *L. braziliensis*, *L. guyanensis*, *L. panamensis* and *L. peruviana*, all of which cause human diseases (Killick-Kendrick, 2002; Schuster and Sullivan, 2002).

2.3.2. Geographical Distribution

According to the available literature, human leishmaniosis is endemic in four continents, in 22 New World and 66 Old World countries and has an estimated yearly incidence of 1-1.5 million cases of cutaneous leishmaniosis (CL) forms and half a million cases of visceral leishmaniosis (VL) forms (Desjeux, 1996). Among 15 well-recognised *Leishmania* species known to infect humans, 13 have zoonotic nature, which include pathogens of visceral, cutaneous and mucocutaneous forms of the disease (Tab. 2) in both the Old and the New Worlds (Gramiccia and Gradoni, 2005). In the Old World, throughout the Mediterranean Basin of North Africa, the Middle East and Southern Europe, the CL forms occur predominantly. It is partly endemic in sub-Sahara Africa, Southern Asia, the western parts of India and China. In the New World, it can be found from Texas (USA) to Argentina in South America. Mucocutaneos leishmaniosis (MCL) occurs mostly in the New World – Brazil and Central America. The most severe form, VL, is found in the Mediterranean Basin and occurs in countries of Northern Africa, the Middle East and Southern Europe as well as in Eastern Africa, South Central Asia and China (Lawyer and Perkins, 2004). The incidence of leishmaniosis is not uniformly distributed in endemic foci. About 90 % of CL cases appear in Afghanistan, Algeria, Brazil, Iran, Peru, Saudi Arabia and Syria. In terms of VL, 90 % of those cases occur in rural and suburban areas of Bangladesh, India, Nepal, Sudan and Brazil (Gramiccia and Gradoni, 2005).

Tab. 2: Overview of clinical manifestations of leishmaniosis in humans (Neuber, 2008).

Clinical forms	Pathogens	Region
Visceral leishmaniosis (kala-azar, dumdum fever)	*L. d. donovani*	China, India, Iran, Sudan, Kenya, Ethiopia
	L. d. infantum	Mediterranean countries
	L. d. chagasi	Brazil, Columbia, Venezuela, Argentina
Cutaneous leishmaniosis (oriental sore, tropical sore)	*L. tropica*	Mediterranean countries, Afghanistan
	L. major	Middle East, Western and Northern Africa, Kenya
	L. aethiopica	Ethiopia
	L. mexicana	Central America, Amazon regions
Mucocutaneous leishmaniosis (espundia)	*L.-braziliensis complex*	Brazil, Peru, Ecuador, Columbia, Venezuela

2.3.3. Situation in Europe

In Europe (Turkey not included), leishmaniosis is essentially associated with one etiologic, pathogenic form of *Leishmania*, ***L. infantum***, which is transmitted by phlebotomine sand flies. Five major species of the subgenus *Larroussius* (*P. ariasi, P. neglectus, P. perfiliewi, P. perniciosus* and *P. tobbi*) are proven competent vectors in the Mediterranean area of Europe (Tab. 3). Although they are not equally distributed, more than one species of this subgenus may transmit the parasite at the same place (Killick-Kendrick, 1990). Some phlebotomine sand flies are suspected to be potential vectors, but none of their detected female specimens were infected. Their vector competence has not been proven yet.

Until recently, cutaneous leishmaniosis of the western Mediterranean was thought to also be caused by *L. tropica*. However, isolation and typing of parasites from patients with cutaneous leishmaniosis in France, Spain, Italy and Malta revealed only one species of *Leishmania, L. infantum* (Killick-Kendrick, 1990). Currently, only in Greece (continental and insular) two pathogenic forms of *Leishmania*, ***L. infantum*** and ***L. tropica***, are present at the same time (Tzamouranis et al., 1984; Chaniotis and Tselentis, 1994). *Phlebotomus sergenti* is the most

probable vector of *L. tropica* in Greece (Killick-Kendrick, 1990). According to a very recent report, the first autochthonous cases of ***L. donovani*** in Europe have been detected in Cyprus (Antoniou et al., 2008).

Tab. 3: Competent vectors of *L. infantum* in Europe (Turkey excluded).

Vector	Country	Reference
*P. neglectus**	Albania	Velo et al., 2003
P. tobbi, P. neglectus	Croatia	Bosnić et al., 2006
P. tobbi	Cyprus	Léger et al., 2000 b
P. ariasi, P. perniciosus, P. papatasi, P. sergenti**	France	Rioux et al., 1984 ba; Dereure et al., 1999; Killick-Kendrick and Killick-Kendrick, 1999
P. neglectus, P. tobbi, P. perfiliewi, P. sergenti, P. similes, P. papatasi**	Greece	Léger et al., 1988; Papadopoulos and Tselentis, 1998; Killick-Kendrick, 1990; Killick-Kendrick and Killick-Kendrick, 1999
P. perfiliewi, P. perniciosus, P. ariasi, P. neglectus*, P. tobbi**	Italy	Bettini et al., 1986; Maroli et al., 1987; Killick-Kendrick and Killick-Kendrick, 1999
P. perniciosus, P. neclectus, P. perfiliewi*, P. sergenti*, P. papatasi**	Malta	Adler and Theodor, 1935; Gradoni and Gramiccia, 1990; Killick-Kendrick and Killick-Kendrick, 1999
*P. neglectus, P. kandelakii**	Montenegro	Ivović et al., 2004
P. ariasi, P. perniciosus	Portugal	Pires, 1984; Campino et al., 2006
P. ariasi, P. perniciosus, P. langeroni,P. papatasi, P. sergenti*,P. longicuspis**	Spain	Killick-Kendrick, 1990; Killick-Kendrick and Killick-Kendrick, 1999; Aransay et al., 2004; Martín-Sánchez et al., 2006

* suspected potential vectors

2.3.4. Life Cycle

Leishmania are transmitted by the bite of female phlebotomine sand flies (*Phlebotomus* spp.). While biting, females inject saliva into the skin, causing allergic reactions, and inject the infective stages, promastigotes, during blood meals (Fig. 9). In the mammalian host, *Leishmania* parasites occupy an intracellular niche and transform into amastigotes, mainly within macrophages of the host. They exist within a parasitophorous vacuole protected from and unaffected by cellular digestive enzymes. Amastigotes proliferate in infected cells and affect different tissues, depending on the *Leishmania* species. When a female sand fly bites an infected vertebrate host, amastigotes forms of the parasite are ingested with the blood meal. In the sand fly's midgut, the parasites differentiate into elongated, motile promastigotes, which rapidly divide by binary fission, migrate to the proboscis and are finally transmitted to the next host during the following blood meal (Killick-Kendrick, 2002; Schuster and Sullivan, 2002).

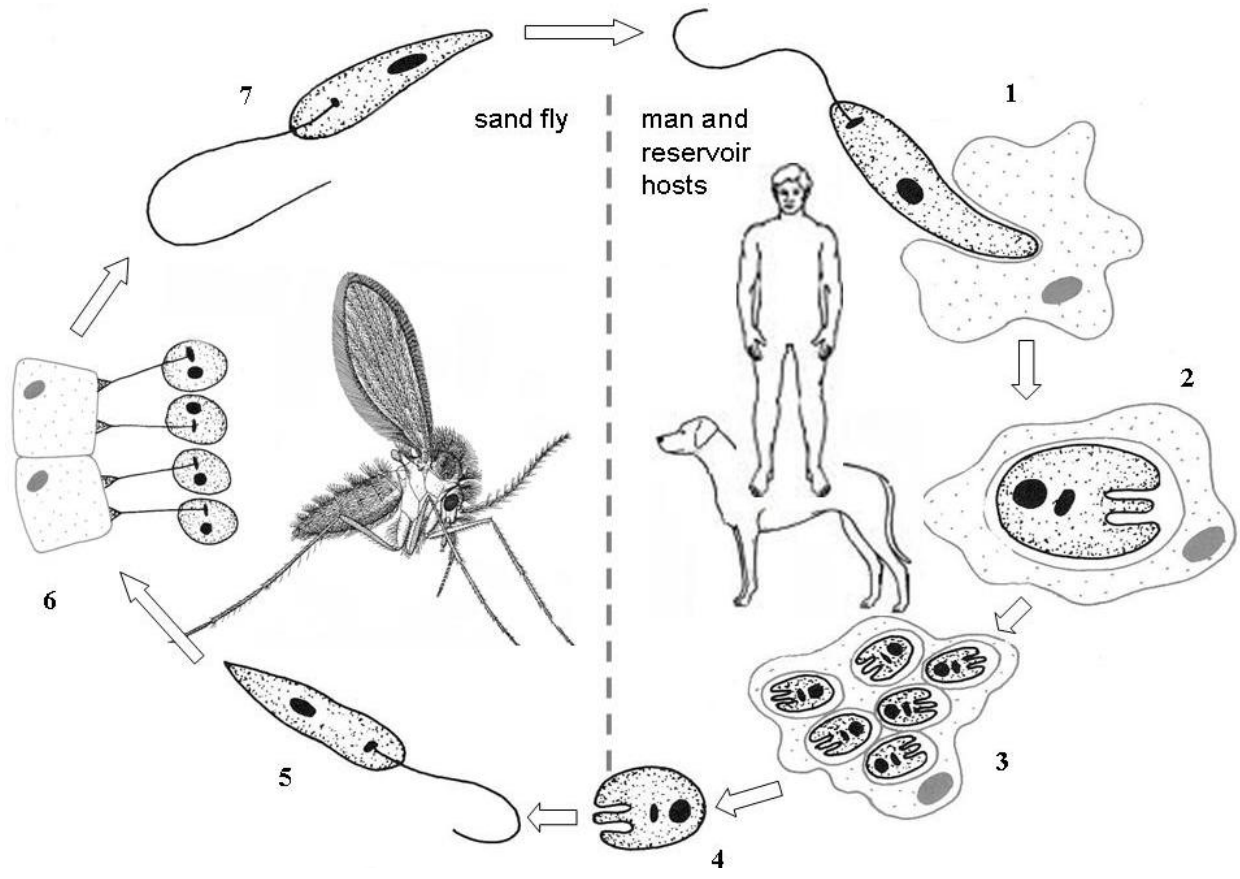

Fig. 9: Life cycle of *Leishmania* spp. (1) invasion of promastigote into macrophage, (2) transformation into amastigote, (3) multiplication of amastigote, (4) free amastigote, (5) differentiation into promastigote, (6) attachment of promastigotes to the midgut, (7) metacyclic promastigote (adapted after Lucius and Loos-Frank, 1997).

2.3.5. Vectors, Transmission and Reservoirs

As described in chapter 2.2.7., phlebotomine sand flies are the only known active vectors of *Leishmania*. Suggestions that transmission may take place through the bite of other haematophagous invertebrates other than sand flies have not been confirmed experimentally (Killick-Kendrick, 2002). In humans, several different, albeit extremely rare transmission-routes are thought to lead to venereal infections (Symmers, 1960), transplacental transmission (Rosypal et al., 2005), blood transfusion (Bruce-Chwatt, 1972; Owens et al., 2001) and contaminated needles used by drug addicts (Cruz et al., 2002; Molina et al., 2003). Slappendel and Teske (1999) reported about infected dogs that have never been to endemic areas but have co-habited with *Leishmania*-positive dogs. There is still a lack of knowledge how transmission takes place from one animal to another, but it is presumed to be by direct contact.

The three possible mechanisms of transmission of leishmaniosis by the bite of an infected sand fly are as follows: (1) regurgitation of metacyclic promastigotes from the thoracic midgut into the skin during the act of biting (Schlein et al., 1992); (2) deposition of metacyclic promastigotes from the proboscis into the skin (Killick-Kendrick, 1979); (3) inoculation of metacyclic promastigotes from the salivary glands into the skin with the saliva (Killick-Kendrick et al., 1996). Of these, the first mechanism seems to be the most common way by which *Leishmania* spp. are usually transmitted.

Dogs are the main peridomestic reservoirs for zoonotic leishmaniosis in the Mediterranean area with a seroprevalence ranging from two to almost 80 % (Miró et al., 2007; Solano-Gallego et al., 2007). Foxes, jackals and wolves are considered to be the sylvatic ones in other countries, additionally sloths and armadillos in Latin America (Gramiccia and Gradoni, 2005). It is also well known that rodents play a role as reservoirs of *Leishmania*. The black rat, *Rattus rattus*, in particular is claimed to be a possible reservoir of *Leishmania infantum* in Italy, Spain and Saudi Arabia (Gradoni et al., 1983). In Southern Italy, *Rattus norvegicus*, *R. rattus* and *Mus musculus* were found to be serologically positive for *Leishmania* (Di Bella et al., 2003). Reports of Petrovic et al. (1975) indicated the presence of *L. donovani* infections in *R. rattus* and *R. norvegicus* in some areas of former Yugoslavia. Additionally, some reports suggested the possibility that other animals like the red fox, *Vulpes vulpes*, and rodents are involved in the epidemiological cycle of the protozoon (Ashford and Bettini, 1987). The role of cats as reservoirs of *Leishmania* is still controversial. It is hypothesized that cats are secondary

reservoirs, rather than accidental ones (Gramiccia and Gradoni, 2005; Solano-Gallego et al., 2007).

2.3.6. Manifestations of Disease

Leishmaniosis is manifested in humans in various clinical forms: cutaneous (CL), mucocutaneous (MCL) and visceral (VL). The characterisation of the disease depends on the species of *Leishmania*, the immunological responses of the individual and other factors (Lawyer and Perkins, 2004). In general, during the course of the disease the parasite spreads into the following organs: the lymph nodes, spleen, bone marrow, liver, kidneys, pancreas, testicles, lungs, eyes, joints and bones. All these organs can develop a granulomatous reaction with variable numbers of amastigotes. Due to a complex immune reaction vasculitis, polyarthritis, uveitis and glomerulonephritis can be caused. Cachexia, lymph node enlargement and hepatosplenomegaly are common. Cutaneous lesions are also very prevalent (Ferrer, 2002).

The clinical symptoms of CL are typically non-healing nodulo-ulcers. Usually lesions emerge within weeks or months after being bitten by a sand fly and slowly evolve from papulae to a nodule and finally to an ulcer. These ulcers are found on areas of the skin that are typically uncovered such as hands, the face, or lower legs. Cutaneous lesions resolve quickly after two to three months without treatment or become chronic, lasting months or years. Scarring accompanies the healing process (Lawyer and Perkins, 2004; Neuber, 2008). The clinical manifestation of MCL is the development of ulcerative or granulomatous lesions of the nasal, oral and pharyngeal mucosa. The disease results in disfiguration and tissue destruction (Lawyer and Perkins, 2004). The most severe and life-threatening form of leishmaniosis is VL, with a mortality as high as 95 % in untreated cases. It is a chronic disease characterized by fever, lymphadenopathy, hepatosplenomegaly, anaemia, plus progressive emaciation and weakness caused by the parasite proliferation in macrophages and organs associated with the reticuloendothelial system (Lainson, 1983; Gramiccia and Gradoni, 2005).

In the Mediterranean area, leishmaniosis in dogs is referred to as canine leishmaniosis (CanL) and occurs as cutaneous or cutaneous-visceral form. Clinical symptoms usually develop three months to seven years after infection. Clinical signs involve generalised lymphadenomegaly, splenomegaly, a pale mucous membrane and weight loss. The disease is often accompanied by skin abnormalities with dry exfoliative dermatitis, ulcers, periorbital or diffuse alopecia and onychogryphosis. The most important laboratory findings consist of an

increase in gammaglobulins, hypoalbuminaemia, hyperproteinemia and anaemia. Some dogs show no systemic clinical signs but can have severe renal failure (Ciaramella et al., 1997; Moritz et al., 1999). A few articles report about leishmaniosis (FL) in cats, which is quite uncommon due to a believed high degree of natural resistance. In Europe, clinical cases have been described in Portugal, France, Spain and Italy. Scant clinical cases appear with typical signs of cutaneous forms, including ulcerocrusted dermatitis and nodular lesions on the nose, lips, ears, eyelids and alopecia. VL showing visceral involvement of liver, spleen, lymph nodes and kidneys is less common (Mancianti, 2004; Gramiccia and Gradoni, 2005). Other animal species such as goats, cattle and horses, which can act as incidental reservoirs of leishmaniosis, do not play a role in transmission. Few sporadic cases of equine leishmaniosis (EquL) have been reported in Europe, appearing as a self-healing, skin-dwelling disease (Mancianti, 2004; Gramiccia and Gradoni, 2005).

2.3.7. Diagnostic of *Leishmania* parasites

Diagnose of *Leishmania* spp. in dogs and humans is challenging due to the diverse and non-specific clinical manifestation of the infections and on the variation in research methods employed (e.g. IFAT, ELISA, PCR). Blood smears are a direct and simple technique of detecting parasites by examination of a Giemsa-stained blood smear under the microscope. *Leishmania* amastigote stages are found in the macrophages verified through the blue cytoplasm with pinkish nucleus and kinetoplast (Schuster and Sullivian, 2002). In addition, the Immune Fluorescence Antibody Test (IFAT) is a common diagnostic method to identify polyvalent antibodies against *Leishmania* parasites. The 1:32 dilution showing fluorescent promastigotes is taken as threshold antibody titre. PCR is a useful and rapid technique in terms of specificity and sensitivity for the detection of *Leishmania* DNA. Roura et al. (1999) and Fisa et al. (2001) therefore developed and applied a conventional and nested PCR to diagnose the pathogen more effectively. A new specific real-time PCR now replaces the conventional PCR providing the ability to perform very sensitive measurements of specific DNA of *Leishmania spp.* parasites (Francino et al., 2006). DNA probes for identifying *Leishmania* have targeted the kinetoplast DNA (kDNA) sequences. This particular kDNA consists of both maxicircles and minicircles, present in each cell in multiple copies. Species-specific and highly conserved sequences are located on small regions of some minicircles (Rodgers et al., 1990; Weiss, 1995; Wilson, 1995).

3. Materials and Methods

3.1. Collection of Psychodidae and Phlebotominae

3.1.1. Entomological surveys

The collection of Psychodidae and Phlebotominae is based on three different entomological field surveys in Germany. Firstly, an investigation of the local existing Psychodid-fauna was started with a trial termed "Latin Square" (see 3.1.1.2.), conducted at a local horse ranch (Reitschule München) and the Zoological garden in Munich during July and August 2007. Secondly, strategic sampling of Psychodidae and Phlebotominae took place with different trap types (see 3.1.1.1.) on various locations in the states of Bavaria, Baden-Wuerttemberg, Rhineland Palatinate and Saarland over a period of four months (June to September) in 2007 and 2008. Thirdly, three locations of different case scenarios of suspected autochthonous leishmaniosis were included in the strategic investigation of phlebotomine sand flies to find the appropriate vector associated with canine leishmaniosis in Bad Breising (2007), in Gehrweiler (2008) and equine leishmaniosis in Garching (2007) in Germany (see 3.1.2.). Additionally, an *ad hoc* field study was conducted to compare the applied methodology at a *Leishmania* endemic and phlebotomine-positive site, in a suburb of Rome in Italy in July 2008. In collaboration with the Istituto Superiore di Sanità in Rome (Italy), another field survey was carried out to investigate the presence of sand flies in new territories, formerly known as phlebotomine-negative areas, in the region of Bolzano in Northern Italy in August 2008 (see 3.1.3.).

3.1.1.1. Traps

Generally, collections were carried out by using three different types of traps (Fig. 10) to sample sand flies in a given habitat during the set time period. The first two types, battery operated light-suction traps, were used to trap sand flies actively by the attraction of light (380 nm black light, 580 nm yellow light). CDC miniature light traps (John W. Hock Company, Gainesville, Florida, USA), containing a photo switch option that automatically turns the trap

and fan activity on and off, were developed by the U.S. Centers for Disease Control to provide reliable and portable sampling devices for the collection of mosquitoes and sand flies used in taxonomic studies. Another trap type, BG-Sentinel™ traps (Biogents AG, Regensburg, Germany) was used for the survey and varied with different parameters of attractants (Biogents, chemical compound not published), carbon dioxide, light and fan. In contrast to most other traps, the BG-Sentinel™ trap does not catch beneficial insects such as honeybees, ladybugs and butterflies. Traps were set from dusk to dawn outside or inside animal shelters with a little container filled with soap water to keep all trapped insects, which were collected after 24 to 48 hours. All traps were placed at each location for a minimum of three to a maximum of five consecutive nights. Due to their convenient operation, it is easy to place those traps at different field sites.

Additionally, a third trap type (sticky traps) was used to intercept sand flies actively in a certain habitat (Alexander, 2000). Sticky traps, standard white DIN A4 paper coated with castor oil for 24 hours (Rioux et al., 1969), were placed in appropriate wall holes or hung up along animal shelters (inside or outside) where sand flies are likely to be active and recovered after one week. These traps are very selective and catch only small insects that glue to the paper. Adhering sand flies were removed with a small brush and alcohol, benefiting to the low viscosity of castor oil.

Fig. 10: Types of traps used for surveillance: (left) CDC miniature light trap, (middle) BG-Sentinel™ trap, (right) sticky traps.

3.1.1.2. Latin Square trial

A Latin Square is a statistical method consisting of an *n* x *n* grid with a set of *n* symbols appearing exactly once in each row and column (Wolf, 1989). The logic-based puzzle "Sudoku" is a special case of a Latin Square. Integers and colours in Figure 11 visualize the design. The Latin square design is used to control variation in two different directions at the same time.

1	**2**	**3**
2	**3**	**1**
3	**1**	**2**

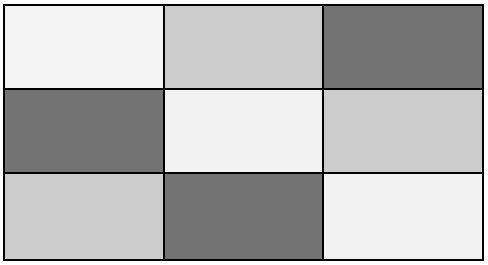

Fig. 11: Typical design of a Latin Square. Each symbol appears only once per row and column.

The first collection assay named Latin Square trial (modified after the statistical method) was carried out from the 9th of July to the 3rd of August 2007. The aim was to get an overview of the local existing Psychodid-fauna and to test different trap parameters to determine the most effective trap combination. Four different trap variations (Tab. 4) were tested at four different locations within a local horse ranch in Munich (Fig. 12) on consecutive days. The trial was repeated four times within four weeks resulting in a multiple Latin Square trial with at total of 16 trapping days. The following parameters were chosen for trapping: attractiveness of light, fan, chemical attractants and carbon dioxide. The same trial was performed at the local Zoological garden at the same time.

Tab. 4: Variations of trap parameters used during the Latin Square trial.

	Trap	**Parameter**
A	BG-Sentinel™	light + fan
B	BG-Sentinel™	light + attractant[1]
C	BG-Sentinel™	light + carbon dioxide
D	CDC Trap	light + fan

[1] unknown chemical composition (Biogents AG, Regensburg, Germany)

Location / Day	I	II	III	IV
1	A	B	C	D
2	B	C	D	A
3	C	D	A	B
4	D	A	B	C

Fig. 12: Design of the Latin Square trial at the local horse ranch in Munich. I-IV are the trap locations within the horse ranch (I: rondel; II: treadmill; III: dunghill; IV: staircase), 1-4 are the four consecutive experimental days, A-D are the trap parameters tested (Tab. 4).

3.1.2. Study area in Germany

The strategic entomological survey was carried out in Bavaria in the city of Munich (48°8'N, 11°34'E, 519 m a.s.l.) and its surroundings. The same entomological survey was furthermore carried out at the Lake Constance in Baden-Wuerttemberg and along the Rhine valley, close to the border of France, from villages surrounding Freiburg (48°0'N, 7°51'E, 278 m a.s.l.) proceeding northwards to Karlsruhe (49°1'N, 8°24'E, 115 m a.s.l.). Moreover, the strategic entomological survey was carried out in the surroundings of Saarbruecken (49°14'N, 7°0'E, 230 m a.s.l.) in the state of Saarland and in two villages and their surroundings in Rhineland Palatinate in Gehrweiler (49°35'N, 7°46'E, 250 m a.s.l.) and Bad Breisig (50°31'N, 7°18'E, 70 m a.s.l.). A total of 44 field sites ranging from an altitude between 47 to 680 m a.s.l., were monitored for the presence of phlebotomine sand flies. Figure 13 presents the different trapping locations (1-39) in Southern Germany. The five different field sites of Bad Breising are not included in the map and table. Further details about geographical and environmental characteristics of each location are presented in Table A of the annex.

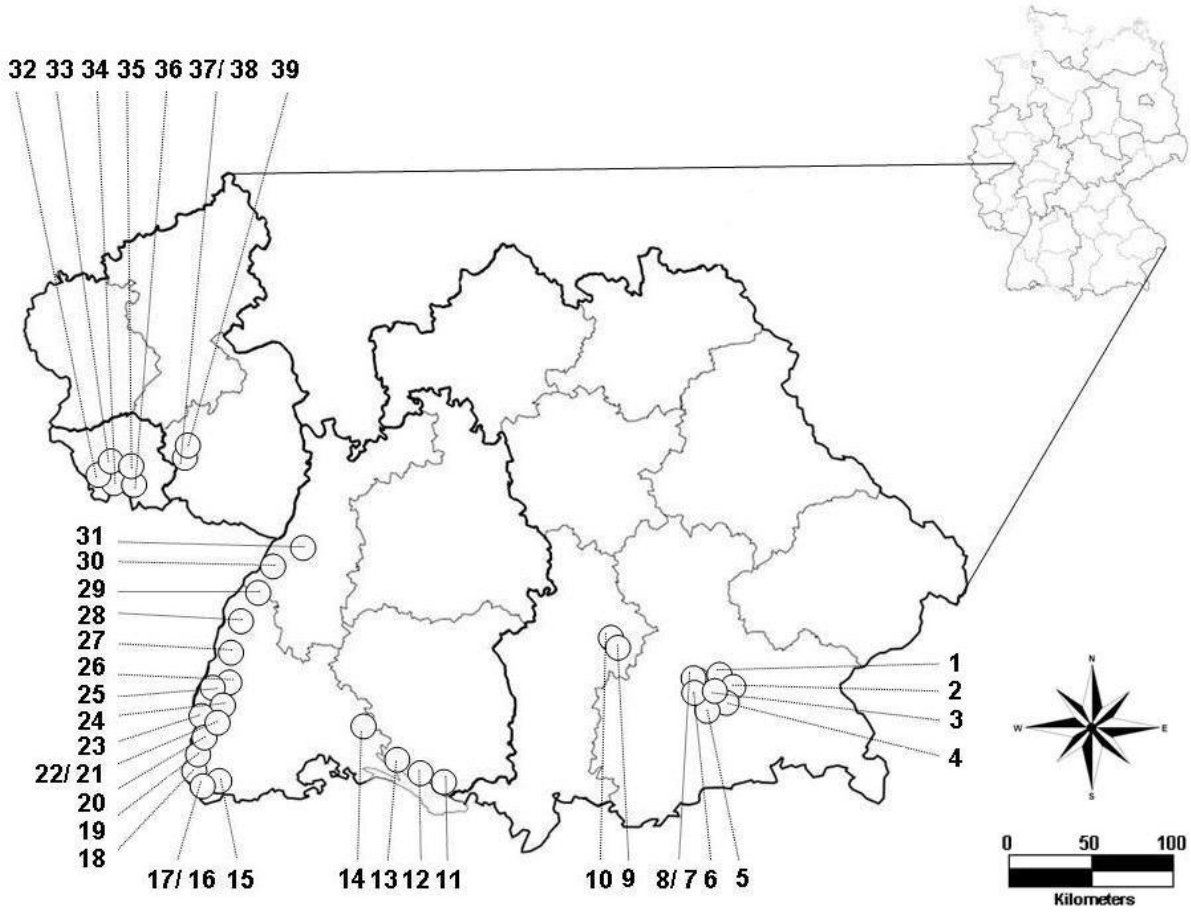

Fig. 13: Map providing the outlines of the federal states of Southern Germany and the locations (1-39) of the collecting sites (circles). Characteristics of each collecting site are presented in Tab. A of the annex. The five different field sites of Bad Breising are not included.

Fig. 14: Sampling site (left) sticky traps inside a cow stable, (right) sticky traps and CDC trap inside a chicken house.

Two types of sampling sites were chosen. Both were ecological niches within cities or farms at the periphery of cities, in small villages or on farmland. (1) Locations of primarily entomological interest were small animal farms (Fig. 14, 15), which harbor domestic animals such as horses, cattle, poultry, dogs and cats, but no intensive mass animal farming. Farms were operated in natural conditions without using pesticides or chemicals on or around the farm site. (2) The other site of investigation were brick or concrete walls, preferably facing southwards, with occurring cavities or pipes as potential resting areas for phlebotomine sand flies (Fig. 16).

Fig. 15: Sampling site (left) BG-Sentinel™ trap inside cow stable, (right) CDC trap semi-outside horse stable.

Fig. 16: Sampling site (left) stone wall with pipes, (right) concrete wall with pipe holes.

Three additional locations were chosen for an extra intensive entomological survey of phlebotomine sand flies due to the reported occurrence of autochthonous leishmaniosis cases. The first location was Bad Breising in the federal state of Rhineland Palatinate, where a case of suspected autochthonous canine leishmaniosis occurred in a husky (information given to our Institute by the local veterinarian). Traps were set up around the dog kennel and at different field sites where the family walked the dog. A total of five different sites were monitored from the 12th to 15th of August 2007. Additionally, blood was taken by the local veterinarian at the same time and analysed in our Diagnostic laboratory with IFAT and real-time PCR. The second field site was located in Gablingen close to Augsburg, where a case of suspected autochthonous equine leishmaniosis was reported in a horse (Koehler et al., 2002). Traps were placed at two different field sites from the 24th to 27th of August 2007. The last location was situated in Gehrweiler (Rhineland Palatinate), where a case of suspected autochthonous canine leishmaniosis was reported in a Rottweiler-Berner Senner-Mix (information provided by the owner). Traps were set up around the dog kennel and within the garden. The monitoring took place from the 23rd to 26th of July, the 12th to 15th of August and the 11th to 14th of September 2008.

3.1.3. Study area in Italy

Sand fly collection was implemented in two different areas of Italy. During these two surveys only Phlebotominae were analysed. The first one was carried out in an endemic *Leishmania* focus in Central Italy located in Canile (41°50'50"N, 12°42'03"E, 192 m a.s.l.), a suburb southeast of Rome, from the 14th to 20th of July 2008. Two brick walls at a distance of 200 m and 5 m close to a dog kennel (Fig. 17) and the area around the kennels (Fig. 18) were investigated with sticky traps and CDC miniature light traps over a period of six nights. A few dogs of the kennel were known to be *Leishmania* positive (personal communication with the owner).

Fig. 17: Sampling site (left) brick wall 200 m from the kennel, (right) brick wall 5 m from the kennel.

Fig. 18: Sampling site (left, right) CDC traps outside the kennel.

The second field investigation was performed in an apparently non-endemic *Leishmania* focus located in the territories of Bolzano province from the 18th to 22nd of August 2008 (Fig. 19). This province is the northern-most region in Italy, bordering Switzerland to the west and Austria to the east and north. The survey was conducted at 15 locations, ranging in altitude from 229 m to 725 m a.s.l., starting from Bolzano along the upper Adige valley to Merano city proceeding into the lower Venosta valley of the pre-alpine territories. Details about geographical and environmental characteristics of each location are presented in Table B of the annex. Brick walls and animal farms were again favourable sampling sites, which were monitored with sticky traps and CDC miniature light traps (Fig. 20, 21).

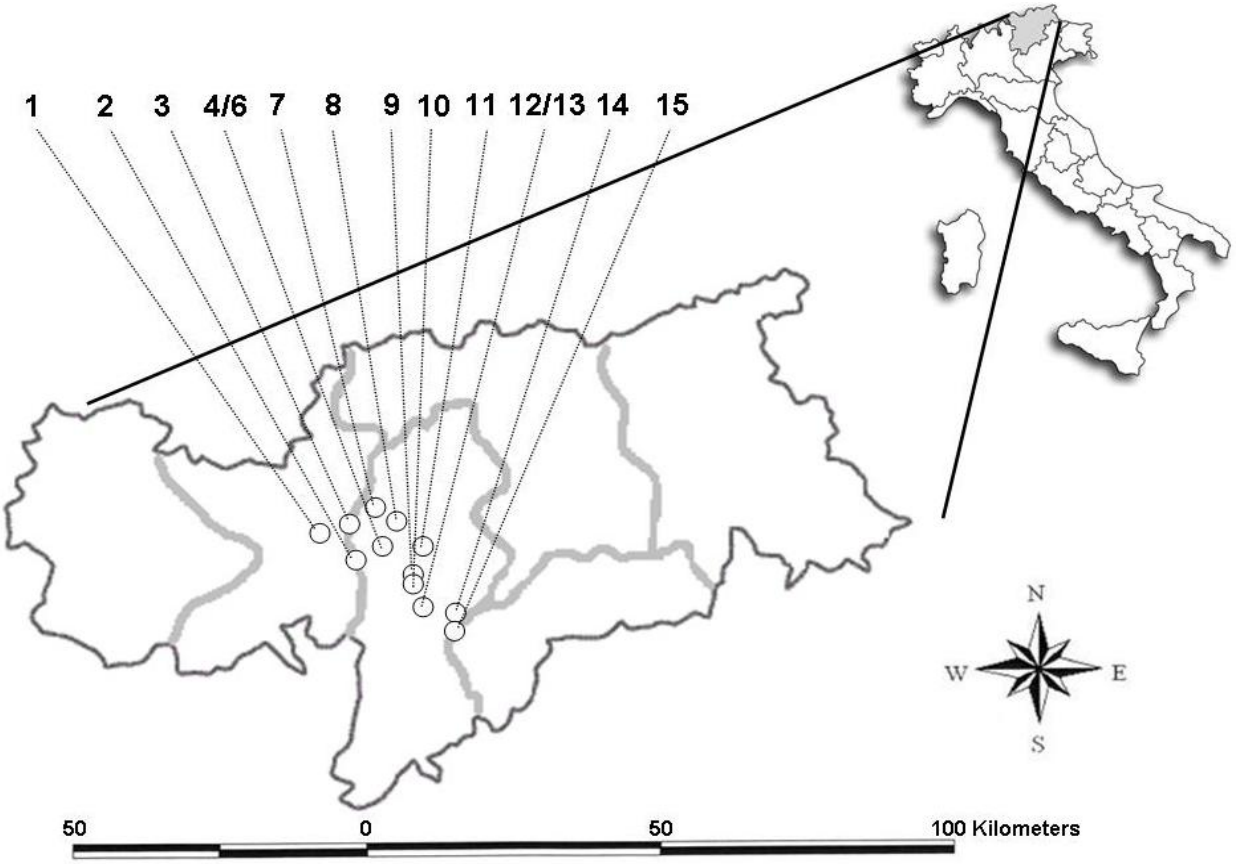

Fig. 19: Map providing the outlines of the state of Bolzano in Northern Italy and the locations (1-15) of the collecting sites (circle). Characteristics of each collecting site are presented in Tab. B of the annex.

Fig. 20: Sampling sites in Bolzano province (left) typical brick wall in the Mediterranean area (right) brick wall with pipes examined with sticky traps.

Fig. 21: Sampling sites in Bolzano province (left, right) farms with different animals.

3.2. Data of Climate

Weather conditions (e.g. sky cover, sun and rain), minimal and maximal temperatures and the relative air humidity at each location were recorded with a digital thermohygrometer (P 330, Carl Roth GmbH, Karlsruhe, Germany) during the entomological survey. In addition, the average temperature and relative humidity of three different sampling locations (Munich, Freiburg and Saarbruecken) during the 24 hours of each day in June, July, August and September were obtained from the online meteorological weather service (www.dwd.de/WESTE). This was done to determine whether existing climatic conditions would be appropriate for the survival of phlebotomine sand flies. Whenever possible, traps were placed at calm field sites. Furthermore, climate maps about the annual average temperature in Germany of the last pentade (2004-2008), decade (1999-2008) and the last two decades (1989-2008) were received from the meteorological weather headquarter in Offenbach. These climate maps were used to evaluate possible climatic changes and prefered sites for phlebotomine sand flies in Southern Germany (see Annex, Fig. A).

3.3. Laboratory Examination

Every specimen of Psychodidae and Phlebotominae was kept in 1.5 ml Eppendorf tubes containing 70 % ethanol and an attached label that recorded the site and day of sampling. Female Phlebotominae were stored in 95 % ethanol until DNA extraction. External morphological analyses of the habitus of different species were carried out by the use of a stereomicroscope (Wild – Photomakroskop, M 400, Heerbrugg, Switzerland). Internal morphological analyses of different organ structures were performed using a light microscope (Zeiss – Axioskop, Oberkochen, Germany).

Identification of sand flies is based mainly on internal structures and therefore requires specimens to be mounted on microscope slides. For detailed morphological studies of the head and abdomen, specimens were cleared in a 10 % potash solution for 30 min at 75°C. Afterwards, they were transferred into pure acetic acid for five minutes and then displaced into clove oil for dissection of the caput, thorax, wings and genitalia. Finally, all four parts were mounted in Canada balsam (Merck, Darmstadt, Germany) on one microscope slide and covered with round cover glasses (Wagner et al., 2008).

3.3.1. Identification of Psychodidae

Adults of the family of Psychodidae were determined on the basis of the "Key to subfamilies; Key to tribes" characterising specific morphological features (Wagner, 1997). Additionally, each of the male specimens was identified with the help of the "Key to the males of tribes and genera of Psychodidae - Psychodinae" provided by Wagner (see Annex, Fig. G). Female specimens can often be determined only to tribe level.

3.3.2. Identification of Phlebotominae

Three randomly selected males and females of each trapped sand fly species were mounted on microscope slides and identified by their morphological characteristics (head and genitalia) to species level by standard taxonomic keys according to Theodor (1948, 1958) and Léger et al., 1983.

3.4. PCR for detection of *Leishmania spp.*

3.4.1. DNA-Extraction

DNA was obtained from 39 single female *P. perniciosus* by using a QIAamp® DNA Mini Kit 250 (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's instruction, albeit with slight modifications. The samples were transferred from absolute alcohol (95 % ethanol) into separate sterile 1.5 ml Eppendorf tubes with 200 µl PBS, where they remained for 24 hours. Afterwards, phlebotomes were bifid with a sterile scalpel and incubated in a water bath to dissolve tissue by adding buffer and proteinase K (QIAGEN) at 56°C for three hours. Subsequently samples were treated according to QIAGEN protocol (QIAamp® DNA Mini and Blood Mini Handbook 11/2007). The final elution volume was 200 µl. To verify the quantity and quality of extracted DNA from *P. perniciosus*, each sample was measured in a fullspectrum (220-750 nm) spectrophotometer (NanoDrop®ND-1000, PeqLab, Erlangen, Germany) according to the manufacturer's instructions (NanoDrop® User Manual, 2004).

3.4.2. Positive control and standard curve

Genomic DNA extracted from a continuous *in-vitro* culture of *Leishmania donovani infantum* (212. passage, 28°C) cultured in our Bio II – laboratory, was used as positive control for each PCR experiment. To achieve a quantitative sample with a well-defined number of *Leishmania*, parasites were counted in a haemocytometer (improved Neubauer counting chamber). This sample was used to perform a standard curve for DNA quantifications. A five-step dilution series was prepared to achieve the minimum of *Leishmania* detected in the real-time PCR (Tab. 5). The performance of the standard curve was carried out with the same PCR protocol as described in chapter 3.4.3. Each dilution series was applied trifold in the PCR experiment.

Tab. 5: 10-fold dilution series of *L. d. infantum*.

Sample	**A**	**B**	**C**	**D**	**E**
Leishmania/µl	4×10^4	4×10^3	4×10^2	4 x 10	4 x 1

3.4.3. Quantitative real-time PCR

A real-time PCR as described by Francino et al. (2006) was performed with AB 7500 Real Time PCR System (Applied Biosystems, Darmstadt, Germany). TaqMan®-MGB probe (18 bp) and PCR primers Leish-1 (23 bp) and Leish-2 (17 bp) as shown in Table 6, target conserved DNA regions of the kinetoplast mini circle DNA from *L. infantum*. The reaction and cycling conditions used are displayed in Table 7 and Table 8.

Tab. 6: Primers and probe for real-time PCR detection of the mini circle DNA of *L. infantum*.

Primer	Oligonucleotide sequence	Reference
Leish – 1	5'-AAC TTT TCT GGT CCT CCG GGT AG-3'	Francino et al.,
Leish – 2	5'-ACC CCC AGT TTC CCG CC-3'	2006
Probe		
TaqMan®-MGB	FAM-5'-AAAAATGGGTGCAGAAAT-3'-non-fluorescent quencher-MGB	Francino et al., 2006

Leishmania primers and probe were added to achieve a final concentration of 900 and 200 nM. Each sample was amplified in a 25 µl final volume reaction mixture including the TaqMan® Universal PCR Master Mix with UNG AmpErase® to avoid carry-over contamination. Each amplification run contained a positive control and a negative control with sterile water.

Tab. 7: Reaction condition for real-time PCR detection of the mini circle DNA of *L. infantum*.

Reagent	Volume
Forward Detection Primer [10 µM]	1.125µl
Reverse Detection Primer [10 µM]	1.125µl
TaqMan® MGB Probe [10 µM]	0.5µl
TaqMan® Universal PCR Master-Mix	17.25µl
Template DNA	5µl
Total Volume	25µl

Tab. 8: Cycling condition for real-time PCR detection of the mini circle DNA of *L. infantum*.

Cycle	Step	Temperature	Duration
Cycle 1: 1x	Initial	50.0 °C	2 min
Cycle 2: 1x	Activation	95.0 °C	10 min
Cycle 3: 40x	Denaturation	95.0 °C	15 sec
	Annealing		
	Extension	60.0 °C	1 min

3.5. Retrospective study

To achieve an overview of possible acute or autochthonous leishmaniosis cases in Southern Germany, a retrospective data acquisition of incoming diagnostic material (blood, sera and lymph node aspirates) of dogs, cats and rabbits was performed in our Diagnostic laboratory. Data were collected over a period of three years from January 2006 until December 2008. A total of 6494 samples, sent in from veterinarians and local or European animal shelters, were screened for requested diagnosis of *Leishmania* and analysed. The blood and lymph node aspirates were examined with real-time PCR and the sera with IFAT. Each request was recorded, including the requesting number, date of receipt, veterinarian, owner and name of the dog, origin of the dog and results of the laboratory examination. Detailed analyses of all data with negative, positive and doubtful results were carried out for each year. Concerning the IFAT, the 1:32 dilution showing fluorescent promastigotes was taken as threshold antibody titre. Positive results, detection of *Leishmania* antibodies, were received by the IFAT dilution series (1:64, 1:128; 1:256 and 1:512), which confirm an ongoing infection. There was either a positive or a negative result for the real-time PCR. In total 400 different dogs, positive tested for *Leishmania* infections, have been evaluated.

3.6. Statistical Analysis

The statistical analysis of this study was assisted by the StabLab (Statistical Consulting Unit) of the Department of Statistics, Ludwig-Maximilians-University, Munich. Evaluation of

the "Latin-Square" was performed in "SPSS for Windows" (Version 16.0) with a compiled syntax file. To investigate the efficiency of the CDC trap (CDC miniature light trap, Florida, USA) compared to the BG-Sentinel™ trap with different parameters a statistical analysis based on a t-test was performed. Prior to the analyses, homogeneity of variances was tested using the Levene-test. Values of $p < 0.05$ were regarded as significant. Corresponding 95 % confidence intervals were computed by a parametric bootstrap conditioning on the parameters of traps and incidence of each species group during the trial. Data of the requested *Leishmania* examinations in our Diagnostic laboratory were analysed using Microsoft Office Excel (2003) and R (version 2.5.0) to investigate monthly variations and frequency of the tested samples, the development of *Leishmania* infections over a long time period and seroprevalence of infected animals.

3.7. Image Acquisition

Macroscopic and microscopic digital pictures were taken with a microscopic camera (Zeiss - AxioCam MRC, Oberkochen, Germany) and the processing program AxioVision Rel. 4.7 according to the manual's instructions 2009.

4. Results

4.1. Publication

Two new Psychodidae (Trichomyiinae and Psychodinae) from Germany bound to decaying wood

Zootaxa (2010), Vol. 2386, 59-64

PDF file available from

http://www.mapress.com/zootaxa/list/2010/2386.html

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Abstract

Two new species of Psychodidae, *Trichomyia stephani* nov. spec. (subfamily Trichomyiinae) and *Telmatoscopus thuringicus* nov. spec. (subfamily Psychodinae) collected in Germany are described and figured. The larvae, like those of their close relatives are most probably bound to decaying wood. Cutting down in particular old trees finally axes habitats of many endangered red data list species.

Key words: Diptera, Psychodidae, Trichomyiinae, Psychodinae, new species, Germany

Introduction

Within the Psychodidae *sensu lato* all species of one subfamily that should be ranked as separate family, the Trichomyiinae, seem to be bound exclusively to wood. The few larvae discovered were all taken from decaying wood of dead or moribund trees or from tree holes filled partly with rain water and the bottom covered with fine brownish remnants of dead wood. In Europe, in the last two decades specimens were only discovered in protected areas with old tree populations (Withers 2004) and particular methods of forest management. The discovery of a new species of this family was thus surprising, but it was not surprising at all that it was found in a nature conservancy area with coppice management and in the English Garden in Munich with "enclaves" of very old trees. The discovery of the second species was also surprising, but again it was discovered in the National Park 'Hainich', a National Park in Thuringia with stands of old trees. Although only adults were collected it is most probable that the larvae of both species live in the habitats mentioned above.

With the so-called 'imperative of modern forest management' in times of global change the amount of 'old' trees in forests decreases worldwide. However, because a large amount of species, not only insects but even vertebrates are in need of 'naturally developing' forests, modern forest management endangers biodiversity on earth. Forests, in particular old-growth forests, have an urgent need for protection, as do single remaining trees. We were lucky to collect in such areas in Germany and to find two new species for science of these rare and remarkable species.

Material and methods

Adults were trapped with CDC miniature light traps (John W. Hock Company, Gainesville, Florida, USA) fixed with a little container filled with soap water and stored in 70-80% ethanol. For discrimination specimens were cleared in hot KOH, transferred through acetic acid, a 1:1 mixture of acetic acid and clove oil, and pure clove oil. They were finally slide mounted in drops of Canada balsam, head, wings, thorax and abdomen under individual cover slips. Drawings were prepared with a drawing mirror on a Leitz Dialux 20 EB at 45x, 100x, and 200x magnification.

Psychodidae - Trichomyiinae

Trichomyia stephani sp. nov.

.Material : 1♂ (holotype) Germany, Rhineland-Palatinate, Kirchheimbolanden, nature reserve Albertskreuz (coppice management), 400 m a.s.l. 23. May -7. June 2002, malaise-trap, leg. D. Doczkal; 1♂ (paratype) 12. July 2007, Germany, Bavaria, Munich, Reitschule [48°09'18" N, 11°35'22" E], 511 m, CDC-light trap, leg. B. Beran; 1♂ (paratype) 19. July 2007, Germany, Bavaria, Munich, Reitschule [48°09'18" N, 11°35'22" E], 511 m, CDC-light trap, leg. B. Beran; 1♀ (allotype) (paratype) 12. July 2007, Germany, Bavaria, Munich, Reitschule [48°09'18" N, 11°35'22" E], 511 m, CDC-light trap, leg. B. Beran.

Derivatio nominis: Dedicated to the grandfather of the senior author, Stephan Beran.

Male description : Head with round eyes, no eyes-bridge, as typical of the subfamily. Antenna 15 segmented (broken in all specimens during preparation, fig 1). Scape short, barrel-shaped, pedicel spherical, shorter than scape. Flagellomeres elongate, slightly asymmetric with a pair of elongate simple ascoids, about 1.3 times longer than a flagellomere. Relative size of basal antennal segments: 28-24-53-46-42- further distal segments missing; scape length 0.1 mm. Palpus 3-segmented (fig 2) 0.24mm long. Basal segment with a circular pit (vesicle), with sensory rods. Relative length of the palpus segments: 29-19-21.

Thorax and legs elongate without specific features. Wing venation is typical of the genus with only a single vein (R_{4+5}) between the forks R_2/R_3 and M_1/M_2. It is not clear whether sc terminates into costa or into R_1 and what is the cross-vein. Radial fork slightly distal of medial fork, a cross-vein between the stems of M_{1+2} and M_3/CuA_1. CuA_2 elongate, with basal cross-vein

to basal stem of M_3/CuA_1, anal vein short. Wing length 1.88 mm (holotype); 1.93, 2.04 mm (paratypes); 2.02 mm (allotype).

Male abdomen with tergites 7 and 8 strongly reduced to thin clasps, sternites of usual size. Segment 8 with a torsion of about 90° to segment 7, genitalia with another torsion of 90° to segment 8 so that the genitalia become inverted (fig 5). Genitalia complicatedly structured; gonocoxite basally with an elongate dorsal apodeme (***da***); distally with a long prominent ventral process, along its inner margin covered with a row of strong bristles; at about middle height lies a short and broad process with several tips and with strong setae. Above it the slightly bent and flattened simple gonostyle articulates. Aedeagus consists of a laterally flattened strongly sclerotized aedeagus apodeme and a distal, slightly sclerotized portion (figs 4, 5, 6). The distal portion maybe bend ventrally at an about median position, where the lateral 'arms' of the aedeagus (***la*** in Figures 5 and 6) are markedly thinner. In a basal position of the aedeagus broad distal sclerites are visible (fig 5), in a distal position these are merely visible because they are fold up and appear as if one looks at a blade tip (fig 4). At the end of the basal aedeagus apodeme a pair of sperm ducts opens into a wide chamber, flanked by the faint sclerites. Tergite 9 rectangular, cerci are in a horizontal plane, elongate triangular, quite large and setose, best visible in lateral view.

Female description: specimen of similar size and coloration as male, palpus three-segmented, also with a depression and sensory rods on the basal segment. Sternite 8 triangular with a slight basal incision, the tip loosely covered with setae, cerci oval (fig 7). The inner genitalia consist of a broad ventral apodeme, and an elongate slightly sclerotized plate. Above the plate is a thin long apodeme that is probably joined to the plate. Further, two short sperm ducts that evolve from a single opening are visible. They end in a pair of holes with well sclerotized circular entrance. Whether these are openings of spermathecae remains dubious. Further structures cannot be sufficiently interpreted at the moment.

Remarks: Six European species of *Trichomyia* Haliday, 1839 are known so far: *T. urbica* Haliday (widespread in Europe), *T. parvula* Szabó (Hungaria, Germany, Great Britain) (Szabó 1960), *T. malickyi* Wagner (Greece: Islands of Kefallinia and Euböa; Wagner, 1982, Ježek, 1990), *T. kostovi* Ježek (Bulgaria; Ježek 1990), *T. carlestolrai* Wagner (Spain: Barcelona; Wagner 2001) and *T. minima* (England; Withers 2004). Relations to the already described

European species appear minor. The construction of the genitalia, especially the male gonostyli is unique among European taxa.

It is still unknown whether specific relationships between certain tree species and Trichomyiinae do exist. However it is remarkable that the discovery sites of recently described European species are related to old forests and single old trees and remainder of old trees with slowly decaying trees and traditional careful forest management. Thus we speculate that quite a number of European Trichomyiinae still remains undiscovered. We will find them probably in the minor remnants of old forests with slowly decaying trees and in nature reserves.

Psychodidae - Psychodinae

Telmatoscopus thuringiacus sp. nov.

Material : 1♂ (holotype) Germany, Thuringia, National Park Hainich, Weberstedt, Birkensee (trap2) 12 - 20 May, Malaise-trap, leg. F. Dziok; 1♂ (paratype) same locality, 26 June – 3 July 2001 leg. F. Dziok; 1♂ (paratype) same locality, 3 - 12 July 2001 leg. F. Dziok.

Derivatio nominis: Dedicated to the German Federal Land Thuringia that established the National Park Hainich.

Male description: Head with reniform eyes. Eye bridge with four rows of facets, distance between eyes about 1 facet diameter. Antenna with barrel-shaped scape, spherical pedicel shorter than scape, and 14 flagellomeres, 1.77 mm long. These are bottle-shaped with long sickle shaped ascoids on (probably) all segments (fig 8). Relative length of antennal segments (assembled from left and right flagellum): 37-26-45-45-46-44-43-42-42-42-40-39-38-37-36-33. Palpi lost in holotype. In paratype: absolute length: 0.85 mm, relative length of segments: 34-56-56-58.

Wing length 2.1 mm. Wing venation: forks R2/R3 and M1/M2 at about middle of wing; R5 terminates just before wing tip (fig 9).

Genitalia (fig 10): Sternal band thin, slightly broader at middle. Gonocoxites cylindrical, slightly bent, more than two times longer than the greatest diameter. Gonostyles longer than gonocoxites, about 4 times longer than wide with sharp tip poiting towards dorsally, so that the real length of the gonostylus is often difficult to judge – Fig 11 shows realistic length ratio. Tergite 9 rectangular as wide as long, lateral margins slightly convergent. Cercopodia slightly bent, longer

than tergite 9, distally with an oval group of approximately 30 tenacula, further tenacula scattered along their whole length. Tergite 10 conical, setose.

Aedeagal apodeme elongate and Y-shaped; at the distal end two stout, complicatedly built distal sclerites, about sickle-shaped with articulate tips curved outward. The entire distal portion of the aedeagus is visible through a diagonally cut 'pipe'. The pipe laterally has articulations to the gonocoxites (probably the ventral bridge).

Female are unknown.

Remarks: The new species belongs to a group of dendrolimnobiontic taxa with several (probably synapomorphic) features in larvae and adults. At least the European species are seemingly difficult to distinguish. The species group includes to date *Telmatoscopus advenus* (Eaton, 1893), *Telmatoscopus seguy*i (Vaillant, 1990) and probably some Nearctic species such as *Telmatoscopus dendrophilus* Vaillant, 1983 (USA, Tennessee). Vaillant confused the 'true' *T. advenus* Eaton with *T. seguyi*, a species he had described in 1990. However, the new species is clearly distinguished from all others by the large number of tenacula, distributed all over the entire length of the cercopodia. The parameres are short and stout, but elongate, long and thin in the other species. Although the larva remains unknown the habitat probably is also the decaying wood. The collections were made in an area with old trees in the National Park Hainich.

Acknowledgments: Sincere thanks to the people from the Reitschule Munich giving us the opportunity to conduct an entomological survey. Thanks to Phil Withers for examining linguistics.

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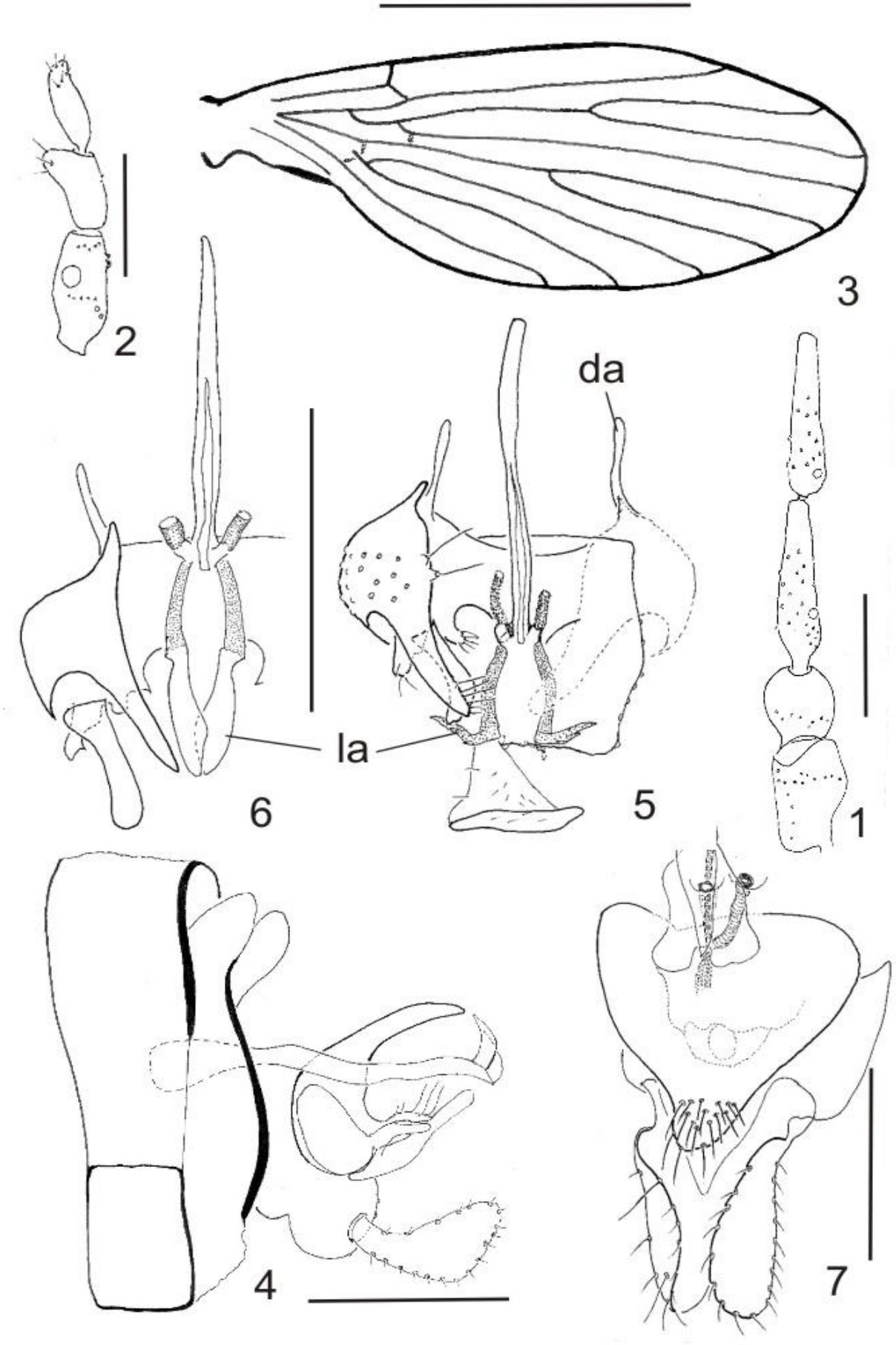
Legend to Figures:

Trichomyia stephani nov. spec. 1-6 male, 7 female. 1-basal antennal segments, 2 -palpus, 3-wing, 4-tip of abdomen, lateral view, 5-6 male genitalia, ventral view with upright (6) and distally pointing ***la*** – lateral arms of aedeagus; ***da*** – dorsal apodeme of gonocoxite) 7-abdomen tip lateroventral view. [Scale: 1, 2= 0.1 mm; 3=1 mm; 4, 5, 6= 0.2 mm 7=0.1 mm].

Telmatoscopus thuringicus nov. spec. 8-11 male, 8-distal antennal segments, 9-wing, 10-genitalia ventral view, 11-gonocoxite and gonostylus, real form and length. [Scale: 8=0.1 mm; 9=1mm; 10=0.2 mm; 11=0.1 mm].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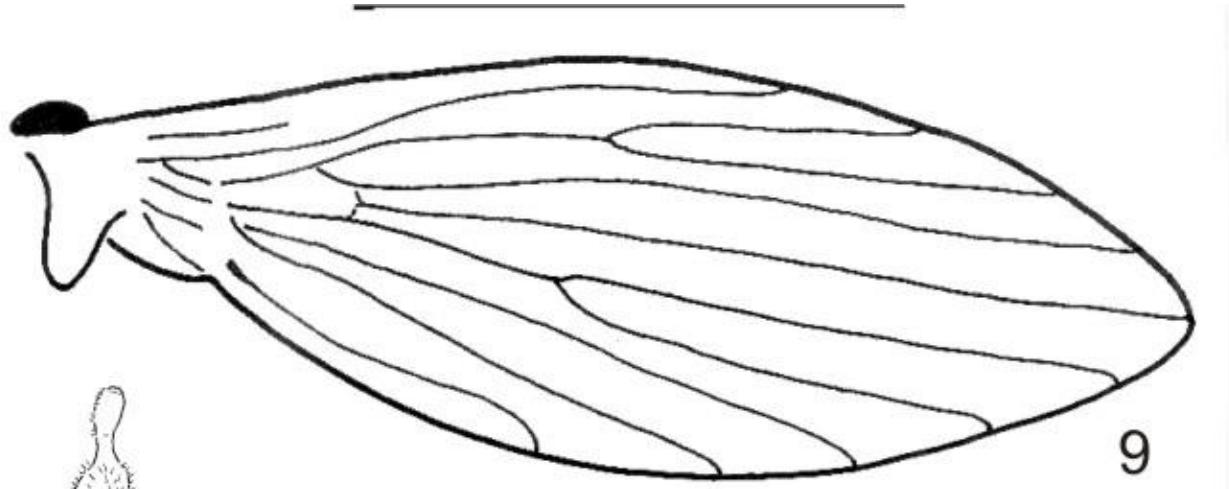

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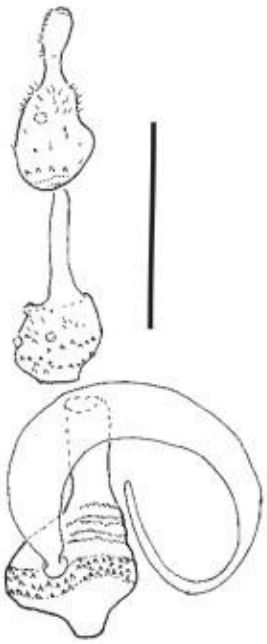

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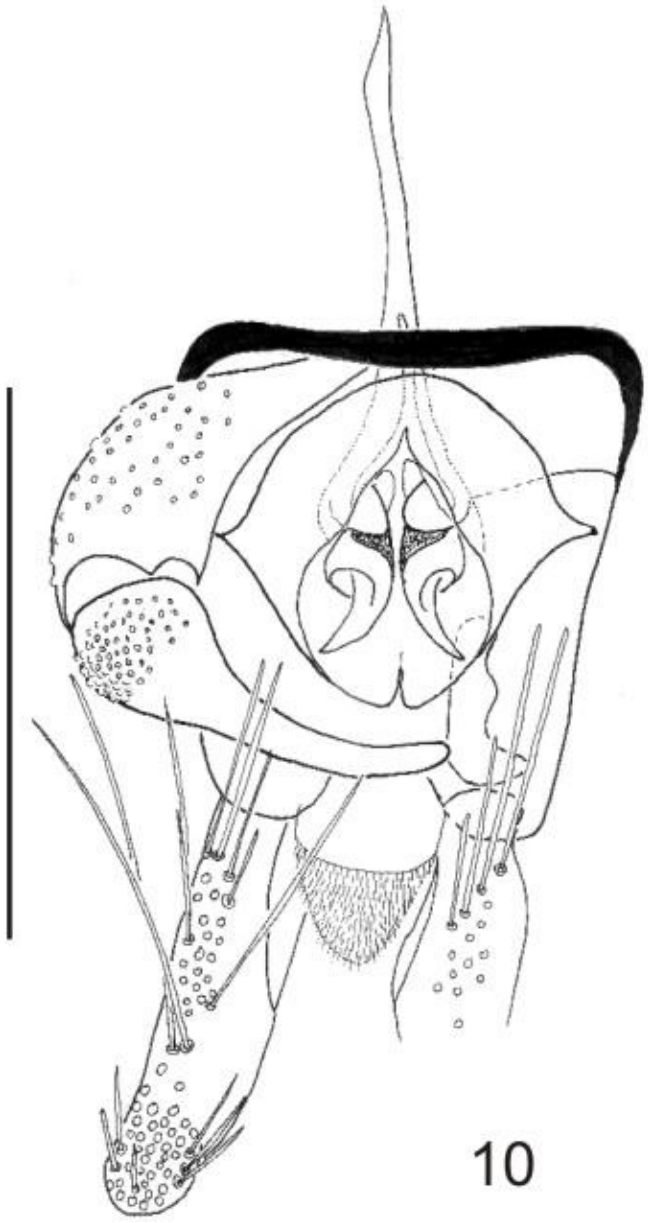

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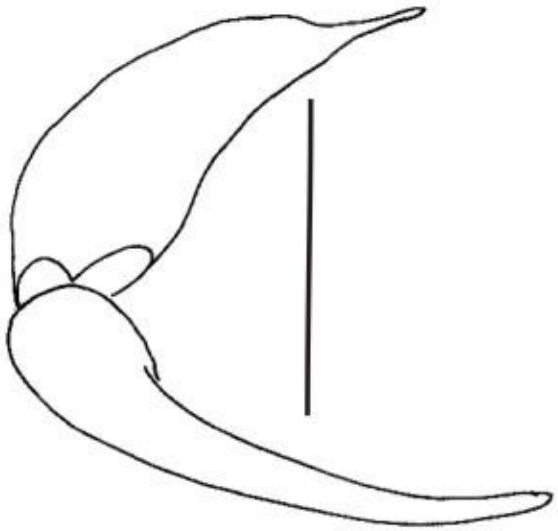

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4.2. Further results of the collection of Psychodidae and Phlebotominae

4.2.1. Latin Square

The first inventory of Psychodidae and Phlebotominae was carried out in a trial testing four different trap parameters and two different trap types. In 16 consecutive nights, a total of 16.237 specimens (see Annex, Tab. D, E) of the family of Psychodidae were trapped at the local horse ranch (16.60 %) and the Zoological garden (83.40 %) of Munich. They were analysed under a stereomicroscope. Determination of all specimens revealed two subfamilies, on the one hand the subfamily of Psychodinae (99.81 %) and on the other hand the subfamily of Trichomyiinae (0.19 %). Five groups of the subfamily of Psychodinae were identified to genus level and five groups to species level (Tab. 9). Two different species, *Trichomyia urbica* and *Trichomyia stephani* nov. spec., were determined for the Trichomyiinae. *Trichomyia stephani* nov. spec. is an unknown species that has not been described in the literature yet (see 4.1. publication). No specimens of the subfamily of Phlebotominae were found at either sampling sites.

Tab. 9: Determination of the subfamily of Psychodinae caught during the Latin Square trial at the horse ranch in Munich. The author of the first description is mentioned in parentheses.

Genus level	Species level
Psychoda spp.	*Tinearia alternata* (Say 1824)
Trichopsychoda spp.	*Mormia apicealba* (Tonnoir 1922)
Philosepedon spp.	*Mormia furva* (Tonnoir 1940)
Pericoma spp.	*Paramormia ustulata* (Walker 1856)
Clytocerus spp.	*Telmatoscopus rothschildi* (Eaton 1912)

The data of the horse ranch (location 3 of Tab. 11) were used to analyse the results of the Latin Square trial (see Annex, Tab. F). The data received at the Zoological garden (location 4 of Tab. 11), however, showed very limited biodiversity of the Psychodid-fauna and was therefore not appropriate for graphical representation. Statistical analyses were conducted but the four different trap locations within the horse ranch were not taken into account, as the variation between them was too large. Therefore, all the data were pooled and considered as one location.

The analysis of the attractiveness of the different trap parameters used for the trapping of Psychodidae at the horse ranch showed a positive result for all four trap variations. Three groups of species, *Psychoda* spp., *Trichopsychoda* spp. and *Philosepedon* spp., and one species *Tinearia alternata* were attracted to all four trap types. All of the less frequent groups from Table 9 appeared in very low numbers and were subsumed under "Other species" in the diagram (Fig. 22). A total of 2696 specimens of Psychodidae were trapped during the Latin Square trial. The light + fan trap caught a total of 326 specimens (12.09 %), the light + attractant trap caught 926 specimens (34.29 %), the light + CO_2 trap caught 499 specimens (18.50 %) and the CDC trap caught 947 specimens (35.12 %). The lowest attraction rate was obtained by the light + fan and the light + CO_2 trap, the highest trap attraction was achieved by the light + attractant and the CDC trap.

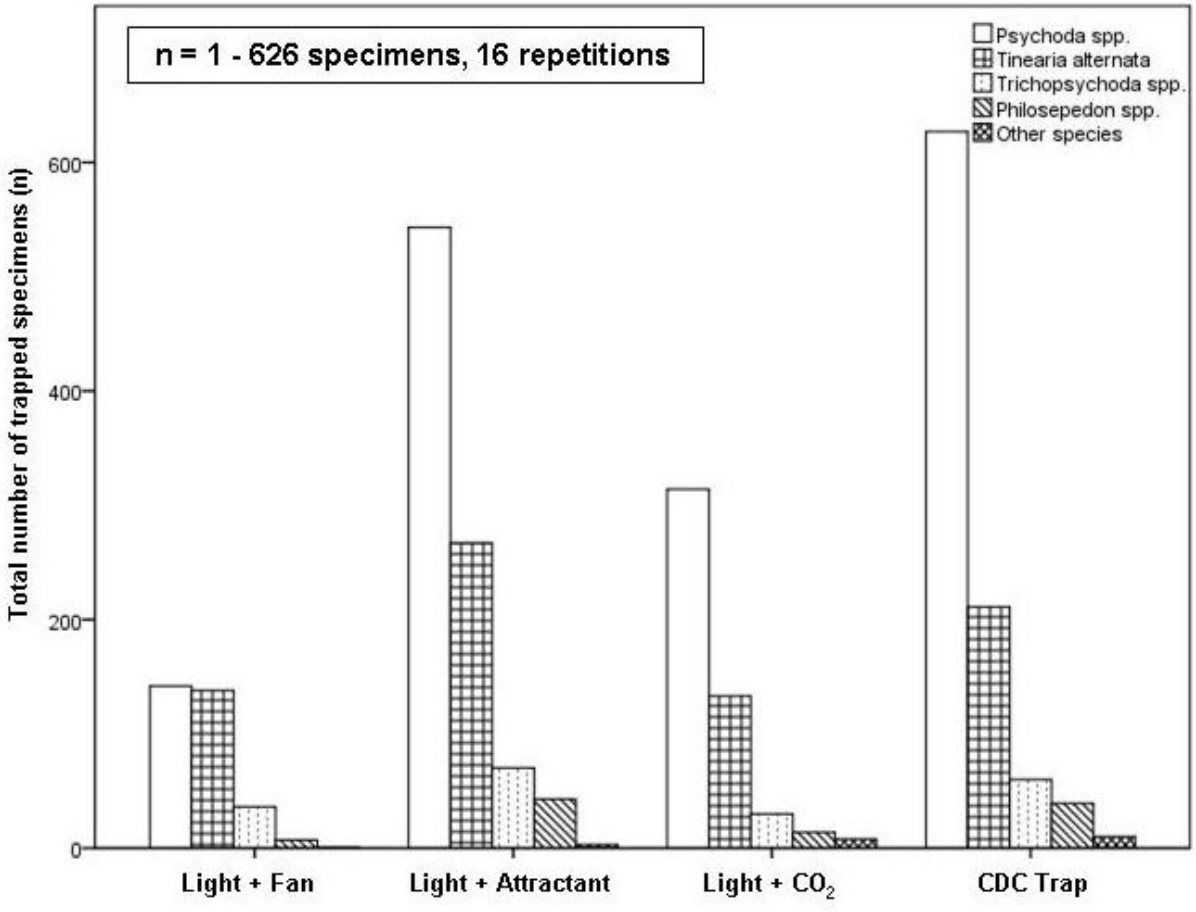

Fig. 22: Absolute trapped specimens of the family of Psychodidae at the local horse ranch in Munich testing different trap parameters.

Comparing the frequency of all trapped species-groups within all four trap variations, each of the mentioned species-group was trapped a minimum of one and maximum of 16 times within the trial (Fig. 23). The species-group and species trapped most often in all trap variations, 13 to 16 times respectively, were *Psychoda* spp. (100 %) and *Tineraria alternata* (∅ 90.62 %), which were omnipresent. *Trichopsychoda* spp. (∅ 56.25 %) and *Philosepedon* spp. (∅ 34.37 %) were trapped less frequently ranging from two to eleven times. "Other species" (∅ 21.88 %) were trapped very infrequently, ranging from one to six times. The CDC trap and the light + CO_2 trap showed a higher frequency (34.37 %) of "Other species", very rare species, compared to the fan, attractant and light parameter (12.50 %).

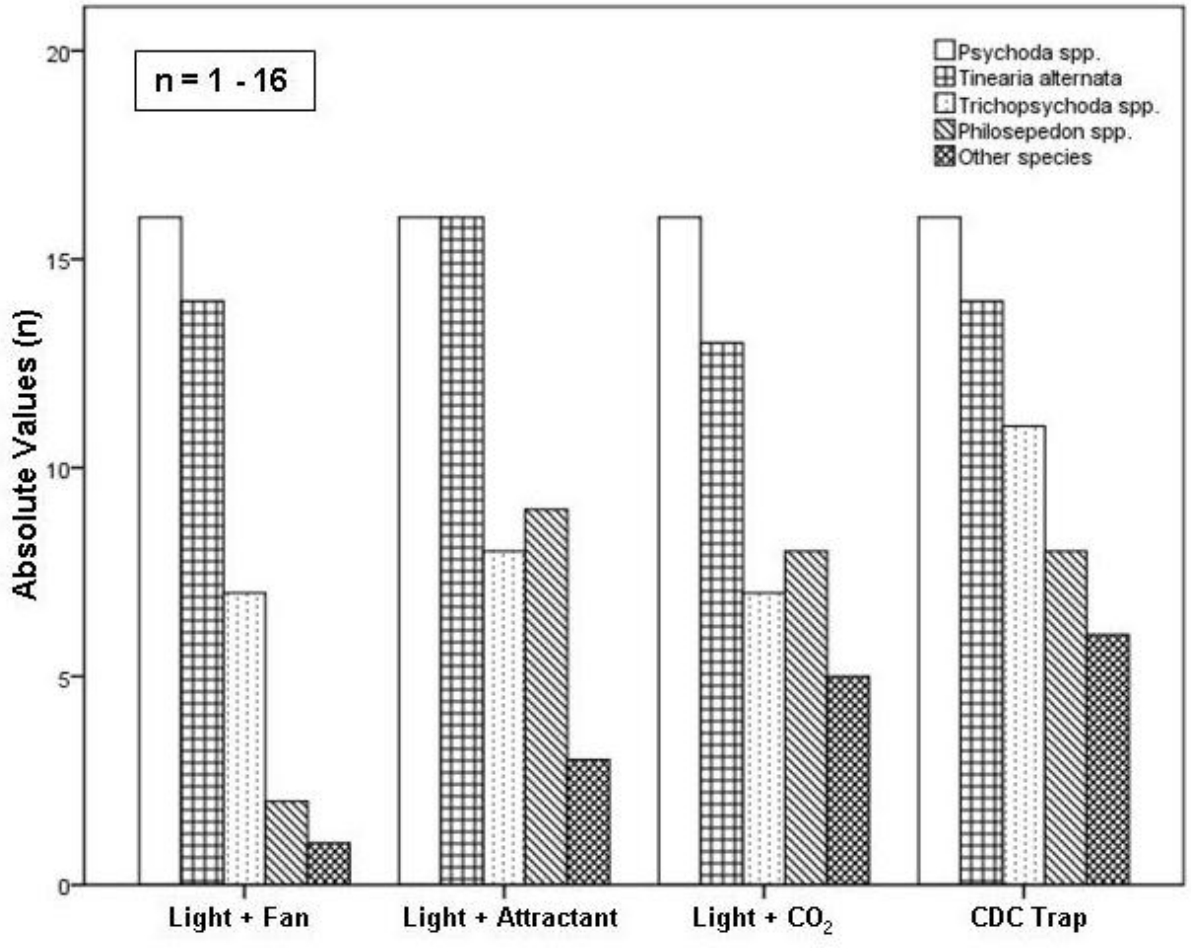

Fig. 23: Frequency of trapped species-groups within 16 repetitions of the Latin Square trial.

Figure 24 displays the evaluation of the trapping success by frequency of the quantity of different species. All species subsumed under "Other species" are separately included in this

Figure. The data are shown in Table 10. During the Latin Square trial, up to six different species were trapped at the same time. Two and three species were commonly trapped up to nine times during the trial. It was rare (one to two times) to trap only one species. Especially the light + attractant, light + CO_2 and CDC trap contained five different species two to three times. Only the CDC trap out of all trap variations caught six different species (two times) and showed the highest biodiversity of different species at the same time.

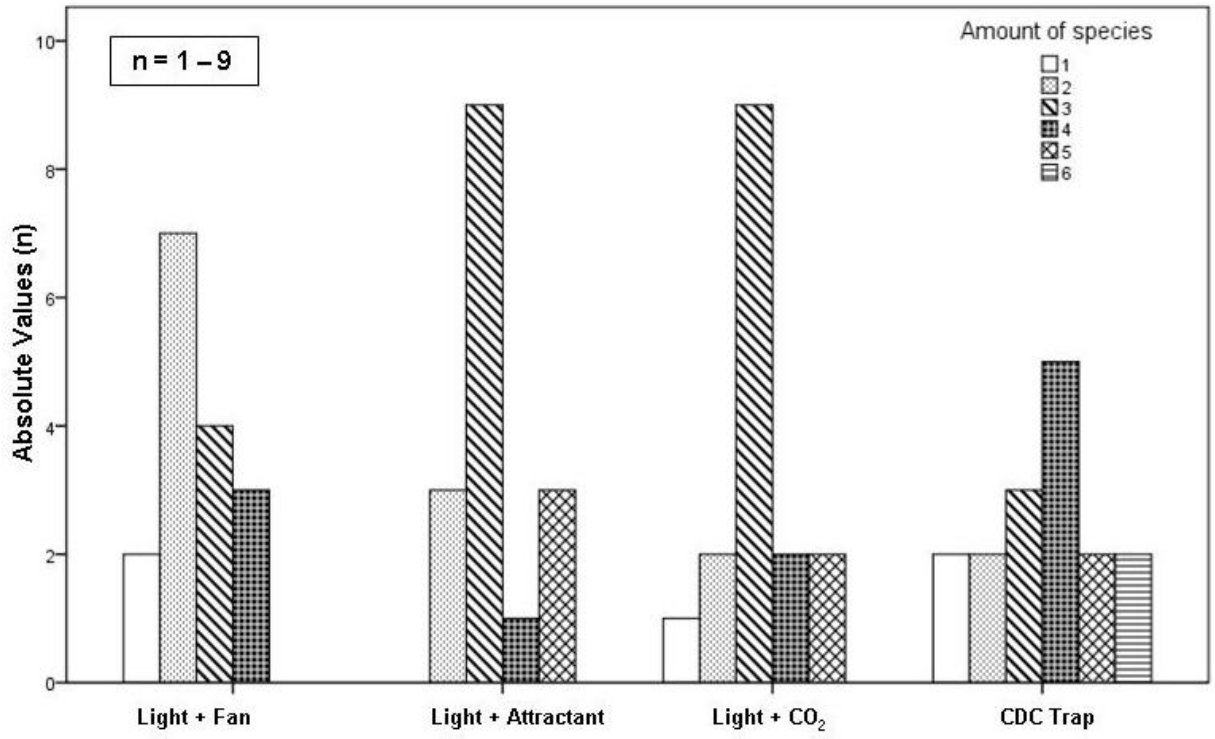

Fig. 24: Absolute frequency of the quantity of different species during the Latin Square trial.

Tab. 10: Cross-classified Table of the trapping success by frequency of different species during the Latin Square trial. Each column presents above the number (n) of trapped species and the frequency of trapped species in each row.

Quantity species (n)		1	2	3	4	5	6	Total
Trap	Light + Fan	2	7	4	3	0	0	16
	Light + Attractant	0	3	9	1	3	0	16
	Light + CO_2	1	2	9	2	2	0	16
	CDC Trap	2	2	3	5	2	2	16
	Total	5	14	25	11	7	2	64

Evaluating the mean average values of all trap types by the number of different species (Fig. 25), the data show that the CDC trap caught the highest variation of species compared to all other trap variants. However, the difference of the efficiency between the different traps is not statistically significant ($p > 0.161$).

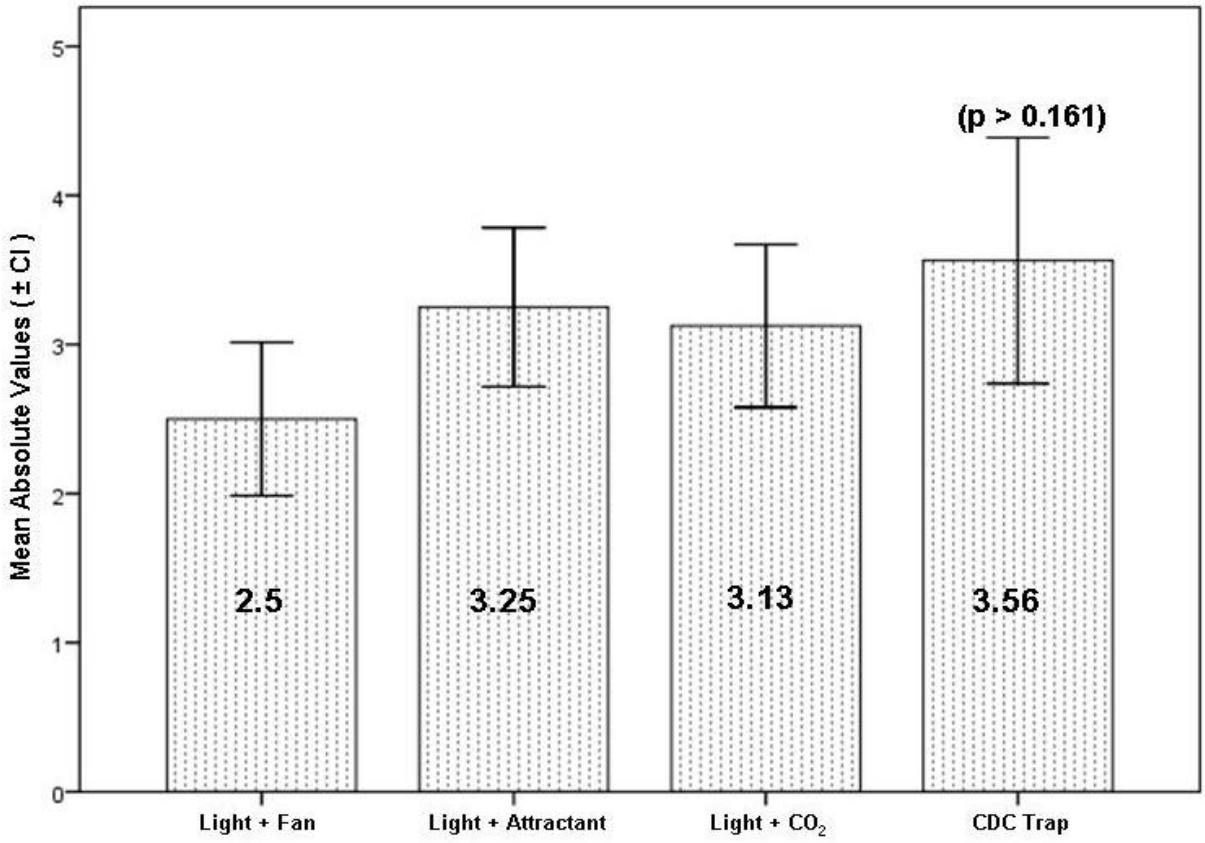

Fig. 25: Mean of quantitative trapped species, data in columns present the mean value of each trap type; CI = confidence Interval, 98 %.

4.2.2. Field collection in Southern Germany

A strategic entomological survey was conducted at 44 different field sites in Southern Germany during 2007 and 2008. Different families of diptera of the suborder of Nematocera (e.g. Ceratopogonidae, Cecidomyidae, Culicidae, Psychodidae, Sciaridae and Tipulidae) and the suborder of Brachycera (e.g. Calliphoridae, Muscidae and Drosophilidae) were caught. Approximately 40.933 specimens of Psychodidae were examined (Tab. 11). No phlebotomine sand fly was found. Additionally, intensive entomological trapping did not reveal phlebotomine sand flies at any of the three locations, for which autochthonous cases of leishmaniosis had been reported before.

Tab. 11: Data present the quantity of insects trapped during the sand fly survey at all different trapping locations in Southern Germany during the summer period of 2007/08. * Trapped insects included different families of diptera of the suborder of Nematocera (e.g. Ceratopogonidae, Cecidomyidae, Culicidae, Psychodidae, Sciaridae and Tipulidae) and the suborder of Brachycera (e.g. Calliphoridae, Muscidae and Drosophilidae). Other insects were not determined.

No.	Locality	Trapped insects* (n)	Psychodidae (n)	Phlebotominae (n)
1	Oberwiesenfeld	6400	1626	0
2	Riem	7680	1513	0
3	Schwabing	10600	2686	0
4	München	23400	13551	0
5	Leutstetten	440	90	0
6	Hummersberg	1380	725	0
7	Eckhof	1420	605	0
8	Eckhof	1210	346	0
9	Augsburg	16740	2953	0
10	Gablingen	10620	2945	0
11	Unterreitnau	620	385	0
12	Schnetzenhausen	540	412	0
13	Laubegg	360	307	0
14	Geisingen	280	211	0
15	Weitenau – Steinen	560	463	0
16	Istein	100	82	0
17	Istein	30	14	0
18	Welmlingen	1240	945	0
19	Schliengen	540	323	0
20	Staufen	220	116	0
21	Au bei Freiburg	440	268	0
22	Au bei Freiburg	4860	1678	0
23	Heuweiler	720	537	0
24	Munzingen	58	29	0
25	Ettenheim	430	298	0
26	Emmersbach	360	142	0
27	Offenburg	3120	1209	0
28	Achern	280	165	0
29	Sinzheim	320	216	0
30	Durmerstheim	230	164	0
31	Ludwigsburg	2350	1298	0
32	Dörrenbach	960	573	0
33	Fürth	860	457	0
34	Hangard	1380	703	0
35	Velsen	2480	1584	0
36	Karlsbrunn	240	196	0
37	Gehrweiler	410	291	0
38	Gehrweiler	820	614	0
39	Gundersweiler	320	213	0
	Result	**105018**	**40933**	**0**

4.2.3. Field collection in Italy

Two different species with a total of 153 specimens of phlebotomine sand flies were trapped in a suburb of Rome during the field collection in the *Leishmania* endemic focus. Morphological examinations determined 55 specimens (36 %) as *P. perniciosus,* including 42 females (10 unfed, 7 blood-fed and 25 gravid) and 13 males. 98 specimens (64 %) were identified as *S. minuta*, including 29 females and 69 males. The two different species presenting males and females are shown in Figure 26 and 27.

Fig. 26: *Phlebotomus perniciosus* (left) male, (right) female.

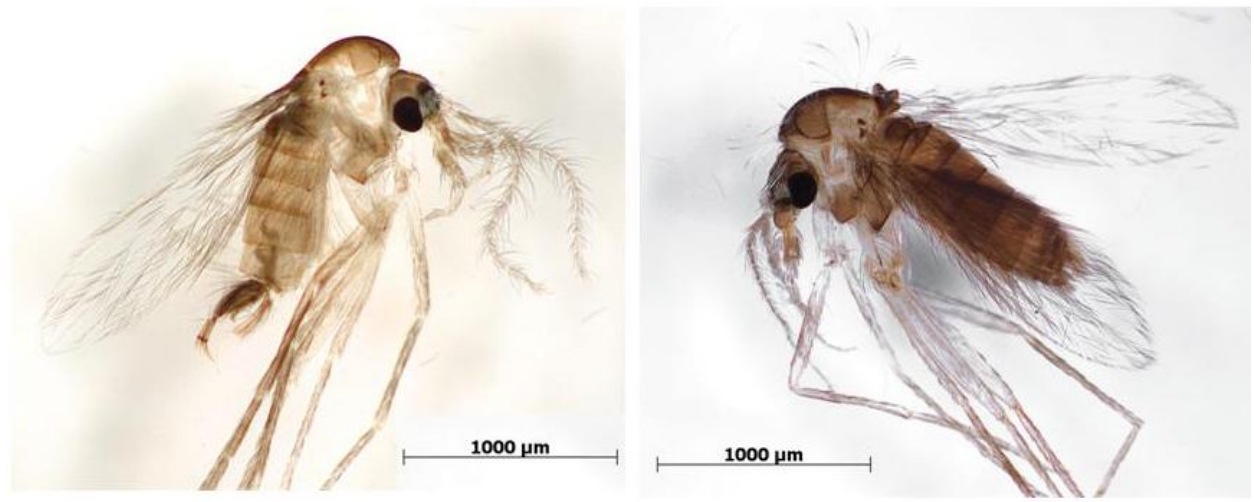

Fig. 27: *Sergentomyia minuta* (left) male, (right) female.

Among the 15 sites monitored in the Bolzano province and the Adige valley, two sites were positive for the presence of phlebotomine sand flies. A total of 30 specimens were captured, five *P. perniciosus* (16.66 %) and 25 *S. minuta* (83.33 %). The results from all positive sites in Italy are summarised in Table 12.

Tab. 12: Cumulative entomological data obtained during the sand fly season 2008 in the suburb of Rome (Canile), the Bolzano province (1) including the Adige valley (2) in Italy.

Locality	*Phlebotomus perniciosus*		*Sergentomyia minuta*	
	male	female	male	female
Canile	13	42	69	29
Guncina (1)	4	1	6	18
Terlano (2)	-	-	-	1
Total (%)	17 (9.3)	43 (23.5)	75 (41.0)	48 (26.2)

To summarise, 60 specimens (32.78 %) of *P. perniciosus* were obtained during the entomological field survey in Italy. Furthermore, 123 specimens (67.22 %) were determined as *S. minuta*, a phlebotomine sand fly that primarily feeds on reptiles. The ratio of males to females was different between the two species.

4.3. Data of Climate

The data received from the meterological weather service showed the monthly average temperature and average relative humidity from June to September 2008 for Munich, Freiburg and Saarbrücken. Additionally, the data of Munich from June to September 2007 were included in the diagram. The average temperature varied between 12.0-19.6 °C during the four months and the average relative humidity ranged from 66 % to 80 % (Fig. 28). The data about climatic parameters (minimal and maximal temperature, relative humidity) of each trapping location are presented in Table C of the annex.

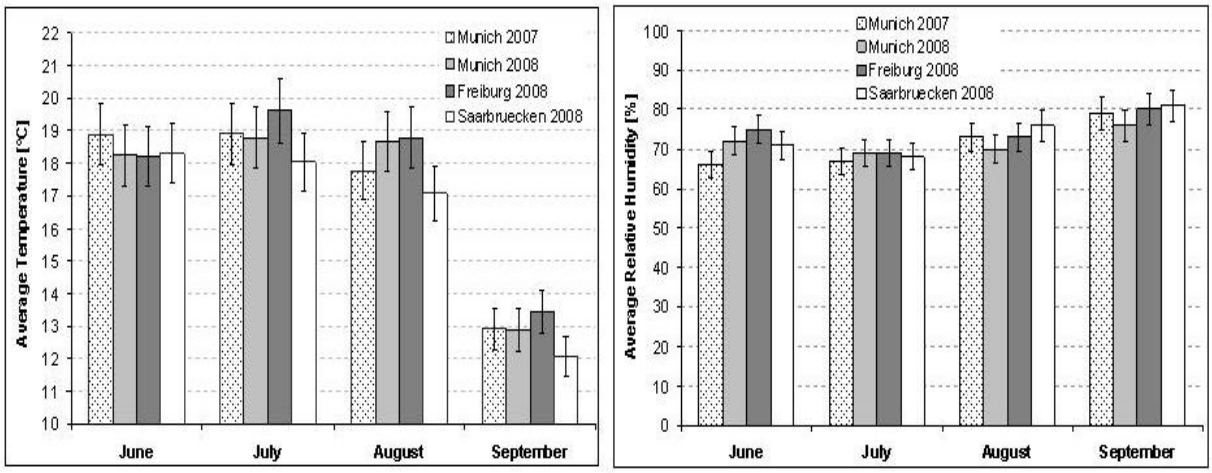

Fig. 28: Climate data (left) average temperature per month, (right) average relative humidity per month. Data provided by the local meterological weather service.

4.4. PCR

4.4.1. Quality and Quantity of extracted DNA

Real-time PCR was used to analyse trapped *P. perniciosus* for the presence of *Leishmania* infections. The amount of DNA extracted from each female *P. perniciosus* measured with NanoDrop® ranged from 2.0 ng/µl to 19.8 ng/µl (see Annex, Tab. H). The positive control contained 10.7 ng/µl.

4.4.2. Standard Curve

The standard curve was performed to determine the efficiency of the PCR assay and the concentration of DNA in a possibly infected female phlebotomine sand fly.

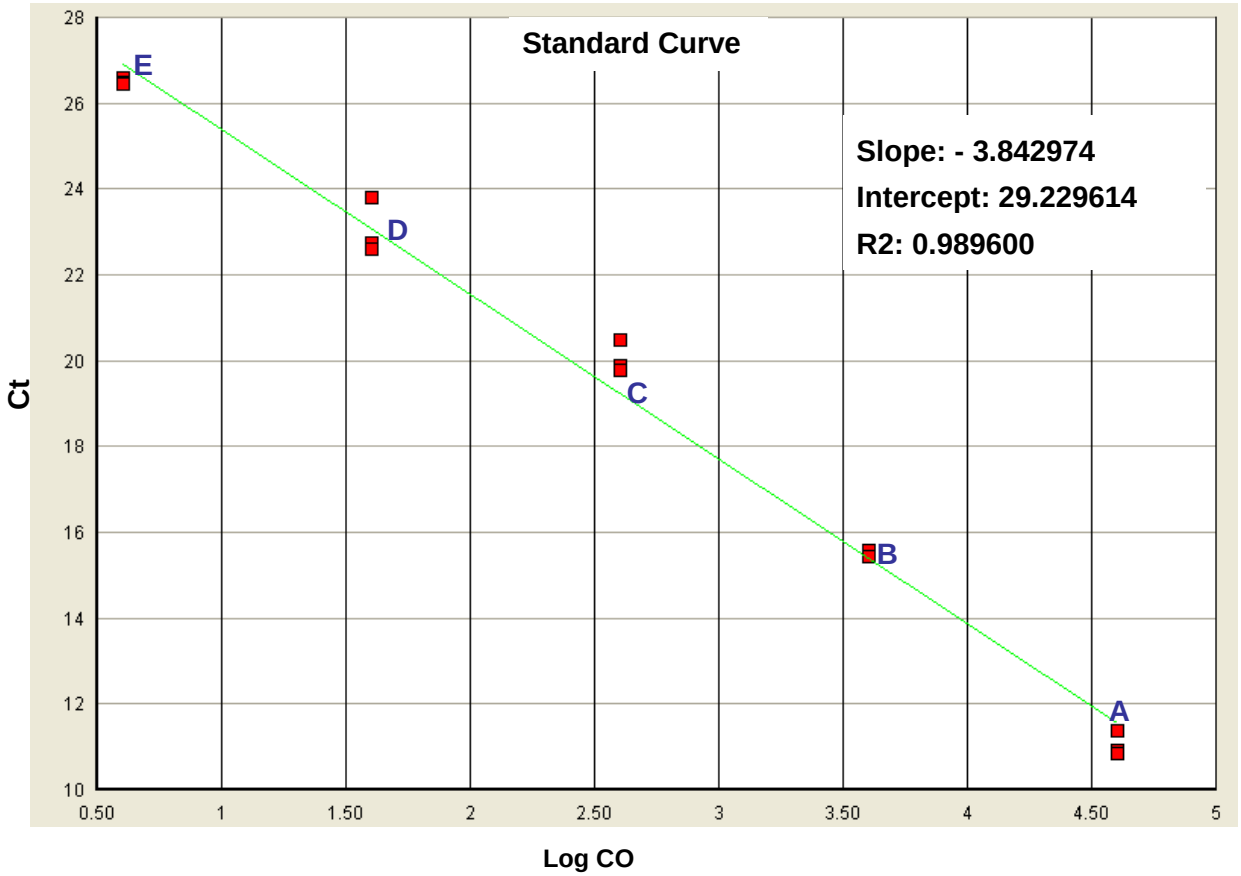

Fig. 29: Standard curve of *Leishmania donovani infantum* **DNA (**Ct: cycle threshold; Log CO: Log copy number, R2: correlations coefficient); Letters indicate the different dilution series (see Tab. 5).

For positive samples, the software calculated the quantity of unknown target sequence from the standard curve for the detector and for the specific target sequence. Figure 29 shows the performed standard curve, a negative linear progression. The lowest dilution series, 4 copies (E) was first detected at cycle 27 in the amplification plot. Therefore, a sample containing only one *Leishmania*, the signal should cross the threshold at cycle 29.

4.4.3. Amplification plot of the real-time PCR

The amplification plot displays Rn (reporter dye fluorescence) as a function of the cycle run (Fig. 30). A total of 40 cycles were run and for the positive control (sigmoidal progression) the fluorescence signal was first detected in cycle 11. Signals positive for *Leishmania* spp. should have been occurred between cycle 13 and 29, plus a variance of two cycles (31). Every signal appearing after cycle 32 was considered to be an unspecific reaction. None of the 39 female phlebotomine sand flies were infected with *Leishmania* spp.

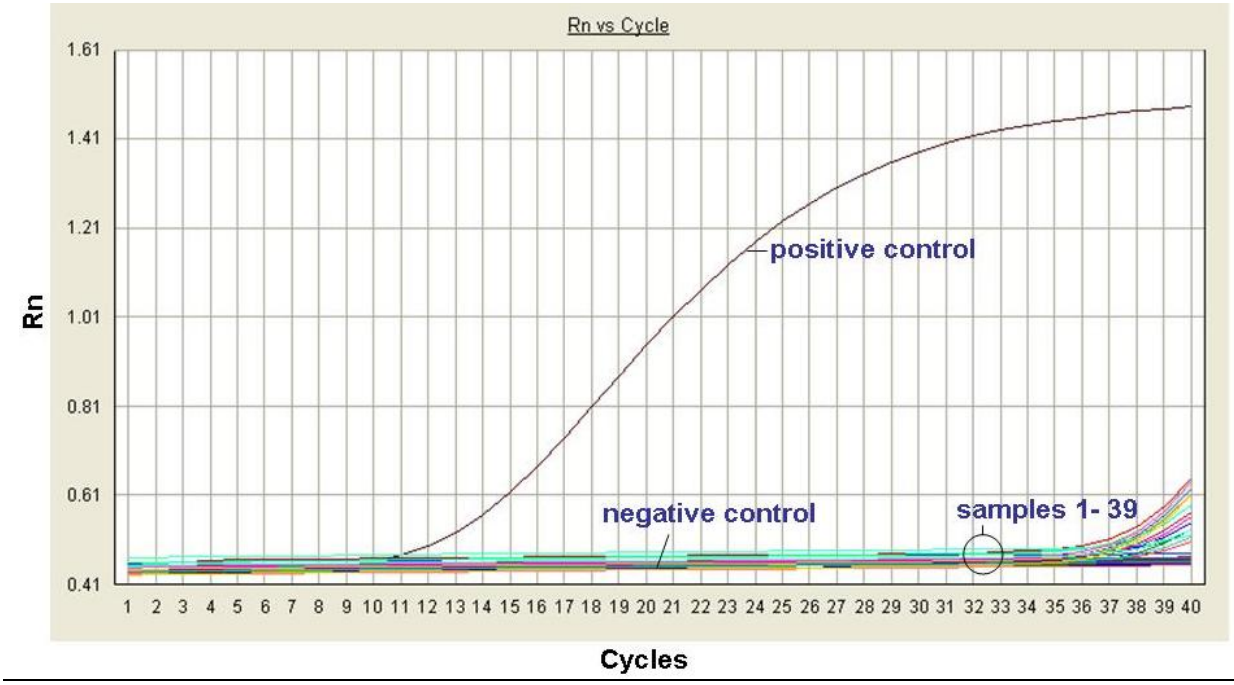

Fig. 30: Amplification plot of the real-time PCR for detection of *Leishmania* spp., (threshold: 0.1748328).

4.5. Retrospective study

Evaluation of retrospective data of dog samples, examined with IFAT and real-time PCR for the infection of *Leishmania*, tested during 2006 and 2008 resulted in a total of 6.494 samples (Tab. 13). During these three years, an increase in the amount of requests was determined. A total of 1759 samples were tested for *Leishmania* in 2006. 2097 samples (an increase of 19 %) were analysed in 2007 and 2638 samples were tested in 2008. This sample amount presents an increase of 49 % compared to 2006. The major part of all analysed samples was negative for

Leishmania. The mean average of all negative samples during the three years was 71.03 %, for the positive results 14.03 %, and for doubtful cases (IFAT titre of 1:32) 14.94 %. Detailed data of each year are listed in Table I of the annex. Besides these samples from dogs, one sample was from a pygmy rabbit, which was negative and three samples were from cats, of which two were negative and one was positive. The positive cat sample originates from one imported from Spain.

Tab. 13: Retrospective evaluation of samples tested for *Leishmania* (IFAT and PCR) in the Diagnostic laboratory during 2006 and 2008.

Year	Total	Negative	Positive	Doubtful
2006	1759	1371	208	180
2007	2097	1420	314	363
2008	2638	1822	389	427
Total (%)	6494 (100)	4613 (71.03)	911 (14.03)	970 (14.94)

The annual distribution of all positive samples (data combined from IFAT dilution series of 1:64 to 1:512 and positive PCR results) in each year is shown in Figure 31. In general, positive samples varied between 10 to 40 samples per month. One exception is given in April 2006, where less than 10 samples were positive. Additionally, in May 2008 the positive samples were exceptionally high.

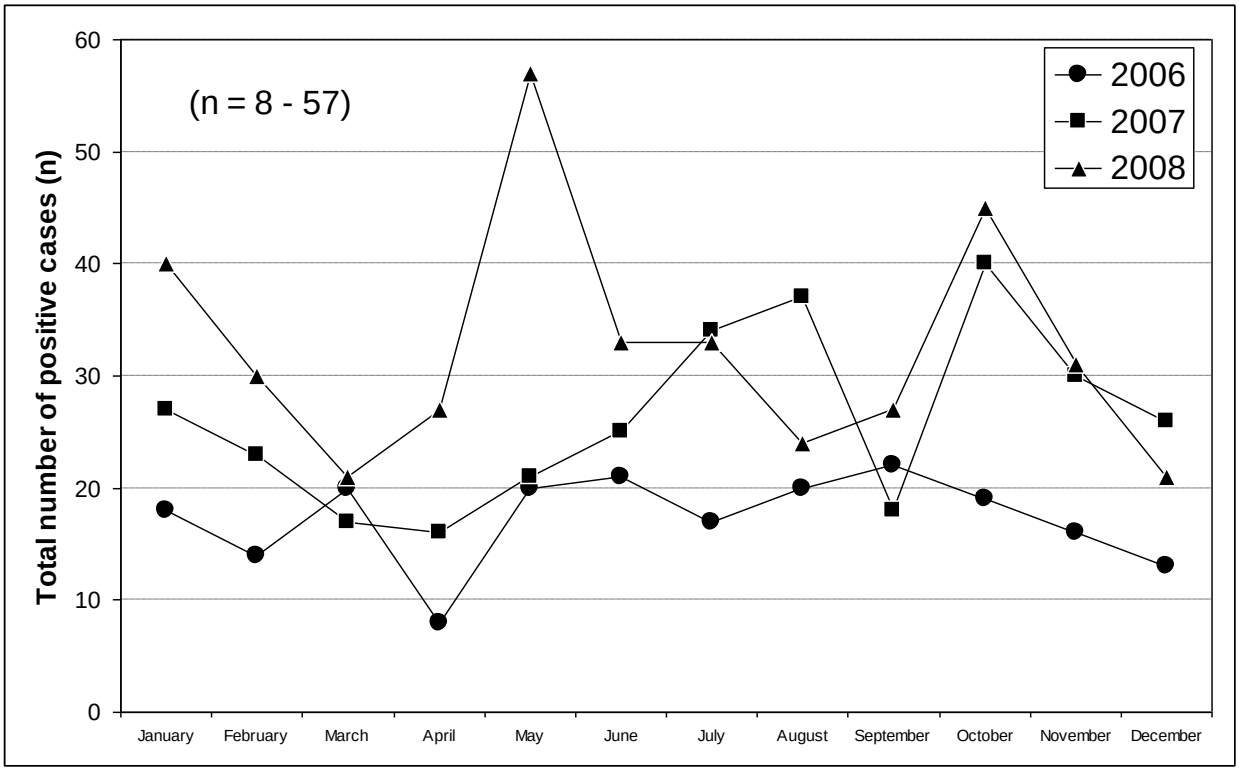

Fig. 31: Annual distribution of positive samples tested for *Leishmania* (IFAT and PCR) in the Diagnostic laboratory during 2006 and 2008.

The 911 positive samples were attributed to 400 different dogs. Samples of those dogs were analysed one to eight times within the three-year retrospective study (Tab. 14). Samples came in for routine check up, direct suspicion of *Leishmania* infection or required tests for travelling diseases. Additionally, in case samples of dogs with doubtful results, it was recommended to send a second sample after three to four weeks to repeat the test. From all *Leishmania* positive dogs, samples were sent in again after six months.

Tab. 14: Frequency of examined samples from 400 *Leishmania* positive dogs.

Examination	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Dogs	244	85	37	19	7	4	1	3

The samples of dogs tested several times in our laboratory were checked for the development of the *Leishmania* titre. For example, three dogs (their samples came in eight times) were chosen to show the progress of the positive titre, which can vary often due to the relapse of leishmaniosis (Fig. 32). Dog 1 showed a stable titre (1:256) over four examinations. At the fifth

examination the titre was dropping down and rising after the sixth test highly positive again. Dog 2 showed first a low positive titre (1:64), then rising slowly and decreasing again after the fifth examination. The last examination, however, indicated a higher *Leishmania* titre (1:128) again. Dog 3 showed a negative titre at first and afterwards a very high titre (1:512). Due to the relapse of the disease, the IFAT titre can fluctuate steadily.

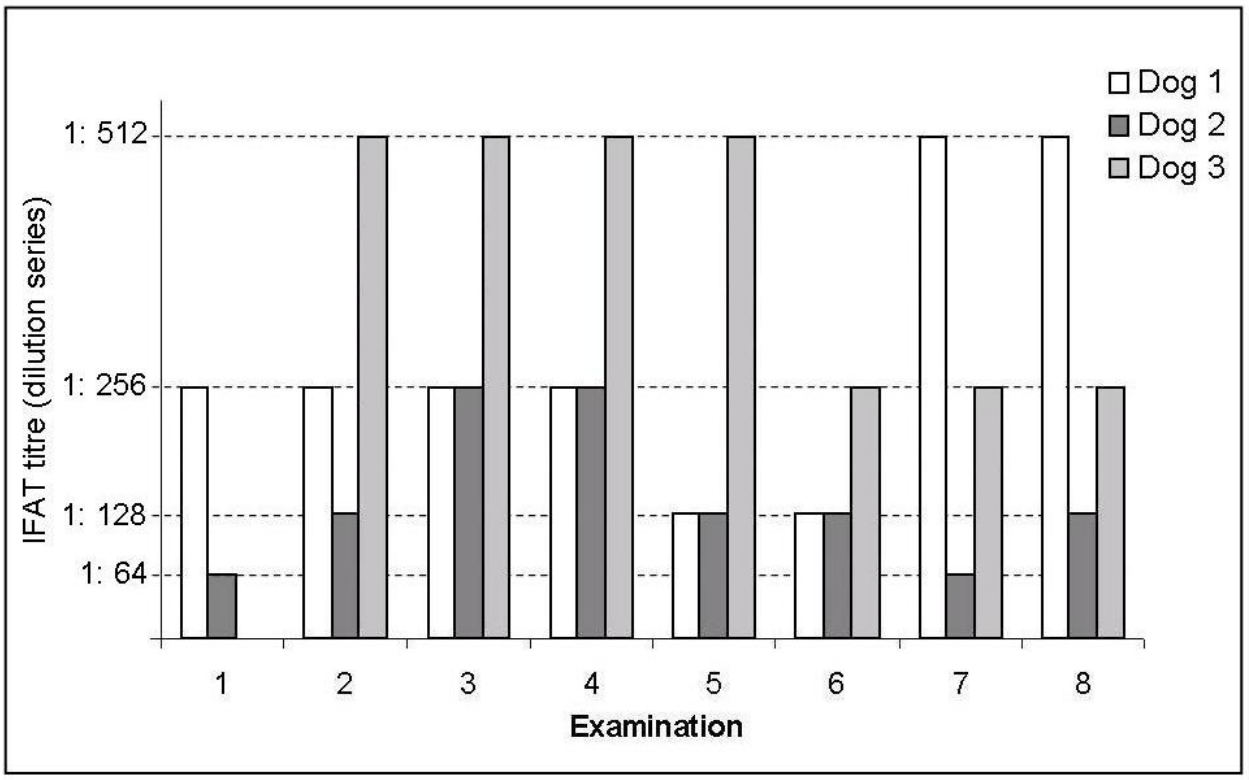

Fig. 32: Fluctuation of IFAT serum titre positive for *Leishmania* during eight tests from three different dogs during 2006 and 2008.

Evaluation of the origin of dogs, samples positive for *Leishmania*, mainly presented dogs imported from the Mediterranean countries. Descriptive analyses of these samples presented ten different countries. The highest number (35.35 %) of samples originated from Spain (Canary Islands - Fuerteventura, Grand Canary, Tenerife; Balearic Islands – Mallorca and Ibiza; Andalusia), 17.67 % came from Greece (Corfu, Crete and Samos) and 3.84 % were from Italy. The others samples (< 2 %) derived from dogs imported from Portugal, Malta, Croatia, Montenegro, Morocco and Turkey. Single cases of positive samples from dogs in Germany occurred too. However, information about the origin or travelling of the owner with the dog was not available.

5. Discussion

5.1. Entomological Survey

5.1.1. Psychodidae

Within the Palaearctic region, moth flies (Psychodidae) assemble a large family of Diptera, which comprises about 500 species (Wagner, 2004). Less attention is paid to the Psychodidae due to their unimportance in medical fields (except Phlebotominae). During the two-year survey in Southern Germany, more than 40.933 specimens of Psychodidae (see 4.2.2., Tab. 11) were captured. Five different species groups and five different species of the subfamily of Psychodinae and two species of the subfamily of Trichomyiinae were successfully determined (see 4.2.1., Tab. 9). The variety of found species might appear rather low compared to the 150 species of Psychodidae known in Germany. The trapping time from dusk to dawn selected only crepuscular and night active species that are attracted by light traps, which reduces the quantity of species but not the high numbers of specimens. Further, the trapping locations were mainly restricted to farms and walls that could decrease the variety of species too. The most abundant species were *Psychoda* spp. and *Tinearia alternata*, which were found at all trapping locations. This indicates that these species are adaptable, omnipresent to different habitats and not limited to certain biotopes. About half of the determined species are very rare species, which were trapped only in few specimens. This result is comparable to known data. In Bavaria, 39 % of Psychodidae are listed on the "Red List of endangered species" (Wagner, 2003). In the present study, captured species such as *Mormia apicealba*, *M. furva*, *Telmatoscopus rothschildi* and *Trichomyia urbica* are very seldom and were found only on selected habitats. One species of the subfamily of Trichomyiinae has even not been described in the literature before. This shows that rare and unknown species of Psychodidae are still present in Southern Germany even though the environment is changing. New species of moth flies are discovered and described in Germany, including this thesis (Wagner and Schrankel, 2005). It seems to be that these species could survive only in nature conservancy areas. Especially, the location Reitschule Munich, being still surrounded by very old trees and a rivulet, seems to be an appropriate habitat for the survival of these rare species like *Trichomyia stephani* nov. spec., which has originally been described there (see 4.1., publication).

5.1.2. Phlebotominae

Many aspects of Phlebotominae biology have not been explored yet. Sand flies are a challenging group to study in the field and very difficult to find in their natural habitats as in the conducted two-year field survey. Even the adults are very small and have strong morphological similarities within the species. Hence, all Psychodidae collected during the survey in Southern Germany were individually screened for Phlebotominae under a microscope, a very laborious and time intensive step. The methods used for trapping focused on adult sand flies that were either resting or active. An attempt to search for larvae was not undertaken during the survey because the collection and sampling of larvae is extremely laborious and time intensive and often remarkably unsuccessful due to a general lack of information about specific breeding sites as shown by Lane (1993) and Alexander (2000). Moreover, it was more important to find adult sand flies first and to prove their existence in Southern Germany. Previously, Naucke and Pesson, (2000) and Naucke and Schmitt (2004) reported the finding of two different species of *P. perniciosus* and *P. mascittii* in Germany. In order to verify the existence of phlebotomine sand flies in Germany more than 105.018 insects were trapped and over 40.933 Psychodidae (Tab. 11) were screened. As for the survey, geographical stratification of 44 different foci of previously reported sand fly occurrences, appropriate habitats or presumably autochthonous cases of Leishmanioses did not succeed. With the methodology used in this entomological survey on the presence and distribution of phlebotomine sand flies, no such specimen could be found in all surveyed areas in Southern Germany. Hence, the previously reported findings could not be confirmed. Neither the interceptive nor the attractive trap provided positive results for phlebotomine sand flies in Southern Germany, although the same traps were successfully applied in Italy. However, this result is rather comparable to that of other studies concerning *P. mascittii*, where this species is reported to be caught in very low abundance, confirming the rarity of this species even in their areas of distribution (Knechtli and Jenni, 1989; Léger et al., 2000 b; Maroli et al., 2002; Rossi et al., 2008). The assumption can be made that this species has to be numerousness to detect single specimens of it. Less information is available about the biology of *P. mascittii*, which makes it difficult to investigate. Therefore, it was questionable whether the population of *P. mascittii* was too little to be detected with the traps or just not existent. Different aspects were considered for the absence or not detectable density of sand flies, which could have influenced the trapping success, namely the methodology, the location, the seasonality of sand flies and the climate influence.

To check the influence of the methodology, different types of traps, interceptive (sticky papers) and attractive traps (CDC miniature light traps) were applied. Due to the recommendation of these standardised traps and the successful application in Italy (Canile and Bolzano) they were also used in Germany but did not work. This goes in line with the results of Killick-Kendrick (1987) and Dye et al. (1987) reporting about *P. perniciosus* that was collected in very few numbers by this trapping method in the South of France. Whereas in Italy, 98 % of all sand flies caught with the CDC light traps were *P. perniciosus* (Rossi et al., 2008). Similarly, Knechtli and Jenni (1989) reported an increasing trapping success with light traps for *P. perniciosus* and *P. mascittii* in Switzerland. Nevertheless, in Germany no phlebotomines were captured with this type of traps. In addition, the most common interceptive trap, sticky paper traps (Lane, 1993), was set up at all appropriate places. Again, no specimens were trapped by this method in Germany. One factor leading to failure could be the high levels of humidity. Lane (1993) showed that during humid periods papers lose their rigidity and hence their effectiveness. In the present study, the relative humidity during the data collection ranged between 65 % and 85 %, which might have interfered with these trap types. Another factor could be a very low density or absence of sand flies. Alexander (2000) raised the hypothesis that non-attractant traps are catching sand flies in a small perimeter and that these therefore yield low numbers of sand flies unless the population densities are high. Moreover, traps can vary their efficacy inter-specifically and intra-specifically by gender, physiological status and the phototropic behaviour of the target species (Davies et al., 1995; Dinesh et al., 2008). As well, moonlight could be the reason to reduce the illumination of the CDC light traps (Alexander, 2000) since sand flies are caught during active flight passing close to the traps. Therefore, traps were set up within a radius of two to five meters as mentioned by Killick-Kendrick et al. (1985). They concluded that *P. ariasi* was attracted to the CDC light traps from a maximum distance of two meters. In addition, during the field surveys in 2007, light traps were combined with dry ice (frozen carbon dioxide) to increase the possibility of trapping phlebotomine sand flies. The catch of haematophagous insects like sand flies can be improved by the addition of carbon dioxide, such as a piece of dry ice. CO_2 is usually a long-range attractant for sand flies and for mosquitoes (Gillies, 1980), whereas light seems to be perceived by a closer distance. Alexander (2000) showed already that the addition of a source of CO_2 could improve catches by increasing the effective sampling area of a light trap. Like this, huge numbers of Psychodinae were trapped in Germany. However, with this method no Phlebotominae were caught. In addition to the use of CO_2 -enhanced light traps, the idea came up to attract sand flies with pheromone-alike volatiles as it is done with mosquitoes. Among

Diptera, the most known chemical signals in adults are sexual pheromones, which operate over a relatively short distance as attractants or stimulants. The sand fly genera *Lytzomyia* and *Sergentomyia* produce male sex pheromones that enhance aggregations of both sexes (Ward and Hamilton, 2002). Females of *Lutzomyia* recognize their partners by pheromones produced by the males with glands on the abdomen (Ward and Morton 1991). Pinto et al. (2001) were able to demonstrate the importance of a combination of carbon dioxide and human kairomone in the attraction for *Lutzomyia intermedia* and *Lutzomyia whitmani* in the field. However, no chemical or biological evidence for similar pheromones have been detected in the genus *Phlebotomus*. The males of *Phlebotomus* spp. appear to have no pheromone-producing abdominal glands (Ward et al. 1991). Therefore, trapping of phlebotomine sand flies with pheromonal traps was not possible during the entomological survey. Nevertheless, traps with chemical attractant (Biogents AG, Regensburg, Germany) that worked perfectly for the Psychodinae were tried to improve the success.

Furthermore, it was important to think of the influence of the location on the trapping success. Sand flies depend on sheltered ecological habitats close to conceivable blood meal sources. Hence, traps were placed at sites of known habitats for sand fly activity, domestic and peri-domestic predominantly inside cow and horse stables, sheep pens, chicken houses, rabbit hutches and dog kennels (Feliciangeli, 2004; Yaman and Dik, 2006; Rossi et al., 2008). In these field sites, the capturing rate was more than 95 % of all species collected by Bongiorno et al., (2003) but negative during the conducted field surveys in Southern Germany. Similar to Grimm et al. (1993), traps were set up at night-time in the inside and/or outside the above mentioned habitats to collect sand flies during their activity. Feeding and mating activity takes place nocturnal and crepuscular. Grimm et al. (1993) reported about trapping success of *P. perniciosus* and *P. mascittii* between 7 pm and 7 am. In addition, adults of those two species were found resting indoors on walls between 10 pm and 3 am. Although, the set up time for the traps was prolonged (5 pm to 9 am) phlebotomine sand flies were not found.

The third possible influence is the seasonality of sand flies, which is limited to one generation per year in temperate regions. Consequently there is only one single peak of activity and transmission per year during the warm months. The life cycle is comparatively long (approximately 30-45 days), depending on the temperature. Species in temperate zones may hibernate in diapause until the following year and thereby can survive cold temperatures (Alexander, 2000; Lindgren and Naucke, 2006). However, the same species can have two or three generations per year in climatically more favourable habitats (Lane, 1993). The

entomological survey was conducted from June to September, according to previously reported activity of sand flies in surrounding countries of Germany. In France, sand fly activity is considered to range from May to the beginning of October, with some species (*P. ariasi*, *P. perniciosus*) being predominantly active in July and August (Rioux et al., 1967). In Switzerland, sand flies are present from end of June to end of August (Knechtli and Jenni, 1989). In Germany, the period of activity is reported to range from June to August (Schmitt, 2002) when climate conditions are favourable. Therefore, sampling months were chosen due to periods of activity during the warm months that are reported in previous studies.

Finally, distribution and density of sand flies is highly dependent on temperature and relative humidity (Haines et al., 2006). Climatic factors distinctly influence their activity and development. The worldwide distribution of phlebotomines is shown to be in regions where the mean temperature is 20°C for at least one month (WHO, 1984). The monthly average temperature from June to September 2008 (data provided by the meterological weather service) varied from 12°C to 19.6°C in the areas studied during the field survey, what seems to be relatively low. However, Naucke (1998) and Schmitt (2002) reported about active specimens of *P. perniciosus* and *P. mascittii* at temperatures of 13°C and 13.5°C in Baden-Wuerttemberg. This suggests that the limit of the European distribution corresponds to the 10°C annual isotherm (Maier et al., 2003). Phlebotomine sand flies could be able to adapt to colder temperatures. Nevertheless, lower temperatures most surely reduce their activity and might decrease the trapping success regardless of any other meteorological factor (Miščević and Milutinović, 1986). In Bavaria, the annual average temperature is below 10°C and therefore not sufficient for the survival of phlebotomine sand flies. Until today, there have been no reports on the presence of sand flies in Bavaria. This circumstance could be confirmed by this study. In addition, there was only one report of *P. perniciosus* in 2001 (Naucke and Schmitt, 2004) and no further findings in Southern Germany until today. Environmental changes are probably responsible for the occurrence and the following disappearance of sand flies as shown at this particular example. Strategic sampling at the site of Gehrweiler (see annex, Tab. A, location 38), where an autochthonous case of canine leishmaniosis including the vector was reported in 2001 (Naucke and Schmitt, 2004), did not reveal any sand flies during a three month consecutive surveillance in 2008. Pre-existing horses (blood meal source) and a gigantic walnut tree (shelter) have been removed (personal communication of the owner). Consequently, the habitat did not provide adequate conditions for the survival of phlebotomine sand flies anymore.

Though no phlebotomine sand flies were trapped during the two-year survey, hypothetically, three ways are possible on which phlebotomine sand flies could enter Germany. Firstly, they could cross the Alps through the passage of Tyrol and appear in Bavaria. However, no reports of phlebotomine sand flies have been presented in Austria until today (Aspöck et al., 2008). The second possible way is a distribution along the corridor of Austria and Hungary and the occurrence in Bavaria in the area of Passau. Very recently, phlebotomine vectors and canine leishmaniosis cases have been recorded in Hungary (Farkas et al., 2009). Thirdly, they could cross the border of France and settle down along the Rhine valley. Since phlebotomine sand flies are very lightweight tiny organisms, there is a high probability of them being dispersed by the wind. The occurrence in France can be explained by the hypothesis that they might be drifted by wind flow and incidentally found in areas close to the border of France along the Rhine valley. Indeed, most reports presented cases of phlebotomes exactly in this region along the border (Naucke and Pesson, 2000; Naucke and Schmitt, 2004). Dispersion seems to be possible and can result in temporary positive findings. However, even though temperatures are temporary and locally high enough to build up possible microhabitats for sand flies along the Rhine valley, this is not sufficient for survival or establishment of new populations of sand flies in this area.

The main objective of the field survey in Central Italy was to show that the applied methodology works perfectly for trapping Phlebotominae in a known endemic area and in a new focus in Italy. Field studies were conducted in July and August, where the activity of sand flies is highest with one peak in June/July and a second peak in August or September/October (Adler and Theodor, 1931; Maroli and Bettini, 1977; Rossi et al., 2008). The successful application of the CDC miniature light traps and sticky paper traps were proven with the survey. In total 183 phlebotomine sand flies, two different species (*P. perniciosus* and *S. minuta*) were identified. This obtained result was identical to the findings of Rossi et al. (2008), who caught 95 % of all sand flies, mainly *P. perniciosus* and *S. minuta*, with these two trap types. Central Italy is known for the presence of different phlebotomine species, especially *P. mascittii, P. papatasi, P. perfiliewi, P. perniciosus* and *S. minuta* (Grassi, 1908; Adler and Theodor, 1931; Corradetti, 1962; Khoury et al., 1992; Maroli and Khoury, 1999). As predicted, two different phlebotomine sand fly species (*P. perniciosus* and *S. minuta*) were trapped in Canile (a suburb of Rome). In addition, sticky paper traps were placed at all possible diurnal resting sites, which are domestic sites, including animal shelters and wall crevices. By this, the possibility was increased to trap *S. minuta*, which inhabits restricted biological niches in stone walls. Similar to Rossi et al. (2008)

and Bongiorno et al. (2003), more than 90 % of all *S. minuta* specimens were trapped in wall crevices. An explanation for this result could be that this species likes to live in close association with lizards and feeds on these reptiles (Adler and Theodor, 1957; Rioux et al., 1969), which inhabit those stone walls. Additionally, in Rome and province of Bolzano the relative humidity (37-60 %) was lower than in Germany (65-85 %), which influences the effectiveness of sticky paper traps and might support a successful catch. Even though, 67 % of the captured species were *S. minuta*, it is not considered to be a competent vector of leishmaniosis. This species is rather a vector of *Leishmania tarentolae* (Parrot, 1935; Maroli et al., 1988) and *Trypanosoma platydactyli* (Gramiccia et al., 1989), which are pathogens in reptiles in the Mediterranean basin. Therefore, it is medically less important as *P. perniciosus*, the main vector of *L. infantum*, which is widespread in the Mediterranean Basin (Bettini and Gradoni, 1986).

Furthermore, an entomological survey was conducted to assess the presence of *Leishmania* vectors in Northern Italy, province of Bolzano. This survey revealed unexpected positive results for phlebotomes. *Phlebotomus perniciosus*, one of the competent vectors of *L. infantum* in Italy was collected at Guncina at 15 field sites. Entomological surveys demonstrated already the presence of *P. perniciosus* in most parts of Italy, with significant density in Central, Southern and insular regions (Coradetti, 1962; Bettini et al., 1991; Maroli et al., 1994; Maroli and Khoury, 1999). However, in Northern Italy evidence of this species has never been reported. Northernmost, the first record of phlebotomine sand flies was reported in South Tyrol in 2008 (Morosetti et al., 2009). Further specimens were found in the present study and confirmed the findings of *P. perniciosus* in August 2008. The northern spread of *P. perniciosus* is comparable to another important potential vector species for *L. infantum* in Italy, *P. neglectus*. In the past its area of distribution was limited to southern regions of Italy too. Now it is spreading throughout the whole country (Gradoni et al., 2004). The same was observed with *P. neglectus* that was found again in several pre-Alpine areas after 78 years of inexplicable disappearance (Maroli et al., 2006). Additionally, *S. minuta* specimens were trapped at two sites in Guncina and Terlano (Tab. 12). In neighbouring areas, the Piedmont and Aosta Valley, sand flies were absent in a survey conducted about 30 years ago (Biocca et al., 1977). Ferroglio et al. (2005) reported the first presence of sand flies in an alpine region (Aosta valley) during a survey conducted in the years 2000 to 2001. In addition, phlebotomine sand flies were found in Italy at a wide range of altitudes (190-700 m) approving the various habitats in different altitudes. *P. perniciosus* has been reported up to even 1070 m a.s.l. in Abruzzo in Italy (Maroli et al., 1991) and together with *S. minuta* up to 1400 m a.s.l. in Morocco (Guernaoui et al., 2006). Hence, it seems that the

appearance of phlebotomine sand flies is not a matter of altitude but a matter of climate. The distribution of phlebotomine sand flies to colder areas seems to be possible. *Phlebotomus perniciosus* is now widespread in Italy and found at high densities in hilly areas and low mountain ranges. A northward spread of phlebotomine sand flies from endemic Mediterranean countries to Central Europe has been proposed to be associated with global warming (Killick-Kendrick, 1996; Harms et al., 2003; Lindgren and Naucke, 2006; Aspöck et al., 2008). This seems to be a big issue in terms of the possible spread of vector borne diseases like leishmaniosis.

Another species, *P. mascittii*, that is no proven vector of leishmaniosis, has been spreading northward throughout Europe. Specimens are well presented in the Mediterranean area e.g. Spain (Rioux et al., 1984 b), Italy (Grassi, 1908; Maroli et al., 2002), France (Rioux et al., 1967; Léger et al., 1985), and also close to the border of Germany in the district of Alsace (Callot, 1950) and have been reported in Switzerland (Knechtli and Jenny, 1989; Grimm et al., 1993). Though *P. mascittii* was not trapped during the two-year survey, most northerly, specimens have been reported in Germany in 1999 for the first time (Naucke and Pesson, 2000). They have been found in Belgium in 2001 (Depaquit et al., 2005) for the first time ever.

Although, *P. perniciosus* is found in southern part of Switzerland, leishmaniosis is not present in this country (Grimm et al., 1993). A few putative autochthonous cases of canine leishmaniosis have been reported in Germany (Gothe, 1991; Moritz and Steuber, 1999; Kellermeier et al., 2007), in regions with higher latitudes, and in the Netherlands (Diaz-Espineira and Slappendel, 1997). It remains questionable whether those cases originated from sand flies already living in surrounding habitats. The attained results in this thesis seem to confirm the absence of phlebotomine sand flies. However, a very low and not detectable quantity of this species can not be excluded.

5.2. PCR

The applied real-time PCR (Francino et al., 2006) worked perfectly as seen in the standard curve and positive control in the amplification plot (Fig. 30). This method was used to detect possible infections with *Leishmania spp*. in the specimens of *P. perniciosus*, which were trapped in Canile (Rome). None of the 39 samples were tested positive. All types of females (unfed, blood-fed and gravid) from the above mentioned vector species were analysed.

Theoretically, unfed females could carry DNA fragments of *Leishmania* from previous blood meals that are not digested yet or fragments from non-established infections (Gradoni, 2002). However, these sand flies were probably freshly emerged nulliparous females without any infections. Blood-fed females probably took their blood meal on healthy, non-infected mammals, mainly dogs. Furthermore, gravid females were studied because a blood meal is needed for the development of eggs. The use of gravid females for the evaluation of prevalence of *Leishmania* is proposed by Torina et al. (2008), finding the highest prevalence of *L. infantum* infections in those females. Analysing the infection rate in these 39 samples, the probability of an infected phlebotomine sand fly would be less than one specimen compared to results from Gradoni (2002). He found out, that in endemic areas in Southern Europe, the average rate of infections in the vector is about 1.0 %, as tested in more than 9000 females of this vector species. Moreover, the infection rate of *Leishmania* in phlebotomine sand flies in the Mediterranean countries varies from 0.4 % in Spain (Guilvard et al., 1996; Martín-Sánchez et al., 2006) up to 10.5 % in South Sardinia (Bettini et al., 1986). Due to the small sample size (39 specimens), one can draw no final conclusion.

5.3. Retrospective data acquisition

The evaluation of retrospective data showed a steady increase of samples tested for *Leishmania* in the Diagnostic laboratory that could be caused by the higher awareness of canine leishmaniosis. The topic climate change and "re-emerging diseases", is currently very present and dog owners are more concerned about travel related diseases of their dogs, especially because they often travel to *Leishmania* endemic areas, as the Mediterranean countries. Furthermore, people like to save dogs from these endemic countries and all dogs that are legally brought and imported to Germany have to be tested for *Leishmania* by the importing sanctuaries. As shown by Solano-Gallego et al. (2001) prevalence of *Leishmania* infection in dogs is high and reached up to 67 % in Majorca (Spain) and 80 % in Marseille (France). In addition, serological data of the European Mediterranean countries suggests an estimated 2.5 million dogs (16.7 %) that are infected with *L. infantum* (Moreno and Alvar, 2002). This all will presumably lead to a further increase of examinations for *Leishmania*.

The data about the dogs used in the retrospective study were very marginal and therefore impeded a correct interpretation of the results. The samples of dogs were randomly selected and

sent to the laboratory in irregular intervals by veterinarians and animal shelters (domestic and abroad). Therefore, sample volumes varied each month and no correlation could be found between the amount of samples and the samples that were tested positive. Furthermore, no evaluation was made about the influence of gender, age and breed of the dogs to the susceptibility of infections with *Leishmania*. However, studies of *Leishmania* infected dogs in endemic areas showed no correlation between the gender and breeds, which seem to be similarly susceptible. Only age seems to be a good indicator for the degree of infection (Moreno and Alvar, 2002). Prevalence is rising up to the age of three and declining until the age of seven to eight years (Moreno and Alvar, 2002). A follow up examination of doubtful cases (~ 15 %) with an IFAT titre of 1:32 did not show any significant conversion or increase of the titre. Due to the few examinations, often only one analysis and short time intervals no change in titre could be observed for long-term control.

In Northern Europe, canine leishmaniosis is restricted to dogs that accompany travellers to the Mediterranean area or originate from endemic areas around the Mediterranean basin. This is in line with the fact that about 60 % of the 400 of positive dogs tested in the Diagnostic laboratory originated from Mediterranean countries. No information was available about the origin of the other dogs, but it can be assumed that these dogs also originated from Southern countries. In particular, three dogs of the present study were assumed to be cases of autochthonous leishmaniosis in Germany. It was possible to prove that this was not the case. On the one hand, the phlebtomine vector could not be found in the shelter or neighbourhood of these dogs and on the other hand, other ways of infection or wrong diagnosis can be presumed. Since leishmaniosis is a disease with a long incubation time, owners of the dogs often forget where they have travelled to with their pets. In contrast, Gothe, (1991), Moritz and Steuber, (1999) and Kellermeier, et al., (2007) reported about proven cases of autochthonous leishmaniosis in Germany. In each of these cases, they could identify the *Leishmania* infection with the help of diagnostic methods. One of the dogs was presumed to have been congenitally infected via trans-placental infection (Moritz and Steuber, 1999). They excluded that dogs have travelled to endemic areas.

The increasing number of imported stray dogs is an important issue. More than 50 % of stray dogs from Greece were found to be infected with *L. infantum* (Sideris et al., 1996) and more than 20 % of dogs with clinical symptoms of leishmaniosis in Germany originated from animal shelters in Southern Europe (Melchers, 2009). No precise conclusion could be drawn about the prevalence of *Leishmania* in dogs from different countries analysed in the Diagnostic

laboratory due to the lack of relevant data. It was noticeable that more than 35 % of positive samples resulted from dogs originating from Spain. The same result was observed by Mettler et al. (2005). Canine leishmaniosis is widespread in the European Mediterranean countries with reported seroprevalences ranging from 1.7 % to 48.4 % (Fisa et al., 1999; Gradoni, 1999).

In healthy dogs, asymptomatic carrier status of *L. infantum* is diagnosed best by serological tests, because parasitic loads seem to be very low compared to those observed during the acute phase of the disease (Berrahal et al., 1996). IFATs were done in the Diagnostic laboratory. The 1:32 dilution was taken as threshold antibody titre and the results were considered doubtful for *Leishmania* antibodies and infections. This titre is not indicative of an ongoing established infection, but may be an indicator for previous contact with the parasite. 6494 samples of dogs were tested in the Diagnostic laboratory and it was not known whether these dogs are immune resistant animals or whether they will develop the disease subsequently. At the time of the examination, dogs did not show a seroconversion. However, one has to keep in mind that leishmaniosis is a disease with a long incubation period of up to several years and dogs can relapse again.

At the present, the risk of contracting leishmaniosis in Southern Germany is not existent, even though *Leishmania* infected dogs are imported to Germany every year. The natural presence of phlebotomine sand flies in Southern Germany could not be confirmed and reported phlebotomes are apparently not persistent in those areas yet. Further entomological surveys have to be conducted to address questions about the veritable risk factors on contracting leishmaniosis in Germany. This includes questions of survival of phlebotomes in Germany, their population dynamics as well as reproduction and finally the question about the actual potential of *P. mascittii* as a vector of *L. infantum*. Hence, at the moment the risk factors are very improbable.

6. Summary

Leishmaniosis is a worldwide occurring, zoonotic, vector-borne disease and is in Europe endemic in the Mediterranean countries. Sporadic reports on the occurrence of autochthonous leishmaniosis cases and records of phlebotomine sand flies (*Phlebotomus mascittii* and *P. perniciosus*) in Germany initiated a study to find out whether populations of sand flies have become endemic and whether there is a risk of infection with leishmaniosis in Southern Germany. In order to search for sand flies, an entomological survey was conducted in the summer months of 2007 and 2008 at 44 sites, including ecological preferable habitats and locations of previous case reports. Additionally, the applied methodology was tested for suitability in an endemic focus in Central Italy and in a non-endemic area in Northern Italy. Furthermore, all samples of dogs sent to the institute's Diagnostic laboratory for leishmaniosis diagnosis in the years 2006 to 2008 were retrospectively analysed. The aim was to achieve an overview of possible acute or autochthonous leishmaniosis cases in Germany.

About 105.018 insects were trapped with CDC miniature light traps and sticky paper traps. More than 40.933 specimens of the family of Psychodidae were determined. Particular focus was paid to the subfamilies of Phlebotominae (vectors of *Leishmania* spp.), Psychodinae and Trichomyiinae. Though, no specimen of the vector family Phlebotominae was caught in Germany. However, a new Trichomyiin-species (*Trichomyia stephani* nov. spec.) was discovered and described in this work. In the comparative study in Italy, phlebotomine sand flies were caught in the endemic area (*P. perniciosus* n = 55, *S. minuta* n = 98) and in the so far non-endemic area (*P. perniciosus* n = 5, *S. minuta* n = 25). These potentially infected female specimens of *P. pernicious* (n = 39) were analysed with real-time PCR and found to be negative for *Leishmania* DNA. The data obtained in this study did not confirm previous reports of the presence of phlebotomine sand flies in Southern Germany. At present, the risk of contracting leishmaniosis in Southern Germany seems not existent due to the lack of the vector, although *Leishmania* infected dogs are imported to Germany every year. Fundamental knowledge about the existence of phlebotomine sand flies in the area surveyed could help to understand the dissemination of these insects, their preferred ecological niches and help to identify important hot spots in case of acute autochthonous leishmaniosis. Further studies should be conducted to survey the continuous spreading of sand flies from neighbouring countries and their potential migration towards Germany.

7. Zusammenfassung

Leishmaniose ist eine weltweit auftretende, zoonotische, vektorübertragende Krankheit, die in Europa in den Mittelmeerländern endemisch ist. Sporadische Mitteilungen über das Auftreten von autochthonen Leishmaniosefällen sowie Berichte von Sandmücken (*Phlebotomus mascittii* und *P. perniciosus*) in Deutschland waren der Anlass für diese Arbeit. Die Fragestellung war, ob bereits endemische Sandmückenpopulationen vorhanden sind und ein Infektionsrisiko für Leishmaniose in Süd-Deutschland gegeben ist. In den Sommermonaten 2007 und 2008 wurden zur Untersuchung der Sandmücken in Deutschland entomologische Feldstudien an 44 Standorten durchgeführt. Gesucht wurde in ökologisch, bevorzugten Sandmücken-Habitaten und Orten mit bereits berichteten Vorkommen. Die angewandte Methode wurde in einem endemischen Gebiet in Mittelitalien und einem nicht endemischen Gebiet in Norditalien auf ihre Eignung getestet. Des Weiteren wurden retrospektive Daten von eingegangen Hundeproben aufbereitet, die im Diagnostiklabor des Institutes zur Untersuchung auf Leishmaniose zwischen 2006 und 2008 eingesendet wurden. Ziel war es, einen Überblick möglicher akuter oder autochthoner Leishmaniosefälle in Deutschland zu erhalten.

Mehr als 105.018 Insekten wurden mit Hilfe von Lichtfallen (CDC miniature light traps) und Klebefallen (sticky traps) gefangen. Über 40.933 Exemplare der Insekten wurden der Familie der Psychodidae zugeordnet. Spezielle Aufmerksamkeit galt den Unterfamilien der Phlebotominae (Vektoren von Leishmaniose), Psychodinae sowie Trichomyiinae. Während der Untersuchung wurden keine Phlebotominae (Sandmücken) in Deutschland gefangen. Unerwartet war die Entdeckung einer neuen Trichomyinen-Art (*Trichomyia stephani* nov. spec.). In der durchgeführten Vergleichsstudie in Italien wurden Sandmücken sowohl in der endemischen Region (*P. perniciosus* n = 55, *S. minuta* n = 98) als auch in dem bis dato nicht endemischen Gebiet (*P. perniciosus* n = 5, *S. minuta* n = 25) gefangen. Die potentiell infizierten weiblichen Exemplare von *P. perniciosus* (n = 39) wurden mit Hilfe der real-time PCR auf Leishmanien-DNA untersucht und für negativ befunden. Die während der entomologischen Untersuchungen in Deutschland gewonnenen Daten bestätigen nicht die bisherigen Berichte über das Vorkommen von Sandmücken in Süd-Deutschland. Derzeit scheint das Risiko einer Leishmanieninfektion wegen des Fehlens des Vektors in Süd-Deutschland nicht gegeben, obgleich leishmanieninfizierte Hunde jedes Jahr nach Deutschland importiert werden. Grundlegendes Wissen über die Existenz der Sandmücken im Untersuchungsgebiet könnte helfen die Ausbreitung dieser Insekten und ihre bevorzugten ökologischen Nischen zu finden, sowie lokal

auftretende Fälle einer akuten autochthonen Leishmaniose zu identifizieren. Weitere Untersuchungen sollten durchgeführt werden, um ihre stetige Ausbreitung in den Nachbarländern sowie ihre potentiellen Wanderrouten nach Deutschland zu überwachen.

8. References

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9. Abbreviations

a.s.l.	above sea level
bp	base pair
°	Grade
°C	degree Celsius
CanL	canine leishmaniosis
CL	cutaneous leishmaniosis
CO_2	carbon Dioxide
DNA	deoxyribonucleic acid
EquL	equine leishmaniosis
et al.	et alii
Fig.	Figure
FL	feline leishmaniosis
IFAT	immune fluorescence antibody test
kDNA	kinetoplast deoxyribonucleic acid
km	kilometre
MCL	mucocutaneous leishmaniosis
m	metre
min	minute
ml	millilitre
mm	millimetre
m/sec	meter per second
µl	micro litre
µm	micro metre
µM	micro molar
nm	nano metre
nov. spec.	nova species
PBS	posphate-buffered saline
PCR	polymerase chain reaction
R	radial vein
St	sternite
T	tergite

Tab.	Table
TagMan® MGB	TagMan® fluorescent labelled probe with reporter and quencher combined with a minor groove binder
UNG AmpErase®	Uracil-N-Glycosylase AmpErase®
VL	visceral leishmaniosis

10. Figures

11. Tables

12. Annex

Tab. A: Characteristics of collecting sites (1-39) monitored for the phlebotomine sand fly survey in Southern Germany during the summer period of 2007/08. The five different field sites of Bad Breising are not included.

No.	Locality	Altitude [m] a.s.l.	Latitude Longitude	Placement traps	Site place	Environment
1	Oberwiesenfeld	515	48°09'48" N 11°32'55" E	Animal farm	Side of town	Mixed trees
2	Riem	520	48°09'23" N 11°40'04" E	Animal shelter	Outside of town	Mixed trees
3	Schwabing	511	48°09'18" N 11°35'22" E	Horse ranch	Inside of town	Park side, mixed trees
4	München	529	48°06'01" N 11°33'07" E	Zoo	Inside of town	Park side, mixed trees
5	Leutstetten	600	48°01'46" N 11°22'02" E	Horse ranch	Rural area	Agriculture, mixed trees
6	Hummersberg	533	48°23'12" N 11°13'44" E	Horse ranch	Rural area	Agriculture
7	Eckhof	492	48°21'06" N 11°16'23" E	Horse ranch	Rural area	Agriculture
8	Eckhof	500	48°21'01" N 11°16'24" E	Horse ranch	Rural area	Agriculture, mixed trees
9	Augsburg	485	48°20'55" N 10°54'56" E	Zoo	Inside of town	Park side, mixed trees
10	Gablingen	47	48°27'07" N 10°48'50" E	Horse ranch	Rural area	Agriculture
11	Unterreitnau	466	47°35'24" N 09°39'47" E	Farm	Rural area	Agriculture, apple trees
12	Schnetzenhausen	439	47°40'35" N 09°26'15" E	Old farm	End of rural area	Agriculture
13	Laubegg	510	47°50'17" N 09°03'37" E	Farm	Rural area	Fruit trees, mixed trees
14	Geisingen	680	47°56'43" N 08°37'18" E	Old farm	End of village	Agriculture
15	Weitenau – Steinen	374	47°40'37" N 07°45'54" E	Small farm	Rural area	Agriculture, mixed trees
16	Istein	246	47°39'04" N 07°31'48" E	Wall	End of village	Vineyards, mixed trees
17	Istein	251	47°39'40" N 07°32'27" E	Wall	Begin of village	Vineyards
18	Welmlingen	282	47°41'13" N 07°33'43" E	Farm	End of village	Mixed trees
19	Schliengen	340	47°44'34" N 07°34'26" E	Farm	Rural area	Vineyards, cornfields
20	Staufen	288	47°53'15" N 07°43'43" E	Wall	Side of town	Vineyards, agriculture
21	Au bei Freiburg	306	47°57'17" N 07°49'41" E	Old farm	Side of town	Agriculture
22	Au bei Freiburg	345	47°57'30" N 07°49'16" E	Farm	Side of town	Agriculture

23	Heuweiler	257	48°02'43" N 07°54'08" E	Farm	End of rural area	Agriculture, mixed trees
24	Munzingen	230	48°07'45" N 07°49'39" E	Wall	Side of town	Park side
25	Ettenheim	180	48°15'05" N 07°49'12" E	Farm	End of rural area	Agriculture
26	Emmersbach	236	48°20'04" N 08°00'39" E	Farm	Valley, rural area	Cornfields, mixed trees
27	Offenburg	150	48°30'56" N 07°57'41" E	Horse ranch	Side of town	Agriculture
28	Achern	140	48°37'54" N 08°02'75" E	Small horse ranch	Rural area	Agriculture
29	Sinzheim	138	48°46'72" N 08°10'09" E	Small chicken farm	End of rural area	Agriculture
30	Durmerstheim	119	48°56'61" N 08°15'83" E	Horse ranch	Outside of town	Agriculture
31	Ludwigsburg	269	48°54'55" N 09°11'42" E	Animal shelter	Side of town	Mixed trees
32	Dörrenbach	283	49°26'06" N 07°14'03" E	Small horse ranch	Rural area	Agriculture
33	Fürth	322	49°25'05" N 07°13'10" E	Farm	Rural area	Agriculture
34	Hangard	323	49°25'03" N 07°13'10" E	Farm	Rural area	Agriculture
35	Velsen	260	49°12'47" N 06°48'40" E	Old farm	Rural area	Agriculture, mixed trees
36	Karlsbrunn	233	49°10'28" N 06°48'39" E	Wall	Side of town	Mixed trees
37	Gehrweiler	255	49°57'33" N 07°77'87" E	Wall	Side of road	Agriculture, blackberries
38	Gehrweiler	284	49°34'22" N 07°46'19" E	House, garden	Hillside	Mixed trees
39	Gundersweiler	230	49°59'35" N 07°78'38" E	Old house	Side of town	Garden, fields

Tab. B: Characteristics of collecting sites monitored for the phlebotomine sand fly survey in Alto Adige - Province of Bolzano (Italy) from 18th to 22nd of August 2008.

No.	Locality	Altitude [m] a.s.l.	Latitude Longitude	Placement traps	Site place	Environment
1	Tschars	602	46°38'12" N 10°56'10" E	Wall	End of village	Aggriculture, Apple trees
2	Pawigl	725	46°36'20" N 11°06'29" E	Wall near farm	Rual village	mixed forest, Apple trees
3	Partchins	635	46°40'50" N 11°04'15" E	Wall	Rual village	Chestnuts, Apple trees
4	Lana	499	46°37'04" N 11°07'58" E	Wall	Rual village	Vineyards, Mixed trees
5	Lana	340	46°37'07" N 11°08'23" E	Wall	Urban area	Gardens, Apple trees
6	Lana	450	46°37'02" N 11°08'08" E	Wall	Rual village	Chestnuts, Vineyards

7	Tirol	389	46°40'42" N 11°10'08" E	Wall	Rual village	Apple trees, Fig trees
8	Labers	520	46°39'28" N 11°11'25" E	Wall	Urban area	Aggriculture, Vineyards
9	Burgstall	270	46°35'48" N 11°12'00" E	Wall	Urban area	Aggriculture, Apple trees
10	Gargazon	270	46°34'33' N 11°12'47" E	Wall	Rual village	Aggriculture, Apple trees
11	Freiberg	439	46°38'36" N 11°11'14" E	Wall	Rual village	Aggriculture, Apple trees
12	Terlano	650	46°32'36"N 11°15'21" E	Wall	Rual village	Vineyard, Fruit trees
13	Terlano	696	46°32'43" N 11°15'28" E	Wall	Rual village	Vineyards, Mixed forest
14	St. Genesio	490	46°30'36" N 11°19'44" E	Wall near farm	End of village	Vineyards, Oak mix, Chestnuts
15	Guncina	460	46°30'40" N 11°20'26" E	Wall	End of village	Vineyards, Oak mix

Tab. C: Climate data of each location during the entomological survey in Southern Germany during summer period of 2007/08. Data are obtained during the set-up of traps.

No.	Locality	Date	Temperature min [°C]	Temperature max [°C]	Relative Humidity [%]
1	Oberwiesenfeld	06.08.2007	25.8	29.1	67.4
		12.08.2007	21.3	24.4	68.7
		29.07.2008	22.6	25.6	43.6
		04.08.2008	22.9	26.0	59.4
2	Riem	23.06.2008	24.3	27.6	36.8
		31.07.2008	28.4	32.9	30.3
		21.08.2008	26.2	30.3	33.4
3	Schwabing	09.07.2007	17.5	19.2	65.4
		15.07.2007	27.8	31.5	51.9
		23.07.2007	25.5	27.8	58.3
		30.07.2007	16.7	20.4	54.4
		01.08.2008	22.4	25.6	59.8
		07.08.2008	28.1	31.5	55.3
4	München	08.07.2007	22.2	26.4	64.7
		17.07.2007	24.3	27.1	51.3
		21.07.2007	23.4	26.7	66.8
		30.07.2007	18.1	21.6	65.7
5	Leutstetten	03.09.2007	17.2	20.5	68.9
6	Hummersberg	01.08.2007	24.6	27.4	55.7
		06.08.2007	25.3	28.6	56.6
		29.07.2008	25.3	29.7	59.9
7	Eckhof	01.08.2007	23.5	26.8	63.2
		06.08.2007	24.9	28.4	62.8
		29.07.2008	25.1	29.5	56.1

8	Eckhof	01.08.2007	23.4	26.7	64.8
		06.08.2007	24.5	28.4	64.5
9	Augsburg	24.08.2007	21.7	25.4	67.6
10	Gablingen	24.08.2007	22.6	26.8	65.3
11	Unterreitnau	03.08.2008	21.3	24.8	64,1
12	Schnetzenhausen	03.08.2008	28.4	31.2	28.4
13	Laubegg	03.08.2008	27.9	30.7	29.1
14	Geisingen	03.08.2008	23.9	26.4	67.2
15	Weitenau - Steinen	03.08.2008	23.5	26.7	49.9
16	Istein	04.08.2008	21.0	23.8	58.1
17	Istein	04.08.2008	20.8	23.7	52.6
18	Welmlingen	04.08.2008	21.9	24.4	55.0
19	Schliengen	04.08.2008	22.8	25.3	63.4
20	Staufen	03.08.2008	23.5	26.7	50.0
21	Au bei Freiburg	03.08.2008	24.8	27.9	52.1
22	Au bei Freiburg	03.08.2008	27.2	30.4	48.8
23	Heuweiler	27.08.2008	26.4	28.6	48.7
24	Munzingen	27.08.2008	27.5	30.2	43.3
25	Ettenheim	27.08.2008	26.2	28.9	42.3
26	Emmersbach	27.08.2008	25.1	27.5	49.2
27	Offenburg	27.08.2008	24.6	26.7	54.8
28	Achern	27.08.2008	26.7	28.1	50.2
29	Sinzheim	28.08.2008	21.9	24.0	49.8
30	Durmerstheim	28.08.2008	23.4	25.2	52.4
31	Ludwigsburg	23.07.2008	25.4	28.0	43.8
		12.08.2008	20.5	23.3	64.2
32	Dörrenbach	11.09.2008	18.7	22.1	52.6
33	Fürth	12.08.2008	18.5	22.4	54.9
34	Hangard	12.08.2008	18.1	22.2	58.4
35	Velsen	23.07.2008	22.8	25.2	68.9
		12.08.2008	26.4	29.3	67.5
36	Karlsbrunn	24.07.2008	20.4	23.5	58.7
37	Gehrweiler	23.07.2008	21.5	24.6	51.9
		12.08.2008	19.8	22.3	57.5
		11.09.2008	17.9	20.1	61.4
38	Gehrweiler	23.07.2008	21.9	24.8	57.4
39	Gundersweiler	23.07.2008	20.4	23.8	62.3

Tab. D: Total number of trapped Psychodidae in the Zoological garden in Munich. I-IV are the trap locations within the Zoo (I: horses; II: buffalos; III: camels; IV: sheep and goats), 1-4 are the four consecutive experimental days.

Latin Square trial 1	I	II	III	IV
1	64	34	1897	0
2	94	11	61	0
3	52	310	24	2
4	0	270	37	1
Total [n]	210	625	2019	3
				2857

Latin Square trial 2	I	II	III	IV
1	81	927	271	0
2	1131	90	409	1
3	421	1079	120	2
4	814	195	3	0
Total [n]	2447	2291	803	3
				5544

Latin Square trial 3	I	II	III	IV
1	382	379	41	1
2	34	17	1	0
3	46	107	468	0
4	545	413	89	0
Total [n]	1007	916	599	1
				2523

Latin Square trial 4	I	II	III	IV
1	7	316	73	0
2	321	21	144	0
3	268	202	3	0
4	118	1099	55	0
Total [n]	714	1638	275	0
				2627

$Total_{(Latin\ Square\ trial\ 1\text{-}4)} = 13.551$

Tab. E: Total number of trapped Psychodidae at the local horse ranch in Munich. I-IV are the trap locations within the horse ranch (I: rondel; II: treadmill; III: dunghill; IV: staircase), 1-4 are the four consecutive experimental days.

Latin Square trial 1	I	II	III	IV
1	3	9	23	4
2	2	8	17	4
3	4	46	19	35
4	5	44	106	39
Total [n]	14	107	165	82
				368

Latin Square trial 2	I	II	III	IV
1	17	32	143	134
2	13	41	161	231
3	9	39	106	27
4	6	72	57	52
Total [n]	45	184	467	444
				1140

Latin Square trial 3	I	II	III	IV
1	48	46	27	20
2	40	15	85	111
3	19	11	16	45
4	43	30	31	49
Total [n]	150	102	159	225
				636

Latin Square trial 4	I	II	III	IV
1	10	4	10	9
2	4	6	10	2
3	41	18	33	76
4	59	76	82	102
Total [n]	114	104	135	189
				542

$Total_{(\text{Latin Square trial 1-4})} = 2.686$

Tab. F: Number of different species trapped at the local horse ranch in Munich during the Latin Square trial 1-4. A-D are the different trap types (Tab. 4). ♀ = female, ♂ = male.

Species	*Psychoda* spp.		*Tricho-psychoda* spp.		*Philosepedon* spp.		*Tinearia alternata*		Other species #		Total	
Latin Square 1	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	n	
1 A	2	0	0	0	0	0	1	0	0	0	3	
1 B	15	4	0	0	0	3	1	0	0	0	23	
1 C	8	1	0	0	0	0	0	0	0	0	9	
1 D	4	0	0	0	0	0	0	0	0	0	4	
2 A	1	1	0	0	0	0	0	0	0	0	2	
2 B	15	0	0	0	0	0	2	0	0	0	17	
2 C	5	1	0	1	0	0	0	1	0	0	8	
2 D	3	1	0	0	0	0	0	0	0	0	4	
3 A	1	0	0	0	0	0	3	0	0	0	4	
3 B	10	0	1	0	0	0	7	1	0	0	19	
3 C	30	9	0	0	0	0	7	0	0	0	46	
3 D	29	0	0	2	0	0	3	0	0	1	35	# 6
4 A	2	1	1	0	0	0	1	0	0	0	5	
4 B	28	3	26	7	0	0	42	0	0	0	106	
4 C	21	0	13	0	0	0	8	2	0	0	44	
4 D	10	6	1	0	0	12	6	1	2	1	39	# 5, 7
Total [n]	184	27	42	10	0	15	81	5	2	2	**368**	

Latin Square 2	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	n	
1 A	14	1	0	0	0	0	2	0	0	0	17	
1 B	94	5	3	0	6	2	29	4	0	0	143	
1C	15	6	1	1	3	0	4	1	0	1	32	# 4
1 D	61	8	9	1	0	2	50	3	0	0	134	
2 A	2	1	3	0	4	0	3	0	0	0	13	
2 B	118	2	4	1	8	1	23	3	1	0	161	# 6
2 C	21	2	0	0	0	0	17	0	1	0	41	# 1
2 D	182	2	9	0	2	1	33	2	0	0	231	
3 A	2	0	1	0	1	2	3	0	0	0	9	
3 B	50	3	1	1	12	0	26	12	1	0	106	# 1
3 C	12	0	3	0	1	0	22	1	0	0	39	
3 D	8	0	1	0	12	0	4	1	0	1	27	# 7
4 A	4	0	0	0	0	0	0	2	0	0	6	
4 B	17	0	19	1	2	0	16	1	0	1	57	# 4
4 C	63	4	0	0	2	1	0	0	2	0	72	# 6, 2
4 D	24	3	12	0	1	1	11	0	0	0	52	
Total [n]	687	37	66	5	54	10	243	30	5	2	**1140**	

# 1	Clytocerus	# 5	Pericoma
# 2	Mormia apicealba	# 6	Telmatoscopus rotschildi
# 3	Mormia furva	# 7	Trichomyia stephani
# 4	Paramormia ustulata	# 8	Trichomyia urbica

Species	*Psychoda* spp.		*Tricho-psychoda* spp.		*Philosepedon* spp.		*Tinearia alternata*		Other species #		Total	
Latin Square 3	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	n	
1 A	23	1	5	0	0	0	16	3	0	0	48	
1 B	9	0	0	0	1	0	14	3	0	0	27	
1 C	33	1	0	0	0	1	10	1	0	0	46	
1 D	13	0	0	0	0	0	6	1	0	0	20	
2 A	10	0	1	0	0	0	28	1	0	0	40	
2 B	58	1	0	0	0	1	22	3	0	0	85	
2 C	11	1	0	1	0	0	1	1	0	0	15	
2 D	72	13	1	1	0	0	17	4	2	1	111	# 6, 8
3 A	13	1	0	0	0	0	3	2	0	0	19	
3 B	11	1	0	0	0	0	4	0	0	0	16	
3 C	4	0	0	0	2	0	3	1	0	1	11	# 3
3 D	37	1	2	0	0	0	3	2	0	0	45	
4 A	7	3	10	3	0	0	13	7	0	0	43	
4 B	21	4	0	0	1	4	1	0	0	0	31	
4 C	24	1	0	0	0	1	3	0	0	1	30	# 5
4 D	22	1	0	0	1	0	23	2	0	0	49	
Total [n]	368	29	19	5	5	7	167	31	2	3	**636**	

Latin Square 4	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	n
1 A	1	0	0	0	0	0	8	1	0	0	10
1 B	2	0	0	0	1	1	6	0	0	0	10
1 C	3	0	0	0	0	1	0	0	0	0	4
1 D	1	0	2	1	3	0	2	0	0	0	9
2 A	4	0	0	0	0	0	0	0	0	0	4
2 B	2	0	0	0	0	0	8	0	0	0	10
2 C	1	1	0	0	0	2	2	0	0	0	6
2 D	1	0	0	0	0	0	1	0	0	0	2
3 A	29	4	0	0	0	0	7	1	0	0	41
3 B	7	0	3	2	0	0	19	2	0	0	33
3 C	1	0	2	1	0	0	12	2	0	0	18
3 D	57	5	3	0	0	0	11	0	0	0	76
4 A	13	1	11	1	0	0	30	3	0	0	59
4 B	59	4	1	0	0	0	13	5	0	0	82
4 C	35	0	7	0	0	0	30	4	0	0	76
4 D	58	1	13	2	2	1	22	3	0	0	102
Total [n]	274	16	42	7	6	5	171	21	0	0	**542**

Total [n]	1513	109	169	27	65	37	662	87	9	7	**2686**

# 1	Clytocerus	# 5	Pericoma
# 2	Mormia apicealba	# 6	Telmatoscopus rotschildi
# 3	Mormia furva	# 7	Trichomyia stephani
# 4	Paramormia ustulata	# 8	Trichomyia urbica

Tab. G: Key to the males of tribes and genera of Psychodidae – Psychodinae.

KEY TO TRIBES:

1 - Flagellar segments barrel-shaped ……. **PERICOMINI**

- Flagellar segments bottle-shaped ……..... 2

2 - Antenna with 14 - 16 segments, distal antennal segments reduced in size or even missing, a pair of Y-shaped ascoids on each of the non reduced flagellomeres, cerci of male genitalia with 1 or 2 (far apart standing) tenacula ..... **PSYCHODINI**

- Antenna 16 segmented, ascoids variously (not Y -) shaped, male genitalia usually symmetric, cercopodia almost straight or slightly curved inward with 2, 3 or more tenacula (in one line at the distal end of the cercopodia) ..... **TELMATOSCOPINI**

- Antenna with 16 segments, distal segment reduced in size, flagellar segments 1 and 2 often fused, ascoids rake-shaped, aedeagus asymmetricor symmetric, cercopodia curved distally ……..……..... **BRUNETIINI**

Key to the genera of Psychodini (males)

1 - tips of longitudinal veins with dark spots …….. genus **Tinearia** Schellenberg

Tinearia alternata (Say) (Cosmopolitan)

Tineria lativentris (Berden) (Holarctic Region)

- tips of longitudinal veins without dark spots ..... genus **Psychoda** Latreille

Key to the genera of BRUNETTIINI (males)

1 - Male aedeagus symmetric, wing elongate to broad, flagellomeres with a pair of simple, elongate ascoids ……. **Brunettia** (Annandale)

[no taxon recorded yet from Russian Far East]

- Male aedeagus asymmetric, wing elongate, flagellomeres with complex ascoids, tibia with elongate spine ……**Neoarisemus** Botoseneanu & Vaillant

Key to the genera of Pericomini (males)

1 - Head with cornicula, antenna 15 segmented, scape at least 3 times longer than wide, postpedicel with a tuft of long androconia ……... **Clytocerus** Eaton

- Head without cornicula, antennae 16 segmented …… 2

2 - Scape 2-3 times longer than wide, pedicel spherical, postpedicel often fused with one or several of the following segments and with several string spines …….. 3

- Scape short, postpedicel unmodified ……..... 4

3 - Basistyles elongate cylindric, dististyles regularly bent, distally decreasing in diameter …….. **Thornburghiella** Vaillant

- Basistyles cubic, often broadly fused dististyles of complicated shape, cercopodia with numerous tenacula ……... **Bazarella** Vaillant

4 - Distal antennal segment without a dustal neck, cercopodia with few strong tenacula, orophilous …… **Saraiella** Vaillant

- Distal antennal segment with a neck 5

5 - Thorax with permanently everted patagia ……. **Ulomyia** Walker

- Thorax without, or with "hidden" patagia (scent organs) ……... 6

6 - Small species, eye-bridge with 4 facet-rows …….... **Pericoma** Walker

- Larger taxa, eye-bridge with more than 4 facet-rows …… **Satchelliella** Vaillant

Key to the genera of Telmatoscopini (males)

1 - Cercopodia with 2 or 3 tenacula or 2 groups of differently shaped tenacula 2

- Cercopodia with more than 5 uniformly shaped tenacula 3

2 - Cercopodia with 2 tenacula in one row …… **Philosepedon** Eaton
[females without large cerci to oviposit, larvipar]
- Cercopodia with 3 tenacula in one row ……... **Threticus** Eaton
- Cercopodia with 2 groups and 2 forms of (paddle-shaped medial. umbrella-form distal) tenacula …….. **Trichopsychoda** (Tonnoir)

3 - Head with cornicula, genitalia with siccle-shaped 'parameres', wing, head and thorax heavily covered with black androconia …… **"Panimerus"** Eaton
- Head with cornicula or cornicular extensions, genitalia without 'parameres', appearance brownish, without androconia ……. **Jungiella** Vaillant
- Head without cornicula, genitalia without 'parameres' …… 4

4 - Dark specimens, wing, head and thorax often heavily covered with black androconia, ascoids often of complicated build, R2/3 and R2+3/4 nearby ..... **Mormia** Enderlein
- R2/3 and R2+3/4 at 'normal' distance, coloration brown, ascoids simple, mainly finger-shaped ……..……..... 5

5 - Antennal segments with numerous simple ascoids in 1 or 2 or irregular rows …….. **Paramormia** Enderlein

6 - Large specimens with 2 simple ascoids on antennal segments, wing with dark spots at distal ends of longitudinal veins ……. **Clogmia** Enderlein
[only *Clogmia albipunctata* (Williston), cosmopolitan]
- Antennal segment with a pair of large and many small simple ascoids ……
..... ***"Telmatoscopus" sensu lato***

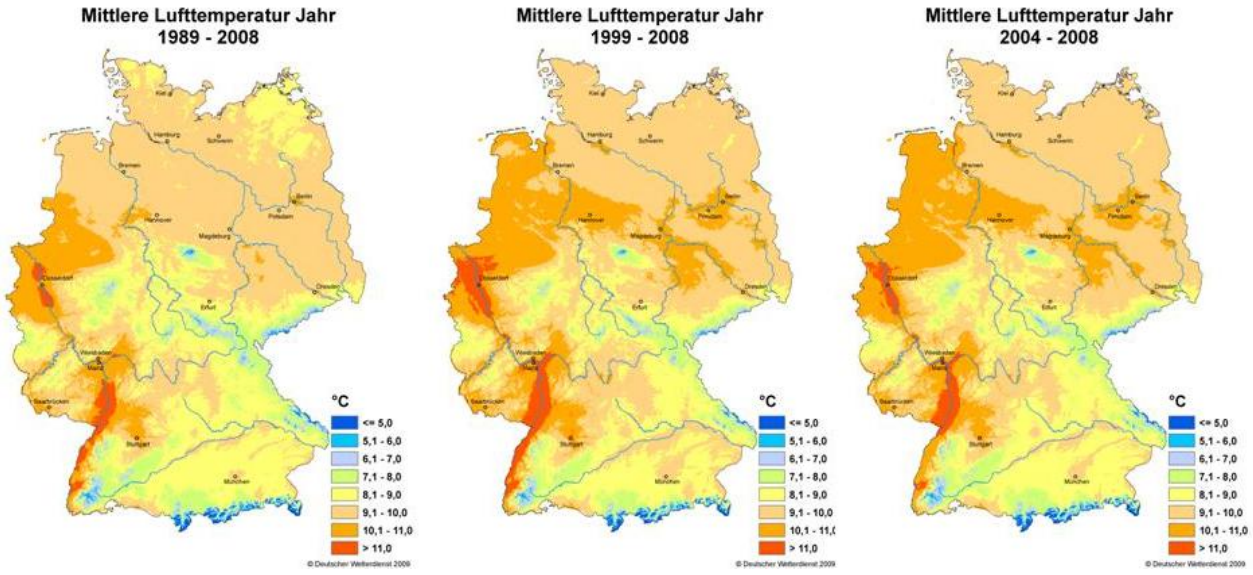

Fig. A: Climate maps provided by the Deutsche Wetterdienst (DWD). © Deutscher Wetterdienst. These charts were produced on June 08, 2009 using data of all stations of the networks of DWD.

Tab. H: Amount of extracted phlebotomine DNA measured with NanoDrop® in all 39 samples.

Sample No.	**1**	**2**	**3**	**4**	**5**
DNA [ng/µl]	4,9	5,3	3,5	5,1	7,6
Sample No.	**6**	**7**	**8**	**9**	**10**
DNA [ng/µl]	5,5	5,7	8,8	5,8	5,4
Sample No.	**11**	**12**	**13**	**14**	**15**
DNA [ng/µl]	2,0	7,9	17,2	6,9	5,1
Sample No.	**16**	**17**	**18**	**19**	**20**
DNA [ng/µl]	14,4	9,0	9,7	10,2	10,4
Sample No.	**21**	**22**	**23**	**24**	**25**
DNA [ng/µl]	19,6	8,3	3,2	6,3	4,5
Sample No.	**26**	**27**	**28**	**29**	**30**
DNA [ng/µl]	15,6	6,7	19,8	5,5	4,1
Sample No.	**31**	**32**	**33**	**34**	**35**
DNA [ng/µl]	3,4	15,0	27,2	5,1	4,1
Sample No.	**36**	**37**	**38**	**39**	
DNA [ng/µl]	2,7	5,0	8,9	6,1	

Tab. I: Data of the monthly analyses of *Leishmania* samples in the Diagnostic laboratory during 2006 and 2008. IFAT dilution series and positive or doubtful results of the *Leishmania* PCR are listed.

Month	Number of Examinations	Negative	1:32	1:64	1:128	1:256	1:512	Positive	Doubtful
January '06	150	121	11	7	3	4	4	0	0
February '06	110	92	4	9	2	1	2	0	0
March '06	148	122	6	8	7	3	2	0	0
April '06	114	92	14	1	2	3	2	0	0
May '06	185	139	27	8	1	6	4	0	0
June '06	181	151	9	3	5	3	10	0	0
July '06	158	128	13	5	4	1	6	1	0
August '06	153	104	28	7	5	4	3	1	1
September '06	124	72	30	4	3	5	10	0	0
October '06	160	140	1	4	4	4	7	0	0
November '06	155	130	9	2	6	3	5	0	0
December '06	121	80	28	2	3	3	5	0	0
Total [n]	**1759**	**1371**	**180**	**60**	**45**	**40**	**60**	**2**	**1**
Proportion [%]	**100,00**	**77,94**	**10,23**	**3,41**	**2,56**	**2,27**	**3,41**	**0,11**	**0,06**

Month	Number of Examinations	Negative	1:32	1:64	1:128	1:256	1:512	Positive	Doubtful
January '07	137	70	40	10	9	2	5	1	0
February '07	137	89	24	1	4	7	11	1	0
March '07	158	125	15	3	3	6	6	0	0
April '07	155	121	12	4	2	2	7	1	6
May '07	155	120	14	1	9	2	9	0	0
June '07	111	67	20	4	7	5	8	0	0
July '07	176	111	31	7	4	7	14	2	0
August '07	270	175	58	9	9	7	12	0	0
September '07	156	103	35	4	2	5	6	1	0
October '07	272	194	38	5	7	4	23	1	0
November '07	209	150	29	9	8	2	10	1	0
December '07	160	93	41	6	2	4	14	0	0
Total [n]	**2096**	**1418**	**357**	**63**	**66**	**53**	**125**	**8**	**6**
Proportion [%]	**100,00**	**67,65**	**17,03**	**3,01**	**3,15**	**2,53**	**5,96**	**0,38**	**0,29**

Month	Number of Examinations	Negative	1:32	1:64	1:128	1:256	1:512	Positive	Doubtful
January '08	272	177	55	18	9	3	9	1	0
February '08	178	118	30	12	1	6	11	0	0
March '08	183	118	44	5	2	1	13	0	0
April '08	226	160	39	6	5	5	9	2	0
May '08	296	196	43	24	8	4	20	1	0
June '08	175	128	22	5	3	5	12	0	0
July '08	186	117	36	12	5	3	13	0	0
August '08	225	166	35	9	4	1	10	0	0
September '08	229	178	24	1	3	8	14	1	0
October '08	264	182	38	9	12	4	19	0	0
November '08	198	135	32	10	8	6	7	0	0
December '08	174	129	24	4	1	4	11	1	0
Total [n]	**2606**	**1804**	**422**	**115**	**61**	**50**	**148**	**6**	**0**
Proportion [%]	**100,00**	**69,22**	**16,19**	**4,41**	**2,34**	**1,92**	**5,68**	**0,23**	**0,00**

13. Acknowledgements

First of all, I would like to thank Prof. Dr. Kurt Pfister for giving me the opportunity to work on this thesis, a very challenging field survey, in his institute and laboratories. I am grateful for his support and advice throughout this very formidable doctorate.

I also wish to show my appreciation to Prof. Bernard Pesson from Strasbourg for helping me to understand phlebotomine sand flies better and for showing me living specimens, which I had never seen before.

I am very thankful to Dr. Michele Maroli from Rome for his knowledge, tremendous help during the excursions in Italy and practical advice for the fieldwork in Germany. His insights were very helpful in understanding appropriate habitats better.

A special thank goes to Prof. Dr. Rüdiger Wagner from Kassel for his excellent support with the determination of the new Psychodid-species and his help in dissecting these tiny insects.

I am grateful for the useful instructions of Monia Mahling of the Department of Statistics (STABLAB), Ludwig Maximilians-University for the statistical analysis of the received data of the Latin Square trial and the retrospective data acquisition.

Additionally, I would like to thank all farmers for their cooperativeness during the two-year survey in Southern Germany and in Italy.

Furthermore, I am particularly grateful to all my colleagues especially Dr. Miriam Scheuerle, Daniela Ruffing and Dr. Conny Silaghi for their help during the fieldwork, for joining me on numerous excursions and informative advices of writing my dissertation. A big cheers goes to all the other colleagues I have not named here for the collaboration at the Institute.

Additional thanks goes to Dr. Sabine Bork-Mimm and Laura Chaudhuri for always having an encouraging word for me even in stressful situations at work.

I thank Katharina Schuhmann from New York so much for the final proofreading of my thesis and Stephan Bender for his advice on layout.

In addition, I am very grateful to my family, especially my brother Stefan and all my very good friends Hilda, Sonja and Ari for motivating me all day long to finish this work, no matter what. They always believed in me that I could manage this big challenge.

Finally, I am extremely thankful to my Sweetheart “GL” for providing me his daily trust and love, encouraging me in all the stages “ups and downs” of my dissertation and for always bringing a big smile to my face with his many lovely surprises.