



***Lutzomyia longipalpis* (Diptera: Psychodidae: Phlebotominae): a review**

RODRIGO P.P. SOARES and SALVATORE J. TURCO

Department of Biochemistry, University of Kentucky Medical Center, Lexington, Kentucky 40536 (USA)

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ABSTRACT

Lutzomyia longipalpis is the most important vector of American Visceral Leishmaniasis (AVL) due to *Leishmania chagasi* in the New World. Despite its importance, AVL, a disease primarily of rural areas, has increased its prevalence and became urbanized in some large cities in Brazil and other countries in Latin America. Although the disease is treatable, other control measures include elimination of infected dogs and the use of insecticides to kill the sand flies. A better understanding of vector biology could also account as one more tool for AVL control. A wide variety of papers about *L. longipalpis* have been published in the recent past years. This review summarizes our current information of this particular sand fly regarding its importance, biology, morphology, pheromones genetics, saliva, gut physiology and parasite interactions.

Key words: sand flies, vector biology, *Lutzomyia longipalpis*, *Leishmania*.

INTRODUCTION

DISTRIBUTION AND EPIDEMIOLOGY OF AVL

Lutzomyia longipalpis, Lutz and Neiva 1912 is the best studied and most important vector of American Visceral Leishmaniasis (AVL) in Latin America. Brazil alone contributes to 90% of the cases. AVL due to *Leishmania chagasi*, Cunha and Chagas 1937 in the New World is widely distributed from Mexico to Argentina (Grimaldi et al. 1989), thus indicating a strong association of this parasite with the sand fly throughout its geographical range (Young and Duncan 1994). The first report of *L. chagasi* in Brazil was made by Penna (1934) during histological examination of liver specimens through post-mortem viscerotomy. Soon after, Chagas et al. (1937, 1938) observed cases of AVL in domes-

tic dogs and *L. longipalpis* was suspected to be the primary vector. Later, wild reservoirs represented by the foxes *Lycalopex vetulus* (Deane and Deane 1954a,b) and *Cerdocyon thous* (Lainson et al. 1969, Silveira et al. 1982) were also reported although the role of opossums as peridomestic hosts was also considered (Sherlock et al. 1984). Clinical, pathological, ecological, diagnostic methods, treatment and control on Leishmaniasis were reviewed by Deane and Grimaldi (1985). A detailed historic description of Leishmaniasis in the Americas is also provided by Lainson and Shaw (1992).

After the description of *L. chagasi* as the agent of AVL in the Americas, the taxonomic position of this species has been controversial due its similarity to *L. infantum*, Nicolle 1908, a Mediterranean species. Lainson and Shaw (1972, 1979) accepted it as a separate species, while not excluding the presence of *L. infantum* in Brazil. With the development

Correspondence to: Salvatore J. Turco
E-mail: turco@uky.edu

of molecular techniques, many researchers continue to address the taxonomy of those two species (for more details, see a review of Maurício et al. 2000). Although the finding of *L. longipalpis* in close association with places where AVL occurs, the vector status was finally established by Lainson et al. in 1977.

A wide variety of studies with *L. longipalpis* has contributed to a better understanding of its biology as well as other parameters. Because of the urbanization of AVL, which has been increasingly reported in many Latin American cities, new alternative methods are needed to control the sand fly. This article reviews current studies involving this vector and discusses the perspectives of its relevance as an insect model.

BIOLOGY

BIOLOGICAL CYCLE AND LABORATORY MAINTENANCE

Sand flies are holometabolous insects proceeding from egg through four larvae stages, pupae and adults (Ward 1990, Killick-Kendrick 1999). In the natural environment, larvae instars feed on organic material from the soil (Ferro et al. 1997), while adults from both sexes can feed on sugar from plant sources (Chanotis 1974). Only female adults need blood prior to oviposition, although some species such as *L. lichyi* can lay the first batch of eggs in the absence of a blood meal (Montoya-Lerma 1992). Due to its importance as a vector of Leishmaniasis, many attempts to establish laboratory reared colonies of *L. longipalpis* and other sand fly species have been reported (Mangabeira 1969, Deane and Deane 1955, Sherlock and Sherlock 1959, Killick-Kendrick et al. 1973, 1977).

L. longipalpis is considered a species complex (Lanzaro et al. 1993) and therefore the productivity of different colonies may vary. For this reason the Lapinha Cave colony (Minas Gerais State, Brazil; longitude 43°57'W; latitude 19°03'S) has been chosen as reference in this review as it is the best studied. Killick-Kendrick et al. (1973) established a

colony in England from field collected insects from Lapinha in 1972. Later, they described in detail the methods of rearing of those sand flies that were in their 24th consecutive generation producing 800-1000 sand flies per week (Killick-Kendrick et al. 1977). The first description of a simple technique for mass rearing phlebotomine sand flies (4000-5000 adults per week) was reported by Modi and Tesh (1983) using *L. longipalpis* and *Phlebotomus papatasi*. Since many experiments in various fields of study require a very high number of sand flies, there continues to be an ongoing pursuit of improved mass-rearing techniques (Wermelinger et al. 1987, Lawyer et al. 1991). Subsequently, Rangel et al. (1986), studied the biological cycle of the Lapinha colony and *L. intermedia* under different conditions and showed that the completion of biological cycle from egg to adult for *L. longipalpis* ranged from 28 to 36 days, depending on the blood source. The productivity of sand flies improved when fed blood from hamster and chick compared to man and dog. Oviposition usually starts on the fifth day after blood meal and varies from 24 to 52 eggs per female. Similarly, Ready (1978, 1979) also observed differences in the feeding behavior in *L. longipalpis* and a nutritional superiority of the hamster blood compared to human blood while studying egg production in two Brazilian *L. longipalpis* colonies. According to Rangel et al. (1986), egg hatching usually takes place after 6-9 days with the development of larvae and pupae stages at approximately 14-19 and 8-9 days, respectively. The total developmental period from blood meal to emergence of adults using hamster blood was 35 days (25-42). For adults, both male and females could feed on sugar sources and approximately 70% of the *L. longipalpis* females could survive up to seven days without a blood meal. For the larval stages, the authors have tested many types of food (vegetable and mixed origin) and observed the preference for fish food, which also prevents the fungal development. The conditions promoted by the humidity, temperature (around 25°C and 80% relative humidity) and food quality may enhance fungal growth. Consequently, killing of

the immature stages due to entrapment in the food particles or excessive fungal growth is likely to occur. Recently, additional data on larvae feeding of *L. longipalpis* and *L. intermedia* was provided by Wermelinger and Zanuncio (2001). They tested different types of food for the larvae, including industrialized food for rabbits, dogs, hamsters and aquarium fishes, as well as liver powder, cooked lettuce, wheat germ, beer yeast, oat and wheat bran. In general, most diets provided adequate development for both species.

It is well known that maintenance of a closed sand fly colony for many years may alter many parameters by genetic selection, thus interfering with productivity and changing the initial features of a given colony (Mukhopadhyay et al. 1997). A combination of many factors, such as number of generations, colony founders and selection of genes, is likely to be occurring in *L. longipalpis* colonies as already observed in *Drosophila* and mosquitoes (Munstermann 1994). Consequently, variations may occur according to the generation observed. Santos et al. (1991a) showed that the male:female ratios may range from 1:0.92 (first generation) to 1:63 (tenth generation) until complete disappearance of males in the eleventh. Changes in sex ratios during laboratory maintenance may drastically affect colony productivity and this could be possibly related to the sexual chromosome X (Santos et al. 1991b). Recently, Luitgards-Moura et al. (2000) described the productivity of four generations of another colony of *L. longipalpis* from Roraima State, Brazil showing that maximum productivity and fecundity rates were greatest in the F2 generation, decreasing in the subsequent ones. Thus, many factors are involved in the productivity of colonies, including sex ratio, egg production and number of emerged adults, parasitism and others.

Despite all the advances in the mass rearing of these insects, the processes are still very labor intensive and time consuming, therefore improved procedures to reduce handling without compromising the productivity are always needed.

BASIC MORPHOLOGY AND TAXONOMY

The species *L. longipalpis* was first described by Lutz and Neiva 1912 from captured insects in the Brazilian states of São Paulo and Minas Gerais. *L. longipalpis* males present paramere with dorsal curved setae inserted directly on the paramere, i.e., not a well developed tubercle. Females present shorter spermathecae; its length being 4X greater than its width and with fewer annulations (for more information on taxonomic characters see Young and Duncan 1994). Since *L. longipalpis* is widely distributed, a considerable degree of natural geographical barriers may exist among various populations. These variations were first observed by Mangabeira (1969) studying the pale patches (one or two spot phenotypes) of the third and fourth abdominal tergites of sand flies from Pará and Ceará States in Brazil. Later, those differences were also observed by Ward et al. (1985) using species from Minas Gerais and Ceará, leading to the proposal of two different taxa. Extension variation was also observed when comparing specimens from South and Central America countries, with the one-spot phenotype being more distributed than the two-spot phenotype. Although those two phenotypes may result in insemination barriers during reproduction, many crosses could also occur not only justifying the separation of species (Ward et al. 1988); but also having no impact in parasite transmission efficiency (Dujardin et al. 1997). The basic morphology of different sand fly stages and its use for taxonomical purposes are reviewed by Young and Duncan (1994).

ULTRA STRUCTURAL STUDIES

Ultra structural approaches have become a useful way to study in detail the morphological features of different *L. longipalpis* stages. The first descriptions of immature stages using scanning electron microscopy (SEM) were made by Ward and Ready (1975) for the egg exocorion. Later, Leite et al. (1991) and Leite and Williams (1996, 1997) described the pupae, fourth and first instar stages, respectively. Later, Secundino and Pimenta (1999)

described the first instar larva, which could be distinguished from the subsequent instars based on the number of caudal setae, and gave additional information on pupae and eggs. The advantages of these studies include observing a number of structures that are not visible using standard microscopy allowing for a better understanding of its biology, physiology, behavior and also as an additional tool for taxonomy. Also, using the fourth instar larva (Fausto et al. 1998), described the structure of the larval spiracular system in eight *Lutzomyia* species, including *L. longipalpis* using light and SEM. This structure can assume a great variety of forms and therefore can be used as a taxonomical tool for grouping different species. In *L. longipalpis* as well as some other Diptera, the fourth instar larva is amphipneustic, having two pairs of spiracles in the thorax and abdomen. In *L. longipalpis*, the number of the papillae in the thoracic spiracle is nine and 19 in the abdominal spiracle. This species also presented the largest thoracic and abdominal spiracular structures compared to *L. youngii*, *L. ovallesi*, *L. evansi*, *L. trinidadensis*, *L. migonei*, *L. absonodonta* and *L. venezuelensis*.

Recently, more data on external morphology have been reported on the posterior spiracles (Pessoa et al. 2000) and external sensory structures (Pessoa et al. 2001) also in fourth instar larvae of *L. longipalpis*. The former structure had been already described by Fausto et al. (1998) but showed no intraspecific variation for Brazilian strains of *L. longipalpis* (Pessoa et al. 2001), although Venezuelan species of *L. longipalpis* and *L. migonei* presented variation (Fausto et al. 1998). The sensory structures included the antennae, maxillary palps and caudal setae in seven *Lutzomyia* species. The antennal structures of these species exhibited considerable variation in the morphology and position. Regarding *L. longipalpis*, each antenna has a basal tubercle (socket), a small and cylindrical segment fused at a second ovoid distal segment. The maxillary palps for all species examined bear a maxillary organ, a small circular saliency, lightly sclerotized, and are endowed with seven oniporous papilliform

sensillae and three knob papillae. Finally, the caudal setae, which are located in the last abdominal segment of larvae, presented for *L. longipalpis* transversal furrows with very small and scattered pores. For additional details on the other species see Pessoa et al. (2001).

Recent information about egg, larvae and pupae structure using SEM was provided by Secundino and Pimenta (1999) using specimens of *L. longipalpis* from the Lapinha Cave. The external surface of the eggs is covered with an exochorion characterized by arrangements of a series of parallel, discontinuous, longitudinal ridges, which converge at egg ends. There are no lateral connections between the ridges, allowing *L. longipalpis* to be included in the group that presents unconnected parallel ridges (Ward and Ready 1975).

In contrast to the immature stages, the external morphology of adults using SEM is partly understood. Spiegel et al. (2000) observed the sensilla on the male terminallia of four species of sand flies including *L. longipalpis*. The sensilla of *L. longipalpis* could be morphologically identified as small coeloconica sensilla varying in number from 10-15. Interestingly, despite their basic morphology, the sensilla appear to be functionally very complex sensory organs modified for different purposes. Although the function of these sensilla is not completely known, they are believed to act as mechanoreceptors during the mating activity.

PHEROMONES, OVIPOSITION AND COURTSHIP

The number of pale patches (one or two spot phenotypes) observed in the abdomen of *L. longipalpis* males consisted of secretory glands and were suggested to produce sex pheromones after SEM (Lane and Ward 1984). This hypothesis was confirmed by Lane et al. (1985) using gas chromatography/mass spectrometry (GC/MS). The mass spectrum of one spot phenotype gave a molecular ion of 218, which was consistent with a formula of $C_{16}H_{26}$ (and possibly related to a farnesene/homofarnesene $C_{15}H_{24}$). Two spots phenotype tergal glands gave a molecular ion of 257, which was considered $C_{20}H_{32}$ and

was believed to have a diterpenoid structure. The two compounds were similar to pheromones found in other insects. Additional populations of *L. longipalpis* exhibiting both phenotypes have been studied (Phillips et al. 1986). It was not possible to establish a relationship between the number of tergal spots and the pheromone type. Nevertheless, it could be used as a potential marker for characterizing reproductively isolated vector populations, since they differ in the chemicals present and in the ratio of these compounds to each other. Later, a study of Ward and Morton (1991) showed that different Brazilian populations of *L. longipalpis* were able to react against male pheromones in a conspecific way (Jacobina, Bahia State). Another population from Sobral, Ceará State, reacted after stimulation with Jacobina pheromone, but preferentially selected conspecific sexual partners. The sexual preferences among different populations that were reproductively isolated may result in failure of copulation and/or viability of the offspring. Santos et al. (1991b) were unable to establish a colony crossing populations from Abaetetuba (Pará State) and Rio Acima (Minas Gerais State). Though crossings among different populations of the *L. longipalpis* complex can sometimes lead to viable and fertile offsprings, the extent of the pheromone influence in this work is subject to speculation. Other comparisons increased the number of populations and the distance among the localities. Hamilton and Ward (1991) studied the pheromone profiles of five Brazilian populations and included one population from Colombia and one from Venezuela. Based on the observed results, the authors suggested that there are at least six different populations of *L. longipalpis*, which exhibit three chemically distinct classes of pheromones in the species complex.

The full description of pheromone glandular structures was accomplished by Lane and Bernardes (1990). Briefly, each gland consisted of numerous large columnar secretory cells, with two distinct parts (one with vacuoles and other so-called end-apparatus), being connected to the exterior via a small duct. Each papule is 3-3.5 μ m in diameter with

a central pore 0.25 μ m in diameter. These structures are widely distributed in male sand fly pale patches and can be subdivided into three groups: those that produce terpenes and have cuticular papules; those that do not produce terpenes but still have associated papules; and those that have neither terpenes nor papules (Hamilton et al. 2002). Since the morphological data on the tergal spots in *L. longipalpis* were consistent with a pheromone gland, these compounds were tested as a means of attracting females. Little or no abdominal contact is made during courtship, therefore for the pheromone to be effective; the rapid wing beating by males enables the propagation of the substance to attract females. Morton and Ward (1989) reported the epiphenomenal response of female *L. longipalpis* sand flies to a hamster host and male pheromone source over distance. Later, the same authors studied the response of female sand flies to pheromone-baited sticky traps in the laboratory with the aim of a possible use of these traps in a future field collection (Morton and Ward 1990). Nigam and Ward (1991) showed the attractant effect of male pheromones and host factors on *L. longipalpis* females. Later, Oshaghi et al. (1994) showed under laboratory conditions the response of *L. longipalpis* to sticky traps baited with host odor with or without a host presence (hamster). Both males and females were attracted to traps by host odor alone, therefore host cues or male pheromones must also be considered. Subsequently, another experiment by Hamilton and Ramsoondar (1994) showed the same response using human skin odors. Both males and females were attracted but virgin females exhibited higher response. Field and laboratory experiments indicate that attraction to a host might be regulated by many host-produced factors, such as heat and CO₂, which may act in a synergistic way with male pheromones. Experiments on mating were carried out in order to evaluate behavioral patterns of aggregation and courtship in *L. longipalpis* sand flies. Jones and Hamilton (1998) observed male mating success and the quantity of pheromones in the glands of copulated and non-copulated males. Mated males had significantly

more remaining pheromones in their glands than unmated males. However, due to the experiment limitations and the importance of pheromones on female attraction, it seems that the successful males had at the beginning more pheromones and even after copulation, they still had a higher quantity when compared to unmated males. Further experiments are thus needed to clarify this issue.

The courtship in *L. longipalpis* sand flies include a series of behavioral patterns, such as male wing fanning either to a female or a male; fights, where a male clashes its abdomen to another male; female wing fanning to an approaching male and the female rejection by moving away from as approaching or wing fanning male. In *L. longipalpis*, male mating success does not seem correlated to fight winners. In contrast, male wing fanning prior to or after introduction of females was positively correlated to mating success (Jones and Hamilton 1998). In the field, it is common to see lek-like aggregations of males and females assembled on or near hosts where blood feeding and mating occur (Quinnell and Dye 1994). According to Kelly and Dye (1997) semiochemical factors in both the sand flies (pheromones) and the host (kairomones) are involved in attraction. It was observed that males first arrived over a host site, followed by the females. Male sand flies are often seen over the host where they form leks, thus attracting females for a blood meal and increasing their chance for mating (Jarvis and Rutledge 1992). It was observed that trans-beta-farnesene, the aphid alarm pheromone, had a stimulatory effect on feeding for both sexes in *L. longipalpis* (Tesh et al. 1992). The presence of farnesene/homofarnesene related substances was observed in male tergal spots (Lane et al. 1985), thus reinforcing the role of these substances in the sand fly aggregation. Additionally, behavioral and electrophysiological responses after exposure to Canid host odor kairomones were observed in *L. longipalpis* (Dougherty et al. 1999).

To examine which of the glandular extract components could be the sex pheromone, Hamilton et al. (1994) used HPLC to establish that the largest

peak (F3) was responsible for most the female attraction activity in the bioassays. The proposed chemical was later described as a novel homosesquiterpene named 3-methyl- α -himachalene (C₁₆H₂₆) for *L. longipalpis* from Jacobina, Bahia State, Brazil (Hamilton et al. 1996a). The same analysis was applied to specimens from Lapinha Cave, Minas Gerais State, Brazil (Hamilton et al. 1996b) and the substance was also a homosesquiterpene with a proposed structure of 9-methylgermacrene-B (E,E)-8-(1-methylethylidene)-1,5,10-trimethyl-1,5-cyclodecadiene. Comparisons on the presence of sex pheromone components in *L. longipalpis* populations were also reported. Honduran populations had no variations in the sex-pheromone, which was structurally the same terpene (9-methylgermacrene-B) previously observed for the Lapinha Cave population. However, Costa Rican specimens showed three types of terpenes in the sex pheromone components leading to an existence of at least two or three different populations in this country (Hamilton et al. 1996c). Finally, Hamilton et al. (1999a, b) confirmed the stereochemistry of 9-methylgermacrene-B as *S* by comparing physical and biological properties of the synthetic enantiomers. The relative stereochemistry of 3-methyl- α -himachalene was defined as *1RS,3RS,7RS* by comparing the natural product with four synthetic diastereoisomers. Recently, the distribution of putative male pheromones among different *Lutzomyia* species was correlated with the presence or absence of papules in the abdomen. Interestingly, some species did not have papules and the pheromone, while others had papules but did not have the pheromone, thus indicating the structure to be vestigial and non-functional (Hamilton et al. 2002).

Besides the pheromones involved in sexual activity, data on the oviposition pheromones have been reported by ElNaiem and Ward (1990). In this study, females were offered either to lay eggs in a test site containing eggs or a blank test site. The egg-containing site was clearly chosen for oviposition. Later, the same authors studied the same phenomenon using two sand fly populations of *L.*

longipalpis from Jacobina (Bahia State, Brazil) and L'Agua (Tolima, Colombia). The results again indicated a predisposition for the females to lay eggs in places containing eggs, independent of the age of the eggs (1-2 day-old compared to 5-6 day-old-eggs). Eggs were washed with organic solvents and the attraction for egg sites and control sites was the same, indicating that a possible attractant could be involved. Finally, female sand flies preferred to lay eggs in oviposition sites containing more than 80 conspecific eggs. Sites containing 20 or 40 eggs had no difference compared to blank control sites (ElNaiem and Ward 1991). To confirm the hypothesis that a pheromone could be involved in the attraction and/or stimulation, sites containing hexane extracts of conspecific eggs were exposed to *L. longipalpis* females (ElNaiem et al. 1991). The GC-MS analysis of the extracts confirmed the presence of non-polar substances that could account for attractiveness including squalene and cholesterol. These two substances in subsequent experiments did not induce an oviposition or stimulation, suggesting that other compounds could be involved. Besides pheromones, ElNaiem and Ward (1992a) tested the effect of surfaces containing frass (colony remains), larval rearing medium and rabbit feces as attractants or stimulants for oviposition. All female sand flies were attracted to those sites compared to blank test controls. An attractant effect of the rabbit feces on ovipositing females was observed, whereas water extracts of rabbit feces showed that the water extract had both attracting and stimulating effect on oviposition. Further, two oviposition attractants apneumones were isolated from rabbit feces consisting of 2-methyl-2-butanol and hexanal (Dougherty et al. 1995). Other factors including thigmotropic response also affect oviposition since female sand flies prefer to lay eggs in surface crevices rather than on flat surfaces (ElNaiem and Ward 1992b). Evidence for the accessory glands as the site of the production of the oviposition attractant/stimulant pheromone was reported by Dougherty et al. (1992), being secreted onto the eggs during oviposition. Soon after that, these authors also demonstrated that extract of

rabbit food and oviposition pheromone had a synergistic effect on sand fly egg laying (Dougherty et al. 1993). This association increased the female survival after oviposition, which is one of the biggest problems in laboratory reared sand flies (Killick-Kendrick et al. 1977, Chaniotis 1986) and also could be used for the development of a laboratory oviposition trap. Finally, Dougherty et al. (1994) identified, isolated and quantified a semiochemical with a suggestive structure of a caryophyllene oxide as the oviposition pheromone in *L. longipalpis*. The complete characterization of the egg pheromone was accomplished by Dougherty and Hamilton (1997). Its structure consisted of dodecanoic acid, which could be acquired from the blood as the non-active compound hexadecanoic acid (palmitic acid) and metabolized into the active pheromone over a 4-day period.

Recently, another parameter related to courtship was described based on the "lovesong" males produced prior to mating. These sounds are produced when the males vibrate their wings and it is believed that this acoustic communication signal is also different among populations of the same species. Two genes involved with "lovesong" were already studied in *Drosophila* and were named *cacophony* (*cac*) and *period* (*per*) (Hall 1994). The former codes for voltage-gated calcium channel α -1 subunit (Smith et al. 1996) and the latter seems to control biological rhythms that could contribute to the reproductive isolation between sibling species of *Drosophila* (Wheeler et al. 1991). Oliveira et al. (2001a) cloned and sequenced two putative *L. longipalpis*' song gene homologues *per* and *cac*. The authors observed a high degree of polymorphism in the *cac* gene due to insertion/deletion and point mutations within the *cac* intron, showing differences between the populations of Natal and Lapinha. Analysis of *per* gene between Lapinha and another population (Jacobina) also indicated that these populations are also quite different. The comparison of the male courtship songs of these three populations were recorded and showed remarkable differences (Souza et al. 2002). Analysis of the recorded profiles and

acoustic signals shows that different monocyclic and polycyclic pulses enable a clear separation of those sibling species. Additionally, those three populations have three different types of sex-pheromones.

Sexual behavior and pheromones in sand flies are very interesting and promising issues. A better understanding of the chemical ecology in *L. longipalpis* can lead to the development of strategies for monitoring field populations. Since many examples of control against agricultural pests are already available, the effect of these pheromones on sand fly biology could possibly be used as a novel alternative method for its control.

GENETICS

KARYOTYPE

The first chromosomal observation of *L. longipalpis* was partly studied by White and Killick-Kendrick (1975, 1976) using specimens from Lapinha Cave (Minas Gerais, Brazil), which presented giant polytene chromosomes in its salivary glands. Later, Kreutzer et al. (1987, 1988) observed the brain cell karyotypes of various species of New World sand flies including *L. longipalpis*. This species has four pairs of chromosomes ($2N=8$) and did not present heteromorphic chromosomes. Later, Yin et al. (1999) compared the mitotic metaphase chromosomes from brain cells of fourth instar sand fly larvae of four geographical strains of the *L. longipalpis* complex (Costa Rica, Colombia and Brazil: Jacobina and Lapinha Cave populations). Major differences of G-banding and/or position of the centromere were observed in the chromosome 4 and enabled the separation of four putative sibling species. Costa Rican and Colombian populations presented the karyotype formula $2n=8M$ (M =metacentric). Brazilian populations of Jacobina and Lapinha exhibited $2n=6M + 2SM$ (SM = submetacentric) and $2n=6M + 2ST$ (ST =subtelocentric).

ISOENZYMES, MORPHOMETRY AND MOLECULAR BIOLOGY

Due to its wide distribution from Mexico to South Brazil, interpopulation studies with *L. longipalpis*

have been reported in the past years. Many geographical and climatic barriers are responsible for keeping the populations isolated since the flight migration is very limited in sand flies. According to Alexander (1987) estimations about flight range in the genus *Lutzomyia* do not exceed 100 meters in 24-hr period. This isolation can lead to genetic drift and or natural selection pressure depending on the local habitats, allowing each population to have specific characteristics. The evidence that *L. longipalpis* could be a species complex is based on morphological (one or two-spot phenotypes) and crossing experiments (Mangabeira 1969, Ward et al. 1985, 1988). Lanzaro et al. (1993) studied 27 enzyme loci and did not obtain sterile offspring after experimental hybridization of three *L. longipalpis* populations, thus considering them to be a species complex, with at least three different siblings. Many studies with isoenzymes have provided information on the variability of *L. longipalpis* populations from Bolivia (Bonney et al. 1986, Dujardin et al. 1997), Brazil and Colombia (Mukhopadhyay et al. 1997), Central America and Colombia (Mutebi et al. 1998), Colombia (Morrison et al. 1995, Munstermann et al. 1998), Colombia, Brazil and Central America (Lanzaro et al. 1998), Venezuela (Lampo et al. 1999, Arrivillaga et al. 2000, Marquez et al. 2001) and Brazil (Mukhopadhyay et al. 1998, Mutebi et al. 1999). It is very important to address the influence of laboratory maintenance in the genetic background of some of the populations used in the previous studies (Mukhopadhyay et al. 1997). Morrison et al. (1995) observed rapid genetic homogenization while comparing specimens from a laboratory colony and from the field collected from the same site four years later. This variation according to Lanzaro et al. (1993, 1998) could not be discounted since a higher heterogeneity estimate (0.037) was observed compared to the value observed by Bonney et al. (1986) using field-collected insects. Together with the isozymic data, Dujardin et al. (1997) also observed metric variations in wing morphometry among populations of *L. longipalpis* from Bolivia, Brazil, Colombia and Nicaragua. Recently,

De la Riva et al. (2001) provided additional information on the use of wing geometry as a tool to distinguish members of the *L. longipalpis* complex. These authors established two groups of populations, even separating Bolivian populations, and the metric variation was found to be independent of the one or two spot phenotype as well as ecological behavior (sylvatic, peridomestic). Collectively, their results indicate that *L. longipalpis* is a complex when populations from Central and South America are compared. Nevertheless, morphological and morphometrical studies using Brazilian populations from many different regions do not support the idea that the species could be a complex in this country in spite of some differences (Azevedo et al. 2000). For more detailed information on the *L. longipalpis* species complex status see Uribe (1999).

Molecular biology studies with sand flies are also a reliable tool to address the species complex subject. Dias et al. (1998) used Random Amplified Polymorphic DNA PCR (RAPD-PCR) to compare populations of *L. longipalpis* from Brazil (Lapinha Cave, MG and Marajó Island, PA), Colombia and Costa Rica, and thus were able to distinguish the population from Marajó Island from the others. Sequence analysis showed that the RAPD-PCR fragments differed in the number of internal repeats. Uribe Soto et al. (2001) studied the speciation and population structure in the *L. longipalpis* complex based on the analysis of the mitochondrial ND4 gene and also confirmed the findings of previous studies (Lanzaro et al. 1993). Using Single Strand Conformation Polymorphism PCR (SSCP-PCR), Hodgkinson et al. (2002) showed differences in *L. longipalpis* populations using the mitochondrial cytochrome B haplotype.

All molecular tools described above have reinforced the status of *L. longipalpis* as a complex of species, while comparing populations from Central and South America. Those data are also reinforced by the pheromone data previously discussed. Bolivian (De la Riva et al. 2001) and Venezuelan (Lampo et al. 1999, Arrivillaga et al. 2000) populations could be distinguished as sibling species. Re-

cently, additional molecular markers were provided by Peixoto et al. (2001). These authors studied two genes (*cacophony* and *period*) known to be involved in "lovesongs" during courtship. Variations in these genes were useful in population genetics and evolutionary studies (Lins et al. 2002, Mazzoni et al. 2002). Bauzer et al (2002a) reported molecular divergence in the *period* gene between two putative sympatric species of *L. longipalpis* from Sobral, Ceará State, Brazil (S1 with one-spot and S2 with 2-spot phenotype). Polymorphisms in this gene were also observed comparing the populations of Jacobina (BA), Lapinha Cave (MG) and Natal (RN) (Bauzer et al. 2002b). Although the isozymic, morphological and morphometrical data were not sufficient to consider the Brazilian populations as sibling species, it is possible that these new molecular tools could provide a more sensitive way to address the real status of sibling species in Brazil as well.

GENE EXPRESSION

The studies of gene expression in *L. longipalpis* are still in the beginning stages and in the future may provide a reliable means of gene manipulation towards a modified insect. Genetically modified insects can provide an alternative tool not only for biological control but also for blocking parasite transmission that those vectors harbor. Two expression libraries from the abdomen and head/thorax from the female *L. longipalpis* fed on sugar were obtained by Ortigão et al. (1997). Later, Ramalho-Ortigão et al. (2001) characterized constitutive and putative expressed mRNAs identifying 37 cDNAs with homology in the GeneBank. Three sequences were differentially expressed in blood-fed or *Leishmania* infected females, and were identified as a chitinase (discussed below), a V-ATPase subunit C (found in epithelial membranes in insects) and a MAP kinase (known to participate in cellular signaling process in the insect innate immune system). Those enzymes are part of the gut physiology, therefore, a better understanding about their regulation can help to develop new mechanisms for blocking or decreasing *Leishmania*-sand fly infections.

Another study by Saraiva et al. (2000) demonstrated that *L. longipalpis* cell lines were also able to express heterologous promoters of the luciferase reporter gene. The authors successfully expressed this gene using the promoters from *Drosophila melanogaster* heat shock protein 70 (hsp70), human cytomegalovirus (CMV), simian virus (SV40) and *Junonia coenia* densovirus P9 (JcDENV). All systems were recognized by the transcriptional machinery of *L. longipalpis* and expressed the luciferase, providing a tool for possible manipulation and genetically transformation of these insects in the near future.

SALIVA

SALIVARY GLANDS AND *Leishmania* INFECTIVITY

The saliva of blood feeding arthropods has a variety of substances that are responsible for the success of the blood meal (reviewed by Ribeiro 1987). Those compounds include vasodilator peptides, anti-inflammatory, anti-histaminic and many others, that when working together will enable the insect to feed, minimizing the perception of the vertebrate host and hemostasis (Titus and Ribeiro 1990, Ribeiro 1995). Sand fly saliva has also been showed to have a potent immunomodulatory effect, enhancing the infection by *Leishmania* and stimulating the production of many cytokines (see a review on Old World species by Sacks and Kamhawi 2001). Also, a cytostatic effect on *Leishmania* grown in the presence of salivary gland homogenates was observed by Charlab and Ribeiro (1993), indicating a possible role of saliva in parasite differentiation in the sand fly midgut.

Preliminary information about *L. longipalpis* saliva was first provided by Ribeiro et al. (1986). Salivary gland lysates were able to enhance *L. major* (Titus and Ribeiro 1988) and *L. amazonensis* (Theodos et al. 1991) infectivity in mice even with 1/10 of a gland. Warburg and Schlein (1986) demonstrated that inclusion of the salivary gland material from *Phlebotomus papatasi* allowed infections to be established with as few as 10-100 parasites, a dose which is poorly infective when the *L. major* parasites are injected alone. While investigating

which specific salivary component could enhance *Leishmania* infectivity, a novel and potent vasodilatory peptide from *L. longipalpis* (probably related to the calcitonine gene related peptide CGRP) was reported (Ribeiro et al. 1989). Salivary gland material has been shown to exacerbate infection (Theodos et al. 1991) and inhibit *in vivo* macrophage antigen presentation to T cells (Theodos and Titus 1993). CGRP has been demonstrated to have 100-fold less enhancing activity than whole saliva itself (Theodos et al. 1991). Later, a substance was isolated from *L. longipalpis* saliva that was shown to have 500 times the vasodilatory activity of CGRP (previously the most potent vasodilator peptide known) and was so called Maxadilan (Lerner et al. 1991). Lerner and Shoemaker (1992) cloned and expressed the Maxadilan gene in *Escherichia coli*, with the recombinant Maxadilan having the same properties as the natural one and sharing similarity with CGRP. Maxadilan is a 63 amino acid peptide which undergoes C-terminal cleavage and amidation to a 61 amino acid peptide, containing four cysteine residues involved in the formation of disulfide bonds between amino acid positions 1-5 and 14-51. Modulation of the immune response by the saliva enables the parasite to survive and infect the host. Salivary gland lysates from *L. longipalpis* has also shown to suppress the immune response of mice to sheep red blood cells *in vivo* as well as concanavalin A (Titus 1998). Zer et al. (2001) recently confirmed that saliva exacerbates *Leishmania* uptake by macrophages and also had a chemotatic effect over these cells.

Most of the work with sand fly saliva involves Old World Species of *Leishmania*. Belkaid et al. (1998) exposed mice to metacyclic *L. major* plus salivary gland sonicate (SGS) of *P. papatasi* and observed an exacerbation effect on the development of the lesion, this phenomenon was not observed in mice preexposed to SGS, indicating that saliva exposure may influence the outcome of the infection during the transmission of the parasites. Similarly, Kamhawi et al. (2000) observed protection against *L. major* in mice exposed to bites of *P. papatasi*. In

a survey in Brazil, it was observed that children with high titers of anti-salivary protein IgG had also high anti-*Leishmania* IgG titers, indicating that individuals exposed to *Leishmania* recognize salivary gland antigens of *L. longipalpis*, and also suggesting also a role in which salivary contents could be protective in the development of the disease (Barral et al. 2000). Accordingly, Gomes et al. (2002) showed that the appearance of an anti-saliva humoral response and anti-*L. chagasi* cell-mediated immunity could be an indication of the use of SGS to induce a protective response against leishmaniasis. Nevertheless, Castro-Sousa et al. (2001) showed dissociation between vasodilation due to Maxadilan and enhancement of the infection with *L. braziliensis* in mice. The authors did not observe consistent differences among the two groups exposed to parasites in the presence or absence of saliva. Similarly, Melo et al. (2001) observed slight differences with or without saliva in *L. major*-like infected hamsters. Thus, the use of salivary gland proteins as potential vaccine candidates is a promising subject and must be studied carefully, since differences in hosts and parasite strains may be responsible for discrepancies while comparing available data.

PHARMACOLOGY OF MAXADILAN

Vasodilatory properties of recombinant Maxadilan were studied in detail by Jackson et al. (1996). They showed that arterial relaxation in rabbit thoracic and abdominal aorta was dose-dependent and independent from endothelium. Relaxation was found to be cAMP dependent, reducing the intracellular levels of calcium. High-affinity class receptors for Maxadilan were expressed on selected neural crest and smooth muscle-derived cells (Moro et al. 1996). Competition studies showed that Maxadilan does not interfere with receptors for CGRP, amylin or adrenomedullin and suggest that this peptide may bind to a novel receptor whose endogenous ligand remains unknown. Later, Moro and Lerner (1997) demonstrated that Maxadilan is a specific Pituitary Adenylate Cyclase Activating Peptide Type I receptor agonist (PACAP-R), although it does not share

significant sequence homology with this neuropeptide. PACAP binds to at least two classes of seven-transmembrane G-coupled receptors (types I and II). Soares et al. (1998) observed that Maxadilan PACAP-R type I was also present in mouse macrophages and treatment with the antagonist PACAP 6-38 blocked Maxadilan activities in macrophages, resulting in decreased levels of cAMP. Maxadilan was also able in this study to inhibit TNF- α and induce IL-6 production, with those cytokines and cAMP having a possible role in certain inflammatory responses. Bozza et al. (1998) showed that Maxadilan protected mice against lethal endotoxemia, and this could be partially dependent on IL-10. The PACAP receptors were also identified in human SH-SY5Y neuroblastoma cells, and Maxadilan was considered a PAC₁ receptor specific agonist. Maxadilan was also able to specifically stimulate PAC₁ receptor, but not VPAC receptors in SK-N-MC neuroblastoma cells (Eggenberger et al. 1999). Guilpin et al. (2002) showed that Maxadilan was able to stimulate hematopoiesis through IL-6 production and activation of PACAP-R in mice bone marrow stromal cells, which could be another mechanism of enhancing *Leishmania* susceptibility.

MAXADILAN AND *L. longipalpis* SPECIES COMPLEX

Warburg et al. (1994) observed differences in saliva composition and capacity to enhance leishmaniasis among populations of Brazil, Colombia and Costa Rica. The authors observed that the saliva from Brazilian and Colombian sand flies had 10-40 times more Maxadilan than the Costa Rican population, where the disease caused by *L. chagasi* is characterized by non-ulcerative cutaneous sores. Genetic analysis using single strand conformation polymorphism (SSCP) showed differences in the primary DNA sequence of the Maxadilan gene. Thus, the differences in saliva can also modulate different long-term pathology of the disease depending on the vector population. Lanzaro et al. (1999) showed differences in Maxadilan from species of the *L. longipalpis* complex. They observed up to a 23% extensive amino acid sequence differentiation

among the populations. The authors suggested that although peptides from different populations share the same vasodilatory activity, they exhibit different antigenic properties, and therefore trigger different skin reactions after the bite of the sand fly. Besides that, Yin et al. (2000) reported that sibling species in *L. longipalpis* complex differ in the levels of mRNA expression for Maxadylan.

OTHER SALIVA COMPOUNDS

In addition to its vasodilatory properties, other substances and activities have been reported for *L. longipalpis* saliva. A series of nine genes encoding salivary proteins in *L. longipalpis* were cloned and expressed by Charlab et al. (1999). From those, five genes that were blood-feeding related had similarities to the bed bug *Cimex lectularius* apyrase, a 5'-nucleotidase/phosphodiesterase, a hyaluronidase, a protein containing a carbohydrate-recognition domain (CRD) and an RGD-containing peptide. The biochemical properties of *L. longipalpis* apyrase are very similar to those of *C. lectularius*. This work was the first to identify a hyaluronidase activity in a hematophagous insect salivary gland and 5'-nucleotidase was only found in *L. longipalpis* but not in *P. papatasi*. The CRD-protein and the RGD-containing peptide are involved in anticlotting activities.

The work described above was followed by a series of papers describing many active components in *L. longipalpis* saliva. Ribeiro et al. (2000a) studied a 5'-nucleotidase that was found to be associated with a phosphodiesterase in *L. longipalpis* saliva. During the blood meal the presence of a nucleotidase may be required due to release of nucleic acids after tissue destruction. Also, 5-nucleotidase may convert AMP to adenosine, a potent vasodilator and anticlotting component necessary for establishment of blood meal intake. Later, the specific activity of the adenosine deaminase was described by Charlab et al. (2000), showing that this enzyme was responsible for conversion of adenosine into inosine, a possible anti-inflammatory/suppressor agent. A role of hyaluronidase was also reported (Ribeiro et

al. 2000b), with its possible involvement in spreading the salivary antihemostatic agents in the vicinity of bite site and also in virus transmission. Phosphodiesterase, 5'-nucleotidase, hyaluronidase and adenosine deaminase secretion was decreased after each blood meal, indicating that they were secreted during blood feeding. Comparisons between *P. papatasi* and *L. longipalpis* saliva contents made by Katz et al. (2000) showed that *L. longipalpis* had high levels of protein phosphatase-1/2A-like activities. However, *L. longipalpis* saliva did not inhibit nitric oxide production (NO) and did not contain AMP and adenosine, which were present in *P. papatasi* salivary glands. Finally, an amylase activity was reported from male and female salivary glands (Ribeiro et al. 2000c). Amylase activity was also observed in the crop and midgut of the *L. longipalpis* females. These findings are consistent with sugar feeding behavior.

The salivary proteins and glycoproteins in different species of sand flies including *L. longipalpis* were studied by Volf et al. (2000). Different gel profiles were observed for different species and populations of the same species, similar to those observed for Maxadylan (Lanzaro et al. 1999). Some *L. longipalpis* salivary proteins reacted with Con A and WGA lectins and were found to be mannosylated, indicating a complex type of N-glycans in the glycoproteins. Hyaluronidase activity was also different in many species of sand flies, with *L. longipalpis* having the lowest activity compared to *Phlebotomus* spp (Cerna et al. 2002). Recently, Cavalcante et al. (2003) have demonstrated a novel function of salivary gland extracts from *L. longipalpis*, which was able to inhibit both the classical and the alternative pathways of the complement cascade. A partial characterization of the inhibitor indicates a high resistance to denaturation by heat and a molecular weight of 10-30 kDa. Salivary components are part of a D7 subfamily of proteins that is widespread among blood sucking Diptera and belonging to a superfamily of pheromone/odorant binding proteins (Valenzuela et al. 2002), thus representing a rich field for research.

L. LONGIPALPIS INFECTIONS

VIRUS AND BACTERIA

A wide variety of organisms has been observed in *L. longipalpis* sand flies ranging from virus to helminthes. Viral infections have been observed in many species of phlebotomine sand flies, several of which have been well described by Young and Duncan (1994). Early descriptions of viral infections in *L. longipalpis* were also made by Jennings and Boorman (1980a, b). These authors observed the susceptibility to infection by three viruses of the Phlebotomus fever group, tested through intrathoracic inoculation and membrane feeding. Only one virus (Pacui) was able to be transmitted by *L. longipalpis*. These authors also tested the susceptibility to bluetongue virus (BTV), genus *Orbivirus*, which was able to infect only by intrathoracic inoculation. After a 6-9 day period, transmission by *L. longipalpis* occurred, though it is unlikely that this species would be important in the maintenance of this virus in the natural environment. Later, using for the first time a continuous *L. longipalpis* cell culture line (LL-5), Tesh and Modi (1983) tested the susceptibility of these cells to 29 arboviral infection including representatives of the genera *Vesiculovirus*, *Orbivirus*, *Flavivirus*, *Alphavirus*, *Bunyavirus*, and *Phlebovirus*. Within this cell line, they were able to replicate 13 of the arboviruses; surprisingly however, most of the phleboviruses did not replicate. Sand fly transmitted *Vesiculovirus* were also incriminated in the outcome of vesicular stomatitis in Colombia (Tesh et al. 1987). Vesiculoviruses were also found in sand flies in Pará State, Brazil (Travassos da Rosa et al. 1984). Hoch et al. (1984, 1985), using the Rift Valley Fever Virus (another member of the *Phlebovirus* group) were able to replicate and mechanically transmit this virus using *L. longipalpis* as a host. Cytoplasmic polyhedrosis virus (CPV) was found in *L. longipalpis* specimens from the Marajó Island in Brazil (Warburg and Pimenta 1995). This virus was shown to disrupt *Leishmania* infections in *P. papatasi*. Using *L. longipalpis* populations, the authors also observed elimination of

L. chagasi infections after day 7. However, bacteria were also observed with CPV infected sand flies and antibiotics were added to sugar. After that, the sand flies sustained *Leishmania* infections longer, indicating that bacteria could be responsible in part for the clearance of *L. chagasi* in CPV-infected insects. A dual role of bacteria plus CPVs is likely accounting for the resolution of *Leishmania* infections in *L. longipalpis*, an interaction which certainly warrants further investigation.

In another study that tested the susceptibility of the cell line Lulo to arboviral infection (Rey et al. 2000), Lulo was susceptible to infection by three viruses from the Togaviridae, Reoviridae and Rhabdoviridae arboviral families. The Rhabdoviridae family includes the genus *Vesiculovirus*, responsible for the vesicular stomatitis, which showed very good replication in Lulo. While the mechanisms of pathogen/host interactions occurring during the viral infection of sand flies remains poorly understood, development of sand fly cell cultures may provide an invaluable tool for the study of these unique interactions, thus providing information about transmission and the true status of sand fly as vectors of viral transmission.

Bacterial infection of sand flies may include Bartonellosis (reviewed in Young and Duncan 1994), however this organism was never isolated from *L. longipalpis*. Recently, bacteria infections in *L. longipalpis* from field (Oliveira et al. 2000) and laboratory reared colonies (Oliveira et al. 2001b) of the Lapinha Cave, Brazil were examined. In the field, the presence of gram negative non-fermenting bacteria including, *Acinetobacter lowfii*, *Stenotrophomonas malthophilia*, *Pseudomonas putida* and *Flavimonas orizihabitans*, was observed in *L. longipalpis*. Fermenting species found were *Enterobacter cloacae* and *Klebsiella ozaenae*, and gram positive bacteria identified were *Bacillus thuringiensis* and *Staphylococcus* spp. After colonization, bacterial species infecting the sand fly can change due to modification of micro-environmental conditions including sugar and blood feeding. Oliveira et al. (2001b) observed the presence of En-

terobacteriaceae of the genera *Serratia*, *Enterobacter* and *Yokenella*, as well as *Pseudomonas*, *Acinetobacter* and *Stenotrophomonas*. Sugar plus blood fed females had similar infections, however an additional genera *Burkholderia* was observed. It is not known to which extent bacterial infections can affect colony viability and productivity, but the rearing process can increase the possibility of infection and mortality due to changes in the natural microbiota. According to Schlein et al. (1985), the microbiota can interfere with the development of *Leishmania* in *P. papatasi*, but this remains to be studied in *L. longipalpis*.

Recently, Ono et al. (2001) examined the presence of *Wolbachia* infections and many species of sand flies, including field and laboratory reared *L. longipalpis* from numerous locations. *Wolbachia* are maternally transmitted intracellular symbionts found in many arthropods and nematodes, and are known to affect host reproduction. The presence of this organism was not observed in all *L. longipalpis* colonies, although it was present in *L. shannoni* and *L. whitmani*. How *L. longipalpis* can control *Wolbachia* infections has yet to be determined, though specific humoral responses against *E. coli* and *Micrococcus luteus* in the hemolymph of this species after bacterial challenge has been reported (Nimmo et al. 1997).

FUNGUS AND HELMINTHES

Laboratory experiments with entomopathogens of phlebotomine sand flies were first conducted by Warburg (1991) using viruses, fungi, bacteria and protozoa (reviewed by Warburg et al. 1991). Under natural conditions, a wide variety of organisms were observed infecting sand flies, including a non-fluorescent *Pseudomonas*, a trypanosomatid (probably *Leptomonas*), gregarines, fungi and nematodes. Also noted was 100% mortality of *L. longipalpis* on day 4 after exposure to *Beauveria bassiana* spores smeared on a filter paper. Exposure to fungus also diminished oviposition. Entomopathogenic fungi penetrate the insect cuticle by a combination of mechanical pressure and enzymatic degradation to sub-

sequently infect internal host tissues (Ferron 1978). This fungus has been shown to be an alternative biological control method against many insects, including *Hypothenemus hampei* (Coleoptera) in coffee plantations in Colombia where sand flies also occur. Reithinger et al. (1997) tested this fungus against phlebotomine sand flies in coffee plantations and observed a significant reduction in the mean survival time.

Helminthes infections in sand flies were already observed by McConnell and Correa (1964), Killick-Kendrick et al. (1989), Warburg (1991) and Poinar et al. (1993). A wide variety of worms, including spirurid, filarid, tylenchid and tetradonematid, have been recovered from the body cavities of sand flies. Poinar et al. (1993) described a new genus and species of nematode infecting *L. longipalpis* in Colombia (*Anandrema phlebophaga*). Recently, Secundino et al. (2002) described a new entomoparasitic nematode (Rhabditida) infecting *L. longipalpis* from Lapinha Cave, Brazil. Although the contamination rates in the field seem to be very low, the productivity of laboratory colonies is readily affected by these helminthes. Nevertheless, the use of these worms as a potential biological control method in the field must be carefully evaluated.

PROTOZOA OTHER THAN *Leishmania*

Infections with protozoa other than *Leishmania* have been reported in *L. longipalpis*, including *Endotrypanum* spp. and *Ascogregarina chagasi*. Brazil et al. (1991) infected *L. longipalpis* from the Lapinha Cave with *Endotrypanum* under laboratory conditions. Different populations of the *L. longipalpis* complex exhibit susceptibility or refractoriness to *Endotrypanum* depending on the origin (Franco et al. 1997). The morphology and life cycle of *A. chagasi* in *L. longipalpis* were described by Adler and Mayrink (1961). During the life cycle, parasite oocysts are found in the sand fly accessory glands and are ingested by larvae after egg hatching. Wu and Tesh (1989) tried to infect a variety of New and Old World sand fly species with *A. chagasi* with no success, indicating a preference of this parasite only

for *L. longipalpis*. During the study of the biological cycle of *A. chagasi* in *L. longipalpis*, Warburg and Ostrovskaya (1991) showed a positive tropism for specific tissues of the sand fly depending on the stage (sporozoite, gamont and gametocyst). Because *A. chagasi* is known to reduce longevity and egg production in *L. longipalpis* colonies, Dougherty and Ward (1991) described a method to reduce *A. chagasi* infections in laboratory-reared colonies based on egg cleaning procedures and found that a 0.1% formalin solution was the most efficient in controlling the parasite infection.

The use of *A. chagasi* as a control method in the field, however, would not be efficient, as the parasite seems to have a limited range and a minimal effect on the sand fly biology under natural conditions.

Leishmania spp

Although *L. longipalpis* is the proven vector of *L. chagasi* (Lainson et al. 1977), it has been shown to be a very permissive sand fly being easily infected by many *Leishmania* species. During earlier studies, Coelho et al. (1967a, b) were able to infect *L. longipalpis* from Lapinha Cave with *L. tropica* and *L. mexicana*, respectively. Infections with *L. mexicana* in *L. longipalpis* were also accomplished by Abdulrahman et al. (1998), Stierhof et al. (1999) and Rogers et al. (2002). *L. amazonensis* development in *L. longipalpis* was reported by Molyneux et al. (1975). *L. longipalpis* was also infected with strains of *L. guyanensis*, *L. amazonensis* and *L. mexicana*, although demonstrating different degrees of susceptibility depending on the strain (Silva et al. 1990). Walters (1993) studied many unnatural life cycles with many species and was able to infect *L. longipalpis* with *L. major*. Later, Walters et al. (1993) studied this association in detail using transmission electron microscopy (TEM), considering *L. longipalpis* a successful host for *L. major*. Although many species of *Leishmania* can infect *L. longipalpis*, to be considered a vector, other factors have to be considered. For example, the distribution of the sand fly has to be coincident with the human disease, the insect must be found infected in

the peridomestic or domestic areas, and it has to feed avidly on man and many hosts (Killick-Kendrick and Ward 1981). Recently, Montoya-Lerma et al. (2003) showed that *L. longipalpis* was more efficient as a vector of *L. chagasi* than *Lutzomyia evansi*. Infection success was dependent on the establishment of the parasite in the midgut, which was very irregular in *L. evansi*. Consequently, these results explain the irregularity in the AVL transmission where *L. evansi* occurs.

The results previously reported here were made under laboratory conditions and extrapolations to the natural situations are limited. For more information of New and Old World phlebotomine sand flies vector incrimination see a review by Killick-Kendrick (1990).

GUT PHYSIOLOGY

BASIC MORPHOLOGY

Leishmania development in the sand fly midgut is a complex process, and many reports have been published detailing morphological, molecular and biochemical aspects required for interaction. However, most of the works involve Old World species such as *L. major* and *L. donovani*. The digestive tube starts in the mouthparts (proboscis) and continues through the cibarium and the pharynx to the gut, which is divided into three portions (anterior, medium and posterior) differing in embryological origin. The cibarium valve separates the cibarium and pharynx, and the cardiac valve separates the pharynx and the anterior gut. The simultaneous pumping of the cibarium and cardiac valves is important for the process of suction and *Leishmania* injection during blood meals. In the junction between the medium and posterior gut are the Malpighian tubules. In most of the insects, digestion and nutrient absorption occur in the midgut, with the feces and urine passed into the posterior gut, where water and salt are also absorbed (Chapman 1985). Rudin and Hecker (1982) studied the midgut epithelium of female *L. longipalpis* using (TEM) morphometry in presence of sugar or blood meals. The morphological structure of the *L.*

longipalpis midgut epithelium was very similar to *P. papatasi*, consisting of a single layer of high polarized cylindrical epithelium cells, which were covered towards the midgut by densely packed microvilli. A fine basal lamina separated the stomach epithelium from the hemocoel. No desmosomes or hemidesmosomes were observed. After the blood meal a flattening of the epithelium occurred. The attributed functions of the midgut cells include formation of the peritrophic matrix (PM), secretion of digestive enzymes, and absorption and transport of digestive products. Most of the regulation of the digestive events remain to be established. However, ultrastructural study using TEM identified for the first time two types of endocrine cells in the midgut of *L. longipalpis* (Leite and Evangelista 2001). Morphology of these cells indicated a presence of granules probably involved in the secretion of peptide-like substances during digestive processes. The ultrastructure of the stomodeal valve and adjacent cardia region of *L. longipalpis* was recently described by Tang and Ward (1998a) using TEM and SEM. The stomodeal valve was found to have chemosensory activity due the presence of typical basiconic sensilla on the inner side of the esophagus at the junction of the estomodeal valve, being able to direct fluids to the crop or midgut portions of the sandfly. This valve is important for the suction process during feeding, and was shown to be damaged by *Leishmania*, resulting in regurgitation of the parasites and thereby facilitating transmission during the bite (Schlein et al. 1992).

SUGAR METABOLISM

It is well known that sand flies in the natural environment feed on plants as their source of sugars. This phenomenon was first suggested by Chanotis (1974), and phytophagy in *P. papatasi* was observed by Schlein and Warburg (1986). Cameron et al. (1995) described some sugar sources for *L. longipalpis* in Ceará State, Brazil. Availability of sugars also has an impact in the biology of laboratory reared *L. longipalpis* (Souza et al. 1995). A preference of *L. longipalpis* for the nectar of the wax plant

(*Hoya* sp) rather than fresh honeydew from *Aphis craccivora* or sucrose solution was reported by Petts et al. (1997). Survivorship and oviposition were also greater in the group of sand flies fed on nectar. The ingestion of sugar by sand flies is suggestive of the presence of enzymes that could metabolize them into monosaccharides when necessary. There are very few studies regarding sugar metabolism in sand flies, and much less with *L. longipalpis*. Gontijo et al. (1998) studied the pH in the gut and presence of digestive glycosidases that could be involved in sugar metabolism in *L. longipalpis*. It was observed that sand flies fed solely on sugar had only α -glucosidase activity (specifically classified as sucrose α -glucohydrolase), a membrane-bound enzyme involved in sucrose digestion. Following a blood meal, however, three other enzymes were synthesized in the midgut, including *N*-acetyl- β -D-glucosaminidase (probably involved in the digestion of peritrophic matrix), *N*-acetyl- β -D-galactosaminidase and α -L-fucosidase. In addition, it was observed that in the gut of unfed sand flies the pH was mildly acidic (6.0), which is coincident with the optimum pH for α -glucosidase activity. A soluble protein with sucrase activity was also identified in *L. amazonensis* (Gontijo et al. 1996). During the biological cycle of *Leishmania*, both sucraes from the parasite and from the sand fly could cleave sucrose and be responsible for sugar availability. *L. amazonensis* is able to sustain infection *L. longipalpis* (Molyneux et al. 1975) and it is tempting to speculate that this phenomenon could possibly occur with *L. chagasi*. Identification of sucrase activity in *L. chagasi* could clarify the role of exoglycosidases in the interaction with *L. longipalpis*.

In the interior of the sand fly, strong evidence of sugar destination was provided by Tang and Ward (1998b). At the onset of feeding, a small amount of sugar was observed in the thoracic midgut, then soon after a blocking occurred with a preferential accumulation of the sugar meal in the crop. Fluid destination was shown to be controlled by the pumping of the stomodeal valve, the sensilla of which could be involved in the chemosensory activity (Tang and

Ward 1998a). Furthermore, the presence of an α -amylase was reported in the saliva (Ribeiro et al. 2000c), suggesting possible involvement in sugar metabolism in the crop.

While a few exoglycosidases have been identified in sand flies from the Old and New World, the biological functions of sand fly and *Leishmania* glycosidases during their interaction remains an open and interesting field (see Jacobson et al. 2001).

Leishmania INTERACTION – DIGESTIVE EVENTS

Other structural studies were conducted in an attempt to understand the interactions between *L. longipalpis* with many species of *Leishmania* including *L. major* (Walters et al. 1993), *L. amazonensis* (Molyneux et al. 1975) and *L. chagasi* (Walters et al. 1989). In general, after the blood meal, the amastigote forms inside the macrophages differentiate into the procyclic dividing promastigotes, which attach to the microvilli to avoid elimination with the digested blood meal. Most of the knowledge concerning molecular interactions during specific steps of the digestion process was obtained using Old World species. During digestion, the blood meal is surrounded by a peritrophic matrix (PM), which is made of chitin, a polymer of N-acetylglucosamine (GlcNAc), which is also present in the exoskeleton of the insects. PM compartmentalizes the digestive events, allowing a trypsin gradient to form from the epithelium to the inner part of digesting material containing blood cells and parasites. Under these conditions, the parasites are protected from destruction by the digestive enzymes, and have time to differentiate into procyclic promastigotes. Subsequently, the parasites produce a chitinase which further digests the PM, thus exposing the epithelium, where the parasites can then attach and remain there until differentiation (Pimenta et al. 1997). A putative *L. longipalpis*-derived chitinase was recently characterized by Ramalho-Ortigão and Traub-Cseko (2003) and was also shown to be involved in PM digestion. This chitinase is produced after the blood meal, reaching peak production in 72h. The formation and destruction of the PM seems to be a

concomitant and well-synchronized event of chitin deposition and degradation. Pascoa et al. (2002) proposed that the PM could also be a binding substrate for heme, a toxic byproduct of the digestion of blood in the mosquito *Aedes aegypti*.

In its life cycle, *Leishmania* undergo many morphological, physiological and biochemical modifications within the sand fly midgut. The polymorphism in the procyclic forms of *L. chagasi* inside *L. longipalpis* is very evident and was fully described in detail by Walters et al. (1989). Briefly, differentiation of the parasite progresses from the promastigote (two sequential forms I and II) to the nectomad, which adheres to the midgut, followed by detachment and differentiation to the pear-shaped haptomonad, which migrates towards anterior parts of the midgut. The haptomonad appears to be the precursor of the heart-shaped paramastigote, which also attaches to the esophagus and pharynx. Free and very active swimming forms are observed late, and are considered to be the infective and metacyclic promastigotes, which are injected in the vertebrate host while by the sand fly during a blood meal. Although multiple bloodfeeding in *L. longipalpis* between each gonotrophic cycle has been observed (ElNaiem et al. 1992c), it does not seem to interfere with the dynamics of metacyclogenesis, being the parasites able to develop normally with extra blood intake (ElNaiem et al. 1994). During the interaction of *L. mexicana* with *L. longipalpis*, Stierhof et al. (1999) described the formation of a gel-like structure composed of secreted proteophosphoglycans. This structure obstructed the digestive tract of the sand fly, disrupting the feeding mechanism during the next blood meal, thus favoring regurgitation rather than blood meal intake. According to Rogers et al. (2002), the plug formation had 75% metacyclic parasites, thus increasing the probability of transmission of *Leishmania* after the next blood meal.

Leishmania INTERACTION – THE ROLE OF LIPOPHOSPHOGLYCAN (LPG)

During its life cycle, *Leishmania* parasites must sur-

vive under extremely adverse conditions represented by the digestive hydrolases present in the midgut, have to avoid passage with the blood meal and must digest PM in order to attach to the insect epithelium (Borovsky and Schlein 1987, Pimenta et al. 1997). The recognition of receptors in the microvilli by the *Leishmania* lipophosphoglycan (LPG), the dominant cell surface glycoconjugate, is a crucial step for *Leishmania* survival, as mutants in LPG synthesis are unable to sustain infection in the sand fly (Butcher et al. 1996). In addition, LPG is not only responsible for specificity of pairing among the sand flies and parasites, but also undergoes biochemical modifications from dividing procyclic stage to infective metacyclic stage (Pimenta et al. 1992, 1994). All data mentioned here were obtained from Old World species of *Leishmania* and *Phlebotomus* species (reviewed by Sacks 2001).

The LPG structure consists of a conserved glycan core region of Gal(α 1,6) Gal(α 1,3) Gal_f(β 1,3) [Glc(α 1)-PO₄] Man(α 1,3) Man(α 1,4)-GlcN(α 1) linked to a 1-*O*-alkyl-2-lyso-phosphatidylinositol anchor. Another conserved domain of LPG is represented by the Gal(β 1,4)Man(α 1)-PO₄ backbone of repeat units followed by a terminal structure cap. Variations in the composition of the sugars that branch off from the repeat units are responsible for the intra- and interspecific variations in the *Leishmania* species (reviewed by Turco and Descoteaux 1992). Concerning *L. chagasi*, there is only one report regarding the role of LPG in its interaction with *L. longipalpis* (Soares et al. 2002). In this species, the procyclic promastigote side chains consist of one β -Glc. Therefore, after metacyclogenesis, metacyclic promastigote increases in size, downregulates β -Glc side chains and detaches from the microvilli. The biochemical modifications of *L. chagasi* LPG are very similar to the Indian strain of *L. donovani* (Mahoney et al. 1999), which causes Visceral Leishmaniasis in the Old World. A receptor for the LPG in *P. papatasi* midgut was identified (Dillon and Lane 1999), but not in *L. longipalpis*. Since this sand fly is a very permissive species, this ligand is probably a molecule present in

large amounts in the microvilli. Many lectins in the midgut have been reported and are able to agglutinate *Leishmania* and in a species-specific way (Svobodova et al. 1996). High agglutination titers were observed in *L. chagasi* exposed to midgut lysates from *L. longipalpis*. The possible role of these lectins as receptors is yet to be determined. Recently, Evangelista and Leite (2002) reported the histochemical localization of N-acetyl-galactosamine in the midgut of *L. longipalpis*, which was widely present in the microvilli during and after digestion. Although its role as the LPG receptor was suggested, it also remains to be elucidated.

CONCLUSIONS

L. chagasi in the Americas, transmitted by *L. longipalpis*, has been increasingly reported in urban areas where until recently, the disease did not occur. Control of leishmaniasis is hampered by the diversity of vectors, parasites, and reservoir hosts and the interventions must take into account these differences. It is crucial to understand the biology of the leishmaniasis in the New World as well, since Brazil is responsible for 90% of AVL and also contributes to a great incidence of the cutaneous and mucocutaneous forms of the disease. The exploration of host-parasite interactions between New World species of *Leishmania* and respective vectors are still in its infancy, representing a wide and important field for basic and applied research as well. A variety of *Leishmania* species and vectors remain to be studied with respect to physiological, biochemical and ecological aspects, providing tremendous opportunities for the research of sand flies and *Leishmania* species in the Americas.

RESUMO

Lutzomyia longipalpis é o vetor mais importante da *Leishmania chagasi*, agente etiológico da Leishmaniose Visceral Americana (AVL), no Novo Mundo. A AVL, uma doença predominante em zonas rurais, tem aumentado sua prevalência, tornando-se urbana nas grandes cidades no Brasil e em outros países na América Latina. Embora a

AVL seja uma doença tratável, medidas de prevenção devem ser utilizadas, como a eliminação dos cães infectados e o uso de inseticidas. A melhor compreensão da biologia do vetor poderia ser mais uma medida para o controle da AVL. Um grande número de artigos sobre *L. longipalpis* foi publicado recentemente. Esta revisão sumariza as pesquisas atuais em *L. longipalpis* em relação a sua importância, biologia, morfologia, feromônios genética, saliva, fisiologia do intestino e interações com diferentes parasitas.

Palavras-chave: biologia de vetor, *Lutzomyia longipalpis*, *Leishmania*.

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